Sp3 Represses Transcription When Tethered to Promoter DNA or Targeted to Promoter Proximal RNA*

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Sp3 is a member of the Sp family of transcription factors, and it binds to the GC box with an affinity and specificity comparable with that of Sp1. Previous studies have shown that Sp3 repressed Sp1-mediated transcriptional activation, suggesting that Sp3 is an inhibitory member of the Sp family. The experiments described here demonstrate that Sp3 contains a portable repression domain that can function independently from the zinc finger DNA-binding domain. We found that the amino-terminal region of Sp3 tethered to a promoter DNA by connecting to a heterologous DNA-binding protein domain represses transcriptional activation by different positive regulators. Moreover, we determined that Sp3 targeted to a promoter-proximal RNA sequence acts as a transcriptional repressor. Taken together, our results suggest that Sp3 functions as a repressor by protein-protein interaction with components of the general transcription complex.

Originally identified as a cellular transcription factor required for SV40 gene expression, Sp1 stimulates transcription by binding to a GC box present in a wide variety of cellular and viral promoters (1). Recently, three Sp1-related genes (Sp2, Sp3, and Sp4) have been cloned based on their homology with the Sp1 DNA-binding domain (2, 3). The DNA-binding domains of the Sp3 and Sp4 proteins are highly conserved, and they recognize GC boxes with specificity and affinity closely similar to that of Sp1. Contrary to these factors, Sp2 seems to have different DNA binding specificities (3). It has been shown that both Sp1 and Sp3 proteins are ubiquitously expressed at a high level in many mammalian cell lines, whereas Sp4 expression appears to be restricted to certain cell types of the brain (2, 4, 5). Functional analysis of Sp3 and Sp4 in direct comparison of Sp1-responsive promoters, whereas Sp3 represses Sp1-mediated transcription (4–7). These results suggest that Sp3 is an inhibitory member of the Sp family.

The intriguing finding that Sp3 represses Sp1-mediated transcription prompted us to embark on an analysis of the transcriptional properties of Sp3. We performed in vivo transfections, in which the non-finger region of Sp3 repressor and a defined activating domain were both targeted to a promoter. We found that Sp3 contains a portable repressor activity, which can function independently from the zinc finger DNA-binding domain. In addition we demonstrated that the Sp3 amino terminus region is a transcriptional repressor of several activators, and the repression is not influenced by the arrangement of basal promoter elements. Finally, we determined that the Sp3 repressor is functional when targeted to a promoter-proximal RNA sequence. Our results indicate that Sp3 acts as a transcriptional silencing of RNA polymerase II promoters either when tethered to DNA by fusion to a DNA-binding protein domain or when targeted to a promoter-proximal RNA sequence.

EXPERIMENTAL PROCEDURES

Reporter Plasmids—The HIV-1 LTR-based reporters G5–83HIV and G5–83HIV-ΔTAR have been described (6, 8). The T7GS-TATA was constructed by deleting the 7x tetO operator sequences, obtained by digestion of the plasmid pHUC13-3 (9) with XhoI and Smal, upstream of the GAL4 DNA-binding domain of G5E1b (10) digested with Xhol and HindIII (filled). The plasmid T7GS1 was constructed by substitution of the TATA sequences of the T7GS-TATA with double-stranded oligonucleotide (upper strand, 5′-CTAGACCGTCTCTCACTCTCTTCGCGAGCCTAC-3′ containing the AdMLP 1sr sequence flanked by the Xbal and KpnI sites and cloned into Xbal-KpnI sites of T7GS-TATA. The plasmid constructions were analyzed by DNA sequencing. E. coli strains were prepared as described (6, 8, 10–12). To construct the Tet-Sp3 effector plasmid the TetR DNA-binding coding region (aa 1–206) was polymerase chain reaction-amplified from pHOD15-1 (9) with 5′-HindIII and 3′-EcoRI adapter primers (5′-CCCGCAAGCTTGCCGCAGATTCGCGACC-3′, 5′-CGGGAATTCGGACCCACTTTC-3′). The PCR product was subcloned into the plasmid pSG424 digested with HindIII and EcoRI to substitute the Gal4 coding region, resulting in pTetR. The Tet-Sp3 clone was obtained by inserting the Sp3 non-finger region (aa 1–527) derived from the digestion of the pBS A31416 plasmid (2) with Clal-Acc51I (both filled) in pTetR EcoRI (filled). The Tet-Sp3 clone was constructed by inserting the aforementioned Sp3 non-finger region (aa 1–527) into pSVTat22/37 EcoRI (filled). All plasmids were analyzed by DNA sequencing to confirm correct construction. 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. Transfection was performed by calcium phosphate precipitation using subconfluent cell cultures with different amounts of reporter and effector plasmids. For normalization of transfection efficiencies a β-galactosidase plasmid was included in the cotransfections (psvβ-gal expression plasmid, Promega). CAT assays were performed with a different amount of extract to ensure linear conversion of the chloramphenicol with each extract, and results are presented as the means ± S.D. of a least four duplicated independent transfection experiments. The CAT activity was quantified using the Molecular Dynamics PhosphorImager System™.

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RESULTS AND DISCUSSION

Transcriptional Activation by Different Positive Regulators Is Suppressed by Sp3—It has been suggested that Sp3 is an inhibitory member of the Sp family (4). Transfection experiments into mammalian cell lines and Drosophila SL2 cells lacking endogenous Sp factors have demonstrated that Sp3 failed to activate Sp1-responsive promoter constructs and enforced expression of Sp3-repressed Sp1-mediated transcriptional activation (4, 6, 7). We now wished to determine whether Sp3 may interfere with the activating function of different defined activators. To address this point, we developed an in vivo transcription assay in which various well characterized transcriptional activation domains were fused to the yeast GAL4 DNA-binding domain and their activity assayed on a reporter plasmid. To evaluate the Sp3-mediated repression the Sp3 non-finger region (aa 1–527) was connected in frame to the COOH terminus of the prokaryotic TetR encoded by Tn10 from Escherichia coli (9). Thus, the Tet-Sp3 chimeric protein was able to bind to the tet operator (tetO) sequences. Two different reporter plasmids were constructed and used as templates: the T7G5-TATA and the T7G5-Inr. The two reporters are isogenic except for the presence of the core promoter element. The T7G5-TATA reporter contains the CAT gene under the control of the E1b TATA box with five GAL4 DNA-binding and seven tetO sequences, whereas the T7G5-Inr bears as core promoter element the AdMLP Inr element. Relevant features of the effectors and reporter plasmids are outlined in Fig. 1. First the effect of the various activator domains present in the GAL4 chimeric proteins was determined. Accordingly with previous results (13) we found that both TATA and Inr promoters responded to the GAL4-based activators, with the exception of the E1a-activating domain, which as previously reported is strictly dependent upon the presence of the TATA box (10). Therefore, the GAL4-E1a fusion protein is unable to transactivate the T7G5-Inr reporter whereas the T7G5-TATA was fully responsive.

To determine the Sp3-mediated repression of the activation function of each GAL4 derivative, the reporter plasmids described in Fig. 1A were co-transfected into HeLa cells with the indicated GAL4 fusion proteins in the presence of an increasing amount of the Tet-Sp3 expression vector. Co-expression of Tet-

Fig. 1. A, schematic representation of the T7G5-TATA and T7G5-I reporter plasmids containing the E1b TATA or the AdMLP Inr sequences as core promoter element, respectively. B, reporter plasmids T7G5-TATA (2 μg, open bars) and T7G5-I (2 μg, solid bars) were cotransfected into HeLa cells with the GAL4 derivatives (1 μg) as indicated. The transcriptional activity of each GAL4 derivative relative to the sample without activator is diagrammed at the right. The values are representative of four independent duplicated experiments; vertical lines indicate the standard deviations.

Repression Functions of the Transcription Factor Sp3

Fig. 2. Sp3 represses transcription in the presence of defined activators. On the top a schematic representation of the chimeric protein Tet-Sp3 is shown. A, reporter plasmids T7G5-TATA (2 μg, open bars) and T7G5-I (2 μg, solid bars) were cotransfected into HeLa cells together with 1 μg of the indicated GAL4-activator plasmid in the presence of increasing amounts (1, 5, and 10 μg) of Tet-Sp3 expression vector in a total of 20 μg adjusted with the parental plasmid. CAT activities relative to the samples without repressor (taken as 100%) are shown. The values are representative of four independent duplicated experiments; vertical lines indicate the standard deviations. B, repression specifically depends upon DNA binding. T7G5-TATA (open bars) and GSE1b (striped bars) reporter plasmids (2 μg each) were transactivated with 1 μg of GAL4-VP16 expression plasmid. Coexpression of CMV-Sp3 DBD (10 μg) or Tet-Sp3 (10 μg), respectively, did not affect transactivation.
Sp3 protein was found to repress in a dose-dependent manner GAL4 chimeric protein-mediated activation of both the TATA and Inr-containing promoters (Fig. 2). The specificity of Sp3-mediated repression was demonstrated by the results reported in Fig. 2B, showing that neither the Tet-Sp3 fusion protein affected the activation function of a reporter lacking the tetO sequences nor did the pCMV-Sp3DBD, encoding the fingerless portion of Sp3 lacking the DNA-binding domain (6), influence significantly promoter activity.

Taken together the data from transfection experiments demonstrate that Sp3 suppresses transcription when allowed to bind next to the activator, and the extent of repression appears to be independent of the type of activator domain and of the presence of a specific core promoter element.

Sp3 Repressor Is Functional When Targeted to a Promoter-proximal RNA Sequence—We have previously shown that the non-finger region of Sp3 tethered to the HIV-1 promoter by a GAL4 DNA-binding domain repressed transcription in a distance-independent manner (6). Repression at a distance supports a mechanism requiring protein-protein interactions between the distantly bound Sp3 repressor domain and proximal factors looping out intervening DNA (14, 15). Alternatively, the Sp3 domain tethered to DNA may alter (bending) promoter topology, such as described for the YY1 transcription factor (16). We reasoned that if repression is due to a local topological alteration of DNA the Sp3 repressor must be tethered to the template via fusion to a DNA-binding domain.

To clarify this point we chose to attempt regulation of HIV-1 promoter activity by designing a chimeric Tat negative transcriptional mutant fused to the non-finger region of Sp3. We used the previously described pSVTat22/37, in which cysteines 22 and 37 have been substituted with glycine and serine, respectively (8). It has been shown that these point mutations in the cysteine-rich region abolished the transacting effect of the protein (17). The non-finger region of Sp3 (aa 1-527) was fused to the COOH terminus of Tat22/37 resulting in the pSVTat22/37-Sp3 expression vector. As reporter, the G5-83HIV-CAT plasmid, which contains five GAL4 DNA-binding sites located at position -83 of HIV-1 LTR (6), was used. Using a similar reporter it has been shown (18) that the GAL4-VP16 fusion protein can potently activate expression from an HIV-1 LTR bearing multiple GAL4 DNA-binding sites. The G5-83HIV-CAT plasmid was transfected into HeLa cells together with the GAL4-VP16 effector plasmid in the presence of increasing amounts of pSVTat22/37 or pSVTat22/37-Sp3 effectors (see Fig. 3A). Accordingly with reported results the GAL4-VP16-mediated trans-activation of G5-83HIV-CAT reporter was very high (18), and the presence of Tat22/37 did not change the promoter activity. Conversely, a dose-dependent repression of GAL4-VP16-mediated activation was observed in the presence of the Tat22/37-Sp3 fusion protein (Fig. 3A).

To further substantiate the notion that Sp3 targeted to nascent RNA is able to repress HIV-1 LTR promoter activity we analyzed the Sp3-mediated repression of the LTR basal activity. Dose-dependent repression of the HIV LTR basal activity was mediated by Tat22/37-Sp3 fusion protein, whereas no significant effect was observed in the presence of the Tat22/37 mutant (Fig. 3B). Moreover, Tat22/37-Sp3-mediated repression was dependent on the presence of TAR element (Fig. 3C), since the activity of the reporter plasmid G5-83HIV-ΔTAR in which the TAR sequences have been deleted was not affected by the presence of the Tat22/37-Sp3 fusion protein. These data clearly indicated that the Sp3 repressor domain can efficiently repress transcription from nascent RNA target and demonstrated that Sp3-mediated repression does not require a stable interaction with the promoter DNA.

Several models can be envisaged to explain how Sp3 might repress transcription. For example, Sp3 may function by inactivating or squelching a protein that normally activates polymerase II expression (14, 15, 19). This possibility seems very unlikely because Sp3-mediated repression is strictly dependent on binding in cis to the promoter or binding to a promoter-proximal RNA target. A number of repressors appear to use quenching as their mechanism. For example, the Drosophila Kruppel protein displays the ability to quench some activators but not others (20). However, the quenching mechanism does not account for the ability of Sp3 to repress different types of activators, and the repression is not influenced by the presence of a specific core promoter element. Therefore, it appears that Sp3-mediated repression may act directly on the general transcription machinery.

The observation that Sp3 repressor function does not neces-
sarily require an interaction with a DNA target sequence appears inconsistent with a repression model involving the formation on the DNA template of multiprotein complexes composed of Sp3 and other factors. Instead, it appears more likely that the RNA-bound Sp3 may interact directly with a component of the general transcriptional machinery and prevent an isomerization or disassembly step. Alternatively, Sp3 could load a putative "corepressor(s)" into the general transcription complex, which may then modify the initiation complex so that the rate of transcription is repressed. However, it remains to be demonstrated whether the mechanism of Sp3-mediated repression is the same when it is targeted to nascent RNA as it is when it is bound to promoter DNA.

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