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Sp1 Transcription Factor Is Required for In Vitro Basal and Tat-Activated Transcription from the Human Immunodeficiency Virus Type 1 Long Terminal Repeat

CARLOS SUÑÉ[1] AND MARIANO A. GARCÍA-BLANCO[1,2]*

Department of Molecular Cancer Biology[1] and Departments of Microbiology and Medicine,[2] Duke University Medical Center, Durham, North Carolina 27710

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Sp1-DNA binding sites have been reported to be essential for basal and Tat-activated transcription of the human immunodeficiency virus type 1 long terminal repeat (LTR). The role of the Sp1 transcription factor itself in regulation of the retroviral LTR, however, has not been clearly defined. It is now known, for instance, that the Sp1-DNA binding sites function also as thyroid hormone receptor response elements (V. Desay-Yajnik and H. H. Samuels, Mol. Cell. Biol. 13:5057–5069, 1993). In this report, we present data that demonstrate a strict requirement for Sp1 for both basal transcription and Tat-mediated *trans* activation of the human immunodeficiency virus type 1 LTR in vitro.

The regulation of transcriptional activation is accomplished via a complex network of interactions between *cis* elements and *trans*-acting factors. The DNA *cis*-acting regulatory sites that control human immunodeficiency virus type 1 (HIV-1) gene expression reside in the long terminal repeat (LTR). The HIV-1 LTR is activated potently by the virally encoded Tat protein. Tat exerts its action through binding to an RNA element designated TAR (for *trans* activation response element) (9, 47). The binding of Tat to TAR and the structural features of this RNA element have been investigated thoroughly (2, 7, 12, 13, 16, 17, 21, 25, 39, 43, 44, 46, 53). At present, the mechanism by which Tat activates gene expression is still unknown. In vitro and in vivo studies have been interpreted as demonstrating an effect of Tat on both the initiation and elongation phases of transcription (4, 8, 15, 18, 31–33, 37, 38, 45, 49, 54). Whatever the precise mechanism of the *trans* activation by Tat, there are now strong data that suggest the involvement of unique coactivators (5, 23, 35, 48, 50, 54). Further characterization of Tat-mediated transcriptional activation awaits the identification and purification of the specific components involved in this process.

The HIV-1 LTR contains two tandem NF-κB elements followed by three tandem Sp1-binding sites and the TATA box sequence (19, 27, 52). Deletion and mutational studies have shown specific contributions of these sites to basal and Tat-activated transcription. In particular, NF-κB sites are required for basal transcription, and Sp1-binding sites and the TATA box sequence are required for basal transcription and Tat-mediated *trans* activation (1, 3, 20, 24, 28, 29, 34, 40, 42, 49). In fact, physical interaction between Tat and the TATA-binding protein (TBP) (30) and Tat and Sp1 (26) have been recently reported. The functional implications of these interactions remain unclear. Although *trans* activation by Tat requires Sp1-binding sites, these can be functionally replaced by other enhancer-binding sites (1, 3, 6, 36, 49), suggesting that HIV-1 transcription activation can be maintained in the absence of specific upstream elements. These findings make the precise role of Sp1 in HIV-1 transcriptional regulation difficult to assess.

To better understand the regulation of HIV-1 gene expression, we initiated a biochemical dissection of the factors involved in basal HIV-1 transcription and *trans* activation by Tat. Column chromatography was used to generate fractions that were assayed for the ability to support basal and Tat-activated transcription of HIV-1 as well as the adenovirus type 2 major late (AdML) promoter. To analyze transcription, we used an in vitro cell-free system described previously (50). Purification of biologically active recombinant Tat protein and RNA polymerase II-dependent transcription of the DNA templates were also performed as described previously (50). Tat was able to specifically *trans* activate the HIV-1 promoter. A nonfunctional K41A Tat mutant was unable to *trans* activate the HIV-1 LTR (50). *trans* activation by Tat was TAR dependent because the behavior in response to Tat of a deletion mutant in the TAR region was indistinguishable from that of the AdML promoter control template (50 and data not shown).

HeLa cell nuclear extract was prepared as previously described (11) and fractionated by using the chromatographic steps illustrated in Fig. 1. The first step was DEAE-Sepharose chromatography. Forty-five milliliters of HeLa extract (16 mg/ml) was loaded on a 31.85-ml DEAE-Sepharose column (cross-sectional area, 1.25 cm^2; height, 6.5 cm) and chromatographed at a flow rate of 2.5 ml/h/cm^2. The flowthrough fraction from this column was unable to carry out specific transcription from the AdML promoter; in contrast, specific AdML transcripts were obtained when the 0.5 M KCl fraction was used (Fig. 2A). Surprisingly, basal or Tat-activated transcription from the HIV-1 promoter was not obtained with either fraction alone (Fig. 2A). The combination of these two fractions restored both basal transcription and Tat-mediated *trans* activation from the HIV-1 promoter (Fig. 2A, lanes 7 and 8). We concluded that the DEAE-Sepharose flowthrough contained one or more factors that allow HIV-1-specific transcription by RNA polymerase II but are not required for AdML transcription.

We next used carboxymethyl (CM)-Sepharose chromatography to further separate the proteins present in the flowthrough

* Corresponding author. Phone: (919) 613-8632. Fax: (919) 613-8646. Electronic mail address: garci001@mc.duke.edu.

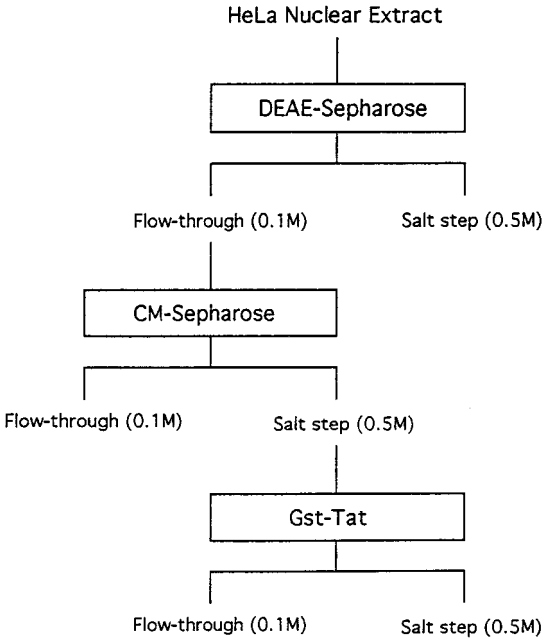

FIG. 1. Scheme for the purification of factors involved in HIV-1 transcription used in this study. Analysis of the fractions at each stage of purification is shown in Fig. 2.

from the DEAE-Sepharose column (Fig. 1). Twenty-five milliliters of this material was chromatographed on an 11-ml CM-Sepharose column (cross-sectional area, 1.77 cm^2; height, 6.2 cm) at a flow rate of 5 ml/h/cm^2. The activity required for specific transcription and Tat-mediated *trans* activation of the HIV-1 promoter was found in the 0.5 M KCl eluate fraction (Fig. 2B, lanes 9 and 10), whereas the CM-Sepharose flowthrough fraction had little effect on transcription of the HIV-1 promoter in the absence or presence of Tat (Fig. 2B, lanes 7 and 8).

To further investigate the factor(s) responsible for this activity, we subjected the CM-Sepharose 0.5 M KCl fraction to affinity chromatography through a glutathione *S*-transferase (GST)–Tat column. The GST-Tat fusion protein used to build this column was able to specifically activate the HIV-1 promoter (see Fig. 5A and reference 50). Three-tenths milliliter of the CM-Sepharose 0.5 M KCl fraction was chromatographed on a 0.1-ml GST-Tat (1.5 mg/ml) column. As shown in Fig. 2C (lanes 9 and 10), the 0.5 M KCl eluate fraction from the affinity column was able to complement the DEAE-Sepharose 0.5 M KCl step and activate the HIV-1 promoter.

As discussed above, Sp1-binding sites are important for basal and Tat-activated transcription, and thus Sp1 itself was a candidate for the factor that restores HIV-1 transcription. To analyze the chromatographic properties of Sp1, the proteins contained in the column fractions were subjected to immunoblot analysis using Sp1-specific antibodies (kindly provided by Jonathan Horowitz, Duke University). As shown in Fig. 3, the antibodies recognize polypeptides of the expected sizes (95 to 105 kDa) in each of the fractions that recovered transcription from the HIV-1 promoter (DEAE flowthrough, CM-Sepharose, and GST-Tat salt fractions). TBP-specific antibodies (Upstate Biotechnology Inc.) were also used in this assay to control the chromatographic separation. TBP was found in the flowthrough from the DEAE and CM-Sepharose columns as well as in the DEAE 0.5 M KCl step (Fig. 3). The heterogeneous behavior of TBP on chromatographic columns has been

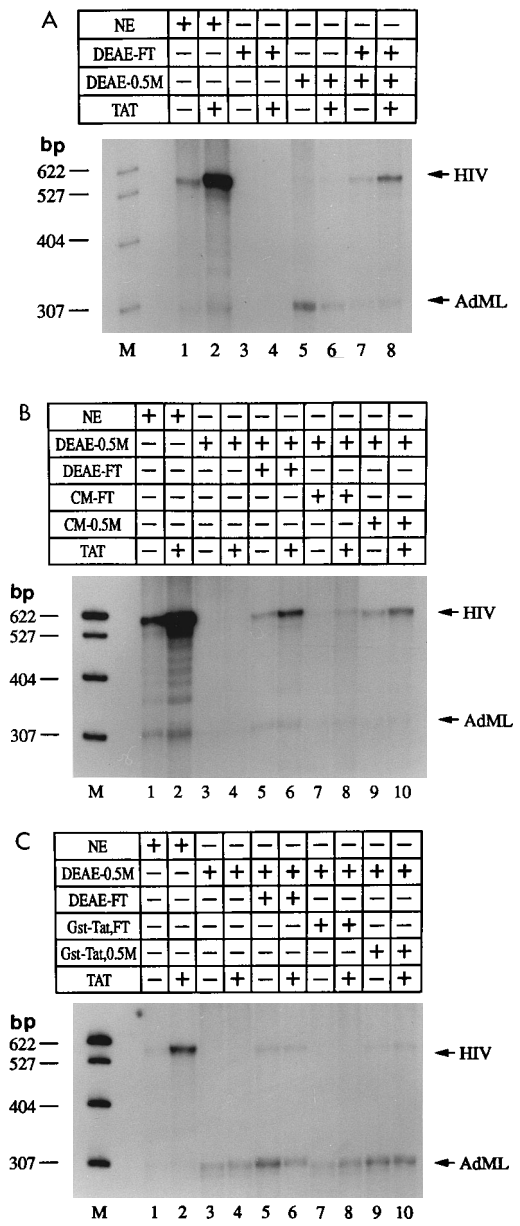

FIG. 2. Analysis of chromatographic fractions in an in vitro transcription system. (A) DEAE-Sepharose chromatography. Ten microliters (16 μg/μl) of unfractionated HeLa nuclear extract (NE; lanes 1 and 2), 6 μl (12 μg/μl) of DEAE-Sepharose flowthrough (DEAE-FT; lanes 3 and 4), 6 μl (11 μg/μl) of DEAE-Sepharose 0.5 M KCl step (DEAE-0.5 M; lanes 5 and 6), and 6 μl of DEAE-Sepharose flowthrough plus 6 μl of DEAE-Sepharose 0.5 M KCl step (lanes 7 and 8) were assayed for transcription activity in the absence (−) or presence (+) of 50 ng of Tat protein. The positions of the HIV- and (AdML-specific) transcripts are indicated. Molecular size markers (lane M) were the pBR322 DNA-*Msp*I digest (New England BioLabs) labeled with [α-^{32}P]dCTP. (B) CM-Sepharose chromatography. The experiment described for panel A was carried out with 10 μl of unfractionated HeLa nuclear extract (lanes 1 and 2), 6 μl of DEAE-Sepharose 0.5 M KCl step (lanes 3 and 4), or the combination of 6 μl of DEAE-Sepharose 0.5 M KCl step plus 6 μl of DEAE-Sepharose flowthrough (lanes 5 and 6), plus 3.5 μl (6 μg/μl) of CM-Sepharose flowthrough (CM-FT; lanes 7 and 8), or plus 10 μl (2 μg/μl) of CM-Sepharose 0.5 M KCl step (CM = 0.5M; lanes 9 and 10). (C) GST-Tat chromatography. Lanes 1 to 6 are as in panel B. The remaining lanes contained 6 μl of DEAE-Sepharose 0.5 M KCl step plus 10 μl (2 μg/μl) of GST-Tat flowthrough (Gst-Tat, FT; lanes 7 and 8) or plus 10 μl (2 μg/μl) of GST-Tat 0.5 M KCl step (Gst-Tat, 0.5 M; lanes 9 and 10) added to the transcription reaction mixtures in the absence (−) or presence (+) of 50 ng of Tat protein. The low *trans* activation level by Tat with the fractions in panel C was not reproduced in other experiments (not shown).

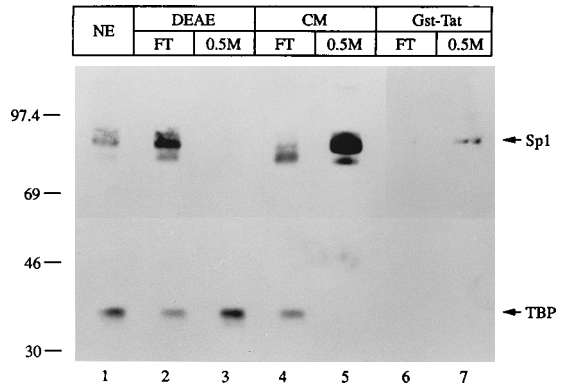

FIG. 3. Chromatographic separation of Sp1 and TBP. Forty micrograms of unfractionated HeLa cell nuclear extract (NE; lane 1), 40-μg aliquots of the DEAE (lanes 2 and 3) and CM-Sepharose (lanes 4 and 5) fractions, and 8 μg of the GST-Tat chromatographic steps (lanes 6 and 7) were subjected to immunoblotting analysis with Sp1-specific and TBP-specific antibodies. Positions of Sp1 and TBP proteins are indicated on the right; molecular masses (in kilodaltons) of the molecular weight markers are shown on the left. Lanes 6 and 7 are from a different experiment. FT, flowthrough; 0.5 M, 0.5 M KCl step.

previously described and is consistent with it being complexed with other proteins (22).

The immunoblotting results prompted us to test directly whether Sp1 protein was the factor responsible for the recovery of HIV-1 specific transcription during the chromatography. Recombinant human Sp1 (rhSp1; Promega) was added to the DEAE-Sepharose salt fraction, and transcriptional activity was measured in the in vitro transcription system. As shown in Fig. 4, rhSp1 was able to restore basal transcription and Tat-mediated *trans* activation of the HIV-1 promoter in the salt fraction to the same extent as the flowthrough from the DEAE-Sepharose column (compare lanes 5 and 6 with lanes 7 and 8). Transcription directed by the AdML promoter was unaltered (Fig. 4, lanes 7 and 8) or slightly diminished (not shown), which could be accounted for by promoter selection by Sp1, a phenomenon that has been reported previously (14). The rhSp1 preparation used was shown to be greater than 90% pure on silver-stained gels (data not shown).

To investigate whether the binding of Sp1 to the GST-Tat affinity column was due to a direct interaction between Sp1 and Tat, 200 ng of rhSp1 was mixed with GST-Tat (Fig. 5A; see also reference 50) or GST alone in buffer D (11) containing bovine serum albumin at 1 mg/ml and Nonidet P-40 at 0.05% (binding solution) and incubated on ice for 1 h. Glutathione-agarose (10 μl of a 50% suspension) was added to each reaction mixture for a further 1 h to retrieve the GST fusion proteins. The suspension was washed three times with 1 ml of cold binding solution. GST proteins were specifically eluted with 10 mM glutathione, and the final supernatant was analyzed for the presence of Sp1. As shown in Fig. 5B, GST-Tat, but not GST, was able to interact specifically with Sp1. GST-Tat did not interact in vitro with other regulatory factors (such as the transcription factor p53 or the splicing factor PRP18) (data not shown).

Previous data based on mutations of the Sp1-DNA binding sites have implicated Sp1 protein in the regulation of HIV-1 gene expression. However, these data could not rule out the presence of other *trans*-acting factors capable of binding the Sp1-DNA binding sites. For example, it has been reported that thyroid hormone (T3) receptor binds directly to a region contained within the viral Sp1-DNA motifs (10). HIV-1 Tat converts a functionally inactive T3 receptor-binding sequence in the Sp1 element to an active T3 response element (10). In this study, we have taken advantage of a HeLa cell-free system to demonstrate that Sp1 protein is required for both HIV-1 basal and Tat-activated transcription. The studies presented here and elsewhere (4, 18, 31, 33, 37, 50) showed that transcription and *trans* activation by Tat of the HIV-1 LTR could be studied in vitro with HeLa nuclear extracts. However, we cannot distinguish between initiation and elongation effects of Tat in the experiments described in this report.

We previously showed that *trans* activation by Tat was suppressed in HeLa cell nuclear extracts that had been passed through GST-Tat affinity columns (50). This effect was not seen when a nonfunctional mutant K41A (51) Tat column was used. Sp1 protein was shown to be retained in both columns, and Sp1 was not able to complement Tat-dependent *trans* activation in these GST-Tat-depleted extracts (50, 50a). These data suggest the presence of cofactors that interact specifically with the activation domain of Tat. Although Sp1 is required for Tat-activated transcription (this report), this could be a consequence of an earlier effect of Sp1 on HIV-1 basal transcription. The reconstitution of Tat-mediated *trans* activation when the Sp1-binding sites are replaced with other enhancer DNA-binding sequences (1, 3, 6, 36, 49) is in agreement with this hypothesis. The interaction between Sp1 and Tat detected in vitro (reference 26 and this report), however, might suggest a separate and direct role of Sp1 in the *trans* activation by Tat.

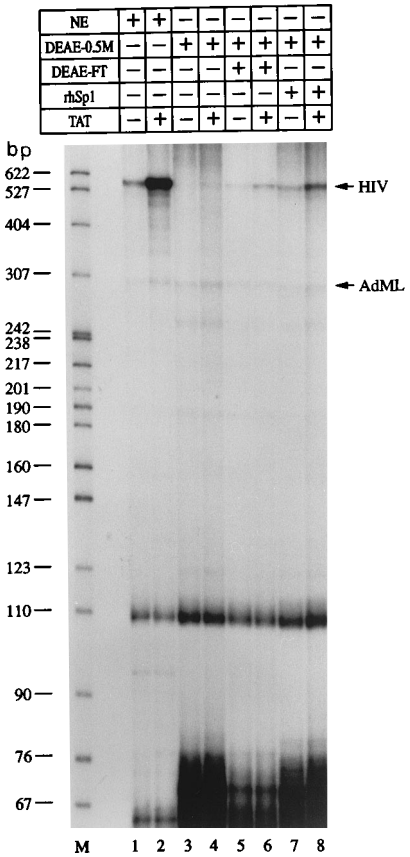

FIG. 4. rhSp1 reconstitutes HIV-1 transcription. Lanes 1 to 6 are as in Fig. 2B. In lanes 7 and 8, 6 μl of DEAE-Sepharose 0.5 M KCl step (DEAE-0.5 M) plus 1 μl of rhSp1 (50 ng/μl) was added to the transcription reactions in the absence (−) or presence (+) of 50 ng of Tat protein. Positions of molecular size markers (lane M) are shown at the left. DEAE-FT, DEAE-Sepharose flowthrough.

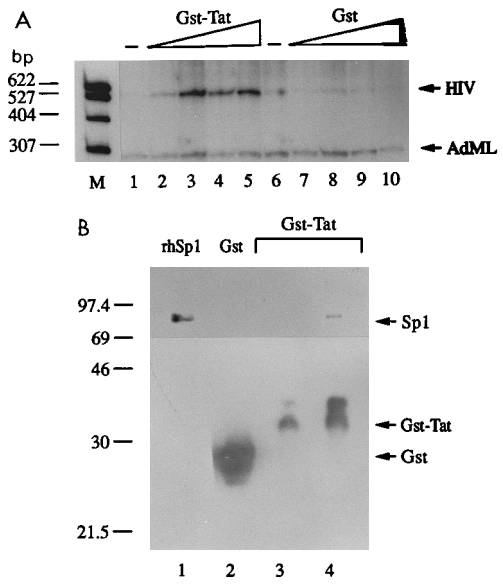

FIG. 5. (A) Biological activity of the recombinant GST-Tat protein used in this study. Transcription reactions were carried out as described previously (50) in the absence (lanes 1 and 6) or presence of 50, 100, 250, and 500 ng of GST-Tat (lanes 2 to 5) or GST alone (lanes 7 to 10). Runoff transcripts resolved on a 6% polyacrylamide gel are marked by arrows. Positions of the molecular size markers (lane M) are also indicated. (B) Direct binding between Tat and Sp1. Fifty nanograms of rhSp1 (lane 1) and the supernatants from the incubation of Sp1 with 5 μg of GST (lane 2) and with 2.5 μg (lane 3) or 5 μg (lane 4) of GST-Tat was analyzed by using Sp1-specific antibodies. As an internal control, GST proteins in these lanes were detected with a specific antiserum. The band above the GST-Tat position is a cross-reacting bacterial product. The positions of the proteins (Sp1, GST, and GST-Tat) and the molecular masses (in kilodaltons) of the molecular weight markers are indicated on the right and left, respectively.

Perkins and coworkers have demonstrated an interaction between Sp1 and NF-κB that may be important in the regulation of the transcription of HIV-1 genes (41). Sp1 may be also involved in the recruitment of Tat to the promoter or in the stabilization of a preformed Tat-cofactors complex. Purification of the putative Tat cofactors so far identified (23, 48, 50, 54) should provide a better understanding of the Tat-mediated *trans* activation as well as the role of Sp1 in this process.

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