Human immunodeficiency virus-1 Nef protein interacts with Tat and enhances HIV-1 gene expression

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Abstract The human immunodeficiency virus (HIV-1) Nef protein is now regarded as a regulatory protein responsible not only for establishment of infection and increased pathogenesis but also for enhancement of viral replication. However, the mechanism of Nef-induced activation of viral replication remains to be clearly understood. Using transient transfection assay, co-immunoprecipitation and pull-down analysis, we demonstrate in this report that the HIV-1 Nef protein physically interacts with Tat, the principal transactivating protein of HIV-1. Our observations with single cycle replication experiments further indicate that this interaction results not only in enhancement of Tat-induced HIV-1 long terminal repeat-mediated gene expression but also in virus production.

Key words: Human immunodeficiency virus; Nef; Tat; Replication; Expression; Virus production

1. Introduction

The Nef protein of the human immunodeficiency virus (HIV) is a 25–30 kDa cytoplasmic phosphoprotein expressed abundantly at all stages of HIV-1 infection and is known to be a crucial element in determining viral pathogenicity [1–3]. The contribution of Nef protein to the induction of acquired immunodeficiency syndrome (AIDS) can be explained by at least three properties that have been ascribed to Nef, namely: (i) down-modulation of cell surface proteins such as CD4 and MHC I, (ii) enhancement of viral infectivity and (iii) induction of T cell activation pathways [4]. The identification of cellular proteins interacting with Nef both in vitro and in vivo has strengthened the concept that Nef acts as a modulator of intracellular activation pathways [4,5].

Upon infection of susceptible cells and integration into the host genome, transcription of the HIV-1 genes is dependent on the concerted action of the cellular transcription machinery and of the viral transactivating protein, Tat [6]. Tat is an 86–104 residue protein essential for HIV-1 replication as it acts as a potent activator of viral gene expression. Tat binds to a bulge sequence within the transactivating region, a highly structured RNA element located at the 5′ ends of all viral transcripts, and acts by a dual mechanism. It increases both the rate of initiation and transcriptional elongation by augmenting the activity of RNA polymerase II [7]. HIV-1 Tat stimulates the growth of some cell types and induces apoptosis in others in addition to inducing the expression of various cytokines [8,9].

The role of Nef in the viral replication process has been a subject of debate over the years. In early studies, Nef was reported to have a negative effect on viral replication in T cell lines [10] but it was later shown to have a positive effect in peripheral blood mononuclear cells [11]. Ahmad and Venkatesan [12] reported a transcriptional suppressor activity for Nef, however, two other groups could not corroborate this negative effect [13,14] and at least one group has shown transcriptional enhancing activity for Nef in macrophages [15]. Some recent reports have shown clear evidence of Nef being an enhancer of viral replication in many cell types [16–18], though the molecular mechanism of this effect has not been elucidated. Studies to date have tried to characterize long terminal repeat (LTR)-mediated gene expression with respect to either Tat or Nef individually but not in the presence of both, which we believe is very important for HIV-1 LTR-mediated gene expression as both are early proteins. The current study was therefore undertaken to understand the role of Nef in viral gene expression, in the presence of Tat, and also to determine if these two proteins interact and thereby influence HIV replication and pathogenesis.

2. Materials and methods

2.1. Expression vectors, cell lines and antibodies

Tat expression vector was constructed by cloning the complete *tat* open reading frame from pCV1, a HXB3 *tat* and *rev* coding vector [19], in pCDNA 3.1 (Invitrogen, USA). The Nef expression vector was constructed by polymerase chain reaction amplification of the Nef fragment from the HXB3 proviral DNA [20] and cloning into pCDNA 3.1. Nl4.3 Nef cloned in pCDNA was a kind gift of Dr. M. Federico. A frameshift mutant of Nef at amino acid 33 due to insertion of an *Xho*I site was cloned in pCDNA3.1 from the molecular clone [21]. pCDNA-CAT reporter vector containing the chloramphenicol acetyltransferase (CAT) gene under the cytomegalovirus (CMV) promoter was used as a control. pEGFPN-Tat and pDsRed-Nef were constructed by sub-cloning the *tat* and *nef* gene fragments as N-terminal fusion to green fluorescent protein (GFP) and red fluorescent protein (RFP) in pEGFPN1 and pDsRed1N1 respectively (Clontech, USA). All the constructs were confirmed by nucleotide sequencing and protein expression. Jurkat and CEM cells ($CD4^+$ human T cell lines), U87MG cells (human astroglial cell line) and 293T cells (human embryonic kidney) were obtained from the NCCS cell repository,

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India. H9/HTLV-IIIB [22] and Jurkat 1G5 cells [23] were obtained from the NIH AIDS repository. Polyclonal antibody to Tat was obtained from the NIH AIDS repository [7] and Autogen Bioclear, UK. Monoclonal Tat antibodies were a kind gift of Dr. V. Ovod (Finland). Polyclonal Nef antibody was obtained from the NIH AIDS repository [24].

2.2. Transfection, viral infection and quantitation

Transient transfections in Jurkat and U87MG cells with Tat and/or Nef expression vectors along with the reporter vector pU3R3 [25] were performed using Lipofectamine or Lipofectamine 2000 reagent respectively (Gibco BRL, USA). Transfection efficiency was normalized by co-transfecting CMV β-gal plasmid (Clontech, USA), and measuring the β-gal activity prior to CAT estimation as previously described [26].

293T cells were transfected with Tat and Nef expression vectors using calcium phosphate precipitation and harvested 48 h post transfection followed by immunoprecipitation and immunoblotting [27]. Single cycle replication studies were done in 293T cells, which were transfected using Lipofectamine 2000 with the molecular clones of wild type NL4.3 or a Nef-deleted clone of NL4.3 [28]. The transfection efficiency was monitored by co-transfection of GFP-expressing vector pEGFPN1. The cells were harvested 36 h post transfection for the CAT assay. The culture supernatants collected at the same time were used to determine virus production by $p24^{gag}$ antigen capture enzyme-linked immunosorbent assay (ELISA; NEN, USA). CEM cells were infected with HIV-1 NL4.3 virus using 100 ng of p24 units per 2×10^6 cells as described previously [29].

2.3. Protein analysis

The Tat and Nef proteins were generated by coupled in vitro transcription and translation as per the manufacturer's instructions (Promega, USA). The [^{35}S]methionine-labeled Tat and Nef proteins were incubated together in binding buffer (20 mM HEPES pH 7.4, 1 mM dithiothreitol, 2 mM $MgCl_2$, 100 μg/ml bovine serum albumin) at 4°C and immunoprecipitated using polyclonal antibodies against either Tat or Nef [7,24]. Protein A-Sepharose (Amersham, USA)-immobilized complexes were washed extensively and resuspended in Laemmli's sample buffer followed by resolution on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was prepared for fluorography as described elsewhere [30] and then exposed to X-ray film.

pGEX-Tat-transformed BL21DE3 *Escherichia coli* cells [31] were induced with isopropyl-β-D-thiogalactose to produce GST-Tat protein, which was then purified using glutathione-Sepharose beads (Amersham, USA). CEM cells (5×10^6) were harvested 9 days post infection and lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris–HCl pH 7.2, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS and 1% sodium deoxycholate) with protease inhibitor cocktail (Roche, Germany). The clarified supernatant ($10\,000\times g$) was incubated with either glutathione *S*-transferase (GST) or GST-Tat immobilized on glutathione-Sepharose beads at 4°C and subjected to five washes with RIPA buffer. The complexes were resuspended in Laemmli's sample buffer, boiled, and resolved on 15% SDS–PAGE.

H9-IIIB cells were activated with phorbol 12-myristate 13-acetate(PMA) for 72 h prior to immunoprecipitation. Cells were lysed in Dignam buffer (20 mM HEPES pH 7.4, 25% glycerol, 0.42 M NaCl, 1.5 M $MgCl_2$, 0.2M EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) on ice for 30 min. Clarified lysates were incubated with polyclonal antibody to the Tat protein (Autogen). The antigen–antibody conjugate was pulled down by binding with a mixture of protein A and protein G agarose beads followed by resolution on 15% SDS–PAGE. The blots were probed with antibody to the Nef protein [24] and developed using the enhanced chemiluminescence (ECL Plus) system (Amersham, USA).

2.4. Confocal microscopy

293T cells grown on coverslips were transfected using either pEGFPN1-Tat and/or pDsRed1N1-Nef by the calcium phosphate method. Cells were harvested 24 h post transfection and fixed in 3.7% paraformaldehyde prior to viewing with an Ultraview confocal imaging system (Perkin-Elmer) using an Olympus X70 microscope.

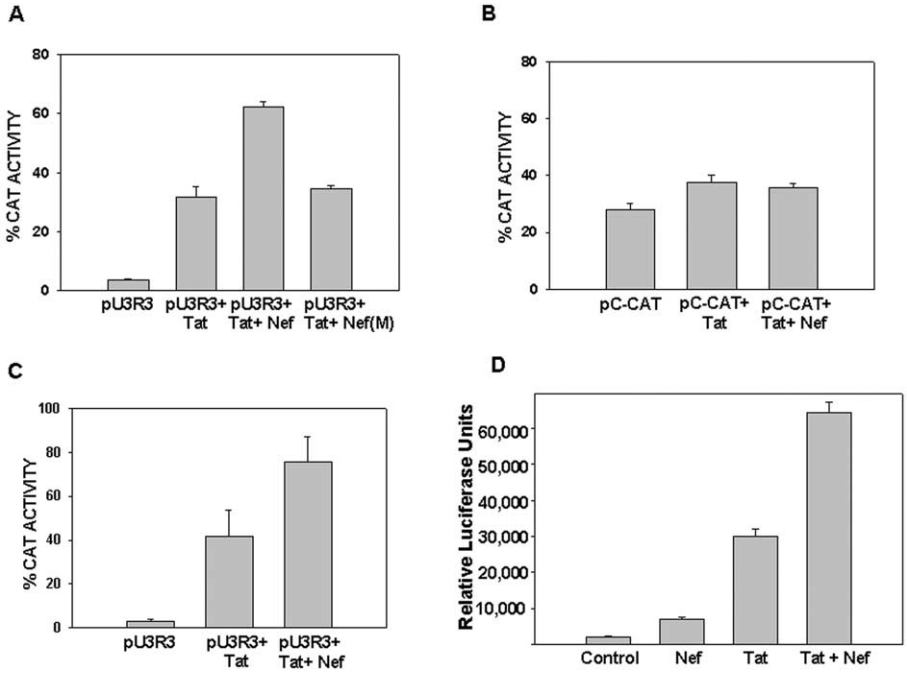

Fig. 1. Enhancement of Tat-mediated transactivation by the Nef protein from the HIV-1 LTR in Jurkat, U87MG and Jurkat 1G5 cells. A: Jurkat T cells were transfected with the vectors pU3R3 (1 μg), pCDNA-Tat (0.3 μg) and pCDNA-Nef (1 μg) or pCDNA-Nef (M) mutant (1 μg) as indicated below each bar and as described in detail in the text. CAT activity was assayed 36 h post transfection, after normalization of β-gal activity. B: Jurkat T cells were transfected with the vectors pCDNA-CAT (1 μg), pCDNA-Tat (0.3 μg) and pCDNA-Nef (1 μg) as indicated. CAT activity was assayed 36 h post transfection, after normalization of β-gal activity. C: U87MG cells were transfected with the vectors pU3R3 (1 μg), pCDNA-Tat (0.1 μg) and pCDNA-Nef (1μg) as indicated. CAT activity was assayed 36 h post transfection after normalization of β-gal activity. D: Jurkat 1G5 cells were transfected with vectors pCDNA-Tat (0.5 μg) or pCDNA-Nef (2 μg) or both as indicated. Luciferase assays of the lysates were performed after 24 h following β-gal normalization as described in the text. The histograms show the means of three independent experiments with error bars representing S.E.M.

3. Results

3.1. Nef enhances Tat-mediated transactivation from the HIV-1 LTR

There have been conflicting reports about the role of the Nef protein in transactivation of HIV-1 LTR-driven gene expression. To reconcile the divergent observations, we revisited the ability of Nef to transactivate the HIV-1 LTR in the presence of Tat. Co-transfection of Jurkat cells with equal amounts of Tat and Nef expression vectors showed a definite increase in Tat-mediated transactivation as compared to Tat or Nef alone (data not shown). In an attempt to delineate the Nef-mediated transactivation in the presence of Tat, we reduced Tat to sub-optimal levels in later transfections. As shown in Fig. 1A, at least a two-fold increase in CAT activity was repeatedly observed in the presence of Nef and Tat, as compared to Jurkat cells transfected with Tat alone. Nef from different HIV-1 isolates, i.e. SF2, NL4.3, NA7, and subtype C 93IN904 on co-transfection with Tat showed a similar enhancement of transactivation (data not shown). However, this Nef-mediated enhancement of transactivation was not observed in the presence of mutated Nef (Fig. 1A). A control experiment with the CMV promoter did not show any effect of Nef, although Tat seemed to enhance the gene expression to some extent (Fig. 1B) as reported in the literature [32]. In order to test whether the same effect is observed in cells of non-lymphoid origin, the microglial cell line U87MG was used to study the role of Nef in Tat-induced transactivation. Similar results were observed in U87MG cells where again a consistent two-fold increase in transactivation was seen upon co-transfection of Nef with Tat (Fig. 1C) as compared to Tat alone. To substantiate this effect, we further studied this phenomenon in 1G5 cells, which contain a stably integrated luciferase reporter gene under the control of the HIV-1 LTR promoter. Our data with co-transfection of Nef and Tat plasmids in 1G5 cells show a similar two-fold increase in luciferase activity as compared to Tat alone (Fig. 1D). As the two cell lines, Jurkat and U87MG, differ greatly in their origin, there is a possibility that this observed transactivation is important for gene expression from the HIV-1 LTR. This led us to explore the possibility of an interaction between the two proteins, Tat and Nef.

3.2. The Nef protein interacts with HIV-1 Tat in vitro and in vivo

Coupled in vitro transcription and translation provides a powerful and easy tool to study proteins in a pure state. Therefore, [^{35}S]Met-labeled Tat and Nef proteins were incubated together and immunoprecipitation carried out using either anti-Nef or anti-Tat antibodies (Fig. 2B). To rule out the possibility of a non-specific interaction between two proteins, labeled CAT protein was used as a control. Fig. 2A clearly shows the absence of any non-specific interaction of Tat or Nef with CAT protein (lanes 2–3) and also demonstrates that the Tat and Nef antibodies do not cross-react with each other (lanes 4–5). The results obtained from Tat–Nef interaction clearly showed that Tat co-immunoprecipitated with the Nef protein and vice versa (Fig. 2B, lanes 3 and 5). Thus the data indicate that the Nef protein directly interacts with Tat in vitro. The slower-migrating band of Nef with a molecular mass of ~27 kDa seems to be the full-length Nef protein whereas the faster-migrating ~25 kDa band corresponds to

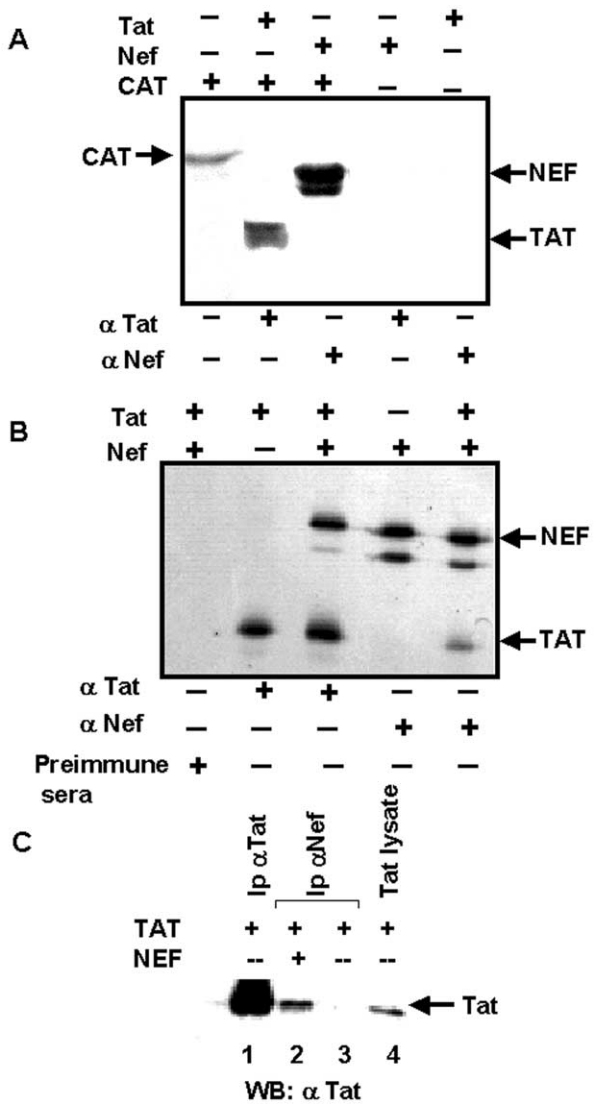

Fig. 2. Interaction of Tat and Nef proteins in vitro and in vivo. A: [^{35}S]Met-labeled Tat and Nef proteins were synthesized using in vitro coupled transcription and translation as described in the text. The two proteins were incubated with CAT protein, followed by immunoprecipitation with Tat or Nef antisera as a control experiment. The antibodies were also tested for cross-reactivity with each other. The proteins were resolved on 15% SDS–PAGE and autoradiographed as detailed in the text. B: The labeled Tat and Nef proteins were incubated with each other followed by immunoprecipitation with Tat or Nef antisera as shown. The immunoprecipitated complexes were analyzed on SDS–PAGE as described in A. C: 293T cells were transfected with Tat expression vector or both Tat and Nef as described in the text. After 36 h, the cells were lysed, immunoprecipitated with anti-Nef antibody followed by immunoblotting with anti-Tat antibody as indicated.

the Nef protein synthesized as a result of translation initiation from an internal methionine start site [1]. Our data further indicate that the Nef protein which associates with the Tat protein could be the full-length Nef species, since the lower molecular mass band was almost absent when immunoprecipitation was done with anti-Tat antibody (Fig. 2B, lane 3). In contrast, when anti-Nef antibody was used to immunoprecipitate, both the Nef bands were pulled down along with Tat, as the antibody recognizes both forms of Nef (Fig. 2B, lane 5).

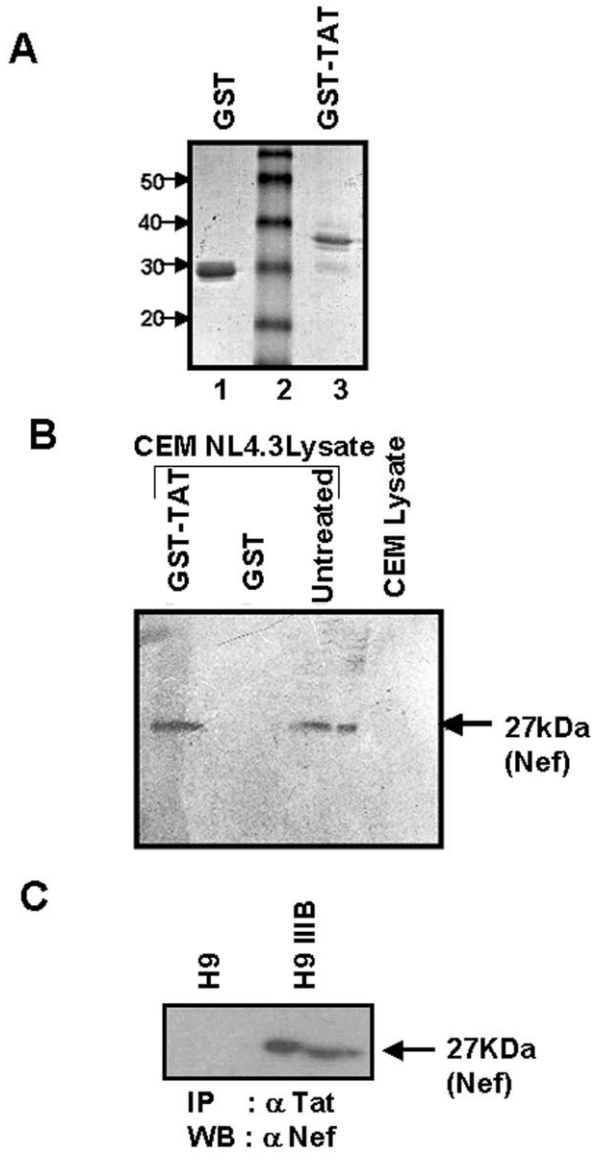

Fig. 3. Interaction of Tat and Nef: pull-down analysis using GST-Tat and co-immunoprecipitation in infected cells. A: Coomassie-stained SDS–polyacrylamide gel showing relative mobility of GST and GST-Tat proteins. Protein markers are indicated on the left (in kDa). B: GST-Tat (lane 1) or GST (lane 2) beads were incubated with infected CEM cell lysate followed by SDS–PAGE and immunoblotting with Nef antiserum as mentioned in the text. The NL4.3-infected (lane 3) and uninfected CEM (lane 4) cell lysates were run in parallel and probed with Nef antiserum as controls. The arrow indicates the position of the Nef protein. C: PMA-activated H9-IIIB cells were lysed and subjected to immunoprecipitation using polyclonal antibody to the Tat protein, followed by immunoblotting with antibody to Nef. Lanes 1 and 2 depict the control H9 cell lysate and the infected cell lysate respectively.

The binding of Tat and Nef proteins in vitro could not be ruled out due to localized concentrations of the two proteins. Hence transfections in 293T cells were performed with either expression plasmid or a combination of both. Fig. 2C shows that the Tat protein co-immunoprecipitates with the Nef protein. This is significant as both these proteins are known to interact with a number of cellular factors involved in transcription and cellular signaling and Nef has been hypothesized to act as an adapter molecule.

3.3. GST-Tat pull-down analysis and co-immunoprecipitation using HIV-1-infected cells

The Tat and Nef proteins are reported to be early HIV proteins synthesized in an infected cell and thus provide an established system to show Tat and Nef association. We utilized a precipitation assay wherein cell lysates of HIV-1-infected CEM cells were incubated with GST-Tat beads (Fig. 3A) for pull-down analysis. The results (Fig. 3B, lane 1) depict that the Nef protein is specifically pulled down from infected CEM cell lysates by GST-Tat beads. As expected, the empty GST-agarose beads did not precipitate Nef (Fig. 3B, lane 2). Lanes 3 and 4 show the presence of the Nef protein in the infected cell lysate and not in the uninfected cells. These data provide clear evidence of an interaction between the Tat and Nef proteins in the context of HIV infection.

Phorbol esters such as PMA stimulate the transcription and translation machinery of T cells and thus also enhance transcription from the HIV-1 LTR [17]. H9-IIIB, a chronically infected T cell line, was stimulated with PMA for 72 h prior to harvesting. Lysates were subjected to immunoprecipitation with antibody to Tat, followed by immunoblotting with polyclonal antibody against the Nef protein. Fig. 3C shows the presence of the Nef protein in only the chronically infected cell lysates and not in H9 cell lysate. Thus the viral proteins Tat and Nef do exist as a complex even in infected cells, thereby confirming our in vitro data of Tat and Nef physically interacting with each other.

3.4. Co-localization of Nef and Tat proteins in transfected cells

Nef has been shown to activate T cells or inhibit their activation depending upon its subcellular location [33] and it would seem pertinent to analyze the localization of Nef in relation to Tat. Tat, although regarded primarily as a nuclear protein, has been reported to interact with an array of cellular proteins and subvert signaling pathways. Nef has been shown

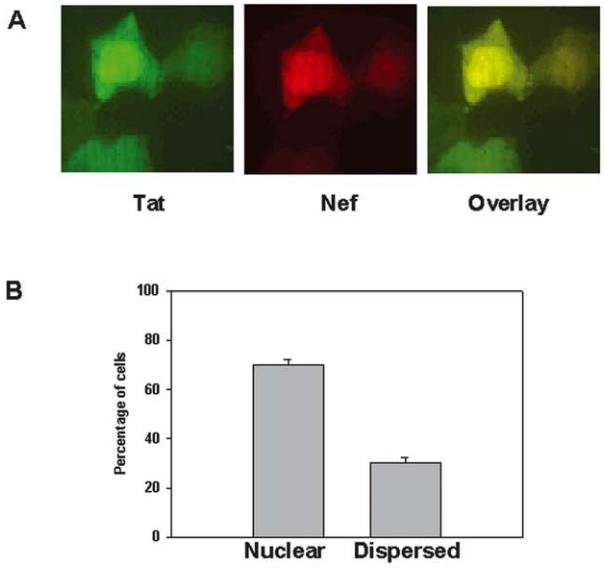

Fig. 4. Localization of Tat and Nef in co-transfected 293T cells. A: 293T cells were grown on coverslips and transfected with pEGFPN1-Tat (2 μg) and pDsRed1N1-Nef (2 μg). The transfected cells were visualized after 24 h by confocal microscopy as detailed in the text. B: Graph depicts cells positive for expression of both Tat and Nef proteins localized in the nucleus or having a dispersed localization.

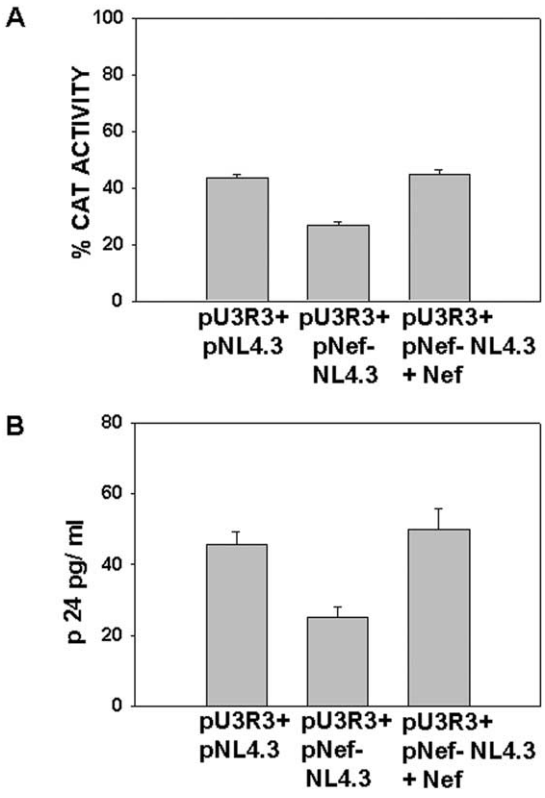

Fig. 5. Role of Nef on transactivation and virus production in 293T cells. A: 293T cells were transfected with pU3R3 reporter vector along with viral clones (100 ng) and Nef expression vector (1 μg) as indicated. CAT activity was determined 36 h post transfection as described in the text. B: Determination of virus production in culture supernatants of transfected 293T cells described in A, using HIV-1 p24 antigen capture ELISA. The histograms represent means of three independent experiments with error bars indicative of S.E.M.

as a predominantly cytoplasmic protein in transfected cells; however, it has also been shown to be present in the nucleus of HIV-1-infected and transfected cells [34–36]. Expression of GFP-Tat and RFP-Nef was utilized to study the cellular localization of these proteins in context with each other. Expression of these fusion proteins independently of each other showed a very dispersed localization within the cells. However, there was a marked difference in the localization pattern when these fusion proteins were co-expressed in 293T cells as shown in Fig. 4A. At least 70% of cells expressing both proteins showed a marked nuclear localization and the remaining 30% of cells showed a dispersed localization pattern (Fig. 4B). This observation is of significance as our earlier data clearly show that Nef physically interacts with Tat, which is involved in transcriptional transactivation in the nucleus. Nuclear co-localization of these two proteins in a majority of cells could be due to the interaction and their role in enhancement in gene expression as indicated by our transactivation experiments.

3.5. Role of Nef in LTR-mediated gene expression and virus production in vivo

To determine the role of this observed interaction between Tat and Nef in vivo, we designed an assay to measure the levels of transactivation from the HIV-1 LTR and virus production in a single cycle replication experiment with 293T cells which are $CD4^-$ and are thus refractory to infection. Molecular clones of HIV-1 were used to determine the levels of transactivation using the CAT assay and p24 levels were used to determine virus production after normalizing the transfection using GFP expression. The Nef-deleted clone shows lower levels of transactivation as compared to the intact NL4.3 clone (Fig. 5A), which are restored to wild-type levels by co-transfecting cells with the Nef expression construct, thereby indicating the importance of Nef in viral gene expression. The p24 values also show a similar pattern with an increase in virus production in cells co-transfected with the Nef^- NL4.3 and pCDNA3.1-Nef, as compared to the Nef-deleted viral clone (Fig. 5B). These data clearly indicate that expression of the Nef protein even in *trans* can confer an increased level of gene expression as well as virus production. Similar results were also obtained using pR7-GFP [37], a Nef-deleted molecular clone, which indicates that it is not an allele-specific phenomenon and is an integral part of the viral infection strategy (data not shown). This experiment also points towards a direct correlation between activation of viral gene expression and viral replication.

4. Discussion

Although Nef has been shown to enhance the infectivity and replication of HIV-1, the molecular basis of this enhancement is not clearly elucidated. Specifically, the role of Nef in HIV-1 gene expression as measured by its effect on the HIV-1 promoter LTR has been a subject of controversy. Nef was earlier characterized with a capacity to silence the LTR, followed by neutral and later with positive effects on LTR-based transcription [10–15]. Therefore the effects of Nef on the LTR are diverse, which could be due to differences in the cell lines used, the strains of Nef used and the cellular localization of the Nef protein. Some recent studies have clearly shown that Nef induces Tat-mediated HIV-1 LTR-driven gene expression [17,33,38] which the authors attribute to activation of T cells and activation of some transcription factors. The data presented in the current study reflect the importance of Nef protein in the viral transcription along with Tat protein. Our observations of enhanced transactivation of Tat-induced HIV-1 LTR-mediated gene expression in the presence of Nef corroborate the recent reports mentioned above [17,33,38]. Our experiments for the first time show that the two regulatory proteins, Tat and Nef, interact amongst themselves and our data also indicate that this enhancement of transactivation is probably mediated by a functional interaction between these two important regulatory proteins. The co-localization of Nef and Tat in the nucleus as visualized using confocal microscopy points toward a possible role of Nef in the Tat transcription complex and confirms earlier observations of Nef localization in the nucleus of transfected and infected cells [34–36]. The present study also suggests that interaction between these two proteins exists in HIV-1-infected cells which leads us to postulate that there lies an important regulatory platform engineered by these two proteins, which determines optimal viral infection.

Finally, our data show a direct correlation between LTR-mediated gene expression and virus production and thus provides a molecular basis for the Nef-induced enhancement of viral replication as shown directly by a number of studies in a

variety of cell types [39,40]. Our single cycle replication data match perfectly time course replication data observed in Jurkat cells using different Nef mutants [18]. Finally, the data presented here clearly indicate that Nef may enhance viral replication by binding to Tat in addition to the activation of T cell function reported earlier. The interaction between these two proteins lays open the entire cell machinery whether it be activation, signal transduction, transcription or effector function to manipulation, thereby leading the cell to whichever fate the infection status deems fit.

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