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EXPRESSION OF A NOVEL NUCLEAR PROTEIN IN ACTIVATED AND IN *tat*-I EXPRESSING T CELLS[1]

ANNA BIELINSKA,* LESLIE BAIER,* NABIL HAILAT,† JOHN R. STRAHLER,† GARY J. NABEL,[2]* AND SAM HANASH†

*From the *Howard Hughes Medical Institute, Departments of Internal Medicine and Biological Chemistry, and †Department of Pediatrics and Communicable Diseases, University of Michigan Medical Center, Ann Arbor, MI 48109*

The intracellular events that occur in T lymphoid cells after activation or after infection with HIV-1 are not well defined. In the case of HIV-1 infection, it is unknown whether the *tat*-I gene, an essential gene for viral replication, affects host cell nuclear factors. Using two-dimensional PAGE, we have identified a novel nuclear protein, designated nuclear protein-28,000 (NP-28), which is induced in Jurkat T cells by stimulation with PMA and/or PHA or ionomycin. This nuclear protein has an apparent molecular mass of 28,000 Da and an isoelectric point of 4.6. Interestingly, Jurkat cells transfected with *tat*-I express higher levels of NP-28 constitutively, without added stimulation. Incubation of Jurkat cells expressing *tat*-I with PMA and/or PHA or ionomycin causes superinduction of NP-28. We have therefore identified a novel lymphoid nuclear protein induced by T cell activation that occurs in *tat*-I expressing cells in the absence of activating agents.

Whereas lymphoid cell activation can be induced with extracellular stimulation by a variety of agents, the intracellular events associated with lymphoid cell activation and proliferation are still not well defined. Several approaches are available for the identification of genes or gene products associated with lymphoid activation and proliferation. One approach that we have found useful is the direct analysis of polypeptide constituents of lymphoid cells using 2-D[3] PAGE. This approach allows the identification of changes that could occur in a large number of relatively abundant proteins as a result of gene activation or as a result of post-translational modification such as phosphorylation. Because the extent to which HIV alters host gene expression has not been well defined, we have also been interested in identifying protein changes in lymphoid cells that express a particular gene of HIV.

Primate viruses encode diverse transactivator proteins that alter host cell gene expression and promote viral replication. These transactivators control cellular gene expression by various mechanisms, including modification of host cell transcription factors, specific binding to *cis*-acting regulatory sequences, or interaction with host cell regulatory proteins. Although the *tat*-I gene is essential for HIV replication, its action is not completely understood. This protein is found in the nucleus (1–5), and exerts its effects on a target sequence within the HIV genome located near the transcriptional initiation site (6–8). Although several DNA binding proteins bind to this region, they do not contribute to activation by *tat*-I (8, 9), and a putative stem-loop structure at the 5′ end of the viral transcript has been implicated as a likely target (7, 10–13). Whereas *tat*-I likely regulates viral mRNA, it is controversial whether it acts directly on this target region, or whether it regulates transcriptional elongation (9, 14–16), transcriptional initiation (6, 10, 11, 16–22), or other processes (7, 17, 23–25). Regardless of the mechanism, there is increasing evidence to suggest that cellular proteins either participate in *tat*-I activation or may be affected by *tat*-I (25–27). We report in this study the identification of a cellular protein enriched in nuclear extracts that is selectively activated in cells stably expressing the *tat*-I gene and is also induced with other proteins after transient transfection. In addition, this factor is expressed, along with other proteins, in Jurkat cells after stimulation by activating agents.

MATERIALS AND METHODS

Cell culture. Cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin (50 U/ml), and streptomycin (50 μg/ml) with freshly added glutamine in a humidified incubator containing 5% CO_2.

Cell lines and vectors. The Jurkat T lymphoma cell stably expressing *tat*-I gene (28) was obtained by infection of an amphotropic replication-defective retroviral vector pZIPNeoSV(x)I (29) containing the *tat*-I gene under regulation of the Moloney murine leukemia long terminal repeat (28, 30). This stable transfectant was used in the experiments presented in Table I and Figures 1, 2, and 3. The following additional plasmids were used to generate independent Jurkat transfectants. Plasmids expressing *tat*-I or *tat*-II linked to a histidinol selectable marker (kindly provided by P. Robbins and R. Mulligan) were designated pHisBgl II-*tat*-I or pHisBgl II-*tat*-II. These plasmids were electroporated into Jurkat T cells to generate stable lines expressing *tat*-I or *tat*-II genes. Stable lines were selected for their ability to grow on l-histidinol. Plasmid pHisBgl II-*tat*-I or *tat*-II contains the *hisD* histidinol resistance gene from *Salmonella typhimurium* (31) downstream of the Moloney long terminal repeat, and the *tat*-I or *tat*-II genes were introduced into the *Bgl*II site downstream of the simian virus 40 enhancer and promoter. Another *tat*-I expressing line was obtained by cotransfection of the pHD101-*tat* (32) and pSV_2Neo (33) plasmids followed by selection using G418 (1.5 mg/ml). Finally, an additional *tat* expression vector was kindly

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[2] Address correspondence and reprint requests to Dr. Gary J. Nabel, Medical Science Research Building-I, Room 4510, Howard Hughes Medical Institute, University of Michigan Medical Center, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0650.
[3] Abbreviations used in this paper: 2-D, two-dimensional; CAT, chloramphenicol acetyl transferase; NP-28, nuclear protein-28,000.

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provided by M. L. Hammarskjold. This plasmid, designated pCMV-*tat*-I, is a pBR 322 derivative containing a 359-bp *Sal*I, *Bam*HI fragment of *tat*-I from pCV-1 (1) under regulation of the CMV immediate early promoter/enhancer (34) and the polyadenylation sequences from rabbit β-globin. Plasmids pHisBgl II *tat*-I, pHD101, and pCMV *tat*-I were used in transient transfections of Jurkat cells and to generate the stable lines used to confirm NP-28 expression described in Figures 2 to 4.

Preparation of nuclear extracts. Nuclear extracts were prepared according to the method of Dignam et al. (35). Activated Jurkat cells were incubated in RPMI 1640 containing 10% FCS for 2 to 24 h before preparation of nuclear extract, which was evaluated initially using the electrophoretic mobility shift assay. For transient transfections, nuclear extracts were prepared from 1 to 2×10^7 cells using a modification of the Dignam procedure (36).

2-D gel electrophoresis analysis. Cell pellets were solubilized by addition of lysis buffer consisting of (per liter) 8 M urea, 20 ml of Nonidet P-40 surfactant, 20 ml of ampholytes (pH 3.5 to 10), 20 ml of 2-ME, and 0.2 mM PMSF in distilled deionized water. 2-D PAGE was performed as described previously (37). In most cases, 25-μl aliquots containing solubilized cells (2.5×10^6) or nuclear extracts were immediately applied onto isofocusing gels. First-dimension gels contained 50 ml of ampholytes per liter (pH 3.5 to 10). Isofocusing was done at 1200 V for 16 h and 1500 V for the last 2 h; 20 gels were run simultaneously. For the second-dimension separation, an acrylamide gradient of 11.4 to 14.0 g/dl was used. Protein spots in gels were visualized by the silver-staining technique of Merril et al. (38). Unsolubilized aliquots were frozen as pellets at −80°C.

Quantitative analysis of NP-28 was conducted as follows. Spot detection and quantitation was performed as described previously (39). Each gel was scanned in a 1024 × 1024 pixel format, giving 160 microns as the pixel width. The integrated intensity of NP-28 and 20 other reference proteins in the gel were measured in units of optical density × mm^2. The reference spots were used to adjust the NP-28 spot-integrated intensity for gel to gel variation due to any difference in gel staining or amount of protein loaded (39).

Phosphoprotein labeling. Jurkat T cells or PBL were analyzed for phosphorylation content by labeling with ^{32}P-orthophosphate. Briefly, cells were preincubated in RPMI phosphate-free medium (GIBCO) for 30 min, and ^{32}P (Amersham) at 480 μCi/2.5×10^6 cells/ml added for 2 h. PMA or PMA/PHA was added to the cells for 10 min, allowing detection of constitutive or inducible phosphoproteins. The ^{32}P incorporation was inhibited by incubating the cells in cold PBS for 10 min. Proteins were analyzed by 2-D PAGE and autoradiography.

CAT assay. Cell extracts were prepared 44 h after transfection, protein concentrations normalized, and conversion of chloramphenicol to its acetylated forms assayed by standard methods (40, 41). Results are representative of at least two independent transfections, and SD for each CAT determination was <10%. Percentage conversions of ^{14}C-chloramphenicol to its acetylated forms are indicated.

Cell transfections. Cells (10^7) were transfected with 10 to 30 μg of plasmid using DEAE-dextran and maintained in RPMI 1640 containing 10% FCS and penicillin-streptomycin for 24 h. Extracts were prepared for 2-D gels according to the methods of Dignam et al. (35).

RESULTS

To identify cellular proteins that are expressed in Jurkat cells expressing *tat*-I, we initially examined cellular protein extracts from Jurkat cells or from sublines that stably expressed *tat*-I. Proteins were resolved using 2D-PAGE (42–44) and visualized by silver staining (39). The protein pattern of solubilized, unfractionated Jurkat cells expressing *tat*-I revealed approximately 1200 polypeptide spots (Fig. 1). There were no detectable qualitative differences between the patterns found in whole cell extracts of Jurkat cells that could be related to *tat*-I expression. In contrast, examination of soluble nuclear proteins revealed patterns with fewer spots, and a polypeptide with a molecular mass of ~28,000 Da and an isoelectric point value of ~4.6 was consistently observed in the *tat*-I lines as a prominent spot but was virtually undetectable in Jurkat cells, which do not contain *tat*-I (Fig. 2, *A* vs *B*). Because of its low level of expression and aberrant migration on SDS-PAGE (7), *tat* protein was not easily detected by 2-D PAGE, and therefore not visible on these gels.

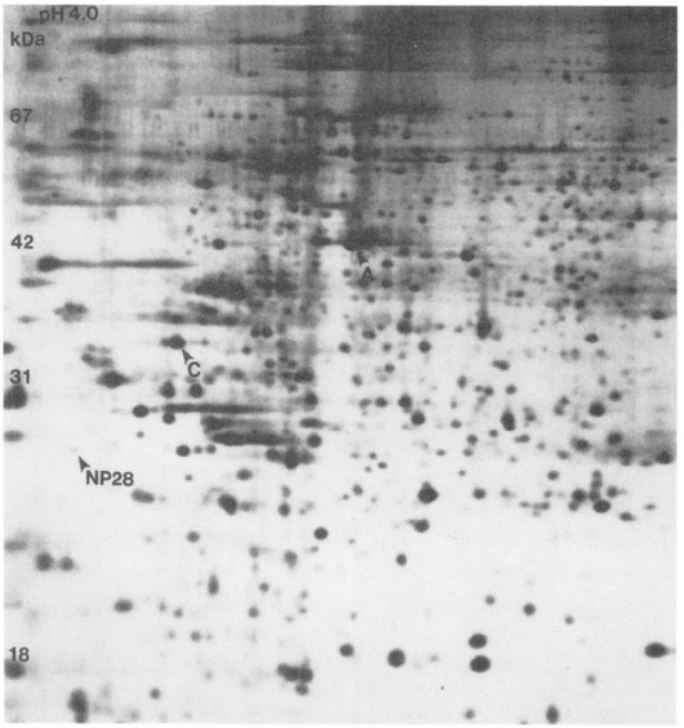

Figure 1. 2-D gel electrophoresis pattern of polypeptides from Jurkat cells expressing *tat*-I. *Arrows* denote location of actin (*A*), proliferating cell nuclear Ag/cyclin (*C*), and NP-28. NP-28 is at the limit of detectability in total cell extracts. Cell extracts were prepared by standard methods (35) and resolved as described previously. The pH is indicated on the *horizontal axis*, and molecular mass on the *vertical axis*.

To determine whether NP-28 could be produced in the Jurkat T leukemic cells in the absence of *tat*-I, cells were incubated in the presence of known T cell activators. Treatment of Jurkat cells with PMA and PHA, or PMA and ionomycin resulted in the detection of a polypeptide spot that corresponds in location to NP-28 (Fig. 3, Table I). This protein appeared after incubation for only 2 h in the presence of these activators. However, the occurrence of NP-28 in Jurkat cells treated with activating agents was associated with changes in other proteins, notably phosphoproteins, detectable in 2-D gels, that we characterized previously. They include a novel 65-kDa nuclear phosphoprotein, designated NP-65, which undergoes phosphorylation after treatment with activating agents (N. Hailat, J. Strahler, R. Melhem, J. Jones, B. C. Richardson, G. J. Nabel, and S. Hanash, manuscript in preparation) (44) and phosphorylation of a major cytosolic polypeptide, designated Op-18, which is up-regulated in proliferating cells (45). When Jurkat cells expressing the *tat*-I gene were incubated with these agents, changes involving the same proteins were observed. In addition, NP-28 became more prominent, with a fourfold increase in relative integrated intensity, suggesting that this protein is superinduced in stimulated Jurkat cells expressing *tat*-I (Fig. 3, Table I).

Analysis of the phosphoprotein pattern of the soluble nuclear protein fraction by γ^{32}P-ATP labeling and autoradiography failed to reveal radiolabel in the position of NP-28 (data not shown), suggesting that the protein observed is not phosphorylated. In other T cell lines, such as EL-4, this protein was observed at low levels, and it was detectable in nuclear extracts from nonlymphoid

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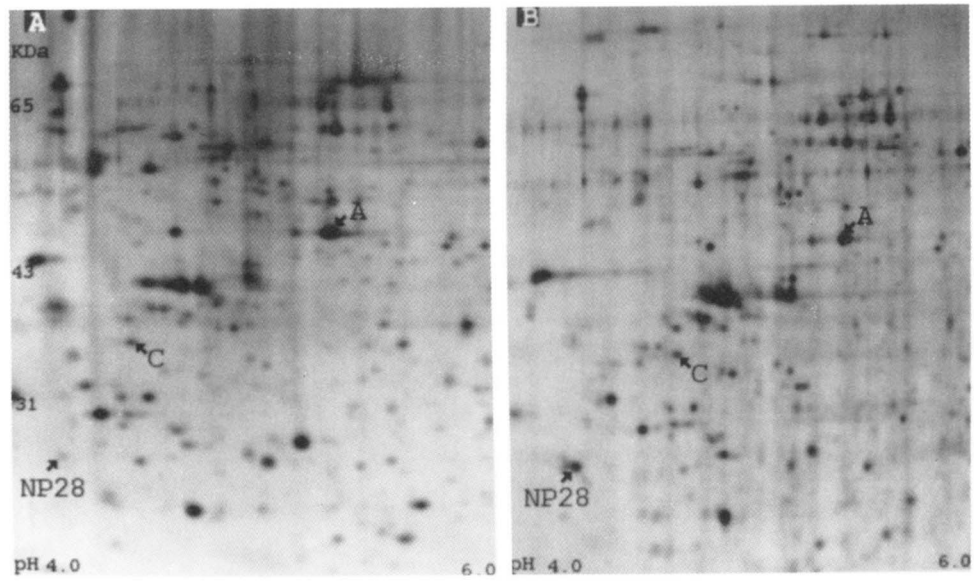

Figure 2. Representative 2-D gel electrophoresis pattern of soluble nuclear polypeptides from Jurkat cells or a derivative that stably expresses *tat*-I. Nuclear extracts were prepared from (*A*) Jurkat cells or (*B*) Jurkat cells stably expressing the *tat*-I gene. *Arrows* indicate the location of NP-28 and the nuclear protein proliferating cell nuclear Ag/cyclin (*C*). Actin, designated by *arrow A*, is present in reduced amounts compared with total cell patterns. Results pertaining to NP-28 induction are representative of at least five independent samples.

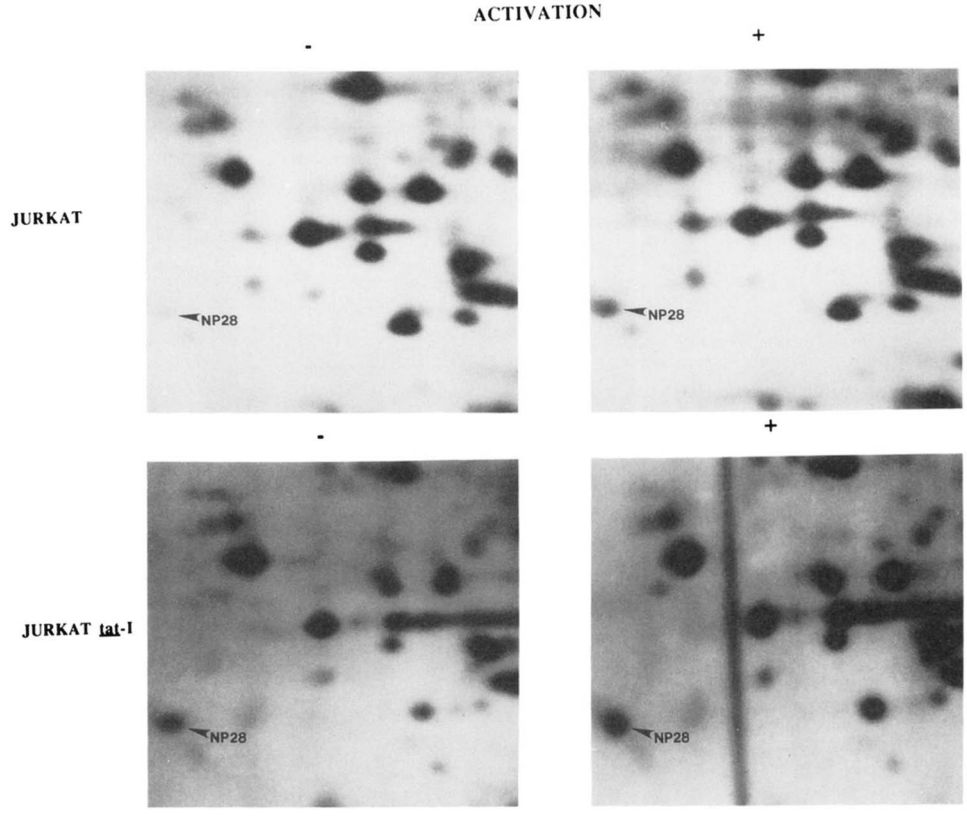

Figure 3. Effect of cellular activation on NP-28 levels in unstimulated and activated Jurkat cells or a derivative that stably expresses *tat*-I. Nuclear extracts were prepared from Jurkat cells (*upper panels*) incubated in medium alone (−) or with PMA (40 nM) and PHA (2 μg/ml) (+) for 4 h. These patterns were compared with nuclear extracts derived from Jurkat cells expressing the *tat*-I gene (*lower panels*) incubated in medium alone (−) or after treatment with PMA (40 nM) and ionomycin (1 μM) (+) for 4 h. No differences were noted in patterns induced by PMA and PHA compared with PMA and ionomycin. *Arrows* denote the location of NP-28.

cells, including the K562 erythroleukemia and the HL-60 myeloid leukemia cell lines.

Jurkat cells stably expressing *tat*-I differed from the parent line in their expression of this gene product and their ability to stimulate HIV-CAT plasmids containing the *tat*-I target region, as demonstrated previously (6, 40). This finding was confirmed in the present study by transfection with 5 μg of HIV-CAT by standard techniques (40). Unstimulated Jurkat cells showed 0.47% conversion of chloramphenicol to acetylated forms, which increased 16-fold after incubation with PMA. Jurkat cells stably expressing *tat*-I showed 19.8% conversion (42-fold higher than Jurkat cells), which increased an additional 11-fold after stimulation. Thus, changes in NP-28 expression correlated with induction of HIV-CAT activity by *tat*-I and mitogens. Despite these differences in activation of the HIV enhancer, these lines were similar in other respects. Both synthesized comparable amounts of IL-2 in response to mitogenic stimulation, as described in other Jurkat lines (45). IL-2 activity was detectable only when cells were incubated with both PMA (40 nM) and PHA (2 μg/ml), synthesizing from 20 to 100 U/ml at cell concentrations of 2×10^5/ml, comparable with previous descriptions of this line (46). These cells also displayed similar cell surface glycoproteins, including CD3, CD4, and WT31, and class I MHC (data not shown). In contrast to one report (47), no significant difference in CD4 expression was observed between Jurkat cells that do or do not

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TABLE I

Integrated intensity of NP-28[a]

tat-I Expression	Activation	
	−	+
−	0.15 (4)[b]	0.9 (4)[c]
−	0.10 (1)	3.33 (1)[d]
+	1.50 (1)	4.67 (1)[c]
+	0.60 (5)	1.03 (5)[d]

[a] The integrated intensity of NP-28 in 2-D gels of untreated or stimulated Jurkat cells and Jurkat cells expressing *tat*-I was determined as described previously (39). The variation in the unstimulated *tat*-I expressing Jurkat cells reflects different amounts of soluble nuclear protein loaded onto the gels. Values below 0.3 usually correspond to spots that are at or below the limit of visual detectability.

[b] Values in parentheses indicate the number of samples averaged.

[c] Cells were incubated for 4 h with PMA (40 nM) and PHA (2 μg/ml).

[d] Cells were incubated for 4 h with PMA (40 nM) and ionomycin (1 μM).

TABLE II

Phosphorylation changes in Jurkat cells transiently transfected with tat-I[a]

Polypeptide Number	Molecular Mass (kDa)	Isoelectric Point
116	65	5.2
126	65	5.7
581	36	4.2
698	33	4.7
723	30	5.5
758	31	6.4
1119	18	5.7
1120	18	5.8

[a] Summary of proteins changed after an introduction of the *tat*-I gene into Jurkat cells by transient transfection. The relevant isoelectric points and molecular masses of altered spots are indicated. These changes were documented in at least two independent experiments.

contain *tat*-I, including four independent stably transfected lines. These findings suggest that the decreased expression reported in the previously published clone (47) may have represented a clonal variant, and this observation is not generalizable to all cells that express *tat*-I. Among these four stable transfectants derived from three different *tat*-I expression plasmids, we also found evidence of NP-28 in nuclear extracts, and NP-28 was also detected in nuclear extracts from a Jurkat transfectant expressing *tat*-II, derived from HIV-2 (data not shown).

When Jurkat cells were transfected transiently with a *tat*-I expression plasmid using DEAE-dextran, NP-28 displayed a threefold increase in relative integrated intensity. This induction was observed reproducibly in five independent experiments (range of two- to fivefold) despite an increase in background NP-28 expression observed in the control transfections with pUC 13 (Fig. 4, *A* vs. *B*) and the relative inefficiency of transient transfection. There was also a substantial increase in HIV-CAT activity (Fig. 4*C*). Interestingly, analysis of cellular proteins from transient transfectants revealed changes in other polypeptides in 2-D patterns of *tat*-I transfectants compared with control transfected cells (Fig. 4). Some of these changes were observed reproducibly, including the phosphorylation of some proteins, such as NP-65 and Op-18, as well as increased expression of cyclin/proliferating cell nuclear Ag (48–50), which are also seen after PMA/PHA stimulation of lymphoid cells. In some instances, changes, including those observed in the vicinity of NP-28 in stable transfectants (Fig. 2), were not reproducibly observed.

It is important to note that induction of NP-28 was observed in transient and stably transfected cell lines. In contrast to this T cell activation protein, several other spots were evident on 2-D gel electrophoresis after transient transfection, as summarized in Table II. In these instances, changes were consistent with phosphorylation of previously existing proteins, based on metabolic labeling of cells with ^{32}P-orthophosphate into these proteins on the 2-D gel (data not shown). These changes differ from NP-28 activation, which was found in stably transfected lines and was unrelated to phosphorylation. Because transient transfection was associated with higher levels of *tat*-I transactivation, it is possible that proteins such as those described in Table II are relevant to the transactivation mediated by the *tat*-I gene.

DISCUSSION

We have identified a nuclear protein that is expressed in T cells after activation with a variety of agents. This nuclear protein appears to be novel on the basis of its isoelectric point and apparent molecular mass. Attempts at N-terminal sequence analysis of this protein did not succeed, presumably because of N-terminal blockage. Whereas this protein is produced in activated T cells along with other nuclear and cytosolic proteins detectable in 2-D gels, in cells stably transfected with *tat*-I, this protein is consistently and uniquely expressed relative to other proteins that we have previously identified as activation- or proliferation-related.

Although its action on HIV gene expression is mediated by a *cis*-acting region within HIV, the general effects of *tat*-I on cellular factors that regulate specific gene expression are not understood. In this study, we have shown that Jurkat cells stably transfected with *tat*-I express at least one host cell nuclear protein that is part of a set of proteins expressed after activation. The exact relationship between *tat*-I expression and production of this protein in T cells remains to be determined. For example, it is possible that *tat*-I directly activates the gene for NP-28, consistent with changes in NP-28 levels observed after transient transfection. Alternatively, NP-28 expression in *tat*-I transfected cells could be a secondary event. Because this protein is superinduced in *tat*-I expressing cells by PMA and/or ionomycin, it is likely that *tat*-I allows an increase in expression beyond levels normally seen after T cell activation. Although its role in transactivation of HIV or cellular genes is not known, its presence correlates with stimulation of HIV-CAT activity dependent on the *tat*-I target region (Fig. 4) and is associated with T cell activation signals that lead to HIV induction and secretion of IL-2 and other lymphokines (45). Although there is a difference between levels of NP-28 expression and the magnitude of *tat*-I transactivation, its significance is not yet clear. Small changes in cellular proteins can stimulate large changes in gene expression, but it is also possible that other cellular proteins, such as those observed in transiently transfected *tat*-I containing cells, contribute to transactivation by *tat*-I, since these transiently transfected cells exhibit 10- to 20-fold higher levels of *tat*-I transactivation than the stable lines.

Other proteins that were identified initially by 2-D gel electrophoresis have been implicated in important regulatory processes. Such proteins include cyclin/proliferating cell nuclear Ag (48–50) and HIVen 86 (51). In the case of NP-28, whether it participates in *tat*-I transactivation or is an independent target of this transactivation must still be determined. In either case, NP-28 might

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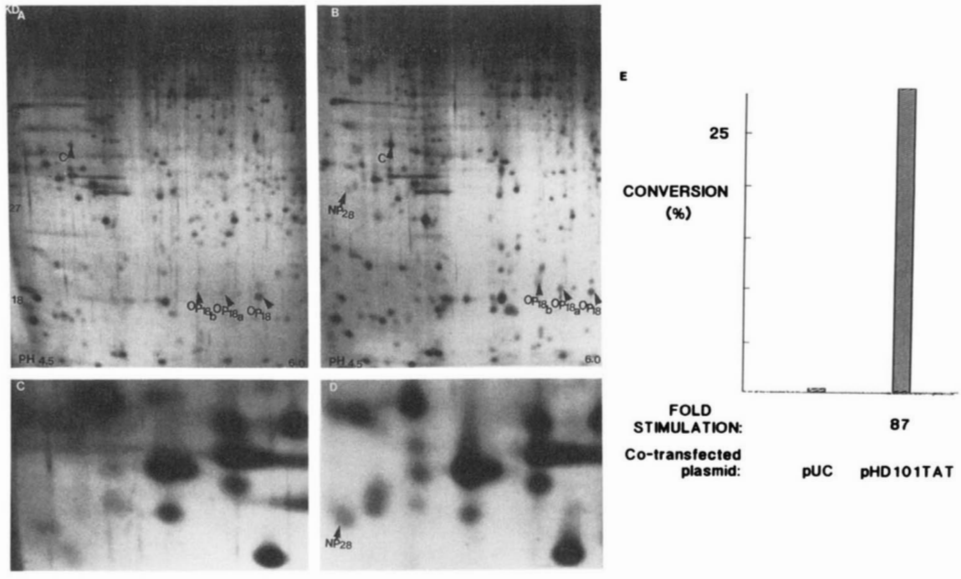

Figure 4. Expression of NP-28 and stimulation of HIV-CAT activity after transient transfection of *tat*-I expression plasmids. Jurkat cells (1 to 2 × 10^7) were transfected using DEAE-dextran with 30 μg of a (*A*) control pUC13 plasmid or (*B*) pHD101 TAT (32), which expresses the *tat*-I gene from the CMV promoter (gift of Dr. Michelle Davis) and analyzed by 2-D gel electrophoresis. Higher magnification of the NP-28 regions for each transfection is shown, respectively (*C* and *D*). CAT activity in parallel cultures (*E*) transfected with HIV-CAT (5 μg) was also determined. Nuclear extracts were prepared for 2-D gel electrophoresis 24 h after transfection as in Figure 1, using a mini-nuclear extract protocol (36). *Arrows* denote the location of NP-28. *Insert* represents region of gel containing NP-28. CAT activity in cells transfected with the indicated plasmids are shown as percent conversion of chloramphenicol to its acetylated forms.

serve as a marker for T cell activation or virus induction from quiescent HIV-infected cells and could contribute to changes in gene expression induced by *tat*-I. Isolation of the gene encoding NP-28 and other associated cellular activation proteins will allow additional definition of their role in T cell activation and HIV infection.

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