mere-binding factors. Altered spatial organization in the nucleus, or an altered telomere structure, could also make mutant telomeres inaccessible to factors that normally act to separate sister chromatids. For example, the yeast DNA untangler topoisomerase II is required for anaphase chromatid separation (*24*), as is proteolysis of noncyclin proteins such as Cut2 (*18*, *25*). The *cut2* and *cut1* mutant phenotype (*26*) is strikingly similar to that of *Tetrahymena ter-43AA*: The main bodies of the sister chromosomes are pulled apart, but the telomeres remain localized late in anaphase, while the cell cycle continues (*20*). Further investigations into cells with altered telomere structure will help define the mechanism by which sister chromatids cohere and separate in a timely manner.

REFERENCES AND NOTES

1. E. H. Blackburn and C. W. Greider, Eds., *Telomeres* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1995).
2. L. L. Sandell and V. A. Zakian, *Cell* **75**, 729 (1993).
3. M. J. McEachern and E. H. Blackburn, *Genes Dev.* **10**, 1822 (1996).
4. J. G. Gall, Ed., *Molecular Biology of Ciliated Protozoa* (Academic Press, New York, 1986).
5. K. E. Kirk and E. H. Blackburn, *Genes Dev.* **9**, 59 (1995).
6. G.-L. Yu, J. D. Bradley, L. D. Attardi, E. H. Blackburn, *Nature* **344**, 126 (1990).
7. P. J. Bruns, T. B. Brussard, E. V. Merriam, *Genetics* **104**, 257 (1983).
8. The template sequence of the *T. thermophila* WT telomerase RNA gene (*ter1*) was mutated from 5′(43)-**C**AACCCCAA-3′ to 5′-(43)**AA**AACCCCAA-3′, by means of polymerase chain reaction, and the mutated gene (*ter1-43AA*) was cloned into the vector pRD4-1 [D. Gilley and E. H. Blackburn, *Mol. Cell. Biol.* **16**, 66 (1996)]. *Tetrahymena thermophila* strains CU428 and B2086 were mated and transformed by electroporation [J. Gaertig and M. A. Gorovsky, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9196 (1992)], with pter1-43AA. Control cells were transformed by electroporation with the WT telomerase RNA gene [prTer1; G.-L. Yu, J. D. Bradley, L. D. Attardi, E. H. Blackburn, *Nature* **344**, 126 (1990)]. Selection was initiated 16 hours after electroporation by the addition of paromomycin (100 μg/ml; Sigma). Four days after electroporation, 20 individual *ter1-43AA* transformant cell lines were pooled, and growth was continued for two additional days. Total DNA was isolated [D. P. Romero and E. H. Blackburn, *J. Eukaryot. Microbiol.* **42**, 32 (1995)]. Telomeric DNA was cloned into pBluescript (Stratagene), and the library then screened with a G_4T_4-specific oligonucleotide. Because the transformed cells also retain their endogenous chromosomal copy of the WT telomerase RNA gene, both WT and mutant repeats are synthesized onto the telomeres. A total of 115 telomeric repeats were sequenced from three different macronuclear telomere clones, 23% of which were mutated. The mutated repeats were confined almost exclusively to the most distal region of the telomere.
9. K. E. Kirk and E. H. Blackburn, unpublished data.
10. Micronuclear phenotypic quantitation was performed in over 700 cells from 14 independent transformant cell lines in two separate experiments from 5 to 7 days after transformation. On average, 55 random micronuclei per cell line per time point were assessed for anaphase by 4′,6′-diamidino-2-phenylindole (DAPI) stain. Cells with no visible micronuclei were excluded from the determination. Length was judged by eye to be roughly equivalent to that observed in WT (elongated) or at least 50% longer than that found in WT, with no apparent separation at the midzone (hyperelongated). The proportion of micronuclei in anaphase varied with respect to cell line and time point (standard deviations for elongated and hyperelongated micronuclei at 7 days were ±7% and ±16%, respectively).
11. Y. Hiraoka, J. R. Swedlow, M. R. Paddy, D. A. Agard, J. W. Sedat, *Semin. Cell Biol.* **2**, 153 (1991).
12. In the *ter1-43AA* mutant, the macronucleus was never observed to divide while the micronucleus was arrested in anaphase. Hence, the onset of macronuclear division may require completion of micronuclear division. Therefore, although mutant telomeres also impair macronuclear division (*6*), possibly independently of the micronuclear telomere phenotype, such impairment of macronuclear division may not become evident while the micronucleus is mitotically blocked.
13. S. M. Adl and J. D. Berger, *J. Eukaryot. Microbiol.* **43**, 77 (1996).
14. T. A. Weinert, G. L. Kiser, L. H. Hartwell, *Genes Dev.* **8**, 652 (1994).
15. T. A. Weinert, *Radiat. Res.* **132**, 141 (1992).
16. C. Holm, *Cell* **77**, 955 (1994).
17. W. Y. Miyazaki and T. L. Orr-Weaver, *Annu. Rev. Genet.* **28**, 167 (1994).
18. M. Yanagida, *Bioessays* **17**, 519 (1995).
19. A. F. Dernberg, J. W. Sedat, W. Z. Cande, H. W. Bass, in (*1*), pp. 295–338.
20. H. Funabiki, I. Hagan, S. Uzawa, M. Yanagida, *J. Cell Biol.* **121**, 961 (1993).
21. E. Henderson, in (*1*), pp. 11–34.
22. G. Fang and T. R. Cech, in (*1*), pp. 69–106.
23. L. Chong *et al.*, *Science* **270**, 1663 (1995).
24. W. C. Earnshaw and A. M. Mackay, *FASEB J.* **8**, 947 (1994).
25. H. Funabiki *et al.*, *Nature* **381**, 438 (1996).
26. T. Hirano, S.-I. Funahashi, T. Uemura, M. Yanagida, *EMBO J.* **5**, 2973 (1986).
27. Cells were also imaged under wet buffer conditions without drying and were found to be quantitatively and qualitatively identical to dried cells: Anaphase micronuclei lengths were on average 10.46 μm by 2.06 μm by 2.49 μm for nondried (n = 7) and 10.95 μm by 1.98 μm by 2.23 μm (n = 3) for dried; nondried chromosomes were 0.41 ± 0.09 μm wide (n = 69) versus 0.40 ± 0.07 μm wide for dried chromosomes (n = 35).
28. D.A. Agard, Y. Hiraoka, P. Shaw, J. W. Sedat, *Methods Cell Biol.* **30**, 353 (1989); Y. Hiraoka, J. W. Sedat, D. A. Agard, *Biophys. J.* **57**, 325 (1990).
29. H. Chen, D. D. Hughes, T.-A. Chan, J. W. Sedat, D. A. Agard, *J. Struct. Biol.* **116**, 56 (1996).

30. We thank J. C. Prescott, A. Bhattacharyya, and H. W. Bass for manuscript review. Supported by NIH grant GM26259 (to E.H.B.), an American Cancer Society postdoctoral fellowship (to K.E.K.), and by an NSF predoctoral fellowship (to B.P.H.).

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Immune Hyperactivation of HIV-1–Infected T Cells Mediated by Tat and the CD28 Pathway

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Human immunodeficiency virus–type 1 (HIV-1) infection is characterized by a chronic state of immune hyperactivation in patients. Infection of human peripheral blood lymphocytes with HIV-1 in vitro resulted in increased interleukin-2 (IL-2) secretion in response to T cell activation via the CD3 and CD28 receptors. Expression of the HIV-1 transactivator Tat recapitulated this phenotype and was associated with increased IL-2 secretion in response to costimulation with CD3 plus CD28. IL-2 superinduction by Tat occurred at the transcriptional level, was mediated by the CD28-responsive element in the IL-2 promoter, and was exclusively dependent on the 29 amino acids encoded by the second exon of Tat.

Symptoms of immune hyperactivation in HIV-1–infected individuals are noted throughout the course of infection and include spontaneous lymphocyte proliferation; expression of T cell activation antigens; lymph node hyperplasia; increased cytokine expression; and elevated levels of neopterin, β_2-microglobulin, acid-labile interferon, and IL-2 receptors (*1*, *2*). Immune hyperactivation is probably critical for the maintenance of the infectious process, as HIV-1 cannot infect resting T cells (*3*, *4*) and therefore depends on a pool of activated T cells in the host. Proposed mechanisms for this immune hyperactivation include the persistence of virus and viral antigens throughout the course of the disease, the presence of a superantigen encoded by HIV or another microbe, and the presence of autoimmune phenomena (*2*).

We considered the possibility that HIV-1 infection itself could lead to the superactivation of infected T cells. Peripheral blood lymphocytes (PBLs) were infected with the primary viral isolate HIV-$1_{89.6}$ (*5*) after activation with antibodies to CD3. Cells were then restimulated with antibodies directed against the CD3 and CD28 molecules. These two receptors play a critical role in T cell activation; the CD3

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complex is associated with the T cell receptor and is its signal-transducing subunit, whereas the CD28 receptor has recently emerged as a costimulatory receptor necessary for the full program of T cell activation (6). Restimulation with antibodies to CD3 plus CD28 (anti–CD3+CD28) at day 3, when reverse transcriptase (RT) activity indicated active viral replication, induced a marked superinduction of secreted IL-2 in infected cells as compared with that in uninfected PBLs (Fig. 1). The same results were obtained when intracellular IL-2 production was measured by flow cytometry (Fig. 1). This superinduction of IL-2 secretion was strictly dependent on CD28 costimulation and did not occur with anti-CD3 alone (7). This initial experiment indicated that HIV-1 infection alone is associated with a hyperresponsiveness of T cells to costimulatory activation signals.

Because the HIV-1–encoded Tat protein is a potent transcriptional activator of the HIV-1 promoter, it might also affect the transcription of T cell–specific genes. Two groups of workers found an increase in IL-2 transcription (8, 9), whereas two other reports described an inhibition of IL-2 transcription (10, 11) in response to Tat. These contradictory results, the absence of data on viral infection, and the unphysiological reagents used to stimulate IL-2 transcription have limited the impact of these observations.

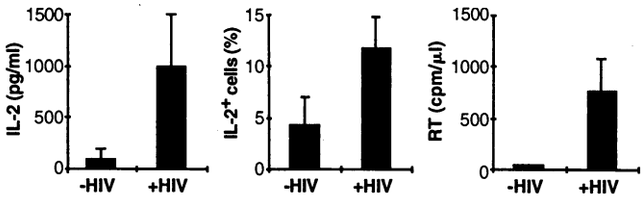

Fig. 1. Hypersensitivity of HIV-1–infected T cells to CD3+CD28 costimulation. PBLs were obtained from Ficoll-Hypaque (Pharmacia)–purified PBMCs after three cycles of adherence-mediated depletion of macrophages. Nonadherent cells were treated with immobilized anti-CD3 (33) and infected with $HIV_{89.6}$ (5) (5×10^5 pg of p24 antigen per 10^7 cells). Forty-eight hours later, cells were stimulated with anti–CD3+CD28 (1 μg/ml) (33) or an isotype control. IL-2 production was analyzed by measurement of the secreted IL-2 concentration in supernatants by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) or by flow cytometry analysis of intracellular IL-2 production with the use of brefeldin A treatment and a phycoerythrin-labeled antibody to IL-2 (Pharmingen) (15). Viral production was estimated by measurement of RT activity in culture supernatants. Data averaged from three independent donors are shown (mean ± SEM).

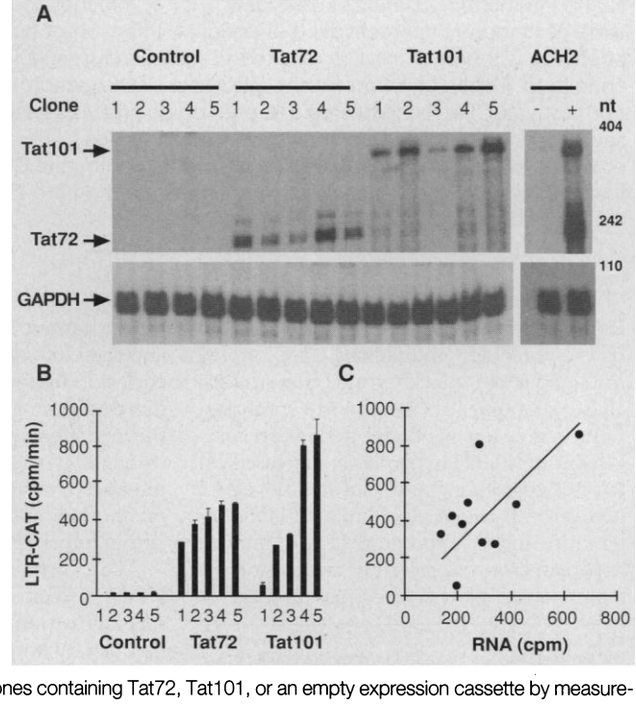

Fig. 2. Characterization of HIV-1 Tat-expressing clones. (**A**) RNase protection analysis. Five representative clones containing Tat72, Tat101, or an empty expression cassette were analyzed by RNase protection assay with an antisense probe corresponding to Tat101 mRNA (14). This probe protects two fragments of 234 and 327 nt that correspond to Tat72- and Tat101-specific transcripts, respectively. RNA from ACH2 cells, untreated (−) or treated (+) for 8 hours with tumor necrosis factor–α (800 U/ml; Gibco-BRL), was analyzed with the same probe for comparison. All samples were also analyzed with an antisense probe corresponding to the mRNA for the GAPDH gene as an internal control (30). (**B**) Transactivating activity. Tat activity, measured by the Tat-dependent transactivation of the HIV promoter, was tested in five clones containing Tat72, Tat101, or an empty expression cassette by measurement of CAT activity in cell lysates after transfection of pLTR-CAT. This construct contains the HIV-1 LTR driving the expression of the CAT gene (30). Average values (±SEM) of two transfections performed in duplicate are shown. (**C**) Correlation between Tat expression and CAT activity. Basal Tat-specific mRNA levels, quantified by phosphorimager analysis (InstantImager, Packard, Downers Grove, Illinois) of Tat-specific protected bands shown in (A) are plotted against Tat activity, measured as LTR-CAT activity.

To examine the role of Tat in T cell activation, several Jurkat cell lines that stably expressed either the one-exon form of Tat [72 amino acids (Tat72)] or the two-exon form of Tat [101 amino acids (Tat101)] were established (12). Tat101 is expressed both early and late in the virus life cycle, whereas Tat72 is expressed solely in the late phase (13). Five Jurkat clones in each group (Tat72, Tat101, or control clones containing an empty expression cassette) were selected and further characterized. Flow cytometry analysis showed similar amounts of cell surface expression of the following markers: CD3, CD4, CD25, CD26, CD28, CD45, and human leukocyte antigen DR (7). Expression of Tat mRNA was demonstrated in these clones by ribonuclease (RNase) protection assays with an antisense riboprobe corresponding to the full-length Tat (14) (Fig. 2A). The amount of Tat mRNA in these clones falls within the range observed in HIV-1–infected cells as shown by comparison with ACH2 cells (Fig. 2A). Tat was detected in these clones with a fluorescein isothiocyanate–labeled antibody directed against Tat (Intracel, Cambridge, MA) and flow cytometry analysis after permeabilization of the cells (7, 15). Tat proteins produced in these clones were functional, as shown by their ability to activate transcription of a transiently transfected construct consisting of the HIV-1 long terminal repeat (LTR) placed upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (16) (Fig. 2B).

To assess the response of these Tat-expressing clones to T cell activation signals, we measured the secretion of IL-2, a crucial cytokine in T cell differentiation and proliferation. Clones stably expressing Tat101 secreted 10 times more IL-2 than did Tat72 or control clones in response to costimulation by anti–CD3+CD28 (Fig. 3A). Amounts of secreted IL-2 positively correlated (correlation coefficient $r^2 = 0.92$) with the Tat101 activity present in the cells (Fig. 3B). This correlation was not observed for Tat72-expressing clones (Fig. 3B). Tat101-mediated IL-2 superinduction was sensitive to low doses of rapamycin (1 nM), an inhibitor of CD28-mediated signaling. Tat101 clones were hypersensitive to CD28 signaling, as demonstrated by the increase in IL-2 secretion and by their ability to respond to reduced doses of CD28 (0.1 μg/ml versus 3 μg/ml) (Fig. 3C).

The same superinduction of IL-2 secretion in response to anti–CD3+CD28 was observed in PBLs treated with exogenous Tat101 but not with Tat72 (Fig. 3D). The superinduction was proportional to the amount of Tat101 (5 versus 10 μg) and did

not occur in PBLs stimulated with anti-CD3 alone (Fig. 3D). We cannot completely exclude the possibility that Tat101 was more readily taken up by these cells, which could account for the difference observed between Tat72 and Tat101. These observations therefore confirm, in nontransformed primary cells, the results observed with the stable Tat-expressing clones. However, because Tat concentrations in infected individuals are lower than the concentrations used here (*17*), it is likely that Tat cannot function as an extracellular mediator to increase CD28 responsiveness and that its activity is limited to the infected cell.

CD28 costimulation induces IL-2 secretion by stimulating IL-2 gene transcription and by increasing IL-2 mRNA stability (*6*). In agreement with these observations, amplification of reverse-transcribed mRNA by the polymerase chain reaction (RT-PCR) with IL-2–specific primers demonstrated that Tat101 clones contain five times more IL-2 mRNA than do controls after CD3+CD28 costimulation (*7*). To examine the effect of Tat101 at the transcriptional level, a reporter construct with the IL-2 promoter cloned upstream of the reporter gene luciferase (pIL-2luc) was transfected into clones expressing Tat72 or Tat101 or into control clones. In Tat101 clones only, the IL-2 promoter was superinduced in response to CD3+CD28 costimulation and, to a lesser extent, in response to anti-CD3 stimulation alone (Fig. 4A). No promoter activity was detected in the absence of stimulation (*7*). A similar superinduction of IL-2 promoter activity in response to CD3+CD28 costimulation was also observed when an expression vector for Tat101 was cotransfected with pIL-2luc (*7*).

The mechanism of action of the Tat101 protein on IL-2 transcription was examined by determination of the binding of transcription factors to regulatory elements within the IL-2 promoter. Gel retardation experiments were done with double-stranded oligonucleotides corresponding to binding sites within the IL-2 promoter for the transcription factors nuclear factor of activated T cells (NF-AT), AP-1, NF kappa B (NF-κB), and the CD28-responsive element (CD28RE) (*18*). Specific and inducible binding of nuclear factors to all four sites was detected as early as 1 hour after CD3+CD28 costimulation (Fig. 4B). Although factors binding to the CD28RE [nucleotide (nt) –154 to nt –143] were significantly superinduced in Tat101-expressing clones when compared to controls (Fig. 4B), factors binding to other sites were unaffected by Tat101 expression (Fig. 4B). Superinduction of CD28RE was weak in response to CD3 stimulation alone and did not occur in Tat72-expressing clones (*7*). In addition, factors binding to the CD28RE of the IL-8 promoter (*19*) were similarly superinduced by Tat101 expression (*7*). As the CD28RE is related to a NF-κB binding site (*20*), the absence of change in factor binding to the IL-2 NF-κB site was unexpected. Nevertheless, examination of two additional NF-κB sites from the lymphotoxin and the HIV-1 promoter (*21*) confirmed that Tat101 does not affect NF-κB binding (*7*). Additionally, using specific antisera, we have confirmed previous observations that the CD28RE binds a complex composed of members of the NF-κB family: c-Rel, p50, and RelA (*22*).

Mutation of the CD28RE in the IL-2 promoter (plasmid pIL2-lucmut) abolished Tat101-mediated superinduction in response to anti–CD3+CD28 (Fig. 4A), demonstrating that the CD28RE is critical for Tat101-mediated increased transcription of IL-2. The CD28RE is also sufficient for the Tat response, because a minimal construct containing three multimerized CD28REs cloned upstream of a minimal promoter (thymidine kinase) was responsive to Tat101-mediated superinduction in response to CD3+CD28 costimulation (*7*).

To prove that the superinduction of IL-2 secretion observed during the course of HIV-1 infection (Fig. 1) was indeed caused by the second exon of Tat, we generated a mutant infectious clone of HIV-1 by introducing a stop codon at amino acid 72 in the Tat open reading frame (ORF) (HIVΔtatexon2). This mutation does not affect the sequence of Rev, which is encoded in another ORF in the same region. Wild-type HIV and HIVΔtatexon2 replicated in PBLs with almost identical kinetics (*23*). Both viruses were used to infect PBLs, and the response of these infected populations to different T cell activation signals was measured in terms of IL-2 secretion. Wild-type HIV-1 infection was associated with an increase in sensitivity to CD3+CD28 signaling reflected in greater IL-2 secretion in comparison to that of uninfected PBLs (Fig. 5). This increase in IL-2 was strictly

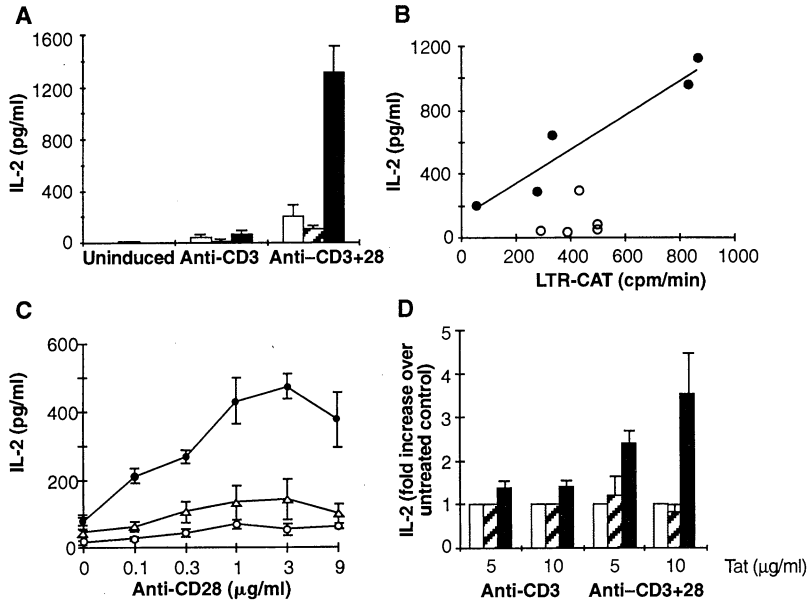

Fig. 3. Up-regulation of IL-2 secretion by Tat101 in response to CD3+CD28 costimulation. Exponentially growing Jurkat clones were activated with monoclonal anti-CD3 in the presence or absence of costimulatory monoclonal anti-CD28 or a control isotype antibody (*33*). Supernatants were harvested 24 hours after the addition of antibodies, and IL-2 concentrations were determined by ELISA (R&D Systems). (**A**) IL-2 concentrations (mean ± SEM) in response to CD3, CD3+CD28 (1 μg/ml) costimulation in control clones (white bars), Tat72-expressing clones (hatched bars), and Tat101-expressing clones (black bars). (**B**) Correlation between IL-2 secretion in response to CD3+CD28–mediated costimulation and Tat transactivating activity measured by CAT assay after transient transfection of a LTR-CAT reporter construct (see Fig. 1B) in Tat101 (solid circles) and Tat72 clones (open circles). (**C**) Clones stimulated with anti-CD3 were treated with increasing concentrations of anti-CD28 (0 to 9 μg/ml). Mean IL-2 values (±SEM) of Tat101 clones (solid circles), Tat72 clones (open circles), and control clones (open triangles) are shown. (**D**) Effect of exogenous Tat on IL-2 secretion in human PBLs in response to anti-CD3 or anti–CD3+CD28. PBLs (10^6 cells) were incubated with no Tat (white bars), 5 or 10 μg of Tat72 (*34*), (hatched bars), or Tat101 (*34*) (black bars) in the presence of sulfated protamine (100 μg/ml) for 15 min at 37°C (*35*). Cells were then diluted into regular culture medium and stimulated with anti-CD3 or anti-CD28 as described above for 24 hours. Results, averaged from three independent experiments, are expressed as fold increase over untreated controls.

dependent on the second exon of Tat, as infection with HIVΔtatexon2 actually resulted in a suppression of IL-2 secretion in comparison to that of uninfected PBLs (Fig. 5). Modulation of T cell activation by HIV-1 was dependent on the successful completion of the virus life cycle, as no effect was observed on IL-2 secretion when the infection was performed in the presence of the reverse-transcriptase inhibitor azidothymidine (AZT) (Fig. 5).

The function of Tat101 described here fulfills many of the proposed activities of an HIV-encoded non-specific activator of the immune system. Because signs of immune hyperactivation are present in HIV-1–infected individuals, we propose that HIV-1, in part through the second exon of Tat, directly modifies the degree of immune activation in infected individuals. Increased IL-2 concentrations in the lymph nodes of HIV-infected patients might prime resting T cells for infection by HIV-1 in an antigen-independent fashion. Such a possibility is supported by the observation that increased IL-2 levels have been observed in the lymph nodes of HIV-1–infected individuals (*24*, *25*) and that IL-2 treatment alone of peripheral blood mononuclear cells (PBMCs) is sufficient to prime these cells for HIV-1 infection (*22*, *26*). Taken together, these observations suggest that increased production of IL-2 by HIV-1–infected cells within the confined environment of the lymph node creates a permissive environment for the propagation of the infectious process by priming unactivated (or partially activated) bystander T cells for infection.

Because CD28 costimulation is a critical modulator of the expression of other genes important in T cell differentiation besides that encoding IL-2 (*19*, *27*), it is probable that Tat causes the up-regulation of several genes in addition to that encoding IL-2, thereby contributing to the chronic immune hyperactivation observed in HIV-infected individuals (*1*, *2*). The fact that the CD28 pathway is specifically targeted by HIV during the infectious process reemphasizes the relevance of CD28 costimulation as an important therapeutic target for modulation of HIV infection and pathogenesis (*28*).

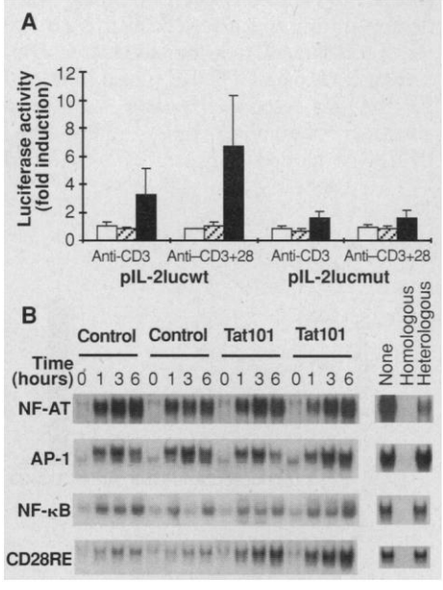

Fig. 4. Activation of IL-2 production by Tat101 occurs at the transcriptional level and is mediated by the CD28-responsive element. (**A**) Transient transfection assays of pIL-2luc into Jurkat Tat72, Tat101, and control clones. DNA from pIL-2luc (in which the human IL-2 promoter drives the reporter gene *luciferase*) or from pIL-2lucmut [a mutated construct lacking the CD28-responsive element (*36*)] were transfected into Jurkat clones expressing either Tat72 or Tat101 (0.3 μg of DNA per 5×10^6 cells) cells with the use of the standard DEAE-dextran technique followed by an incubation with 0.1 mM chloroquine. Twenty-four hours after transfection, equal numbers of viable cells, as determined by Trypan blue exclusion, were induced with either anti-CD3 or anti-CD3+CD28. Cells were harvested 18 hours later, and luciferase activity was analyzed according to the Luciferase Assay System (Promega) with a luminometer (Turner Designs Luminometer Model 20, Promega). Values, normalized to protein concentrations, are expressed in fold increase over unstimulated controls. The mean (±SEM) of three control clones (white bars), three Tat72 clones (hatched bars), or three Tat101 clones (black bars) in one representative experiment is shown. (**B**) Gel retardation assay for factors binding to the IL-2 promoter. Double-stranded oligonucleotides corresponding to NF-AT (nt −283 to nt −250), AP-1 (nt −149 to nt −133), NF-κB (nt −202 to nt −185), and CD28RE (nt −154 to nt −143) binding sites in the human IL-2 promoter were synthesized (Genset, San Diego, California) and were ^{32}P-radiolabeled (*37*). Nuclear extracts were prepared with the use of a rapid protocol (*38*) from two Jurkat-control clones and two Jurkat-Tat101 clones stimulated for 0, 1, 3, and 6 hours with anti-CD3+CD28. Gel retardation assays were performed as previously reported (*30*, *37*). For competition studies, a 50-fold molar excess of unlabeled double-stranded oligonucleotide either homologous or heterologous to the probe was co-incubated with the ^{32}P-labeled probe before nuclear extract was added.

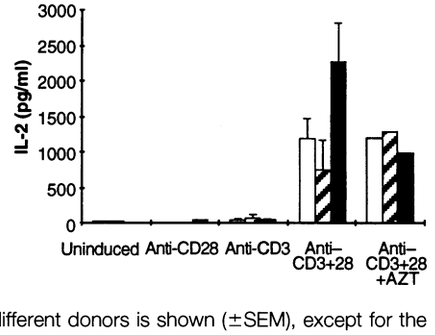

Fig. 5. The second exon of Tat is critical for HIV-1–mediated T cell hypersensitivity to CD28 signals. PBLs were isolated from normal donors, stimulated with anti-CD3 (3 μg/ml precoated for 24 hours) and infected with wild-type HIV-1 (solid bars) or HIVΔtatexon2 (*39*) (hatched bars) or were left uninfected (white bars) (5×10^5 pg of p24 antigen per 10^7 cells). Three days after infection, cells were restimulated with anti-CD28, anti-CD3, or anti-CD3+CD28 for 24 hours, and secreted IL-2 was measured in culture supernatants. As a control, one group was infected in the presence of AZT (1 μM) and stimulated with anti–CD3+28. The average of four independent experiments performed with four different donors is shown (±SEM), except for the AZT control, which was performed in a single experiment.

REFERENCES AND NOTES

1. G. Pantaleo and A. S. Fauci, *Annu. Rev. Immunol.* **13**, 487 (1995).
2. A. S. Fauci, *Science* **262**, 1011 (1993).
3. J. A. Zack *et al.*, *Cell* **61**, 213 (1990).
4. M. Stevenson, T. L. Stanwick, M. P. Dempsey, C. A. Lamonica, *EMBO J.* **9**, 1551 (1990).
5. R. Collman *et al.*, *J. Virol.* **66**, 7517 (1992).
6. P. S. Linsley and J. A. Ledbetter, *Annu. Rev. Immunol.* **11**, 191 (1993).
7. M. Ott *et al.*, data not shown.
8. M. O. Westendorp, M. Li-Weber, R. W. Frank, P. H. Krammer, *J. Virol.* **68**, 4177 (1994).
9. A. Vacca *et al.*, *Biochem. Biophys. Res. Commun.* **205**, 467 (1994).
10. S. F. Purvis, D. L. Georges, T. M. Williams, M. M. Lederman, *Cell Immunol.* **144**, 32 (1992).
11. R. K. Puri, P. Leland, B. B. Aggarwal, *AIDS Res. Hum. Retroviruses* **11**, 31 (1995).
12. A PCR-amplified cDNA fragment corresponding either to the first exon (Tat72) or both exons (Tat101) of the HIV-1 Tat gene was amplified with the use of the HIV-1 cDNA clone pCV1 (29) as a template and primers containing an additional Bam HI site for cloning. The amplified fragment was cloned into the unique Bam HI site of the episomal vector pRep9 (Invitrogen, San Diego, CA) under the control of the Rous sarcoma virus promoter. Because pCV1 cDNA encodes a truncated 86–amino acid form of Tat, a mutation (T**A**G [→] T**C**G, changing codon 86 from a stop to a serine) was introduced in the 3′ primer used in the PCR reaction to reopen the Tat reading frame to its full length. Recombinant plasmids were transfected into Jurkat cells by electroporation, followed by selection with Geneticin (600 μg/ml, Gibco-BRL) and cloning by limiting dilution. All clones were frozen rapidly after identification, and aliquots were periodically thawed (every 6 weeks) to maintain the identity of the clones. During that interval, all phenomena described in this report (Tat mRNA, Tat activity, and IL-2 hyperresponsiveness) were stable and reproducible. All cell lines used in this study were negative for *Mycoplasma* (MycoTest, Life Technologies).
13. M. H. Malim, J. Hauber, R. Fenrick, B. R. Cullen, *Nature* **335**, 181 (1988).
14. RNase protection analysis was performed on 15 μg of total RNA purified by TRIZOL (Gibco-BRL) (Lysate Ribonuclease Protection Kit, USB), with a ^{32}P-labeled antisense riboprobe synthesized in vitro with SP6 polymerase and a plasmid containing the 2-exon Tat cDNA. A ^{32}P-labeled antisense riboprobe for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (*30*) was hybridized in a separate reaction with 2 μg of total RNA.
15. P. Openshaw *et al.*, *J. Exp. Med.* **182**, 1357 (1995).
16. Transient transfection of supercoiled plasmid DNA was performed with the use of DEAE-dextran, and

CAT activity was measured with a diffusion assay as previously reported (*30*).
17. M. O. Westendorp *et al.*, *Nature* **375**, 497 (1995).
18. E. Serfling, A. Avots, M. Neumann, *Biochim. Biophys. Acta* **1263**, 181 (1995).
19. A. S. Wechsler, M. C. Gordon, U. Dendorfer, K. P. Le Clair, *J. Immunol.* **153**, 2515 (1994).
20. 25. P. Ghosh, T. H. Tan, N. R. Rice, A. Sica, H. A. Young, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1696 (1993).
21. G. Nabel and D. Baltimore, *Nature* **326**, 711 (1987).
22. M. Ott and E. Verdin, unpublished observation.
23. PBLs were infected with HIV infectious stocks generated after transfection of molecular clone $HIV_{89.6}$ or a mutant containing a stop codon at amino acid 72 of Tat (5×10^4 pg of p24 antigen per 10^7 cells). Culture supernatants from cells stimulated with antibodies directed against either CD3 or CD3+CD28 were harvested at different times after infection and analyzed for RT activity.
24. C. Graziosi *et al.*, *Science* **265**, 248 (1994).
25. C. Graziosi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4386 (1996).
26. A. L. Kinter, G. Poli, L. Fox, E. Hardy, A. S. Fauci, *J. Immunol.* **154**, 2448 (1995).
27. J. D. Fraser, B. A. Irving, G. R. Crabtree, A. Weiss, *Science* **251**, 313 (1991).
28. O. K. Haffar *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11094 (1993).
29. S. K. Arya, C. Guo, S. F. Josephs, F. Wong-Staal, *Science* **229**, 69 (1985).
30. C. Van Lint, S. Emiliani, M. Ott, E. Verdin, *EMBO J.* **15**, 1112 (1996).
31. F. Pages *et al.*, *Nature* **369**, 327 (1994).
32. M. Adams *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3862 (1994).
33. Anti-CD3 (454.3.21) was provided by N. Chiorazzi (North Shore University Hospital, Manhasset, NY) and precoated on tissue culture wells with 3 μg of antibody per milliliter of buffer [35 mM bicarbonate and 15 mM carbonate (pH 9.6)] at 4°C overnight. Anti-CD28 (clone 28.2) was provided by D. Olive (INSERM, Marseilles, France) (*31*).
34. To express the different forms of Tat in *Escherichia coli*, Bam HI–Bam HI fragments corresponding to either Tat72 or Tat101 cDNAs (*12*) were cloned into the unique Bam HI site of pTrcHisB behind the Trc promoter (Invitrogen). *E. coli* (TOP10 strain, Invitrogen) containing the recombinant plasmids were induced for 16 hours with isopropyl-β-D-thiogalactopyranoside (1 mM) at 37°C. Pelleted cells were resuspended in buffer A [6M guanidine hydrochloride, 0.1M NaH_2PO_4, and 0.01M tris (pH 8.0)] at 5 ml per gram wet weight and stirred for 1 hour at room temperature. Lysate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was collected. Four milliliters of a 50% slurry of Ni–nitrilotriacetic acid resin (Invitrogen) equilibrated in buffer A was added to the cell suspension and incubated at room temperature for 1 hour. This mixture was loaded on a column and the flowthrough was collected. The resin was washed sequentially with 10 ml of buffer A, 15 ml of buffer B [8 M urea, 0.1M NaH_2PO_4, and 0.01 M tris (pH 8.0)], and 15 ml of buffer C (same composition as buffer B, but at a pH of 6.3). The recombinant protein was eluted with 15 ml of buffer C containing 250 mM imidazole. This eluate was loaded directly on a high-performance liquid chromatography C4 column run in a gradient of acetonitrile (0 to 100%) in 0.1% trifluoroacetic acid. Fractions containing Tat were lyophilized in small aliquots and stored at −70°C under anaerobic conditions to prevent Tat oxidation. Tat was resuspended in degassed phosphate-buffered saline plus 0.1 mM dithiothreitol immediately before use. The biological activity of Tat purified according to this protocol was tested by incubating it with U1 cells, which contain a virus exhibiting a Tat-defective phenotype (*32*). Tat treatment of these cells caused a 50-fold induction of viral expression as detected by p24 secretion into culture supernatant. Endotoxin contamination of Tat prepared with this protocol was below the detection limit of the assay (<59 EU/ml; Limulus Amebocyte Lysate Assay, Biowhittaker, Walkersville, MD).
35. D. A. Mann and A. D. Frankel, *EMBO J.* **10**, 1733 (1991).
36. pIL-2luc was constructed by cloning a 390–base pair Kpn I–Hind III fragment containing the human IL-2 promoter (nt −340 to nt +50) (a gift from R. Gaynor) into the corresponding sites of pGL2-BASIC (Promega, Madison, WI). This plasmid was used as a substrate for the mutagenesis of the CD28RE by means of the Transformer site-directed mutagenesis method (Clontech, Palo Alto, CA). The oligonucleotide (5′-GGGTTTAAAGAA**GCCT**CAAAGAGTCAT-CA-3′) was used to introduce four mutations within the CD28RE (mutations are highlighted in bold), abolishing binding of the CD28RC to the element (*27*). The oligonucleotide (5′-CTTATCATGTCT**GA-CGTC**GTCGACCGATGC-3′) changes a Bam HI into an Aat II restriction site (highlighted in bold) and was used for selection during mutagenesis. The region corresponding to the IL-2 promoter was fully resequenced to confirm the mutation, and the resequenced fragment was subcloned into pGL2-BASIC as described above.
37. C. Van Lint, J. Ghysdael, P. Paras Jr., A. Burny, E. Verdin, *J. Virol.* **68**, 2632 (1994).
38. L. Osborn, S. Kunkel, G. Nabel, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2336 (1989).
39. A point mutation (C → T) was introduced into a molecular clone of $HIV_{89.6}$, p89.6 (*5*) to change codon 72 of the Tat ORF from CAG into TAG. The mutation was introduced with the use of the Transformer kit (Clontech) with the following two oligonucleotides: 5′-CTCTATCAAAGTAGTAAGTAGTAC-3′ (Tat mutation) and 5′-GTGCCACCTGATATCTA-AGAAACC-3′ (selection primer). The presence of the mutation was verified by sequencing, and a fully resequenced Sal I–Stu 1 fragment containing the mutation was subcloned back into p89.6. Supernatants from CEM × 174 cells transfected with this DNA were harvested and their RT titer was measured. To confirm that virus stocks had not reverted to wild type, virus stocks were centrifuged and purified RNA was used in RT-PCR to amplify a fragment containing the mutated Tat gene. This PCR fragment was cloned with the TA cloning kit (Invitrogen), 10 individual clones were resequenced, and all contained the original mutation in the Tat gene.
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A Plastid of Probable Green Algal Origin in Apicomplexan Parasites

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Protozoan parasites of the phylum Apicomplexa contain three genetic elements: the nuclear and mitochondrial genomes characteristic of virtually all eukaryotic cells and a 35-kilobase circular extrachromosomal DNA. In situ hybridization techniques were used to localize the 35-kilobase DNA of *Toxoplasma gondii* to a discrete organelle surrounded by four membranes. Phylogenetic analysis of the *tufA* gene encoded by the 35-kilobase genomes of coccidians *T. gondii* and *Eimeria tenella* and the malaria parasite *Plasmodium falciparum* grouped this organellar genome with cyanobacteria and plastids, showing consistent clustering with green algal plastids. Taken together, these observations indicate that the Apicomplexa acquired a plastid by secondary endosymbiosis, probably from a green alga.

Apicomplexan parasites contain two maternally inherited extrachromosomal DNA elements (*1*). The mitochondrial genome is a multicopy element of ~6 to 7 kb encoding three proteins of the respiratory chain and extensively fragmented ribosomal RNAs (*2*). In addition, these parasites contain a 35-kb circular DNA molecule with no significant similarity to known mitochondrial genomes. The 35-kb element is similar to chloroplast genomes, containing an inverted repeat of ribosomal RNA genes and genes typically found in chloroplasts but not mitochondria (*rpoB/C*, *tufA*, and *clpC*) (*3*). The 35-kb DNA is also predicted to encode a complete set of tRNAs, numerous ribosomal proteins, and several unidentified open reading frames (*3*).

We used in situ hybridization to determine whether the 35-kb DNA is found within the parasite nucleus, mitochondrion, or cytoplasm or, alternatively, whether this

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