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22. The cytoplasmic region of NpSRII lacks a nucleation site for the development of a hydrogen-bonded network of water molecules [H. Luecke *et al.*, *J. Mol. Biol.* **300**, 1237 (2000)]. Also, the extracellular region lacks proton release machinery; that is, a pair of glutamic acid residues connected to the Schiff base region by a 3D hydrogen-bonded network of side chains and water (*9*) [R. Rammelsberg, G. Huhn, M. Lübben, K. Gerwert, *Biochemistry* **37**, 5001 (1998)] that prevents the return of the proton from the extracellular side in bacteriorhodopsin [S. P. Balashov, E. S. Imasheva, R. Govindjee, T. G. Ebrey, *Biophys. J.* **70**, 473 (1996); H.-T. Richter, L. S. Brown, R. Needleman, J. K. Lanyi, *Biochemistry* **35**, 4054 (1996)]. A similar situation arises in the $Asp^{96} \rightarrow Asn^{96}$ mutant of BR at a pH below the pK for proton release; but unlike NpSRII, this protein exhibits transport. In BR, although at pH < 6 protonation equilibrium between the Schiff base and its counterion ensures that the proton can pass to the extracellular side [L. S. Brown, A. K. Dioumaev, R. Needleman, J. K. Lanyi, *Biophys. J.* **75**, 1455 (1998)], at the low pH the reprotonation from the cytoplasmic side is strongly accelerated [A. Miller, D. Oesterhelt, *Biochem. Biophys. Acta* **1020**, 57 (1990)]. In contrast, the cytoplasmic region of NpSRII is likely to be a permanent barrier to protons. If directionality is conferred on the movement of the transported ion by the changing ion conductivities of the extracellular and cytoplasmic regions [L. S. Brown, A. K. Dioumaev, R. Needleman, J. K. Lanyi, *Biochemistry* **37**, 3982 (1998)], reprotonation of the Schiff base in NpSRII will be, of necessity, from the extracellular rather than the cytoplasmic side.
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Selective Transcription and Modulation of Resting T Cell Activity by Preintegrated HIV DNA

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The quiescent nature of most peripheral T cells poses an effective limitation to human immunodeficiency virus (HIV) replication and, in particular, to viral integration into the host chromatin. Two HIV proteins, Nef and Tat, increase T cell activity, but a requirement of integration for viral gene expression would preclude a role for these proteins in resting cells. Here, we report that HIV infection leads to selective transcription of the *nef* and *tat* genes before integration. This preintegration transcription in quiescent cells leads to increased T cell activation and viral replication.

Replication of HIV predominates in the CD4 T cell population (*1*), and the level of replication is highly prognostic for the development of acquired immunodeficiency syndrome (AIDS) (*2*). However, viral infection is limited by the quiescence of most circulating T cells, which is nonsupportive of viral replication. As a retrovirus, the RNA genome of HIV undergoes reverse transcription to a DNA intermediate, followed by integration into the host chromatin. Formation of the integrated provirus is essential for HIV replication (*3–5*), and yet the most prevalent form of HIV DNA during the asymptomatic phase of infection is full-length unintegrated DNA (*6*). The barrier to integration seen in peripheral T cells can be overcome by stimulatory increases in the cellular metabolic state through either mitogenic (*3*, *7*, *8*) or submitogenic (*9*, *10*) stimulation. Recent findings suggest that HIV may have evolved functions to overcome this threshold. Expression of the earliest HIV gene products, Nef and Tat, can increase T cell activity (*11–15*), and in particular, Nef can lower the activation threshold in T cells (*14*). However, it is unclear how these HIV proteins could promote the early and limiting steps of infection (and integration) if provirus formation is required first.

To define the capacity of HIV to affect the activation state of quiescent primary CD4 T cells, we explored the earliest events in HIV infection. Quiescent CD4 T cells were infected with the NL4-3 strain of HIV-1 at 0.5 infectious virions per cell. After 5 days of incubation, during which no measurable viral replication occurred (*16*), cells were stimulated through their T cell receptors with CD3 plus CD28 (CD3-CD28) immobilized antibody beads and were measured for interleukin-2 (IL-2) (Fig. 1). IL-2 is the definitive indicator of CD4 T cell activation, a condition optimal for viral integration and replication. The expression of IL-2 is mediated by transcription factors also responsible for the HIV long terminal repeat (LTR) promoter activity (*17*). We observed a stimulus-dependent increase in IL-2 generation in resting cells exposed to HIV (Fig. 1).

The binding of the HIV envelope to cellular receptors (*18*, *19*) or the inclusion of Nef in the virion (*20*) could alter the activation state of the T cell before integration. To distinguish between activation through these existing virion components or newly synthesized viral products, we incubated purified resting CD4 T cells with HIV in the presence or absence of the reverse transcriptase inhibitor 3′-azido-3′-deoxythymidine (AZT). The addition of AZT to the T cells did not affect the level of IL-2 generated by CD3-CD28 bead activation (*21*). However, the HIV-enhanced IL-2 response was lost in the presence of AZT (Fig. 1, donors 3 and 4). This implies that the reverse transcription and its downstream products, and not virion-cellular interactions, were responsible for the enhancement of T cell activation.

We then examined the state of the HIV DNA and the transcriptional activity of the DNA in quiescent cells. Resting T cells were

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infected as above, except at a lower multiplicity of infection (MOI = 0.02). To determine whether full-length viral DNA was synthesized and translocated into the nucleus, we purified nuclear DNA from infected cells at 5 days after infection and then amplified the nuclear DNA by polymerase chain reaction (PCR) with primers targeting the late product of the reverse transcription (F1/B1, Fig. 2A). Full-length HIV-1 DNA was easily detectable from as few as 1×10^3 to 2×10^3 infected resting T cells (Fig. 2B).

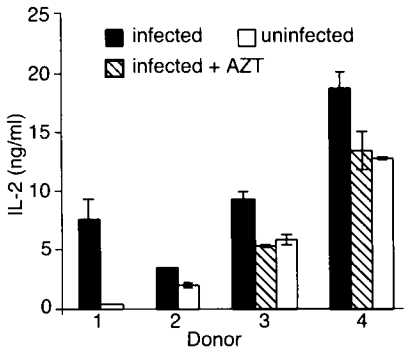

Fig. 1. HIV infection of resting T cells enhances the cellular response to T cell receptor stimulation. Purified CD4 T cells from four representative healthy donors were infected with HIV-1_{NL4-3} at a MOI of 0.5 (*21*). Five days after exposure to HIV, 10^5 cell aliquots were stimulated with CD3-CD28 beads (at five beads per cell) for 18 hours and IL-2 was measured (*14*). The data represent the mean of three independent determinations (±SD) for activated cells either infected (solid bars) or not infected (open bars). Eleven of 12 donors displayed IL-2 enhancement. To prevent reverse transcription, 50 μM AZT was added (donors 3 and 4, hatched bars).

To detect HIV integration into the host genome, we used an Alu sequence–based PCR strategy. The Alu sequence is a ubiquitous repeat element found in the human genome but absent in HIV. Thus, by amplifying the junction between the nearest Alu sequence and the HIV LTR (Alu/L1, Fig. 2A), proviral DNA was specifically demonstrated. With a sensitivity capable of detecting less than five cells with a single integrated HIV genome (8E5 cells, Fig. 2C), no HIV integration was seen in 5×10^4 resting CD4 T cells 5 days after infection (Fig. 2C).

Under conditions where integration was undetectable, we looked for viral transcription. All HIV transcripts are derived from a common full-length precursor, which also serves as the mRNA for the *gag-pol* genes and the viral genomic RNA. By alternative splicing, this full-length precursor generates about 30 different messages, including singly spliced transcripts coding for Env, Vpu, Vpr, and Vif, and multiply spliced transcripts coding for Nef, Tat, and Rev (*22*). We took advantage of variable regions in splicing donors and acceptors to amplify a specific set of transcripts with reverse transcriptase PCR (RT-PCR) (Fig. 2A). Among the multiply spliced transcripts, *nef* was prominent in quiescent cells, along with lesser levels of *tat* message (Fig. 2D, lane 1). No other multiply spliced transcripts were evident. By comparison, activated cells generated abundant levels of *nef*, *tat*, and *rev* transcripts (Fig. 2D, lane 2). Our data are consistent with previous work by Spina *et al.* (*23*), who found that *nef* is the predominant transcript in HIV-infected resting T cells. Of singly and unspliced transcripts, only one transcript was detected. This transcript is one of the predicted singly spliced *env* transcripts, which share the splice acceptor with *nef* (Fig. 2D, lane 4). Activated cells generated all the predicted products, including the full-length unspliced transcript (Fig. 2D, lane 5).

To ensure that the transcriptional activity in resting cells was not the result of undetected integration, we also examined the activity of a nonintegrating mutant of the NL4-3 strain, HIV-$1_{IN/D116N}$, which carries the point mutation $Asp^{116} \rightarrow Asn$ (D116N) in the viral integrase catalytic domain (*24*). This point mutation disables the viral integrase activity and renders this virus nonreplicative. The incubation of equivalent viral levels of wild-type HIV-1_{NL4-3} and HIV-$1_{IN/D116N}$ with resting CD4 T cells resulted in similar levels of full-length viral DNA (Fig. 3A) and an identical pattern of transcription, including a

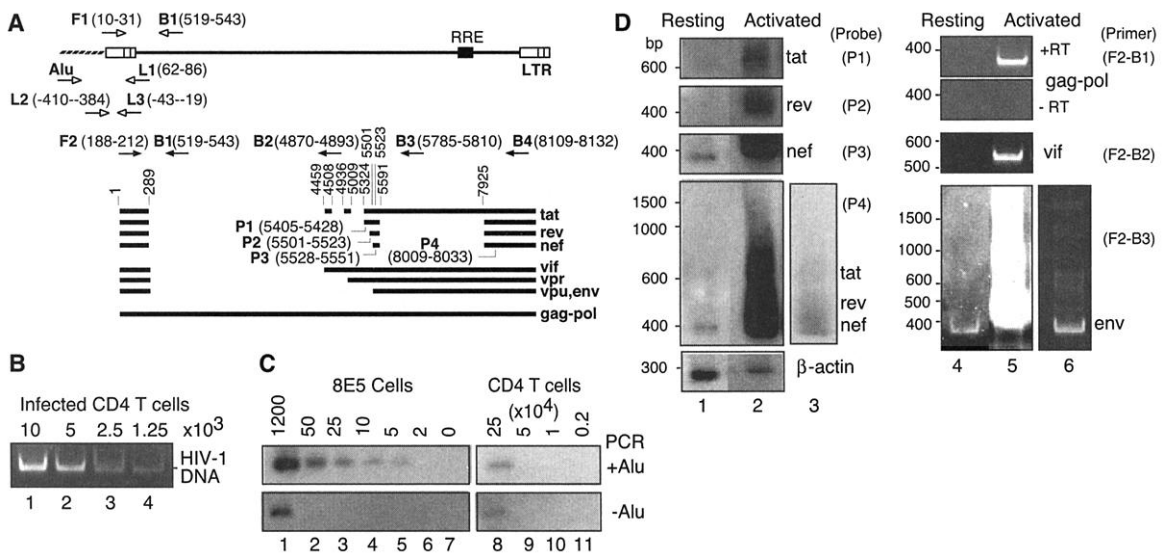

Fig. 2. Unintegrated HIV DNA in resting T cells transcribes early genes. (**A**) Schematic representation of PCR amplification of the HIV proviral genome and its transcripts. Primers used for detection of full-length viral DNA are indicated by open arrows (F1/B1), whereas those for RT-PCR amplification of viral mRNAs (*21*), doubly spliced (F2/B4), singly spliced (F2/B2 and F2/B3), or unspliced (F2/B1), are indicated by solid arrows. The direction of the arrows indicates primer orientation, and the numbers in parentheses indicate the locations of these primers. Splice donors and acceptors are marked by vertical numbers. The probes used for hybridization of the PCR products are indicated by P1 to P4. (**B**) Viral DNA synthesis was detected by PCR amplification of serially diluted nuclear DNA from infected resting CD4 T cells (MOI = 0.02; 5 days after infection). (**C**) Infected resting T cells lack integrated HIV DNA. Resting CD4 T cells were infected (MOI = 0.02) and incubated further for 5 days in a resting state. PCR amplifications (Alu/L1) of existing Alu-HIV LTR junctions were subjected to a second round of PCR with HIV-1 LTR–specific primers (L2/L3) (*21*). 8E5 cells [obtained through the AIDS Research and Reference Reagent Program from T. Folks (*33*)], used here as a positive control, possess a single integrated HIV genome. (**D**) *nef* and *tat* genes are transcribed in resting T cells. Resting (lanes 1 and 4) or activated (lanes 2 and 5) CD4 T cells were infected (MOI = 0.02) and harvested at 5 days after infection. Messenger RNA from 10^4 cells was treated with deoxyribonuclease I and subjected to RT-PCR amplification with primers F2/B4 (lanes 1 and 2). Products were hybridized with digoxingenin-labeled probes specific for *tat* (P1), *tat/rev* (P2), *tat/rev/nef* (P3), or multiple viral transcripts (P4). The human β-actin transcript was coamplified for relative quantification (β-actin). Lane 3 is a short exposure of lane 2. The same reverse-transcribed mRNA was further amplified by primers F2/B3, F2/B2, or F2/B1 to detect singly spliced and unspliced transcripts (lanes 4 and 5). The lack of product when the mRNA was directly amplified with F2/B1 in the absence of reverse transcriptase (–RT) indicated that there was no DNA contamination.

predominant *nef* transcript, along with the *tat* and singly spliced *env* message (Fig. 3B). No full-length viral transcript was detected. These data support the earlier conclusion that in resting T cells, where integration of viral DNA does not occur, viral gene transcription is initiated.

Given the presence of the transcripts, we then looked for the Nef protein. Immediately after the infection of resting T cells, Nef was not detected by Western analysis (Fig. 3C, lane 2). However, 5 days after infection with wild-type (wt) virus (Fig. 3C, lane 3), Nef protein synthesis became evident. Additionally, Nef protein was detected in HIV-$1_{IN/D116N}$–infected quiescent T cells (Fig. 3C, lane 4), and the protein level of Nef appeared to be comparable to that generated by wt virus. Although *tat* transcription was present in all infections of quiescent cells, we were unable to detect Tat protein by these methods. This is consistent with the poor sensitivity of Tat Western analysis.

Thus, the absence of integrase activity did not affect patterns of gene expression in viral infection of resting T cells. As shown in Fig. 3D, the enhancements in T cell activation were also unaffected by the integrase mutation. This finding establishes that the infection of quiescent CD4 T cells with HIV can affect the response of that cell to stimuli before integration of viral DNA.

Because *nef* is a prominent transcript, but dispensable for viral replication, we then directly evaluated the contribution of *nef* to cell activity by infecting resting T cells with a Nef-negative virus. The preparation of viral stocks in the absence of *nef* diminishes the virion infectivity (*25–27*), but this can be corrected for single-cycle infection by expression of Nef in trans in the cell generating the virus (*20*, *28*, *29*). Quiescent CD4 T cells were infected with equivalent doses of either wt or Nef-negative [nef/DS (double stop mutant; two stop codons in *nef* open reading frame); Nef in trans] HIV (*21*). Five days later, the infected resting T cells were activated by CD3-CD28 beads and IL-2 was measured. As in the previous activation

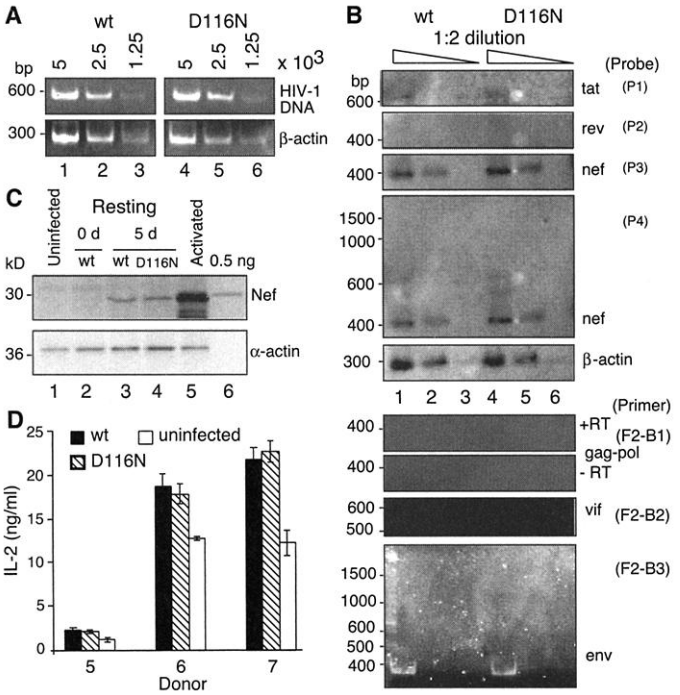

Fig. 3. HIV DNA synthesis, RNA transcription, Nef synthesis, and T cell activation enhancement are independent of integration; a comparison of wt HIV-1_{NL4-3} (wt) and integrase mutant HIV-1_{D116N} (D116N). (**A**) Detection of viral DNA synthesis was carried out by PCR amplification of serially diluted nuclear DNA from infected resting CD4 T cells (equal virion levels, wt or D116N; examined 5 days after infection). Viral-specific F1/B1 primers and cellular β-actin primers (β-actin) were used (*21*). (**B**) Quantitative RT-PCR analysis of HIV-1 transcripts. Messenger RNA from 10^4 infected resting T cells was serially diluted (1:2), then subjected to RT-PCR amplification as in Fig. 2D. (**C**) Detection of Nef protein in resting T cells. Quiescent T cells were infected as in Fig. 1 with wt or D116N, and the equivalence of 10^6 cells was examined by Western blot for Nef protein (*21*). Uninfected cells (Uninfected), cells harvested immediately after infection (0 days), and activated infected cells (Activated) were examined. Cellular β-actin protein and 0.5 ng of recombinant Nef protein were also measured on the same blot. (**D**) IL-2 secretion by cells infected with an equivalent virion level of HIV-1_{NL4-3} (wt, solid bars) or the integrase mutant (D116N, hatched bars). Infection of resting T cells, activation, and IL-2 measurement were done as in Fig. 1.

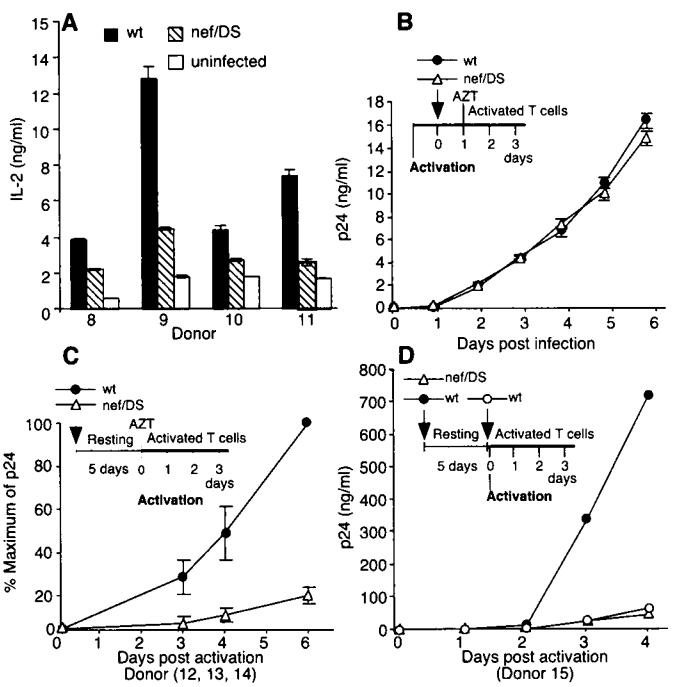

Fig. 4. The HIV *nef* gene enhances IL-2 secretion and viral output after the stimulation of infected resting T lymphocytes, a comparison of wt and Nef-negative (nef/DS) virus. (**A**) IL-2 secretion by CD4 T cells infected with equivalent p24 levels of HIV-1_{NL4-3} (wt, solid bars) or HIV-$1_{nef/DS}$ (nef/DS, hatched bars). The results are the average (±SE) of three independent determinations. Open bars, uninfected T cells. (**B**) Infectivity is identical for HIV-1_{NL4-3} (wt, solid circles) and HIV-$1_{nef/DS}$ (nef/DS, open triangles) generated with Nef expressed in trans in prestimulated [with phytohemagglutinin (3 μg/ml), and IL-2 (5 U/ml)] T lymphocytes. Equivalent doses (p24) of virus were used for infection of 10^6 cells, and at 24 hours after infection, AZT (50 μM) was added. Results are the average p24 production from three independent determinations (±SE). A second set of experiments with AZT addition at 12 hours yielded equivalent results (*16*). Infectivity equivalence was also demonstrated by the infection of a HeLa CD4 indicator line (*21*). (**C**) The HIV *nef* gene increases viral output from infected resting T cells. T cells from three donors were infected as in (B) and stimulated with CD3-CD28 beads at 5 days after infection. AZT (50 μM) was added at this time to limit replication to a single cycle, and secreted p24 was measured. Results are the mean value (±SE) of p24, normalized to 100% of the maximal levels at day 6 for infection by wt virus. The maximal HIV p24 values for the three donors were 82, 347, and 544 ng/ml. (**D**) Enhancement of viral output by *nef* requires incubation before T cell stimulation. Purified CD4 T cells were infected as in (B). Cells infected with wt HIV were activated with CD3-CD28 beads either 5 days after infection (wt, solid circles) or immediately after infection (wt, open circles). The *nef*-negative HIV (nef/DS; triangles) infected cells were activated after a 5-day incubation. In this experiment, AZT was not added.

studies, the absolute level of IL-2 varied between donors. For each of the cell populations from four donors (Fig. 4A), mutation of the *nef* gene resulted in diminished T cell sensitization. There remained a varied viral-mediated sensitization with the Nef-negative virus, presumably because of Tat. Both HIV infection and either Tat or Nef expression in primary cells result in increased T cell activity as defined by IL-2 (*12*, *14*, *15*), and these enhancements have been shown to vary with the donor by yet-unidentified mechanisms.

The ability of HIV to promote an active state in quiescent T cells would be expected to also positively influence viral replication from infected resting cells. Compared to the wt HIV, the Nef-negative virus had a similar infectivity in preactivated T cells for single-cycle viral production (Fig. 4B). However, the infection of quiescent cells, followed by a 5-day resting state before activation, resulted in an increase in viral replication when a functional *nef* gene was present (Fig. 4C). This increase in viral synthesis is due to Nef alone, and unlike the IL-2 study above, the comparison does not include the effect of Tat expression on viral replication from resting T cells. It also differs from the IL-2 study in that the generated data do not include the activity of uninfected cells. We also found that if the 5-day preactivation incubation, during which the viral gene products are synthesized, is eliminated, the enhancement is lost, with wt and Nef-negative virions yielding similar viral production (Fig. 4D).

This Nef-mediated effect is in addition to the previously characterized increase in virion infectivity (*25*–*27*). Whereas the increase in infectivity is manifest before viral gene expression in the newly infected cell (*20*, *28*, *29*), the positive effect on viral output from quiescent T cells is dependent on viral gene activity in the newly infected cell.

Our ability to detect two of the multiply spliced transcripts, *nef* and *tat*, but not the third, *rev*, suggests that the demonstrated singly spliced transcript for *env* in resting T cells (Fig. 2D) is not likely to become transported to the cytosol (*30*). About 80% of the singly spliced *env* message is spliced at the *nef* site (*22*), and in our system this *env* transcript may be a precursor to the doubly spliced *nef* transcript. Our findings are in part corroborated by previous work, in which reverse-transcribed DNA or gene transcription by integrase mutants has been indicated (*3*, *5*, *24*, *31*). Because cell-cycle progression of primary T cells past the G_{1a} stage is essential for HIV reverse transcription (*32*), we presume that our population, although not supportive of viral replication, includes cells at various stages as found in vivo.

Beyond the potential to alter resting T cells in vivo, the capacity of preintegration transcription by HIV raises other issues. HIV may be able to affect cell function in the absence of productive infection, such as in nonlymphatic cells where binding and entry (but not integration) can occur. Moreover, the extensive presence of unintegrated HIV DNA in T cells of infected individuals may have an underappreciated bioactivity. Last, with the ability to transcribe in the absence of proviral formation, HIV could induce cytotoxic T lymphocyte recognition and destruction of a cell that is not replicating virus particles.

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21. Supplementary material, including information on T cell purification, virus infection, quantitative RT-PCR, hybridization, cloning, Nef Western blotting, and detection of integrated DNA, as well as Web table 1 and Web figs. 1 and 2 are available on *Science* Online at www.sciencemag.org/cgi/content/full/293/5534/1503/DC1.
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Representation of Perceived Object Shape by the Human Lateral Occipital Complex

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The human lateral occipital complex (LOC) has been implicated in object recognition, but it is unknown whether this region represents low-level image features or perceived object shape. We used an event-related functional magnetic resonance imaging adaptation paradigm in which the response to pairs of successively presented stimuli is lower when they are identical than when they are different. Adaptation across a change between the two stimuli in a pair provides evidence for a common neural representation invariant to that change. We found adaptation in the LOC when perceived shape was identical but contours differed, but not when contours were identical but perceived shape differed. These data indicate that the LOC represents not simple image features, but rather higher level shape information.

A central goal for any theory of human visual object recognition is to characterize the internal representations we extract from visually presented objects. Recent findings from neuroimaging in humans suggest that the LOC (Fig. 1) plays a critical role in object recognition. These studies (*1*–*6*) further suggest that the LOC may represent object shape independent of the particular visual features (e.g. luminance, motion, texture, or stereoscopic depth cues) that define that shape. However, previous results are also consistent with the possibility that the LOC instead represents low-level information about visual contours. The two hypotheses are difficult to distinguish because contours are always present in images of objects. However, contour and shape information are not the same thing: A given shape can be represented by more than one set of local contours (Fig. 2A), and a given set of contours can represent more than one shape (Fig. 2B). The present