39. M. M. Clark, *U.S. Geol. Surv. Prof. Pap. 787* (1972), p. 55.
40. J. E. Ebel and D. V. Helmberger, *Bull. Seismol. Soc. Am.* **72**, 413 (1982).
41. T. H. Heaton and D. V. Helmberger, *ibid.* **67**, 315 (1977).
42. T. C. Hanks and M. Wyss, *ibid.* **62**, 561 (1972).
43. H. Kanamori and P. C. Jennings, *ibid.* **68**, 471 (1978).
44. M. D. Petersen *et al.*, *Tectonics* **10**, 1187 (1991).
45. W. Thatcher and R. M. Hamilton, *Geol. Soc. Am. Bull.* **63**, 647 (1973).
46. A. L. Bent, D. V. Helmberger, R. J. Stead, P. Ho-Liu, *Bull. Seismol. Soc. Am.* **79**, 500 (1989).
47. H. Magistrale, L. Jones, H. Kanamori, *ibid.* p. 239.
48. S. A. Sipkin, *ibid.* p. 493.
49. R. V. Sharp *et al.*, *ibid.*, p. 252.
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# Detection of HIV-1 DNA and Messenger RNA in Individual Cells by PCR-Driven in Situ Hybridization and Flow Cytometry

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**Human immunodeficiency virus type–1 (HIV-1) DNA and messenger RNA sequences in both cell lines and blood obtained directly from HIV-1–infected patients were amplified by polymerase chain reaction and hybridized to fluorescein-labeled probes in situ, and the individually labeled cells were analyzed by flow cytometry. After flow cytometric analysis, heterogeneous cell populations were reproducibly resolved into HIV-1–positive and –negative distributions. Fluorescence microscopy showed that the cellular morphology was preserved and intracellular localization of amplified product DNA was maintained. Retention of nonspecific probe was not observed. Analysis of proviral DNA and viral messenger RNA in cells in the blood of HIV-1–infected patients showed that the HIV-1 genome persists in a large reservoir of latently infected cells. With the use of this technique it is now possible to detect single-copy DNA or low-abundance messenger RNA rapidly and reproducibly in a minor subpopulation of cells in suspension at single-cell resolution and to sort those cells for further characterization.**

Interactions between HIV-1 and its host cell extend across a wide spectrum, from latent to productive infection. The virus can persist in cells as unintegrated DNA (*1*), as integrated DNA with alternative states of viral gene expression (*2*), or as a defective DNA molecule (*3*). Determining the fraction of cells in the blood that are latently or productively infected is important for the understanding of viral pathogenesis and in the design and testing of effective therapeutic interventions. Determining the number of infected cells in a heterogeneous cell population and the proportion of those cells that are transcriptionally quiescent requires the unambiguous identification of low-abundance proviral DNA and viral mRNA at single-cell resolution (*4, 5*).

Detection of rare cells containing specific nucleic acid sequences has been confounded by the low copy number of the target sequence, variation in sequence specificity, and the inability to isolate these cells from a heterogenous population. Rare target sequences can be detected in vitro with quantitative polymerase chain reaction (PCR) (*6, 7*), but the product DNA signal is averaged for the number of input cells that are lysed; thereby the association with individual cells is lost. Although conventional in situ hybridization can unambiguously identify target sequences in a single cell, a low copy number target sequence may not be detected. The combination of PCR with in situ hybridization allows the target sequence to be amplified above the limit of detection while maintaining the cellular architecture (*4, 5*), but a large number of microscopic fields need to be surveyed by a trained observer to demonstrate a rare affected cell.

Flow cytometric detection of DNA sequences in nuclei has been attained by means of fluorescein-linked DNA probes complementary to total genomic DNA (*8*) or highly repetitive chromosome-specific sequences (*9*). Flow cytometry has also been used to detect fluorescein-labeled probes hybridized in situ to high-abundance α-actin mRNA (*10*) or ribosomal RNA (*11*) in populations of cells. The *bcr-abl* fusion gene has been detected in the human K562 myeloid leukemia cell line by flow cytometry after in situ nested-PCR amplification using fluorescein-labeled primers (*12*). Unfortunately, multiple amplification reactions, poor retention of product DNA within the cell, and the potential for mispriming by this technique frequently compromise both sensitivity and specificity. Detection of rare nucleic acid sequences in a minor subpopulation of cells in suspension by flow cytometry has not been demonstrated.

To detect single-copy proviral DNA or low-abundance viral mRNA in a subpopulation of cells in suspension, we used PCR-driven in situ hybridization with fluorescein-linked oligonucleotide probes and flow cytometry. Intracellular DNA and RNA sequences were preserved in situ with a nonaldehyde, noncross-linking, water-soluble fixative [Streck Tissue Fixative (STF); Streck Labs, Omaha, Nebraska], and the cell membrane was permeabilized with proteinase K (1 μg/ml). Specific intracellular nucleic acid sequences were amplified by a PCR protocol in which bulky digoxigenin-linked deoxyuridine 5′-triphosphate (dUTP) was used to produce a product DNA that remained in place in situ. Product DNA was then hybridized in situ with an internally conserved, fluorescein-labeled oligonucleotide probe, and the cell suspension was analyzed by flow cytometry.

To demonstrate PCR-driven in situ amplification of viral sequences in cells in suspension, we amplified proviral DNA sequences in HIV-1–infected 8E5/LAV cells (*13*), which contain a single copy of proviral DNA, by PCR with HIV-1–specific primers and digoxigenin-linked dUTP (*14*). The cell suspension containing known ratios of 8E5/LAV cells and uninfected peripheral blood mononuclear cells (PBMCs) was centrifuged onto microscope slides that had been treated with Denhardt's solution. Cells were visualized by light microscopy after immunohistochemical staining (*15*) with alkaline phosphatase–conjugated anti-digoxigenin (Fig. 1A). A 100% sensitivity and a 98% specificity was attained on the basis of duplicate 500-cell counts. Each experiment was done in triplicate. Nonspecific alkaline phosphatase–conjugated anti-digoxigenin staining was not detected.

The procedure was optimized for hybridization of fluorescein-labeled oligonucleotide probes to cells in suspension under conditions of high stringency (*10*). Oligonucleotides with fluorescein-linked nucleotides positioned at 10- to 12-nucleotide intervals were synthesized by phosphoramidite chemistry (*16*). Intracellular product DNA was hybridized to either the specific viral probe or a noncomplementary probe in situ (*17*). Intracellular HLA-DQα product DNA sequences hybridized to either complementary

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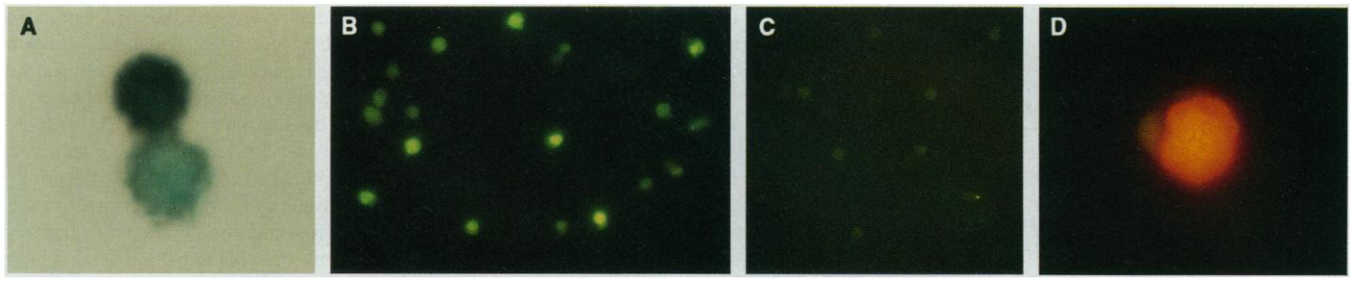


**Fig. 1.** Detection of product DNA in the 8E5/LAV cell line by PCR-driven in situ hybridization. Intracellular localization of HIV-1 product DNA in cells was detected by immunochemistry with conventional light microscopy (magnification, ×1000). Positive cells are stained brown and negative cells are stained green (**A**). Amplified HIV-1 DNA in cells detected by fluorescence-linked oligonucleotide solution hybridization and visualized by fluorescence microscopy with a monochromatic blue filter (magnification, ×200). (**B**) Positive cells are bright fluorescent green and (**C**) negative cells are dim fluorescent green. (**D**) Intracellular localization of amplified HIV-1 *tat* cDNA in a cell detected by fluorescence-linked oligonucleotide hybridization, counterstained with propidium iodide, and visualized with fluorescence microscopy with a monochromatic blue filter (magnification, ×1000).

or noncomplementary probes were used as positive and negative controls, respectively. Proviral DNA or HLA-DQα DNA sequences were amplified by PCR in situ in mixtures of 8E5/LAV cells and uninfected PBMCs in suspension (*18*). The heterogeneous cell population was centrifuged onto microscope slides that had been treated with Denhardt's solution and visualized by fluorescence microscopy, which showed that the cellular morphology was preserved, cellular debris was minimal, and intracellular localization of product DNA was maintained (Fig. 1B). The noncomplementary probe did not hybridize to the product DNA in situ (Fig. 1C). Nonspecific binding was not observed.

To demonstrate that PCR-driven in situ hybridization of heterogeneous cell populations in suspension can be analyzed by flow cytometry, we allowed virus-specific product DNA to hybridize in situ to either a complementary or noncomplementary fluorescein-linked probe. The cells were then counterstained with propidium iodide (0.01 μg/ml) and sorted (*19*). To determine the sensitivity and specificity of this technique, we used the HIV-1–infected 8E5/LAV cell line and HIV-1–infected CEM cells (*20*). Figure 2A shows a standard curve calculated from serial dilutions of 8E5/LAV cells and uninfected PBMCs that were mixed and then analyzed by amplification, in situ hybridization, and flow cytometry. Using a least squares fit linear regression analysis, we found a linear relation for the detection of a single copy of intracellular proviral DNA over a wide range of HIV-1–infected cell concentrations (correlation coefficient, $r = 0.99$). A representative histogram of the logarithm of green fluorescence (Fig. 2B) illustrates a clear distinction between the positive and negative cell populations. Amplification of viral sequences in 8E5/LAV cells by PCR with the virus-specific primers and hybridization to the HLA-DQα 5′-fluorescein–labeled probe in situ did not produce a fluorescence signal above background. Similarly, 8E5/LAV cells amplified in the absence of *Thermus aquaticus* (*Taq*) DNA polymerase and hybridized with the virus-specific probe also did not produce a signal above background. Virus-specific product DNA recovered from the cellular lysates of the STF-treated cells and analyzed by Southern (DNA) blot hybridization was the predicted size (*21, 22*).

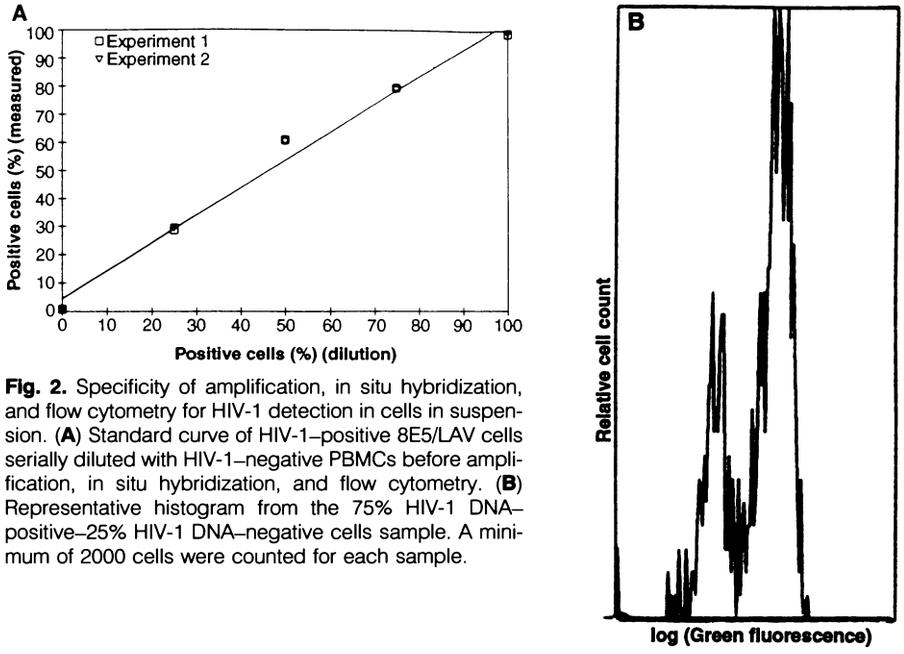


**Fig. 2.** Specificity of amplification, in situ hybridization, and flow cytometry for HIV-1 detection in cells in suspension. (**A**) Standard curve of HIV-1–positive 8E5/LAV cells serially diluted with HIV-1–negative PBMCs before amplification, in situ hybridization, and flow cytometry. (**B**) Representative histogram from the 75% HIV-1 DNA–positive–25% HIV-1 DNA–negative cells sample. A minimum of 2000 cells were counted for each sample.

Flow cytometry also was used to analyze mRNA sequences in the cytoplasm of cells in suspension (*23*). CEM cells productively infected with the HIV-1 NL4-3 strain (CEM/NL4-3) (*24*) and uninfected CEM cells were used as positive and negative controls, respectively. Viral transcripts were reverse-transcribed to cDNA, and the multiple-spliced *tat* mRNA was amplified with MF5869-MF8760, which flanks the splice junction between nucleotides 6083 and 8431 (numbered according to the HIV-1 HXB2 strain) (*25*). To detect the low-abundance *tat* mRNAs, we allowed product DNA to hybridize in situ to a *tat* mRNA-specific fluorescein-labeled probe that spans the splice junction (*25*). Fluorescence micrographs of cells that were centrifuged onto microscope slides showed specific intracellular localization of the fluorescein-labeled probe (Fig. 1D). CEM/NL4-3 cells amplified by PCR with MF5869-MF8760, hybridized in situ with the transcript-specific probe, and analyzed by flow cytometry showed a replicative experimental range of 28 to 35% positivity. Hybridization with the noncomplementary provirus probe did not produce a signal above background, nor did amplification without rTth reverse transcriptase followed by hybridization with the complementary probe.

To determine the fraction of cells in blood of HIV-1–infected patients that had

**Table 1.** Quantification of cells harboring amplified HIV-1 proviral DNA or tat cDNA. Blood was obtained by phlebotomy from nine HIV-1–infected and two uninfected study participants enrolled in the Multicenter AIDS Cohort Study in Chicago. Each HIV-1–infected study participant had a stable $CD4^+$ number for at least 18 months before analysis and had not received any antiretroviral therapy during this time interval. PBMCs were separated by Histopaque 1077 discontinuous density gradient centrifugation and were processed for flow cytometric analysis as described (*14, 18, 23*). Blood was also obtained for a white blood cell count, $CD4^+$ and $CD8^+$ T lymphocyte subset analysis, and p24 antigen testing with standard techniques (*33*). One subject (patient 6) was p24 antigen–positive. The values are an arithmetic mean (±SD) for at least duplicate determinations.

| Patient | CD4 count | HIV-1 DNA (%) | HIV-1 tat mRNA (%) |
|---|---|---|---|
| 1 | 96 | 15 ± 3.0 | 1 ± 0.2 |
| 2 | 113 | 7 ± 0.6 | 1 ± 0.4 |
| 3 | 196 | 10 ± 2.7 | 3 ± 0.9 |
| 4 | 234 | 11 ± 3.8 | 2 ± 0.6 |
| 5 | 452 | 4 ± 1.0 | 2 ± 0.2 |
| 6 | 516 | 8 ± 1.3 | <1 ± 0.3 |
| 7 | 615 | 13 ± 3.0 | 8 ± 0.3 |
| 8 | 652 | 8 ± 0.1 | <1 ± 0.2 |
| 9 | 945 | 8 ± 0.6 | <1 ± 0.2 |
| 10 | 1012 | 0 | 0 |
| 11 | 1275 | 0 | 0 |

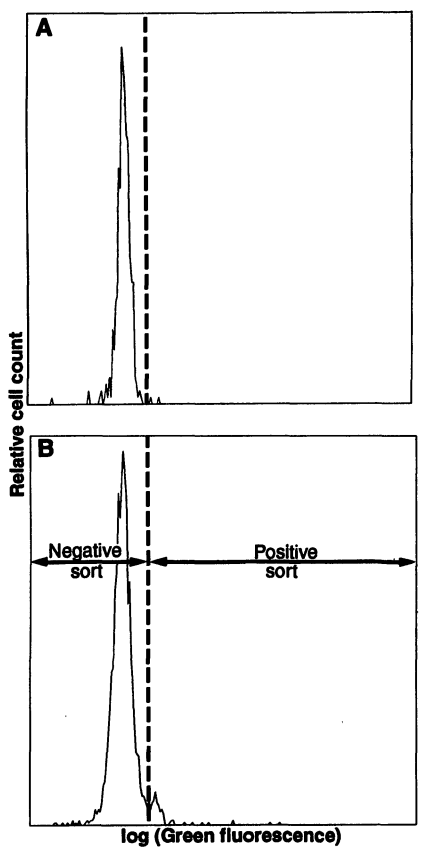


**Fig. 3.** Representative histograms from an uninfected (**A**) and an HIV-1–infected (**B**) patient. The cursor position is indicated by a dashed line. Sorted positive and negative cell populations are indicated by the horizontal bars.

either latent or transcriptionally active viral infection, we analyzed coded samples of PBMCs from nine HIV-1–infected patients with stable $CD4^+$ counts (range, 96 to 945 per microliter) who were not receiving any antiretroviral therapy and from two uninfected subjects for both proviral DNA and viral mRNA by PCR-driven in situ hybridization and flow cytometry. A portion of the cell suspensions was also examined visually by light microscopy after immunohistochemical staining, and the fraction of HIV-1–positive cells was determined. Figure 3, A and B, shows representative histograms for the DNA flow cytometric analyses of the cell populations from an uninfected and an HIV-1–infected patient, respectively. The specificity of the product DNA hybridized in the sorted positive and sorted negative cell lysates was validated by fractionation of a single characteristic peak in the sorted positive cell lysate with the use of fluorescence-hybridization electrophoresis (*21, 26*). Nonspecific hybridization of the noncomplementary probe to the virus-specific product DNA or binding of either the complementary or noncomplementary probe to the negative cell population was not observed.

Table 1 shows the range of the HIV-1–host cell interactions among these nine HIV-1–infected and two uninfected subjects. The percentage of PBMCs with HIV-1 proviral DNA varied from 4 to 15%, whereas the percentage of PBMCs with *tat* mRNA varied from <1 to 8%. These fractions corresponded to the numbers of HIV-1–positive cells detected by amplification and immunohistochemical staining and observed by light microscopy. The percentage of PBMCs that harbor proviral DNA is similar to some (*4, 7*), but not all (*6, 27*), previous estimates. Similarly, the level of transcriptionally quiescent virus that we found is consistent with that obtained from an analysis of an HIV-1–infected lymph node biopsy specimen by amplification and in situ hybridization (*4*), although it is higher than that observed by others using conventional in situ hybridization (*28*).

Several immunopathogenic mechanisms of HIV-1 infection have been proposed to explain the functional impairment and quantitative depletion of cells expressing the $CD4^+$ surface antigen (*29*). Indirect cytopathic mechanisms such as superantigen stimulation (*30*), inappropriate cell signaling (*31*), and apoptosis (*32*) have been postulated to account for the observed disparity between a low viral burden and the degree of immune cell dysfunction and destruction. Our data indicate that a significant proportion of PBMCs are infected with HIV-1. In the majority of the PBMCs that harbor HIV-1, the virus is in a latent state. These latently infected cells constitute a reservoir of persistent infection that cannot be targeted by the host's immune surveillance mechanism. Virus sequestered in this immunologically privileged focus could be disseminated within the infected individual despite natural or vaccine-induced immune stimulation (*4*). Because of the magnitude of HIV-1 infection, immune dysregulation and the depletion of lymphocytes and monocytes may be a consequence of viral gene expression in this population of infected cells. Although the relation between viral persistence and gene expression is complex (*1–4*), the level of infection in this population of cells is sufficient to account for the devastation of the immune system in people with advanced disease.

## REFERENCES AND NOTES


1. M. Stevenson, T. L. Stanwick, M. P. Dempsey, C. A. Samonica, *EMBO J.* **9**, 1551 (1990); M. I. Bukrinsky, T. L. Stanwick, M. P. Dempsey, M. Stevenson, *Science* **254**, 423 (1991); J. A. Zack *et al.*, *Cell* **61**, 213 (1990).
2. T. Seshamma, O. Bagasra, D. Trono, D. Baltimore, R. Pomerantz, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10663 (1992).
3. Y. Li *et al.*, *J. Virol.* **65**, 3973 (1991).
4. J. Embretson *et al.*, *Nature* **362**, 359 (1993).
5. O. Bagasra, S. P. Hauptman, H. W. Lischner, M. Sachs, R. J. Pomerantz, *N. Engl. J. Med.* **326**, 1385 (1992); A. T. Haase, E. F. Retzel, K. A. Staskus, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4971 (1990); G. J. Nuovo, P. MacConnell, A. Forde, P. Delvenne, *Am. J. Pathol.* **139**, 847 (1991).
6. S. M. Schnittman *et al.*, *Science* **245**, 305 (1989).
7. K. Hsia and S. A. Spector, *J. Infect. Dis.* **164**, 470 (1991).
8. G. Dudin *et al.*, *Hum. Genet.* **76**, 290 (1987).
9. H. van Dekken, G. J. A. Arkesteijn, J. W. M. Visser, *Cytometry* **11**, 153 (1990); D. Pinkel, J. W. Gray, B. Trask, *Cold Spring Harbor Lab. Symp. Quant. Biol.* **51**, 151 (1986).
10. E. A. Timm, Jr., and C. C. Stewart, *BioTechniques* **12**, 363 (1992).
11. J. G. J. Bauman and P. Bentvelzen, *Cytometry* **9**, 517 (1988).
12. M. J. Embleton, G. Gorochov, P. T. Jones, G. Winter, *Nucleic Acids Res.* **20**, 3831 (1992).
13. The 8E5/LAV established cell line [obtained from T. Folks through the AIDS Research and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD 20892; T. M. Folks *et al.*, *J. Exp. Med.* **164**, 280 (1986)], containing a single copy of integrated HIV-1 proviral DNA per cell, was used as the HIV-1–infected cell copy number control. The 8E5/LAV cells, at a low passage number, were maintained in RPMI 1640 media (Gibco) containing 20% fetal bovine serum (Gibco), 2 mM L-glutamine, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) in a humidified incubator with a 5% $CO_2$ atmosphere.
14. Cell samples were adjusted to a concentration of $1 \times 10^6$ cells per milliliter, and 400 μl of each sample was pelleted at 1500 rpm for 2 min. The supernatant was removed and the cells were resuspended in 50 μl of STF and incubated at room temperature for 15 min. Cells were again pelleted at 1500 rpm for 2 min, resuspended in 25 μl of proteinase K (1 μg/ml) in 0.1 M tris-HCL, 50 mM EDTA (pH 8.0), and incubated at 37°C for 15 min. Cells were pelleted as above, washed with phosphate-buffered saline (PBS), and resuspended in 190 μl of PCR reaction mixture [10 mM tris-HCL (pH 8.3), 50 mM KCL, 1.5 mM $MgCl_2$, 0.25 mM each of deoxyadenosine 5′-triphosphate (dATP), deoxycytidine 5′-triphosphate (dCTP),

and deoxyguanosine 5′-triphosphate (dGTP), 0.14 mM deoxythymidine 5′-triphosphate (dTTP), 4.3 μM dUTP-11-digoxigenin, 100 pmol each of forward and reverse primer, 1.0 μl (5 U) of *Taq* polymerase (Amplitaq, Perkin-Elmer, Norwalk, CT), and 0.001% gelatin (w/v)]. The reaction mixture was amplified in a 48-well thermocycler (Perkin-Elmer Cetus, Norwalk, CT) programmed for 40 cycles of thermal denaturation (94°C for 1 min), reannealing (58°C for 2 min), and extension (74°C for 1.5 min), with 5 s added for each of the 40 cycles.

15. Cycled cells were centrifuged onto microscope slides that had been treated with Denhardt's solution, washed with PBS, and incubated with a 1:1000 dilution of antibody to digoxigenin conjugated with alkaline phosphatase for 2 hours at 37°C. Slides were washed with PBS and incubated with NBT/X-phosphate substrate for 10 min at ambient temperature. Cells were counterstained with Fast green, mounted on cover slips, and visualized under light microscopy.
16. Sequence-specific oligonucleotide probes containing either single or multiple fluorescein-tagged nucleotides were synthesized on a 380B DNA synthesizer (Applied Biosystems, Foster City, CA) with carboxyfluorescein phosphoramidites. Fluoresceinated probes were as follows: MFA-1 (5′-XTTCTCTATCAAAGCAACCCACCTC-CCAATC-3′; nucleotides 6069 to 6083 and 8431 to 8445, numbered according to the HIV-1 HXB2 strain); human lymphocyte antigen (HLA) GH64 (5′-XTGGACCTGGAGAGGAAGGAGACTG-3′), where X represents the position of a 6-carboxyfluorescein amidite (Applied Biosystems); and SK19-3 (5′-ATCCYGGGATTAAATAAAATYGTAA-GAATGTATAGYCCTAC-3′), where Y represents the position of a 5-carboxyfluorescein phosphoramidite [P. Theisen *et al.*, *Tetrahedron Lett.* **33**, 5033 (1992)]. The synthesized material was alkaline-deprotected and purified by elution through an oligonucleotide purification cartridge. 5-Carboxyfluorescein phosphoramidite incorporation was verified by ultraviolet spectroscopy.
17. A 400-pM sample of the viral or HLA-DQα sequence–specific fluorescein-tagged oligonucleotide probe and sonicated herring sperm DNA (10 μg/ml; Sigma) was added to the PCR reaction tube; the sample was denatured at 95°C for 2 min and was then hybridized at 56°C for 2 hours. The cells were washed for 30 min with 2× SSC–50% formamide–bovine serum albumin (BSA; 500 μg/ml) at 42°C, 30 min with 1× SSC–50% formamide–BSA (500 μg/ml) at 42°C, 30 min with 1× SSC-BSA (500 μg/ml) at ambient temperature, and briefly with PBS at ambient temperature. Cells were resuspended in PBS (pH 8.3) and counterstained with propidium iodide (0.01 μg/ml) for flow cytometric analysis.
18. PBMCs were isolated from fresh heparinized blood layered on a Histopaque 1077 (Sigma) discontinuous density gradient and centrifuged for 30 min at 1600 rpm at ambient temperature. The turbid layer was removed and was washed twice with 3 volumes of RPMI and once with PBS. Cells were divided into portions and treated with STF and proteinase K as described (*14*).
19. The cell suspension was filtered through a 37-μm nylon mesh and analyzed by flow cytometry with an EPICS PROFILE II flow cytometer. Laser excitation was 15 mW at 488 nm. Instrument sensitivity was standardized before each experiment with Immuno-Bright calibration beads (Coulter Source, Marietta, GA). The percentage of fluorescence-positive cells was determined by integration over a range of 0.1% positive counts on the identically treated negative sample (100% uninfected PBMCs). At least 2000 events within the propidium iodide–positive window gate were counted for each sample.
20. The established acute lymphoblastic leukemia cell line CEM was obtained from T. Minowada through the AIDS Research and Reagent Program, Division of AIDS, NIAID, NIH. The cells were maintained in RPMI 1640 medium containing 2.5 mM Hepes buffer (pH 7.4), L-glutamine, 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 μg/ml) in a humidified incubator with a 5% $CO_2$ atmosphere.
21. B. K. Patterson *et al.*, unpublished data.
22. Southern blot hybridization was performed as described previously (*25*).
23. After proteinase K treatment (*14*), 40 μl of a reaction mixture containing 10.0 U of thermostable rTth reverse transcriptase (Perkin-Elmer Cetus), 90 mM KCL, 100 mM tris-HCl (pH 8.3), 1.0 mM $MnCl_2$, 200 μM each of dGTP, dATP, and dCTP, 125 μM dithiothreitol, 4 μM dUTP-11-digoxigenin (Boehringer Mannheim), 40 U of RNasin (Promega, Madison, WI) RNase inhibitor, and 100 pmol of the appropriate downstream primer was added to each sample. Samples were incubated for 15 min at 70°C and then placed on ice. Cells were resuspended in 160 μl of a PCR reaction mixture containing 100 mM KCl, 10 mM tris-HCl (pH 8.3), 0.75 mM EDTA, 0.05% Tween-20, 5.0% (v/v) glycerol-chelating buffer (Perkin-Elmer Cetus), 2 mM MgCl, 100 pmol of upstream primer, and 0.5 μl of *Taq* polymerase. Samples were then cycled in an automated thermal cycler. All solutions were made with diethyl pyrocarbonate–treated analytical reagent water (Mallinkrodt).
24. The pNL4-3–infectious molecular clone was obtained from M. Martin through the AIDS Research and Reagent Program, Division of AIDS, NIAID, NIH. Transfections were performed as described previously (*25*).
25. M. R. Furtado, R. Balachandran, P. Gupta, S. M. Wolinsky, *Virology* **185**, 258 (1990).
26. HIV-1–positive and –negative cell populations from the HIV-1–infected patients were sorted (EPICS C, Coulter), and the cells were lysed with proteinase K (100 μg/ml) at 56°C for 45 min. The probe:product DNA heteroduplex was resolved on a 6% nondenaturing polyacrylamide gel and analyzed by a 373 DNA sequencer with GeneScan 672 software (Applied Biosystems).
27. S. M. Schnittman *et al.*, *Ann. Intern. Med.* **113**, 438 (1991); G. Pantaleo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9838 (1991).
28. M. E. Harper, L. M. Marselle, R. C. Gallo, F. Wong-Staal, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 772 (1986).
29. G. Pantaleo, C. Graziosi, A. S. Fauci, *N. Engl. J. Med.* **328**, 327 (1993).
30. C. Janeway, *Nature* **349**, 459 (1991).
31. A. Amadori *et al.*, *J. Immunol.* **148**, 2709 (1992).
32. L. Meyaard *et al.*, *Science* **257**, 217 (1992).
33. K. MacDonell *et al.*, *Am. J. Med.* **89**, 708 (1990).

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# Crystal Structure of Domains 3 and 4 of Rat CD4: Relation to the $NH_2$-Terminal Domains

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**The CD4 antigen is a membrane glycoprotein of T lymphocytes that interacts with major histocompatibility complex class II antigens and is also a receptor for the human immunodeficiency virus. The extracellular portion of CD4 is predicted to fold into four immunoglobulin-like domains. The crystal structure of the third and fourth domains of rat CD4 was solved at 2.8 angstrom resolution and shows that both domains have immunoglobulin folds. Domain 3, however, lacks the disulfide between the beta sheets; this results in an expansion of the domain. There is a difference of 30 degrees in the orientation between domains 3 and 4 when compared with domains 1 and 2. The two CD4 fragment structures provide a basis from which models of the overall receptor can be proposed. These models suggest an extended structure comprising two rigid portions joined by a short and possibly flexible linker region.**

The CD4 antigen is present on the subset of T lymphocytes that recognize foreign antigens in the presence of major histocompatibility complex (MHC) class II antigens [reviewed in (*1*)]. It interacts with MHC class II molecules (*2*) and is also the receptor by which the human immunodeficiency virus (HIV) binds its host (*3*, *4*). The extracellular portion of CD4 has been predicted, on the basis of sequence analysis, to fold into four immunoglobulin (Ig)-like domains, although the evidence for this organization in domain 2 (D2), D3, and D4 is weaker than in D1 (*5*–*8*).

Attempts to determine the structure of the full extracellular portion by protein crystallography have so far been unsuccessful (*9*, *10*); but the structures for the two $NH_2$-terminal domains in which the HIV-binding site is located have been determined, and both have an Ig fold (*11*, *12*). We report the crystal structure, determined at 2.8 Å resolution, of the third and fourth domains (D3D4) of rat CD4. Rat D3D4 shows considerable sequence homology to the equivalent domains from human CD4; however, crystallization of human D3D4, which contains an additional glycosylation site, has not been reported.

A protein consisting of CD4 D3 and D4

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