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T-Cell Activation Influences Initial DNA Synthesis of Simian Immunodeficiency Virus in Resting T Lymphocytes from Macaques

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The relationship between T-cell activation and early events in the replication cycle of simian immunodeficiency virus (SIV) was analyzed in resting T lymphocytes from macaques. We used the polymerase chain reaction to detect an early product of reverse transcription (R/U5) and almost complete viral DNA (long terminal repeat/*gag*). We found that SIV can enter resting T lymphocytes and initiate replication but that the reverse transcription process is not efficient and proceeds slowly in resting cells. Cross-linking the CD3/T-cell receptor complex with monoclonal antibodies, unlike cross-linking either the CD28 or CD2 accessory receptor and like phorbol myristate acetate, induced a rapid increase in viral R/U5 DNA detected within 3 to 6 h postinfection. Anti-CD3 or phorbol myristate acetate induced replication of full-length viral DNA within 6 to 9 h postinfection, but full-length SIV DNA was not detectable at earlier time points. We then compared various inhibitors of T-cell activation for their effects on viral initiation and complete replication. Cyclosporin A, an inhibitor of a distal step in T-cell activation, blocked anti-CD3-induced T-cell proliferation and completion of SIV DNA replication but had no effect on induced increases in SIV R/U5 DNA. By contrast, initial SIV DNA synthesis was partially blocked by inhibitors of very early steps in T-cell activation, including herbimycin A, an inhibitor of protein tyrosine kinases, and by two different inhibitors of protein kinase C, H7 and staurosporine. Since resting T cells do not efficiently complete SIV DNA synthesis and cyclosporin A can block the formation of complete viral DNA induced in activated T cells, a cellular factor(s) present in activated T cells appears to be required for the formation of full-length SIV DNA.

The simian immunodeficiency viruses (SIVs) are primate lentiviruses that have been isolated from a number of African and Asian primates. They are the closest known relatives to human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (12). SIV shares extensive amino acid sequence similarity with HIV-2 and, to a lesser extent, HIV-1 (6, 14). Like HIVs, SIVs are highly tropic for cells expressing CD4 receptors (10, 11, 26, 32).

The early molecular events that occur after infection of CD4 T cells with SIV are not yet well defined (26, 40). After HIV enters host cells, viral DNA is synthesized and can be integrated into the host genome. In vivo, most T cells are in a quiescent state and are activated to proliferate in response to specific antigen. Therefore, blood-borne virus may encounter $CD4^+$ T cells that are not activated. In vitro, HIV can infect resting lymphocytes, but activation of the T cells is required for productive infection showing a block in the complete reverse transcription (61) or at the level of proviral integration (4, 50). In vivo, SIV (17, 60) and HIV (45) are principally found in CD45 RA^- (or $R0^+$) T cells, which are thought to include activated and/or memory T cells (57). When the polymerase chain reaction (PCR) assay and primers detecting HIV initiation were used, the products of viral reverse transcription were detectable in resting cells (61). However, completion of viral DNA synthesis in the cytoplasm and movement of fully replicated virus to the nucleus may be defective or incomplete in resting T cells (4, 50). HIV-1 virions also carry some partial reverse transcripts (31, 53), which may help in the maintenance of the virus in a stable form during latency (31).

Several factors, including mitogens, cytokines, and anti-CD3 antibodies (21, 42), up-regulate HIV expression in vitro in infected cells. Certain T-cell activation signals, while not mitogenic alone, are nevertheless sufficient to induce viral transcription. For instance, signals generated by cross-linking either CD28 (25, 55) or CD2 (3), while not mitogenic, can induce transcription from the HIV long terminal repeats (LTRs).

In this study, we investigated the susceptibility of resting macaque T cells to SIV infection and examined at what level initial virus replication is controlled. We stimulated resting T cells at the time of infection with either phorbol myristate acetate (PMA), an inducer of protein kinase C (PKC), monoclonal antibody (MAb) to the CD3/T-cell receptor (TCR) complex, or MAb to the CD28 or CD2 accessory receptor on T cells or with the recombinant cytokine tissue necrosis factor alpha (TNF-α) (41, 58) or interleukin-2 (IL-2) (33). Our results show that exogenous stimulation of T cells may not be required for initiation of SIV replication. SIV can enter resting T cells and begin viral DNA synthesis, but the reverse transcription process is not efficient and proceeds slowly or incompletely in these cells. Viral replication is enhanced by cross-linking TCRs with anti-CD3 MAb or by PMA. However, inhibitors of T-cell activation, including cyclosporin A (CSA) or PKC inhibitors like H7 or staurosporine (STP), blocked SIV DNA synthesis in activated cells, suggesting that cellular proteins expressed in activated T cells but not resting T cells may be required for replication of full-length SIV DNA.

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MATERIALS AND METHODS

Cells and inhibitors. Uninfected human Hut-78 cells and clone E11S cells derived from Hut-78 cells infected with SIV/Mne (1, 2) were grown in complete RPMI 1640 media (GIBCO, Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah) and supplemented with glutamine (R10 medium). Both E11S and the molecular clone derived from it have been shown to be infectious and pathogenic in multiple macaques species (24, 39). Peripheral blood was obtained from normal *Macaca nemestrina* by venipuncture. Resting T lymphocytes were isolated by centrifuging heparinized blood samples over 95% Ficoll-Hypaque, followed by nylon wool fiber column separation to deplete B cells and macrophages and separation (8) on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient (40 to 60%). The resting T cells in the dense fraction (60% Percoll) were collected and cultured in R10 at 37°C in a 5% CO_2 humidified atmosphere until use. The PKC inhibitor H7 (Seikagaku America, St. Petersburg, Fla.) was stored at 10 mM in distilled water at 4°C. STP (Kamiya Biomedical Company, Thousand Oaks, Calif.) was prepared as a stock solution in dimethyl sulfoxide at 1 mM and stored at 4°C. CSA (Sandoz, Hanover, N.H.) stock was prepared at 10 mg/ml in ethanol and stored at −20°C. A protein tyrosine kinase (PTK) inhibitor, herbimycin A (Kamiya Biochemical), was prepared at 1 mg/ml in dimethyl sulfoxide and stored at 4°C, and 3′-azido-3′-deoxythymidine (zidovudine; AZT) from Burroughs Welcome was prepared at 1 mM and stored at −20°C.

[^{3}H]thymidine incorporation. Activation of resting T cells was also assessed by [^{3}H]thymidine incorporation. First, 10^5 resting T cells were incubated for various times in triplicate in 96-well plates in R10, either alone or in the presence of anti-CD3. Then each well was pulsed for 16 h with 1 μCi of [^{3}H]thymidine, and incorporation of radioactivity was determined with a beta counter after cells were isolated with a Skatron cell harvester.

Viral preparations and infection. Virus used for infection was derived from the cell culture supernatant of a cell clone of SIV/Mne (clone E11S) (1). Cells were removed by centrifugation, and supernatants were stored in aliquots in liquid nitrogen. In experiments for which PCR was performed, the SIV inoculum was filtered through a 0.45-μm-pore-size filter and treated with 8 U of RNase-free DNase (Worthington, Freehold, N.J.) per ml for 30 min at 37°C in the presence of 10 mM $MgCl_2$. The SIV inoculum was pretreated with DNase to remove any SIV-related DNA that might contaminate subsequent DNA preparations, and a control of heat-inactivated (60 min at 60°C) SIV was run in each assay to monitor the efficiency of this treatment. After infection, cells were washed with medium three times to remove residual free virus. In some experiments, SIV-infected cells were incubated in R10 only (unstimulated) or stimulated with 10 ng of PMA (Sigma Chemical Co., St. Louis, Mo.) per ml or with anti-macaque CD3 MAb FN18 (37) at 20 μg/ml, a dose previously shown to activate macaque T cells (16). Stimulation via CD28 or CD2 was carried out under similar conditions, using 20 μg of MAb 9.3 (22) or MAb 9.6, respectively, per ml. Recombinant IL-2 (Cetus, Berkeley, Calif.) was used at 25 to 50 U/ml, and TNF-α (Genzyme, Cambridge, Mass.) was used at 20 μg/ml.

PCR. Total DNA for amplification was prepared by lysing cells in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 150 mM NaCl, and 0.4% sodium dodecyl sulfate. Digestion with 100 μg of proteinase K per ml overnight at 37°C was followed by phenol extraction and ethanol precipitation. The DNA concentration of each sample was quantitated by spectrophotometry, and the DNA samples were also run in agarose gels and visualized by ethidium bromide staining prior to use. DNA from cells infected with live and heat-inactivated virus was extracted at the same time. One microgram of DNA of each sample was amplified in a PCR mixture that contained 0.2 or 1 μM each primer, 200 μM each of the four deoxynucleoside triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and 1.0 U of *Taq* polymerase (Perkin-Elmer Cetus, Branchburg, N.J.). The final volume was 50 μl. A DNA thermal cycler (Perkin-Elmer Cetus) was used to cycle the temperature. The reaction was subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 60°C, and elongation for 3 min at 70°C.

The oligonucleotide primers used for SIV DNA detection were derived from the nucleotide sequence of SIV/Mac (1) and correspond to the nucleotide numbers as follows: (i) for the LTR region-specific oligonucleotide primer pair RO1 (positions 61 to 84 [sense]) and U51 (228 to 251 [antisense]), the amplified product is 191 bp; (ii) for a primer pair specific for the LTR-*gag* region, S1 (228 to 251 [sense]) and S8 (536 to 559 [antisense]), the product is 330 bp, and for S12 (178 to 199 [sense]) and S81 (586 to 618 [antisense]), the product is 440 bp; (iii) for a primer pair specific for the envelope region, env10 (7191 to 7211 [sense]) and env12 (7541 to 7561 [antisense]), the amplified product is 370 bp. A pair of primers complementary to the human β-globin gene (30), PCO4 54-73 and GH20 195-176 (Perkin-Elmer Cetus), that each differ at only one nucleotide from macaque β-globin (44), was run separately in most experiments. This oligonucleotide pair amplifies a 268-bp product. One oligonucleotide of each complementary pair was 5′ end labeled with [^{32}P]ATP by using polynucleotide kinase (New England Biolabs, Inc. Beverly, Mass.). The ^{32}P-labeled PCR products obtained by amplification were analyzed by electrophoresis on 8% nondenaturing polyacrylamide gels and visualized by direct autoradiography of the dried gels exposed to X-Omat AR (Eastman Kodak Co., Rochester, N.Y.) films with an intensifying screen at −70°C.

Quantitation of SIV DNA during PCR amplification was performed by analyzing a standard curve of dilutions of DNA of E11S cells. The copy numbers of SIV included in the standard curve generally ranged from 100 to 10,000 in the reaction, and the individual standard curves used are indicated in the figures.

To quantify our DNA standard, we analyzed DNA of E11S cells by using the conditions described above with an unlabeled pair of primers, SIV-gag1 (positions 586 to 618 [sense]) and SIV-gag2 (positions 1504 to 1537 [antisense]) (43). The PCR product was analyzed on a 1% agarose gel. The target band amplified a 950-bp fragment from the *gag* region, which was then purified, concentrated, and quantified (34). The mass of one copy of this fragment was estimated, and 10-fold dilutions were prepared. All samples were adjusted with salmon sperm DNA to a final concentration of 1 μg. The samples were amplified along with a DNA of E11S cells and a negative control (DNA of Hut-78 cells) in a thermal cycler under the conditions described above. The ^{32}P-labeled PCR products obtained were visualized by acrylamide gel electrophoresis and autoradiography and were quantified by excision of bands and scintillation counting. The efficiency of amplification with primers SIV-gag1 and SIV-gag2 was linear in the range of 10^2 to 10^4 copies. The amount of total DNA (1 μg) in the reaction had no effect on the detection of SIV in the samples, and it was possible to estimate the relative number of copies present in each fraction. In this way, we estimated the copy number of *gag* genes present in the total DNA sample of the E11S cells that were persistently infected with SIV.

We quantified the bands revealed by autoradiography, using the System Visage 2000 (Bio Image Co., Ann Arbor, Mich.), which is a video densitometry and analysis system. The value obtained represents the integrated optical density (IOD) of those elements that lie within the border of the band. In every experiment, we calculated the IOD of every sample including the one for heat-inactivated virus, and this value (background) was subtracted from values for the other samples. The control in each PCR assay was set to 1.00, and the other sample values were related to this value. Values higher than 1.00 indicate an increase, and values below 1.00 indicate a decrease, in the amount of DNA.

Flow cytometric analyses. Flow cytofluorimetric analysis of cell cycle distribution using propidium iodide (PI) and cell surface immunofluorescence was quantified by using a FACScan flow cytometer and Lysis II software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). For DNA content analysis, 5×10^5 T lymphocytes were washed once in cold RPMI 1640 medium and processed as described previously (51). Briefly, cell pellets were resuspended in 125 μl of a hypotonic solution (10 mM Tris-HCl [pH 7.4], 20 mM NaCl, 20 mM $MgCl_2$ [solution A]) containing 2.5 μg of RNase (DNase free; Boehringer Mannheim, Indianapolis, Ind.) and kept on ice for 5 min. Then, 125 μl of a lysis solution (solution A plus 1% Nonidet P-40) was added, and the suspension was kept on ice for another 5 min. Next, cells were incubated for an additional 30 min at 37°C, after which 150 μl of a PI solution (100 μg of PI per ml in phosphate-buffered saline [PBS] containing 0.1% Triton X-100) was added. Nuclei were kept in the dark at 4°C, and fluorescence of individual nuclei was measured during the same day. Ten thousand events were collected, and fluorescence emission above 585 nm was displayed as a linear plot of a channel number on the horizontal axis (red fluorescence intensity) versus number of nuclei on the vertical axis. The fluorescence intensity from cell nuclei stained with PI is proportional to the cellular DNA content (15). Resting cells in G_0 or G_1 phase of the cell cycle have a normal, diploid quantity of DNA per cell, replicating cells in G_2 or M contain twice that amount of DNA, and S-phase cells, which are synthesizing DNA, have an intermediate quantity of DNA per cell.

To quantify IL-2 receptors (IL-2R), 5×10^5 activated T cells were washed in cold PBS plus 3% fetal bovine serum and 0.02% sodium azide (SB buffer), resuspended in 50 μl of SB buffer containing fluorescein-conjugated MAb to the p55 IL-2R (CD25) (54) (Becton Dickinson), and then incubated for 20 min on ice. The stained cells were washed once with cold SB buffer, fixed in 0.5 ml of PBS containing 1% paraformaldehyde (Sigma), and kept at 4°C in the dark until further analysis by flow cytometry. Five thousand cells were evaluated, and the data were plotted as logarithmic green fluorescence intensity (horizontal) versus cell number (vertical).

RESULTS

SIV enters dense resting T cells. We used the PCR assay to determine the ability of SIV to infect dense resting macaque T cells (see Materials and Methods). First, using flow cytometry (15), we analyzed resting T cells for their DNA content. As expected, very dense T cells had a single unimodal and uniform peak of red fluorescence, demonstrating that virtually all T cells used were in the G_0 phase of the cell cycle (Fig. 1A). The PCR assay then was used to specifically amplify SIV DNA sequences in infected resting T cells. We chose a primer pair of the R/U5 region of the LTR (R01/U51) to detect an early step in initiation of reverse transcription (56, 61), a pair of the

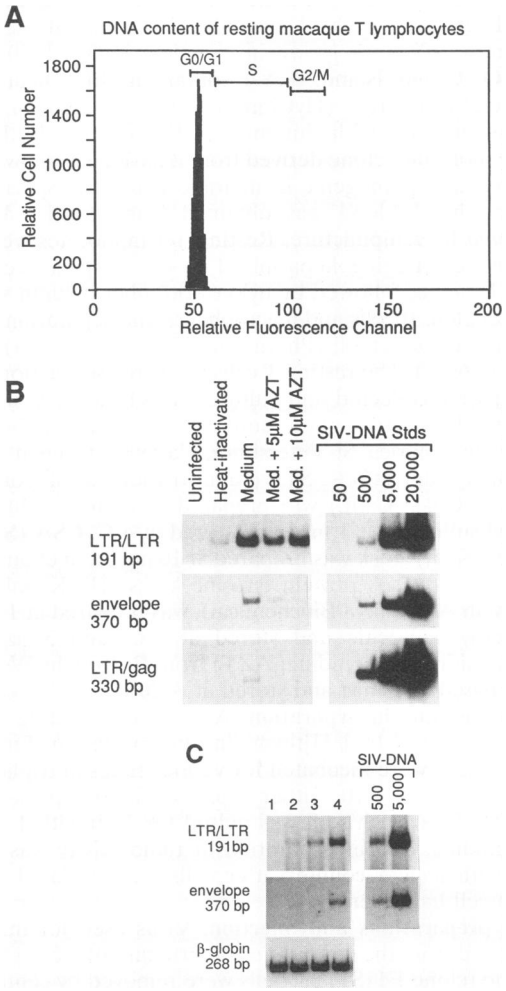

FIG. 1. (A) DNA content of resting macaque T lymphocytes. Cell nuclei were stained with PI and analyzed by flow cytometry. The fluorescence intensity of PI on stained cells is shown and expressed in arbitrary units of a linear scale (channel number) on the horizontal axis versus the cell number on the vertical axis. The coefficient of variation of the G_0 peak in the DNA distribution was less than 5%. (B) AZT pretreatment inhibits reverse transcription of SIV in resting macaque T cells. Resting T lymphocytes (dense fraction) were isolated by centrifugation over a Percoll gradient and then were cultured in the presence or absence of 5 or 10 μM AZT for 4 h prior to infection with SIV/Mne (clone E11S). Heat-inactivated controls were infected in parallel. After 28 h, total cellular DNA was harvested and PCR was performed with 1 μg of DNA, using a primer pair specific either for the LTR region (R01/U51), envelope (env10/env12), or LTR/*gag* (S1/S8) as indicated. Uninfected cell controls were included in the reaction. SIV DNA standards (SIV-DNA Stds) were run in parallel. (C) Resting T lymphocytes were infected with SIV/Mne (clone E11S). After 3 h of infection, the cells were washed exhaustively. Half of the washed cells were DNA extracted at this time (lane 3); the other half were placed in RPMI medium and left for another 15 h, and then DNA was extracted (lane 4). Cells also were infected with heat-inactivated virus during 3 h, and DNA was extracted at that time (lane 2). Uninfected cells (control) are in lane 1. PCR was run on 1 μg of DNA with primers of the LTR region (R01/U51) or envelope (env10/env12). Primers for β-globin were also run concurrently. SIV DNA standards (SIV-DNA) were run in parallel.

envelope region (env10/env12) to detect partially transcribed DNA, and primers of the LTR/*gag* region (S1/S8) that reflect almost completely replicated virus. Because the PCR assay is sensitive to reaction conditions and the amount of target DNA being amplified, it was necessary to compare each PCR measurement with a known standard, amplified by PCR under the same conditions. To determine approximate SIV DNA copy numbers, we ran in parallel an external standard of known SIV copy number.

Some purified preparations of HIV have been found to contain virus-specific DNA that resulted from reverse transcription in newly assembled virus particles (31, 53). To test whether the DNA that we detected by PCR in unstimulated T cells infected with SIV was newly reverse transcribed, we infected unstimulated T cells in the presence or absence of AZT. Total DNA was isolated 28 h postinfection, and PCR was performed on 1 μg of DNA with the LTR (R01/U51), envelope (env10/env12), and LTR/*gag* (S1/S8) primers. The number of SIV DNA copies present in the samples was compared with PCR levels of known amounts of SIV DNA amplified in parallel.

As shown in Fig. 1B, in cells pretreated with 5 or 10 μM AZT, there was a reduction in the amount of DNA detected by PCR (with primers to the envelope or LTR/*gag* region) compared with the level in untreated cells. In a representative experiment (Fig. 1B) using primers of the envelope region, we could detect 500 copies in cells cultured 28 h in medium only and 50 to 100 copies in cells cultured with 10 μM AZT. Using primers of the LTR/*gag* region, we did not detect SIV DNA in the presence of AZT. The levels of PCR product representing initiated viral DNA (LTR/LTR) were similar in untreated and AZT-treated infected cells. However, AZT blocked elongation of newly synthesized SIV reverse transcripts, consistent with previous studies showing that AZT is much more efficient in blocking later HIV products than early ones (53, 62). In the presence of AZT, we were not able to discriminate between possible preexisting virus versus newly synthesized viral DNA detected with primers of the LTR/LTR region. Therefore, we infected unstimulated T cells with SIV in the absence of AZT (Fig. 1C); 3 h after we added the virus, we washed the cells extensively. At that time, DNA was extracted from one half of the infected cells (Fig. 1C, lane 3). The other half was placed in RPMI medium and left for 15 h at 37°C, and then DNA was extracted (Fig. 1C, lane 4). An increase in viral DNA replication was evident in these cultured and unstimulated cells (Fig. 1C, lane 4) compared with LTR/LTR or envelope fragments detectable 3 h postinfection (lane 3), while the amount of LTR/LTR DNA in cells exposed to live virus for 3 h was slightly higher than that detected in the cells exposed to heat-inactivated virus for 3 h (Fig. 1C, lane 2). This difference between cells exposed to live virus and cells exposed to heat-inactivated virus could be due to heat degrading of some preexisting viral DNA or to some initiation occurring within 3 h of infection with live virus. In repeat experiments, viral LTR/LTR DNA levels were 3 to 4.5 times higher in cells cultured in medium overnight after viral infection than in infected but uncultured controls. Thus, reverse transcription of SIV DNA was initiated in unstimulated macaque T cells (Fig. 1B and C), as has been reported for HIV-1 (50, 61).

We next examined whether stimulating T cells with either PMA or anti-CD3 cross-linking MAb would affect the levels of SIV DNA amplified compared with unstimulated T cells. We consider PMA just a positive control and not a physiologic stimulus. Total DNA was isolated 24 to 28 h after addition of SIV in unstimulated or stimulated T cells, and PCR was performed on 1 μg of each DNA sample. As shown in Fig. 2,

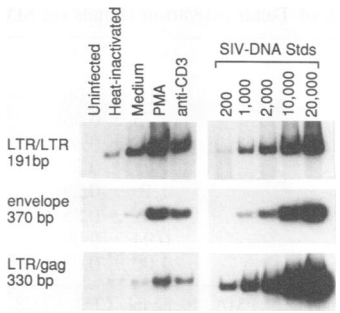

FIG. 2. Detection of SIV DNA in resting and activated T lymphocytes. Resting T lymphocytes maintained in medium or stimulated with PMA (10 ng/ml) or anti-CD3 MAb (20 μg/ml) were infected with SIV/Mne (clone E11S). After 24 h, total cellular DNA was prepared and 1 μg of DNA was analyzed by PCR with the three pairs of primers indicated in Fig. 1B. Standards for each PCR product are shown on the right (SIV-DNA Stds).

stimulation of the cells with PMA or CD3 MAb increased the relative amount of R/U5 SIV DNA compared with R/U5 DNA in unstimulated cells (see also Table 1). A comparison of the number of copies detected with LTR/LTR, envelope, and LTR/*gag* primers suggests that in this macaque system, the efficiency of reverse transcription is <10%. Significant initiation of SIV DNA was detected in total DNA from both stimulated and unstimulated T cells. However, very low levels of LTR/*gag* were present in resting cells 24 to 28 h postinfection, and LTR/*gag* products were not detectable at earlier time points in unstimulated cells (e.g., Fig. 3). Again, induced increases in envelope or LTR/*gag* products could be inhibited with AZT (data not shown). During a 24- to 28-h culture, $CD4^+$ T cells may either activate spontaneously or be activated by factors in serum.

Cross-linking the CD3/TCR complex increases SIV replication in resting T cells. To determine the signal transduction requirements for optimal SIV DNA replication, we stimulated resting T cells exposed to SIV by either cross-linking the CD3, CD2, or CD28 receptor or by treatment with the cytokines TNF-α and IL-2. The signal transduction pathways induced after cross-linking the CD3/TCR complex have been extensively studied (7, 9); PTK, PKC, and calcium-dependent kinases are rapidly activated. Cross-linking CD28 on resting T cells alone does not lead to T-cell proliferation but does activate IL-2 mRNA expression and stability (25). Stimulation via CD28 is an important second signal that augments proliferation induced by anti-CD3 (25) and prevents apoptosis of T cells from HIV-infected individuals (20).

As shown in Table 1, SIV-DNA was enhanced in T cells stimulated for 24 h with CD3 MAb or with PMA three- to fivefold above the level for untreated cells. However, resting T cells treated with TNF-α or IL-2 were not different from controls, even 24 h postinfection. Stimulation via CD28 consistently induced a modest increase of viral DNA, while stimulation via CD2 had little or no effect. The most effective inducers of increased SIV DNA synthesis, anti-CD3 and PMA, were also the most effective in inducing T cells to express CD25 IL-2R (data not shown).

Kinetics of anti-CD3-induced increases in SIV DNA. We next determined how rapidly anti-CD3 or PMA could induce increases in SIV DNA levels. Resting T lymphocytes were infected with SIV, and total DNA was extracted at 3, 6, and 9 h postinfection for PCR analysis of the LTR region (Fig. 3A). Within 3 h postinfection, PMA induced high levels of SIV

TABLE 1. Effects of T-cell activation signals on SIV DNA synthesis

Stimulation[a]	Mean ± SEM[b]	No. of expts
Medium	1.00 ± 0.00	6
PMA	4.73 ± 0.50	6
Anti-CD3	3.88 ± 0.80	5
Anti-CD28	1.77 ± 0.35	4
Anti-CD2	1.10 ± 0.29	3
Anti-CD3 + anti-CD28	4.01 ± 0.85	2
IL-2	0.94 ± 0.09	2
TNF-α	1.00 ± 0.02	2

[a] Doses of reagents used: PMA, 10 ng/ml; CD3, CD28, or CD2 MAb, 20 μg/ml; IL-2, 30 U/ml; TNF-α, 20 μg/ml.

[b] Mean of the enhancement of SIV DNA detected by LTR/LTR primers achieved compared with the unstimulated control value. IOD was obtained as described in Materials and Methods. DNA was extracted 24 to 28 h postinfection.

initiation, nearly 10,000 copies compared with standards. Anti-CD3 MAb induced increases in SIV DNA that were detectable within 3 h but were higher after 6 h of culture with virus.

When primers to the LTR/*gag* region were used (Fig. 3B), no SIV DNA could be detected at 3 h, but it was detectable after 6 to 9 h of stimulation with PMA or after 9 h of stimulation with anti-CD3, only at copy numbers 10 to 100 times less than LTR/LTR numbers. We did not detect SIV-DNA with the LTR/*gag* primer pair in unstimulated cells at these early time points. Similar results were obtained after 7 h of infection (data not shown): full-length SIV DNA was evident only in the cells activated with PMA, not in unstimulated or anti-CD3-activated T cells. Thus, either the time needed to produce LTR/*gag* DNA is greatly delayed in unstimulated cells or complete virus is not produced in unstimulated cells.

Effect of inhibitors of T-cell activation on SIV infection. We next tested certain inhibitors for the ability to affect anti-CD3-induced increases in SIV DNA. CSA is a potent immunosuppressive agent that inhibits T-cell activation by binding to cyclophilin, which is required for a Ca^{2+}-dependent signaling pathway normally leading to increases in IL-2 mRNA (46). As shown in Fig. 4A, CSA profoundly inhibited anti-CD3-dependent T-cell proliferation even at a dose of 0.1 μg/ml. Low doses of CSA also blocked the induction of IL-2R (Fig. 4B), although very high doses toxic to T cells (100 μg/ml) were needed to completely inhibit anti-CD3 induction of IL-2R (Fig. 4B). The numbers of copies of initiated SIV were the same in T cells stimulated with anti-CD3 only and in those stimulated with anti-CD3 in the presence of CSA (Fig. 4C). Concentrations of CSA (1 μg/ml) that inhibited anti-CD3-driven proliferation (Fig. 4A) had no effect on the ability of anti-CD3 to augment initiation of reverse transcription (Fig. 4C; see also Table 3). Thus, T-cell proliferation is not required for anti-CD3 to induce increases in SIV initiation.

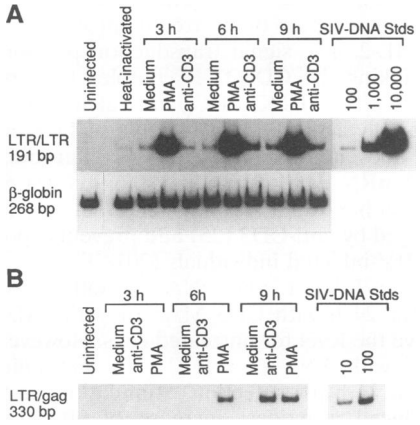

FIG. 3. Kinetics of anti-CD3 activation of SIV-DNA. (A) Resting T cells were infected with SIV/Mne and stimulated with PMA (10 ng/ml) or CD3 MAb FN18 (20 μg/ml) or maintained in medium. Total cellular DNA was extracted at 3, 6, and 9 h postinfection, and 1 μg was analyzed by PCR. Primers of the LTR region (RO1/U51) were used. (B) Detection of SIV DNA at 3, 6, and 9 h postinfection. PCR was run with 1 μg of total DNA. Two rounds were done, the first with the LTR/*gag* region primer pair S12/S81 (30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) and the second with primer pair S1/S8 (25 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C). SIV-DNA Stds, SIV DNA standards.

When we analyzed the effect of CSA on the extension of SIV DNA synthesis induced by anti-CD3 MAb, a different result was obtained. In the presence of anti-CD3, the ratio of initiation to elongation was about 2 (elongation/initiation = 0.5). In the presence of CSA, this ratio shifted to about 4 (0.25), indicating that CSA could reduce elongation by 50% (Fig. 4C). Table 2 shows three additional experiments done in conditions similar to those used for Fig. 4C in which CSA blocked SIV DNA elongation detected with primers of the LTR and envelope regions. The ratio of envelope signal to LTR signal is shown for each sample.

CSA blocks an early step in T-cell activation occurring within hours after T cells are stimulated with anti-CD3 (27, 46) and also inhibits a Ca^{2+}-dependent step in T-cell activation, but it does not inhibit PKC (46). Thus, the data with CSA suggest that anti-CD3 may augment initiation of SIV via an early step in the signaling pathway proximal to the step blocked by CSA. Regulation of T-cell activation is controlled in part by the action of PTKs that are activated within seconds after TCR-CD3 complex binding (7). To determine whether PKC or PTK may influence efficient SIV infection and initiation, we monitored the initiation of reverse transcription in SIV-infected T cells pretreated with kinase inhibitors (Table 3), including herbimycin A, an inhibitor of PTK, and H7 or STP, both which are inhibitors of PKC (23, 48). Incubation of resting T cells with these inhibitors for 2 to 6 h had no effect on the levels of expression of CD4 (data not shown). Treatment with these inhibitors at the doses used also had no effect on cell viability, which remained >95%.

Pretreatment of the cells stimulated with anti-CD3 with herbimycin A consistently reduced the level of R/U5 DNA to one-fourth the level seen in anti-CD3-stimulated T cells after 20 to 24 h postinfection (Table 3). As noted above, CSA had no effect on the initiation of reverse transcription. H7 had no effect on the initiation of reverse transcription, while STP was a more effective inhibitor. H7 at the same dose did effectively block the increase of initiation by PMA (data not shown), indicating that it was actively inhibiting PKC in T cells. This finding suggests that the stimulation of increase in viral DNA via CD3 may not require PKC and that STP, unlike H7, is an effective inhibitor because it affects enzymes other than PKC (47). In summary, these results suggest that a PTK and perhaps another STP-sensitive protein may be required for replication of SIV at an early stage during the infection of T cells by SIV.

DISCUSSION

In this study, we found that SIV can enter and begin reverse transcription in resting T cells and that replication of SIV is influenced by cellular factors. In an early study using Southern blotting, Gowda and coworkers (19) reported that HIV-1

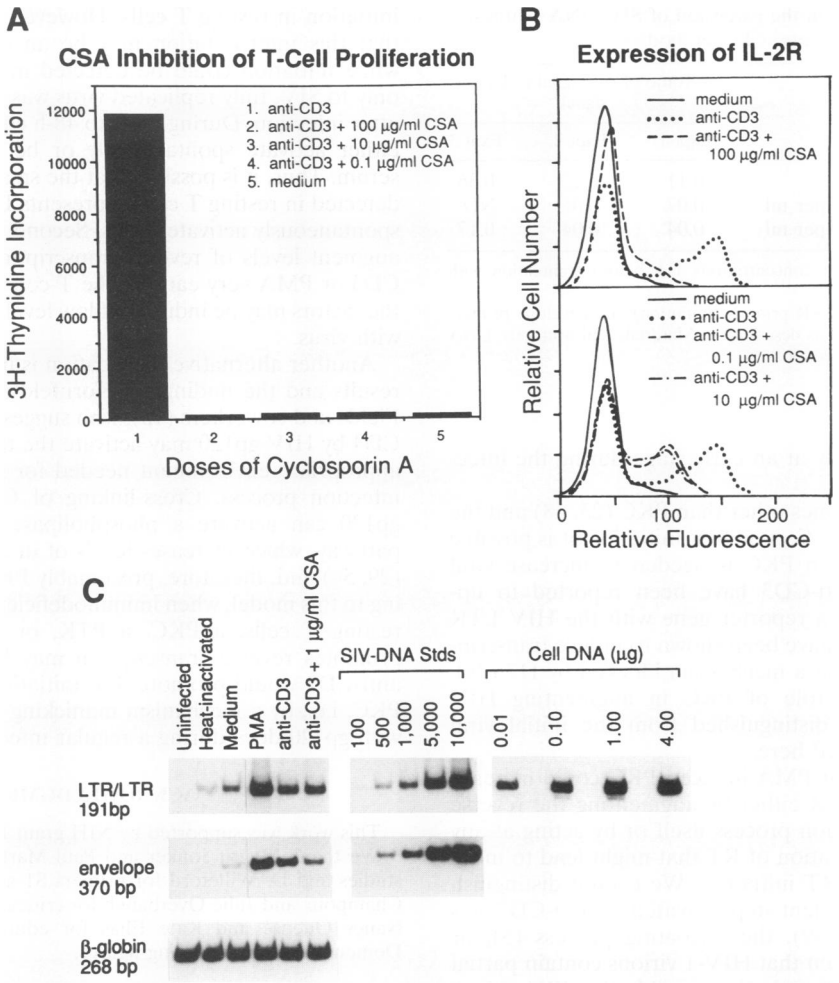

FIG. 4. Effect of CSA on T-lymphocyte stimulation induced by immobilized CD3 MAb. (A) T-lymphocyte proliferation. T lymphocytes were stimulated by immobilized CD3 MAb (20 µg/ml in the presence of 0.1 µg, 10 µg, or 100 µg of CSA per ml). [^{3}H]thymidine incorporation was measured on day 3. (B) Expression of IL-2R. T lymphocytes were stained and analyzed as described in Materials and Methods after 24 h of stimulation with anti-CD3. CSA was used at 0.1, 10, 100 µg/ml. (C) SIV infection. Resting T lymphocytes that had been purified and infected as described in the text were stimulated with PMA (10 ng/ml), anti-CD3 (20 µg/ml), or anti-CD3 plus 1 µg of CSA per ml. Total DNA was prepared 24 h postinfection. PCR was run on 1 µg of DNA with primers of the LTR region (R01/U51) or envelope (env10/env12). Primers for β-globin were also run concurrently. SIV DNA standards (SIV-DNA Stds) and cell DNA standards were run in parallel.

reverse transcribes viral DNA in activated T cells but not in resting T cells examined 48 h after exposure to HIV-1. More recently, using a sensitive PCR assay, two groups have found that while penetration and reverse transcription occur in resting T cells infected with HIV-1, complete transcription of full-length viral DNA is much more efficient in activated T cells (38, 61). Our results for SIV are in accord with these studies using HIV-1.

Cross-linking TCR molecules on resting T cells with CD3 MAb induced detectable increases in SIV DNA within 3 h postinfection and higher levels within 6 h. By contrast, unstimulated T cells had relatively little detectable viral LTR/LTR 6 h after infection. Thus, while SIV can enter resting T lymphocytes and start initiation, this process takes longer than in T cells activated by CD3 MAb.

CSA did not effectively inhibit the effect of anti-CD3 on SIV initiation yet it did block a step induced within hours after T-cell activation, thereby inhibiting T-cell proliferation (27, 46). When we analyzed the effect of CSA on the extension of SIV DNA synthesis induced by anti-CD3, we found that CSA consistently reduced the amount of elongated SIV detected with primers of the envelope region (Fig. 4 and Table 2). These results strongly suggest that efficient replication of complete virus may require additional cellular factors induced by T-cell activation. CSA may simply reduce the overall efficiency of DNA synthesis or, alternatively, could block a step from initiation to elongation. One testable possibility is that CSA blocks increases in the size of the deoxyribonucleoside triphosphate pool in activated cells and thereby reduces overall SIV DNA synthesis. Our data also suggest that anti-CD3 may augment SIV replication via an early step in the signaling pathway proximal to the step blocked by CSA and possibly requiring the activation of kinases such as PKC or PTKs. To determine whether PKC or a PTK may influence efficient SIV infection and initiation, we monitored the initiation of reverse transcription in SIV-infected T cells pretreated with kinase inhibitors. Pretreatment of anti-CD3 stimulated cells with herbimycin A reduced the level of R/U5 DNA fourfold compared with the effect of anti-CD3 (Table 3). Thus, a PTK may be required for anti-CD3 to increase SIV initiation and

TABLE 2. Effect of CSA on the extension of SIV DNA synthesis induced by anti-CD3 antibodies

Stimulation[a]	Ratio of *env* signal to LTR signal[b]		
	Expt 1	Expt 2	Expt 3
Anti-CD3	0.11	0.12	0.36
Anti-CD3 + 0.1 μg of CSA per ml	0.07	0.05	ND[c]
Anti-CD3 + 1.0 μg of CSA per ml	0.04	0.04	0.17

[a] CSA was added at different concentrations 1 h before stimulation with anti-CD3 (20 μg/ml).
[b] SIV DNA was detected by LTR primers (initiation) and envelope primers (elongation). IOD was obtained as described in Materials and Methods. DNA was extracted 20 to 24 h postinfection.
[c] ND, not done.

perhaps required normally at an early stage during the infection of T cells by SIV.

As STP can affect enzymes other than PKC (23, 48) and the PKC inhibitor H7 had no effect on SIV initiation, it is possible that an enzyme other than PKC is needed to increase viral initiation. PMA and anti-CD3 have been reported to upregulate transcription of a reporter gene with the HIV LTR (52), and PKC activators have been shown to induce transcription of new HIV RNA via a mechanism blocked by H7 (28). However, the potential role of PKC in augmenting HIV transcription should be distinguished from the initial viral DNA replication described here.

Theoretically, a PTK or PMA-induced PKC could increase the level of SIV LTR/LTR either by augmenting the reverse transcriptase (RT) initiation process itself or by acting at any step proximal to the activation of RT that might lead to more SIV being available for RT initiation. We cannot distinguish whether the kinase-dependent step activated by anti-CD3 acts to promote viral entry (49), the uncoating process (5), or reverse transcription. Given that HIV-1 virions contain partial reverse transcripts (31, 53), it is also possible that SIV virions contain partial transcripts as well. Thus, any step affecting viral entry theoretically could affect levels of LTR/LTR DNA within the cells even before reverse transcription begins. It is interesting to note Gaulton and coworkers (18) found that while STP completely blocked phorbol dibutyrate-induced phosphorylation of CD4, it only partially blocked anti-CD3-induced phosphorylation of CD4. Thus, it is possible that STP in their experiments was partially reducing SIV initiation by affecting CD4 phosphorylation and internalization. The role of phosphorylation of CD4 in viral entry, however, remains controversial (13, 29, 35).

Our results are consistent with the interpretation that T-cell activation is not required for SIV entry and DNA synthesis initiation in resting T cells. However, several findings suggest that this interpretation may be an oversimplification. First, while initiation could be detected in resting T cells exposed only to SIV, fully replicated virus was detected only 24 to 48 h after infection. During a 24- to 48-h culture, $CD4^+$ T cells may either activate spontaneously or be activated by factors in serum. Thus, it is possible that the small amount of viral DNA detected in resting T cells represents DNA being replicated in spontaneously activated cells. Second, the cellular factors that augment levels of reverse transcription are induced by anti-CD3 or PMA very early in the T-cell activation process; thus, the factors may be induced at low levels in T cells cultured only with virus.

TABLE 3. Effects of inhibitors of PTK (herbimycin A) and PKC (STP) on SIV DNA initiation

Stimulation[a]	Mean ± SEM[b]	No. of expts
Medium	0.57 ± 0.13	7
Anti-CD3	1.00 ± 0.00	7
Anti-CD3 + 50 μM herbimicyn A	0.27 ± 0.09	5
Anti-CD3 + 1 μg of CSA per ml	1.00 ± 0.08	4
Anti-CD3 + 100 μM H7	0.97 ± 0.02	2
Anti-CD3 + 1 μM STP	0.60 ± 0.06	4

[a] Inhibitors were added 1 h before stimulation with anti-CD3 (20 μg/ml).
[b] Mean of the reduction of SIV DNA detected by LTR/LTR primers achieved compared with values for cells stimulated with anti-CD3. IOD was obtained as described in Materials and Methods.

Another alternative explanation is not inconsistent with our results and the findings of Kornfeld and coworkers (29) and Fields and coworkers (13), who suggested that cross-linking of CD4 by HIV gp120 may activate the target T cell to create an appropriate environment needed for subsequent steps in the infection process. Cross-linking of CD4 by either MAb or gp120 can activate a phospholipase C-dependent signaling pathway, which increases levels of inositol 1,4,5-trisphosphate (29, 59) and, therefore, presumably PKC as well (36). According to this model, when immunodeficiency viruses bind CD4 on resting T cells, a PKC, a PTK, or another enzyme which promotes reverse transcription may be activated. PMA and anti-CD3 would promote SIV initiation by activating PTK or PKC, i.e., by a mechanism mimicking what cross-linking CD4 with gp120 does during a regular infection.

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