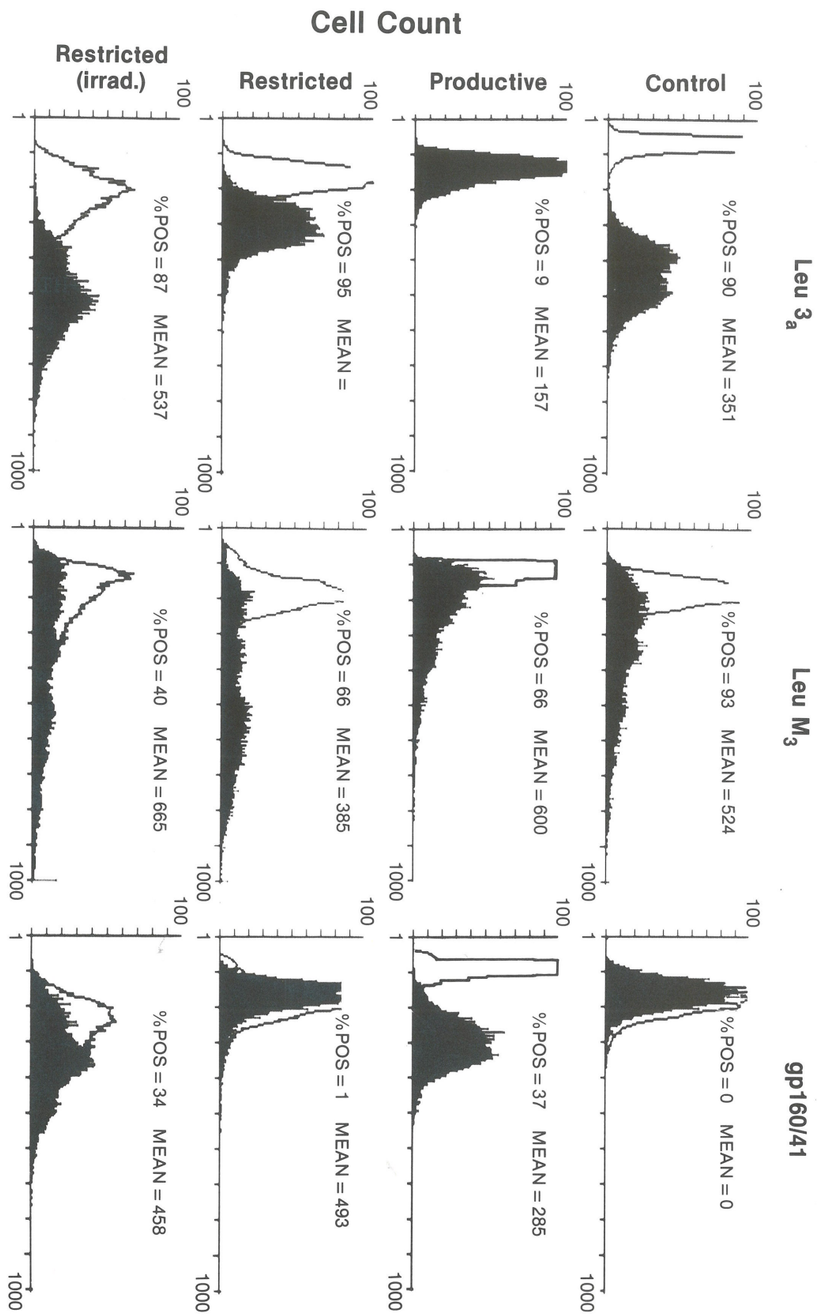


and surface antigens recognized by anti-gp 160:41 (Table 1, Figure 3).

In contrast, 45-60 days after the onset of acute infection, entire cultures of these previously productively infected cells produced little or no extracellular virus (Table 1). Cell surface analysis showed that CD4 antigen could be recognized again while the presence of viral antigens could not be detected (Figure 3). Presence of virus as measured by RT, infectivity, T cell cytopathology and syncytia formation was not detected in the media of these cells.

However, the media of some of these cultures contained p24 core antigen at a concentration of <20 ng/ml as compared to 200-500 ng/ml for productive cultures. These cultures remained HIV-infected as shown by increased expression following treatment of the cells with LPS, (Table I) or irradiation (Figure 3). 72 hours following activation of the cells, extracellular virus was present, as measured by all the criteria used; and viral antigens could again be detected on the cells. These data suggested that viral expression was restricted in these cells. Interestingly, several nonproducer THP-1 cultures remained nonproducers after activation (Table 1). This absence or low level viral production in infected THP-1 cells was reproducibly seen with two other isolates, HIV-1 ADA, a monocytoid isolate and HIV-2 Rod (Table 1).

Conditions for Establishing Restricted Viral Production in THP-1 Cells. To better understand the conditions necessary for the establishment of restricted expression, experiments were done to examine the effect of MOI on subsequent viral expression in THP-1 cells (Table 2). At high MOI, productive infection with cell cytotoxicity was evident with no restricted expression of HIV seen. As the MOI was lowered, initial productive infection was followed by development of several cultures with restricted or no viral expression. Infection of THP-1 with the BP-1 isolate which infects both T cells and monocytes resulted in cultures with restricted expression approximately 40% of the time. Of these cultures with restricted expression, one in three cultures was had no viral expression. Interestingly, infection of THP-1 with the monocytotropic ADA strain of HIV-1, resulted in considerably more cytotoxicity and restricted expression could be seen only at MOIs a log lower than BP-1. Furthermore, the restricted expression obtained was only the nonproducer cell. Infection of THP-1 with HIV-2 Rod resulted in only restricted expression. This was probably due to an inability to obtain a viral preparation with a higher titer, as well as the less cytopathic nature of HIV-2.

TABLE 2

EFFECT OF MOI ON HIV INFECTION OF THP-1 CELLS

Viral strain	MOI*	Days to infection†	Cytolysis§	No. productive/ no. total	No. restricted‖/ no. total
BP-1	10/1	4-7	+/-	10/10	0/10
	0.1	14	-	9/10	1/10
	0.01	14-18	-	6/10	4/10
ADA	10/1	4	+++	3/3	0/3
	.1	7-10	+	3/3	0/3
	0.01	14	+/-	1/3	1/3
ROD	1	14	-	0/2	2/2
	0.1	21-24	-	0/2	2/2

*MOI is the number of $TCID_{50}$ U/cells. THP-1 was infected with HIV as described.
†Number of days post infection that detectable virus was present in extracellular media as assayed by viral p24 antigen.
§Cytolysis was observed microscopically. ADA at high MOI caused complete death.
‖Analysis of the restricted viral expression was made 60 d post infection using the criteria of no syncytia formation and low or absent extracellular p24 as in Table I. Restricted and nonproducer cultures are included in total.

Kinetics of expression of p24 in THP-1 cells was also followed after HIV infection. Until day 21, both restricted and productive HIV cultures contained the same number of viral p24 positive cells (Table 3). However, by day 60 >95% of the cells were positive in the productive cultures whereas only 32% were positive in the cultures with restricted expression. It seems likely that restricted HIV-1 expression also leads to decreased viral spread in the cultures. At no time, after the development of nonproducer cultures were any cells in the cultures positive for HIV-1 p24 antigen.

Characterization of THP-1 Cells with Restricted Expression. Since little or no detectable extracellular virus was found in some HIV-infected THP-1 cultures, the status of viral expression was examined in these cells by northern analysis. High levels of viral RNA were found in the productively infected THP-1 (Figure 4) with prominent peaks at the genomic 9.1 kb size along with subgenomic 4.4 and 2.0 kb mRNAs. However, THP-1 cultures with restricted expression contained markedly less viral RNA than the productively infected cultures (Figure 4) with subgenomic RNAs of 7.5 and 4.4 kb preferentially accumulating over full length 9.1 kb RNA (Figure 4). A 7.5 kb RNA (Figure 4) species has not been previously reported and its presence may be related to the mechanism of restricted expression in these cells. Surprisingly, the nonproducer cultures

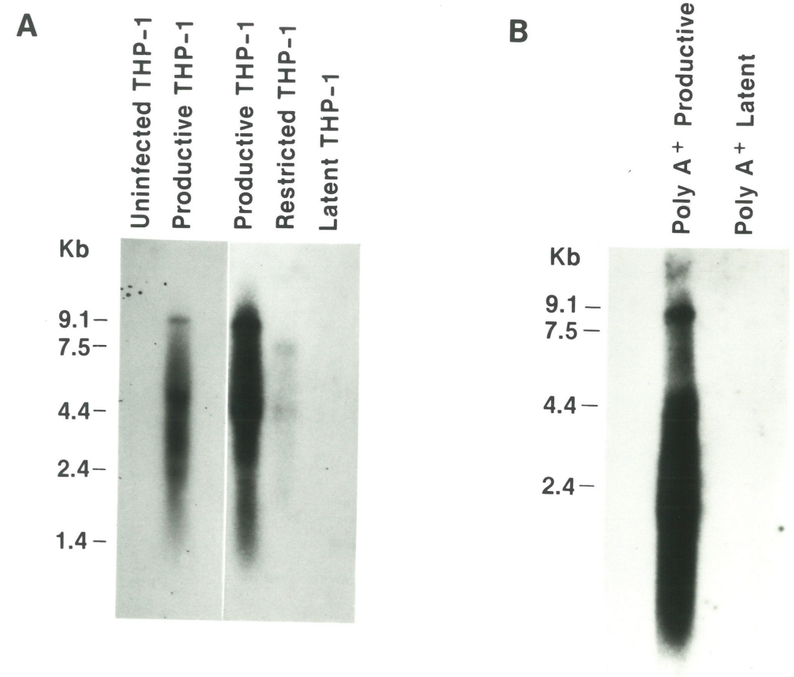
TABLE 3

EXPRESSION OF HIV p24 ANTIGEN DURING INFECTION OF THP-1 Cells

	p24 Positive cells (%)	
Days after infection	Producer	Restricted
0	0	0
7	0	0
14	18	17
21	38	35
35	75	36
50	>95	33
60	>95	32

THP-1 cells were grown and infected with HIV strains at an MOI of 0.05 as described in the Materials and Methods. Analysis of the intracellular p24 antigen was performed as described in the Materials and Methods.

Figure 4. Analysis of viral RNA in infected THP-1 cells. Northern transfer and hybridizations on RNA were performed as described in the Materials and Methods. Panel A: total RNA; lanes 1 and 2, 30 min exposure; lanes 3, 4 and 5, 2hr exposure B: poly A+ RNA, lane 1, 20μg; lane 2, 60μg overnight exposure.



produced no detectable viral RNA even if 60 μg of poly A^+ selected RNA (Figure 4) was analyzed suggesting that HIV expression in these cells was truly latent (i.e. complete absence of viral expression).

Since HIV-1 infected THP-1 with restricted expression had a low level of viral RNA, the level of proviral DNA in the cultures was assessed by Southern analysis (Figure 5). Using ecoR1 digestion to visualize a highly conserved 1.6 kD internal band in the pol-env region (Figure 5) (20) and BamH1 to visualize 4.5 and 6.5 kD bands (Figure 5), there was little difference observed between the amount of provirus present in productively infected THP-1 or Hut-78 and THP-1 with restricted expression even after six months in culture. This indicates that while cells with restricted expression reduce the spread of HIV in the culture, the restricted expression in these cultures cannot be attributed to a selective loss of the infected cell population.

Since the THP-1 cultures with restricted expression possessed a low level of viral RNA and p24 production, the level of viral particles was studied by EM. In those cultures with restricted viral expression, intracellular assembly of numerous viral particles was observed. Most of the virions were seen within intracytoplasmic vacuoles. Many immature and mature forms as well as virions budding into the vacuoles could be seen (Figure 6). The ultrastructural virus present in these cultures with

Figure 5. Southern analysis of DNA in HIV-1 infected THP-1. High molecular weight DNA (20μg) was digested with BamHI (20 units/16 hr.) Panel A; and ecoR1 (20 units/16 hr) Panel B; then transferred to nitrocellulose as described in Materials and Methods.

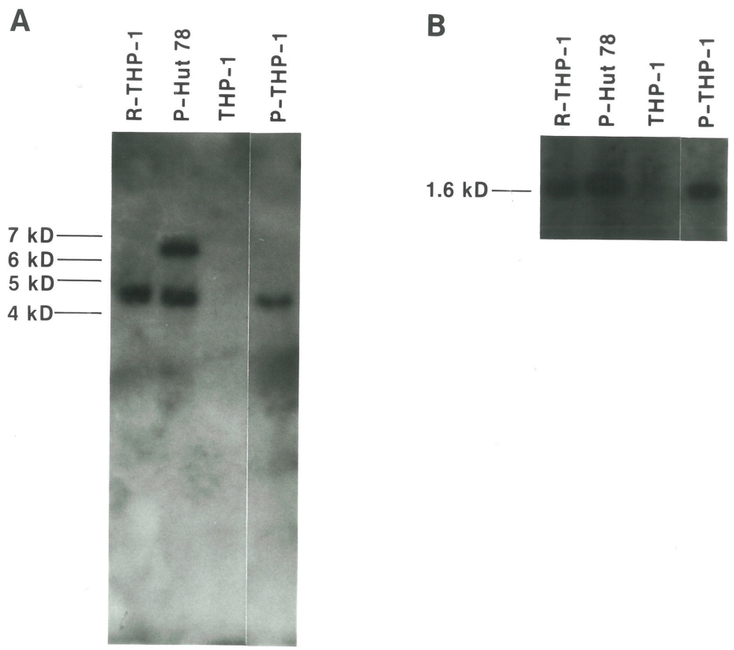
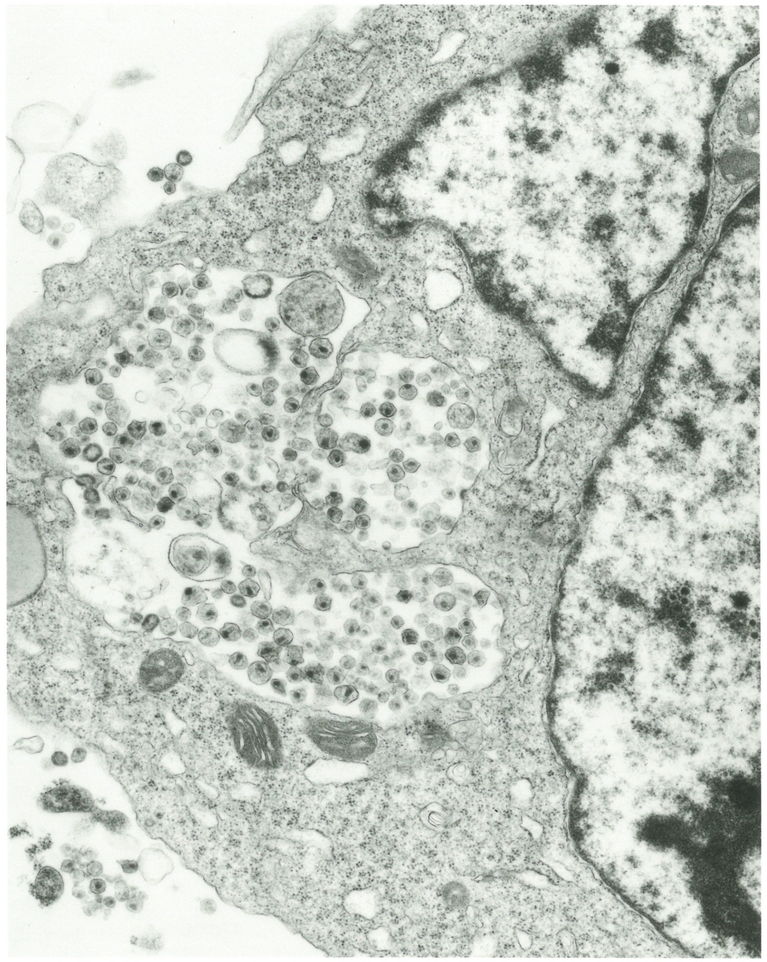


Figure 6. EM of HIV infected THP-1 cells with restricted expression. HIV particles are observed budding into intracellular structures. Electron micrographs were prepared as described in the Materials and Methods. Intracytoplasmic budding (mag = 45,000x).



restricted expression was identical to previous descriptions of HIV (4). As seen in freshly infected macrophages (115,118), these vacuoles were predominantly found in the perinuclear golgi region. Surprisingly, few if any virions were found associated with the plasma membrane or in the extracellular media (Figure 7). Approximately 30% of the cell sections were associated with viral particles. The electron micrographs suggest at least one of the mechanisms of restricting HIV expression occurs at the level of viral release.

Viral and Cellular Biology of THP-1 with Restricted HIV Infection. The morphological, phenotypic and functional characteristics of these THP-1 cells with restricted expression of HIV-1 and productively infected THP-1 cells were essentially the same as uninfected cells (Table 4). The functional characteristics of THP-1 cells were assayed with and without PMA induced differentiation. Phagocytosis of yeast particles, accessory cell function for T cell activation, IL-1 production, and development of anchorage dependence were all essentially normal. Phenotypic analysis of cell surface showed no difference in modulation of Leu-M3 or HLA-DR compared to uninfected THP-1 cells. Since these cells were functionally normal, it was important to determine whether they were recognized as normal by the immune system. As had been shown by previous studies (76), IL-2-activated LGL can recognize and lyse HIV-infected

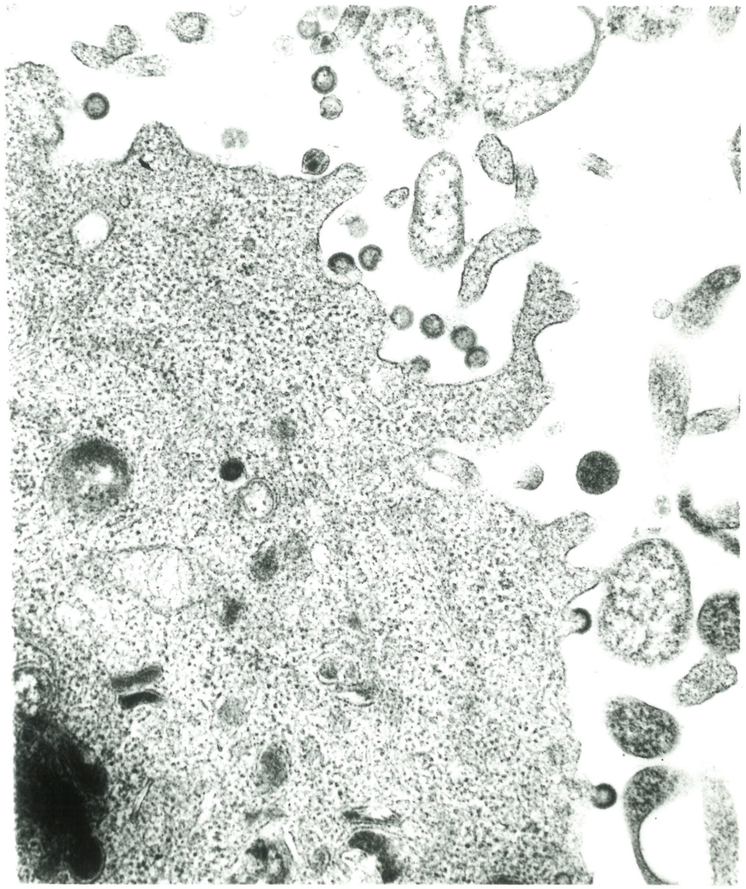


Figure 7. EM of budding from the plasma membrane HIV-1 infected THP1 (mag =18,000). Electron micrographs were prepared as described in Materials and Methods.

Table 4

Effect of HIV-Infection on Normal THP-1 Cell Function

	Viral status		
Function	None	Productive	Restricted
Doubling time (h)	20-24	20-24	20-24
Viability (%)	>95	70-80	>95
Cytolysis	-	+	-
Adherence (PMA) (%)	>95	>95	>95
Phagocytosis (%)	10-15	15-20	15-25
Phagocytosis (PMA) (%)	35-45	45-50	45-60
Accessory cell function (T cell growth)	+	ND	+
HIV-1 Ag-positive cells (%)	0	>95	31-38
Viral T cell cytotoxicity (%)	0	>95	0

HIV-infected cells with productive and restricted expression were assayed 45-60 days post infection. Functional assays were performed as described in the Materials and Methods plus or minus PMA (50 ng/ml for 24 hours) as indicated. 5 day supernatants were assayed for T cell cytotoxicity. Values are for at least two experiments.

cells. Therefore, IL-2-activated LGL were incubated with infected and uninfected THP-1 cells and cytotoxicity was measured using a 1 day chromium release assay (Table 5). Specific LGL-mediated cytotoxicity was observed only on the HIV-1 producing THP-1 cells. IL-2 stimulation increased the magnitude of LGL mediated cytotoxicity. In contrast, LGL-mediated cytotoxicity was not seen against the cells with restricted expression, suggesting that such cells can avoid recognition by specific immune mechanisms.

Next, the functional capabilities of the virus residing in THP-1 cells with restricted expression were determined. T cell cytopathology was measured using an assay where 100 virus-producing cells will cause cytolysis of 10,000 MT-2 cells (Figure 8). Using HIV-1 infected HUT-78 cells, 50% of the target cells are killed by <100 cells, while all the cells are killed by 250 cells. Using the THP-1 cells with restricted expression, 50% of the target cells are killed by 250 cells. This cell cytotoxicity is blocked when azidothymidine (AZT) is added to the cultures showing that it is HIV mediated. The supernatant of these cells with restricted expression cannot induce MT-2 cytotoxicity (Figure 9), while supernatant from cells productively infected kill very efficiently. Supernatant from THP-1 cells with restricted expression treated with PMA, cytokines such as TNF, GM-CSF, M-CSF, IL-6 or TGFβ, or γ irradiation will also kill MT-2. Consistent with this is the

Table 5

Role of NK Cells in Direct Lysis of HIV Infected THP-1 Cells

Effector	Target	Percent Specific lysis (mean ± SE)
LGL-control	uninfected	3.7 (1.3)
LGL-control	HIV restricted	2.1 (1.3)
LGL-control	HIV productive	9.4 (0.8)
LGL-IL-2 activated	uninfected	22.6 (9.8)
LGL-IL-2 activated	HIV restricted	17.3 (1.0)
LGL-IL-2 activated	HIV productive	53.4 (3.8)

LGL were purified and activated and the cytotoxicity assay was performed as described in the Materials and Methods. 3 replicates per experimental group were used and the percentage of specific lysis was calculated as mean +/- SD was determined as previously described (76). Values are representative of two experiments.

Figure 8. Cell-cell cytotoxicity as measured by viral mediated T-cell cytotoxicity assays. HIV infected cultures were tested for induction of cytolysis of MT-2 and MTT was used to measure in vitro growth by cell mediated reduction of tetrazolium as described in Materials and Methods. The OD at 540 nm is directly proportional to the number of viable cells. THP-1 cells with restricted HIV expression (▲), control THP-1, HUT-78 (□), and HIV-I infected HUT-78 (●) are shown.

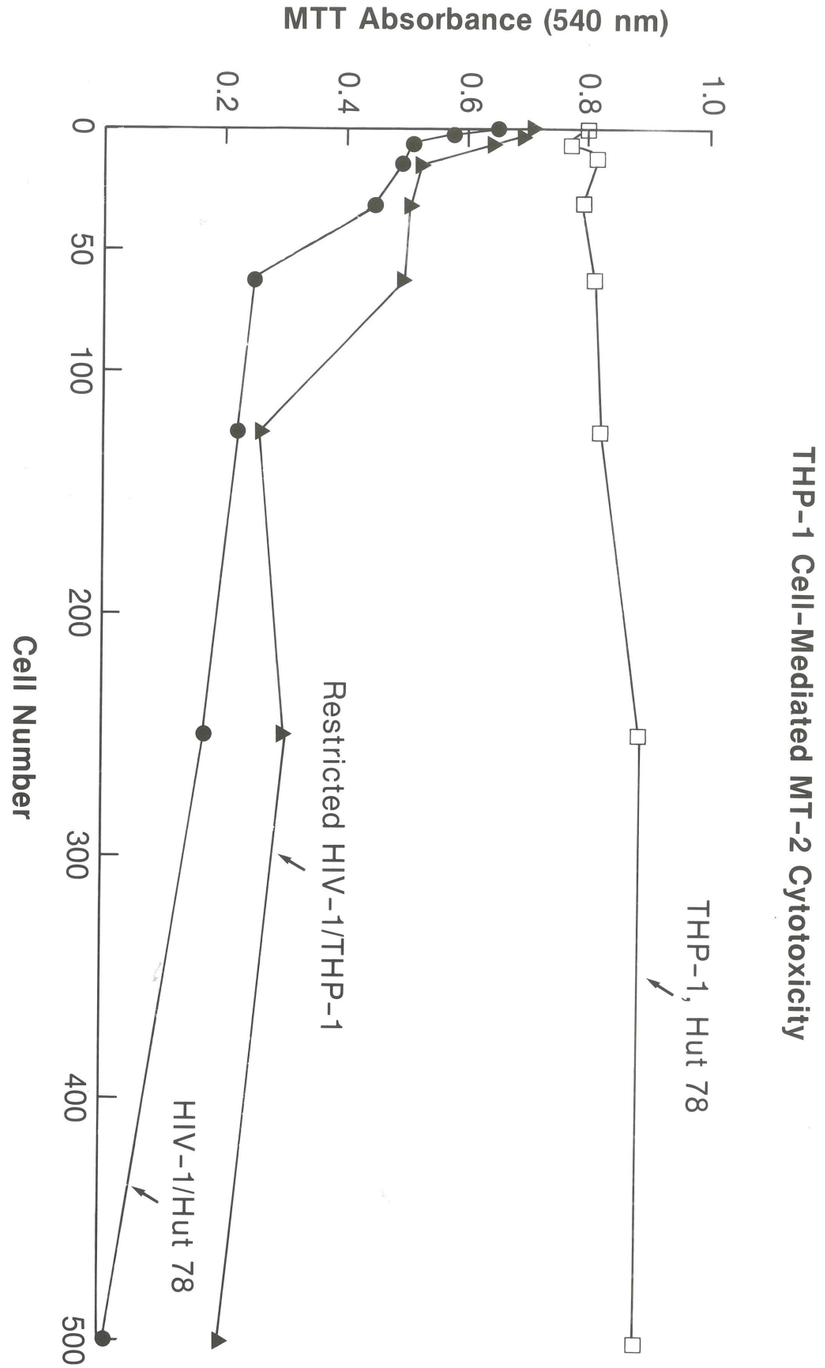
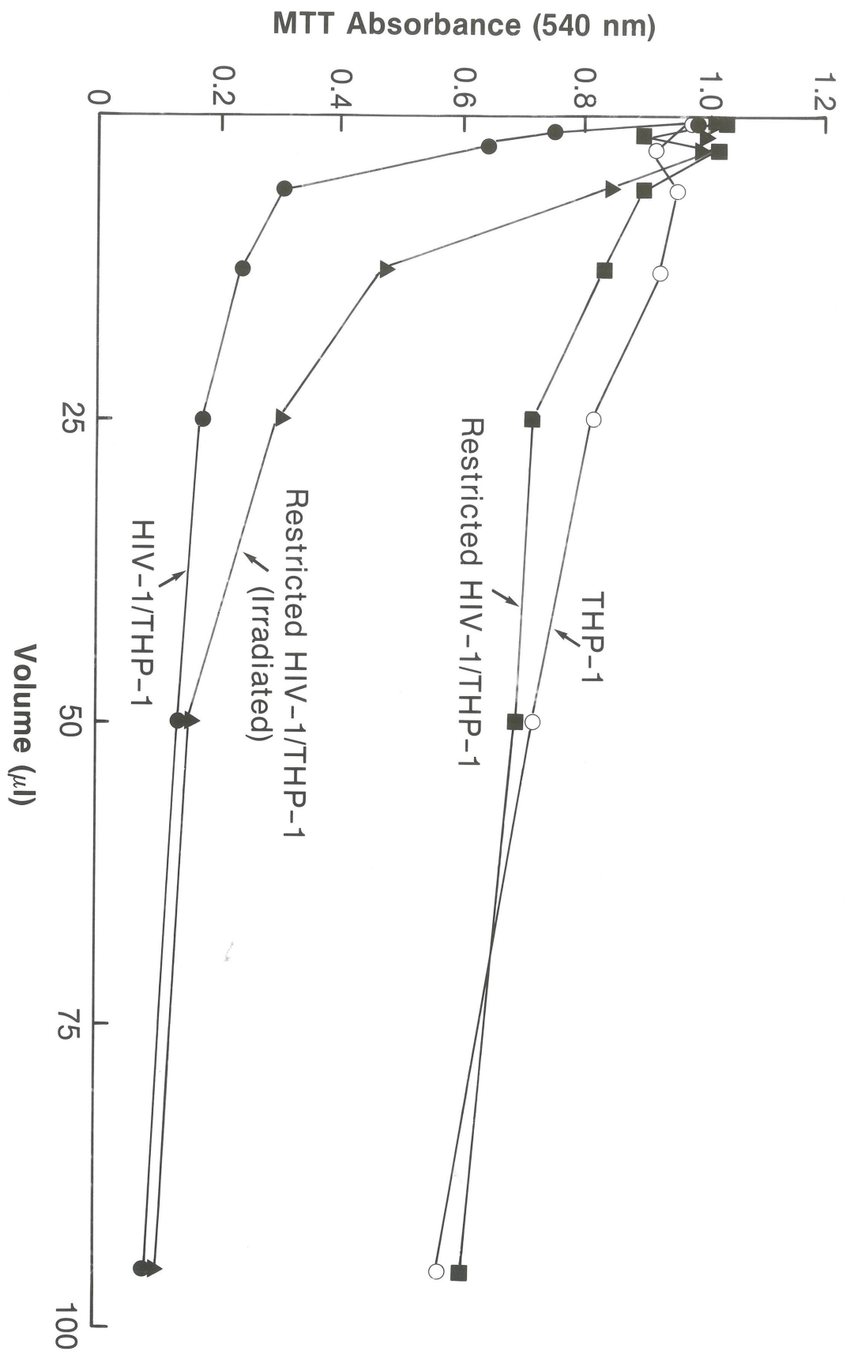


Figure 9. Cell free cytotoxicity as measured by viral mediated T-cell cytotoxicity assays. HIV infected cultures were tested and MTT was used to measure in vitro growth by cell mediated reduction of tetrazolium as described in Materials and Methods. The OD at 540 nm is directly proportional to the number of viable cells. 5 d old culture supernatants from THP-1 cells with restricted HIV-1 expression (■), THP-1 cells with restricted expression following irradiation (▲), control THP-1 (○), and HIV-1 infected THP-1 (●) are shown.



observation that THP-1 cells with restricted expression will not kill MT-2 cells when they are separated by a permeable barrier. These studies demonstrate that HIV-1 infected THP-1 cells with restricted expression are able to function normally during an immune response. The low level of HIV expression and lack of expression of viral surface antigens allows them to escape immune surveillance and cause T cell cytopathicity through cell-cell contact.

Negative Regulation of HIV Expression in THP-1 with Restricted Expression. Characterization of HIV-1 infected THP-1 cells with restricted expression revealed that the accumulation of viral RNA in the HIV restricted cells is many fold lower than in the HIV producing cells (Figure 4). In addition, these cells produced much less extracellular virus than productively infected monocytes or T cells. Furthermore, THP-1 with restricted HIV expression are able to sequester virus intracellularly. These results suggest the involvement of several levels of regulation. The fact that significant levels of viral cytotoxic activity were seen in these cells with restricted expression following activation with irradiation, PMA or cytokine treatment suggest the viral expression can be regulated in these cells. Therefore, to determine if THP-1 cells with restricted expression were capable of accurately initiating transcription of HIV-1 in a manner similar to T cells, in vitro transcription assays were performed using nuclear

extracts from THP-1 with various states of HIV expression. Using a template DNA derived from a 199 base pair region of the HIV-1 LTR containing the enhancer and RNA start site, few transcripts were seen using extracts from cells with restricted HIV expression compared to transcripts stimulated by extracts from productively infected cells (Figure 10). However, after LPS treatment, nuclear extracts from cells with restricted expression had similar levels of transcripts (Figure 10). Since these cells could support correct initiation of transcription, a direct quantitative comparison of the level of transcription between productively infected cells and those with restricted expression was made using a nuclear runoff transcription assay (Figure 11). In this assay, RNA transcripts are not initiated, but transcripts that have already initiated are faithfully elongated. Nuclei from HELA and uninfected THP-1 showed a small basal level of transcription. Nuclei from the THP-1 cells with restricted expression showed a small increase in the level of transcription over control HELA and uninfected THP-1 (Figure 11) but the transcription was much reduced from levels of transcription observed using nuclei from productively infected cells. To ascertain whether factors in restricted nuclei were affecting LTR-directed transcription, nuclei from productively infected cells were incubated with various mixtures of nuclei (Figure 11). Mixing nuclei from either uninfected THP-1 or from HELA in a

Figure 10. In Vitro transcription of HIV-1 infected THP-1 cells. Nuclear extracts and reactions were prepared as described in Material and Methods. The protein concentration of the extracts were adjusted to approximately 10 - 15 mg/ml for uninfected and infected cells.

In Vitro Transcription of HIV-Infected Monocytes

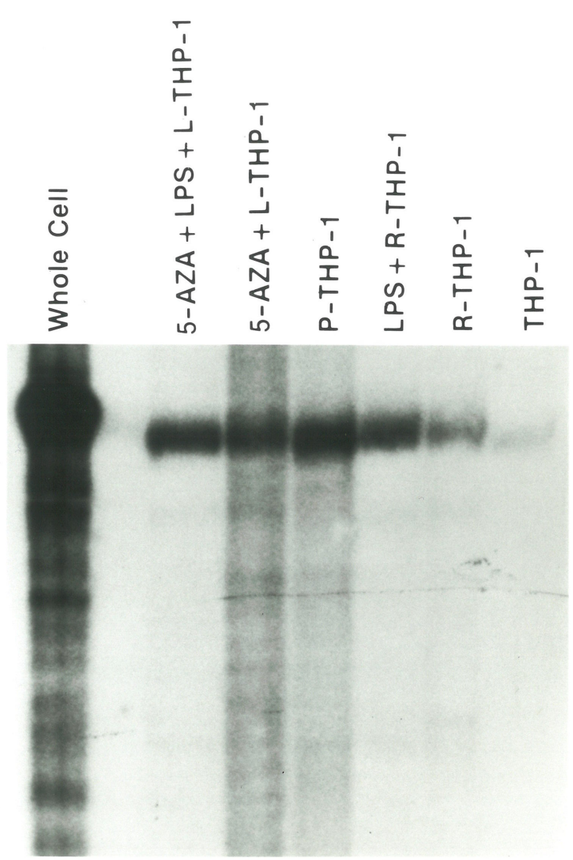
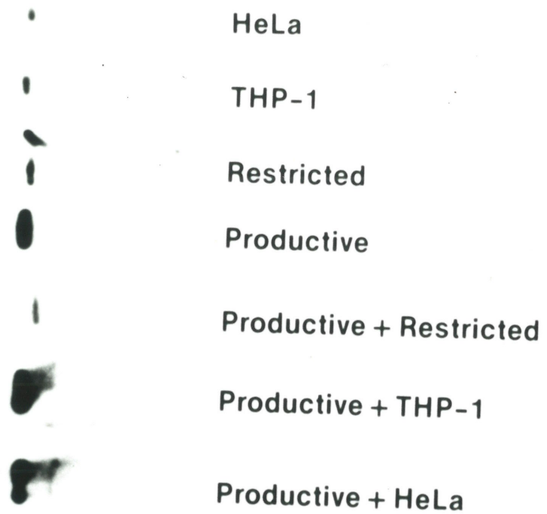


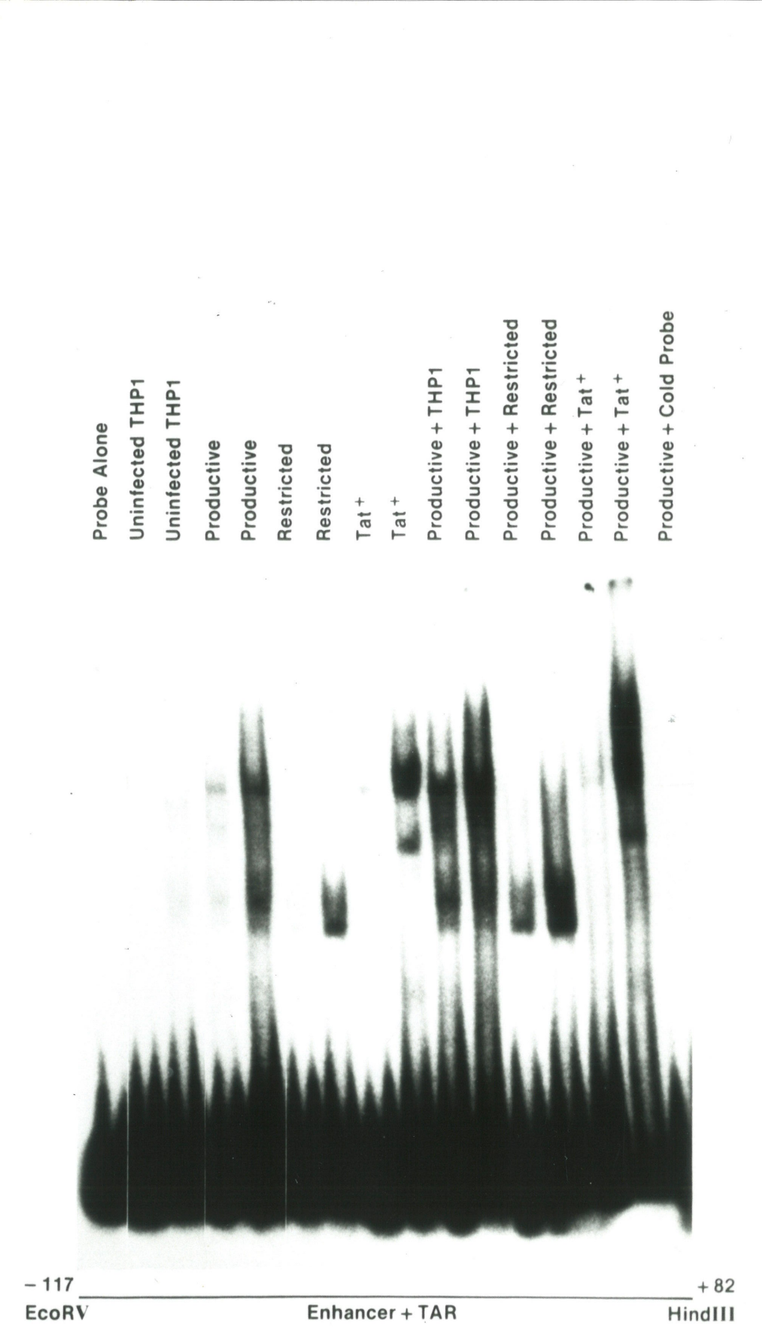
Figure 11. HIV-1 LTR-directed nuclear run-on competition experiments. Analysis of RNA transcripts was performed by nuclear transcription run-on assay as described in the Materials and Methods. Unless indicated, reactions contained 75 μl nuclei and 25 μl buffer or 75μl of productive extract and 25μl of indicated extract in mixing experiments.



1:4 ratio with nuclei from productively infected THP-1 cells did not affect the rate of transcription. However, there was a marked decrease (5-10-fold) in the transcriptional level of nuclei from HIV-producing THP-1 cells when the same ratio (1:4) of competing nuclei from THP-1 cells with restricted expression were added (Figure 11). This nuclear material would also inhibit LTR-directed transcription of nuclei from HIV-infected T cells but not HTLV-1 LTR-directed transcription in HTLV-1 infected T cells.

Many cellular transcription factors mediate their effects by binding specific sequences in the DNA (23). To determine whether the negative transcriptional regulation of THP-1 cells with restricted HIV expression was mediated at the level of DNA binding complex formation, gel mobility shift assays were employed using the enhancer-tar region (-117 to +82) of the HIV-1 LTR (Figure 12). Two concentrations of each nuclear extract were used. Extracts from HIV-1 producing THP-1 cells and a tat-producing mouse cell line, gives the same complex formation while the extract from the restricted cell line did not have any DNA binding in the area where extracts from both the productively infected THP-1 cells or the tat-producing cells bound the DNA. A second (lower) complex was found using extracts of both productive and restricted cells but not the tat-producing cells. Mixing an extract from uninfected THP-1 with an extract from productively infected cells did not

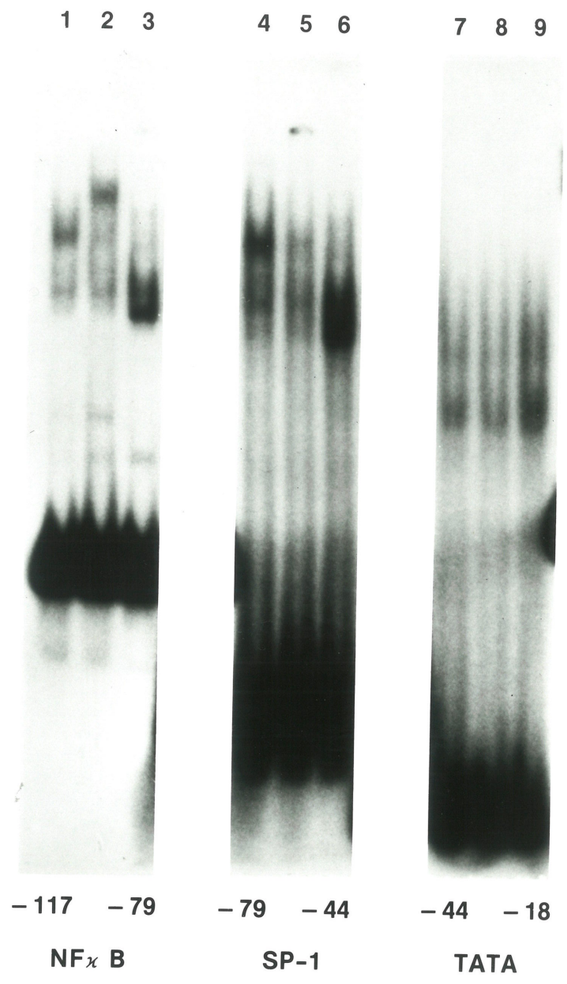
Figure 12. Gel mobility shift analysis of protein binding from infected THP-1 cells to HIV-LTR. A [^{32}P]-labeled oligonucleotide spanning the enhancer-tar region (-117 to +82) of the HIV-1 LTR was incubated with two concentrations (5 and 10 μg) of nuclear extracts prepared from various cells as described in the Materials and Methods. For mixing experiments, 1 and 2 μg of the indicated extracts were incubated with 4 and 8 μg of extracts from productively infected cells.



affect binding. In contrast, when an extract from cells with restricted expression was mixed with an extract from productively infected cells, it completely eliminated the binding of productive cell extract to the LTR. This suggests that one mechanism of restricted HIV-1 expression was at the level of transcription by blocking binding of factors with the HIV-1 LTR-DNA. Thus, HIV expression in cells with restricted expression may be actively suppressed by the sequestration of DNA binding transcription factors.

The HIV-1 enhancer region contains two copies of the 11-base pair (bp) repeat which binds NF-κB, three SP1 sites and the TATA box (Figure 2). Therefore, gel mobility shift experiments were performed using separate DNA probes for each defined enhancer element (Figure 13). While some differences in binding were observed when a specific probe for SP1 was used, no difference in binding was seen when a specific probe to TATA box sequences was used. However, using the sequences specific for NF-κB, a significant difference was noted. As previously shown (70), uninfected THP-1 cells as well as freshly isolated monocytes and macrophages constitutively produce active NF-κB leading to two binding complexes, a higher 65 + 50 Kd heterodimer and a lower 50 Kd homodimer (144). Both complexes were seen in the nuclear extract from the productively infected cells but the higher band (the heterodimer) was absent using extracts from cells with restricted expression (Figure 13) suggesting

Figure 13. Gel mobility shift analysis of protein binding from infected THP-1 cells to TATA, SP-1, and NFκB regions of HIV-1-LTR enhancer. Mobility shift assays were performed using ^{32}P labeled probes to the separate enhancer elements of NF-kB, SP-1, and TATA. The same nuclear extracts were used for uninfected THP-1, (lanes 1,4,7), THP-1 with productive (lanes 2,5,8) and restricted expression (lanes 3,6,9) as indicated.

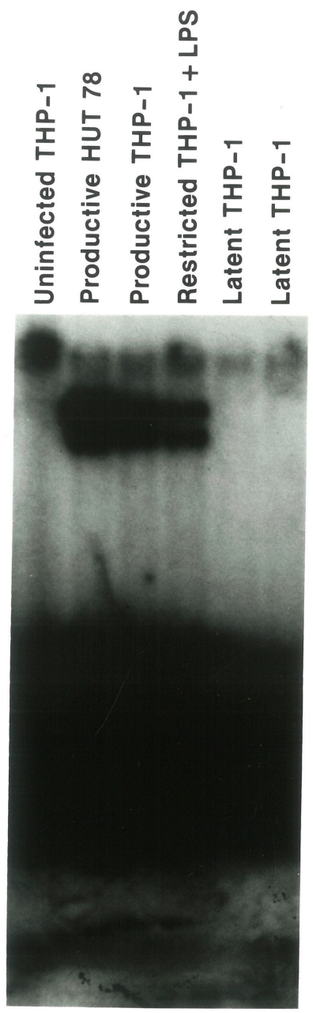


the presence of an inhibitor of this NFκB transcriptional activator complex in THP-1 cells with restricted expression. As an out growth of this observation, it has recently been shown that THP-1 cells with restricted expression contain an IkB-like factor in the nucleus which specifically inhibits the binding of the 65+50 kD NFκB heterodimer, necessary to upregulate transcription to the two NFκB sequences in the HIV promoter (145).

Identification of THP-1 with a Latent HIV-1 Infection. Since some HIV infected THP-1 cultures exhibited no detectable viral expression, these cells were next examined by polymerase chain reaction (PCR), the most sensitive technique available to detect nucleic acids, to determine if viral expression could be detected in these cells. Primer pairs to the nef and tat regulatory regions in addition to the gag region, which has been shown to be the most sensitive region for HIV detection (132) were used. Two non-producing HIV infected cultures (Figure 14) showed no detectable viral RNA specific gag sequences after 2 h or 24 h of exposure. For comparison, productively infected T cells, productively infected THP-1 and THP-1 with restricted expression, which as in the northern analysis has much less RNA, are shown. Similar results were obtained with both tat and nef (Figure 15). These results are consistent with the result of the northern analysis. Therefore, no viral expression could be detected by northern analysis of Poly A+

Figure 14. PCR analysis of gag RNA from infected THP-1 cells under various conditions. RNA was isolated as described in the Materials and Methods and amplified using gag primers following an initial reverse transcription step using 1μg and 5μg of RNA and MuLV RT. At the end of 35 cycles, aliquots were hybridized to a [^{32}P]-end labeled oligonucleotide probe spanning the region between the primer pairs. PCR products were analyzed on a 20% polyacrylamide gel using the gag probe. 24 h exposure.

Figure 15. PCR analysis of nef RNA from infected THP-1 cells with different states of HIV expression. RNA was isolated from $1x10^7$ LTHP-1 as described in the Materials and Methods and amplified using nef (132) primers following an initial reverse transcription step using 1μg and 5μg RNA. At the end of 35 cycles, aliquots were hybridized to a [^{32}P]-end labeled oligonucleotide probe spanning the region between the primer pairs. PCR products were analyzed on a 20% polyacrylamide gel using the appropriate probe.



RNA nor by PCR analysis for the gag, tat or nef, regions of HIV-1.

Since no viral expression could be detected, the HIV DNA status of these cultures was assessed using PCR analysis. Primer pairs specific for the nef region of HIV-1 (Figure 16) showed low but significant levels of HIV specific DNA were detected in both of these nonproducing THP-1 cell lines. These results suggested that HIV expression is latent in these cells. We next studied under what conditions a latent virus could be reactivated. Neither LPS nor IUdR (Figure 17) could induce viral expression from cells with latent virus. Since previous work has shown that the HIV-LTR stably transfected in fibroblasts was methylated (146), 5-azacytidine was used and found to be an inducer of virus expression (Figure 17) even after the virus was latent for 10 months. Sufficient amounts of infectious virus were produced to get a massive infection of the T cells line, HUT-102B2, within 10 days of transmission (Figure 18). Electron micrographs of reactivated latent virus transmitted into HUT-102B2 show typical morphological characteristics of HIV assembly and budding (Figure 19).

Interestingly, virus reactivated from latently infected THP-1 cells (LTHP-1) after months of continuous culture maintained the biological characteristics of the original infecting strain of HIV-1. That is, the monocytotropic

Figure 16. PCR analysis of nef DNA from infected THP-1 cells under various conditions. DNA was isolated as described in the Materials and Methods and amplified using nef primers (132). At the end of 35 cycles, aliquots were hybridized to a [^{32}P]-end labeled oligonucleotide probe spanning the region between the primer pairs. PCR products were analyzed on a 20% polyacrylamide gel using the nef probe.

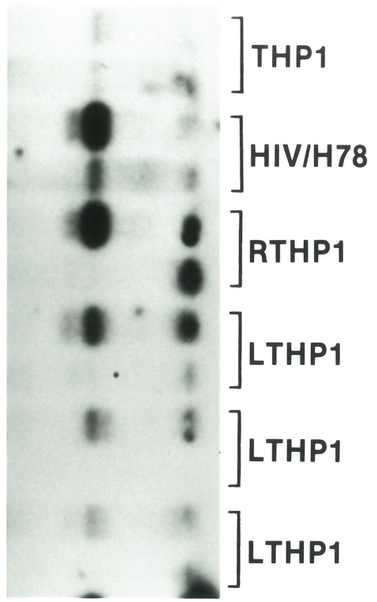


Figure 17. PCR analysis of viral gag RNA from latent THP-1 cultures after activation by various agents. RNA was isolated as described in the Materials and Methods and amplified using gag primers. At the end of 35 cycles, aliquots were hybridized to a [^{32}P]-end labeled oligonucleotide probe spanning the region between the primer pairs. PCR products were analyzed on a 20% polyacrylamide gel using the gag probe.