Dependence on host cell cycle for activation of human immunodeficiency virus type 1 gene expression from latency

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Abstract

Human immunodeficiency virus type 1 (HIV-1) establishes latent infection of a certain population of CD4+ host cells which could be long-term reservoirs for HIV-1. The expression of viral genes in such long-term infected cells is strongly regulated by cellular status, such as the phase of the cell cycle or stage of cell differentiation. Here, viral gene expression in synchronized U1 cells, a monocytic cell clone latently infected with HIV-1, was characterized. The expression of HIV-1 antigens was detected exclusively at G2/M phase in U1 cells, irrespective of phorbol myristate acetate (PMA) treatment. The induction of HIV-1 gene expression in PMA-treated cells was due to the recruitment of NF-κB with DNA-binding activity at G2/M phase. Activated NF-κB was induced only by PMA treatment during the late G1 to S, but not after entering G2 phase, indicating that the transcriptional factor(s) involved in viral gene expression is also largely regulated by the host cell cycle. In contrast, the enhancement of antigen expression by treatment with tumour necrosis factor-alpha (TNF-α) was cell cycle-independent. In fact, NF-κB was activated 2 h after TNF-α treatment at all stages of the cell cycle. Thus, the mechanisms of HIV-1 activation from latency in U1 cells by PMA and TNF-α treatment are different. The model system using U1 cells shown here may provide insight into the mechanisms responsible for HIV-1 gene expression from latency.

Introduction

After infection of CD4+ cells with human immunodeficiency virus type 1 (HIV-1), the viral DNA copy is integrated into the host chromosome as a provirus. The expression of HIV-1 provirus is governed by complex regulatory mechanisms involving host trans-acting factors (Gaynor, 1992) and the viral regulatory proteins Tat and Rev (Cullen, 1991; Jones & Peterlin, 1994), as well as the site of integration in the chromosome (Lind et al., 1996).

Active virus replication occurs in HIV-1 carriers throughout all clinical stages (Emmetson et al., 1993; Pantaleo et al., 1993; Piatak et al., 1993). However, the clinical stage of disease is significantly associated with virus load (Cao et al., 1995; Michael et al., 1995). Most of the HIV-1 in plasma is derived from short-lived infected cells with a half-life of only a few days (Ho et al., 1995; Wei et al., 1995; Perelson et al., 1996, 1997). In addition, there is evidence that a large proportion of cells carrying the HIV-1 genome establishes latent infection (Bukrinsky et al., 1991; Emmetson et al., 1993; Patterson et al., 1993), although only a small fraction of latently infected resting CD4+ T-cells carry replication-competent, integrated provirus (Chun et al., 1997a; Perelson et al., 1997). Recently, the importance of such latent reservoirs for HIV-1 was highlighted. Potent antiretroviral drugs that block new rounds of infection produce a marked drop in plasma virus levels within 2 weeks, which is followed by a slower second-phase decay of plasma viraemia, which seems to be mainly derived from such long-lived latently infected cells (Perelson et al., 1997). In fact, it was recently demonstrated that highly purified CD4+ T-cells from patients receiving highly active antiretroviral therapy with undetectable plasma viraemia carried integrated proviral DNA and were capable of producing infectious virus upon cellular activation in vitro (Chun et al., 1997b). Thus, the mechanisms that stimulate the activation of HIV-1 from latency are essential for understanding the pathogenicity of the disease.

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Latency in HIV-1 can be described as an interaction between cellular and viral factors. The mechanisms of HIV-1 latency have been examined in several cell lines expressing no or low levels of viral messages and proteins, referred to as postintegration latency (Pomerantz et al., 1992a; McCune, 1995). Activation from postintegration latency in such model cells was induced by several reagents which lead to the induction of cellular transcription factors (Gaynor, 1992; Nabel, 1993). However, the detailed mechanisms of postintegration latency are still largely obscure.

The U1 cell line was established by rescuing and cloning a human promonocytic cell line, U937, which had been infected with HIV-1 LAI strain (Folks et al., 1987). Under unstimulated conditions, the level of viral transcripts in U1 cells is very low (<5% of total cell population; Pomerantz et al., 1990) because the hallmark of the latent state is the expression predominantly of doubly spliced transcripts encoding viral regulatory proteins such as Tat, Rev and Nef (Butera et al., 1993). However, the detailed mechanisms of postintegration latency are still largely obscure.

Induction of NF-κB has been shown to be transduced via several independent pathways (Baueuerle & Baltimore, 1996), through the activation of protein kinase C (PKC) by diacylglycerol or phorbol esters (Heller & Krönke, 1994), the sphingomyelin pathway in TNF-α, phorbol esters or other reagents, the levels of viral transcripts in U1 cells significantly increase, with the appearance of singly spliced and unspliced transcripts encoding viral structural proteins leading to virus production (Bednarik & Folks, 1992; Fujinaga et al., 1995). These reagents stimulate a number of cellular transcriptional enhancers, especially NF-κB, which is believed to be the factor most responsible for reactivation of HIV-1 from latency (Nabel & Baltimore, 1987).

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Methods

- **Cell culture.** U1 (Folks et al., 1987), a latently HIV-1-infected cell clone isolated from the promonocytic cell line U937 after infection with HIV-1 LAI strain, was kindly supplied by T. M. Folks (Center for Disease Control and Prevention, Atlanta, USA). The cells were cultured in complete medium (RPMI 1640 medium supplemented with 5% foetal bovine serum). The cell number was adjusted to 5 × 10^5/ml in fresh complete medium every 3 days.

- **Cell cycle arrest.** The cells at 5 × 10^5/ml in fresh complete medium were cultured for 1 day, then adjusted again to 7.5 × 10^5/ml. After further cultivation in the presence of 0.3 mM hydroxyurea (HU; Sigma) for 16 h, the cells were washed twice with complete medium. Again, the cells at 7.5 × 10^5/ml were cultured for 8 h, then for a further 16 h in the presence of 0.3 mM HU. After washing twice with complete medium, the cells were designated as 0 h post-synchronization (p.s.). Further results, were further cultured in the presence of absence of PA (15 ng/ml; LC Services Corporation) or TNF-α (200 units/ml; Upstate Biotechnology).

- **Western blotting.** The cells (1 × 10^7) were lysed in 100 µl sample buffer (20 mM Tris-HCl pH 6.8, 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol). The proteins in the cell lysates (10 µl), after denaturation by boiling, were separated on 12% SDS-PAGE gels and were blotted onto nylon membranes. The proteins were electrotransferred onto PVDF membranes (Millipore), then incubated with anti-Gag p24 MAb (V107) (Ikuta et al., 1995). When described previously (Kishi et al., 1989), the cells (1 × 10^7) were lysed in 100 µl sample buffer (20 mM Tris-HCl pH 6.8, 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol). The proteins in the cell lysates (10 µl), after denaturation by boiling, were separated on 12% SDS-PAGE gels and were blotted onto nylon membranes. The proteins were electrotransferred onto PVDF membranes (Millipore), then incubated with anti-Gag p24 MAb (V107) (Ikuta et al., 1989). As a control, the same proteins on the membranes were reacted with mouse MAb to human tubulin (Oncogene Research Products). Bound antibodies were visualized with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako), then with enhanced chemiluminescence Western blotting detection reagents (Amersham).

- **Northern blotting.** Total RNAs were extracted from the cells as described previously (Kishi et al., 1993). RNAs were separated on agarose gels and were blotted onto nylon membranes. 32P-labelled HindIII fragments of the full-length of HIV-1 in pNL432 (Adachi et al., 1986) prepared using a Multiprime DNA labelling kit (Amersham) were used as probes to detect HIV-1 transcripts. As a control, β-actin DNA probe solution for hybridization (WAKO Pure Chemical Industries) was used.

- **Nuclear extract preparation and gel mobility shift assay.** Nuclear extracts were prepared by the ‘mini-extract’ method (Schreiber et al., 1989). Briefly, the cells (~10^6) were homogenized in 10 mM HEPES-KOH pH 7.8, 10 mM KCl, 0.1 mM EDTA and 0.1% NP40. After centrifugation of the homogenate in Eppendorf tubes at 5000 r.p.m. for 1 min, the pellet was suspended in 50 mM HEPES-KOH pH 7.8, 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl2 and 20% glycerol, then
HIV-1 activation from latency

Induction of HIV-1 expression in U1 cells by PMA and TNF-α

The U1 cell line is transcriptionally latent in terms of HIV-1 expression (Folks et al., 1987), and is activated by treatment with several reagents (Bednarik & Folks, 1992). Under normal culture conditions, less than 5% of the cells continuously expressed the HIV-1 antigens, as estimated by IF (not shown) or flow cytometry (Fig. 1a) using anti-Gag p24 MAb.

Initially, we examined the possible cell cycle-dependent expression of viral antigens in U1 cells after stimulation with PMA or TNF-α for 8–16 h. The PI staining of unstimulated U1 cells for cell cycle analysis showed that a large population of the cells was in G1/S phase (Fig. 1a). Stimulation of U1 cells with 15 ng/ml PMA significantly increased the population of HIV-1 antigen-expressing cells up to 11.5 ± 3.0% in triplicate assays at 8 h, 12.8 ± 2.2% at 10 h, 16.9 ± 4.3% at 12 h and 20.2 ± 4.1% at 16 h. Similarly, TNF-α stimulation also increased the population of virus antigen-positive cells up to 13.0 ± 1.8% at 8 h, 15.4 ± 2.8% at 10 h, 22.3 ± 2.8% at 12 h and 24.0 ± 1.5% at 16 h. As representatives, the results of PMA and TNF-α stimulation at 10 h are shown in Fig. 1(b and c, respectively). The percentages of the cells in G0/G1 and G2/M phases were as follows: 47.0 ± 4.4% at G0, 24.9 ± 3.2% at G2/M in the cells treated with PMA for 10 h; 57.9 ± 1.0% at G0 and 13.7 ± 1.5% at G2/M in the cells treated with TNF-α for 10 h; and 60 ± 5.5% at G0 and 15.9 ± 4.1% at G2/M in the untreated cells. However, IF staining of the gated cells from U1 cells stimulated with PMA for 10 h revealed that a larger population of the HIV-1 antigen-positive cells were in G2 phase (20.1 ± 3.1% at R4 versus 0% at R3 in Fig. 1b). In contrast, HIV-1 antigen-positive cells were distributed into both G1 and G2 phases in TNF-α-stimulated U1 cells (13.9 ± 2.4% at R5 and 17.5 ± 3.9% at R6 in Fig. 1c). These results clearly indicated that induction of HIV-1 antigen expression from latency by PMA stimulation was dependent on the cell cycle, the mechanism of which seemed to be markedly different from that evoked by TNF-α stimulation.

Cell cycle-dependent HIV-1 antigen expression in U1 cells by PMA stimulation

To clarify the cell cycle-dependent regulation of HIV-1 expression in U1 cells by PMA stimulation, we next examined the increase of HIV-1 antigen expression in synchronized cells (Fig. 2). The U1 cells were cultured in the absence or presence of PMA after G0 arrest by treatment with HU. The maximum number of cells at G2/M phase appeared at 6 h p.s., irrespective of PMA treatment. The cells at G2/M phase gradually decreased and finally returned to the basal level within 12 h p.s. in the absence of PMA stimulation, while the cells at G2/M phase remain at a high level up to 12 h p.s. (Fig. 2) and thereafter most of the cells died (not shown) when stimulated by PMA.

The expression of HIV-1 antigens in the cells at each time-point was analysed by IF and Western blotting using anti-HIV-1 Gag MAb (Fig. 3a and b, respectively). The expression signals in the cells cultured in the presence of PMA were faint by both analyses. Upon stimulation with PMA at 0 h p.s., the population of HIV-1 antigen-positive cells gradually increased up to 42% at 10 h p.s. (Fig. 3a). Similar levels (35–42%) of antigen-positive cells were observed at least until 24 h p.s. (not shown). Similar results were obtained by Western blotting (Fig. 3b), in which the amount of Gag protein showed a maximum at 10 h p.s. Thus, the amount of Gag protein expression increased concomitantly with the increase in proportion of the cells in G2/M phase on stimulation with PMA. The maximum level of Gag expression was observed at the same time as the maximum proportion of cells in G2/M phase (Figs 2 and 3a, b). These results indicated that the activation of HIV-1 expression

Results

Induction of HIV-1 expression in U1 cells by PMA and TNF-α

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Fig. 1. Cell cycle-dependent HIV-1 antigen expression in PMA-stimulated U1 cells. The cells were mock-treated (a) or treated with 15 ng/ml PMA (b) or 200 units/ml TNF-α (c). After incubation for 10 h, the cells were stained with PI (upper left panels) and anti-HIV-1 Gag V107 MAb (upper right panels) for flow cytometric analysis on cell cycle and antigen expression, respectively. The cells at G1 (R1, R3 and R5) or G2 phase (R2, R4 and R6) were gated for further analyses of the viral antigen expression with V107 MAb (lower panels).

Fig. 2. Cell cycle analysis of synchronized U1 cells. The cells were arrested at late G1 phase by treatment with HU. Cell cycle was restarted by removing HU, followed by the immediate addition of PMA (this time-point referred as 0 h). As a control, cell cycle was restarted by removing HU without addition of PMA. The cells were collected at the indicated time-points and permeabilized with ethanol, then incubated with PI. DNA contents were quantified by measuring incorporated PI by flow cytometry. The individual cell cycle phases corresponding to each peak are indicated at the top of the figure.

from latency by PMA treatment occurred predominantly at G<sub>2</sub>/M phase.

**Cell cycle-dependent regulation of HIV-1 transcription in U1 cells by PMA**

HIV-1 latency in U1 cells is determined at transcriptional and/or posttranscriptional levels (Pomerantz et al., 1990; Butera et al., 1994). Results have shown that the viral transcripts are predominantly multiply spliced (2-0 kb) or singly spliced (4-3 kb) RNA species, although their amounts are maintained at low levels. However, the latent state of HIV-1 in U1 cells can

Fig. 3. HIV-1 expression in synchronized U1 cells. The synchronized U1 cells were mock-treated (a) or treated with 15 ng/ml PMA (b). The cells were harvested at the time-points indicated for IF (c), Western blotting using anti-HIV-1 Gag V107 MAb (d) and Northern blotting (c). Three species of viral mRNA (9-2, 4-3 and 2-0 kb) in Northern blots were visualized by using HIV-1-specific probes (see Methods). To confirm that the same amounts of RNA were transferred onto the membrane, cellular β-actin mRNA in the same membrane was also visualized with a specific probe (lower panels).
shift to the productive state following stimulation with agents such as PMA or TNF-α that activate HIV-1 long terminal repeat (LTR) as well as by overexpression of the viral regulatory proteins Tat or Rev (Pomerantz et al., 1992b; Emiliani et al., 1996). Therefore, we next investigated the kinetics of the appearance of viral transcripts in U1 cells at each stage of the cell cycle.

Fig. 3(c) shows the kinetics of viral mRNA appearance in synchronized U1 cells after stimulation with PMA determined by Northern blotting analysis. Consistent with previous reports (Pomerantz et al., 1990; Butera et al., 1994), the viral 2.0 kb and 4.3 kb transcripts in U1 cells were only faintly detected in the absence of PMA treatment. However, we also detected unspliced 9.2 kb RNA (Fig. 3c), which should be derived from > 5% of Gag antigen-positive cells in untreated U1 cells (Fig. 3a). Upon stimulation with PMA, the amounts of 2.0 kb, 4.3 kb and 9.2 kb RNAs gradually increased (Fig. 3c). However, the kinetics of the appearance of such viral transcripts showed two different patterns: transient appearance at 2 h.p.s. at lower levels, and higher levels from 8 h.p.s. Since the maximum levels of expression of HIV-1 antigens were detected by anti-Gag MAb at 10–12 h (Fig. 3a, b), the increased levels of transcripts at 8 h.p.s. seemed to contribute to the viral antigen expression.

The LTR of HIV-1 contains several enhancer sequences for cellular transcriptional factors, and these factors can stimulate viral gene expression (Nabel, 1993). Among them, NF-κB is believed to be the factor most responsible for HIV-1 expression in infected cells (Nabel & Baltimore, 1987). Therefore, we next compared the DNA-binding activities of NF-κB at individual stages of the cell cycle in synchronized U1 cells by gel mobility shift assay (Fig. 4). In PMA-unstimulated cells, NF-κB activation was not significantly detectable throughout all phases of the cell cycle. Upon stimulation with PMA, the induction of NF-κB was first detected 8 h.p.s. and reached the maximum level within a further 4 h of incubation. IF analysis of NF-κB by confocal microscopy also supported these results, i.e. NF-κB staining, which was sequestrated only into the cytoplasm in unstimulated cells, became co-localized with PI staining slightly at 6 h.p.s. and completely at 8 h.p.s. in stimulated U1 cells, indicating the transport of this protein from the cytoplasm to nucleus (Fig. 5). These findings again suggested that the induction of NF-κB occurred predominantly at G₂/M phase. Therefore, NF-κB seems to be one of the cellular factors responsible for the cell cycle-dependent viral gene expression.

Comparative studies on cell cycle-dependent HIV-1 activation from latency between PMA and TNF-α treatment

To determine the cell cycle dependency of virus activation from latency in U1 cells by PMA and TNF-α treatment, we stimulated the synchronized U1 cells with such reagents at several time-points. The U1 cells adjusted to G₁ phase by treatment with HU were cultured as shown in Fig. 2.

Firstly, PMA was added at 0, 2, 4, 6 or 8 h.p.s., as indicated by arrows in Fig. 6(b). The numbers of HIV-1 antigen-positive cells determined by IF with anti-Gag MAb increased at 10 h.p.s. in cells stimulated with PMA at 0 h.p.s. (Fig. 6b), as described above (Fig. 3a). Interestingly, similar effects were also observed even with addition at 2 and 4 h.p.s. However, the increases in the number of IF-positive cells were significantly lower when PMA was added at 6 h.p.s., and no apparent difference was observed between cells treated with
Fig. 5. Confocal microscopic examination for activation of NF-κB in synchronized U1 cells after PMA treatment. The PMA-stimulated U1 cells at 2, 4, 6 or 8 h p.s. were smeared on glass slides, then fixed with acetone. The cells were treated with RNase, then incubated with anti-NF-κB p50 rabbit polyclonal IgG. After incubation with FITC-conjugated second antibody, the cells were stained with PI, then analysed using INSIGHT system.

PMA at 8 h p.s. (Fig. 6b) and control unstimulated cells (Fig. 6a). Since the cells at 8 h p.s. were predominantly in early G2 phase (Fig. 2), these results indicated that the signal transduction involved in virus activation from latency after PMA treatment should be specifically induced at G1/S phase, but not by PMA treatment of the host cells which had already entered G2 phase.

In contrast, significantly faster kinetics of the appearance of HIV-1 antigen-positive cells were observed following treatment with TNF-α (Fig. 6c), compared with PMA (Fig. 6b). Furthermore, TNF-α treatment of synchronized U1 cells at 0, 4 and 8 h p.s. showed no apparent difference; gradual increases were observed in HIV-1 antigen-positive cell numbers in both cases to about 17–30% after an additional 10 h incubation (Fig. 6c). Thus, the mechanism of HIV-1 activation from latency by TNF-α was markedly different from that by PMA in terms of dependence on host cell cycle. Gel mobility shift assays performed as in Fig. 4 revealed that the induction of NF-κB in U1 cells following stimulation with PMA at 2 and 4 h p.s. was similarly detectable at 8 h p.s., whereas the cells stimulated at 8 h p.s. did not induce NF-κB even at 14 h p.s. (not shown). In contrast, the induction of NF-κB in U1 cells by stimulation with TNF-α at 0 and 8 h p.s. was detected with a subsequent 2 h incubation (not shown). These observations suggested that the induction of NF-κB by TNF-α treatment was independent of the host cell cycle.

Discussion

HIV-1 can infect quiescent or nonproliferating cells such as monocytes or macrophages (Weinberg et al., 1991), unlike other subfamilies of retroviruses. However, for effective virus replication HIV-1 requires active lymphocyte proliferation (Zack et al., 1990; Bukrinsky et al., 1991). HIV-1 mRNA could
be detected only in the S phase-enriched fraction of quiescent CD4+ T-cells with a stable, inducible form of HIV-1 (Spina et al., 1995), indicating that the expression of HIV-1 is blocked at the transcriptional level in quiescent cells. However, the process of cell division per se might not be required for active virus replication (Li et al., 1993; Schuitemaker et al., 1994). Thus, a particular activation state accompanied with cell division appears to be essential for active virus replication. In this study, we clearly demonstrated that the induction of HIV-1 gene expression in U1, a latently infected monocytic cell clone, by PMA occurred predominantly at the G2/M phase of the cell cycle. In contrast, TNF-α could activate the latent form of HIV-1 in U1 cells by a cell cycle-independent mechanism.

PMA treatment of the synchronized U1 cells induced viral protein expression only at the late stage of G2/M phase, concomitantly with cell differentiation in a proportion of the cells. Interestingly, the HIV-1 activation level was significantly lower when the cells already in G2 phase were stimulated with PMA (Fig. 6b). Northern blot analysis clearly revealed that the HIV-1 expression in U1 cells was regulated at the transcriptional level (Fig. 3c). Since, among the transcriptional factors, cell cycle-dependent NF-κB is the most plausible candidate for the induction of HIV-1 gene expression (Nabel & Baltimore, 1987), we monitored the activity of NF-κB. The DNA-binding activity of NF-κB was detected exclusively at G2/M phase (Figs 4 and 5). Again, the NF-κB activity was very low in cells which had been stimulated with PMA after entering G2 phase, whereas active NF-κB with DNA-binding activity was detected just 2 h after TNF-α treatment at G1 and even after G2 phase (not shown). The failure of a quick response for NF-κB induction by PMA seemed to be due to the absence of active PKC, since TNF-α can activate NF-κB through another pathway (Meichle et al., 1990). These observations are consistent with the previous results of Duckett et al. (1995), who demonstrated that only TNF-α but not PMA could induce NF-κB in a cell cycle-independent manner in serum-starved NIH3T3 cells and mutant cells at G2 phase (FT210). In addition, Takai et al. (1996) showed that mitosis-specific phosphorylation by PKC was due to the distribution of its activated form. The details of why PMA failed to activate the NF-κB in the cells at G2 phase are currently under investigation.

The results presented here may be inconsistent with those of Baldwin et al. (1991), who reported that the induction of NF-κB was limited to the G2 to G1 transition in serum-starved NIH3T3 cells, and with the results of Griffin et al. (1989), who showed that NF-κB could be activated concomitantly with monocyte differentiation by phorbol ester or TNF-α. Differences in cell type studied (monocytes versus fibroblasts) or stimulant used (phorbol ester versus serum growth factors) might account for the discrepancy in the former case. Differences in the period of the assay might account for the discrepancy in the latter case (within 10 h after stimulation in this study versus more than 20 h after stimulation in the latter case). Another possible explanation is that particular type(s) of NF-κB-like activities might take place at different cell cycle phases, as described (Evans et al., 1993). We could not identify which isotype of NF-κB was induced in G2/M phase. Experiments using antibodies specific to the individual isotypes of NF-κB are now under way in our laboratory. Although HIV-1 gene expression in U1 cells almost paralleled the induction of NF-κB (Figs 3–5), the existence of another pathway for HIV-1 activation cannot be excluded, as a small population (~ 5%) of cells continuosly express HIV-1 antigens without induction by NF-κB under unstimulated conditions (Figs 1, 3 and 4). Such NF-κB-independent activation of HIV-1 gene expression was also detected as an immediate early response to PMA stimulation (Fig. 3c). Viral transcripts immediately detected after stimulation with PMA by Northern blotting (Fig. 3c) seemed to be produced in an autocrine manner by HIV-1 gene product(s) which are present in unstimulated cells (Biswas et al., 1995). Additional studies are required to clarify this point.

The vast majority of T-cells are found in a quiescent state (Cotner et al., 1983). Such cells are good candidates as reservoirs for HIV-1 in the lymphocyte compartment (Bagasra et al., 1993). Activation of HIV-1 from latency in such cells seems to play a key role in regulating the amounts of circulating virus even after treatment with antiretroviral drugs and subsequent progression to the disease. In this study, we clearly demonstrated that the activation of latently infected monocytic cells by PMA but not by TNF-α occurred predominantly at G2/M phase of the cell cycle, and this could be governed mostly by the limited, cell cycle-dependent inducibility of NF-κB. These results will provide insight into the cell cycle-dependent mechanisms of particular gene expression as well as facilitating development of novel approaches to control HIV-1 replication in infected carriers.

We are grateful to Dr T. M. Folks (Center for Disease Control and Prevention, Atlanta, GA, USA) for kindly providing U1 cell line and Dr I. M. Jones (National Environmental Research Centre, UK) for valuable discussion. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture, a Grant-in-Aid for AIDS Research from the Ministry of Health and Welfare, a Special Grant-in-Aid for the promotion of Education and Science at Hokkaido University provided by the Ministry of Education, Science, Sports and Culture in Japan, and the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (M. T. and K. F.).

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Received 17 October 1997; Accepted 23 February 1998