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Influence of cell cycle on HIV-1 expression differs among various models of chronic infection

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Summary. Because of an inherent dependence on host cell second and third messenger signaling pathways for activation of HIV-1 expression, a potential exists for a relationship between the induction of latent HIV-1 and cell-cycle-related events. To investigate this potential relationship, cellular models of latent HIV-1 infection (OM-10.1 promyelocytes, ACH-2 T-lymphocytes, and U1 promonocytes) were chemically treated or γ-irradiated to synchronize cultures at each cell cycle stage and then examined for constitutive and TNF-α-induced HIV-1 expression. Cell cycle synchronization alone had no effect on HIV-1 expression in OM-10.1 and U1 cultures; whereas enhanced constitutive HIV-1 expression was observed in ACH-2 cultures at $G_2 + M$. A 2 hour TNF-α treatment of all synchronized OM-10.1 cultures activated HIV-1 expression to a similar extent as unsynchronized cultures. In contrast, the extent of TNF-α-induced HIV-1 expression in ACH-2 S and G_2 + M cultures and in the Ul G_0/G_1 culture was greater than that in unsynchronized control cultures. However, no delay in the initial response was observed. Thus, the influence of cell cycle on constitutive and induced HIV-1 expression varied in each cellular model and, therefore, may further relate to the different molecular mechanisms maintaining viral latency.

Introduction

The replicative cycle of HIV-1 can conceptually be divided into afferent (pre-integrative) and efferent (post-integrative) phases [4, 28]. The afferent phase holds the cell-free virion as the central element and involves those steps necessary for viral binding, penetration, reverse transcription, and proviral integration into the host genome. The efferent phase encompasses the events necessary for the successful transcription and translation of the integrated

provirus and the assembly, packaging, and release of new progeny virions [4]. Although the division between these conceptual phases of HIV-1 replication may be somewhat artificial, very different cellular and viral factors regulate the pre- and post-integration aspects of the viral life cycle.

While cellular activation and entry into the cell cycle are required for the afferent replication of certain avian and murine onco-retroviruses [17, 21, 22, 44], several observations have raised doubts concerning the absolute requirement for cell division during afferent HIV-1 replication. HIV-1 can productively infect non-dividing cells of the macrophage lineage [15, 20, 32], even after irradiation [47]. Furthermore, productive acute HIV-1 infection has been reported in CD4-positive HeLa cells arrested in cell cycle [29] and in C8166 T-lymphoid cells blocked in cell division [30]. The mechanism by which non-dividing cells become infected has been linked to viral-associated Vpr and matrix proteins which can function to assist in the transport of the HIV-1 pre-integration complex to the nucleus [19, 23]. However, incomplete and inefficient HIV-1 reverse transcription [49] without subsequent viral integration [40] has been observed during infection of T-lymphocytes in the absence of cellular activation.

It is generally accepted that following proviral integration extracellular stimulation via cytokines or the T-cell receptor is required to activate HIV-1 expression [4]. Because viral activation is critically dependent upon normal cellular signal transduction pathways, the possibility exists for a regulatory role of cell-cycle-related factors in post-integrative HIV-1 replication [26]. Furthermore, activation of the HIV-1 provirus involves poorly defined protein kinases, some of which theoretically could be among the group of temporally synchronized kinases involved in the progression of cells through the G_0/G_1, S, and G_2 + M phases of the cell cycle (reviewed in [14, 27, 33]).

To investigate the influence of cell-cycle-related factors on the post-integrative phase of the HIV-1 replication cycle, we performed viral activation studies on several chronically infected cell models synchronized in the individual stages of the cell cycle. These studies demonstrated that regardless of which cycle stage the cells resided at the point of induction, the initiation of HIV-1 expression occurred within a similar time frame. However, basal level of viral expression and the extent of the HIV-1 response to activation varied among the differing models tested and at specific cell cycle stages.

Materials and methods

Cell culture and cell cycle synchronization

OM-10.1 promyelocytes (HL-60 derived) [5], ACH-2 T-cells (A3.01 derived) [7], and U1 promonocytes (U937 derived) [13] were obtained following acute HIV-1_{Lai} infection and clonally contain either one (OM-10.1 and ACH-2) or two (U1) stably integrated provirions. These cultures were maintained in RPMI 1640 basal medium (Gibco, Grand Island, NY)

containing 10% fetal bovine serum, 2 mM L-glutamine, and 1% Pen-Strep (Gibco) at 37 °C in a humidified atmosphere of 7% CO_2 and 93% air. Cell cultures were synchronized to the early S phase of the cell cycle by treatment with 1 mM thymidine [1, 31] for 12 h after which the cells were placed back into complete culture medium. Cultures enriched for cells in the S and G_0/G_1 phases were obtained at 4 and 12 h post-thymidine removal, respectively. To better synchronize cultures at the $G_2 + M$ stage, normal cultures were exposed to 20 μM genistein [42] or γ-irradiated (625–1 000 Rads) by exposure to a ^{137}Cs source. These cultures were used 24 h after treatment. The chemical and γ-irradiation treatments were staggered to simultaneously obtain cultures synchronized at each stage of the cell cycle (Fig. 1A).

Cell cycle analysis

Cell cycle analysis was performed by propidium iodide staining of cellular DNA. Cells were washed once with phosphate-buffered saline (PBS) and resuspended in 0.5 ml PBS containing 50 μg per ml propidium iodide, 2 mg per ml RNase A, and 0.6% Nonidet P-40. Cellular DNA content was analyzed using a Becton Dickinson FACScan system equipped with Cell-Fit DNA quantitation software (Becton Dickinson, San Jose, CA).

HIV-1 induction and detection

HIV-1 induction experiments were performed at 5×10^5 cells per ml using human recombinant TNF-α (Genzyme Corp., Cambridge, MA) at 20 (OM-10.1) or 100 (ACH-2 and U1) U per ml. Exposure to TNF-α was limited to 2 h after which TNF-α was removed by washing and the cells were returned to complete culture medium.

Detection of cellular HIV-1 expression was performed by immunofluorescence analysis (IFA). Briefly, acetone fixed cells were blocked with 10% goat serum in PBS and then stained with FITC-conjugated anti-HIV-1 serum pooled from AIDS patients. The percentage of positive cells was visually determined by UV light microscopy [5]. HIV-1 p24 core antigen was detected in culture supernatants by antigen capture ELISA (Coulter, Hialeah, FL). Northern analysis for HIV-1 specific RNA was performed as described [6]. Equivalent amounts of total purified RNA were denatured and electrophoresed through a 0.8% agarose gel containing formaldehyde. After being transferred to a nylon membrane (Hybond, Amersham Corp., Arlington Heights, IL), the RNA was probed in a 50% formamide hybridization buffer overnight at 42 °C using a ^{32}P-labeled, 2.5-kb XbaI-PstI fragment of pHXB2 [37] containing the 5′-long terminal repeat.

Results

Synchronization of chronically HIV-1-infected cells

To study potential interrelationships between cell cycle and HIV-1 activation, the percentage of cells at each individual stage of the cell cycle had to be substantially increased. Using the approach shown in Fig. 1A, synchronization of chronically HIV-1-infected cultures resulted in an enrichment of cells at the G_0/G_1, S, or $G_2 + M$ cell cycle stage (Fig. 1B, Table 1). No apparent differences were observed in the synchronization of the chronically infected cell models and their respective uninfected parental lines (data not shown).

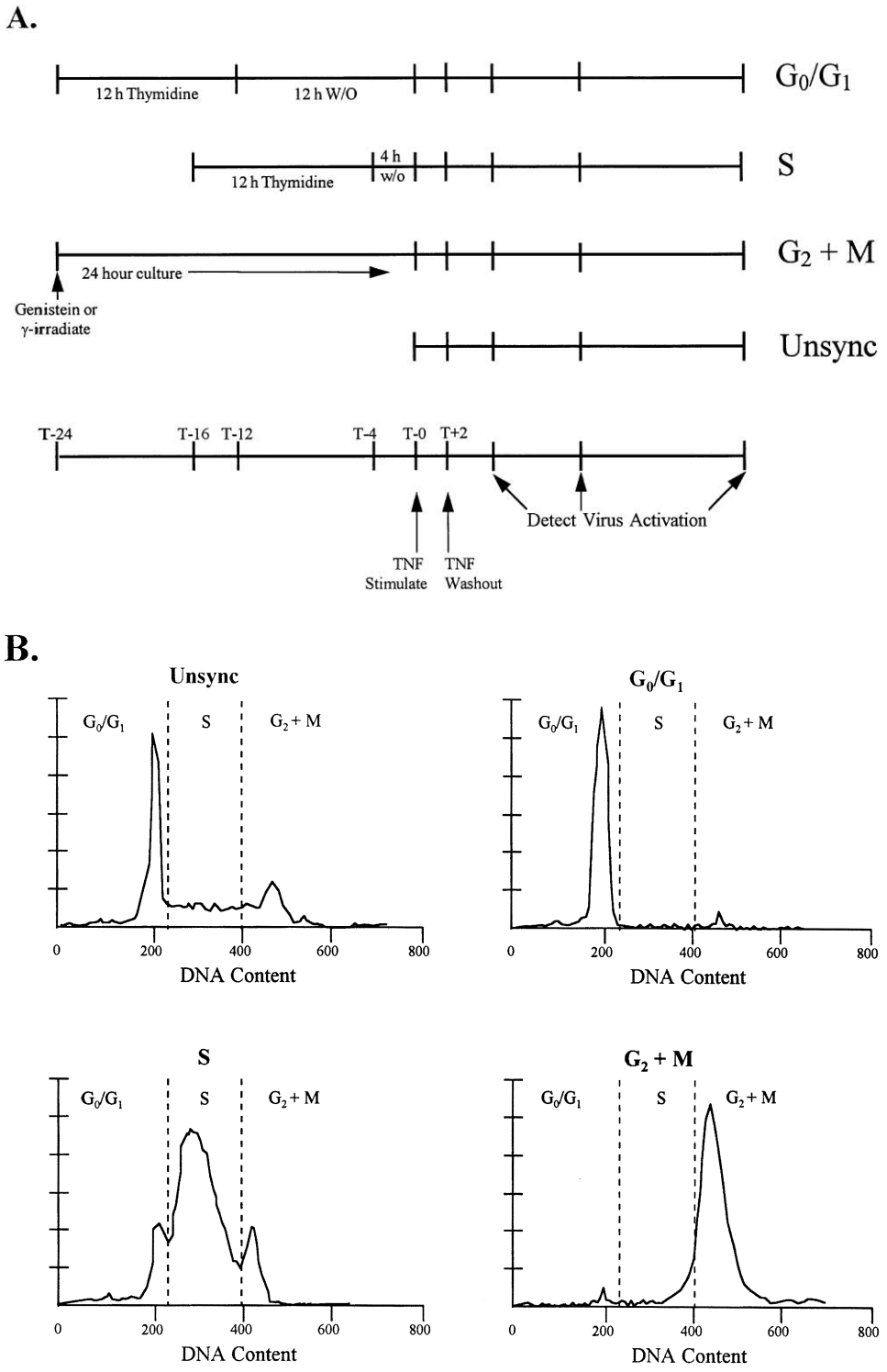

Fig. 1. Synchronization of cultures to enrich for cells in the various cell cycle stages. **A** Schematic time scale for staggered treatments to obtain individual cell cultures synchronized to each stage of the cell cycle as detailed in Materials and methods. **B** Cell cycle analysis of synchronized cells by propidium iodide uptake at T-0. DNA content distinguishes cells in each cell cycle stage and illustrates thymidine and γ-irradiation enrichment of the cell populations. The histogram patterns are representative for all cell types based on several independent synchronization experiments

Table 1. Percentage of cells in each cell cycle stage after synchronization

Cell line	Cell cycle status of culture	Cell cycle stage[a]		
		G_0/G_1	S	G_2 + M
ACH-2	Unsynchronized	47.60 ± 1.73	45.17 ± 1.35	7.23 ± 0.58
	G_0/G_1	68.13 ± 9.85	23.10 ± 13.71	7.85 ± 1.34
	S	3.87 ± 0.64	80.87 ± 15.06	3.63 ± 0.45
	$G_2 + M$ (genistein)	17.10 ± 12.06	47.80 ± 4.30	35.10 ± 16.04
	$G_2 + M$ (γ-irradiation)	9.33 ± 5.79	26.40 ± 6.31	66.43 ± 7.05
OM-10.1	Unsynchronized	40.58 ± 2.33	47.16 ± 1.64	12.28 ± 2.85
	G_0/G_1	62.82 ± 3.88	20.05 ± 0.78	16.25 ± 4.31
	S	11.35 ± 9.83	84.20 ± 5.31	9.82 ± 3.89
	$G_2 + M$ (genistein)	11.45 ± 7.99	48.50 ± 7.63	40.10 ± 0.28
	$G_2 + M$ (γ-irradiation)	4.81 ± 3.88	4.09 ± 1.52	76.91 ± 6.42
U1	Unsynchronized	45.05 ± 2.05	45.70 ± 0.71	9.20 ± 1.31
	G_0/G_1	66.40 ± 0.62	16.63 ± 5.95	17.70 ± 6.07
	S	25.27 ± 6.20	64.53 ± 4.03	8.37 ± 1.27
	G_2 + M (genistein)	17.53 ± 17.79	36.10 ± 30.58	46.40 ± 23.55
	G_2 + M (γ-irradiation)	4.15 ± 3.04	17.00 ± 15.13	76.47 ± 9.48

[a] Mean percentage of cells in each cycle stage from a minimum of three independent experiments $\pm$ standard deviation

Effect of cell cycle synchronization on constitutive HIV-1 expression

Synchronized cultures were first examined for alterations in the level of constitutive HIV-1 expression. In OM-10.1 and U1 cultures, synchronization alone did not alter the low constitutive level of HIV-1 expression. In contrast, ACH-2 cells showed a marked increase in basal HIV-1 expression at the $G_2 + M$ stage as evidenced by both anti-HIV-1 IFA (Table 2) and Northern analysis (Fig. 2B). Because γ-irradiation can directly activate the HIV-1 long terminal repeat promotor [39], the chronically infected cell models were also synchronized to the $G_2 + M$ stage by treatment with genistein. A similar pattern of increased HIV-1 basal expression at the $G_2 + M$ stage was evident in genistein-treated ACH-2 cultures (Table 2). An increase in basal HIV-1 transcription was also observed by Northern analysis in ACH-2 cultures synchronized to the G_0/G_1 stage (Fig. 2B); however, this increase was not reflected by parallel anti-HIV-1 IFA (Table 2).

Induction of HIV-1 expression in synchronized cultures

To examine the relationship between cell cycle and HIV-1 activation from latency, cultures were stimulated with TNF-α for just 2 h so that viral induction occurred only while the cells resided in a specific cell cycle stage. TNF-α treatment had little effect on cell cycle status of the synchronized cultures; however, as expected [45], a transient and modest accumulation of cells in the S phase was evident in unsynchronized cultures (data not shown). The initiation

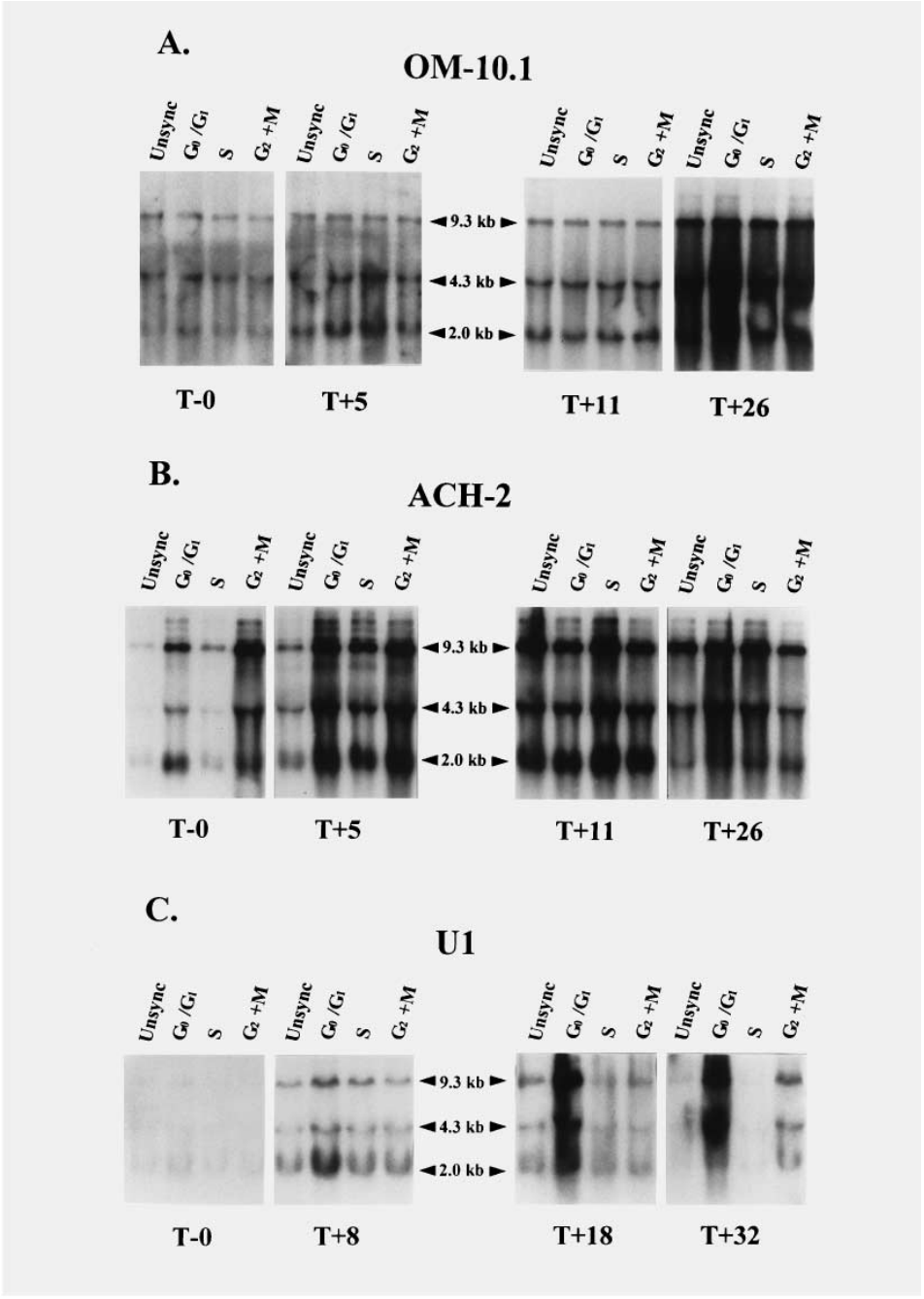

Fig. 2. TNF-α induction of HIV-1 transcription in OM-10.1 (**A**), ACH-2 (**B**), and U1 (**C**) cultures synchronized to the various cell cycle. Northern analysis autoradiographs for activated HIV-1 transcription in OM-10.1 (**A**) at T-0 and T+5 were intentionally over exposed to show detail. Full length (9.3 kb genomic) and spliced HIV-1 RNA species (4.3 kb envelope and 2.0 kb regulatory) are indicated. Ethidium bromide staining of the 28S ribosomal RNA in each lane was used to insure quantity and integrity (not shown)

Table 2. Direct immunofluorescence detection of HIV-1 expression in unsynchronized and synchronized cultures after TNF-α induction

Cell cycle status of culture	Cell line					
	ACH-2		OM-10.1		U1	
	Medium	TNF-α	Medium	TNF-α	Medium	TNF-α
Unsynchronized	10	70	10	75	1	5
G_0/G_1	10	70	10	75	2	25
S	10	70	10	75	1	5
$G_2 + M$ (genistein)	50	80	15	75	1	15
$G_2 + M$ (γ-irradiation)	50	75	15	75	1	15

Percentage of cells expressing HIV-1 at 24 h with medium alone or TNF-α treatment for 2 h. Data is representative of 3 independent experiments

of HIV-1 expression was then monitored by Northern analysis. The extent of viral activation and expression was monitored on a cell-per-cell basis by anti-HIV-1 IFA and on the culture basis by supernatant p24 antigen levels.

In synchronized OM-10.1 cultures, the pattern of HIV-1 transcriptional activation was identical regardless of the predominant cell cycle stage at the time of TNF-α stimulation (Fig. 2A). Furthermore, the amount of HIV-1 RNA that accumulated during the course of TNF-α induction was similar in all OM-10.1 cultures. This finding was further supported by anti-HIV-1 IFA in which approximately 75% HIV-1 expressing cells were observed in all OM-10.1 cultures at 24 h (Table 2). Similarly, TNF-α induction resulted in an equivalent increase (>10-fold) in detectable p24 antigen in each OM-10.1 culture (Fig. 3).

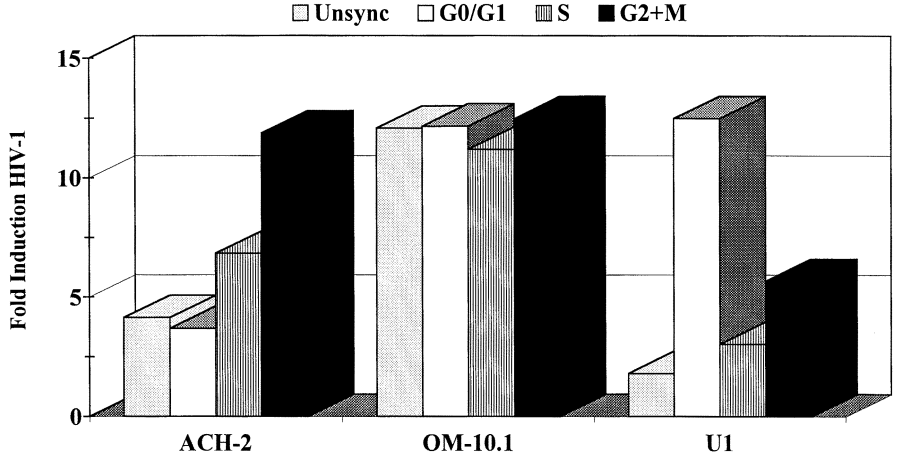

Fig. 3. HIV-1 expression at each stage of the cell cycle 24 h post TNF-α induction. Viral antigen in cell-free culture supernatants was measured by HIV-1 p24 antigen capture ELISA (Coulter). Data represents induced viral production as compared to the corresponding similarily synchronized culture without TNF-α stimulation

TNF-α treatment of ACH-2 cultures activated HIV-1 transcription by 5 h at each cell cycle stage (Fig. 2B), although a greater initial HIV-1 RNA accumulation was observed in the synchronized cultures. By 26 h post-TNF-α induction, all ACH-2 cultures showed similar levels of HIV-1 RNA (Fig. 2B) and approximately 70% HIV-1 expressing cells (Table 2). Interestingly, the amount of supernatant p24 antigen detected after TNF-α induction was dissimilar among ACH-2 cultures. A greater than 10-fold increased was observed in the G_2 + M culture and 5-fold in the S culture; while only a 3-fold increase was observed in both the unsynchronized and G_0/G_1 cultures (Fig. 3).

The pattern of TNF-α-induced HIV-1 expression in U1 cultures was dissimilar to both OM-10.1 and ACH-2 cultures. Northern analysis revealed that all U1 cultures responded to TNF-α induction by 8 h; however, the amount of viral RNA in cultures at the G_0/G_1 stage was markedly increased throughout the course of the experiment (Fig. 2C). HIV-1 transcription was modestly increased in the U1 $G_2 + M$ culture, but this effect was evidenced only late in the induction period. By anti-HIV-1 IFA, unsynchronized and S-stage cultures showed approximately 5% HIV-1-expressing cells, cultures synchronized to the G_0/G_1 stage showed 25% HIV-1-expressing cells, and the $G_2 + M$ culture showed 15% HIV-1-expressing cells (Table 2). Consistent with these results, unsynchronized and S-phase U1 cultures expressed lower amounts of viral antigen and the highest amount of viral expression was detected in the G_0/G_1 culture supernatants (Fig. 3).

Discussion

The influence of cell-cycle-related factors on HIV-1 expression was examined in models of chronic infection. Enhanced constitutive HIV-1 expression was observed in ACH-2 T-lymphocytes synchronized to the $G_2 + M$ stage; while no effect on constitutive viral expression was seen in synchronized OM-10.1 promyelocytes and in U1 promonocytes. The initial response time to TNF-α-induced HIV-1 activation was similar in all synchronized cultures, although the magnitude of response differed among the various models of chronic infection. These results demonstrate that cell cycle-related factors can influence the extent of HIV-1 expression in a cell line dependent manner and further suggests that multiple mechanisms control HIV-1 latency [6, 10].

Productive acute HIV-1 infection of normal T-lymphocytes is dependent on cellular activation. This dependency reflects the need for these cells to enter the cell cycle and permit the stable integration of an HIV-1 provirus [2, 3]. HIV-1 infection of non-dividing macrophages [15, 20, 32, 47] and transformed T-cell lines arrested in cycle [2, 29, 30] does not require activation; however, these cells normally reside at some cell cycle stage outside of G_0. Therefore, cell cycle status is an important component of the acute infection process.

In contrast, our data suggests that cell cycle-related factors are not an absolute requirement for the initiation of viral expression from an integrated

provirus. In accordance with this, TNF-α induction of NF-κB can occur at each stage of the cell cycle with increased binding activity in cells arrested at G_2 [12]. Our studies further indicate that second and third messenger signaling pathways required for TNF-α activation of latent HIV-1 expression do not critically involve kinases or phosphatases whose activity oscillates in a cell-cycle-dependent manner and that this response pathway persists during a variety of cellular influences.

TNF-α cytolysis has been associated with the $G_2 + M$ stage of the cell cycle [8, 9, 38], apparently due to signaling events that occur at the G_1/S interface [38]. Also, the level of TNF receptor surface expression fluctuates in a cell cycle-dependent manner with the highest level expressed at the $G_2 + M$ stage [46]. However, data from this study shows that TNF-α signaling pathways that result in HIV-1 activation are not influenced by factors that oscillate in a cell cycle-dependent manner; therefore, diverge from those resulting in cytolysis. Signaling via other cytokine receptors also activates HIV-1 expression in U1 cells [34]; however, the cell cycle influence on these signaling pathways has yet to be investigated.

In addition to its role in HIV-1 preintegration complex nuclear localization, the HIV-1 regulatory protein Vpr can directly hinder cell cycle progression [36] by blocking the activation of the $p34^{cdc2}$/cyclin B complex required for cells to enter M phase [18, 24, 35]. Blockade of this regulatory pathway could result in a delay of apoptosis and possibly allow for increased viral expression in infected cells while at $G_2 + M$ [24]. Although it is unlikely that the models of chronic infection used in this study harbor wild-type Vpr [36], enhanced viral expression by ACH-2 $G_2 + M$ cultures further supports the possibility that one function of Vpr is to permit increased HIV-1 production at $G_2 + M$ prior to cytolysis.

The cellular protein p53 has been described to regulate expression of cell cycle-related genes (reviewed in [43]). Overexpression of the anti-proliferative form (wt-p53) arrests cells at the G1 stage, while mutant forms of the protein allows cellular proliferation and may be involved in transformation. Additionally, mutant forms of p53 enhance HIV-1 replication in cotransfection studies [11, 16, 41] and could potentially influence viral expression in these models of chronic infection. While endogenous mutant p53 expression is absent in U1 [11] and unlikely in OM-10.1 cells [25, 48], ACH-2 cells do express endogenous mutant p53 [11]. Therefore, the unique pattern of HIV-1 expression in synchronized ACH-2 cultures may result to some extent from mutant p53 interacting with other factors that fluctuate with the cell cycle.

In our studies, enhanced HIV-1 transcription was also observed in ACH-2 cultures synchronized to the G_0/G_1 phase. Interestingly, accumulation of HIV-1 RNA in these cultures did not result in an increased level of viral expression as detected by p24 antigen or IFA. This disparity was not evident in U1 cultures synchronized at G_0/G_1 where increased HIV-1 transcription did correspond to an increase in viral expression. A separation of signals necessary for efficient viral transcription and translation has been previously observed and resulted in

an apparent synergy when TNF-α and IL-6 were used in combination [34]. Thus, in least in some cell types, the factors required for efficient viral transcription and translation may not coordinate in a cell cycle-dependent manner.

It is clear from our studies that TNF-α-induced HIV-1 expression can occur at each stage of the cell cycle in various models of chronic infection. However, cellular factors or other influences on cell cycle progression, including virally encoded factors such as Vpr, may further modify the extent of HIV replication from infected cells.

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