JOURNAL OF VIROLOGY, Apr. 1994, p. 2726–2730
0022-538X/94/$04.00+0

Vol. 68, No. 4

Human Immunodeficiency Virus Type 1 RNA Expression by Four Chronically Infected Cell Lines Indicates Multiple Mechanisms of Latency

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Received 25 February 1993/Accepted 17 December 1993

Recent information has suggested that posttranscriptional mechanisms, whereby human immunodeficiency virus type 1 (HIV-1) RNA exists as multiply spliced transcripts without promoting an accumulation of the larger messages, are responsible for maintaining a stable state of nonproductive viral expression or viral latency. To test the universality of these observations, we compared the patterns of viral RNA splicing and the frequencies of cells actually harboring HIV-1 RNA in four chronically HIV-1-infected cell lines (U1 [promonocytic], ACH-2 [T lymphocytic], OM-10.1 [promyelocytic], and J1.1 [T lymphocytic]). In uninduced U1 and ACH-2 cultures, a high frequency of cells (approximately one in six) contained HIV-1 RNA but mainly as multiply spliced transcripts, again supporting a posttranscriptional mechanism maintaining viral latency. In sharp contrast, only 1 in 50 cells in uninduced OM-10.1 and J1.1 cultures contained HIV-1 RNA, indicating a primary transcriptional mechanism controlling viral expression in these cells. Furthermore, those OM-10.1 and J1.1 cells that did contain viral RNA were in a state of productive HIV-1 expression marked by the presence of both spliced and unspliced transcripts. Even though the total absence of viral RNA in the majority of OM-10.1 and J1.1 cells indicated a state of absolute latency, treatment with tumor necrosis factor alpha induced transcription of HIV-1 RNA in nearly 100% of the cells in all four of the chronically infected cultures. Tumor necrosis factor alpha induction of U1, ACH-2, and OM-10.1 cultures resulted in an initial accumulation of multiply spliced HIV-1 RNA followed by a transition to the larger unspliced viral RNA transcripts. This RNA splice transition was less apparent in the J1.1 cell line. These results demonstrate that host cell-specific transcriptional and posttranscriptional mechanisms are important factors in the control of HIV-1 latency.

During the extended clinically asymptomatic period following human immunodeficiency virus type 1 (HIV-1) infection, the opportunities for medical intervention are potentially immense (7). However, such intervention will require a detailed understanding of the various mechanisms that control HIV-1 latency and expression at a cellular level (1). Advances in this area have been made by studying chronically infected cell models in which constitutive HIV-1 expression is minimal but can be induced physiologically with cytokines (2).

After integration, HIV-1 gene expression can be divided into early and late stages. Initially, the predominance of viral mRNA is multiply spliced to 2.0-kb transcripts that encode the regulatory proteins (including Tat and Rev) necessary to propagate viral expression (8, 11, 15). A transition then occurs to permit the accumulation of singly spliced (4.3-kb) and full-length (9.3-kb) RNA species encoding the viral structural proteins and providing the genomic RNA for budding virions (8, 11). The mechanisms that control this early-to-late-stage transition in viral RNA splicing is reported to involve the action of the Rev protein in the protection and transport of full-length HIV-1 RNA (9, 13).

In chronically infected U1 and ACH-2 cultures, the constitutive pattern of HIV-1 RNA splicing previously showed a predominance of the 2.0- and 4.3-kb viral messages coincident with a relative absence of full-length message (14, 21). In addition, an initial accumulation of multiply spliced viral RNA after induction of these cultures that mimicked the early-to-late-stage transition of HIV-1 replication was observed. Therefore, the nonproductive pattern of constitutive viral expression in these cultures was appropriately described as blocked early-stage latency (22), whereby HIV-1 RNA is present but selectively spliced to restrict productive viral expression. These findings suggested that a primary posttranscriptional mechanism was responsible for maintaining nonproductive HIV-1 expression and that a threshold level of Rev protein was necessary to drive productive viral expression (20, 21). Additional support for the Rev threshold hypothesis has arisen from observations of an identical HIV-1 RNA splice pattern during infection with *rev* deletion mutants (8) and that multiple Rev protein monomers must bind to the Rev response element of the viral RNA to achieve full functional activity (12).

To test the universality of posttranscriptional regulation of HIV-1 expression, we compared the patterns of HIV-1 RNA splicing and the frequency of cells harboring viral RNA in four independently derived, chronically infected cell lines. The promonocytic U1 (10), promyelocytic OM-10.1 (3), T-lymphocytic ACH-2 (5), and T-lymphocytic J1.1 (19) lines were cloned following an acute HIV-1 (LAI) infection of their parental cell lines (U937, HL-60, A3.01, and Jurkat, respectively). All four infected lines harbor one or more integrated provirions and constitutively express very low amounts of HIV-1 proteins when maintained in RPMI 1640 medium containing 10% fetal bovine serum. However, HIV-1 expression can be induced in

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each cell culture using recombinant human tumor necrosis factor alpha (TNF-α).

To determine the frequency of cells harboring viral RNA in uninduced and TNF-α-induced cultures at the single-cell level, reverse transcriptase-dependent PCR (RT-PCR) was performed on limiting cell dilutions. Chronically infected cells were cultured in the presence or absence of TNF-α for 24 h, and then serial twofold dilutions of the cells into their respective uninfected parental cell lines were prepared so that each dilution contained a total of 10^6 cells. Cell dilutions were performed prior to RNA extraction to ensure that the frequency results would not be artifactually influenced by individual cells harboring a relative abundance of viral RNA. Total RNA was extracted from each infected cell dilution by using a commercially available kit (Clontech Laboratories, Inc., Palo Alto, Calif.), digested with DNase (Promega Corp., Madison, Wis.) for 30 min at 37°C, chloroform-isoamyl alcohol extracted, and ethanol precipitated. The RNA pellet was resuspended in 4 μl of diethyl pyrocarbonate-treated water and subjected to reverse transcription at 42°C, using random hexamer primers, as directed by the kit's manufacturer (Perkin-Elmer Cetus, Norwalk, Conn.). Half of the resultant cDNA product was amplified in a 100-μl reaction volume by using the *gag*-based primers SK38 and SK39 (16) as described previously (23), and the other half was amplified by using the 5′ long terminal repeat (LTR)-based primers FPL1 and FPL2 as described previously (14). Ten microliters of each of the final amplification products was analyzed by agarose gel electrophoresis and Southern hybridization to ^{32}P-end labeled probe SK19 (16) for *gag*-based primer reactions or LTRP3 (14) for LTR-based primer reactions. The amplified products of *gag*-based primer reactions were further quantitated by luminometry after liquid-phase hybridization with HIV-1 *gag*-specific, acridinium ester-conjugated probes as specified by the manufacturer (Gen-Probe, Inc., San Diego, Calif.).

Detection of all viral RNA transcripts, regardless of the splicing event, was achieved by using PCR primers and probe specific for sequences within the 5′ LTR (14, 15). As shown in Fig. 1, the frequency of uninduced U1 and ACH-2 cells expressing HIV-1 RNA was relatively high, approximately 20% (>1 in 6). However, by comparison, the frequency of cells in uninduced OM-10.1 and J1.1 cultures transcribing LTR-containing messages was dramatically lower, approximately 1 in 50. Long exposures of the hybridization reaction were used to determine the final end point of reactivity depicted in Fig. 1. Because of the technical variability inherent to dilutions on a single-cell level and RT-PCR, three additional cell titrations were independently prepared and amplified. In every case, the frequency of cells positive for LTR-containing messages in uninduced OM-10.1 cultures was at most 1 in 50, while the frequency remained much higher in uninduced U1 cultures (data not shown). Thus, the vast majority of uninduced OM-10.1 and J1.1 cells contain no detectable viral RNA, indicating a state of absolute latency on a cellular level, while a much higher frequency of uninduced U1 and ACH-2 cells harbor HIV-1 LTR-containing RNA. Dilutional RT-PCR was also performed 24 h after TNF-α induction, and in all four of the cell lines, TNF-α treatment resulted in an accumulation of LTR-containing viral RNA in nearly 100% of the cells (Fig. 1).

A similar approach was used to determine the frequency of cells expressing full-length HIV-1 RNA except that primers and probes targeting the *gag* region were used. Because the *gag* region of the viral RNA is removed during splicing (8), *gag*-based primers will amplify only full-length HIV-1 RNA. Amplification products from the HIV-1 *gag* region of the expected molecular size were confirmed by Southern hybridization and were quantitated by luminometry using acridinium ester-conjugated, *gag*-specific probes (Fig. 2). All four chronically infected cultures when maintained in medium showed a frequency of approximately 1 in 50 cells containing *gag*-encoding viral RNA. However, after TNF-α treatment, the frequency of cells expressing full-length viral RNA increased to nearly 100% in all four cultures (Fig. 2), indicating complete conversion to productive HIV-1 expression.

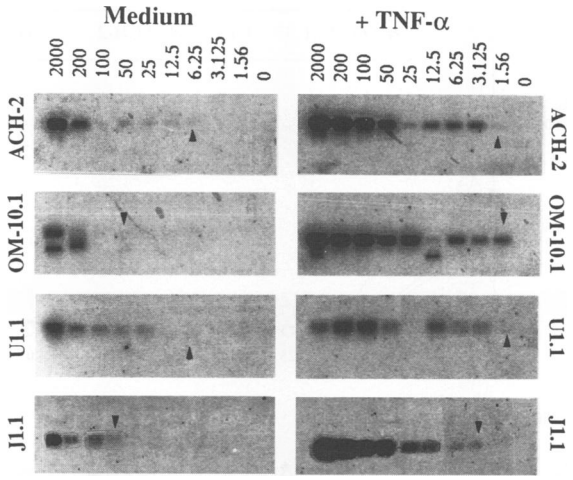

FIG. 1. Limiting-dilution RT-PCR of four chronically infected cell lines either uninduced (Medium) or after HIV-1 induction (+ TNF-α). HIV-1 induction was performed by using human recombinant TNF-α (Genzyme Corp., Cambridge, Mass.) at 20 U/ml for OM-10.1 cultures and at 100 U/ml for U1, J1.1, and ACH-2 cultures for 24 h. The number of infected cells per culture is indicated above each lane. Total cellular RNA was purified from each culture, DNase treated, reverse transcribed, and PCR amplified by using LTR-based primers and probe for detection of all HIV-1 RNA. Amplified products were detected by Southern hybridization and analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) analysis. A series of control reactions was performed to verify that the PCR-amplified products were reverse transcriptase dependent (not shown). In each case, amplified products of the expected molecular sizes were generated; spurious bands were presumably due to partial RNA degradation. Extended autoradiographic exposures of each blot were performed to determine the cellular frequency of detection (marked on each panel by an arrowhead). Results are representative of five independent experiments.

The frequency of RT-PCR-positive cells with use of LTR-based primers was compared with that obtained with use of *gag*-based primers (Table 1). In uninduced U1 and ACH-2 cultures, LTR-based RT-PCR detected a cellular frequency of approximately 20% (>1 in 6), much higher than the cellular frequency detected by *gag*-based amplification (approximately 1 in 50). Therefore, the viral RNA pattern observed with uninduced U1 and ACH-2 cells by RT-PCR mimicked the blocked early-stage latency pattern reported by standard Northern (RNA) blot analysis (14, 21) in that a high frequency of cells expressed spliced HIV-1 RNA in the relative absence of full-length transcripts. In uninduced OM-10.1 and J1.1 cultures, the cellular frequency observed by both LTR-based and *gag*-based RT-PCR was identical, approximately 1 in 50. Thus, the small proportion of uninduced OM-10.1 and J1.1 cells that did contain viral RNA were in a state of productive expression, marked by the presence of both spliced and full-length transcripts. However, the vast majority of uninduced OM-10.1 and J1.1 cells contained no detectable viral

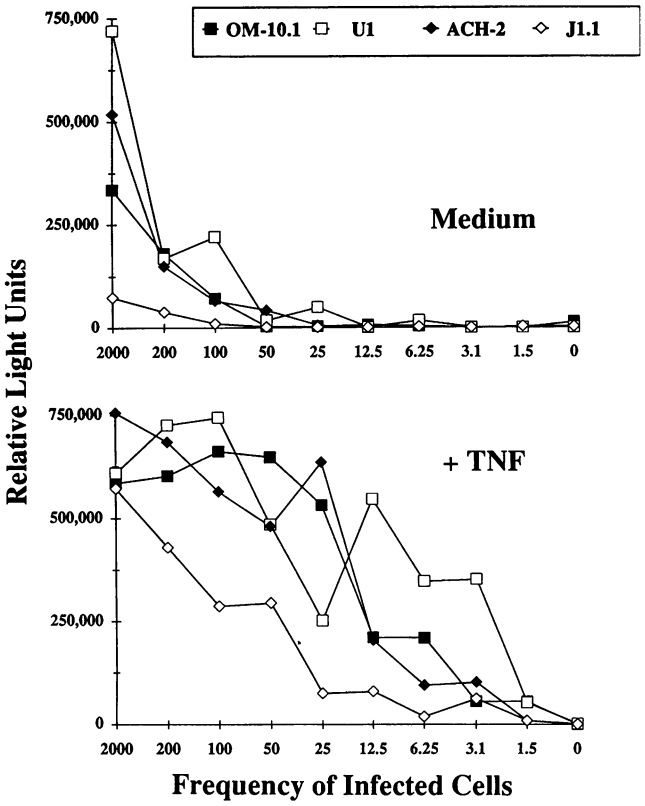

FIG. 2. Limiting-dilution RT-PCR of four chronically infected cell lines either uninduced (Medium) or after HIV-1 induction (+ TNF) as described for Fig. 1 except that RT-PCR was performed with *gag*-based primers. Amplified products of the expected molecular sizes were verified by Southern hybridization and quantitated by luminometry using acridinium ester-conjugated probes (Gen-Probe). Results are reported as relative light units for each frequency of infected cells. Results are representative of two independent experiments.

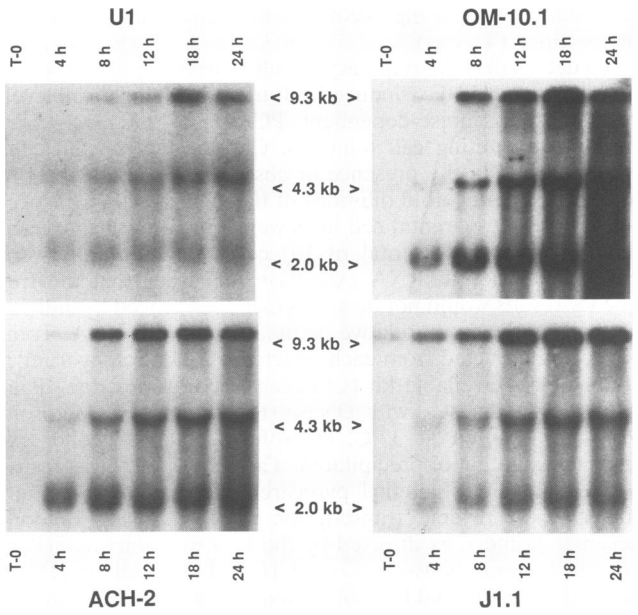

FIG. 3. Kinetic induction of HIV-1 expression from four chronically infected cell lines after TNF-α treatment. HIV-1-specific RNA was detected from 25 μg of total cellular RNA per lane for U1 and 10 μg of total cellular RNA per lane for the other three cell lines. The quantity and integrity of the separated RNA was confirmed by ethidium bromide staining and UV visualization of the ribosomal bands (not shown). A radiolabeled HIV-1 5′ LTR probe was used in hybridization, and the image was developed by autoradiography (7-day exposure). For each cell line, a representative blot depicting the pattern observed in several independent experiments is shown. HIV-1 expression was verified in cell-free culture supernatants by p24 enzyme-linked immunosorbent assay (Coulter Immunologics, Hialeah, Fla.) or by reverse transcriptase assay (29), with induction after 72 h being >100-fold in OM-10.1 cultures, >50-fold in U1 cultures, and 12- to 15-fold in J1.1 and ACH-2 cultures.

RNA, and blocked early-stage latency was not evident in these cultures. After TNF-α induction, HIV-1 RNA could be detected to the single-cell level in all four cell lines when analyzed with either set of RT-PCR primers (Table 1).

We then examined the kinetic pattern of HIV-1 RNA splicing during TNF-α induction at the population level by standard Northern blot analysis (Fig. 3). Total RNA was purified from guanidine thiocyanate cell lysates (26), quanti-

TABLE 1. Numbers of cells expressing HIV-1 RNA in uninduced and TNF-α-induced chronically infected cultures[a]

No. of infected cells	Reaction															
	OM-10.1				ACH-2				U1				J1.1			
	Medium		+TNF-α		Medium		+TNF-α		Medium		+TNF-α		Medium		+TNF-α	
	LTR	*gag*	LTR	*gag*	LTR	*gag*	LTR	*gag*	LTR	*gag*	LTR	*gag*	LTR	*gag*	LTR	*gag*
2,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	−	−	+	+	+	−	+	+	+	−	+	+	−	−	+	+
12.5	−	−	+	+	+	−	+	+	+	−	+	+	−	−	+	+
6.25	−	−	+	+	+	−	+	+	+	−	+	+	−	−	+	+
3.125	−	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+
1.56	−	−	+	+	−	−	+	+	−	−	+	+	−	−	−	−
0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Cultures containing known numbers of infected cells were prepared in 10^6 uninfected parental cells and subjected to RT-PCR, using either LTR-based primers (for the detection of all viral RNA transcripts) or *gag*-based primers (for the detection of full-length viral RNA). Results are reported as a positive or negative RT-PCR reaction after specific Southern hybridization for the LTR reactions or luminometry for the *gag* reactions.

tated, and electrophoresed through formaldehyde-containing agarose gels (3). After Northern transfer, HIV-1-specific RNA was detected by hybridization using a ^{32}P-labeled 2.5-kb *Xba*I-*Pst*I fragment of pHXB2 (25), containing the 5′ LTR, as described previously (4). The viral RNA pattern observed at the population level for each culture before TNF-α induction (T-0) accurately reflected the pattern observed at the cellular level by RT-PCR. Accordingly, uninduced U1 cultures displayed a viral RNA pattern dominated by the spliced transcripts with an absence of full-length message (Fig. 3). The same was true for uninduced ACH-2 cultures when the T-0 pattern was enhanced by longer autoradiographic exposures (data not shown). This pattern is evidently due to the high frequency of cells displaying block early-stage latency in these cultures. In contrast, the viral RNA pattern from uninduced OM-10.1 and J1.1 cultures had evidence of full-length HIV-1 RNA. This pattern is apparently contributed by the small percentage of cells that do contain viral RNA and are in a state of productive expression as evidenced by RT-PCR.

Viral induction in each cell line was evident by an accumulation of HIV-1-specific mRNA after 4 to 8 h of TNF-α treatment (Fig. 3). In U1, ACH-2, and OM-10.1 cultures, the initial viral RNA that arose after induction was predominantly spliced to the 2.0-kb messages encoding the regulatory proteins. In these cultures, a transition to the singly spliced and full-length messages occurred within 12 h of induction, after which all three viral RNA bands continued to accumulate. Laser densitometry verified the initial accumulation of 2.0-kb viral RNA and the transition to the larger messages during the time course of TNF-α induction in these cultures (data not shown). The initial accumulation of the multiply spliced messages and the early-to-late-stage transition in these cultures support the possibility that a threshold level of Rev activity is required to drive HIV-1 expression after activation (12, 14, 20, 21). The kinetic viral RNA pattern observed in J1.1 cultures after induction was different in that no initial predominance of the multiple spliced RNA species was observed (Fig. 3). This may indicate a lower threshold requirement for Rev in these cells or a higher endogenous level of host factors necessary for Rev activity (27, 28).

The mechanisms controlling viral quiescence among these cell systems appear to exist on at least two distinct levels. As observed in a proportion of U1 and ACH-2 cells, one level of control exists posttranscriptionally, since HIV-1 RNA is transcribed but processed via splicing to maintain a nonproductive viral state. This blocked early-stage latency pattern was evident at the cellular level by RT-PCR and at the culture level by standard Northern analysis. The posttranscriptional control mechanisms of U1 and ACH-2 cells may involve additional host cell factors essential for Rev activity (27, 28) that may need to accumulate after cellular stimulation to drive productive HIV-1 expression. Alternatively, viral quiescence in OM-10.1 and J1.1 cells is due to strict transcriptional control. This was clearly evident on a cellular level by RT-PCR because the vast majority of uninduced cells contained no detectable viral RNA. On a population level, the small percentage (approximately 2%) of cells constitutively expressing HIV-1 gave evidence of full-length viral RNA by Northern analysis. Cellular factors may also be critical for maintaining the state of latency in OM-10.1 and J1.1 cells. This regulation could potentially involve factors working at the level of the integrated provirus that prevent transcription or an absence of positive regulatory factors necessary to drive viral transcription.

Furthermore, multiple mechanisms may be controlling HIV-1 expression within U1 and ACH-2 cultures, since only approximately 20% of the uninduced cells exhibited the blocked early-stage pattern of latency. By RT-PCR analysis, the majority of uninduced U1 and ACH-2 cells appeared to contain no viral RNA and may be subject to control mechanisms similar to those exhibited by OM-10.1 and J1.1 cultures. The ability of U1 and ACH-2 cells to transcribe HIV-1 RNA may occur during a discrete stage of the cell cycle or be under the control of other mechanisms influencing gene activity, therefore permitting only a proportion of the uninduced cells to harbor viral RNA at any one time. The same may be true for OM-10.1 and J1.1 cultures because approximately 2% of the cells productively express HIV-1 RNA and protein at any given time.

The relative absence of full-length HIV-1 RNA message in uninduced U1 and ACH-2 cells suggests that a low constitutive level of Rev protein may be a posttranscriptional mechanism responsible for maintaining nonproductive viral expression (12, 14, 21). Our results with OM-10.1 and J1.1 cells suggest that mechanisms at the level of transcription may also control constitutive HIV-1 expression. Because cell lines displaying different mechanisms of control over latency each underwent a selective splice switch to the regulatory messages upon viral induction, the accumulation of Rev to a critical threshold level remains a potential unifying mechanism for the activation of HIV-1 expression. The same may be true for the J1.1 cell line, although the critical Rev threshold appears to be lower due to cell line-specific differences.

The contribution of HIV-1 latency on a cellular level to the clinical progression to AIDS remains poorly defined. Although active HIV-1 replication can be detected at all stages of AIDS progression, including the clinically asymptomatic phase (17, 18), a large number of infected cells within the lymph nodes harbor HIV-1 DNA in the absence of detectable viral RNA (6). This discrepancy between viral DNA and RNA suggests a state of absolute viral latency and is reminiscent of the pattern observed in the OM-10.1 and J1.1 cell lines. From our in vitro observations with these cell lines, it might be expected that when these infected lymph node cells in vivo encounter the proper combination of extracellular stimuli, a conversion to active viral transcription can occur. Furthermore, the blocked early-stage latency pattern observed with the U1 and ACH-2 cell lines has also been detected in the peripheral blood cells of HIV-1-infected individuals (24). These findings suggest that HIV-1 latency on a cellular level is an important aspect of the clinical disease and that HIV-1 activation from latency may contribute to virus dissemination and disease progression.

We report on the HIV-1 RNA patterns in four independently derived, chronically infected cell lines during uninduced and induced viral expression. These cell systems represent at least two different and distinct mechanisms governing HIV-1 expression with various degrees of cellular and viral control and will serve as important systems of comparison. It will be of continued interest and medical importance to investigate the molecular mechanisms involved in controlling the state of repressed viral expression and the pathways responsible for HIV-1 activation.

We thank P. Guenther for technical involvement with the RT-PCR assays and M. Saltarelli and C. Hart for critical reviews of the manuscript.

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