Temporal Aspects of DNA and RNA Synthesis during Human Immunodeficiency Virus Infection: Evidence for Differential Gene Expression

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The kinetics of retroviral DNA and RNA synthesis are parameters vital to understanding viral growth, especially for human immunodeficiency virus (HIV), which encodes several of its own regulatory genes. We have established a single-cycle growth condition for HIV in H9 cells, a human CD4+ lymphocyte line. The full-length viral linear DNA is first detectable by 4 h postinfection. During a one-step growth of HIV, amounts of viral DNA gradually increase until 8 to 12 h postinfection and then decrease. The copy number of unintegrated viral DNA is not extraordinarily high even at its peak. Most strikingly, there is a temporal program of RNA accumulation: the earliest RNA is greatly enriched in the 2-kilobase subgenomic mRNA species, while the level of 9.2-kilobase RNA which is both genomic RNA and mRNA remains low until after 24 h of infection. Virus production begins at about 24 h postinfection. Thus, viral DNA synthesis is as rapid as for other retroviruses, but viral RNA synthesis involves temporal alteration in the species that accumulate, presumably as a consequence of viral regulatory genes.

Synthesis of DNA from the genomic RNA template and transcription of viral RNA from the viral DNA are early steps in the retroviral life cycle. Although these steps have been well studied in simpler retroviruses (for a review, see reference 27), the sequential pathways of DNA and RNA synthesis in human immunodeficiency virus (HIV) have not been investigated. It is clear that these steps are not uniform among retroviruses. The diversity in aspects of DNA synthesis is exemplified by spleen necrosis virus (10) and Rous-associated virus (30), in which a high copy number of unintegrated linear DNA has been correlated with cell death, and by visna virus, which may not integrate its DNA into the genome of host cells, at least in tissue culture (7). It has also been hypothesized that for HIV, unintegrated viral DNA may be used as a template for transcription and that the high copy number of unintegrated viral DNA may result in cell death (15, 23). To examine such possibilities, we have studied the timing and quantity of viral DNA synthesis following HIV infection. In addition, the complex nature of the genome—nine genes in its 9.2 kilobases (kb)—and the action of the two best-characterized trans-acting regulatory genes, tat and rev (for recent articles, see references 4, 5, 11, 12, 19, and 20), indicate that HIV might have an efficient way of regulating its own gene expression during its infection cycle. Therefore, we have studied the species of viral RNA present at different times after infection. We find that viral DNA synthesis is similar to that of standard retroviruses but that there is temporal control of the species of viral mRNA that accumulate.

MATERIALS AND METHODS

Virus and infection. To produce virus, COS cells were transfected with the plasmid pW13. pW13 is essentially the same as RIP7 (14) except that the former contains the full nef coding region derived from pCV-3 (1). The culture supernatant was mixed with H9 cells, a human CD4+ lymphocyte line (18), 2 to 3 days posttransfection. Approximately 70 to 90% of these H9 cells were infected after 7 to 14 days. At this time, a high concentration of virus was prepared by the shaking method (29) and then filtered through the 0.45-μm-pore-size filter. To give the highest rate of initial infection, virus was used immediately for infection of fresh H9 cells. H9 cells were infected with HIV type 1 (HIV-1) W13 as follows. Cells were treated with polybrene at 37°C for 1 h, collected by centrifugation, and exposed to virus at 37°C for 1.5 h. They were then washed with medium and divided among an appropriate number of culture flasks. For most of the experiments described here, each bottle initially contained approximately 107 cells. At each time point, samples were taken for reverse transcriptase assay (17), indirect immunofluorescence assay (9), viable cell count, and preparation of low-molecular-weight DNA. (Times indicated in the figures include 1.5 h of virus exposure time.)

Primary T cells were prepared as described elsewhere (6, 32) and treated with 2 μg of phytohemagglutinin per ml for 2 to 3 days before infection.

DNA blot hybridization. Low-molecular-weight DNA was prepared from infected cells by Hirt extraction (8) and subjected to Southern blot analysis (24). For quantitative measurement of the copy number of unintegrated HIV DNA, the total DNA fraction, including high- and low-molecular-weight DNA, was prepared. Five million cells were treated with 1% sodium dodecyl sulfate and 400 μg of protease K per ml, and 1/60 of these DNA preparations was directly subjected to Southern blot analysis. It has to be noted that this procedure consistently yields a lower ratio of circular to linear DNA than the Hirt procedure. The DNA probe used for hybridization was the SacI fragments of pW13, which includes most of the HIV genomic DNA. The intensities of HIV-specific DNA bands were determined by Ultrascan Laser Densitometer (LKB 2202). X-ray film was preflushed before exposure to the filter.
RNA blot hybridization. Total cellular and cytoplasmic RNAs were prepared by the guanidine thiocyanate-cesium chloride method (2). For cytoplasmic RNA, the cytoplasmic fraction was prepared with the detergent Nonidet P-40 and then mixed with guanidine thiocyanate. The DNA probe used for hybridization was the 511-base-pair $BglII$ fragment of pW13, which includes the polyadenylation signal sequence.

RESULTS

Establishment of a one-step growth of HIV. Investigating the kinetics of DNA and RNA synthesis required the establishment of a single-cycle growth condition for HIV. To achieve this, we used a procedure for producing reliably high-titer HIV-1 stocks from a homogeneous, cloned viral DNA, thus allowing synchronous infection of 10 to 20% of H9 or primary T cells. When H9 cultures were analyzed at various times after infection, a significant number of immunofluorescence-positive cells were first observed 28 to 32 h postinfection, coinciding with an exponential increase in reverse transcriptase activity in cell culture supernatants (Fig. 1). Immunofluorescence indicated the expression of viral structural gene products, while reverse transcriptase measured the active release of virus particles, suggesting the end of a one-step growth cycle. This interpretation is reinforced by the DNA and RNA analysis described below. Therefore, by making observations during the first 32 h after infection, we could approximate one-step growth conditions.

DNA synthesis during a one-step growth of HIV. To study temporal aspects of viral DNA synthesis, low-molecular-weight DNA was prepared from infected cells by Hirt extraction (8) at various times before and after a one-step growth cycle and assayed for viral DNA content by electrophoretic separation and hybridization to a $^{32}P$-labeled DNA probe. The analysis of total low-molecular-weight DNA from cultured cells acutely infected with HIV revealed three major species of HIV-specific DNA. The DNA bands were identified as linear, circular, and nicked circular species (designated L, C, and X, respectively [Fig. 2]) by restriction enzyme digestion as well as by their sizes (data not shown).

HIV-specific linear DNA could be detected 4 h after initiation of infection, while the circular form was first detectable between 8 and 12 h (Fig. 2A; note that two exposures of the same gel are presented). The ratio of circular to linear DNA increased as the viral infection progressed, but linear DNA remained the dominant form throughout the one-step growth cycle. After the first cycle of infection was completed, between 28 and 32 h postinfection, the amount of viral DNA increased greatly as the infection spread through the culture (Fig. 2A).

To localize the different viral DNA species, cytoplasmic and nuclear fractions were prepared with the detergent Nonidet P-40 and low-molecular-weight DNA was made from each fraction. Only linear DNA was evident in the cytoplasmic fraction (Fig. 2B); it was detectable as early as 5 h after infection in both the cytoplasm and nucleus. Circular DNA appeared in the nucleus between 9 and 12 h postinfection. Therefore, the nucleus seems to be the exclusive site of circularization of viral DNA.

To test whether DNA synthesis occurs with similar kinetics in normal T cells, human primary T cells enriched for
CD4+ cells were used for infection. When low-molecular-weight DNA from these cells was analyzed, a linear DNA species was detected 5 h after initial infection, while circular DNA appeared at 12 h (Fig. 2C). These results indicate that the rates of reverse transcription and DNA processing in primary T cells are comparable to those in H9 cells.

**Copy number of unintegrated DNA.** The copy number of HIV-specific DNA was estimated by Southern blot analysis by using known amounts of the initiating plasmid DNA as a standard (Fig. 3). For quantitative measurement, total DNA fractions containing low- and high-molecular-weight DNAs were prepared at various time points. The copy number of linear DNA could be determined by using the observed values for the concentration of free viral DNA (estimated from Southern blot analysis) and the total number of cells. Although only a fraction of the cells were infected productively at early times, we calculated the copy number on the basis of total cells because we assume that all cells are able to bind virus and support reverse transcription. This assumption may not be correct, but it provides a basis for calculation; it is partially justified by the observation that after a few days almost all cells become infected, as measured by indirect immunofluorescence assay, implying that all cells are potentially infectable. Our DNA copy number per cell, thus, represents a minimum estimate of copy number per infected cell.

The time course of viral DNA synthesis in two representative infections is shown in Fig. 3. The changes in the amounts of free viral DNA occurred in two phases. During the first round of infection, representing a one-step cycle, the copy number per cell rose to 1 to 3 and then fell. In all infections studied, there was a reproducible decrease in linear DNA between 12 and 24 h postinfection, which may represent degradation of the earliest reverse transcripts. After the first cycle was completed between 28 and 32 h postinfection, the copy number per cell rapidly increased, reaching 20 and again declining. The increase in the copy number presumably represented infection of uninfected cells left in the culture after the initial round of infection. The exact time of the peak varied from one infection to another, apparently because of the biological potency of the input inoculum, which would determine the fraction of initially infected cells. In the slower infection cycle, the proportion of immunofluorescent cells was 5% after 27 h, for the faster infection it was 8%. After a peak at about 46 or 80 h (Fig. 3), the amount of free viral DNA fell as the infection progressed. After virtually all cells had been infected (150 h postinfection or in chronically infected cultures), the amount of linear DNA fell drastically, but even in chronically infected cultures (3 weeks postinfection), there was detectable linear DNA (Fig. 2A, gel b, lane Ch). The source of this free viral DNA is not clear, but the DNA could come from a low level of continual reinfecion or intracellular activity of reverse transcriptase.

**RNA synthesis during a one-step growth of HIV.** To study temporal aspects of viral RNA synthesis, total and cytoplasmic RNAs were prepared from infected cells at various times before and after a one-step growth cycle. During acute infection, three RNA bands were detected by Northern (RNA) blot analysis (Fig. 4A). The largest band was 9.2 kb and represented the full-size transcript, which acts both as the genome for the virus and as the mRNA for the gag and pol genes. The second band consisted of env mRNA and was 4.3 kb. Upon densitometric analysis of RNA samples, this band showed a broader peak than others (Fig. 4B) and thus could include other messages, such as those for vif, vpr, and the partially spliced tat transcripts (11). The smallest band included a heterogeneous mixture of mRNAs encoding the small regulatory genes such as tat, rev, and nef and was about 2 kb (1.15). During an asynchronous infection, the amounts of HIV RNA gradually increased with the 9.2-kb species at highest concentration (Fig. 4, lanes 8 and 9) and the ratio of radioactivity in 2-, 4.3-, and 9.2-kb mRNAs was approximately 1:0.8:1 (1:0.4:0.5 when corrected to the molar ratio). When RNA from various time points during a one-step growth cycle was analyzed, however, the first species, barely evident at 12 h and significant by 16 h, were the 2- and 4.3-kb RNAs (Fig. 4). They predominated until 24 h, when the ratio of the three RNAs was about 1:0.4:0.3 (equivalent to a molar ratio of 1:0.2:0.06). The 9.2-kb RNA accumulated to a greater extent than the lower-molecular-weight RNAs after 24 h, and the pattern became indistinguishable from that of an asynchronous infection. Between 24 and 36 h, 9.2-kb RNA is the only one whose concentration is markedly increased (Fig. 4). The same pattern of RNA expression was observed when either cytoplasmic or total RNA was analyzed (data not shown).

**DISCUSSION**

Our results suggest that HIV DNA synthesis has the same general form as that for other retroviruses: DNA appears first, probably in the cytoplasm, and rapidly moves to the nucleus, and there some of it circularizes. We have not measured integration, but we can assume that a fraction of the DNA integrates. The drops in concentration of linear DNA are not likely to be due to integration, because that
Once the superinfection barrier is in place, linear DNA content falls, presumably by degradation, and little new DNA is made.

It had been suggested that HIV, like spleen necrosis virus and Rous-associated virus, can make large amounts of viral DNA that might result in toxicity (15, 22, 23). Copy numbers of unintegrated viral DNA in infected cells vary among retroviruses. Even the same virus can show variation, depending on the host cell. For example, spleen necrosis virus synthesizes 200 copies per cell in chicken cells early after infection, showing little reaction to alterations in the multiplicity of infection, whereas 2 copies are made in rat kidney cells (10). Therefore, the 20 copies per cell we observed for HIV is not exceptionally high. H9 cells show little toxicity after infection, implying that 20 copies per cell is not toxic. Perhaps more HIV DNA is made in other cell types (15). If CD4 synthesis is greater than viral glycoprotein synthesis, a complete superinfection barrier might never appear and cells could accumulate very large numbers of reverse transcripts.

Our results indicate that there are early and late transcriptional phases in HIV infection. Early mRNAs are predominantly the smaller ones: presumably the 2-kb spliced mRNAs for the low-molecular-weight regulatory proteins (tat, rev, etc.) and the 4.3-kb spliced mRNAs which represent env and also possibly others such as vif, vpr, and the partially spliced tat (11). The ratio of 2-kb to 4.3-kb RNAs was about 1:0.3 between 16 and 20 h and thereafter changed to 1:0.6 or higher. Therefore, at earlier times, the 2-kb RNA is enriched even relative to the 4.3-kb RNA, suggesting a controlled shift in RNA patterns as the infection process develops. At the earliest point, however, all three species of RNA are evident; thus, the control may involve a gradual shift rather than a qualitative switch from one pattern to another. Such differential RNA expression was also found after DNA transfection of the viral genome into COS-1 cells (31).

The best previous examples of differential gene expression in viruses involve DNA viruses such as simian virus 40, polyomavirus, adenovirus, and herpesvirus (26). For these viruses, certain groups of genes are expressed early after infection and others are expressed later. This differential gene expression is controlled mainly through transcription initiation by using stage-specific promoters. Because HIV contains only one promoter (i.e., the long terminal repeat), however, understanding the regulation of gene expression in HIV requires a different perspective. The best candidate gene for determining differential RNA expression in HIV is rev. Presence of the rev protein favors cytoplasmic accumulation of 9.2-kb RNA over spliced products (3, 19, 31). It may either modulate the processing of mRNA or be involved in the export of the unspliced mRNA from the nucleus to the cytoplasm (11, 12). Whatever the actual mechanism, at least part of the shift in RNA patterns is likely to be due to increased accumulation of rev protein with time after infection. This hypothesis is supported by the fact that the pattern of RNA expression resulting from the mutations in rev (3, 31) is very similar to that observed during the early transcriptional phase. From our data, it would appear that rev action becomes evident at about 24 h postinfection and that thereafter only 9.2-kb mRNA is made. A sequential transcription is not unprecedented among retroviruses: spliced mRNA species selectively appear early in infection by visna virus (28), and one of these early mRNAs appears to transactivate viral transcription (13).

Understanding viral latency is crucial to interpreting the pathogenesis of HIV. Our analysis of DNA and RNA

FIG. 4. Hybridization analysis of HIV-specific RNA from a single-cycle infection culture. (A) RNA blot analysis. Numbers above the lanes are hours after the start of infection, while lane numbers are indicated at the bottom. Lanes 10 and 11 are shorter exposures of lanes 8 and 9. Numbers in the middle of two panels indicate approximate sizes of RNA bands (kilobases). (B) Densitometric analysis of RNA samples. The intensities of HIV-specific RNA bands were determined by laser densitometry by using a shorter exposure of the autoradiogram shown in panel A.

would suggest many integration events per cell. HIV-specific RNA appears first at 12 to 16 h postinfection and increases thereafter. Whether the viral DNA must integrate to provide a transcription template is not known. Integration could be the limiting factor to the efficiency with which a virus preparation can initiate a productive infection, because within 8 h after infection, sufficient linear DNA was present to support transcription in every cell, if that form of the template was the active one.

After the initial cycle of infection, cells that become infected release virus that can then infect surrounding cells. We interpret the secondary 10-fold increase in molecules of viral DNA per cell as indicating that many virus particles produce full-length viral DNA in each cell, implying an absence of a superinfection barrier in most cells. Multiple superinfections have been observed in other retroviral infections (10, 25, 30). For HIV, it appears that 50 to 80 h (or longer at lower input multiplicity of infection) is required before most cells are making sufficient viral glycoprotein to block all of the CD4 receptor molecules on the cell surface.

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synthesis during a one-step growth cycle may provide some insights in understanding how latency might occur. The earliest time at which free viral DNA can be detected during retroviral infection is generally 3 to 4 h after infection (for reviews, see references 21 and 27). This suggests that synthesis of HIV DNA occurs as rapidly as in any other retroviral infection and thus that aspects of DNA synthesis alone are probably not a major factor in establishing or maintaining the latent state. On the other hand, temporal aspects of RNA synthesis show a complex pattern of RNA expression in cells infected with HIV. In particular, it is interesting that there is a significant delay in the full-level expression of the 9.2- and, to a lesser extent, the 4.3-kb mRNA species. After the 2-kb RNA first appears, it takes about 12 h more before steady-state expression of the three major transcripts is achieved. This delay may result from a combined effect of viral and cellular factors. Because the assembly of a whole virus would require the expression of 9.2- and 4.3-kb mRNAs, the early transcriptional phase, when the 2-kb mRNA predominates, could represent the in vitro equivalent of a latent stage. This hypothesis suggests that a controlled shift from the early to the late transcriptional phases may represent the transition from the latent to acute infection in vivo. In this context, it is interesting to note that when SCID-hu mice containing human fetal thymic implants were infected with HIV, the majority of infected thymic cells expressed only RNA, not the viral structural proteins (16). Regardless of detailed mechanisms, the slow progress of clinical symptoms characteristic of HIV infection is not due to HIV being an intrinsically slow-growing retrovirus but is due instead to the complex nature of viral gene regulation and virus-host interactions.

Despite an intense effort to understand the molecular mechanisms involved in the growth of HIV, the functions of many regulatory genes still remain to be clarified. Thus far, most analyses have been done by using heterologous systems or subgenomic pieces of viral DNA in transient fashion. In addition, most studies involving virus have been performed during the period of steady-state infection. The interpretation of such studies may be complicated by asynchronous infections. The one-step growth cycle used in our studies provides a powerful tool for analyzing the life cycle of HIV. The use of a single-step growth cycle establishes conditions that will facilitate the characterization of roles of viral regulatory proteins.

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LITERATURE CITED


