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Persistent Human Immunodeficiency Virus Type 1 Infection of Monoblastoid Cells Leads to Accumulation of Self-Integrated Viral DNA and to Production of Defective Virions

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Cell-free virus preparations from persistently infected monoblastoid cells (HU937) become progressively less infectious during long-term passage. This effect is specific for cell lines derived from U937 and is not observed in persistently infected T-cell lines. Reduced infectivity is correlated with accumulation of unusual, high-molecular-weight, extrachromosomal forms of the human immunodeficiency virus type 1 (HIV-1) DNA. These DNA molecules contain multiple copies of the viral genome, and their structures are highly variable. Of 17 subclones of the HU937 cell line, 15 unique restriction fragment patterns were observed for the HIV-1 viral DNA. Structural analysis of these viral DNA species indicated that they were formed by sequential rounds of long terminal repeat-mediated integration of one circular DNA form into preexisting monomeric or multimeric structures. These viral DNA structures are termed nested self-integrates. Once formed, self-integrates prove to be stable and can be maintained for several months in culture. The unusual structures of HIV-1 DNA in persistently infected monoblastoid cells attest to an alternative to the accepted retrovirus life cycle. The self-integrated viral DNA species reported here may explain some aspects of the mechanism controlling establishment and maintenance of persistent HIV-1 infection in cells of the monocyte/macrophage lineage.

The human immunodeficiency virus (HIV) infects cells of the lymphoid and myeloid lineages bearing the CD4 membrane glycoprotein (3, 6, 15, 22, 23). Cytopathic effects and cell destruction occur when CD4-positive lymphocytes are infected (19, 20), an effect consonant with the observed pattern of cell depletion in seropositive individuals (10, 18, 24). In contrast, HIV type 1 (HIV-1) infection of normal human monocytes and macrophages is noncytopathic (9, 25) and monocyte levels in acquired immunodeficiency syndrome (AIDS) patients are not discernibly different from the levels observed in uninfected individuals (12, 38). HIV infection of brain-associated macrophages (8, 17, 39), of the macrophagelike follicular dendritic cells of lymph nodes (2, 26), and of related cells in the skin and lungs of infected individuals (4, 30) attest to the important role of monocytic cell infection in AIDS.

Two independent pathways of virus entry into monocytes have been previously demonstrated (29). A rapid, CD4-independent pathway was detected in U937 cells, although this route of entry did not lead to infection. Virions internalized via this rapid, CD4-independent pathway enter a phagocytic pathway leading to efficient degradation of the incoming material. A slower, CD4-dependent pathway for virus internalization was also demonstrated; this is the mode of entry that leads to infection (5, 25, 29). Aside from these characterizations of the pathway of entry for infectious HIV, relatively little is known about the retroviral life cycle in monocytes. Unusual patterns of intracytoplasmic virion assembly have been noted (9), although the significance of these findings has not yet been established. It has been noted (5, 28) that HIV-1 infection of U937 cells renders them partially refractile to differentiating agents, and this effect is explained, in part, by an apparent correlation between virus replication and c-*myc* gene expression (28).

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It is particularly important to determine the structure and activity of viral DNA in monocytes and macrophages. Strategies to reduce the contribution of infected monocytic cells to the dissemination of HIV will depend upon precise knowledge concerning the status and activity of these DNA genomes. In addition, monocytes and macrophages are terminally differentiated, nondividing cells, and HIV would not normally be expected to integrate its provirus into the chromosomal DNA of these cells (31, 34). Therefore, established views of the retroviral life cycle do not account for the unusual situation encountered upon HIV infection of monocytes and macrophages. To address these issues directly, we have analyzed the HIV-1 viral DNA in both acutely and persistently infected monoblastoid cells and compared these forms with the products of acute or persistent infection of T cells. Large, multimeric, extrachromosomal viral DNAs were observed uniquely in persistently infected monocytic cells. These structures represent an alternative to the standard retroviral life cycle and might account for the ability of HIV-1 to establish a persistent infection of this cell type.

Terminally differentiated monocytes and macrophages have been proposed as an essential reservoir for HIV in the infected individual (16, 27). It follows then that the obligatory interaction between antigen-presenting macrophages and major histocompatibility complex class II-restricted helper T cells would provide a distinctive mechanism for HIV dissemination from this reservoir to the helper population at large; cell destruction and immune deficiency may well be consequences of this directed pathway of viral transmission (27). Accordingly, concerns about the mode of HIV replication in monocytes and macrophages are related directly to our ability to understand the pathogenetic mechanism in AIDS, and, in turn, will direct our efforts to design and implement novel therapeutic strategies for this disease.

We have reported the mode of virus entry (29) and the control of HIV-1 replication during acute infection (28) of the

U937 monoblastoid cell line. Our continuing efforts to characterize this model system reveal a temporal decrease in infectivity of cell-free virus preparations from persistently infected U937 cells (the uncloned, persistently infected cell line is designated HU937); this pattern is not evident in a persistently infected T-cell line. Further examination of cell populations producing defective virions disclosed the presence of unusual extrachromosomal forms of viral DNA; these forms are heterogeneous, structurally rearranged, and of greater complexity than expected for single HIV-1 genomes. The aberrant viral DNAs in HU937 are composed of multiple HIV-1 genomes that have integrated into each other. On the basis of their structural characteristics, we have termed these molecules nested self-integrates; nested is intended to convey the image of each successive viral DNA becoming embedded in the preceding structure, and self-integrate serves to emphasize a characteristic assembly process involving repeated integration of monomeric units to form complex multimers.

Accumulation of nested self-integrates in HU937 reflects the most compelling example of HIV-1 structural variation in vitro. These viral DNAs accumulate as structurally rearranged forms, and their appearance is correlated with decreased infectivity of cell-free virus preparations from HU937. Structural comparison of extrachromosomal viral DNA in persistently infected lymphoid (CEM) and myeloid (U937) lineage cells reveals that unusual forms occur only in cells of the myeloid lineage. Nested self-integrates have been maintained in stable form in HU937 and subclones of this cell line.

On the basis of this experience, it seems likely that these DNA molecules replicate independently of the normal retroviral life cycle and represent an alternative to proviral integration into the host genome as a means for establishing a substrate for transcription of viral genes. Alternative modes for retroviral persistence in monocytic cell lines might explain the ability of lentiviruses to infect and replicate in nondividing monocytes and macrophages and account for this important aspect of viral pathogenesis in AIDS.

MATERIALS AND METHODS

Cell culture and preparation of virus stocks. U937 and CEM cell cultures were maintained at densities between 10^5 and 10^6 cells per ml in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum supplemented with penicillin-streptomycin-glutamine. The cells were passaged on the day before infection. Cell-free stocks of the $LAV1_{bru}$ strain of HIV-1 (1) were prepared by infection of the T-lymphoblastoid cell line, CEM. The titers were adjusted to 10^7 50% tissue culture infectious doses per ml, as determined by syncytium formation in a terminal dilution assay with MT-2 cells (13). The persistently infected cell lines HU937 and HCEM were first infected in May of 1986 and then maintained for 2 years as uncloned cell lines. Subclones of these cell lines were obtained by diluting the culture to less than 1 cell per 200 μl and plating in microwells.

Virus production in persistently infected cell cultures. Cell-free supernatants were obtained 1 day after passage of each cell culture; typically, the cells were diluted 1:5 at each passage. Viral particles were precipitated by the addition of polyethylene glycol. Reverse transcriptase activity was assayed as described previously (28). The infectivity of HU937 culture supernatants was determined by infecting the T-lymphoid cell line CEM and measuring the subsequent virus production by reverse transcriptase assay. These values are expressed as 50% tissue culture infectious doses by comparing these values with those of standardized infections (13).

Analysis of extrachromosomal viral DNA. Viral DNA was prepared by the method of Hirt (14) and analyzed by restriction enzyme digestion and DNA blotting. For each sample, 12 ml of infected-cell culture was collected by centrifugation, washed once with cold phosphate-buffered saline, and suspended in 2 ml of lysis buffer containing 10 mM EDTA–0.6% sodium dodecyl sulfate–10 mM Tris hydrochloride (pH 7.5). To this cell lysate was added 0.5 ml of 1 M NaCl; protein and chromosomal DNAs were precipitated at 4°C for 12 h. The precipitate was removed by centrifugation in a Beckman J2-21 centrifuge at 4°C at 15,000 rpm for 30 min. The supernatant was decanted, proteinase K was added to a final concentration of 200 μg/ml, and the sample was incubated at 56°C for 2 h. Protein was removed by a single extraction with buffered phenol solution and an additional extraction with $CHCl_3$. Nucleic acids were recovered by two sequential ethanol precipitations. The 12-ml sample of cell culture (from the persistently infected lines) generated sufficient material for two lanes of an agarose gel. The ethanol precipitate was dried, suspended in water, and digested with the appropriate restriction endonucleases, using the conditions suggested by the manufacturer. Digested samples were analyzed by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with the radiolabeled ARV-2 DNA probe (21) as described previously (28).

RESULTS

Loss of infectivity in virus stocks from persistently infected U937 cells. Virus production by persistently infected cell lines was monitored over a 2-year period, with sampling intervals that ranged from 3 to 6 months. Virus production by the persistently infected monoblastoid cell line HU937 and the persistently infected T-cell line HCEM was assessed. Assay of polyethylene glycol-precipitable reverse transcriptase activity measured directly the production of virions or virion components by the HU937 line. These data were contrasted to the capacity for these supernatants to infect productively a sensitive T-cell line. In this way, the relative infectivity of HU937 supernatant preparations was determined (Fig. 1).

Production of polyethylene glycol-precipitable reverse transcriptase activity appeared to remain relatively constant throughout 40 months of growth of the persistently infected HU937 cell line. It merits emphasis that these results are distinct from those reported previously for acutely infected U937 cells. In the situation of acute infection (28), virus replication was productive for the first few days postinfection and then became restricted (as used here, restriction refers to a condition of decreased viral gene expression and reduced levels of virus release without an evident cytopathic effect). The restriction phase continued for approximately 20 days and was supplanted by active, constitutive virus replication and the establishment of a persistently infected culture. Analysis of persistent infection was initiated when restricted replication was no longer evident. Figure 1 also reveals a pattern of decreasing infectivity in cell-free virus preparations derived from the HU937 cell line. This change was gradual with a total loss of viral infectivity about 20 months postinfection. Presently, cell-free virus stocks from the HU937 line, used to infect a T-cell line, neither produced reverse transcriptase nor induced syncytia and consequently

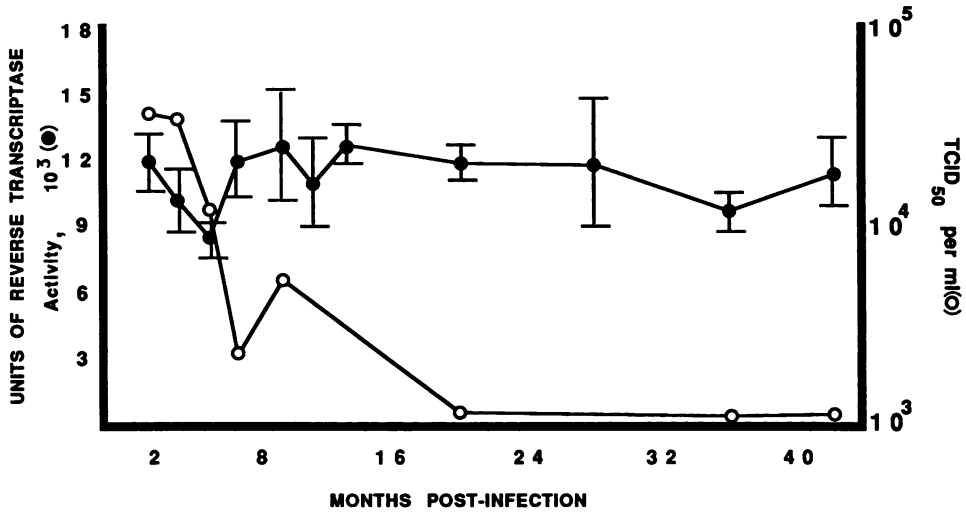

FIG. 1. Decrease in the infectivity of HU937 virus during the initial 42 months postinfection. Steady-state levels of virus production by the HU937 cell line were determined by reverse transcriptase assay of cell-free supernatants. For each interval, three to five independent samples were collected and assayed. The bars indicate the standard error in each determination. The infectivity of HU937 culture supernatants in a standard assay involving the T-lymphoid cell line CEM was also examined; the data are 50% tissue culture infectious doses ($TCID_{50}$) per ml of culture supernatant calculated as described previously (13). Months postinfection documents the interval between the initial establishment of these cultures and the time of sampling.

are considered noninfectious. Cell-free virus preparations from acutely infected U937 cells have been examined previously (28) and are of comparable virulence to virus preparations from T-cell infections. The infectivity of HU937 virus was also examined, using U937 cells as targets; this was done to address the possibility that the loss of infectivity reflected a change in the host range of this virus. There was no evidence for infection of U937 when preparations of defective HU937 virus were used (results not shown). The data for T-cell infections are presented here because of the increased sensitivity of this system compared with the infection of U937 cells (5).

The initial infection of the T-cell line CEM was characterized by substantial cell destruction due to the cytopathic effect of HIV-1. Subsequently, a survivor population arose and was maintained as the persistently infected cell line. In the ensuing 2 years, there was little change in the constitutive level of virus production from the HCEM cell line; the infectivity of these virus preparations remains indistinguishable from that of the original virus isolate.

Structure of the extrachromosomal viral DNA in acutely infected CEM and U937 cells. Actively growing cultures of CEM and U937 cells were infected with cell-free virus. At 3 days postinfection, the cells were harvested and extrachromosomal DNA was prepared by the Hirt extraction procedure. The samples were digested with either *Bam*HI (one site in the LAV1 genome [Fig. 2]) or *Pst*I (one site in the genome) and analyzed by blot hybridization, using radiolabeled ARV-2 DNA (containing the entire viral genome) as the probe (Fig. 3). *Bam*HI digestion of viral DNA from the T-cell infection (Fig. 3A, lane 2) generated the products expected. The highest-molecular-weight form, 9.7 kilobase pairs (kb), is the circular DNA with two long terminal repeat (LTR) sequences. The next lower band, at 9.36 kb, is the circular DNA with one LTR, and the 8.7-kb band represents the larger fragment of the linear form; a right-end fragment of 1.25 kb was released by *Bam*HI digestion. A 1.38-kb end fragment was released from the linear form by *Pst*I digestion. In addition, the one- and two-LTR circular forms migrated to a position identical to that observed after *Bam*HI digestion. Double digestion, with *Bam*HI plus *Pst*I, liberated a 7.1-kb middle fragment, the 1.25- and 1.38-kb ends of the linear molecule, and the circle junction fragments at 2.65 (from the two-LTR circle) and 2.2 kb (from the one-LTR circle). These data are consistent with the published restriction map of LAV1 (1).

The same three DNA forms observed previously in acutely infected T-cell cultures were present in the viral DNA from the acutely infected U937 cells. The larger form appeared at the position expected for the two-LTR circular form, the middle form appeared at the position expected for the one-LTR circular form; linear viral DNA was also detected. Consequently, acute HIV-1 infection of the monocytic or lymphocytic cell lines led to the accumulation of identical forms of extrachromosomal viral DNA.

Radioactive bands were excised from the hybridization membrane, and their specific activities were determined by liquid scintillation counting. This analysis demonstrated that the three forms of extrachromosomal viral DNA in acutely infected CEM cells were present at a ratio of 1:1.5:3 for two-LTR circle, one-LTR circle, and linear forms, respectively. Accordingly, the linear form represents the major species in this infection. This value is in contrast to the

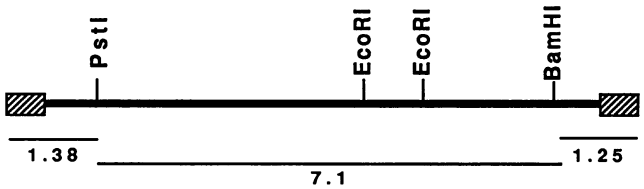

FIG. 2. Restriction map of the two-LTR-containing linear form of the LAV1 strain of HIV-1. The restriction endonuclease recognition sites for *Bam*HI (one site), *Pst*I (one site), and *Eco*RI (two sites) are shown. The sizes, in kilobase pairs of DNA, for three of the restriction fragments are shown. The hatched boxes represent the LTR sequences. This figure is adapted from Alizon et al. (1).

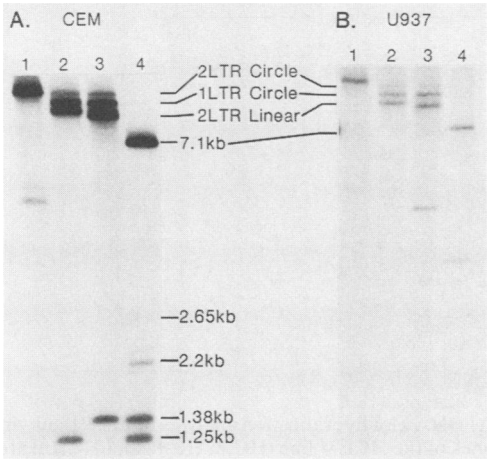

FIG. 3. Presence of extrachromosomal viral DNA in acutely infected T cells (CEM) and monoblastoid cells (U937). Cells were infected with 0.2 50% tissue culture infectious doses per cell. On day 5 postinfection, extrachromosomal DNA was prepared by the Hirt extraction; these samples were digested with the appropriate restriction endonucleases, fractionated by agarose gel electrophoresis, and visualized by hybridization with radiolabeled ARV-2 (32) DNA. Lane 1 contains uncut DNA. DNA samples in lanes 2, 3, and 4 were digested with the restriction endonuclease(s) *Bam*HI, *Pst*I, or *Bam*HI plus *Pst*I, respectively. Positions of the three discrete viral DNA forms, identified as circular or linear and as containing one or two LTR sequences, are indicated in the center; sizes are given as kilobase pairs. The interpretation of the lower-molecular-weight bands from 2.65 to 2.25 kb is elaborated in the text. The faint bands approximately midway down the gel in panel B, lanes 3 and 4, were observed only in this experiment and have been identified as contaminants in the restriction enzyme preparation.

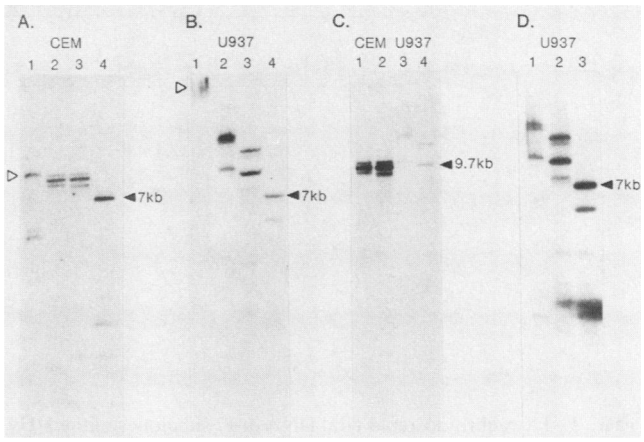

FIG. 4. Structure of extrachromosomal viral DNA in persistently infected monoblastoid (HU937) versus persistently infected T lymphoid (HCEM) cell lines. (A) HCEM viral DNA. Lane 1, uncut DNA; lane 2, *Bam*HI-cut DNA; lane 3, *Pst*I-cut DNA; lane 4, *Bam*HI-plus-*Pst*I-cut DNA. The position of the 7.1-kb middle fragment of LAV1 is indicated at the right, and the uncut viral DNA (lane 1) is indicated (△). (B) HU937 viral DNA. Lane assignments are as described for panel A. The positions of the highest molecular weight bands in lanes 2 and 3 are approximately 35 and 25 kb, respectively; the position of the uncut viral DNA is again indicated (△). (C) Direct comparison between HCEM and HU937 DNA samples; extracts from equivalent numbers of cells were loaded in each lane of the gel. Lanes 1 and 3 show the result of *Bam*HI digestion; lanes 2 and 4 are samples cut with *Pst*I. The position of 9.7-kb molecular weight marker is shown on the right. (D) Viral DNA in persistently infected U937 cells. Lane 1, *Bam*HI-cut DNA; lane 2, *Pst*I-cut DNA; lane 3, *Bam*HI-plus-*Pst*I-cut DNA material.

distribution of viral DNA in the U937 cell line, which is 1:2:2 for the same forms. Thus, the linear form is most abundant in CEM cells and less evident in acutely infected U937 cells. These values represent the average of three particular experiments in which it was possible to observe the linear-form viral DNA in U937 cells. In three other experiments, this species was not detected, even though the circular forms were clearly visible. Consequently, equimolar amounts of one-LTR circle and linear forms in U937 cells most likely represent an upper estimate of the proportion of linear viral DNA in this cell type.

In persistently infected monoblastoid cells, the extrachromosomal viral DNA is a self-integrate of multiple viral genomes. We next examined the structure of extrachromosomal viral DNA in the persistently infected T-cell and monoblastoid cell lines (Fig. 4). Figure 4A depicts the result from analysis of the virus in the persistently infected T-cell line HCEM. Here too, three DNA forms were detected. Molecular weight estimates and restriction mapping data affirm that these species in persistently infected T cells are identical with those observed during acute infection. Figure 4B presents an unexpected result concerning extrachromosomal viral DNA in the persistently infected HU937 cell line. In this instance, very high molecular weight DNA species were found in the uncut DNA preparations and upon *Bam*HI or *Pst*I endonuclease treatment. In this uncloned cell line, the major high-molecular-weight forms after *Bam*HI digestion were approximately 25 to 35 kb. Circular viral DNA species equivalent to single genomes were not encountered in HU937 cells. The bands migrating near the unit molecular weight (approximately 10 kb) do not represent intact viral genomes; intact, circular DNA genomes would be expected to migrate at the same position after either *Bam*HI or *Pst*I digestion (each of these enzymes recognize a single site per genome); these specifications were not met in the case of HU937 proviral DNA. Figure 4C provides a direct comparison of the relative viral DNA content in HCEM and HU937 cells. A greater abundance of extrachromosomal DNA forms were observed for persistently infected T cells, and these results are consistent with similar observations from acute infection experiments (Fig. 2). Figure 4D shows an additional restriction analysis of viral DNA in HU937 cells; here, the increased intensity of hybridization reveals the presence of numerous low-molecular-weight DNA fragments.

The alkaline lysis method of Griffin et al. (11) was employed as an alternative procedure for the preparation of extrachromosomal DNA. This extraction preferentially yields circular DNA molecules and provides a more-critical discrimination between extrachromosomal and chromosomal DNA than is afforded by the Hirt procedure. The distribution of high-molecular-weight DNA forms was identical in the samples prepared by the conventional Hirt extraction and by the alkaline lysis method (results not shown), thereby supporting the contention that these species are indeed extrachromosomal DNA. Control hybridizations, in which a probe for the cellular gene c-*myc* was exposed to blots of total DNA versus Hirt extract DNA, also provided evidence for the relative absence of chromosomal species in these DNA preparations (results not shown).

Figure 4 also attests to the presence of some intact viral DNA sequences in the Hirt supernatant preparations from HU937 cells; these intact viral genomes are integrated into

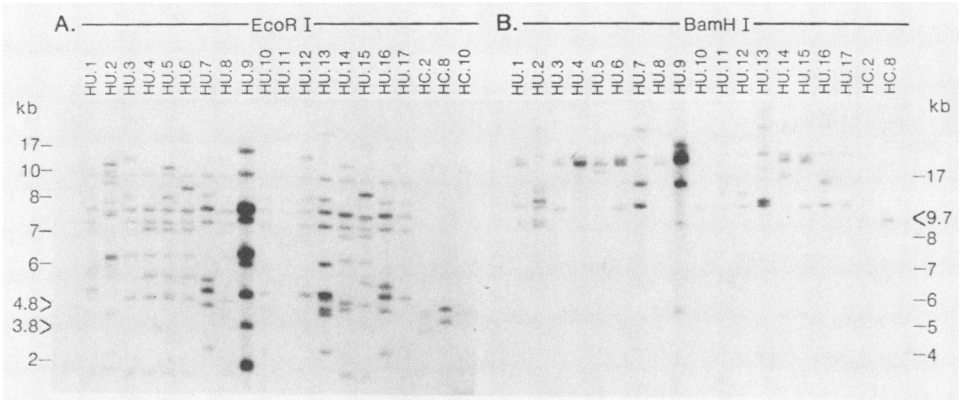

FIG. 5. Extrachromosomal viral DNA in 17 subclones of the HU937 cell line. (A) DNA blot hybridization analysis of *Eco*RI-cut viral DNA from the HU937 subcloned lines HU.1 through HU.17. The results for three subclones of the HCEM line (HC.2, HC.8, and HC.10) are shown for comparison. The positions of known molecular weight markers are indicated as kilobase pairs at the edge of each respective panel. The positions of the 4.8-kb and 3.8-kb fragments, shown at the left, are expected on the basis of the LAV1 sequence and are indicated by the arrowheads (1). (B) The same subcloned cell lines as in panel A were analyzed by *Bam*HI restriction endonuclease digestion. The position of the 9.7-kb, monomeric, viral genome is indicated at the right by an arrowhead.

the larger extrachromosomal species. Digestion of HU937 DNA samples with *Bam*HI plus *Pst*I (Fig. 4B, lane 4, and Fig. 4D, lane 3) released a 7.1-kb fragment, along with numerous lower-molecular-weight fragments. Presumably, this 7.1-kb fragment is the middle portion of the normal viral genome; it was also observed after similar restriction endonuclease digestion of DNA from the persistently infected T-cell line (Fig. 4A, lane 4) and in acute infection experiments as well (Fig. 3). Multimers composed of end-to-end viruses would be expected to release one middle fragment from each viral genome after *Bam*HI plus *Pst*I digestion. Accordingly, restriction analysis of end-to-end multimers would be expected to reveal a very intense band of hybridization at the 7.1-kb size, in contrast to the high-molecular-weight bands which are expected to transfer at reduced efficiency during blotting. In the present experiments, the relative intensity of this 7.1-kb fragment from HU937 DNA proved to be lower than expected from simple end-to-end multimeric viral DNA; thus, the proportion of structurally intact viral genomes seems to be low compared with the total amount of retrovirus-related DNA sequences in the cell. The abundance of lower-molecular-weight fragments indicates that these large DNA forms are composed of multiple retroviral genomes integrated randomly into each other. Together, these are the features defining the nested self-integrate form of HIV-1 extrachromosomal DNA. These DNA molecules are being examined in further detail; their structures will be reported elsewhere. Total genomic DNA was radioactively labeled and used as hybridization probe for DNA blots of the extrachromosomal DNA species. Specific hybridization to viral sequences was not detected (results not shown), thus indicating that highly repetitive sequences of chromosomal origin were not present in the nested self-integrates.

Markedly diverse extrachromosomal viral DNA structures are present among 17 independent subclones of the HU937 parent line. To evaluate the range of structural diversity present in HU937, DNA samples from 17 subclones of the parental cell line were examined; these subcloned lines were isolated initially at month 35 of the passage of HU937. Extrachromosomal DNA samples were prepared by Hirt extraction and mapped, using either *Bam*HI or *Eco*RI restriction endonuclease digestion. This experiment (Fig. 5) revealed the range of structural diversity present in the uncloned HU937 parent cell line. Restriction patterns of these viral DNA samples were markedly divergent and not related directly to the known map of the LAV1 virus used originally to initiate this cell line. The majority of these cloned lines did not contain viral DNA forms of the size expected for single viral genomes; this result is particularly notable in the *Bam*HI digestion experiment in which a single restriction site in the viral genome had been expected to generate viral DNA of approximately 9.7 kb. All of these cell lines released appreciable levels of cell-free HIV-1 particles; these virus preparations were incapable of establishing a productive infection in CEM cells. Accordingly, the high degree of structural degeneracy was correlated with the inability to produce infectious virus. This pattern of structural diversity was not observed in subclones of the T-cell line HCEM. For persistently infected T cells, the extrachromosomal viral DNA in each subline was identical. Accordingly, the band at 8.5 kb (very faint in this exposure) was the product of two *Eco*RI cuts removing a 1-kb fragment from the center of the viral genome, and the 4.8-kb and 3.8-kb fragments were the products of two *Eco*RI cuts in the linear molecule. The band at 9.7 kb has been observed in the parental HCEM line and in most of the sublines; this fragment appears to represent a contaminating viral form that has lost one of its *Eco*RI sites. We do not know, at this time, whether this situation represents incomplete cloning of the HCEM line or a stable accommodation of multiple variants in the same subclones. Repeated subcloning of the HCEM sublines have, so far, failed to separate the two variants (results not shown).

The considerable extent of structural diversity in viral DNA from HU937 and its sublines complicates the analysis of retroviral integration in monoblastoid cell lines. In this cell line, it was not possible to detect restriction fragments unique to unfractionated or chromosomally enriched (Hirt pellet) DNA samples because of contamination of the chromosomal DNA pellet with the complex extrachromosomal DNA molecules. Accordingly, conclusions about the presence of proviruses in monoblastoid cells or the potential role that chromosomal integration might play in the generation and maintenance of self-integrates are, at present, not warranted.

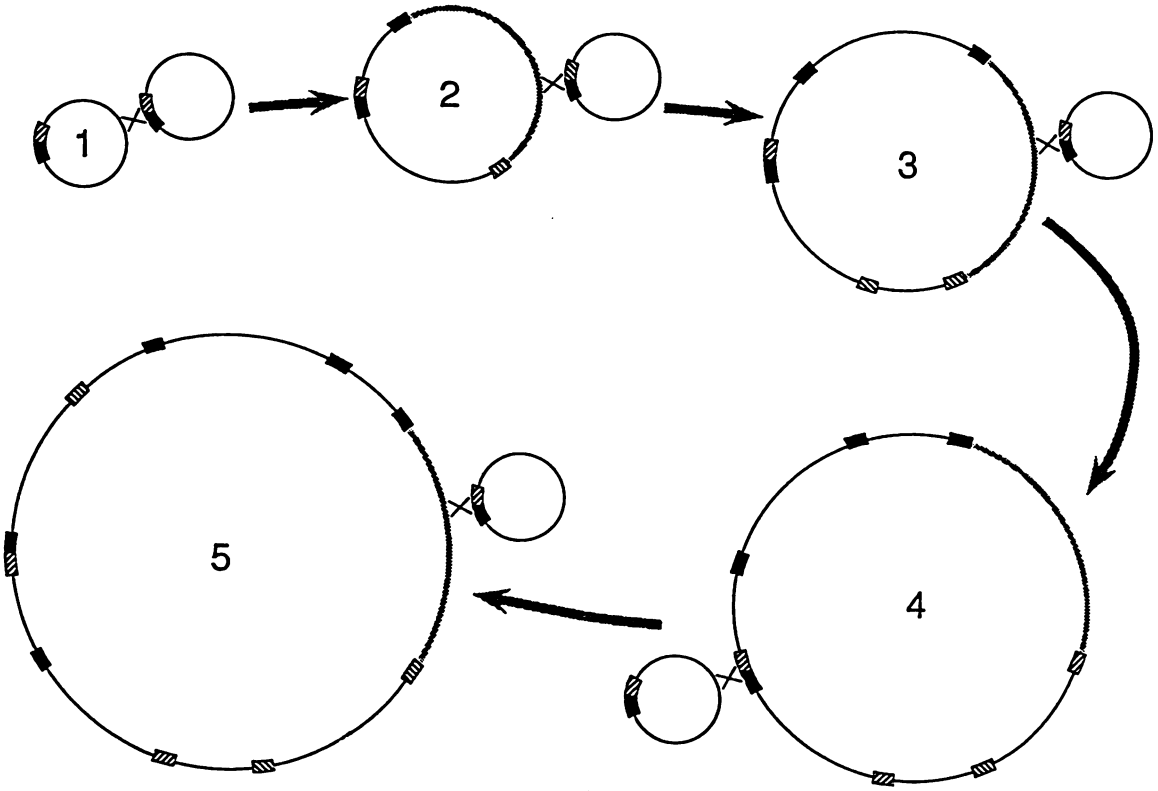

FIG. 6. A hypothetical pathway leading to formation of nested self-integrates. The viral LTRs are shown as hatched and solid boxes. Beginning with step 2, the single intact viral genome present within each multimer is shown as a stippled arc within the circle. The steps of this process are described in the text.

DISCUSSION

Monocytes and related cells constitute an important reservoir for HIV infection. Accordingly, the host-pathogen relationship between HIV and this cell type is a crucial feature in the pathogenetic mechanism leading to AIDS. Our studies continue to be directed toward understanding viral infection (29), replication (28), and persistence in this cell type. An objective of these studies has been to ascertain the features of HIV-1 replication in monocytic cells that could account for retroviral persistence in this nondividing cell population. The discovery of structurally rearranged, extrachromosomal viral DNA is indicative of one possible mechanism for virus persistence in macrophages. Our studies have established that the extrachromosomal viral DNA species in persistently infected monoblastoid cell lines have the following characteristics. (i) The molecules are significantly larger than single genome length. (ii) Structurally intact viral sequences are underrepresented compared with rearranged sequences. (iii) The structures are best explained by the nested self-integrate hypothesis. (iv) Sequence variation, although not yet ruled out as an explanation for the generation of defective viruses, cannot account for the high-molecular-weight structures reported here. (v) The long-term stability of these forms implies that they are independent replicons.

Although the appearance of defective virions is correlated with accumulation of structurally rearranged viral DNA molecules, these data do not yet provide a convincing explanation for the lack of infectivity of HU937 virus. Inasmuch as our model assumes the presence of at least some intact viral sequences, a direct explanation for the defective nature of the cell-free virus is not entirely apparent. Further analysis of the polyethylene glycol-precipitable material from HU937 and its subclones is in progress.

A process of rearrangement involving LTR-mediated recombination of one circular DNA into another is a likely explanation for nested self-integrate formation. This model termed end-to-middle (to distinguish this mechanism from an end-to-end recombinational process or other mechanisms that might lead to concatomer formation) is presented schematically in Fig. 6. The model assumes that the initiating partner in each recombination reaction utilizes LTR sequences and that the target sites are distributed randomly along the length of a preexisting viral genome. For simplicity, the incoming unit in the integration reaction is depicted as a circle, although there is no data as yet to indicate that this is the recombinationally active form of HIV-1. In the initial step, intragenic recombination would occur between two viral DNA molecules and the dimeric structure shown in step 2 emerges. Another unit-length circle would invade this structure, forming the trimer of step 3. Repetition of this process would give rise to a tetramer, and so on. In step 4, we account for the formal possibility that a original LTR junction, preserved in step 1, might itself initiate recombination into other viral genomes. It should be noted that only a single, intact viral genome is present within each nested self-integrate; this finding is consistent with the hybridization results given in Fig. 4. The relatively weak hybridization at 7.1 kb supports the contention that intact middle fragments, including the 7.1-kb portion bounded by the *Pst*I and *Bam*HI sites (Fig. 2), are under represented in these nested self-integrate structures and is consonant with the pathway

outlined in Fig. 6. However, our data do not rule out entirely the possibility that more than one intact genome is present in each self-integrate.

Two predictions have been made for the structure of the nested self-integrate forms of HIV-1 DNA in persistently infected U937; these features will be examined in the test of our hypothesis about their unique structure. First, the LTR sequences should be flanked on both sides by viral sequences (although the sequences on one side will be discontinuous with the known genetic map of HIV); this will provide the strongest evidence for self-integration. Second, if it were possible to characterize the flanking sequences from all of the LTRs in a single self-integrate, then the occurrence of small duplications of the viral sequence at the site of insertion should be apparent (7, 33, 36). It is also of interest to isolate and characterize sequences in these self-integrates that function as origins of replication. It should be noted that the two tandem LTR sequences are organized as directly repeated sequences in one of the extrachromosomal circular DNA forms characteristic of acute infection. In the self-integrated structures described here, it should also be possible to generate inverted repeats of the LTR sequences, a situation observed previously for self-integrates of Moloney leukemia virus (33, 36). This inverted repeat structure is considered the most likely candidate for a surrogate origin of replication because of its dyad symmetry and capacity to be transcriptionally active.

Generally, only the chromosomally integrated form of the provirus is thought able to undergo semiconservative DNA replication (34). On the other hand, it seems unlikely that the large extrachromosomal viral DNA species in HU937 cells would be transcribed into RNA and then subjected to reverse transcription to regenerate their original form. Size estimates for many of these molecules approach the 50-kb range; thus, replication via reverse transcription would necessitate accumulating large RNA molecules and copying them precisely. In addition, this process would require frequent repetition to maintain these DNA forms in the population. Subclones of HU937 have been maintained in culture for approximately 4 months with no evidence for the loss of existing structures. The viral DNA sequences are reasonably stable, as judged by limited restriction endonuclease analyses, and this argues that self-integrate replication is unlikely to involve the error-prone (35) reverse transcriptase pathway; conclusive evidence on these points still requires direct nucleotide sequence analysis of self-integrate DNA. It thus seems most reasonable that these high-molecular-weight retroviral genomes replicate as DNA, without going through an RNA intermediate. Should this indeed prove to be the case, additional issues arise regarding what sequences of these multimeric self-integrates satisfy the requirements for an origin of replication.

Three issues concerning the origin and replication of nested self-integrates remain unresolved. First, the potential involvement of host sequences in the generation or maintenance of these viral DNA forms has not yet been ruled out. Second, other viruses, perhaps present in the U937 cell line previously or latent in its genome, may have recombined to link an origin of DNA replication to HIV-1 and thus immortalize the nested self-integrate structure. Third, the extrachromosomal viral DNAs might have arisen from a provirus that was once integrated into the genome. In the latter case, one might expect to detect cellular sequences in the extrachromosomal DNA form. We intend to address all of these concerns by high-resolution mapping and sequence analysis of these self-integrated viral DNA molecules.

Among the many difficulties confronting research in this field, the principal ones relate to the structure and localization of HIV-1 DNA in monocytic cells. The unique aspects of HIV-1 replication in this cell type are of central importance for our understanding of the mechanisms governing establishment and maintenance of the viral reservoir in AIDS (16, 27), and our studies may help to elucidate some aspects of this subject. It is also important to recognize the limits of our model system. Monocytes, found in the peripheral blood, or macrophages, found in tissues, are nondividing cells derived initially from proliferating promonocytes in the bone marrow (37). Accordingly, immortalized cell lines, such as U937, cannot be compared directly with circulating peripheral blood monocytes. In fact, the continuously dividing cell lines might constitute a better model for myeloid stem cells than for mature monocytes and macrophages. It is appropriate to consider our results as providing a fresh insight into lentivirus replication in cells of the myeloid lineage although they cannot, as yet, be applied directly to considerations of HIV biology in monocytes and macrophages.

HIV infection of monocytes and macrophages involves the very cells normally carrying the burden of host defense against microbial pathogens in the disease process leading to AIDS. This situation represents an important aspect of the immunosuppressing activity of these viruses, although these mechanisms have not yet been defined. Self-integrated extrachromosomal viral DNA is an unique feature of HIV-1 infection that could explain the ability of this virus to persist in monocytes and macrophages. In addition, the progressive loss of viral infectivity due to the formation of nested self-integrates might also be a key feature in viral persistence and pathogenesis. Further exploration of the HIV-1 life cycle in monocytic cells should help to ascertain the significance of these mechanisms and could well reveal an advantageous new target for therapeutic intervention in AIDS.

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