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High Frequency of Isolation of Defective Human Immunodeficiency Virus Type 1 and Heterogeneity of Viral Gene Expression in Clones of Infected U-937 Cells

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Limiting-dilution techniques were employed to derive single-cell clones from U-937 cells that had been chronically infected with human immunodeficiency virus type 1. All clones thus obtained were positive for the presence of viral antigens; however, not all of the clones produced infectious progeny virus, as detected by the presence of reverse transcriptase (RT) activity in culture fluids. Six of these clones were monitored over time to determine whether their phenotype of human immunodeficiency virus type 1 expression was stable. Three clones maintained production of RT activity at a high level and showed a very high percentage of cells positive for viral p24 antigen, as determined by indirect immunofluorescence. The other three clones showed variations in either their levels of RT activity or the number of cells positive for p24, after which they stabilized. Infectious virus could be recovered from only three clones, as assessed by coculture experiments with different cell types. Two other clones were shown to produce noninfectious viruses. Molecular analyses at the DNA, RNA, and protein levels showed extensive variations between the viral isolates recovered from each clone.

Human immunodeficiency virus type 1 (HIV-1) infects cells via its surface glycoprotein gp120, which interacts with CD4 surface receptors found on T-helper lymphocytes (9, 19), B cells (25, 28), and monocytes or macrophages (15, 18, 25). The latter cell type is thought to constitute an important early target of HIV-1 infection in the body and to be a principal reservoir for virus persistence and dissemination. In this way, infection by HIV-1 is analogous to that by animal lentiviruses (16, 41).

Studies of the infection of monocytes by HIV-1 are difficult because of the heterogeneity of these cells, their lack of cell division, and terminal differentiation. We have used a monocytic cell line to facilitate such studies. The U-937 cell line (43) is a handy tool because it shares several functional characteristics of monoblasts and immature monocytes and can be induced to differentiate into adherent phagocytic cells by treatment with several agents (8, 40, 47). U-937 cells possess the CD4 receptor that permits HIV entry, and they have been extensively studied as a model for the infection of monocytes by HIV-1 (3, 25, 32).

Chronic infection of U-937 cells can easily be obtained and is characterized by active viral replication. Chronically infected U-937 cells can remain stable in culture over several months. However, such populations are not homogeneous. For one thing, some cells remain negative for expression of viral antigens over long periods. It is also possible to derive clones of U-937 cells which show differential sensitivity to HIV-1-induced cytopathic effects in direct correlation with their surface CD4 levels (3). In addition, a particular subclone of this cell line was shown to express minimal levels of progeny HIV-1 which could be induced by treatment with phorbol myristate acetate (13).

Expression of HIV-1 in U-937 monocytes is thus a variable phenomenon which may be explained, at least in part, by differences between individual cells. In this regard, a recent work has shown that cell-free preparations from persistently infected U-937 cultures became progressively less infectious during long-term passage (31). The presence of unusual structures of HIV-1 DNA seemed to account for this aberrant expression of viral progeny, a phenomenon which was not observed in persistently infected T-cell lines.

To evaluate the extent of heterogeneity of HIV-1 expression in infected U-937 cultures, we derived clones by limiting dilution from long-term chronically infected cultures. Our results show that such clones of cells display extensive heterogeneity with regard to production of progeny virus. The viral isolates recovered are characterized by either high or low infectiousness for previously uninfected cells, in comparison with parental HIV-1 preparations. Other clones studied were found to produce noninfectious HIV variants whose structural defects were confirmed by electron microscopy. We report also the establishment of a particular subclone possessing integrated viral sequences but negative for expression of viral genomic RNA. Furthermore, this line is not inducible by compounds that have previously been shown to induce latent viruses (13).

MATERIALS AND METHODS

Infection of U-937 cells. The U-937 monocytoid cell line was obtained from the American Type Culture Collection and was maintained as a cell suspension (2×10^5 to 2×10^6 cells per ml) in RPMI 1640 medium (GIBCO Products Ltd., Toronto, Ontario, Canada) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 250 U of penicillin per ml, and 250 μg of streptomycin per ml.

The III_B strain of HIV-1 (kindly supplied by R. C. Gallo, National Institutes of Health, Bethesda, Md.) was propagated on the Molt-3 T-lymphocyte cell line, and a 100× suspension was obtained from clarified culture fluids by ultracentrifugation. Pelleted virus was suspended in small volumes, quantified by plaque assay on MT-4 cells (17), and

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frozen at −70°C. Infection of U-937 cells was performed at a multiplicity of infection of 0.1 for 3 h at a cell concentration of 10^7/ml. After 2 months of culture, a chronically infected population was obtained in which more than 95% of the cells were positive for expression of viral p24 antigen, as determined by indirect immunofluorescence assay (IFA), and the level of reverse transcriptase (RT) activity present in 24-h culture fluids always exceeded 10^6 cmp/ml.

Derivation of U-937 HIV-1-infected clones (UHC). Chronically infected U-937 cells were serially diluted in 96-well microdilution plates to between 0.15 and 0.5 cells per well. The plates were incubated at 37°C in a 5% CO_2 atmosphere and observed daily under an inverted microscope for the presence of microcolonies derived from single cells. After 2 weeks, well-defined colonies (maximum, one to three per well) consisting of 10 to 50 cells were subcloned to ensure clonality. Each colony was harvested by micromanipulation under an inverted microscope by using extra-small-bore Pasteur pipettes, seeded individually into another 96-well plate, and left to expand.

RT assay. For the RT assay, clarified supernatant fluids were ultracentrifuged at 100,000 rpm for 30 min at 4°C in a Beckman TL-100 centrifuge; pellets were then suspended in 20 μl of growth medium. Viral RT activity was measured in 10 μl of suspended viral pellet to which 50 μl of polymerase reaction mixture containing 50 mM Tris (pH 8.0), 150 mM KCl, 5 mM dithiothreitol, 5 mM $MgCl_2$, 0.3 mM glutathione, 0.5 mM EGTA, 1% Triton X-100, 50 μg of poly(rA) · oligo$(dT)_{12-18}$ per ml, and 30 μCi of [^{3}H]TTP per ml (80 Ci/mmol) had been added. After being held for 1 h at 37°C, the reaction was stopped by the addition of 2 ml of sodium pyrophosphate (0.01 M in 1 N HCl). Ice-cold trichloroacetic acid was added to precipitate incorporated [^{3}H]TTP onto Whatman GF/C filters, which were then counted for radioactivity in a Packard Tri-Carb scintillation analyzer.

IFA. About 10^6 cells were collected and suspended in about 50 μl of phosphate-buffered saline (PBS), pH 7.4, for detection of viral antigen by IFA. For this purpose, we employed monoclonal antibodies against viral p24, kindly supplied by R. C. Gallo. Briefly, cells were plated onto glass slides, allowed to air dry, and fixed in acetone-methanol (1:1) for 30 min at room temperature. A 10-μl portion of monoclonal antibody dilution was added to each well at room temperature for 30 min; the cells were then washed in PBS for 10 min and incubated with a 1:100 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin (Cooper Biomedicals). After being held for 30 min at room temperature, the slides were washed again in PBS and rinsed in distilled water. The slides were then dried, mounted, and examined by fluorescence microscopy. At least 10 fields, including 2,000 cells, were studied in each instance.

Electron microscopy. Negative staining of viral preparations was performed by the method of Alain et al. (1). Portions (100 μl each) of clarified, virus-containing culture fluids were loaded into 240-μl nitrocellulose tubes into which an electron microscopy grid had been inserted. The tubes were centrifuged at 120,000 × *g* for 5 min, using an Airfuge (Rotor A-100; Beckman Instruments, Inc., Palo Alto, Calif.); the grids were then recovered, dried, and contrasted with phosphotungstic acid (3%; pH 6.0). Specimens were examined with a Philips 300 electron microscope.

For thin-section analysis, cells were centrifuged at 400 × *g* and the pellet was fixed for 1 h in 2% glutaraldehyde in PBS containing 3% sucrose. Cell pellets were washed three times. The cells were then postfixed in 1% OsO_4 for 30 min, washed in buffer, dehydrated in graded acetone, and embedded in Spurr plastic. Observations were made with a Philips 300 electron microscope.

Western blot (immunoblot) analysis. Viral protein preparations for Western blotting were prepared as follows. A 60-ml portion of clarified cell culture medium from infected cells was centrifuged at 40,000 rpm for 45 min. The resulting virus pellet was suspended in 100 μl of lysis buffer (10 mM Tris hydrochloride [pH 7.4]–100 mM NaCl–1% sodium deoxycholate–0.1% sodium dodecyl sulfate [SDS] containing 1 mM phenylmethylsulfonyl fluoride–0.1 mM leupeptine–20 U of aprotinin per ml). The homogenate was thoroughly vortexed and incubated for 30 min at 37°C.

Viral antigens from whole-cell preparations were obtained as follows. Cells were washed in PBS and suspended in 200 μl of PBS containing 1% Nonidet P-40–2% dimethyl sulfoxyde–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride as protease inhibitor. The lysate obtained was centrifuged at 100,000 × *g* for 1 h in a Beckman air-driven centrifuge. Protein content was standardized by the Lowry method.

Equal amounts of total protein were electrophoresed on 10% SDS-polyacrylamide gels, using the buffer system of Laemmli (22). These gels were then blotted onto nitrocellulose sheets. The electroblots were incubated overnight at 4°C in the presence of a 1:100 dilution of human serum obtained from an HIV-1-positive patient. The serum had been found to contain antibodies against all viral proteins. Fixed antibodies were revealed by incubation for 1 h at room temperature in the presence of ^{125}I-labeled protein A (Na^{125}I, 14.8 mCi/μg; Amersham Corp., Arlington Heights, Ill.). The blots were again washed, dried, and exposed to Kodak X-O MAT films for 16 h with intensifying screens at −70°C.

Northern (RNA) blot analysis. Total cellular RNA was prepared from each UHC clone by the acid-guanidium thiocyanate-phenol-chloroform method (7). For Northern blot analysis, 10 μg of total RNA per clone was electrophoresed through a 1% agarose-formaldehyde gel and blotted onto nitrocellulose (Schleicher & Schuell, Inc.) for 18 h. RNA dot blots were performed as follows. An 8-μg portion of total RNA per clone was suspended in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-7.4% formaldehyde, denatured at 65°C for 15 min, and chilled on ice. The RNA was then blotted onto nitrocellulose by serial dilution of the initial RNA solution, using a dot blotting manifold. Nitrocellulose filters were treated in an identical fashion for both types of RNA blot experiments. They were initially baked for 2 h under vacuum and then prehybridized for 24 h at 42°C in 50% (vol/vol) formamide–Denhardt solution–5× SSC–250 μg of salmon sperm DNA per ml. Hybridization was then carried out for 24 h at 42°C in the presence of 10% dextran sulfate. The probe used for viral RNA was pBH10, a genomic clone of the human T-cell lymphotropic virus type III_B isolate, kindly furnished by F. Wong-Staal, National Cancer Institute, Bethesda, Md. (39). Two probes, pRAH3-2 (2) and pβ-actin (generously supplied by G. Shyamala, Lady Davis Institute), containing mouse H-3 histone and β-actin coding sequences, respectively, were used as internal controls to verify whether equal quantities of RNA were loaded into each well. Probes were prepared to a specific activity of 2×10^8 cpm/μg of DNA, using a commercially available nick translation kit (Boehringer GmbH, Mannheim, Federal Republic of Germany). Filters were then washed in 2× SSC–0.1% SDS followed by 0.1× SSC–0.1% SDS at 60°C and exposed to Cronex film with intensifying screens at −70°C.

Southern blot analysis. High-molecular-weight DNA was isolated by standard methods (26). A 20-μg portion of DNA

TABLE 1. RT activity[a] of UHC clones after subcloning

Clone designation	RT activity (cpm/ml × 10^3)
UHC1	2,219.3
UHC2	1,800.7
UHC3	379.6
UHC4	658.5
UHC6	533.2
UHC9	60.0
UHC10	299.5
UHC12	52.0
UHC13	1,497.0
UHC14	180.3
UHC15	1,588.1
UHC17	973.1
UHC5	4.7
UHC8	2.7
UHC11	2.4
UHC16	2.5
UHC18	2.2

[a] IFA results showed >95% of cells positive for p24 antigen in all cases. A minimum of 500 cells was examined in each instance.

was digested with 40 U of *Xba*I, *Sac*I, or *Kpn*I (Pharmacia Fine Chemicals, Montreal, Canada) and subjected to electrophoresis through 0.8-cm-thick, 0.8% agarose slab gels. Gels were blotted for 20 h in 10× SSC onto 0.45-μm nitrocellulose filters (Schleicher & Schuell, Inc.). Prehybridization was performed for 6 h at 42°C in 50% (vol/vol) formamide–Denhardt solution–5× SSC–250 mg of salmon sperm DNA per ml; hybridization was then performed under the same conditions, with the addition of 10% dextran sulfate. Filters were washed four times for 5 min each with 2× SSC containing 0.1% SDS at room temperature and washed again four times for 10 min with 0.1× SSC containing 0.1% SDS at 65°C. Plasmid pBH-10 was used to detect HIV-1 provirus.

Syncytium assay. To further assess the abilities of infected, parental U-937 cells and various U-937 clones to express biologically active viral gp120, cocultures of such cells (7 × 10^3 cells) were established with 6.3 × 10^4 MT-4 cells (ratio, 1:10) in 0.2 ml in 96-well microdilution plates. These cultures were maintained as described above and examined daily for the appearance of multinucleated giant cells. At various times, the cultures were fixed in methanol and Giemsa stained.

RESULTS

Derivation of chronically HIV-1-infected clones of U-937 cells. When U-937 cells were initially infected by HIV-1, cells positive for p24 as determined by IFA appeared by 14 days postinfection; this was accompanied by the presence of detectable RT activity. After 2 months of culture, more than 95% of the cells in this population were positive for p24 antigen, and RT activity in culture fluids was consistently above 10^6 cpm/ml. We then derived from this population the 17 clones (UHC) which are outlined in Table 1 in terms of RT activity and p24 antigen immunofluorescence at 30 days after initiation. In each instance, more than 95% of the cells continued to be positive for p24 antigen. However, the 17 clones could be separated into two groups distinguished by their abilities to produce RT activity. Twelve clones produced high levels of RT activity, while five others produced only background levels (less than 5,000 cpm/ml). However, the cells of each clone showed greater than 95% positivity for viral p24 as determined by IFA.

TABLE 2. Stability of UHC clones grown in tissue culture over long periods

Clone designation	Expression of RT[a] or IFA[b] at indicated wk after cloning:					
	4		13		21	
	RT	IFA	RT	IFA	RT	IFA
UHC1	2,218.3	>95	193.4	>95	1,221.1	>95
UHC3	379.6	>95	674.0	>95	639.4	>95
UHC17	973.1	>95	1,340.7	>95	2,708.8	>95
UHC8	2.7	>95	4.1	>95	1.6	>95
UHC4	658.5	>95	4.9	>95	2.6	0
UHC15	1,588.1	>95	1,669.1	>95	1,414.9	17

[a] RT expressed as counts per minute per milliliter multiplied by 10^3.
[b] IFA results expressed as percentage of cells positive for p24 antigen.

Stability of UHC clones. We next wished to determine whether the phenotype of each clone was stable in terms of production of high or low levels of RT activity. Toward this end, six rapidly growing clones were monitored for more than 21 weeks for expression of RT activity and p24 immunofluorescence (Table 2). Different groups could be distinguished. The first consisted of three clones (UHC1, UHC3, and UHC17) that were continously expressing high levels of both p24 and RT throughout this period. A fourth clone (UHC8) was distinctive in that it remained negative for production of RT while being highly positive for p24 antigen during this time. Other clones of cells were characterized by changes in levels of both RT production and p24 expression during the period of study. Clone UHC4 was initially characterized as positive for each parameter but became negative for both with time. In contrast, clone UHC15 was a high RT producer throughout but, with time, showed a diminished number of cells positive for viral p24 antigen. This suggests that the population might have been contaminated with cells not expressing HIV. However, similar variations in the numbers of cells positive for HIV antigens were also observed in multiply subcloned UHC15 cells. Thus, it is likely that these various populations may be in equilibrium with one another. IFA performed with monoclonal antibodies against viral p66/51 and p17 yielded results similar to those described above (data not shown). After 21 weeks, all clones were examined on a regular basis for expression of RT and by IFA. Only minor variations were observed after this time, implying that the phenotype was stable after 21 weeks.

Infectiousness of HIV-1 derived from cloned U-937 cells. We next wished to assess the infectiousness of the HIV-1 progeny of these clones and, accordingly, inoculated the respective cell culture fluids onto several cell types. For this purpose, these fluids were adjusted to RT levels of 200,000 cpm/ml, except for the two RT-negative clones, UHC4 and UHC8, for which a 1:2 final dilution was used. Target cells included two T-cell lines (MT-4 and H-9), peripheral blood lymphocytes obtained from healthy volunteers, and parental U-937 cells. The results shown in Fig. 1 show the levels of RT activity recorded on MT-4 cells after infection. In addition to parental infected U-937 cells, three clones (UHC1, UHC3, and UHC17) were found to produce infectious progeny virions, as determined by production of RT activity by the infected MT-4 targets. These three clones were the same as those previously shown to produce high levels of viral RT activity (Table 1). Variations in patterns of infectiousness were also observed among these clones with regard to the kinetics of infection. UHC1 and, to a lesser extent, U-937 supernatants were the most infectious, showing peak levels of RT activity at day 4 and a drop at day 7, by

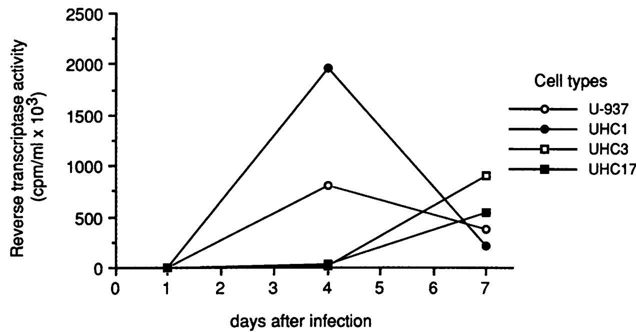


FIG. 1. RT activity present in culture fluids of MT-4 cells at various times after infection with cell-derived supernatants of U-937-infected cells and UHC clones. Results obtained with UHC4, UHC8, and UHC15 culture fluids were negative and are not shown.

which time virtually all MT-4 cells in the culture were lysed. In contrast, culture fluids from UHC3 and UHC17 were slower at establishing active infection, and MT-4 cultures survived for 7 days.

When other targets were used, culture fluids from parental U-937 cells and the UHC1 clone were found to be the most infectious and could infect PBL, H-9, and U-937 cells (Table 3). Culture fluids from UHC17 but not from UHC3 were infectious for PBL. In contrast, those from UHC15 could not establish infection of any of the targets used, despite the fact that the supernatant fluids of this clone were very rich in RT activity, suggesting that this clone may produce defective virus particles. Similarly, RT-negative UHC4 and UHC8 culture fluids from clones UHC4 and UHC8 could not transmit infection. In the cases of these latter two clones, we wished to ensure that infectious virus was not trapped inside

TABLE 3. Infectiousness of culture fluids of HIV-1-infected U-937 cells and UHC clones

Source of culture fluid[a]	Target cell type	RT activity[b] (cpm/ml × 10³)	% Cells fluorescent for p24 antigen[b]
U-937	H-9	38.7	5
	PBL	80.8	7
	U-937	20.8	6
UHC1	H-9	51.6	7
	PBL	93.2	9
	U-937	24.7	8
UHC3	H-9	5.3	0
	PBL	2.6	0
	U-937	2.7	0
UHC17	H-9	3.8	0
	PBL	76.8	4
	U-937	3.2	0
UHC4	H-9	4.8	0
	PBL	5.2	0
	U-937	4.1	0
UHC8	H-9	3.7	0
	PBL	3.9	0
	U-937	3.2	0
UHC15	H-9	3.7	0
	PBL	4.9	0
	U-937	3.2	0

[a] Cell-derived culture fluids were adjusted to a concentration of 200,000 cpm/ml of RT activity and used to inoculate target cells (culture fluids from UHC4 and UHC8 were diluted 1:2 before passage was attempted).
[b] Results of experiments using H-9 and PBL as targets are shown for 14 days after infection. In the case of U-937 targets, assessment of viral RT and p24 activity was performed after 28 days.

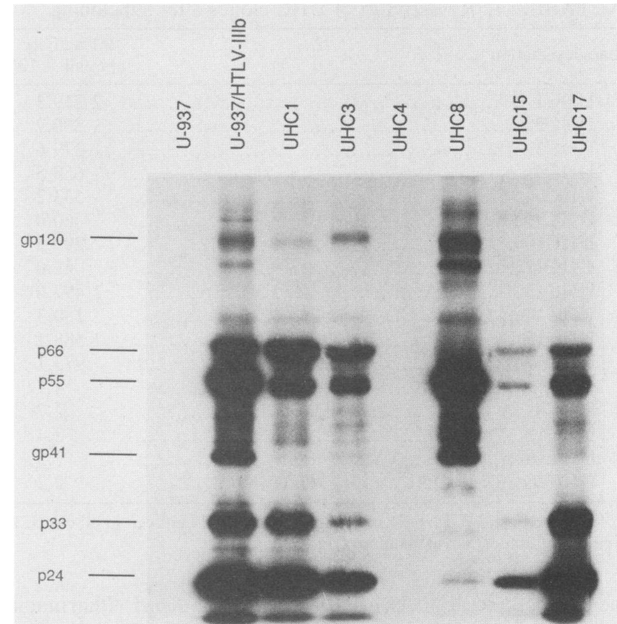


FIG. 2. Immunoblot of material pelleted from culture fluids of UHC clones and parental HIV-1-infected U-937 cells. Lysates, prepared as described in Materials and Methods, were electrophoresed on 10% SDS-polyacrylamide gels and immunoblotted, using sera from HIV-1-infected individuals. HTLV-IIIb, Human T-cell lymphotropic virus type III_B.

intracytoplasmic vacuoles (29) and, accordingly, disrupted 10^7 cells of each type by repeated cycles of freezing and thawing. The lysates thus obtained were clarified and assayed for RT activity and were inoculated onto MT-4 target cells. In neither case did we demonstrate the presence of either RT activity or infectious virus (results not shown).

Synthesis of viral proteins. The results described above indicate extensive heterogeneity among the various U-937-derived clones with regard to expression of viral antigens, production of progeny virus, and levels of infectiousness. We next assessed viral protein synthesis in these various cells by Western blot analysis of both clarified culture fluids and whole-cell preparations, using sera from patients with acquired immunodeficiency syndrome containing antibodies to all major HIV proteins.

The Western blot patterns obtained with culture fluids from each of the UHC1, UHC3, and UHC17 clones were similar to those seen with HIV-1-infected parental U-937 cells (Fig. 2). Although the total amounts of protein loaded into the wells were the same for each preparation, the relative amounts of viral proteins in each preparation were variable, as suggested by the presence of faint gp41 and gp120 bands. Overexposure of the X-ray film confirmed the presence of all major viral proteins in these three preparations. In contrast, culture fluids from nonproducer p24(+) UHC8 clones were apparently devoid of both viral p66 and p33, which represent, respectively, viral RT and endonuclease. However, these fluids did contain a strong band at 115 kilodaltons, which may correspond to the precursor of the *pol* gene, and a large excess of the p55 *gag* precursor. In the case of UHC15 cells which produced RT activity but not infectious progeny virus, the two major glycoproteins gp120 and gp41 were absent in virions recovered from this cell supernatant. This result was further confirmed by radioimmunoprecipitation assay, using patient serum containing

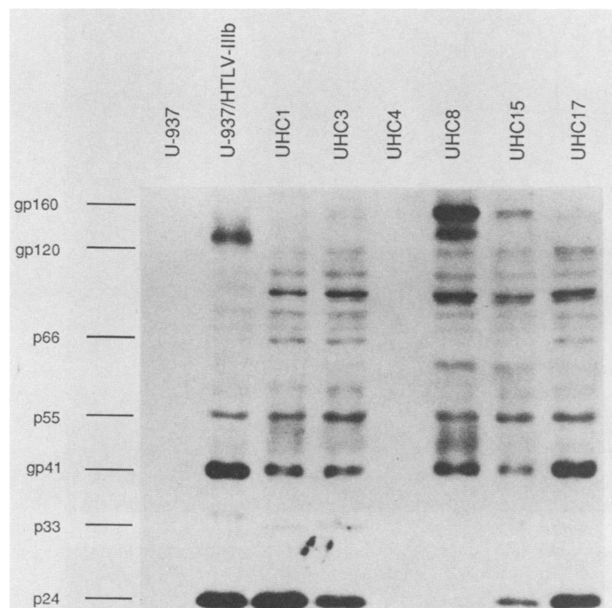


FIG. 3. Immunoblot of whole-cell lysates of UHC clones and parental HIV-1-infected U-937 cells. The same cultures as those used as sources of the cell-free material shown in Fig. 2 were employed. HTLV-IIIb, Human T-cell lymphotropic virus type III_B.

antibodies against viral gp120 and gp41 (data not shown). The UHC4 clone was negative for all viral proteins.

We also performed Western blot analysis on preparations of intact cells (Fig. 3). Again, the UHC4 clone was devoid of any viral proteins. The UHC8 cells apparently synthesized a number of known viral proteins, with the exception of p66 and p24. UHC15 cells were found to contain all viral proteins, including the glycoproteins gp120 and gp41 not seen in culture fluids derived from these same cells and their common precursor gp160; other band patterns were consistent with what had been observed in the respective culture fluids. As in the case of viral pellets, these findings were repeated by radioimmunoprecipitation assay, using a pool of patient sera containing high titers of antibodies against gp120 and gp41 (data not shown). These results confirmed the presence of the 115-kilodalton *gag-pol* precursor protein in both UHC15 and UHC8, as well as the absence of p66 and p33 in UHC8 cells.

Negative staining. The results described above indicate that some clones (i.e., UHC1, UHC3, and UHC17) produced infectious HIV particles, while culture fluids of UHC8 and UHC15 were noninfectious, in spite of the presence of certain viral proteins. We asked whether these clones might somehow assemble these proteins and whether defective viral particles might be present. Negative staining shows that normal-looking viral particles were present in the UHC8 supernatants (Fig. 4C). In contrast, viral structures in UHC15 fluids seem to lack external spikes (Fig. 4E). Examination of thin sections revealed that viral particles present in UHC8 cells contained a ring-shaped nucleoid characteristic of noninfectious viruses with immature cores (Fig. 4D) (33). In contrast, viruslike structures in UHC15 cells contained an electron-dense nucleoid. In both instances, virus was contained inside intracytoplasmic vacuoles.

Syncytium assay. The observation that the UHC8 and UHC15 clones were defective in protein synthesis and viral maturation was further confirmed by cocultivation of these cells with MT-4 target cells as previously described (9). When infected parental U-937 cells were coincubated with noninfected MT-4 cells, large syncytia were seen after 24 h, presumably as a consequence of the interaction of the gp120 at the plasma membrane of the infected parental U-937 cells with surface CD4 on the MT-4 cells (Fig. 5A). In contrast, coincubation of MT-4 cells with UHC8 and UHC15 cells did not result in formation of multinucleated giant cells, even after 72 h (Fig. 5B and C).

Expression of viral RNA. We next assessed whether the expression of viral RNA was normal in these various UHC clones by Northern blot analysis (Fig. 6). The presence of three bands representing the full-length and genomic viral RNA (9.6 kilobases [kb]), singly spliced *env* mRNA (4.3 kb), and multiply spliced messages (2.0 kb) were detected in all cases except for UHC4 cells, which were devoid of any viral mRNAs and which did not express any viral proteins (Fig. 2). HIV mRNA accumulation was also studied in these clones by comparing the relative quantities of viral messages with that of the β-actin gene (Fig. 7). UHC4 and UHC15 were omitted in this study because of a lack of cells expressing HIV. High (UHC1 and UHC17) and low (UHC3, U-937, and UHC8) producers of viral mRNA could be distinguished, suggesting variable levels of mRNA turnover. There was, however, no correlation between the level of HIV-1 mRNA accumulation and the level of infectiousness of virus harvested from these cells.

Integrated viral DNA. Finally, we asked whether the observed heterogeneity among these clones of cells would be reflected at the level of integrated proviral DNA. Toward this end, high-molecular-weight DNA obtained from each cell type was digested with various restriction endonucleases, namely *Xba*I, *Sac*I, and *Kpn*I. *Xba*I, which is a no-cut enzyme within the HIV-1 genome, generated a band of approximately 10 kb or more (Fig. 8) in each case. The band seen in the case of UHC15 cells was fainter than that visualized with the other cells. The UHC1 clone showed a secondary band at around 4.3 kb, which was also seen in parental HIV-1-infected U-937 cells. The UHC17 clone also possessed a unique 2.0-kb band. UHC clones thus show considerable restriction fragment polymorphism. This explains, at least in part, the observed heterogeneity within these U-937 clones, especially insofar as infectiousness of culture fluids is concerned.

Heterogeneity in the Southern blot digests was also apparent after digestion with *Kpn*I. Two bands of 2.8 and 2.3 kb were found in four of the six clones studied. These bands were absent in UHC4 cells. The UHC17 clone was variant and characterized by a strong band at 5.5 kb. Differences among clones were further evidenced by the presence of other bands which differed in intensity and molecular weight. Digestion of the provirus by *Sac*I revealed two prominent bands at 3.6 and 5.5 kb, which were present in all cases except for UHC4, which appeared to possess a light intermediary band at ~4.0 kb. The UHC8 clone was also characterized by two other bands at 2.3 and >23 kb.

DISCUSSION

This report describes the derivation of a number of cell clones from HIV-1-infected U937 cells by limiting dilution. We observed considerable heterogeneity among these various clones with regard to viral gene expression, replication, antigenic profile, and infectivity.

Major differences were observed in the abilities of the culture fluids of these various clones to infect different cell

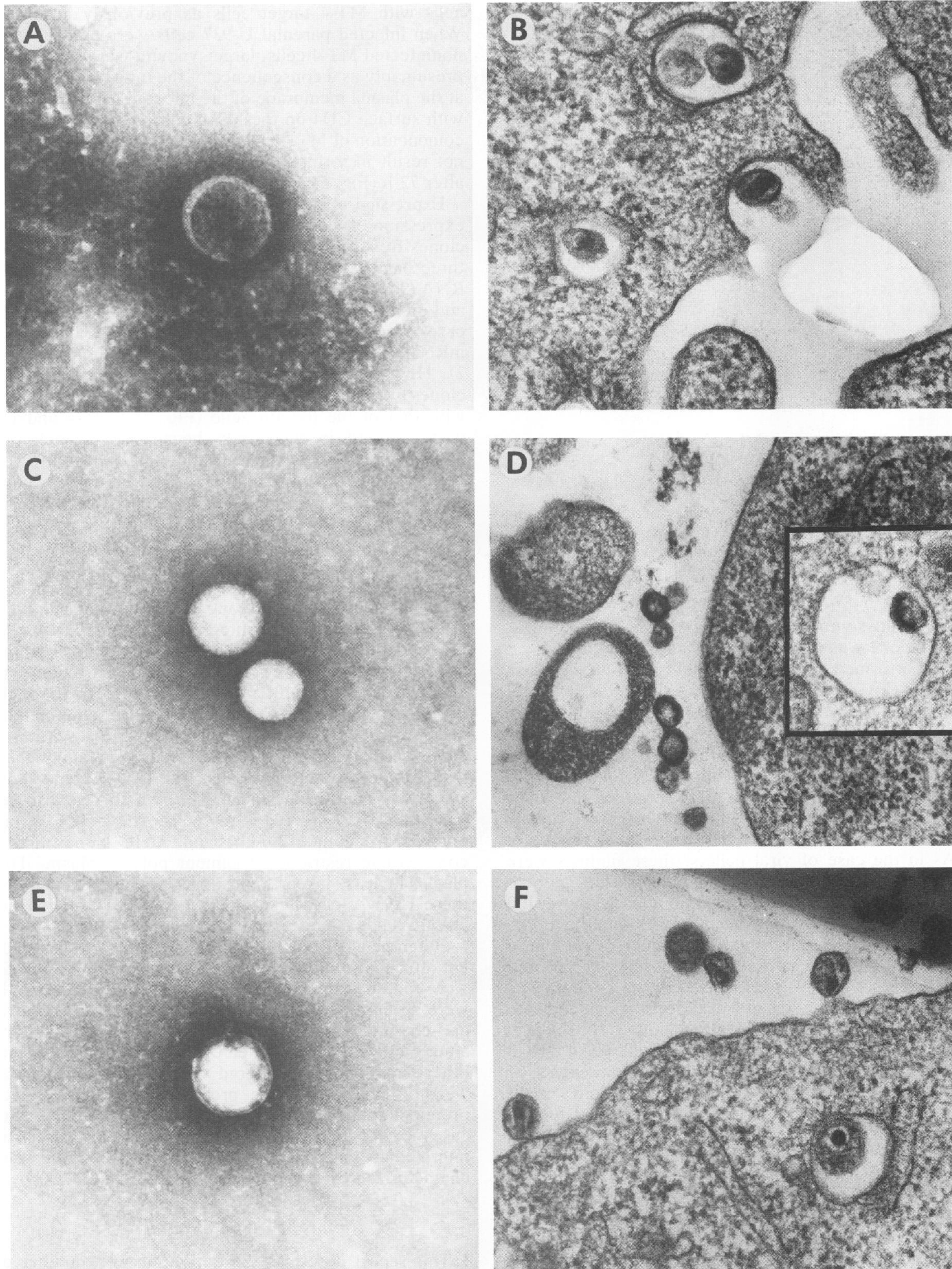
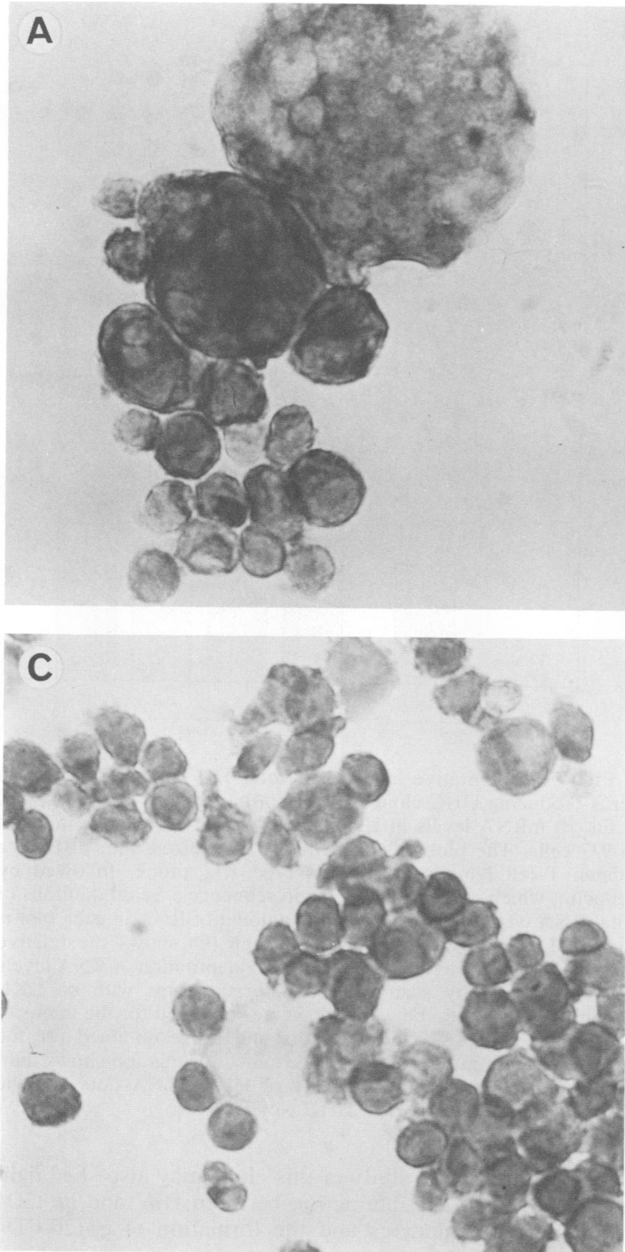


FIG. 4. Negative staining of cell-derived virus from HIV-1-infected U-937 cells (A), clone UHC8 (C), and clone UHC15 (E). Also shown are transmission electron micrographs of infected U-937 cells (B), clone UHC8 (D), and clone UHC15 (F). UHC15-derived particles show a smooth surface devoid of external glycoproteins. Thin sections of U-937, UHC8, and UHC15 cells show vesicles which contain virus particles. Immature ring-shaped nucleoids were observed in the case of UHC8 cells, while UHC15 and U-937 cells seem to contain mature particles. Original magnification: Panels A, C, and E, ×151,000; panels B, D, and F, ×48,000.



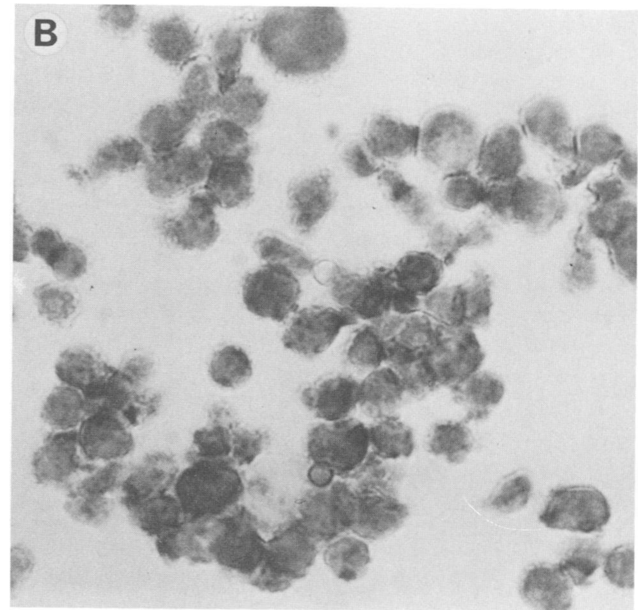


FIG. 5. Cocultures of MT-4 target cells and (A) infected U-937 parental cells, (B) UHC8 cells, and (C) UHC15 cells (10:1 ratio in each case). After 24 h, the cells were fixed in methanol and Giemsa stained.

types (MT-4, H-9, PBL, and U-937) in comparison with parental HIV-1-infected U-937 cells. The culture fluids of these clones were either highly infectious (UHC1), moderately infectious (UHC3 and UHC17), or noninfectious. In individual instances, this lack of infectiousness could be attributed either to the production of defective particles which were devoid of mature RT enzyme (p66) (UHC8) or the two surface glycoproteins gp120 and gp41 (UHC15) or to the absence of viral transcription products (UHC4).

The basis for such heterogeneity in HIV-1 gene and protein expression may be related to the well-known genomic diversity (39, 46) in HIV-1 itself. This is, in part, the result of a lack of fidelity in the action of viral RT (35, 37) and may be reflected in the current study by the high degree of restriction site polymorphism seen in the Southern blots. Modifications of specific viral genes such as *vif* (12, 42) may also affect infectiousness. This could account for the differential susceptibility of the various cell types to infection by culture fluids of UHC1, UHC3, and UHC17. Another gene, *vpu*, was recently shown to be responsible for accelerating viral export (E. A. Cohen, E. Terwilliger, J. Sodroski, and W. Haseltine, Program Abstr. 5th Int. Conf. AIDS, p. 530, 1989) and essential for efficient viral maturation (T. Klimkait, K. Strebel, J. Orenstein, and M. A. Martin, Program Abstr. 5th Int. Conf. AIDS, p. 530, 1989). It is also possible that modifications of unrelated sequences such as those encoding the packaging signals also affect virus maturation. On the other hand, in this study no correlation was seen between the degree of viral infectiousness and relative HIV-1 mRNA levels in each clone.

Biological variations have also been observed on the basis of clinical studies and are consistent with the generation of variants with either increased virulence (6) or preferential tropism for macrophages (15). Of course, the biological diversity present in vivo might be rapidly lost in vitro by the selective outgrowth of HIV strains capable of rapid replication (11). In vitro, however, there is an absence of immunological pressure on less virulent strains. Our results suggest the presence of extensive heterogeneity among viral isolates in the parental HIV-1-infected U-937 population, with the potential to yield noninfectious defective particles at high frequency. It will be of interest to determine whether some of these particles can interfere with the infectious life cycle of normal HIV virions.

The absence of p66 and p33 in clone UHC8 suggests a defect in the processing of the 115-kilodalton *pol* precursor. These cells seem to contain relatively high levels of this 115-kilodalton precursor but lack the RT unit (p66) and endonuclease (p33) normally yielded by the action of viral protease on p115 (33). Mutations of the protease have also been reported to alter RT activity (24). The viral protease is also responsible for the cleavage of the core precursor protein p55 into its smaller components p17, p24, and p15 and is necessary for HIV maturation and infectivity (20, 33).

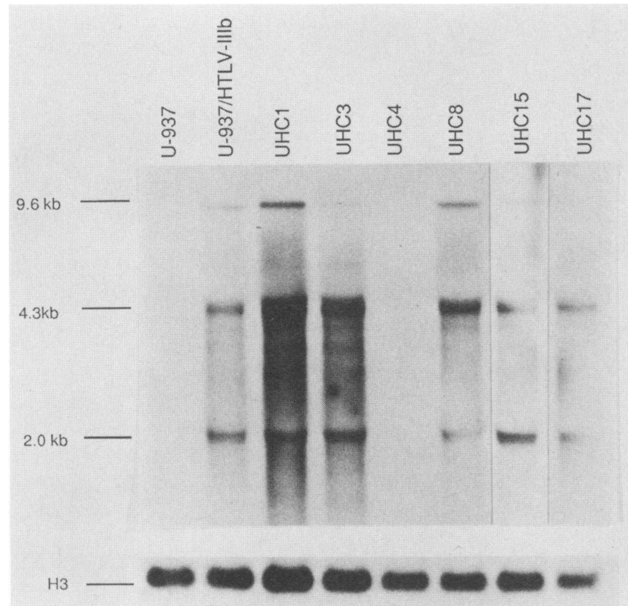


FIG. 6. HIV-1 mRNA expression in UHC clones. A 10-μg portion of total RNA was electrophoresed through a 1% agarose-formaldehyde gel, blotted onto nitrocellulose, hybridized with a ^{32}P-labeled HIV probe (PBH-10), and washed, as described in Materials and Methods. Blots were exposed to Cronex X-ray film for 3 h, except in the case of the UHC15 clone, for which a 15-h exposure was used. As an internal control, blots were rehybridized with a mouse histone H3 probe (pRAH3-2). HTLV-IIIb, Human T-cell lymphotropic virus type III_B.

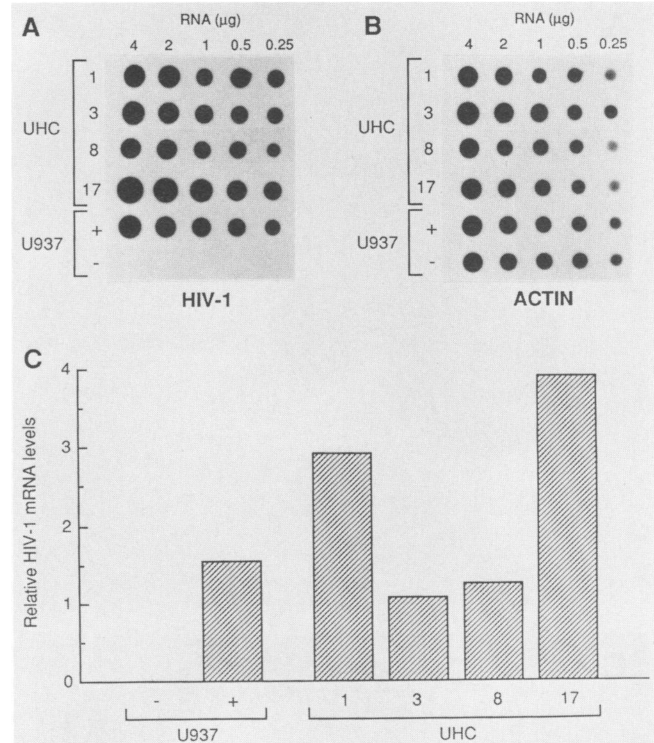


FIG. 7. Quantitative analysis of total HIV-1 mRNA levels in virus-producing UHC clones. RNA dot blots show HIV-1 (A) and actin (B) mRNA levels in UHC clones and in chronically infected U-937 cells. The blot was sequentially hybridized with pBH10, a human T-cell lymphotropic virus type III_B probe, followed by pβ-actin, which contains mouse actin sequences. Serial dilutions of total RNA were performed, and the amount of RNA in each blot is indicated above each column. The graph (C) shows the relative amount of HIV-1 mRNA in each clone. Quantitation of RNA levels was determined by scanning the autoradiograms with an LKB densitometer. A ratio between the values obtained for the intensity of the HIV-1 RNA-associated dots and those obtained for the respective actin intensities was determined. The graph shows individual ratios between the intensity of HIV-1 RNA dots and the respective actin dot for each UHC clone.

Our electron microscopic observations of thin sections and the high ratio of p55 to p24 observed in UHC8 suggest a defect in core protein maturation, which may result from a mutation in the viral protease. The fact that p24 can be detected by IFA by using a monoclonal antibody against p24 indicates either that p24 is, in fact, being synthesized to some extent or that the monoclonal antibody also recognizes p24 in its precursor form, p55. We favor this second possibility, since no p24 was detected in UHC8 cells by Western blot. The faint band seen in the viral preparations may consist of contaminating proteins or degradation products.

Mutations in several domains of the *env* gene coding for gp120 and gp41 may influence viral infectiousness by acting either at the level of entry of virus into cells or at the level of maturation and budding (23, 45). It will be interesting to carry out more extensive analysis of our UHC15 clone, which shows the presence of viral glycoproteins in whole-cell preparations but not in material pelleted from culture fluids, as also shown by electron microscopy (Fig. 4E) and radioimmunoprecipitation assays (data not shown). Current work by surface labeling analysis in our lab demonstrates that neither gp120 nor gp41 is expressed at the cell membrane of these cells, suggesting that these glycoproteins are confined inside smooth-membrane intracytoplasmic vacuoles, possibly originating from the Golgi apparatus. Absence of gp120 was also confirmed in a syncytium assay, using MT-4 cells as targets.

In this regard, it is relevant that one group has described a mutation in the amino termini of gp120 and gp41 which disrupts gp120-gp41 binding (21). This mutant yielded viruses that were devoid of external gp120. Our UHC15 clone represents an interesting tool for the study of mechanisms involved in viral maturation and budding in cells of monocytic origin. Further study of this clone may also shed light on the nature of the interaction between HIV and its CD4 receptors on monocytes and the formation of gp120-CD4 complexes.

Clone UHC4 was the only one studied whose phenotype changed dramatically from that of producer to that of non-producer. Although initially positive for both RT activity and p24 immunofluorescence, UHC4 cells became negative for these markers after 21 weeks. Contamination and subsequent domination of HIV-1-resistant (i.e., CD4-negative) U-937 cells was ruled out by demonstrating the continued presence of the HIV-1 genome in UHC4 cells by Southern blotting (Fig. 8) and polymerase chain reaction (results not shown). We are not sure why the use of *Xba*I led to a signal stronger than those of other restriction enzymes for HIV-1 DNA in UHC4 cells. However, this may be because HIV-1 sequences present in UHC4 cells are deleted with regard to the restriction sites at which the other enzymes used (i.e., *Kpn*I and *Sac*I) act. Attempts to induce infectious HIV-1 in UHC4, UHC8, and UHC15 cells by phorbol esters, azacytidine, and iododeoxyuridine were unsuccessful (not shown). Similar experiments will soon be performed with

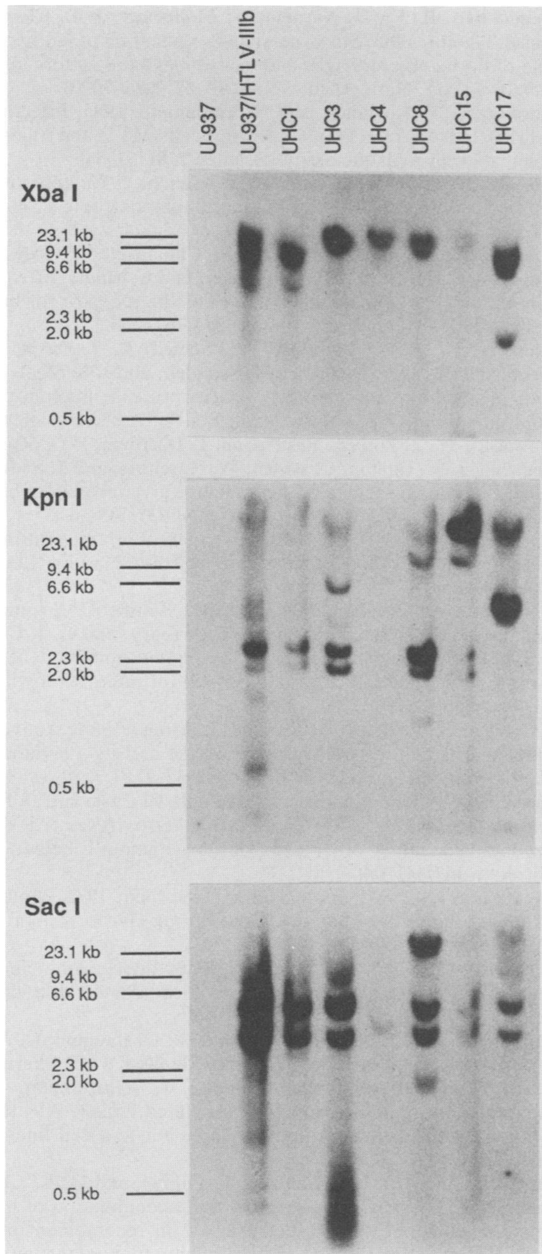


FIG. 8. Southern blot analysis of genomic DNA from UHC clones. DNA was cleaved with *Xba*I, *Sac*I, and *Kpn*I, hybridized with PBH-10, and washed as described in Materials and Methods. The nitrocellulose filters were exposed for 48 h. *Hin*dIII-cleaved lambda DNA fragments were used as molecular weight markers, as indicated in the right margin. HTLV-IIIb, Human T-cell lymphotropic virus type III_B.

tumor necrosis factor α, as this substance has been shown to be a potent stimulus of HIV-1 replication (10). We emphasize, however, that no cellular defect could be identified in the UHC4 clone, since these cells could be reinfected with the same preparation of HIV-1 as that originally used, with kinetics of viral replication identical to those seen with parental U-937 cells. Furthermore, HIV-1 could not be rescued from UHC4 nonproducer cells by heterologous viruses, such as HIV-2, over a culture period of 8 weeks. It thus appears that HIV-1 DNA is probably defective in this clone; however, the mechanisms which underlie the failure of UHC4 cells to produce HIV-1 remain unclear.

Aberrant expression of retroviral genes has also been reported during clonal infection of NIH/3T3 cells with Moloney murine leukemia virus (38). Particles defective in RT activity or viral glycoproteins were found at high frequency. Of the cell clones that produced antigens after infection, one-third showed defective expression of the viral genome. There is also evidence that the defects in murine leukemia virus expression were of viral origin and that infected clones produced viruses which could not form plaques in cell cultures; however, this viral phenotype could be passaged to fresh cells by the addition of culture supernatants (36). The importance of defective viruses in the pathogenesis of acquired immunodeficiency syndrome-like conditions in mice and cats has also recently been reported (4, 5, 34). This raises the possibility that defective HIV-1 particles play some role in human acquired immunodeficiency syndrome. Such viruses could have independent biological activity or could carry scrambled genes of viral and cellular origin that may subsequently be rescued by helper viruses.

Each of our clones, with the exception of UHC4, expressed a particular stable HIV variant. Since it is unlikely that a noninfectious virus such as that derived from UHC8 would have replicated and integrated itself into the cellular genome, genetic alterations must have followed initial replication. In this respect, it has recently been proposed that HIV extrachromosomal proviruses may be found in the form of integrated multimers called nested self-integrates (31), which are formed by sequential integration of monomeric proviral circles into larger preexisting multimers. This novel mode of replication may be peculiar to monocytes and macrophages and may lead to production of defective viruses through deletion and insertion mutations. It may also be that such concatemers are more sensitive to mutations than are integrated proviruses. The high restriction fragment polymorphism observed in the Southern blots also argues in favor of a strong heterogeneity among the integrated proviruses themselves.

U-937 cells are highly dividing cells, and it remains to be proven how this model applies to monocytes and macrophages which show no more than one or two division cycles. On the other hand, replicating myeloid precursor cells of the bone marrow are also susceptible to HIV infection (14). Under clinical circumstances, it is possible that cells of myeloid origin also contain noninfectious variants like those seen in our U-937 cultures. Cells which harbor defective HIV particles may indeed be present in large numbers, after the selective elimination by cytotoxic T lymphocytes and viral cytopathic effects of cells yielding infectious virions. Such cellular hosts of defective HIV might play a role in viral persistence. The remaining cells may contribute to the high level of p24 antigenemia observed in some HIV-infected individuals who remain asymptomatic for several months and do not demonstrate significant $CD4^+$ cell loss and from whom viral isolates are not easy to recover (44).

Such slow or nonreplicating viruses may be able to evolve to a fully infectious phenotype with novel pathogenic potential by genetic switches due to mutational changes or by complementation or recombinational events with the help of a rescue virus. Complementation may be favored by the fusion of two cells through CD4-gp120 interactions or by other factors which might potentiate monocyte and macrophage multinucleated cell formation, such as IL-4 (27). Progression to serious disease may also depend on the

presence of those cells which harbor highly infectious variants which eventually become predominant.

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