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Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector

(retrovirus/AIDS/virus assembly/reverse transcriptase)

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ABSTRACT **Infectious retrovirus particles consist of a core structure containing RNA and gag–pol polypeptides surrounded by a lipid membrane studded with env proteins. A recombinant vaccinia virus was designed to express the entire gag–pol precursor protein of the human immunodeficiency virus type 1. Synthesis and processing of gag proteins occurred in mammalian cells infected with this live recombinant virus, and reverse transcriptase was detected largely in the medium. Electron micrographs revealed immature retrovirus-like particles budding from the plasma membrane and extracellular particles with morphological characteristics of immature and mature human immunodeficiency virus. The latter contained functional reverse transcriptase as well as processed p24 and p17 gag polypeptides. Thus, assembly and maturation of human immunodeficiency virus-like particles can occur in the absence of either infectious RNA molecules or env proteins. The production of noninfectious virus-like particles by expression vectors should be useful for biochemical studies and could provide a safe source of material for the development of vaccines.**

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), contains an RNA genome that encodes gag, pol, and env proteins, as well as additional regulatory proteins (1–4). The primary *gag* translation product is a 55-kDa precursor, p55, that is proteolytically processed to p24, p17, and p15, the major core proteins. The *pol* open reading frame encodes the protease, reverse transcriptase, and integrase (5–11). Expression of the protease, as well as other products of the *pol* gene, requires a relatively inefficient ribosomal frameshifting event within the *gag* gene (12) that leads to the formation of small amounts of the putative gag–pol precursor. A myristic acid residue is present at the N terminus of p17 as well as the gag precursor (13, 14) and by analogy with other retroviruses is likely to be required for transport to the plasma membrane (15), into which the glycosylated envelope proteins are inserted. Studies with defective avian and murine retroviruses, however, have shown that neither infectious RNA nor env protein is required for particle assembly (16–19). In this context, Gheysen *et al.* (20) observed the formation of immature particles containing unprocessed p55 in insect cells that were infected with a baculovirus containing only the *gag* gene of HIV-1. In this communication, we describe HIV-like particle formation in mammalian cells infected with a recombinant vaccinia virus expressing the entire *gag–pol* gene under control of a vaccinia virus promoter. Production of noninfectious HIV-1 particles using expression vectors could be valuable both for studies of assembly and for vaccine purposes.

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MATERIALS AND METHODS

Plasmid Construction. The plasmid sp64/HXB.2, containing an infectious cDNA copy of HIV-1 isolate HXB.2 (21) inserted into the *Xba* I site of plasmid sp64, was provided by F. Wong-Staal and R. C. Gallo (National Cancer Institute) and served as the source of the *gag–pol* gene. A 2300-base-pair *Sac* I–*Eco*RV fragment of sp64/HXB.2 was inserted into the *Sac* I–*Sma* I sites of the replicative form of bacteriophage M13mp18. Oligonucleotide-directed mutagenesis was carried out to place a *Sal* I site followed by a consensus sequence for eukaryotic translation (22) before the start codon of the *gag* gene. The *gag–pol* gene was inserted into a modified form of the vector pSC11 (23) giving rise to pVK3, the final plasmid used to obtain the recombinant virus (vVK1) expressing the HIV *gag–pol* genes. Restriction endonuclease analysis and DNA hybridization were used to characterize the plasmid pVK3.

Viruses and Cells. Vaccinia virus (strain WR) was originally obtained from the American Type Culture Collection. Recombinant vaccinia virus vVK1 was isolated using the plasmid pVK3 and was purified as reported (23). CV-1 monkey kidney cells were propagated as monolayers in minimal essential medium (Quality Biologicals, Gaithersburg, MD) supplemented with 2.5% (vol/vol) fetal bovine serum. Cells were infected with 30 plaque-forming units of virus per cell for 2 hr; the virus was then removed, and the cells were washed and overlayed with fresh medium. The cells were harvested at appropriate times after infection by scraping the monolayers in isotonic phosphate-buffered saline (PBS), washed with PBS, and lysed in 0.5% Nonidet P-40/PBS.

Immunoblotting. The detergent-disrupted cytoplasmic extracts were treated with SDS and 2-mercaptoethanol prior to polyacrylamide gel electrophoresis. The electrophoretically separated polypeptides were transferred by electroblotting onto a nitrocellulose membrane, blocked with 5% (wt/vol) nonfat dry milk/0.3% Tween 20 in PBS, incubated with the antibody in 0.3% Tween/PBS for 1 hr, washed three times with 0.3% Tween/PBS, incubated with ^{125}I-labeled protein A, washed three times with 0.3% Tween/PBS, dried, and subjected to autoradiography.

Reverse Transcriptase Assay. Cells were infected as described above, and reverse transcriptase activity was measured using 5 μl of cell extract or medium, poly(A), oligo(dT), and [α-^{32}P]dTTP in 30 μl as described (24). After 2 hr at 37°C, 10 μl was spotted on DE81 ion-exchange chromatography paper (Whatman), air-dried, and washed four times in 2× standard saline citrate (0.3 M NaCl/0.03 M sodium citrate, pH 7.0). The paper was dried and the radioactivity present was determined in a Beckman scintillation counter.

Purification of Particles. Medium, collected 15 hr after infection of CV-1 cells with vVK1, was clarified by centrif-

Abbreviation: HIV-1, human immunodeficiency virus type 1.

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ugation at 2000 × *g*, and layered on top of a 20% (wt/vol) sucrose cushion. The pellet, formed by centrifugation at 27,000 rpm in an SW 27 rotor for 90 min, was suspended in PBS, and layered on top of a preformed 20–60% sucrose gradient. After centrifugation at 100,000 × *g* for 18 hr in an SW 41 rotor, 12 equal fractions were collected starting at the bottom.

Electron Microscopy. CV-1 cells infected with recombinant vaccinia virus expressing HIV-1 *gag–pol* genes were harvested in PBS and centrifuged at 1500 × *g*. The pellets were fixed in 1.25% (vol/vol) gluteraldehyde and then in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in epoxy resins. Virus pellets were prepared by centrifugation at 100,000 × *g* for 90 min and then processed for electron microscopy as above. Thin sections were cut and stained with uranyl acetate and lead citrate.

RESULTS

Expression and Processing of HIV *gag–pol*. When cells were infected with the recombinant vaccinia virus, synthesis and processing of the gag-pol proteins occurred. In cell extracts prepared 4 hr after infection, the most prominent polypeptides that reacted with HIV-1-specific antiserum were ≈55, 46, and 41 kDa (Fig. 1). Also present were small amounts of high molecular mass material (>100 kDa), presumably resulting from frame-shifting, as also shown by Gowda *et al.* (25). With time, three small polypeptides of 32, 24, and 17 kDa increased in amount and the higher molecular mass polypeptides diminished. Pulse–chase experiments with [^{35}S]methionine were consistent with precursor–product relationships between the higher and lower molecular mass proteins (V.K., unpublished results). Specific antisera were used to confirm the identities of the gag polypeptides p24 and p17 and the integrase polypeptide p32. In addition, the 17-kDa polypeptide (as well as the 41- and 55-kDa polypeptides) was labeled with [^{3}H]myristic acid consistent with its derivation from the N-terminal end of the gag precursor (V.K., unpublished results). Only a faint 66-kDa polypeptide was detected with reverse transcriptase-specific antiserum (V.K., unpublished results).

Secretion of Reverse Transcriptase Activity. Functional assays revealed low amounts of reverse transcriptase in cytoplasmic extracts (Fig. 2). The medium, however, contained much larger amounts of this enzyme that increased with time (Fig. 2). These data suggested that reverse transcriptase was activated just before or after its release from the cell. Control experiments confirmed the absence of detectable reverse transcriptase activity from cells infected with wild-type vaccinia virus. It is also important to note that vaccinia virus (strain WR) particles remain almost entirely intracellular.

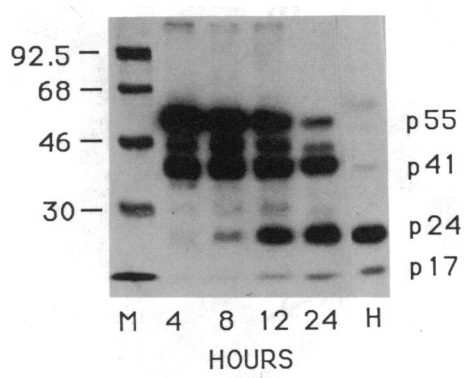

FIG. 1. Synthesis and processing of HIV-1 *gag–pol* gene products. CV-1 cells were infected with vVK1 for 4, 8, 12, or 24 hr, as indicated by lane labels; after which, cytoplasmic extracts were analyzed by polyacrylamide gel electrophoresis and immunoblotting using AIDS patient serum. Detergent-disrupted HIV-infected cell lysates (lane H) were used as positive controls and radioactively labeled polypeptides (lane M) served as size markers. The numbers on the left of the autoradiograph refer to the molecular masses of the marker proteins in kDa; the numbers on the right show the positions of the HIV-1 gag proteins.

Characterization of Purified Particles. To understand the physical nature of the secreted reverse transcriptase, the medium was collected and placed over a sucrose cushion. After centrifugation, all of the activity was found in the pellet fraction, suggesting that it was in a particulate form (Fig. 2). The pelleted material was resuspended and subjected to equilibrium density sucrose gradient sedimentation. Almost all of the reverse transcriptase activity was found in fractions 5, 6, and 7, corresponding to a density of ≈1.16 g/cm^3 (Fig. 3 *Upper*).

Additional samples from each gradient fraction were analyzed by immunoblotting after polyacrylamide gel electrophoresis (Fig. 3 *Lower*). Although material reactive with AIDS patient antiserum was widely dispersed, the processed gag polypeptides p24 and p17 were predominantly located in the fractions with reverse transcriptase as expected for mature HIV particles.

Electron Microscopy of Retroviral Particles. Electron micrographs of thin sections of vVK1-infected cells revealed the presence of 100- to 120-nm particles budding from the plasma membrane (Fig. 4*A*). These retrovirus-like particles were detected at 4 hr and increased greatly in amount from 8 hr on. The morphogenesis of the particles, produced by vVK1, was similar to that of HIV-1 and other retroviruses (26–29). The first indication of particle assembly in infected CV-1 cells was the appearance beneath the plasma membrane of a crescent-shaped variably electron-dense nucleoid that became con-

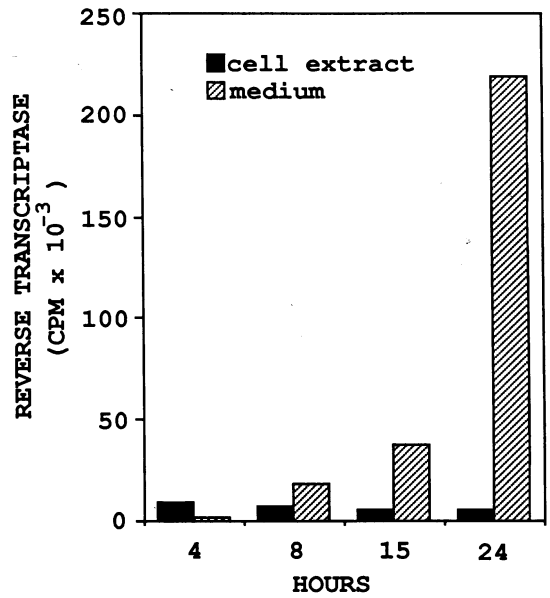

FIG. 2. Reverse transcriptase activity in extracts and culture medium of cells infected with recombinant vaccinia virus expressing HIV-1 *gag–pol* genes. Replicate flasks containing 3 × 10^7 cells were infected with vVK1 at 5 plaque-forming units per cell. At the indicated time, the medium was removed and particulate material was concentrated by centrifugation through a sucrose cushion. The pellet was resuspended in 170 μl of PBS, and 12 μl was assayed for reverse transcriptase. The cpm for each time interval are displayed. The cell monolayers were harvested by scraping, washed, and lysed in a total volume of 500 μl, and 12 μl of the lysate was assayed for reverse transcriptase activity. The resulting cpm were multiplied by 3 to normalize for the various amounts of cell extracts and medium used.

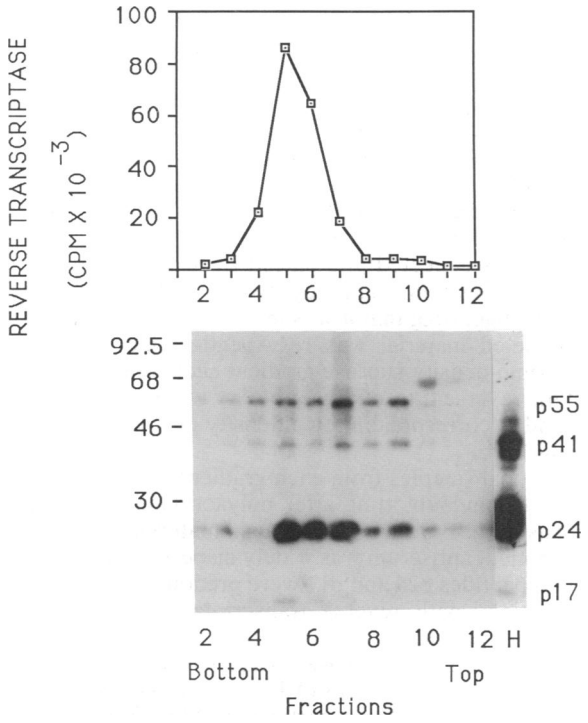

FIG. 3. Purification of reverse transcriptase-containing particles. (*Upper*) Reverse transcriptase activity of fractions collected from equilibrium density centrifugation of medium of vVK1-infected mammalian cells. Samples were removed from each fraction to measure the refractive index and reverse transcriptase activity. (*Lower*) Another sample was precipitated with trichloroacetic acid, washed in 95% ethanol, and analyzed by immunoblotting after polyacrylamide gel electrophoresis of fractions described above. The numbers on the left and right of the autoradiograph and material in lane H are as in Fig. 1.

centric with an electron-lucent center upon budding. The budding particles were easily distinguished from vaccinia virions (not present in Fig. 4), which are much larger, have a distinctly different morphology, and do not form at the plasma membrane (30). The budding retrovirus-like particles were never seen in cells infected with a control vaccinia virus; moreover, their formation was not affected by the presence of rifampicin (Fig. 4*C*), a drug that specifically prevents the assembly and maturation of vaccinia virions (31). HIV-like particles at various stages of maturation were present in the extracellular spaces. The most mature, which had bar- or cone-shaped cores, were also in high-speed pellets of the infected-culture medium (Fig. 4*B*). The presence of maturing extracellular forms was consistent with the observed proteolytic processing of the gag precursor. Electron microscopic analysis of the equilibrium sucrose gradient of Fig. 3 revealed mature extracellular HIV-like particles only in fractions 5, 6, 7, and 8. These fractions, as described above, contained most of the reverse transcriptase activity and processed HIV-1 gag polypeptides; no vaccinia virions were seen in these fractions.

DISCUSSION

The synthesis, assembly, budding, and maturation of HIV-like particles from cells infected with the recombinant vaccinia virus vVK1 appeared to closely mimic the process that occurs during a natural HIV-1 infection. Since the only HIV-1 DNA present in vVK1 was derived from the *gag–pol* genes, the retrovirus-like particles can contain neither infectious RNA nor env protein. There is no need to postulate a specific role for vaccinia RNA or proteins in the assembly process, as studies with avian and murine retroviruses have demonstrated the formation of defective particles that are lacking either infectious RNA or env protein (16–19). Moreover, immature particles also can form in insect cells that were infected with a baculovirus containing only the *gag* gene of HIV-1 (20). Overton *et al.* (32), however, found the baculovirus-produced HIV-1 gag precursor in cytoplasmic inclusion bodies.

The use of expression vectors containing mutated HIV-1 *gag–pol* genes to analyze the steps in the assembly of HIV-1 particles would be a direct and important extension of the present studies. Association of env proteins with the particles may be achieved either by constructing a double-recombinant vaccinia virus that expresses both gag–pol and env or by

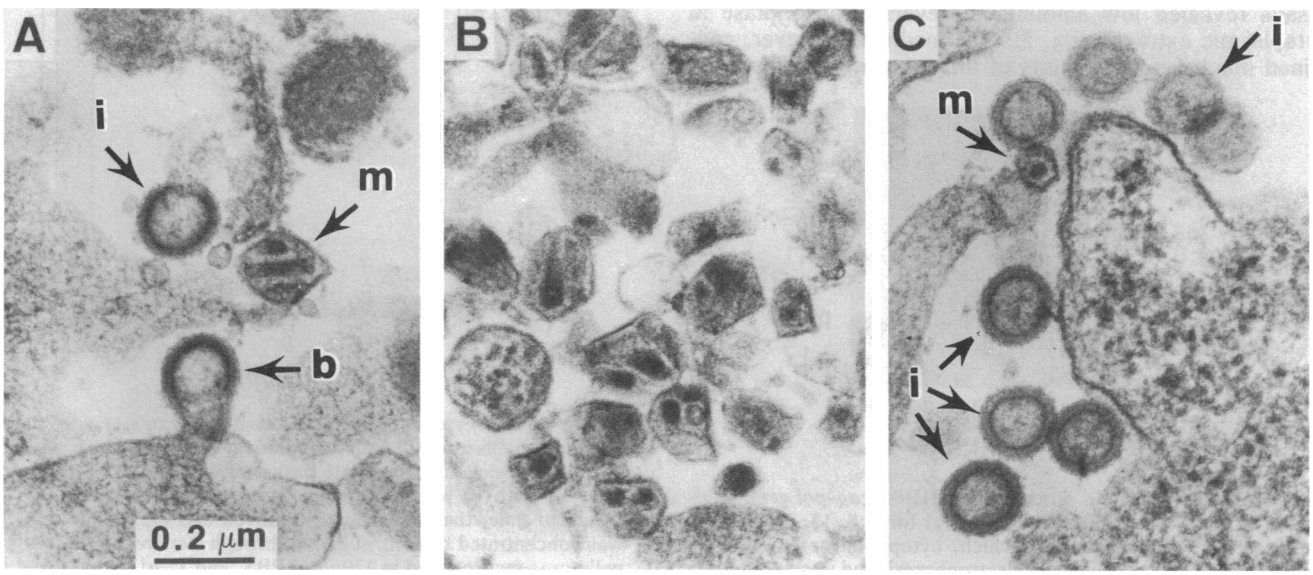

FIG. 4. Electron microscopy of HIV particles obtained from CV-1 cells infected with recombinant vaccinia virus expressing HIV-1 *gag–pol* genes. (*A*) Electron micrograph of cell with budding (b), immature (i), and mature (m) HIV-like particles 24 hr after cells were infected with recombinant vaccinia virus. (*B*) Electron micrograph of virus pellets prepared by centrifugation of medium through a sucrose cushion from cells 24 hr after infection. (*C*) Cells infected with the recombinant vaccinia virus in the presence of rifampicin (100 μg/ml) and fixed at 24 hr. Electron micrograph of a cell producing immature (i) and mature (m) HIV particles.

coinfecting cells with two single recombinants. To evaluate the immunogenicity of purified HIV-like particles, the level of expression needs to be increased. Initial data, however, indicate that mice inoculated with the recombinant vVK1 generate a good antibody response to the gag polypeptides (V.K., unpublished results). In contrast, a previous recombinant virus that synthesized p55 but failed to process gag–pol proteins or assemble particles did not induce gag-specific antibodies in the same mouse strains (33).

The likelihood that HIV particles would be more immunogenic than subunit proteins has led to the testing of inactivated virus for immunotherapy of AIDS patients (34). Nevertheless, the production of large quantities of infectious HIV-1 entails personal risks and the possibilities of incomplete inactivation, reactivation, or recombination are of concern in considering the use of such materials as vaccines in healthy individuals. It would be safer to manufacture particles lacking infectious HIV-1 RNA by using expression vectors. Moreover, recombinant DNA methods provide opportunities for additional modifications designed to enhance immunogenicity.

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