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Expression and Processing of Human Immunodeficiency Virus Type 1 *gag* and *pol* Genes by Cells Infected with a Recombinant Vaccinia Virus

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Human cells infected with a recombinant vaccinia virus containing human immunodeficiency virus type 1 *gag-pol* genes produced large amounts of human immunodeficiency virus *gag* proteins beginning at 1 h and peaking at 48 h postinfection. We show that these polyproteins are processed accurately into mature forms and that the viral polymerase gene is encoded as a 160-kilodalton *gag-pol* fusion protein, most likely by translational frameshifting from the *gag* into the *pol* reading frame.

Molecular characterization of the human immunodeficiency virus (HIV) has demonstrated that the viral genome exhibits the same overall *gag-pol-env* organization as other retroviruses (10, 12, 13, 18). The HIV *gag* region is initially translated as a polyprotein precursor of 55 kilodaltons (kDa) which is then processed into mature p17 (matrix protein), p24 (core antigen [CA]), and p15 *gag* structural proteins (16). In several retroviruses, the proteolytic maturation of *gag* and *gag-pol* polyproteins has been shown to be mediated, at least in part, by a highly specific protease (1, 5, 11, 17, 20). Protease is essential to the retroviral life cycle, as indicated by the production of noninfectious replication-deficient virions by Moloney murine leukemia virus variants mutated in the protease region (5). This suggests that specific inhibitors of retroviral protease could block the maturation and infectivity of HIV and might, therefore, be useful in the treatment of HIV infection. However, there are no sensitive and efficient mammalian test systems that could be used to rapidly screen anti-protease drugs. To this end, we have constructed VV:*gag*, a recombinant vaccinia virus (VV), expressing HIV *gag* and *gag-pol* polyproteins. We report here that VV:*gag* efficiently infects a variety of human lymphoid cell lines and generates high levels of HIV-*gag* proteins within a few hours of infection. Moreover, these proteins are processed into mature *gag* structural proteins.

To insert the *gag-pol* genes of HIV into the genome of VV, we used a generalized method for the construction of recombinant VV (3, 9). Briefly, foreign DNA was first inserted into a plasmid vector (pVV3) downstream from the high-efficiency 7.5K VV promoter. This plasmid was transfected into monolayers of Ltk^- cells which had been infected 2 h previously with wild-type VV at a multiplicity of 0.05 PFU per cell. Homologous recombination between the plasmid and viral DNA occurred in the VV thymidine kinase (*tk*) gene region, thus inactivating the *tk* gene by insertion of the chimeric gene and allowing the enrichment of recombinant populations by low-multiplicity passage through Ltk^- cells in the presence of 25 μg of bromodeoxyuridine per ml. Using this approach, we constructed a recombinant virus, VV:*gag*, which contains the 5.5-kilobase HIV *gag-pol*-coding region (10, 12, 13, 18). The recombinant was plaque purified three times, and the virus stock was prepared as described previously (3, 9).

To test whether the *gag* gene was expressed in cells infected by the recombinant virus, Epstein-Barr virus-transformed B-lymphoblastoid cells (JW-5) were infected at a concentration of 2 PFU per cell for 1 h, washed in phosphate-buffered saline (PBS), and subsequently incubated in Iscove modified Dulbecco medium containing 5% heat-inactivated fetal bovine serum for 48 h. Cells (10^6) were removed from the bulk culture at defined intervals, washed twice in PBS, and solubilized in 0.5% Triton–PBS lysis buffer. The nuclei were separated from the lysate by spinning at room temperature for 3 min in a microcentrifuge, and the quantity of HIV-specific *gag* proteins (55, 39, and 24 kDa) in each lysate was determined with a p24-specific enzyme-linked immunosorbent assay (ELISA) (Du Pont). As shown in Fig. 1, the HIV-specific *gag* proteins were detectable as early as 1 h after infection and continued to increase linearly for up to 48 h. Another recombinant virus (VV:*env*) capable of expressing HIV envelope glycoproteins did not show any reactivity and served as a negative control (Fig. 1). These results show that VV:*gag* expresses *gag*-specific proteins within 1 h of infection. Since the p24 (CA) ELISA can recognize *gag* precursors (55 and 39 kDa) as well as mature p24 (CA), these results do not provide any insights as to whether these polyproteins are being processed to maturation.

To determine whether *gag* polyproteins expressed by the recombinant viruses were being posttranslationally processed to yield mature *gag* proteins, JW-5 cells were infected for 1 h with either VV or VV:*gag* at a concentration of 2 PFU per cell and subsequently labeled with [^{35}S]methionine and [^{35}S]cysteine for 6 h. Cell lysates were prepared and immunoprecipitated with nonimmune rabbit serum or rabbit p24 (CA) antiserum generated against recombinant p24 (CA). Such precipitated proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (7). Three major bands migrating at 55 and 39 kDa (*gag* precursors) and 24 kDa (CA) (Fig. 2, lane 4) were seen. Minor bands at 160, 60, and 45 kDa (Fig. 2, and 4) were also detectable. None of these proteins was precipitated from VV-infected cells by using the rabbit anti-p24 antiserum (Fig. 2, lane 2). Nonimmune rabbit serum failed to precipitate these proteins from either VV or VV:*gag* lysates,

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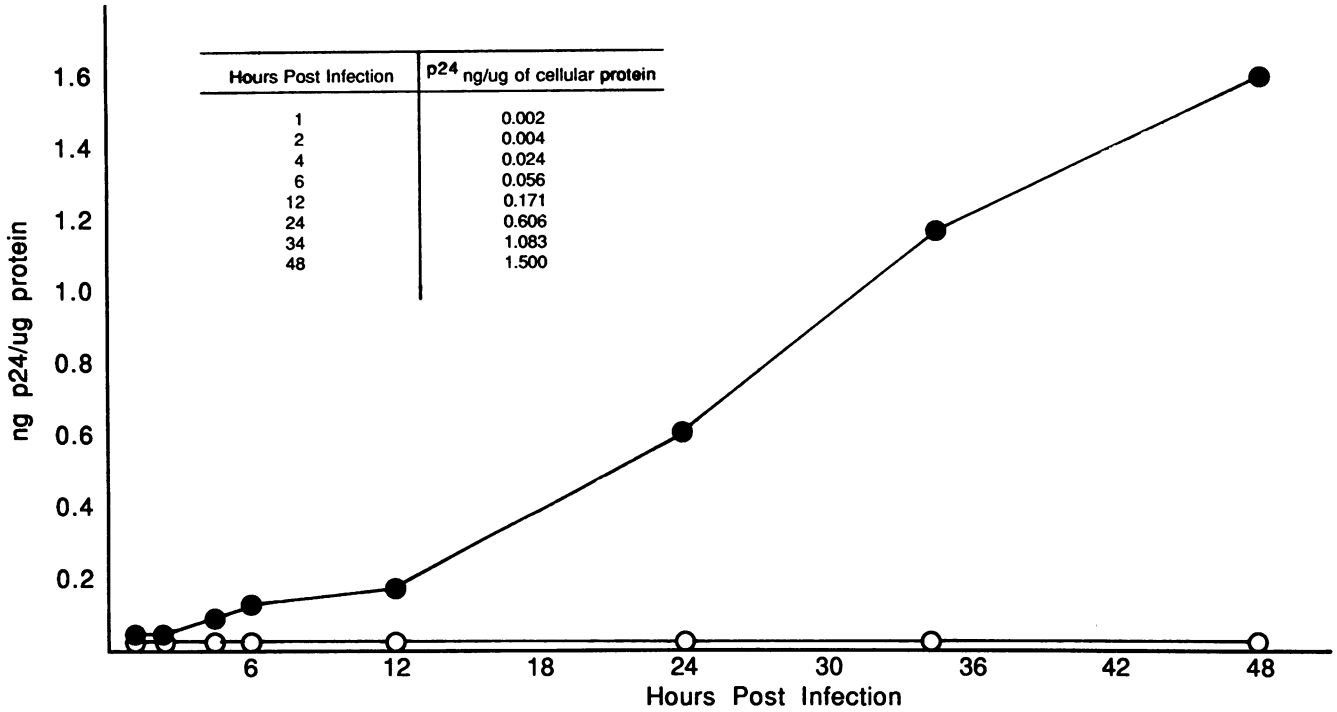

FIG. 1. Kinetics of expression of *gag*-related proteins in a VV:*gag*-infected B-cell line. JW-5 cells (10^7) were infected with either VV:*env*-containing HIV envelope genes (○) or VV:*gag*-containing HIV *gag-pol* genes (●) at a concentration of 2 PFU per cell in 2.5 ml of Hanks balanced salt solution containing 0.1% bovine serum albumin for 60 min. The cells (10^6/ml) were then washed twice in PBS and incubated at 37°C for 48 h with Dulbecco modified Eagle medium containing 5% heat-inactivated fetal bovine serum. Aliquots (10^6 per sample) of cells were removed at defined intervals, washed twice in PBS, and solubilized in 400 μl of 1% Triton X-100 in PBS. The extracts were then centrifuged at 12,000 × *g* for 3 min to remove insoluble material, and each lysate was transferred to a fresh 1.5-ml tube and stored at −20°C until all of the samples were harvested. The lysates were thawed, and an aliquot was used to determine the protein concentration by the method of Lowry (8). The remaining portion was used to detect p24 (CA) by ELISA using the Du Pont p24 specific ELISA kit according to manufacturer recommendations. The protein concentration in each lysate and the quantity of HIV p24 (CA) as determined by ELISA were used to calculate the quantity of HIV p24 (CA)-related proteins per microgram of total protein in each lysate.

confirming specificity of the immune rabbit serum (Fig. 2, lanes 1 and 3, respectively).

The origins of the minor bands precipitated by anti-p24 from lysates of VV:*gag*-infected cells are unknown. A 45-kDa protein has also been detected in HIV-1-infected cells and is thought to originate from an alternate ATG initiation codon downstream from the major ATG initiation codon. The 60-kDa protein seen in the anti-p24 precipitates could represent a posttranslationally modified 55-kDa precursor protein. Jacks et al. (4) have shown by cell-free transcription-translation assay that the precursor of the HIV reverse transcriptase is a 160-kDa polyprotein resulting from ribosomal frameshifting during translation of the *gag* polyprotein; however, a 160-kDa *gag-pol* fusion protein has never been observed in HIV-infected cells.

To determine whether the 160-kDa band present in VV:*gag*-infected cell lysates contains both *gag* and *pol* determi-

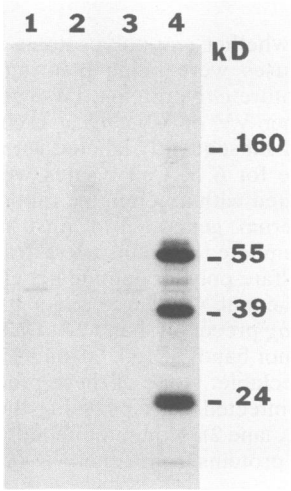

FIG. 2. Radioimmunoprecipitation analysis of HIV *gag*-related protein expressed in VV:*gag*-infected cells. Approximately 2.5 × 10^7 JW-5 cells were infected for 60 min with either wild-type VV (lanes 1 and 2) or recombinant VV:*gag* (lanes 3 and 4) at a concentration of 2 PFU per cell in 2.5 ml of Hanks balanced salt solution containing 0.1% bovine serum albumin. The cells were then washed twice in PBS, metabolically labeled with 0.5 mCi of [^{35}S]cysteine and 0.5 mCi of [^{35}S]methionine for 6 h at 37°C, and subsequently immunoprecipitated as described previously (14). Lane 1, Proteins eluted from nonimmune rabbit serum resin incubated with an extract of radiolabeled wild-type VV-infected JW-5 cells; lane 2, proteins eluted from rabbit anti-p24 resin incubated with an extract of radiolabeled, wild-type VV-infected JW-5 cells; lane 3, proteins eluted from nonimmune rabbit serum resin incubated with an extract of radiolabeled VV:*gag*-infected JW-5 cells; lane 4, proteins eluted from rabbit anti-p24 resin incubated with an extract of radiolabeled VV:*gag*-infected JW-5 cells. kD, Kilodaltons.

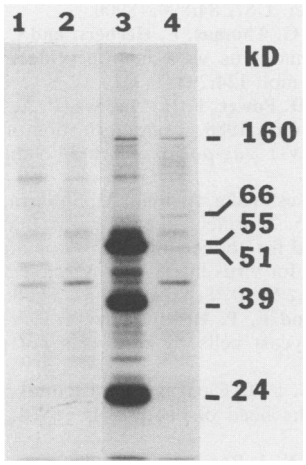

FIG. 3. Radioimmunoprecipitation analysis of HIV-*pol*-related proteins expressed in VV:*gag*-infected cells. Infection of JW-5 cells with VV:*gag*, radiolabeling, and immunoprecipitation were performed as described in the legend to Fig. 2. To precipitate polymerase-specific protein products, extracts were incubated with anti-HIV p66/51 specific murine monoclonal antibody (15) for 16 h at 4°C. The conjugates were subsequently precipitated by using 25 μl of protein A-Sepharose CL-4B coated with 80 μg of goat anti-mouse IgG (Zymed) resin. The subsequent steps were similar to the ones described in the Fig. 2 legend. Lane 1, Proteins eluted from nonimmune rabbit serum resin incubated with an extract of radiolabeled VV:*gag*-infected JW-5 cells; lane 2, proteins eluted from goat anti-mouse IgG resin incubated with an extract of radiolabeled VV:*gag*-infected JW-5 cells; lane 3, proteins eluted from rabbit anti-p24 resin incubated with an extract of radiolabeled VV:*gag*-infected JW-5 cells; lane 4, proteins eluted from goat anti-mouse IgG resin incubated with an extract of radiolabeled VV:*gag*-infected JW-5 cells previously treated with anti-HIV-p66/51 specific murine monoclonal antibody (15) for 16 h at 4°C. kD, Kilodaltons.

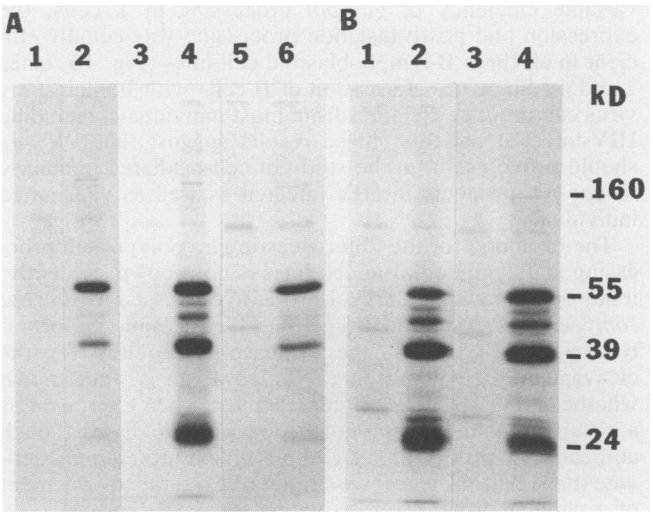

FIG. 4. Radioimmunoprecipitation analysis of *gag*-related proteins expressed in various T- and B-lymphoblastoid cell lines infected with VV:*gag*. VV:*gag* infection of T-lymphoblastoid (A) and B-lymphoblastoid (B) cells and subsequent immunoprecipitation of HIV-*gag* proteins by using anti-p24 rabbit serum were performed as described in the Fig. 2 legend. (A) Lanes 1, 3, and 5, Proteins eluted from nonimmune rabbit serum resin incubated with an extract of radiolabeled VV:*gag*-infected T-lymphoblastoid VB (lane 1), H9 (lane 3), and Jurkat (lane 5) cells. Lanes 2, 4, and 6, Proteins eluted from rabbit anti-p24 resin incubated with an extract of radiolabeled, VV:*gag*-infected T-lymphoblastoid VB (lane 2), H9 (lane 4), and Jurkat (lane 6) cells. (B) Lanes 1 and 3, Proteins eluted from nonimmune rabbit serum resin incubated with an extract of radiolabeled VV:*gag*-infected B-lymphoblastoid SKF (lane 1) and KCH (lane 3) cells. Lanes 2 and 4, Proteins eluted from rabbit anti-p24 resin incubated with an extract of radiolabeled VV:*gag*-infected B-lymphoblastoid SKF (lane 2) and KCH (lane 4) cells. kD, Kilodaltons.

nants, JW-5 cells were infected for 1 h with VV:*gag* at a concentration of 2 PFU per cell and labeled with [^{35}S]methionine and [^{35}S]cysteine for 6 h. Lysates of these cells were subsequently immunoprecipitated with either anti-p24 rabbit serum or anti-p66/51 (reverse transcriptase-specific) murine monoclonal antibody (15). As shown in Fig. 3 (lane 4), a 160-kDa protein was precipitated together with 66- (reverse transcriptase) and 51-kDa proteins by anti-p66/51 murine monoclonal antibody. Anti-p31 rabbit serum against the endonuclease component of the polymerase polyprotein also precipitated the 160-kDa protein (data not shown). When the lysates were subjected to immunoprecipitation by using anti-p24 rabbit serum, the 160-kDa protein was detectable in addition to 60-, 55-, 45-, 39-, and 24- (CA) kDa proteins (lane 3). These proteins were not precipitated by either preimmune rabbit serum or goat anti-mouse immunoglobulin G (lanes 1 and 2, respectively). These results indicate that VV:*gag* produces a 160-kDa *gag-pol* fusion protein most likely by translational frameshifting.

Although the 160-kDa *gag-pol* fusion protein originates as a result of ribosomal frameshifting in transcription-translation assays (4) and is easily detected in lysates of VV:*gag*-infected cells, this protein has never been observed in HIV-1-infected cells. It is possible that in HIV-1-infected cells the 160-kDa protein is processed too rapidly to be detected. It is interesting that the recombinant VV containing HIV-1 *gag* genes used by Walker et al. (19) fails to process the 55-kDa *gag* precursor into mature forms. Neither the production of 160-kDa polyprotein nor the reverse transcriptase activity was detectable in their system (19). The explanation for the disparity between their results and ours is unknown.

To test whether expression and subsequent processing of *gag* polyproteins occur efficiently in both T- and B-cell lineages, we infected three T-lymphoblastoid cells (H9, VB, and Jurkat) and two additional B-lymphoblastoid cells (SKF, KCH) with VV:*gag*. These cells were exposed to VV:*gag* for 1 h at a concentration of 2 PFU per cell and subsequently labeled metabolically for 6 h with [^{35}S]methionine and [^{35}S]cysteine. At the end of the labeling period, the cells were washed, lysed, and immunoprecipitated with anti-p24 rabbit serum. The results are shown in Fig. 4. Of the three T-lymphoblastoid cells tested, all expressed *gag* polyprotein (Fig. 4A, lanes 2, 4, and 6). However, the ability to process *gag* polyproteins into mature *gag* proteins varied among the three cell lines. Whereas H9 cells processed the *gag* polyproteins (55 kDa) very efficiently (Fig. 4A, lane 4), Jurkat and VB cells were deficient in their ability to process *gag* polyprotein (Fig. 4A, lanes 2 and 6). This difference is not due to the relative efficiency of VV:*gag* infection because another VV-HIV recombinant, VV:*env*, infects all of these T-cell lines, resulting in multinucleated giant cells with similar kinetics. Furthermore, the processing pattern seen in VB cells infected with VV:*gag* for 24 h prior to labeling did not differ from the pattern that was seen at 1 h after infection. These results indicate that posttranslational processing of *gag* and *gag-pol* polyproteins may differ among different T-lymphoblastoid cell types. In contrast to the

variable efficiency of *gag-pol* processing in T cells, the expression and posttranslation processing was equally efficient in all three B-lymphoblastoid cell lines (Fig. 4B, lanes 2 and 4). Since transformation of B cells with Epstein-Barr virus can produce cell lines from most individuals, including HIV-infected patients, these results suggest that VV:*gag* should prove useful in the study of cell-mediated immunity to *gag-pol* products in HIV-infected as well as vaccinated individuals.

The reason(s) for the differences in *gag* polyprotein processing in T-lymphoblastic cell lines is not known. Recently, it has been shown that HIV-1 protease expressed in *Saccharomyces cerevisiae* and *Escherichia coli* exhibits autocatalytic activity and that the protease is required for the cleavage of *gag* polyprotein (2, 6). However, it is not known whether HIV-1 protease is autocatalytic when expressed as a component of the *gag-pol* fusion protein. Even though autocatalytic processing of *gag-pol* fusion protein may activate the HIV-1 protease, we cannot rule out the involvement of a cellular protease in activating the HIV-1 protease by releasing it from the *gag-pol* fusion protein. In such a case, the differences in the efficiency of *gag* polyprotein processing among these cell lines may reflect variations in the quantity of cellular protease and/or other host cell factors. It is also interesting that the VB and Jurkat cell lines, which express an immature T-cell phenotype ($WT31^-$, $CD4^+$, $CD8^+$), process *gag* polyprotein inefficiently, whereas the H9 cell line, which expresses a mature T-cell phenotype ($WT31^+$, $CD4^+$, $CD8^-$), processes *gag* polyprotein very efficiently. It is conceivable, therefore, that the processing efficiency may correlate with the state of differentiation of the T cell.

In summary, we have constructed a recombinant VV (VV:*gag*) capable of expressing HIV *gag-pol* genes in VV:*gag*-infected cells. This recombinant is capable of generating high levels of HIV p24 (CA)-related proteins in a very short time after infecting several T- and B-cell lines. We provide evidence for the synthesis of the *gag-pol* fusion protein most likely by ribosomal frameshifting during translation. In addition, *gag* and *pol* precursor polyproteins are accurately processed into mature proteins. This recombinant should prove useful in studies of the cell-mediated immune response to various components of the *gag* polyprotein and may be of value for screening anti-protease drugs in mammalian cell systems.

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