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Synthesis and Processing of Human Immunodeficiency Virus Type 1 Envelope Proteins Encoded by a Recombinant Human Adenovirus

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A recombinant adenovirus was constructed by inserting the human immunodeficiency virus type 1 (HIV-1) envelope gene downstream from the early region 3 (E3) promoter of adenovirus type 5 (Ad5), replacing the coding sequences of E3. The recombinant virus replicated as efficiently as the parent virus in all cell lines tested. Human cells infected with the recombinant virus synthesized the HIV-1 envelope precursor gp160, which was efficiently processed to the envelope glycoproteins gp120 and gp41. A human T-lymphoblast line (Molt-4) infected with the recombinant virus expressed HIV-1 envelope glycoproteins on the cell surface, leading to syncytium formation. The envelope gene was expressed from the E3 promoter at early times after infection and at late times from the major late promoter. When cotton rats were infected with the recombinant virus, antibodies against the HIV-1 envelope glycoproteins could be expressed in an immunoreactive form by the recombinant adenovirus, further illustrating the usefulness of adenoviruses as expression vectors.

Human immunodeficiency virus type 1 (HIV-1) has been implicated as the causative agent of acquired immunodeficiency syndrome and related disorders (1, 10, 25, 36, 43). When HIV infects lymphocytes, multinucleated giant cells are formed as a consequence of the interaction between the virion membrane glycoprotein gp120 and the CD4 receptor on the surface of susceptible T lymphocytes (7). Recognition of this receptor by the virus is the initial event in the infectious cycle, and transduction of the CD4 gene to HIV-1-resistant cells is sufficient to convert them to a susceptible state (28). Monoclonal antibodies to the CD4 receptor are able to block virus infection (7, 23, 31), and antiserum prepared against purified gp120 contains antibodies capable of neutralizing HIV-1 (6, 16, 33, 38). In view of these findings, much of the effort in developing a vaccine against HIV-1 has focused on the potential of the envelope glycoprotein as an antigen that might confer protective immunity. This approach has been successful in blocking primary infections by retroviruses in other species of animals (9, 20–22, 30). Currently, recombinant DNA techniques are being applied to produce HIV-1 protein subunits for use as immunogens (8, 38, 41) and in the construction of hybrid virus variants with the potential to be used as live vaccines (4, 17, 18).

We wanted to determine if hybrid adenoviruses containing HIV-1 genes could serve as a source of HIV-1 proteins to be used for the biologic characterization of the proteins and to analyze their potential use as live vaccines against HIV-1 infection. In this communication, we describe the construction of a recombinant adenovirus type 5 (Ad5) containing the entire HIV-1 envelope glycoprotein gene. The envelope gene was inserted downstream of the early region 3 (E3) promoter of Ad5, replacing the E3 coding sequences which have been shown to be nonessential for virus replication (2, 42). In cells infected with the recombinant virus, the envelope glycoprotein gene was expressed at both early and late during the infectious cycle, with maximal expression occurring during the late phase of infection. Cotton rats (*Sigmodon hispidus*) infected with the recombinant virus produced antibodies against the HIV-1 envelope glycoproteins gp160, gp120, and gp41.

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MATERIALS AND METHODS

Cells and viruses. Monolayers of 293 cells were maintained in Eagle minimal essential medium containing 10% fetal bovine serum, and suspension cultures of HeLa cells were maintained in Dulbecco minimal essential medium containing 5% fetal bovine serum. Molt-4 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum.

Ad5 (obtained from the American Type Culture Collection, Rockville, Md.) and the mutant and recombinant viruses described below were grown, and virus titers were determined by plaque assays using the 293 cell line. The viruses were purified and their DNAs were isolated as described previously (14, 34). In all experiments, viruses were used at a multiplicity of infection of 50 PFU per cell.

Enzymes and reagents. Restriction endonucleases, DNA-modifying enzymes, and DNA linkers were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or New England BioLabs, Inc. (Beverly, Mass.). Protease inhibitors were purchased from Boehringer Mannheim. Radioactive isotopes were obtained from ICN Radiochemicals (Irvine, Calif.) and Radiochemical Centre (Amersham, England). Purified gp160 expressed in a baculovirus system was kindly provided by B. Ericson of MicroGeneSys, Inc.

Construction of plasmids. pBRVR and pBRVRenv were constructed by standard cloning procedures (29). The vector used was pBR322, which had been modified at the *Eco*RI site so that it was resistant to *Eco*RI digestion. pBRVR was constructed by inserting adenovirus DNA from map units (MU) 76.8 to 100.0 (with deletion of sequences from MU 79.4 to 84.0), between the *Hin*dIII and *Sal*I sites of the modified pBR322 vector. The HIV-1 envelope gene from nucleotide 5801 to 9153 (48), derived from plasmid pSC23 (4), was inserted at the unique *Eco*RI site of pBRVR, and a recombinant having the correct orientation was selected and designated pBRVRenv. Additional details of the constructs are provided in Fig. 1.

Generation of mutant and recombinant viruses. Adenovirus DNA from MU 0 to 83.5 was prepared by ligation of *Eco*RI

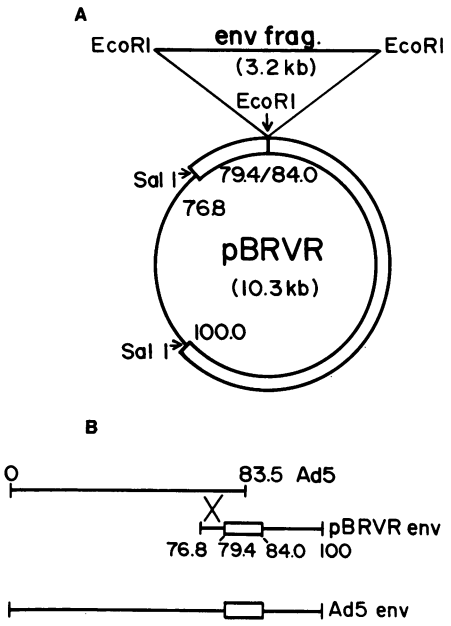

FIG. 1. (A) Schematic diagram showing the structure of plasmid pBRVR. Ad5 DNA (MU 76.8 to 100.0, with a deletion between MU 79.4 and 84.0) is shown as a double line; *Eco*RI-resistant pBR322 DNA is shown as a single line. The HIV-1 *env* gene (nucleotides 5801 to 9153) with *Eco*RI linkers was inserted at the unique *Eco*RI site of pBRVR to generate pBRVRenv. pBRVR was constructed as follows. The covalently attached terminal protein was removed from Ad5 DNA by successive treatment with proteinase K, T4 DNA polymerase, and S1 nuclease as described by Berkner and Sharp (2). The deproteinized DNA was digested with *Eco*RI, and *Eco*RI fragments B (MU 83.5 to 100) and C (MU 76.0 to 83.5) were isolated. The B fragment was further digested with *Sst*I, and a fragment from MU 84.0 to 100 was isolated and engineered to have an *Eco*RI-cohesive end at MU 84.0 and a *Sal*I-cohesive end at MU 100. The C fragment was also digested with *Sst*I, and a fragment from MU 76.8 to 79.4 was isolated and modified to have an *Eco*RI-cohesive end at MU 79.4 and a *Hin*dIII-cohesive end at MU 76.8. These fragments were ligated at their common *Eco*RI ends and then inserted into *Hin*dIII- and *Sal*I-digested *Eco*RI-resistant pBR322. (B) Generation of recombinant Ad5env. *Sal*I-digested pBRVRenv was cotransfected with the Ad5 DNA fragment from MU 0 to 83.5 into 293 cells by the calcium phosphate precipitation method. pBRVRenv and the Ad5DNA fragment have a common DNA sequence of about 2.6 MU (approximately 800 base pairs), where homologous recombination can occur in vivo to generate viable viruses (2).

fragments A (MU 0 to 76.0) and C (MU 76.0 to 83.5) [Ad5(A+C)]. A 3-μg amount of this DNA and 5 μg of *Sal*I-digested pBRVR or pBRVRenv were used to transfect confluent monolayers of 293 cells (2, 13). The characteristic adenovirus cytopathic effect was observed by light microscopy. When maximum cytopathic effect was noted, the cell sheet was scraped into serum-free medium, and viruses were released by three cycles of freezing and thawing and plaqued on 293 cells. Individual plaques were picked and grown on 293 cells, and virus DNA was extracted (15) and analyzed by Southern blotting and hybridization (29, 45). The correct recombinant viruses were selected and further plaque purified.

Analysis of recombinant HIV-1 envelope proteins. Cells were labeled either with [^{35}S]methionine and [^{35}S]cysteine (Tran ^{35}S label; ICN Radiochemicals) in serum-free, methionine-free Dulbecco modified medium or with [^{14}C]glucosamine in Eagle modified minimal essential medium at various times after infection. Cell lysates were prepared as previously described (39), using RIPA buffer (20 mM Tris hydrochloride [pH 7.5], 2 mM EDTA, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, and 0.25% sodium dodecyl sulfate [SDS]) containing 1 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), and pepstatin (0.7 μg/ml), and used for radioimmunoprecipitation with protein A-Sepharose and human serum containing high-titer antibodies to HIV-1. Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (24) and fluorography.

Unlabeled cell extracts were prepared from cells at various times postinfection and analyzed by SDS-PAGE, followed by transfer to nitrocellulose membranes (47) and successive incubation with human serum positive for antibodies to HIV-1 and ^{125}I-labeled protein A.

Expression of envelope glycoproteins in cotton rats. Cotton rats (*S. hispidus*) obtained from the animal facility at the National Institutes of Health (Bethesda, Md.) were bred and maintained at the Georgetown University animal facility.

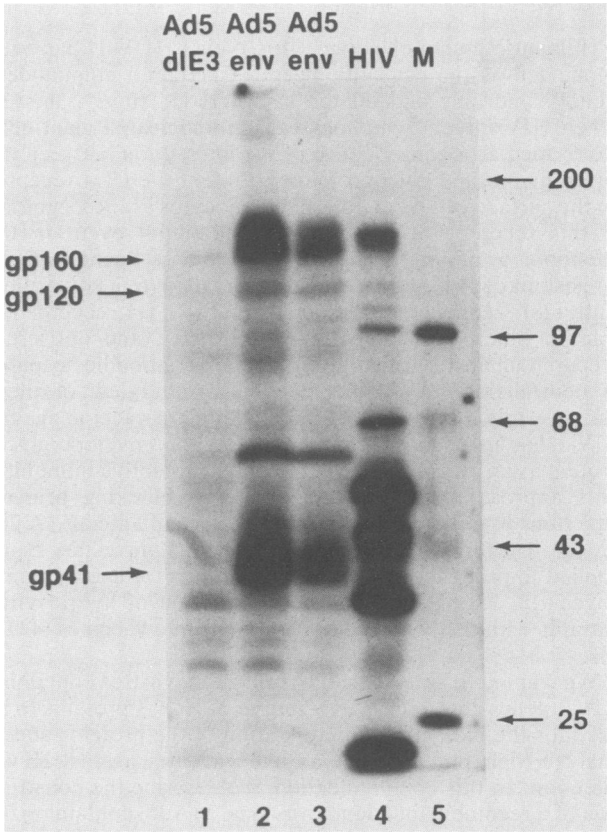

FIG. 2. Expression of envelope proteins in recombinant Ad5env-infected cells. HeLa cells were infected with Ad5dlE3 or Ad5env at a multiplicity of infection of 50. Extracts were made by using RIPA buffer (39), electrophoresed on a 10% SDS-polyacrylamide gel, and electroblotted onto a nitrocellulose membrane. The dried membrane was treated with 6% nonfat dry milk and then incubated with anti-HIV-1 antibody for 2 h at room temperature and with ^{125}I-labeled protein A for 3 h at room temperature. The washed blot was subsequently autoradiographed. Lanes: 1, HeLa cells infected with Ad5dlE3 for 24 h; 2, HeLa cells infected with Ad5env for 24 h; 3, HeLa cells infected with Ad5env for 48 h; 4, human lymphocytes infected with HIV-1; 5, ^{14}C-labeled molecular size markers (in kilodaltons). Positions of gp160, gp120, and gp41 are indicated.

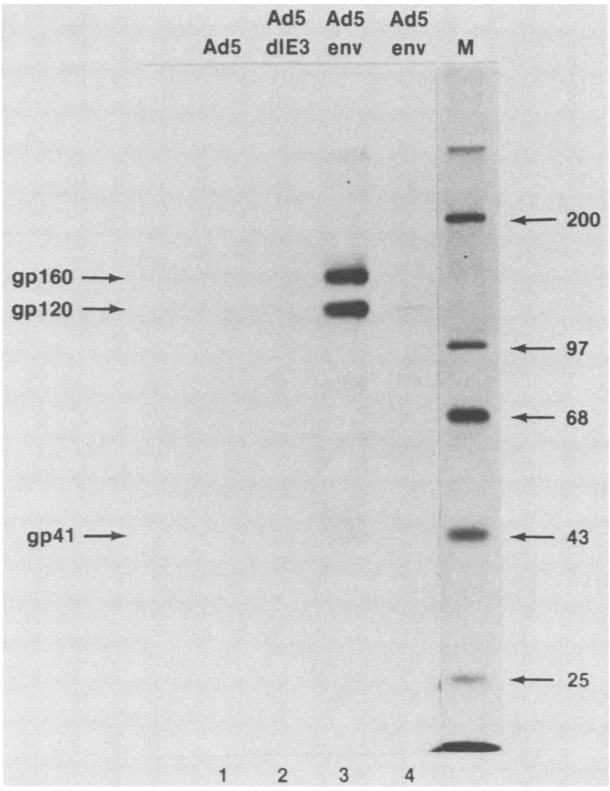

FIG. 3. Glycosylation of envelope proteins. [^{14}C]glucosamine (0.6 μCi/ml) was used to label Ad5-infected (lane 1), Ad5dlE3-infected (lane 2), and Ad5env-infected (lane 3) HeLa cells between 8 and 20 h postinfection. Extracts prepared from these cells were immunoprecipitated with pooled human sera containing antibodies to HIV-1, and the precipitates were collected on protein A-Sepharose beads. The proteins eluted from the beads were electrophoresed on a 10% SDS-polyacrylamide gel and fluorographed. Ad5env-infected cell extract was immunoprecipitated with normal human serum (lane 4); ^{14}C-labeled protein markers (molecular sizes indicated in kilodaltons) are shown in lane M.

One-month-old animals were anesthetized with penthrane (Abbott Laboratories, North Chicago, Ill.) and infected intranasally with 0.1 ml of virus containing 10^8 PFU as described elsewhere (35). Serum samples were obtained from these animals at various times after infection and analyzed for HIV-1 envelope antibodies by a modified enzyme-linked immunosorbent assay. The modified procedure was carried out as described by Prince et al. (37), using plates coated with HIV-1 antigen (Du Pont Co., Wilmington, Del.). Immunoblot analysis, using cotton rat serum, was performed as described above with the addition of rabbit anti-cotton rat immunoglobulin G as the second antibody before the addition of ^{125}I-labeled protein A.

RESULTS

Characterization of recombinant viruses. Confluent monolayers of 293 cells were transfected with either pBRVRenv or pBRVR, along with Ad5(A+C) DNA containing sequences from MU 0 to 83.5. Through homologous recombination between these DNAs (Fig. 1B), viable viruses were generated (as evidenced by the typical adenovirus cytopathic effect in the cultures 6 to 7 days posttransfection). Viruses were harvested and plaque purified; DNAs were prepared by Hirt extraction, digested with *Eco*RI, and analyzed by Southern transfer and hybridization to either ^{32}P-labeled HIV-1 *env* DNA or ^{32}P-labeled Ad5 DNA. An isolate obtained from cells cotransfected with pBRVRenv and Ad5(A+C) had the HIV-1 envelope gene and the expected deletion of E3 sequences and was designated Ad5env. Cells cotransfected with pBRVR and Ad5(A+C) yielded an isolate that had the expected deletion of 2.2 kilobases of DNA from the E3 region (from MU 79.4 to 84.0) and was designated Ad5dlE3. The structures of the Ad5env and Ad5dlE3 DNAs were established by cleavage with a series of restriction endonucleases and yielded the predicted pattern of restriction fragments.

Expression of the HIV-1 envelope gene in Ad5env-infected cells. HeLa cells were infected with either Ad5env or Ad5dlE3, and cell extracts were prepared at various times postinfection (Fig. 2). Expression of the *env* gene product was demonstrated by SDS-PAGE and immunoblotting of infected cell lysates. The *env* gene of HIV-1 encodes a precursor protein (gp160) which is proteolytically cleaved to form an external envelope protein (gp120) and a transmembrane protein (gp41) (40, 48). When HeLa cells were infected with Ad5env, protein bands which comigrated with authentic gp160, gp120, and gp41 were detected at both 24 and 48 h postinfection (Fig. 2). These bands were absent in extracts from Ad5dlE3-infected cells.

The glycoprotein nature of the *env* gene-coding polypeptides was demonstrated by labeling infected cells with [^{14}C]glucosamine from 8 to 20 h postinfection. Lysates were prepared as before, and proteins were separated by SDS-

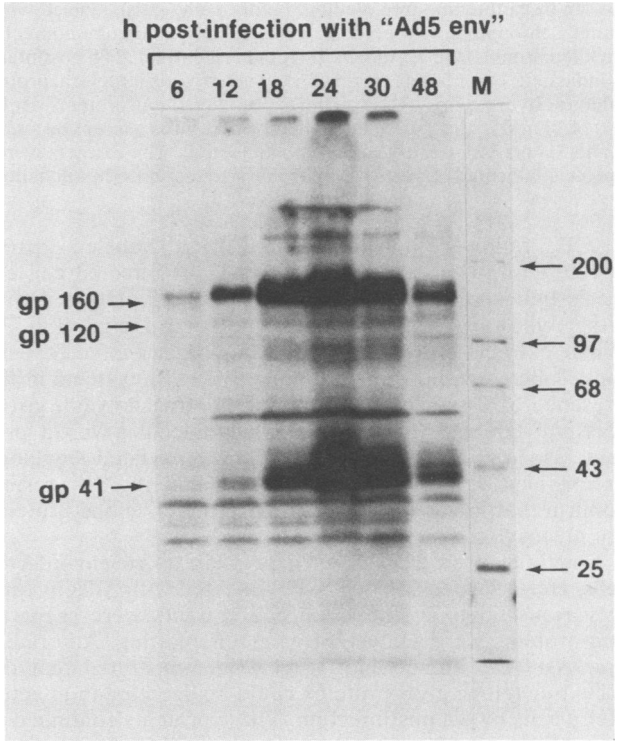

FIG. 4. Western blot analysis showing the accumulation of envelope proteins in Ad5env-infected HeLa cells. Extracts from Ad5env-infected cells were prepared at the indicated times postinfection and electrophoresed. ^{14}C-labeled protein markers (molecular sizes indicated in kilodaltons) are shown in lane M. Positions of the envelope glycoproteins are indicated.

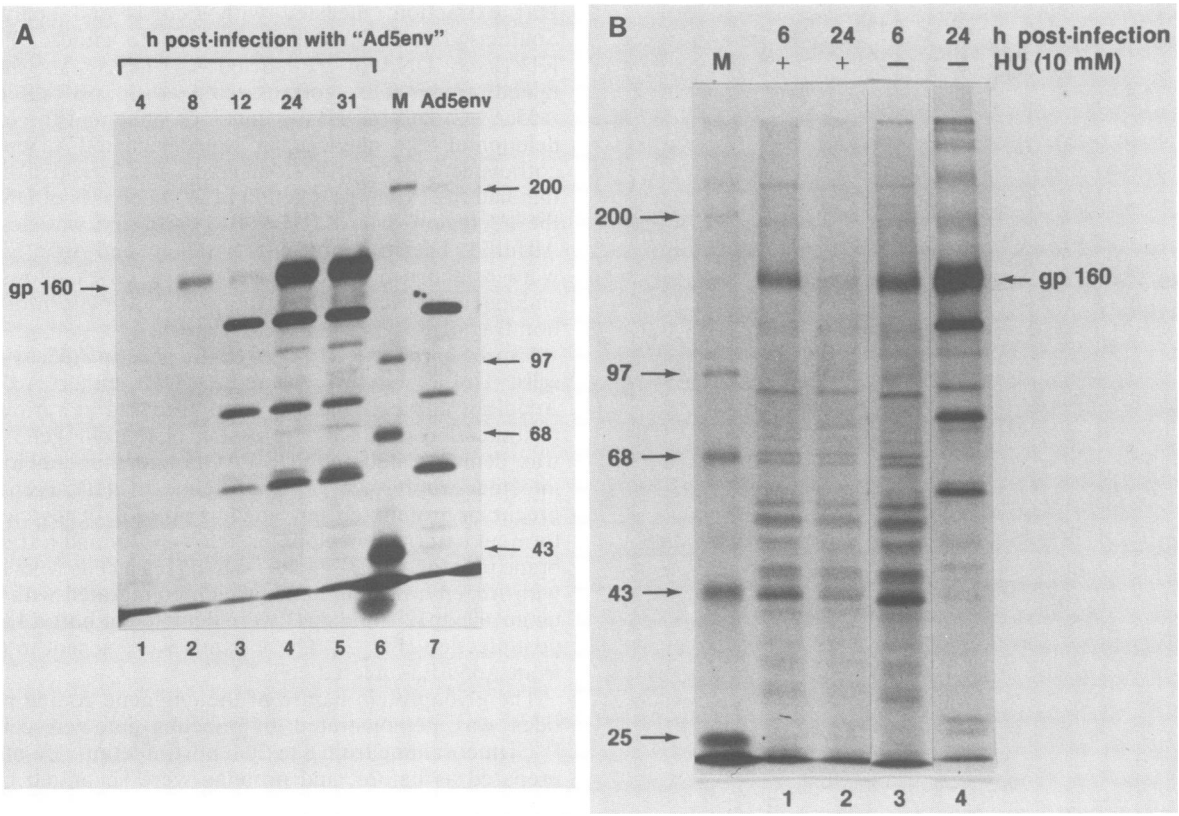

FIG. 5. (A) Time kinetics of expression of the *env* gene in Ad5env-infected HeLa cells. HeLa cells were infected with Ad5env and starved for 1 h in methionine-free medium before being pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min at the time points indicated (lanes 1 through 5). Cell extracts were prepared and immunoprecipitated as described in the legend to Fig. 3. Lane 6, Molecular size markers (in kilodaltons); lane 7, Ad5env-infected cell extract (24 h postinfection) immunoprecipitated with normal human serum. The position of gp160 is indicated; other bands correspond to adenovirus structural proteins that were nonspecifically precipitated. (B) Effects of the DNA synthesis inhibitor hydroxyurea (HU) on the expression of *env* proteins. HeLa cells infected with Ad5env were incubated in the absence (−) or presence (+) of 10 mM hydroxyurea immediately after virus adsorption and pulse-labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine at 6 h (lanes 1 and 3) and 24 h (lanes 2 and 4) postinfection. The extracts were immunoprecipitated as described in the legend to Fig. 3. The position of gp160 is indicated. Lane M, Molecular size markers (in kilodaltons).

PAGE, followed by fluorography. Radiolabeled gp160, gp120, and gp41 were present in Ad5env-infected cell extracts but were absent in extracts from cells infected with either wild-type Ad5 or deletion mutant Ad5dlE3 (Fig. 3). When extracts were prepared from Ad5env-infected cells pulse-labeled with [^{35}S]methionine and [^{35}S] cysteine in the presence of the glycosylation inhibitor tunicamycin, gp160 was not detected. Instead, a 95,000-molecular-weight protein, the size expected for a nonglycosylated envelope precursor (48), was observed (data not shown). These results confirm that in Ad5env-infected cells the envelope proteins are glycosylated.

Kinetic analysis of *env* gene expression in Ad5env-infected cells. HeLa suspension cells were infected with Ad5env, and at various times postinfection cell extracts were prepared and proteins were analyzed by immunoblotting. Autoradiographs of the immunoblot (Fig. 4) demonstrated that the HIV-1 envelope glycoproteins could be detected in infected HeLa cells by 6 h postinfection. These proteins continued to accumulate, reached maximum levels at 24 h postinfection, and declined thereafter. The kinetics of envelope protein synthesis in Ad5env-infected cells were further analyzed by pulse-labeling these cells with [^{35}S]methionine and [^{35}S]cysteine for 30 min at different times postinfection and analyzing envelope proteins by immunoprecipitation.

De novo synthesis of envelope proteins was first observed at 4 h, increased at 8 h, declined at 12 h, and then dramatically increased at 18 h postinfection (Fig. 5A). Treatment of infected cells with 10 mM hydroxyurea immediately after virus adsorption (to inhibit virus DNA replication and therefore late gene transcription, which is initiated from the major late promoter) (5) did not affect synthesis of envelope glycoproteins at early times but blocked synthesis at late times (Fig. 5B). These data suggest that at early times postinfection the *env* gene is expressed from the E3 promoter, whereas at late times the major late promoter is used for expression of the *env* gene. Analysis of mRNA, isolated at early and late times postinfection, by Northern (RNA) blotting and hybridization to an *env*-specific probe (data not shown) confirmed this suggestion.

To examine the precursor-product relationship between gp160 and gp120-gp41 in Ad5env-infected cells, HeLa suspension cells infected with Ad5env were pulse-labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine at 18 h postinfection and chased for 0, 0.5, 3, 5, and 8 h (Fig. 6). Cell lysates were prepared, and equal portions were immunoprecipitated. During the pulse, most of the label was incorporated into gp160; during the chase, the gp160 band diminished in intensity, whereas the intensity of bands

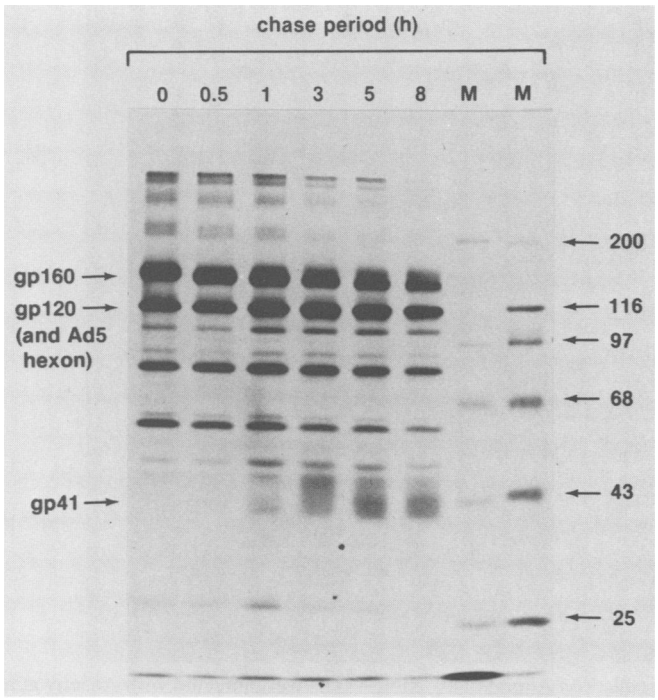

FIG. 6. Synthesis and processing of envelope proteins. At 18 h postinfection with Ad5env, HeLa cells were starved for 1 h in methionine-free medium and then pulse-labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine. The cells were then suspended in medium containing an excess of cold methionine and harvested at 0, 0.5, 1, 3, 5, and 8 h as indicated. Immunoprecipitation and SDS-PAGE were done as described in the legend to Fig. 3. Lanes M, ^{14}C-labeled marker proteins (molecular sizes indicated in kilodaltons). Positions of the envelope glycoproteins are indicated.

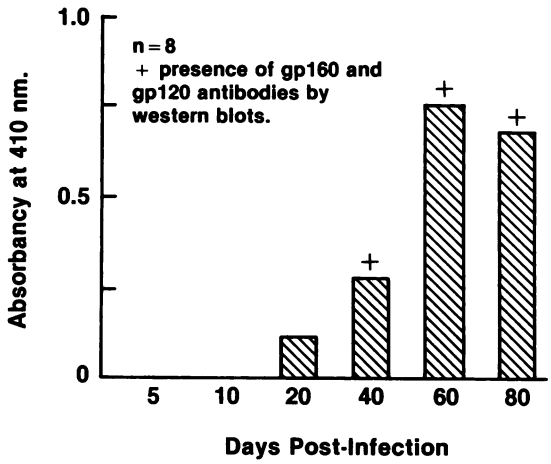

FIG. 7. Antibody response in cotton rats to HIV-1 envelope proteins. One-month-old cotton rats were inoculated intranasally with Ad5env (10^8 PFU per animal), and antibody responses were measured by enzyme-linked immunosorbent assay and Western blotting as described in Materials and Methods. Bars represent mean antibody titers from eight animals in each group infected with Ad5env; + indicates the presence of gp160, gp120, and gp41 antibodies by Western blot analysis.

corresponding to gp120 and gp41 increased, consistent with a precursor-product relationship.

Induction of antibodies to envelope glycoproteins in cotton rats infected with Ad5env. To assess immunogenicity of the Ad5env-encoded envelope glycoproteins. 1-month-old cotton rats were each intranasally inoculated with 10^8 PFU of Ad5, Ad5dlE3, and Ad5env preparations. The animals were bled periodically and screened for production of HIV-1 envelope-specific antibodies by enzyme-linked immunosorbent assay and Western blot (immunoblot) analysis. Antibodies to the HIV-1 envelope proteins were induced in animals inoculated with Ad5env, appearing on day 20 post-inoculation (Fig. 7). The antibody levels increased, reached a peak by day 60, and persisted throughout the study (120 days).

Syncytium formation in Ad5env-infected Molt-4 cells. Molt-4 cells were infected with Ad5env or Ad5dlE3 and maintained in RPMI 1640 medium containing 10% fetal bovine serum. A parallel mock-infected culture was maintained as a control. The cultures were examined microscopically for the presence of syncytia. On day 5 postinfection with Ad5env, syncytia could be detected in the Molt-4 cells (Fig. 8A). No syncytia developed at any time in the uninfected cell culture (Fig. 8B) or in the Ad5dlE3-infected Molt-4 cells (not shown).

DISCUSSION

We have constructed a helper-independent recombinant adenovirus containing the HIV-1 envelope glycoprotein gene. Human Ad5 was used for this construction because of the extensive genetic, structural, and biochemical information available concerning this serotype (11) and also because it is able to replicate in the cotton rat animal model system (35). Ad5 is able to generate viable virus with up to 2 kilobases of additional DNA. To facilitate packaging of DNA, we have deleted about 2.2 kilobases of the nonessential E3 and inserted 3.2 kilobases of DNA, containing the entire coding sequence of the HIV-1 envelope gene, downstream from the E3 promoter.

Envelope protein synthesis was observed in cells at early (6 h) and late (18 h) times after infection, with a decline between these two periods. In the presence of hydroxyurea, however, these proteins were synthesized only at early times, which suggests that expression of the *env* gene during Ad5env infection of human cells is first directed by the E3 promoter and is later mediated by the major late promoter. When expression of the hepatitis B surface antigen (HBsAg) gene located downstream from the E3 promoter of an Ad5 vector was examined, similar results were obtained (32). Both findings parallel patterns of transcription of the E3 genes during adenovirus infection, in which mRNAs were driven from the E3 promoter at early times (4 to 6 h) and transcribed from the major late promoter at late times (18 h) (3; this study).

Use of the endogenous adenovirus promoters probably facilitated the expression of the recombinant gene at both the protein and mRNA levels, since some recombinant adenovirus vectors that use exogenous promoters are transcribed efficiently but are translated with low efficiency. For example, when the HBsAg gene, with its own promoter, was inserted into the Ad5 genome, abundant HBsAg mRNA was made but very little protein was detected (42). This result was probably due to the suppression of HBsAg mRNA translation by adenovirus (12, 46).

We have assayed the amount of immunoreactive envelope protein that is produced by cells infected with Ad5env by immunoblots, using purified gp160 as a standard, and have estimated that in HeLa suspension cell cultures approxi-

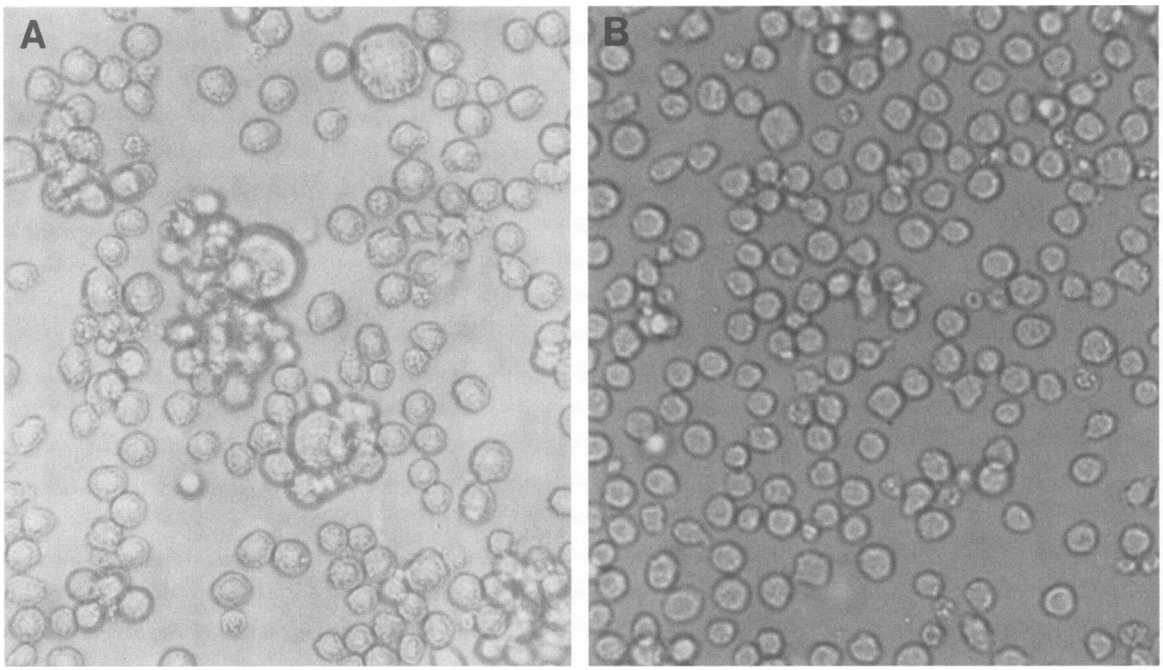

FIG. 8. Photomicrographs of Ad5env-infected and uninfected Molt-4 cells. (A) A total of 10^7 Molt-4 cells were infected with Ad5env at a multiplicity of infection of 50 PFU per cell. The infected culture was maintained in RPMI 1640 medium containing 10% fetal bovine serum. Syncytia were observed beginning at day 5 postinfection. (B) Mock-infected Molt-4 cells maintained in RPMI 1640 medium containing 10% fetal bovine serum. Magnification, ×200.

mately 1 mg of envelope protein per liter is made (data not shown). This finding compares favorably with results obtained with the baculovirus expression system, in which similar amounts of unprocessed gp160 are synthesized (19).

As has been demonstrated in human cells infected with HIV-1 (40, 48), the envelope protein is also synthesized as a glycosylated precursor (gp160) in cells infected with Ad5env and is proteolytically processed to gp120 and gp41. A residual band which migrated slightly faster than gp160 on polyacrylamide gels was observed during pulse-chase labeling of cells infected by Ad5env (Fig. 5A). This band could have arisen from gp160 after the core sugars were trimmed from the precursor molecule before proteolytic cleavage. Indirect immunofluorescence staining of cells infected with Ad5env demonstrated that the envelope glycoprotein is expressed on the surface of these cells. The recombinant-encoded proteins are biologically active, as evidenced by the formation of syncytia when CD4-bearing cells were infected with Ad5env (Fig. 8). This finding also indicates that, as in the case of HIV-1 infection of $CD4^+$ cells (26, 27, 44), the envelope glycoproteins produced by the recombinant Ad5env are capable of binding to the CD4 molecule.

We have used the cotton rat animal model system to determine the immunogenicity of the HIV-1 envelope proteins encoded by Ad5env. The sera from the Ad5env-infected cotton rats recognized gp160, gp120, and gp41 synthesized in HIV-1-infected human cells as well as the glycoproteins synthesized in Ad5env-infected human cells. These data indicate that recombinant adenoviruses expressing HIV-1 genes are a good source of HIV-1 proteins and should be further explored as candidates for vaccines against HIV-1 infection.

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