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Envelope Glycoprotein and CD4 Independence of *vpu*-Facilitated Human Immunodeficiency Virus Type 1 Capsid Export

XIAO JIAN YAO,[1] HEINRICH GÖTTLINGER,[2] WILLIAM A. HASELTINE,[2] AND ERIC A. COHEN[1]*

Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada H3C 3J7,[1] and Division of Human Retrovirology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115[2]

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The effect of *vpu* on the release of human immunodeficiency type 1 capsid proteins was examined in the presence or absence of virus-encoded envelope glycoproteins as well as in cells which constitutively express either the CD4 or CD8 protein. The results show that *vpu*-mediated facilitated export of capsid proteins from HeLa cells does not require expression of the envelope glycoprotein. The experiments also show that export of virus capsid proteins from HeLa cells facilitated by *vpu* is not affected by coexpression of either the CD4 or CD8 protein. The *vpu* protein acts in *trans* to facilitate export of virus capsid proteins from HeLa cells.

The *vpu* protein is encoded by human immunodeficiency virus type 1 (HIV-1) but not by closely related human and simian retroviruses, including HIV-2 and the simian immunodeficiency viruses (4). The *vpu* protein is specified by a short open reading frame that overlaps the 5′ end of the envelope glycoprotein gene (*env*) (4, 15, 21). Previous studies have shown that *vpu* significantly increases the number of virus particles released into the supernatant of infected $CD4^+$ T-cell lines (11, 20–22). The increase in the release of virus particles of Vpu^+ viruses from $CD4^+$ T cells compared with the release of otherwise isogenic Vpu^- viruses is accompanied by a decrease in the accumulation of cell-associated protein rather than by an increase in the total amount of viral proteins made (11, 20, 22). These observations suggest that *vpu* increases the rate of virus export by facilitating either the rate of assembly of the virus particle or the rate of release of the assembled particles from the infected cell surface.

The observation that the *vpu* protein and the envelope glycoprotein are made from a single bicistronic mRNA raises the possibility that coordinate expression of *vpu* and the envelope glycoproteins may be required for an increase in the efficiency of virus export (1, 18). It has also been reported that *vpu* may affect maturation of the envelope glycoprotein by altering the processing of the nascent polypeptide either directly or by the disruption of an intracellular complex formed between the envelope glycoprotein precursor and the CD4 nascent protein (23).

To study the role of the envelope glycoprotein and the HIV-1 receptor, the CD4 protein, on *vpu* export of virus capsids, an experimental system that does not depend on virus replication is needed. The feasibility of using HeLa cells as recipients in transfection assays for these studies was investigated. In the initial experiment, 10^6 HeLa cells were transfected with 10 or 40 μg of DNA of the Vpu^- strain HXBc2 (5), and the Vpu^+ strain HXBH10-vpu^+/env^+ (22), using the calcium phosphate coprecipitation technique (7) (Fig. 1A). Although all sequences of the HXBH10-vpu^+/env^+ strain are derived from the IIIB isolate of HIV-1, the region of viral DNA between the *Sal*I site at nucleotide 5372 (+1 is the transcription initiation site) and the *Kpn*I site at nucleotide 5934 are derived from the BH10 provirus that contains an intact *vpu* open reading frame rather than from the Vpu^- HXBc2 DNA (16) (Fig. 1A).

Virus protein export from HeLa cells was followed by labeling the cells transfected with HXBc2 and HXBH10-vpu^+/env^+ DNA with [^{35}S]cysteine (50 μCi/ml) for 12 h beginning at 48 h posttransfection. The cells were washed and lysed in RIPA buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.25% deoxycholate, 0.2% phenylmethylsulfonyl fluoride), and the virus protein was precipitated with an HIV-1-positive human serum. The virus proteins were visualized by SDS-polyacrylamide gel electrophoresis (PAGE) (11.5% polyacrylamide gel) and autoradiography. The virus-specific proteins present in the cell-free filtered supernatant fraction were detected by precipitation of these proteins with an HIV-1-positive human serum. Additionally, virus particles from the supernatant were collected by high-speed centrifugation onto a dense sucrose cushion. The virus proteins in the pellet were analyzed on SDS-polyacrylamide gels directly without immunoprecipitation.

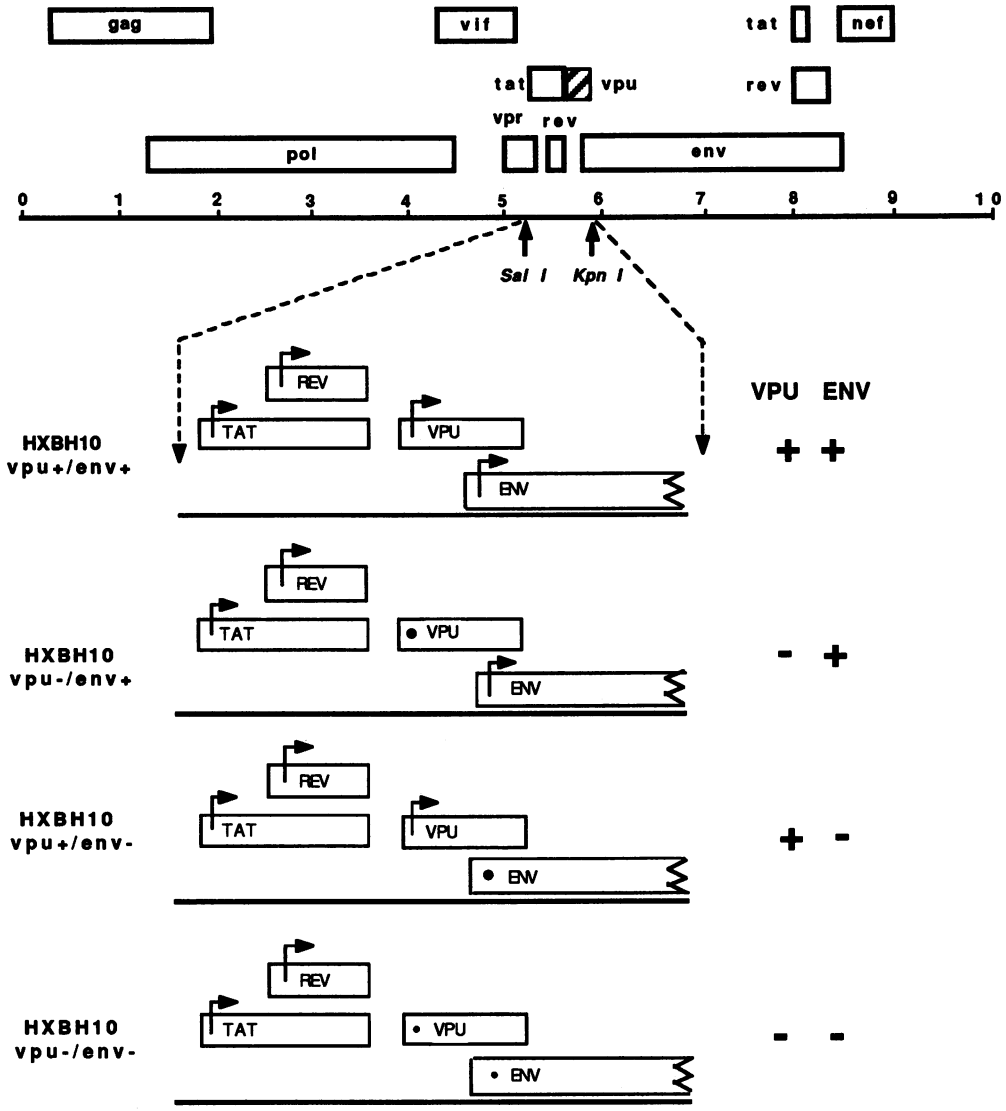
The data in Fig. 2A shows that the amounts of virus-specific protein in HeLa cells transfected with HXBc2 and HXB10-vpu^+/env^+ proviruses are similar. The relative abundance of the capsid precursor p55 and of the processed capsid proteins p17 and p24/25 are similar, as are the relative amounts of the envelope glycoproteins gp160 and gp120 (lanes 1 to 4).

In contrast, the amount of capsid protein released into the cell supernatant as detected by using an HIV-1-positive human serum is markedly different. It is evident that considerably more virus capsid proteins are present in the supernatant fluids of cells transfected with HXBH10-vpu^+/env^+ (Fig. 2A, lanes 7 and 8) than in cells transfected with HXBc2-vpu^- (lanes 5 and 6). There is no reproducible change in the amount of the envelope glycoprotein gp120 present in the supernatant fluids of cells transfected with the two DNAs.

The increased export of virus particles by the HXBH10-

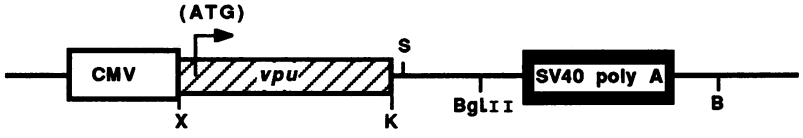
* Corresponding author.

A:

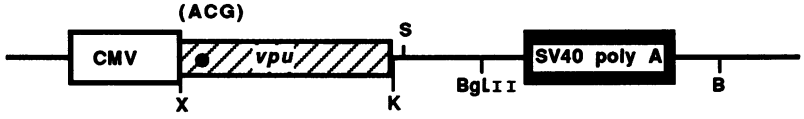


B:

SVCMVvpu+



SVCMVvpu-



vpu$^+$/*env*$^+$ strain is also reflected in the dramatic increase in the amount of virus particles pelleted from these supernatant fluids. Densitometric analysis of the autoradiogram shown in Fig. 2B indicates that at least 10 times more virus particles are released from HeLa cells transfected with the HXBH10-*vpu*$^+$/*env*$^+$ DNA (lane 1) than from cells transfected with the Vpu$^-$ HXBc2 DNA (lane 2).

Role of envelope glycoprotein and CD4 in *vpu*-mediated capsid protein export. The preliminary experiments demonstrate that the effect of *vpu* on export of HIV-1 virion proteins can be assessed in HeLa cells. To examine the role of the envelope glycoprotein in *vpu*-mediated export of virus capsid proteins, a set of four isogenic strains of viral DNAs was made. These four strains are all derived from the HXBH10-*vpu*$^+$/*env*$^+$ DNA clone and are designated the HXBH10-*vpu*$^+$/*env*$^+$, HXBH10-*vpu*$^+$/*env*$^-$, HXBH10-*vpu*$^-$/*env*$^+$, and HXBH10-*vpu*$^-$/*env*$^-$ strains (Fig. 1A). The initiation codons of the open reading frames that specify the *vpu* and envelope glycoproteins were altered from ATG to ACG by site-directed mutagenesis (24).

To assess the role of the CD4 protein in *vpu*-mediated export of viral proteins, HeLa cells that express either the CD4 protein (HeLa-T4) (3) or the CD8 protein (HeLa-T8) (14) were used as recipients in these experiments. The CD4 protein present on the HeLa-T4 cell line has been demonstrated to serve as a functional receptor for HIV-1 (3, 14).

For the initial experiments, the role of *env* proteins and CD4 on *vpu*-mediated capsid export was assessed by transfection of HeLa-T4 and HeLa-T8 cells (10^6) with the set of four viral DNAs (10 μg). Release of capsid antigens was determined by detection of p24 in the culture supernatant fluids, using a commercial radioimmune competition assay kit (NEK-040; New England Nuclear). The amount of capsid antigen in the filtered supernatants was measured in intervals for 60 h posttransfection.

The data in Fig. 2C show that substantially more p24 is present in culture supernatant fluids of HeLa-T4 cells transfected with the HXBH10-*vpu*$^+$/*env*$^+$ viral DNA than in the supernatant of cells which received the Vpu$^-$/Env$^+$ DNA. The amount of p24 released by CD4 cells transfected with the Vpu$^+$/Env$^-$ virus is also greater than that released from CD4$^+$ cells which received the Vpu$^-$/Env$^-$ DNA. The amount of p24 protein released into the cell supernatant is similar for HeLa-T4 cells transfected with Env$^+$ and Env$^-$ viral DNAs.

The data in Fig. 2C also show that the amount of p24 released into the cell supernatant of CD8 cells transfected with the Vpu$^+$/Env$^+$ DNA is significantly greater than the amount released from cells transfected with the Vpu$^-$/Env$^+$ DNA. Similarly, the amount of capsid antigens released into the culture fluid of HeLa-T8 cells is greater for cells transfected with the Vpu$^+$/Env$^-$ DNA than it is for cells transfected with the Vpu$^-$/Env$^-$ DNA. As before, the total amount of p24 released into the supernatant fluids of the HeLa-T8 cells depends on the genotype of *vpu* but not the genotype of *env*. For all virus strains used, the amount of p24 released from HeLa-T8 cells is about twice the comparable amount released from HeLa-T4 cells. This difference probably reflects a consistent increase in the transfection efficiency of the HeLa-T8 cells compared with that of HeLa-T4 cells.

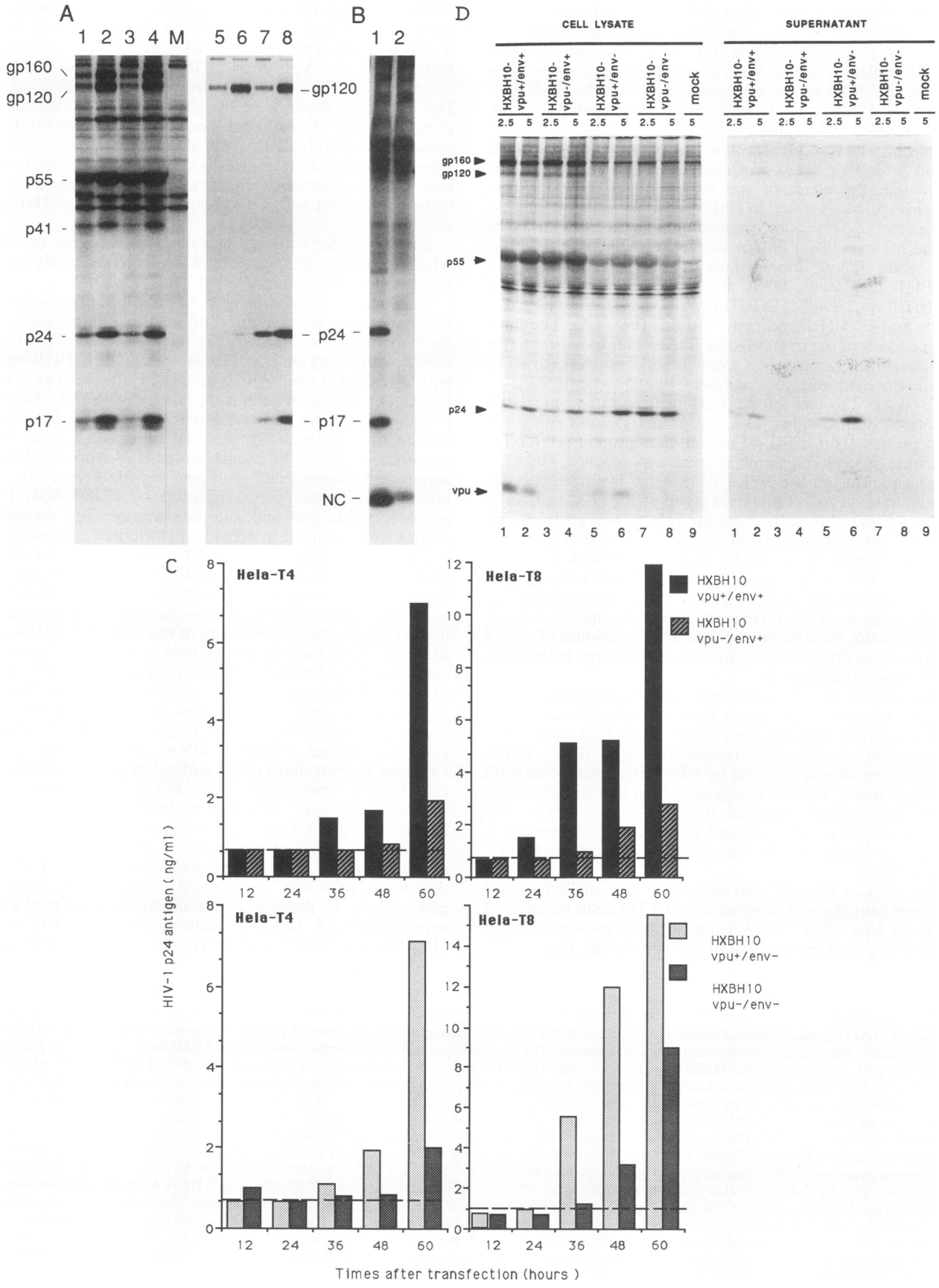
Results similar to those described above were obtained when release of virus-specified RNA-dependent polymerase was measured (data not shown).

The distribution of virus-specific proteins between the cell and the culture supernatant fluid was also determined. For these experiments, HeLa-T4 and HeLa-T8 cells were transfected with 10 μg of HXBH10-*vpu*$^+$/*env*$^+$, HXBH10-*vpu*$^-$/*env*$^+$, HXBH10-*vpu*$^+$/*env*$^-$, or HXBH10-*vpu*$^-$/*env*$^-$ viral DNA. The HeLa-T4 cells were labeled with 100 μCi/ml of [^{35}S]methionine for either 2.5 or 5 h beginning 60 h posttransfection. Both cell lysates and supernatant fluids were immunoprecipitated by using a mix of HIV-1-positive human serum and monospecific anti-*vpu* peptide serum (4). The immunoprecipitates were analyzed by SDS-PAGE (12.5 polyacrylamide gel) and autoradiography. The autoradiographs were scanned to provide quantitative comparisons.

The data in Fig. 2D show that the amount of capsid proteins detected in HeLa-T4 cell lysates is independent of the viral DNA strain used. Densitometric tracing of the gels shows no significant difference in the amount of p24 present in the cells transfected with any of the four viral DNAs. The data also show that the *vpu* protein is present in lysates of cells transfected with the HXBH10-*vpu*$^+$/*env*$^+$ (lanes 1 and 2) and HXBH10-*vpu*$^+$/*env*$^-$ (lanes 5 and 6) DNAs. The *vpu* protein is not detected in lysates of cells transfected with either the HXBH10-*vpu*$^-$/*env*$^+$ (lanes 3 and 4) or HXBH10-*vpu*$^-$/*env*$^-$ (lanes 7 and 8) DNA. The amounts of the envelope glycoproteins gp160 and gp120 present in HeLa-T4 cell lysates transfected with HXBH10-*vpu*$^+$/*env*$^+$ and HXBH10-*vpu*$^-$/*env*$^+$ DNAs are similar in lysates of cells labeled both for 2.5 and for 5 h (lanes 1 to 4).

There is a marked difference in the amount of capsid proteins present in the supernatant fluids of HeLa-T4 cells transfected with Vpu$^+$ as opposed to Vpu$^-$ DNA. Abundant p24 antigen is detected by radioimmunoprecipitation in supernatants of HeLa-T4 cells transfected with either HXBH10-*vpu*$^+$/*env*$^+$ or HXBH10-*vpu*$^+$/*env*$^-$ viral DNA. By

FIG. 1. (A) Oligonucleotide-directed mutagenesis of the initiation codon of the *env* gene of HIV-1. The genetic map of HIV-1 indicating the position of the *vpu* gene in the viral genome is shown. The construction of HXBH10-*vpu*$^+$/*env*$^+$ and HXBH10-*vpu*$^-$/*env*$^+$ was previously described (22). Segments of the HXBH10-*vpu*$^+$/*env*$^+$ and HXBH10-*vpu*$^-$/*env*$^+$ proviruses were cloned into the pTZ19U Genescribe vector by using the *Sal*I and *Kpn*I sites indicated. Alignment of the viral open reading frames within the fragment is shown. Oligonucleotide-directed mutagenesis was carried out to remove the *env* initiation codon, mutating ATG to ACG. These *env* mutants were cloned back into HXBH10-*vpu*$^+$/*env*$^+$ and HXBH10-*vpu*$^-$/*env*$^+$ by taking advantage of the same restriction sites to yield replication-defective proviruses HXBH10-*vpu*$^+$/*env*$^-$ and HXBH10-*vpu*$^-$/*env*$^-$, respectively. Arrowheads indicate intact initiation codons. The scale is in kilobases. (B) *vpu* expressor plasmids. Plasmid SVCMV*vpu*$^+$ was constructed by inserting a DNA fragment encoding *vpu* from the BH10 molecular clone 3′ to the cytomegalovirus (CMV) immediate-early gene promoter (−990 to +66) (10) and 5′ to the simian virus 40 (SV40) 16S RNA splice and poly(A) site (6). SVCMV*vpu*$^+$ also contains a simian virus 40 origin of replication. The 297-bp *Xba*I-*Sac*I DNA fragment (nucleotides 6085 to 6382) encoding *vpu* and the N-terminal portion (42 amino acids) of *env* was generated by polymerase chain reaction (17), using the HXBH10-*vpu*$^+$/*env*$^+$ molecular clone as the template. SVCMV*vpu*$^-$ was derived from SVCMV*vpu*$^+$ except that a point mutation in the initiation codon of *vpu* was introduced by oligonucleotide-directed mutagenesis, replacing ATG with ACG. The open boxes represent cytomegalovirus sequences, the lightly shaded boxes represent HIV-1 sequences, the darker boxes represent simian virus 40 sequences. The arrowhead represents functional ATG. X, *Xba*I; K, *Kpn*I; S, *Sac*I; B, *Bam*HI.


A
1 2 3 4 M 5 6 7 8
gp160
gp120
p55
p41
p24
p17
gp120
p24
p17
B
1 2
p24
p17
NC
D
CELL LYSATE
SUPERNATANT
HXBH10-vpu+/env+
HXBH10-vpu-/env+
HXBH10-vpu+/env-
HXBH10-vpu-/env-
mock
2.5 5
gp160
gp120
p55
p24
vpu
1 2 3 4 5 6 7 8 9
C
Hela-T4
Hela-T8
HXBH10 vpu+/env+
HXBH10 vpu-/env+
HXBH10 vpu+/env-
HXBH10 vpu-/env-
HIV-1 p24 antigen (ng/ml)
12 24 36 48 60
Times after transfection (hours)

contrast, only a very small amount of p24 is detected by radioimmunoprecipitation in supernatant fluids of HeLa-T4 cells transfected with either the HXBH10-*vpu*$^-$/*env*$^+$ or HXBH10-*vpu*$^-$/*env*$^-$ viral DNA (Fig. 2D; compare lanes 1 and 2 with lanes 3 and 4 and lanes 5 and 6 with lanes 7 and 8).

There is no reproducible difference in the amount of envelope glycoprotein gp120 released into the media from cells transfected with the Vpu$^+$/Env$^+$ and Vpu$^-$/Env$^+$ DNAs. The release of gp120 into the supernatant fluid by viruses derived from the IIIB isolate has previously been shown to be independent of the synthesis of capsid proteins (9, 12). No *vpu* protein can be detected in the supernatant fluids of HeLa-T4 cells transfected with HXBH10-*vpu*$^+$/*env*$^+$ (Fig. 2D, lanes 1 and 2) and HXBH10-*vpu*$^+$/*env*$^-$ (lanes 5 and 6) viral DNAs.

Distribution of virus-specific proteins between cells and the culture fluid of HeLa-T8 cells transfected with the set of four viral DNAs showed a pattern similar to the one observed for HeLa-T4 and HeLa cells (data not shown).

These results indicate that the effect of *vpu* on the export of virus capsid proteins from HeLa cells does not require expression of the envelope glycoproteins and is not affected by coexpression of the CD4 protein.

Effect of *vpu* on export of infectious virus. The infectivity of supernatant fluids of HeLa-T8 cells transfected with HXBH10-*vpu*$^+$/*env*$^+$ and HXBH10-*vpu*$^-$/*env*$^+$ viral DNAs for the human CD4$^+$ T-cell line MT4 (8) was determined. For this experiment, 200 μl of cellular supernatant fluids was harvested from HeLa-T8 cells transfected with 10 μg of viral DNA 60 h posttransfection. The supernatant fluids were used to infect 5×10^5 MT4 cells.

The infected cultures were examined for evidence of virus replication 3 to 6 days postinfection. Infection was monitored by determination of the percentage of cells in the culture that are positive for virus-specific antigens, as judged by immunofluorescence using anti-HIV-1 p24 monoclonal antibodies (2). The amount of virus-specified RNA-dependent DNA polymerase activity released into the supernatant fluid of the MT4 cultures was also measured (13), and the cultures were scored for syncytium formation.

The data in Table 1 show that virus in the supernatants of cultures transfected with the HXBH10-*vpu*$^+$/*env*$^+$ DNA initiates infection much more rapidly than does virus in the supernatant fluids of cells which receive the Vpu$^-$/Env$^+$ DNA. Previous experiments have shown that the *vpu* gene itself has little effect on the infectivity or rate of replication of the virus in T-cell lines, as judged by all three assays used (22). These experiments indicate that the level of infectious virus present in the culture supernatants of cells transfected with HXBH10-*vpu*$^+$/*env*$^+$ DNA is greater than the amount present in cells transfected with HXBH10-*vpu*$^-$/*env*$^+$ DNA.

TABLE 1. Effect of *vpu* on export of infectious particles[a]

Days after infection	Infectivity of supernatants from HeLa-T8 transfected with:					
	HXBH10-*vpu*$^+$/*env*$^+$			HXBH10-*vpu*$^-$/*env*$^+$		
	CPE	IF (%)	RT (10^3 cpm)	CPE	IF (%)	RT (10^3 cpm)
3	++	23	200.9	−	0	1.0
4	++++	>90	406.1	+	3	25.6
5	++++	>90	67.3	+++	37	254.4
6	++++	>90	ND[b]	++++	>90	144.2

[a] HeLa-T8 cells were transfected with 10 μg of HXBH10-*vpu*$^+$/*env*$^+$ or HXBH10-*vpu*$^-$/*env*$^+$. Forty-eight hours posttransfection, 200 μl of cell-free supernatant from both cultures was used to infect 5×10^5 MT4 cells. Effects of virus infection within each culture were then monitored at regular intervals. Cytopathic effects (CPE) were measured by daily count of syncytia. Spread of infection and virus production were determined by measuring p24-specific immunofluorescence (IF) and reverse transcriptase activity (RT), respectively. For the latter data, background values have been subtracted.

[b] ND, not determined.

Effect of *vpu* in a human CD4$^+$ T-cell line. The effect of *vpu* on capsid release was also examined in the human CD4$^+$ cell line MT4. For this experiment, 10^7 MT4 cells were transfected with 12 μg of either HXBH10-*vpu*$^+$/*env*$^-$ or HXBH10-*vpu*$^-$/*env*$^-$ DNA by the DEAE-dextran method (19). The amount of capsid proteins transiently released into the supernatant of each transfected culture was determined by measuring the virus-specified RNA-dependent DNA polymerase activity at intervals for 60 h posttransfection (Fig. 3A). By this measure, particle production was higher at all times posttransfection in the MT4 culture transfected with the Vpu$^+$/Env$^-$ DNA than in the MT4 culture transfected with the Vpu$^-$/Env$^-$ DNA. At 36 h posttransfection, the amount of enzyme activity released into the supernatant of MT4 cells transfected with the Vpu$^+$/Env$^-$ DNA was fourfold greater than the amount released from cells transfected with the Vpu$^-$/Env$^-$ DNA. These results indicate that *vpu* increases the release of virus capsid proteins in MT4 supernatant fluid independently of the *env* genotype.

The distribution of viral proteins between the cells and supernatant fluids was also examined. The transfected cells were labeled for 5 h with [^{35}S]methionine (100 μCi/ml) beginning 60 h posttransfection. The labeled cells and supernatant-containing virus were immunoprecipitated with an HIV-1-positive human serum. The immunoprecipitates were analyzed on SDS–12.5% polyacrylamide gels.

The data in Fig. 3B show that the level of p24 immunoprecipitated is threefold higher in the supernatant of MT4

FIG. 2. Role of envelope glycoprotein and CD4 in *vpu*-mediated capsid protein export. (A) Immunoprecipitation of virus capsid and envelope proteins from HeLa cell lysates (lanes 1 to 4) and supernatant fluids (lanes 5 to 8). The cells were transfected with 10 μg (odd-numbered lanes) or 40 μg (even-numbered lanes) of HXBc2 (lanes 1, 2, 5, and 6) or HXBH10-*vpu*$^+$/*env*$^+$ (lanes 3, 4, 7, and 8). Lane M, mock-transfected cells. (B) Sedimentation of viral particles from culture supernatants. HeLa cells were transfected with 40 μg of HBXH10-*vpu*$^+$/*env*$^+$ (lane 1) or HXBc2 (lane 2), and particulate material released into the supernatants was pelleted through a 20% sucrose cushion at 27,000 rpm for 2.5 h at 4°C in a Beckman SW41 Ti rotor. The pellets were disrupted in Laemmli sample buffer and directly analyzed by SDS-PAGE. NC, nucleocapsid protein. (C) Virus export. HeLa-T4 and HeLa-T8 were transfected with 10 μg of HXBH10-*vpu*$^+$/*env*$^+$ or HXBH10-*vpu*$^-$/*env*$^+$ provirus (top). In parallel, HeLa-T4 and HeLa-T8 were transfected with 10 μg of HXBH10-*vpu*$^+$/*env*$^-$ or HXBH10-*vpu*$^-$/*env*$^-$ (bottom). At the indicated times, virus export was determined by measurement of virion-associated p24 in the cell-free supernatants. Data represent the results from one of three identical experiments. (D) Immunoprecipitation. HeLa-T4 cells transfected with the indicated proviruses were pulse-labeled for 2.5 (odd-numbered lanes) and 5 (even-numbered lanes) h with [^{35}S]methionine 60 h posttransfection. Both cells and clarified supernatants were lysed and immunoprecipitated with a mix of HIV-1-positive human serum and rabbit anti-*vpu* peptide serum (1:1 ratio) and then analyzed on an SDS–12.5% polyacrylamide gel. The mock-transfected HeLa-T4 cell control is shown in lane 9 of each panel. The positions of HIV-1 viral proteins are indicated.

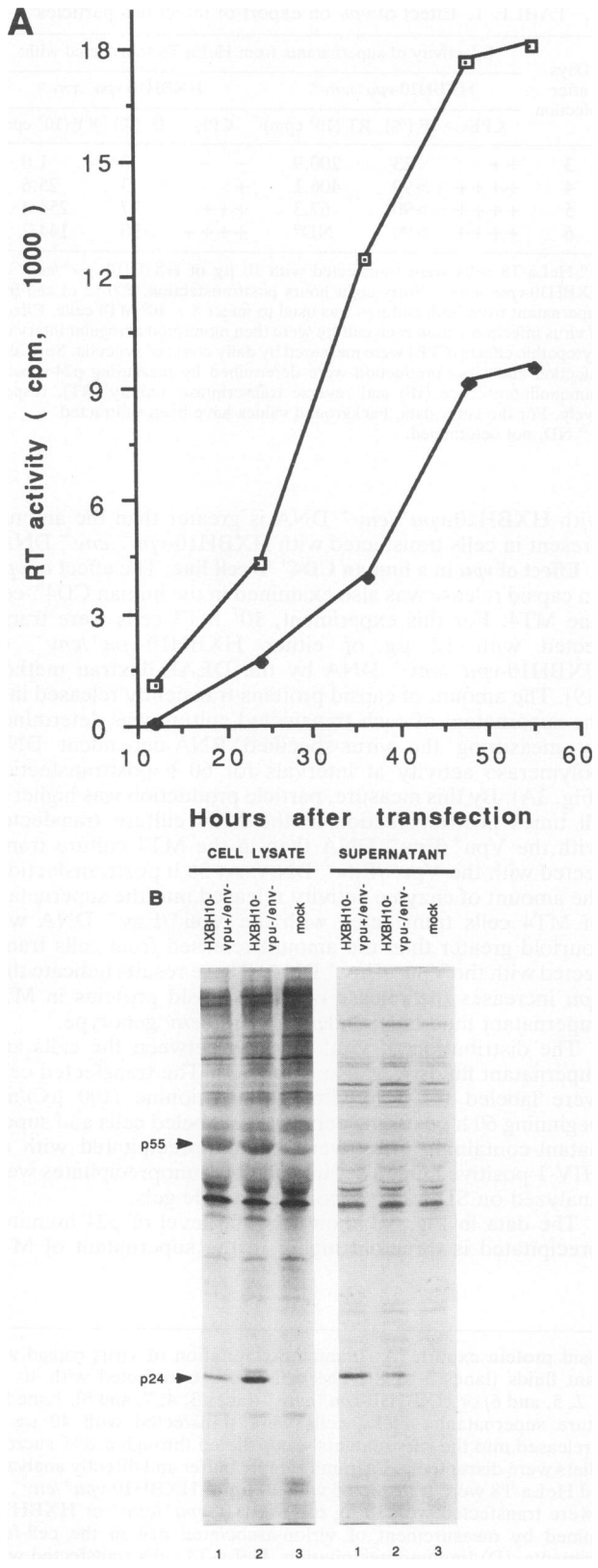


FIG. 3. Role of envelope glycoproteins in *vpu*-mediated capsid protein export in CD4$^+$ T-cell line MT4. (A) MT4 cells (10^7) were transfected with 12 μg of HXBH10-*vpu*$^+$/*env*$^-$ (□) or HXBH10-*vpu*$^-$/*env*$^-$ (●). At each posttransfection time indicated, cell-free supernatants were collected and virion-associated reverse transcriptase (RT) activity was measured. (B) Cells from the MT4 cell cultures transfected with HXBH10-*vpu*$^+$/*env*$^-$ (lane 1) or HXBH10-*vpu*$^-$/*env*$^-$ (lane 2) described above were labeled for 5 h with [^{35}S]methionine 60 h posttransfection. Both cells (left panel) and the corresponding cell-free supernatants (right panel) were lysed, immunoprecipitated with an HIV-1-positive human serum, and analyzed on an SDS–12.5% polyacrylamide gel. The mock-transfected MT4 cell control is shown in lane 3 of each panel. The position of HIV-1 viral protein is indicated.

TABLE 2. Effect of *vpu* on export of viral particles in *trans*[a]

vpu expressor	RT activity (10^3 cpm)	
	HXBH10-*vpu*$^-$/*env*$^+$	HXBH10-*vpu*$^-$/*env*$^-$
SVCMV*vpu*$^+$	14.7	60.1
SVCMV*vpu*$^-$	4.8	14.3

[a] SVCMV*vpu*$^+$ or SVCMV*vpu*$^-$ expression plasmid (7.5 μg) was cotransfected with 10 μg of HXBH10-*vpu*$^+$/*env*$^-$ or 10 μg of HXBH10-*vpu*$^-$/*env*$^-$ (molar ratio of 2:1) in HeLa-T8 cells; 48 h posttransfection, viral particle export was determined by measuring reverse transcriptase (RT) activity in the cell-free supernatant. Background values have been subtracted.

cells transfected with the Vpu$^+$/Env$^-$ DNA than in the supernatant of MT4 cells transfected with the Vpu$^-$/Env$^-$ DNA (compare lanes 1 and 2). As described in previous studies (11, 20, 22), the increase in p24 released in the supernatant is accompanied by a reduction in the amount of cell-associated p24 (Fig. 3B; compare lanes 1 and 2). As expected, the envelope glycoproteins are not detected in the cell lysate or in the cell-free supernatant of MT4 cells transfected with the Vpu$^+$/Env$^-$ and Vpu$^-$/Env$^-$ constructs.

***vpu* acts in *trans*.** To determine whether the *vpu* protein acts in *trans* to increase export of virion proteins, the ability of a plasmid that expresses *vpu* to increase the level of virion protein present in the supernatant of cells transfected with the Vpu$^-$/Env$^+$ and Vpu$^-$/Env$^-$ DNAs was examined. For this experiment, the open reading frame expressing the *vpu* protein was placed 3′ to a cytomegalovirus promoter on a separate plasmid. As a control, the same *vpu* open reading frame in which the initiation codon ATG is altered to ACG was used (Fig. 1B). The *vpu* expression plasmids and the HIV-1 proviruses were introduced by cotransfection into HeLa-T8 cells.

The data in Table 2 show that the *vpu* protein increases the amount of virus exported from cells, as measured by release of the viral DNA polymerase activity into the cell supernatant, three- to fourfold. The Vpu$^-$ expressor plasmid does not alter the amount of viral DNA polymerase present in the supernatant of the expressor cells. This *trans* effect of *vpu* can be observed with both the HXBH10-*vpu*$^-$/*env*$^+$ and HXBH10-*vpu*$^-$/*env*$^-$ constructs.

Determination of the amount of viral protein released in the supernatant of the transfected cells by immunoprecipitation confirms the results obtained by measurement of reverse transcriptase activity (Fig. 4). Cotransfection of the Vpu$^+$ expressor plasmid results in a significant increase of viral protein export in the cell supernatant (lane 1) compared with the level seen after cotransfection with the control plasmid (lane 2). In contrast, the expression of *vpu* does not affect significantly the amount of capsid and envelope glycoproteins present in the cell lysates (lanes 1 and 2).

The experiments reported here demonstrate that *vpu*-

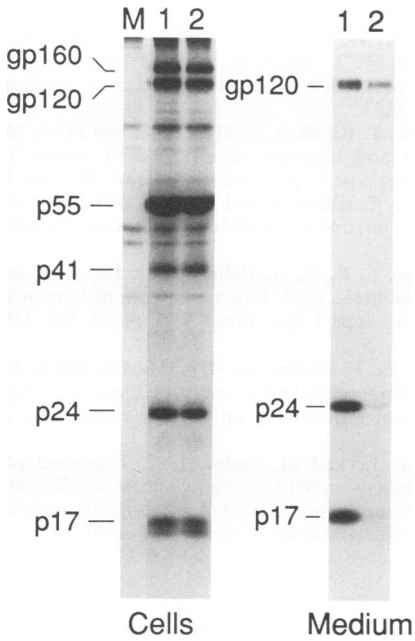


FIG. 4. *trans* effect of *vpu*. Twenty micrograms of SVCMV*vpu*$^+$ (lane 1) or SVCMV*vpu*$^-$ (lane 2) was cotransfected with 30 μg of HXBH10-*vpu*$^-$/*env*$^+$ in HeLa cells. Forty-eight hours posttransfection, cells were metabolically labeled for 12 h with [^{35}S]cysteine. Both cells and the corresponding clarified supernatants were then lysed, immunoprecipitated with an HIV-1-positive human serum, and analyzed on an SDS–11.5% polyacrylamide gel. The mock-transfected HeLa cells are shown in lane M. The positions of HIV-1 viral proteins are indicated.

mediated export of the capsid proteins is independent of the expression of the envelope glycoproteins. The amount of capsid proteins released into the supernatant fluids of cells transfected with viral DNA is dramatically increased in the presence of a functional *vpu* regardless of coexpression of the envelope glycoprotein from the same genome. The dramatic effect of *vpu* on capsid export, independent of *env*, was determined by measurement of the amount of p24 in the culture supernatant by use of radioimmune competition and precipitation assays. The increase in export of virus particles was determined by measurement of the amount of assembled as well as infectious virus particles in culture supernatant fluids.

The experiments reported here also show that coexpression of CD4 in the target cell has no measurable effect on *vpu*-directed increase in export of capsid proteins, at least in this experimental context. Expression of the *vpu* protein significantly increases the level of capsid proteins present in the supernatant of HeLa cells which constitutively express either the CD4 or CD8 protein. An effect of *vpu* on the biosynthesis and export of envelope glycoprotein in cells which express CD4 is not found in these experiments. The levels of cell-associated gp160 and gp120 are similar in HeLa-T4 cells transfected with Vpu$^+$/Env$^+$ and Vpu$^-$/Env$^+$ viral DNAs.

There is a significant difference between the effect on capsid proteins of *vpu* in HeLa cells compared with its effect in CD4$^+$ T-cell lines. In HeLa cells, the amount of capsid protein in the cell lysate is not measurably decreased by *vpu*. In T-cell lines, the increase in export of capsid protein is accompanied by a decrease in the amount of capsid proteins detected in the cell lysates. This difference is probably more apparent than real. In HeLa cells, the ratio of cell-associated to supernatant capsid protein in the presence of *vpu* is only about 20:1. In the absence of *vpu*, almost no capsid protein is released from the cell. In contrast, in T lymphocytes, the fraction of capsid proteins released from the cell increases from about 20% to almost 80% in the presence of *vpu* (22).

The experiments also demonstrate that *vpu* acts in *trans* to facilitate export of capsid proteins made from viral DNA defective in *vpu*. The amount of capsid protein released into the cell supernatant increases by at least fivefold in the presence of *vpu*. This observation should permit characterization of sequences in the *vpu* protein as well as sequences within the capsid proteins and their precursors that are required for *vpu*-facilitated export.

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