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Identification and Characterization of Human Immunodeficiency Virus Type 1 *gag-pol* Fusion Protein in Transfected Mammalian Cells

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Three human immunodeficiency virus type 1 (HIV-1) mutants were constructed with mutations in their protease genes: AH2-pSVL, with an in-phase deletion; BH27-pSVL, with an out-of-phase deletion creating a stop codon immediately after the deletion site; and CA-pSVL, with a point mutation creating an Asp-to-Ala substitution at the putative protease active site. The wild-type, HXB2-pSVL, and the mutated viral genomes were used to transfect COS-M6 cells and to produce virions. Immunoblotting assays with a monoclonal antibody (MAb) specific for p24 showed that all three mutants contained a *gag* precursor, Pr56^{gag}, with AH2 and CA expressing an extra band of about 160 kDa. Similar assays with a MAb specific for HIV-1 reverse transcriptase (RT) also revealed a 160-kDa protein from AH2 and CA virions and two mature p66 and p51 RT subunits from HXB2 virions. In addition, HXB2, AH2, and CA but not BH27 virions exhibited RT activity. The same protein in the 160-kDa band seemed to possess both p24 and RT components, since the MAb against p24 was able to immunoadsorb RT antigen and enzymatic activity. These results indicate that the HIV-1 *gag-pol* fusion protein produced in mammalian cells expressed significant RT activity.

Among the major structural and functional genes in the genome of human immunodeficiency virus type 1 (HIV-1), *gag* encodes a polyprotein, Pr56^{gag}, which is processed by a virus-specific protease to form several mature core proteins [p24, p17, and p15 (p7/p6)] (15, 21, 24, 25, 28, 30). The *pol* gene encodes at least three important products, the protease, reverse transcriptase, and integrase (or endonuclease), which are generated from a presumptive precursor protein, Pr160$^{gag\text{-}pol}$, containing most of the *gag* polyprotein and the entire *pol* product (3, 21). It has been proposed that this fusion protein is synthesized by the −1 ribosomal frameshifting mechanism during translation (9, 10). The *gag-pol* fusion protein, like Pr56^{gag}, is also processed by the HIV-specific protease to produce the mature proteins. Previous studies by us (23) and by other investigators (4, 11) have shown that the processing of HIV *gag* and *pol* precursors by the virus-specific protease is required for the assembly of mature viral particles and for viral infectivity.

Among the *pol* products, reverse transcriptase (RT) is an essential component for viral replication. The mature RT contains two polypeptides, p66 and p51, which share a common N terminus, presumably because p66 is the precursor of p51 and p15 (14, 29). The functional RT molecules are believed to be either homodimers consisting of only p66 or heterodimers consisting of subunits of p66 and p51 (1, 17, 29). In HIV virions, p66 and p51 exist in about equal amounts (17). An in situ examination of the RT activity of renatured proteins in electrophoretic gels has shown that despite the dimer structure, p66 from *Escherichia coli* is the subunit with the majority of RT activity and p51 has little or no detectable RT activity (7, 16, 17, 26, 27). In similar enzyme activity gel analyses examining the extracts of HIV-infected H9 cells, Starnes et al. and Hansen et al. (7, 26) reported that p66 was the only subunit and that there was no other band exhibiting RT activity, whereas Lori et al. (16) reported that in addition to p66 and p51 bands, a band of 165 kDa was found which exhibited weak RT activity and was presumed to be the *gag-pol* precursor.

In the presence of viral protease, the *gag-pol* fusion protein or the *pol* precursor protein exists in a relatively unstable, transient stage (5, 6, 8, 19, 20). Most studies on this precursor protein have been carried out by using bacterial expression systems with defective viral protease. Among them, Le Grice et al. (12, 13) found that the precursor protein from *E. coli* had no RT activity, and they suggested that the intact *pol* product had to be processed to yield p66 and p51 to acquire RT enzymatic activity. In our earlier report (23), we suggested that the protease-defective mutant AH2 contained the putative *gag-pol* precursor and that this fusion protein had RT activity as high as 20 to 50% of that in wild type. Since the *gag-pol* fusion protein produced by mammalian cells is the precursor of several important viral proteins, we have further characterized its antigenic and enzymatic properties in this study.

Protease-defective HIV-1 mutants. Two types of protease-defective HIV mutants were constructed, as described in our previous report (23) and in the legend to Fig. 1. AH2 and BH27 were deletion mutants derived from the genome of the wild-type virus HXB2. AH2, which lost 39 nucleotides in the protease coding region and acquired 12 nucleotides from a *Bam*HI linker, maintained the same reading frame as that of the wild type. In BH27, which lost 71 nucleotides in the protease coding region and also acquired the 12-nucleotide linker, a new translation termination codon was created two amino acids downstream from the mutation site (Fig. 2). The second type of mutant contained a point mutation in a gene

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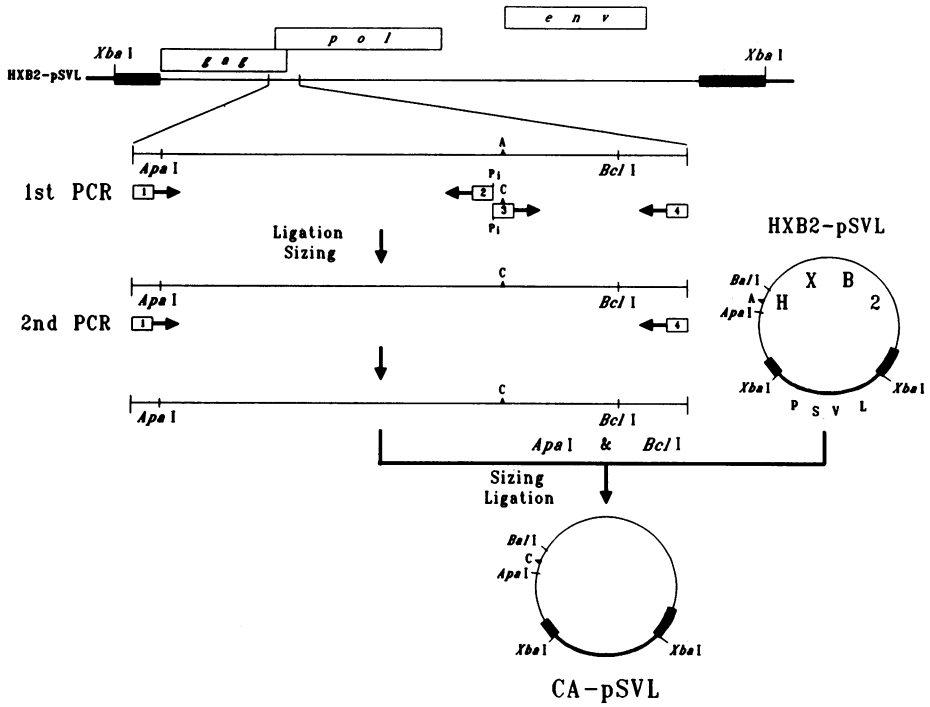

FIG. 1. Construction of mutant CA-pSVL. The HIV proviral genome, HXB2 (thin line), with open reading frames *gag*, *pol*, and *env* (open boxes) and flanking cellular sequences (thick line), was ligated to vector pSVL at the *Xba*I site. The four primers used in polymerase chain reaction (PCR) and their orientations are shown as boxes with arrows. The construction of plasmids, including of wild-type HXB2-pSVL, and the two deletion mutants AH2-pSVL and BH27-pSVL, was described in detail in our previous paper (23). The site-directed mutant, CA-pSVL, was constructed by using the PCR method. The four oligonucleotide primers were made in the Department of Cell Biology, Baylor College of Medicine. The oligonucleotide sequences (from 5′ to 3′) and their nucleotide numbers in the HXB2 genome are (i) AAGGGCACACAGCCAGAAAT (nucleotides [nt] 1981 to 2000), (ii) TAGGGGGGCAACTAAAGGAA (nt 2314 to 2295; complementary), (iii) GCTCTATTAGCTACAGGAGCA (nt 2315 to 2335; at nt 2325, A is replaced by C), and (iv) ACAGTATTAGTAGGACCTAC (nt 2490 to 2471; complementary). Note that at the indicated nucleotide A in HXB2 is substituted by C in primer 3. Primers 2 and 3 were phosphorylated with T4 polynucleotide kinase (Pharmacia, Piscataway, N.J.) (18) before the PCR. In the PCR, the templates were denatured at 94°C for 1 min, primers were annealed at 55°C for 2 min, and enzyme reaction was permitted at 72°C for 2 min, for a total of 30 cycles in a Perkin-Elmer/Cetus DNA thermal cycler. After ligation and fractionation of the ligated fragments by gel electrophoresis, the band with the expected size (about 510 nt) was isolated (18). The isolated DNA was used as the template, and the second round of PCR was performed to eliminate incorrect orientation in the ligated fragments. The amplified fragment as well as plasmid HXB2-pSVL was then cleaved with *Apa*I and *Bcl*I. The pertinent fragments were then isolated and ligated to plasmid HXB2-pSVL, which was isolated from *E. coli* GM161, to prevent methylation of the DNA sequence at the *Bcl*I site. The DNA sequence between the *Apa*I and *Bcl*I sites was confirmed by dideoxy sequencing with denatured double-stranded DNA (2). The constructed plasmid was named CA-pSVL.

segment which corresponded to the putative active enzymatic site of the protease (22). In the mutant CA, a single nucleotide substitution, A to C (substituting amino acid residue Asp for Ala [Fig. 2]), was introduced. The same point mutation and amino acid residue substitution were employed by Le Grice et al. (12). All of the wild-type and mutated viral genomes were cloned into the pSVL vector, which has a simian virus 40 late promoter in front of inserted viral genomes. Constructed plasmids containing viral genomes were transiently expressed in a simian virus 40-transformed monkey kidney cell line, COS-M6. The culture media from transfected cells were collected, and HIV-1 virions were precipitated by polyethylene glycol 6000 and used as the source of HIV variants (23).

	23		70
HXB2	~LLDTGADDTVLEEMSLPGRWKPKMIGGIGGFIKVRQYDQILIEICGHK~		
CA	~LLATGADDTVLEEMSLPGRWKPKMIGGIGGFIKVRQYDQILIEICGHK~		
AH2	~LLDTGADDTVLEEMSLPGRWKPKMIGGIG	*ADPR*	EICGHK~
BH27	~LLDTGADDTVLEEMSLPGRWK	*RGSA*	WT*

FIG. 2. Amino acid sequences at the mutation sites of the constructed HIV-1 mutants. The amino acid sequences are listed from amino acid residues 23 to 70 of the mature HIV protease (12, 15). Mutant CA has a single-amino-acid-residue D-to-A substitution (underlined). AH2 has an in-frame deletion; BH27 has an out-of-frame deletion, which creates a stop codon (*) immediately after the mutation site. The extra amino acid residues shown in italicized letters are created by the *Bam*HI linkers used in the deletion mutations.

Structural analyses of *gag*, *pol*, and *gag-pol* products. Since the *gag-pol* fusion protein was proposed to contain both *gag* and *pol* regions, we used two monoclonal antibodies (MAbs), one specific for p24 (C246; Tanox Biosystems Inc., Houston, Tex.) and one specific for RT (C14120; developed by Karin Moelling of Max-Planck-Institut für Molekulare Genetik), to detect and analyze the fusion protein. By using MAbs specific for p24 and RT as probes in immunoblotting assays, the wild-type viruses were shown to contain processed *gag* p24 protein and *pol* RT of 55 and 66 kDa (Fig. 3).

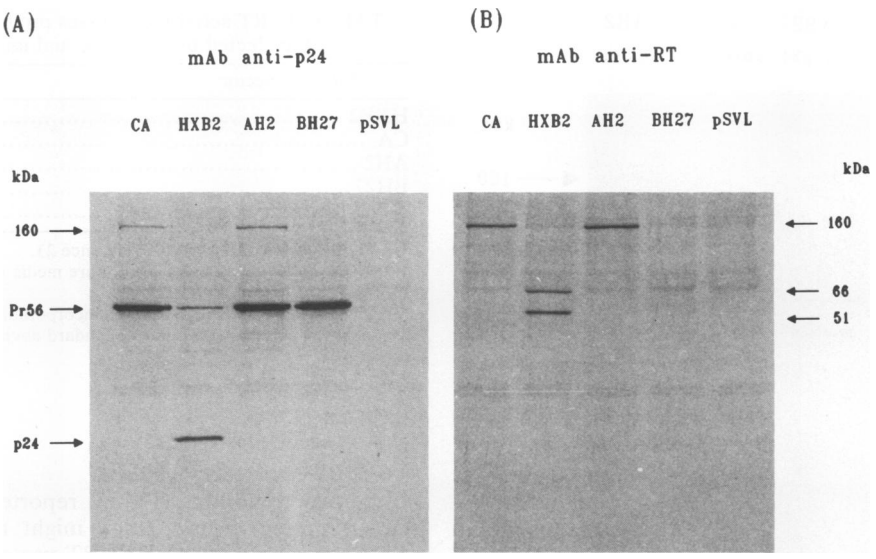

FIG. 3. Immunoblotting analyses of *gag*, *pol*, and *gag-pol* products by using MAbs against p24 and RT. (A) Reactivity with MAb against HIV p24. (B) Reactivity with MAb against RT. The culture medium of COS-M6 cells transfected by HXB2-pSVL, CA-pSVL, AH2-pSVL, BH27-pSVL, or vector pSVL alone was treated with polyethylene glycol to precipitate HIV virions. The precipitates were treated with lysis buffer containing Triton X-100 and were analyzed as described previously (23). HIV p24-specific MAb (C246) was used at 1 mg/ml, and MAb against RT (C14120) was used at a 1:2,000 dilution of mouse ascitic fluid. The second antibody, goat anti-mouse immunoglobulin antibody conjugated with alkaline phosphatase (Promega, Madison, Wis.), was used at a 1:7,000 dilution. The molecular sizes of the bands are indicated at the sides of panels.

The protease-defective mutants AH2, CA, and BH27 contained the precursor of *gag* proteins, $Pr56^{gag}$. In addition, both anti-p24 and anti-RT revealed bands of about 160 kDa in AH2 and CA. However, in BH27, only an extra band of 57 kDa was shown to be reactive with the MAb to p24 (Fig. 3); the *pol* products of 55 and 66 kDa and the 160-kDa bands were no longer detectable.

Although the MAbs specific for p24 and for RT were both reactive with proteins of the same molecular weight, additional evidence was required to indicate that these two antibodies reacted with the same molecule, which was presumably the *gag-pol* fusion product. In subtractive immunoprecipitation experiments, viral particles were lysed and the MAb C246 was added to bind to any viral product with the p24 epitope. Solid-phase adsorbents combining fixed *Staphylococcus aureus* beads and the same beads covalently conjugated with goat anti-mouse antibody were used to remove the immune complexes and to free antibodies. The adsorbed viral lysates were then subjected to Western immunoblot and to detection with the MAb against RT. The results showed that when C246 removed molecules of 160 kDa and other molecules with the p24 epitope, it also eliminated the 160-kDa molecule with reactivity to anti-RT, indicating clearly that the 160-kDa molecule contained both *gag* and *pol* segments (Fig. 4). The bands above 160 kDa seen in AH2 lysates (Fig. 4) probably resulted from nonspecific binding of the high concentration due to the second antibody used in immunoblotting. The fact that the *gag-pol* fusion protein can be produced in the mammalian cell expression system used in our studies provides indirect evidence that the ribosomal frameshifting mechanism also occurs in mammalian cells.

Reverse transcriptase activity of the *gag-pol* product. The viral particles in the culture supernatants of COS-M6 cells transfected by wild-type (HXB2) and mutant (CA, AH2, and BH27) genomes were precipitated by polyethylene glycol, and the RT activities in the virions were assayed. Table 1 shows that CA and AH2 mutant virions contain significant levels of RT activity, while BH27 virions had no activity. Subtractive immunoadsorption experiments using the anti-p24 MAb, C246, were performed to determine whether the *gag-pol* precursor fusion protein in AH2 and CA mutant virions possessed RT activity. After the removal of molecules carrying the p24 epitope with MAb C246, the RT activity of the adsorbed lysates was measured. The RT activity of both the AH2 and the CA mutant was decreased by about 40% by adsorptive treatment with the anti-p24 MAb, while the RT activity from wild-type HIV showed insignificant reduction (Fig. 5). These results indicate that the *gag-pol* fusion protein does have RT activity. Since the anti-p24 MAb does not react with mature processed RT, the results also rule out the possibility that the observed RT activity in the protease-defective mutants is due to mature RT that is generated by nonspecific cellular protease and packaged into mutant virions. It should be noted that the RT activity absorbed by the *S. aureus* beads could not be measured. In control experiments, we found that the *S. aureus* particles suspended in the RT assay mixture inhibited RT enzymatic activity by some unexplained mechanism; when these particles were spun down and removed, the RT activity was again measurable.

In summary, the present study has provided evidence indicating that protease-defective viruses express a relatively stable protein of 160 kDa which is presumptively a component of the *gag-pol* precursor. The 160-kDa protein contains both *gag* and *pol* polypeptides, and the precursor itself exhibits significant RT activity. Although immunoadsorption experiments demonstrated that both p24 and RT

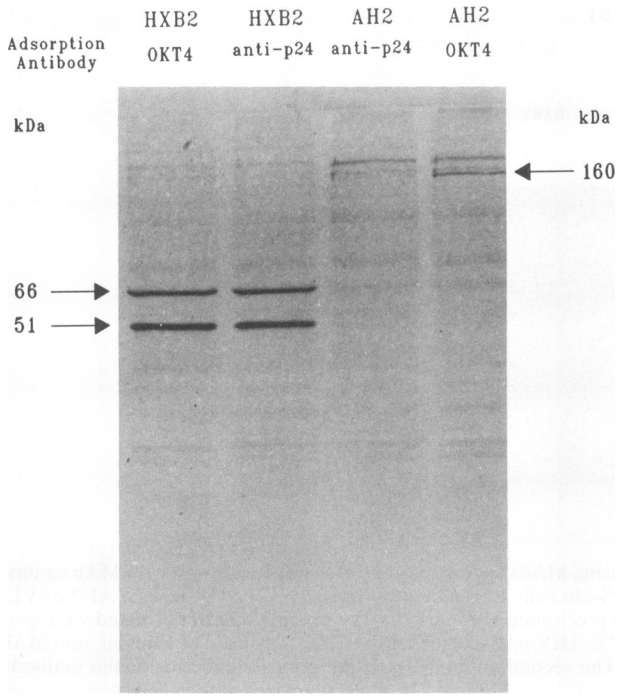

FIG. 4. Subtractive immunoadsorption assay for defining p24 and RT epitopes in *gag-pol*. Ten microliters of viral lysates was incubated with 5 μl of MAb C246 (mouse immunoglobulin G2b against p24) or of protein A-purified MAb OKT4 (mouse immunoglobulin G2b, adjusted to 1 mg/ml) (used as negative control) and 45 μl of 50 mM Tris-HCl (pH 8.0)–2 mM dithiothreitol for 2 h on ice with shaking. The reaction mixture was transferred to *S. aureus* beads (10 μl of 10% suspension and prewashed with phosphate-buffered saline [Pansorbin; Calbiochem, San Diego, Calif.]), resuspended, and incubated on ice for 30 min. After pelleting, the supernatant was transferred to and incubated with prewashed Tachisorb beads (5 μl of goat anti-mouse immunoglobulin antibody conjugated on fixed *S. aureus* beads [Calbiochem]) on ice for another 30 min. The Tachisorb beads were removed by microcentrifugation at 12,000 × *g*, and one-half of the supernatant was used for polyacrylamide gel electrophoresis. After electroblotting onto nitrocellulose membranes and blocking the nonspecific sites with BLOTTO solution (11), the antigens on the membranes were reacted with the MAb C14120 (against RT) and the binding was detected with the second antibody at a 1:4,000 dilution. Molecular size markers are indicated on the sides of the figure.

antigenic epitopes could be removed by a MAb specific for one of the antigens (anti-p24), the MAb specific for RT (C14120) could not be used to remove the *gag-pol* polypeptide from viral lysates because it could bind only to denatured and not to native RT (unpublished observation). The *gag-pol* fusion protein, like the mature RT, has enzymatic activity but does not react with C14120. These analyses suggest that the immunoadsorption method used in our experiments detected the native form of RT components, whereas the in situ enzyme activity gel analyses employed by Lori et al. (16) and Starnes et al. (26) might neither qualitatively characterize the antigenicity of components nor quantitatively detect the RT activity in the *gag-pol* fusion protein produced in HIV-infected T cells.

In studies of the HIV protease by mutational analysis, Le Grice and coworkers (12, 13) reported that intact *pol* precursor protein from *E. coli* might not have RT activity and suggested that the HIV RT protein must be processed from the polyprotein to acquire any enzymatic activity. One explanation for this discrepancy is that the precursor protein produced in the *E. coli* expression system did not have the proper posttranslational modifications to obtain functional structure, as it would have in mammalian cells, and therefore did not have RT activity. It is unlikely that the *gag* component of the *gag-pol* fusion protein would rescue the *pol* component to regain RT activity. Another consideration is that mammalian host cells may contain cellular proteases which nonspecifically process precursor proteins and produce small amounts of mature RT. Our results, however, suggest that the cellular proteases in COS-M6 do not process the *gag-pol* precursor to produce mature RT, because the RT activity in the protease-defective virions could be removed by the MAb against p24, which reacted with the *gag-pol* precursor protein but not with mature RT.

TABLE 1. RT activity of viruses produced on COS-M6 cells transfected by wild-type and mutant genomes[a]

Virus[b] or vector	RT activity[c]
HXB2	33,111.43 ± 610.14
CA	17,190.56 ± 337.52
AH2	17,382.33 ± 625.40
BH27	233.00 ± 53.22
pSVL (negative control)	239.30 ± 57.73

[a] The assay was described in reference 23.
[b] Viruses were harvested from culture media of transfected COS-M6 cells by polyethylene glycol precipitation.
[c] RT activity was expressed as the incorporation of [^{3}H]TTP. The numbers are the mean counts per minute ± standard deviation of triplicate determinations.

Our previous study (23) showed that COS cells transfected with protease gene-defective HIV-1 genomes can still produce HIV-1 virions and that even though these virions contain some RT activity, they are no longer productively infectious. It remains to be elucidated whether the protease-defective mutants actually get into target cells and whether some reverse transcription occurs and, if so, how many other initial events go on in the infected cells before the viral replication cycle ceases to proceed. If the *gag-pol* fusion protein still has some RT activity, the suppressive effects of protease gene defects or of protease inhibitors on HIV-1 replication and viral infectivity may be due to the fact that some other proteolytic processes also depend entirely on the HIV-1-specific protease. Further studies on the processing of core proteins, on the synthesis of viral cDNA, and on the assembly of virions in cells, which employ protease-defective mutants, should generate additional insights on this important part of the HIV-1 replication cycle.

Nucleotide sequence accession number. The GenBank and EMBL accession number for the HXB2 genome is K03455.

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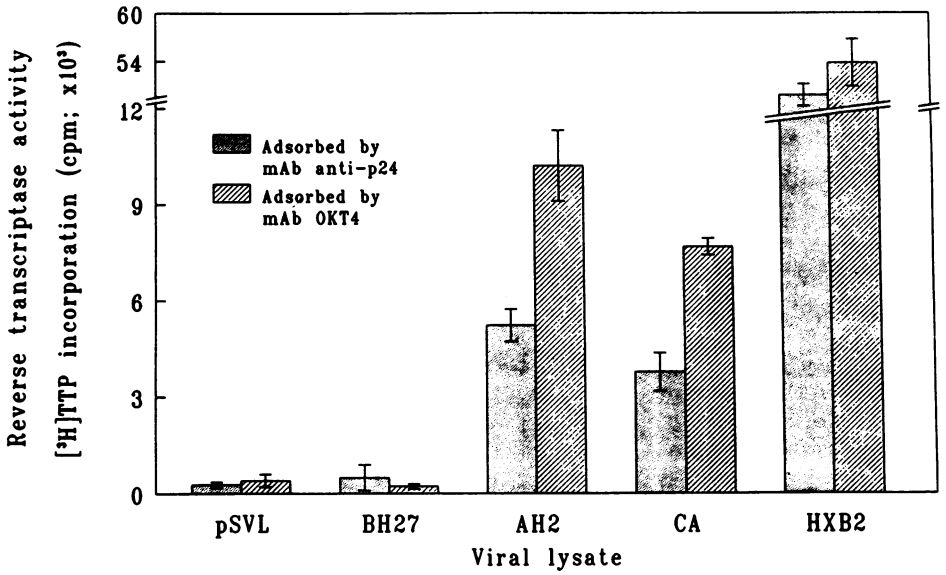

FIG. 5. Subtractive immunoadsorption analyses for identifying the RT activity of the *gag-pol* fusion product. Viral lysates were prepared and reacted with MAb against p24, and the antigen-antibody complexes were removed from the reaction mixture as described in the legend to Fig. 4, except that 5 μl of the viral lysates was incubated with 0.2 μl of MAb C246 or OKT4 in 16 μl of RT assay buffer. Twenty-one microliters of final supernatant was transferred to 4 μl of the template-primer and [³H]TTP mixture. The RT assay was then performed (23). Each value shown is the mean ± standard deviation of three determinations.

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