The Major Homology Region of the HIV-1 Gag Precursor Influences Membrane Affinity†

D. Ebbets-Reed,‡ S. Scarlata,§ and C. A. Carter*,‡

Department of Molecular Genetics and Microbiology and Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11794

Received March 15, 1996; Revised Manuscript Received July 2, 1996⊗

ABSTRACT: Assembly of retroviruses, including HIV-1, involves movement of newly synthesized viral proteins and RNA to the plasma membranes of host cells. The major homology region (MHR, aa 285−304), a highly conserved sequence in the capsid domain of the HIV-1 Gag precursor polyprotein, plays a critical, but unknown, role in infectious particle assembly. Mutations of invariant residues in the sequence have pleiotropic effects: Mutation of Gln287 blocks viral assembly while mutation of Arg299 permits assembly, but blocks formation of infectious particles. In this report, we demonstrate that Gag proteins lacking the entire MHR accumulated in the cytoplasm of transfected COS-1 cells, as did the wild-type protein, but were processed in a defective manner at the cellular membrane resulting in impaired particle assembly. To further examine the role of the MHR in membrane association, membrane binding of unmyristylated recombinant Gag proteins with alterations in the MHR was investigated *in vitro.* The wild-type Gag precursor bound to acidic phospholipid vesicles highly efficiently, as determined by fluorescence spectroscopy or velocity sedimentation. In contrast, deletion of the entire MHR reduced membrane affinity an average of ∼3-fold or greater. Mutation of the invariant Gln287 residue disrupted membrane affinity ∼6-fold relative to the wild-type, which was similar to the level of inhibition obtained by deletion of a membrane-binding signal previously identified in the matrix domain of the Gag precursor. Mutation of the invariant Arg299 residue reduced the affinity to a lesser extent. The results indicate that correct membrane binding is determined not only by signals in the MA domain of the precursor but also by sequences in the CA domain of Gag. We speculate that defects in the highly conserved MHR affect a Gag conformation that is required for productive interactions at the membrane assembly site.

The human immunodeficiency virus (HIV) is a member of a large group of animal viruses which are causative agents of tumors, persistent syndromes, and lytic infections (Dickson *et al.*, 1985). Although they are morphologically, pathologically, and genetically distinct, all members have in common three genes that encode the structural components of the infecting particle: *gag*, *pol*, and *env*. The *gag* gene encodes a precursor, Gag, whose matrix (MA), capsid (CA), and nucleocapsid (NC) domains give rise to these portions of the assembled particle. One of the most highly conserved regions in all retroviral Gag proteins is a stretch of ∼20 amino acids within the CA domain (Wills & Craven, 1991). This sequence is designated as the major homology region (MHR). Genetic analyses have demonstrated that mutations in the MHR, and particularly in the invariant residues of this sequence, alter either the assembly of the virus on the plasma membranes of host cells or postassembly events (Craven *et al.*, 1995; Mammano *et al.*, 1994; Strambio de Castillia & Hunter, 1992). The MHR is predicted to form an amphipathic helix (Momany *et al.*, 1996; see Figure 2 below). Moreover, it has recently been demonstrated that a peptide analog of the region adopts an α-helical structure in the presence of 50% trifluoroethanol, although not in a pure aqueous solution (Clish *et al.*, 1996). Such a structure, if present in the intact protein, could conceivably contribute to membrane binding or stabilize a protein conformation required for assembly on the membrane surface. Defective subunit assembly due to altered membrane association could account for observed impairments in viral particle formation and release since most mutations in the part of the MHR sequence that would constitute the helix affect postassembly functions (Mammano *et al.*, 1994; Craven *et al.*, 1995). In this report, we demonstrate that deletion of the MHR impaired membrane-associated functions and particle release in transfected COS-1 cells. Moreover, using purified recombinant Gag protein, we show that mutation of the most conserved residues in the MHR also decreased membrane binding *in vitro*. Our results suggest that the MHR plays a critical role, either directly or indirectly, on assembly events at the membrane surface.

MATERIALS AND METHODS

DNA Constructs. The HIV-1 sequence [pBH10 nt 333−2130 (Ratner *et al.*, 1985)] encoding the *gag* gene and that part of the *pol* gene encoding proteinase (PR) was cloned into the pET 3 vector (Studier *et al.*, 1990) for T7 RNA polymerase promoter-driven expression in *Escherichia coli* strain BL21 (DE3) using two fragments. One fragment was derived from pHIV-FSII (Krausslich *et al.*, 1988) and contained the sequence between the *Cla*I site at nt 375 and the *Eco*RI restriction site. The *Eco*RI site in FSII follows

† This work was supported by National Institutes of Health Grant GM-48294 to C.A.C.

* Correspondence should be addressed to this author. Phone: (516) 632-8801. Fax: (516) 632-8891. Electronic mail address: carol@asterix.bio.sunysb.edu.

‡ Department of Molecular Genetics and Microbiology.

§ Department of Physiology and Biophysics.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

S0006-2960(96)00639-3 CCC: $12.00

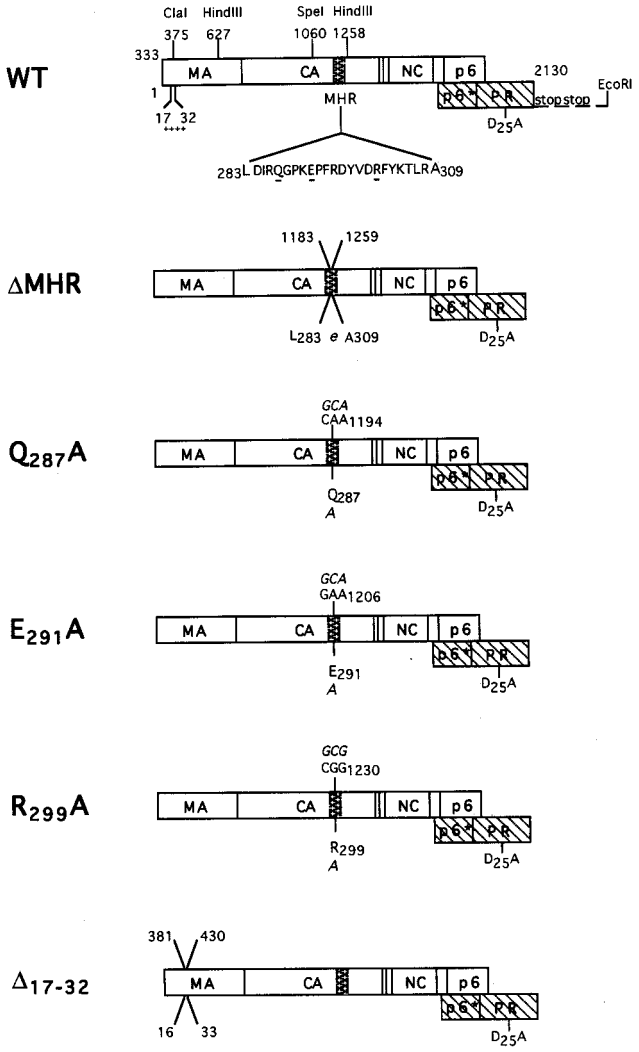

FIGURE 1: Mutants used for analysis of membrane binding. A schematic representation of the parental Gag-PR polyprotein precursor is shown at the top (WT). Open boxes represent the major domains of the *gag* (MA, CA, NC, p6) gene; hatched boxes indicate *pol* gene domains (p6*, PR) encoded by HIV-1 sequences from nucleotides 333 to 2130 of BH10 (Ratner *et al.*, 1985). Numbers above the boxes indicate nucleotide locations of mutations or of restriction enzyme sites used in cloning. Altered nucleotides or new amino acids are italicized. Letters below the boxes indicate locations of amino acids changed by mutation. ++++ represents the stretch of basic residues in MA involved in membrane binding; MHR, major homology region; D25A, inactivating mutation in the catalytic site of PR. All amino acid residues are indicated by the single-letter code.

two stop codons placed immediately downstream of the PR coding region which terminates at nt 2130. The second fragment was derived by PCR and encoded the N-terminal region of the *gag* gene and a 5′ *Bam*HI restriction site. The resulting expression construct, gpVI-ATG (Figure 1), also encodes an inactivating mutation in the catalytic site of PR (D25A) introduced by site-directed mutagenesis (Partin *et al.*, 1990). The ΔMHR mutant was constructed by PCR using pFSII plasmid as a template. The upstream primer (5′GGAAGCTTTAGACAAGATAGAGG3′) was identical to nucleotides 627−649 and included a 5′ *Hin*dIII site. The downstream primer (5′GGGTGAAGCTTCCAGAATGCTGGTAGGGC3′) annealed at nucleotides 1166−1183 and included an engineered *Hin*dIII site (underlined). The PCR product and the pFSII plasmid were digested with *Hin*dIII, and a fragment exchange with gpVI-ATG was performed. The ΔMHR *gag* gene (Figure 1) lacked nucleotides 1184−1258, encoded a Gag polyprotein which lacked amino acids 284−308, and contained a new Glu residue (Gaa) at the site of the deletion ($L_{283}-A_{309}$).

The point mutations Gln287 to Ala, Glu291 to Ala, and Arg299 to Ala were constructed by PCR using the pFSII plasmid as template. All point mutations were constructed using two rounds of PCR. The product of the first PCR reaction, which encoded the point mutation, was used as a primer for a second PCR reaction. The upstream primer in the first PCR reaction encoded the desired point mutation. For the Gln to Ala mutation, the upstream primer in the first reaction (5′GGACATAAGAGCAGGACC3′) was identical to nucleotides 1182−1199 with the exception of a CA to GC change at nucleotides 1192 and 1193. For the Glu to Ala mutation, the upstream primer in the first reaction (5′CCAAAAGCACCTTTTAGAG3′) was identical to nucleotides 1198−1216 with the exception of a A to C change at nucleotide 1205. For the Arg to Ala mutation, the upstream primer in the first reaction (5′GACTATGTAGACGCGTTC3′) was identical to nucleotides 1216−1233 with the exception of a CG to GC change at nucleotides 1228 and 1229. The downstream primer for the first PCR reaction (5′GCCGAGCAAGCTTGGG3′) was identical for all point mutations constructed. The downstream primer annealed to nucleotides 1249−1261 and included a 3′ *Hin*dIII site. The upstream primer in the second PCR reaction (5′GGAAGCTTTAGACAAGATAGAGG3′) was identical for all point mutations constructed. The upstream primer annealed to nucleotides 627−649 and included a 5′ *Hin*dIII site. The products from the first PCR reaction containing the desired point mutation served as the downstream primers in the second PCR reaction. The final PCR product and the pFSII plasmid were digested with *Hin*dIII, and a fragment exchange was performed. All mutations were then introduced into gpVI-ATG for mutant Gag polyprotein expression (Figure 1). A mutant containing a deletion in the MA domain, MAΔ(17−32), was constructed by PCR using the pFSII plasmid as a template. The upstream primer (5′CCATCGATGGCATATAGTATGGGCAAGCAGGGAGC3′) annealed at nucleotides 430−454 and included an engineered *Cla*I site (underlined). The downstream primer (5′GGAACTACTAGTACCC3′) was identical to nucleotides 1045−1060 and included a *Spe*I site. The PCR product and the pFSII plasmid were digested with *Cla*I and *Spe*I, and a fragment exchange was performed. The mutation was then subcloned into gpVI-ATG (Figure 1). The MAΔ(17−32) *gag* gene lacked nucleotides 382−429 and encoded a Gag polyprotein which lacked amino acids 17−32. For expression in COS cells, DNA that encoded Gag lacking nucleotides 1184−1258 (ΔMHR) was subcloned from the pFSII plasmid into pgp-RRE-r (Smith *et al.*, 1990) using a *Spe*I to *Apa*I fragment exchange. All mutations were confirmed by sequencing. The Myr- mutant used in these studies was a kind gift of H.-G. Krausslich (Mergener *et al.*, 1992). This mutant encoded a G_2A mutation and also lacked the reverse transcriptase (RT) and integrase (IN) domains of the *pol* gene.

Expression and Purification of Gag Proteins. Large-scale cultures of bacterial cells carrying wild-type or mutated gpVI-ATG were grown and induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) in a 14 L fermenter (Micro ferm MMF-14) as described (Ehrlich *et al.*, 1990, 1996). Ten

grams of cell paste was resuspended in 90 mL of buffer A (50 mM Tris, pH 7, with 1 mM EDTA) and lysed by French press. The lysate was centrifuged at low speed for 15 min. The supernate was mixed with crystalline NaCl (to 1 M), incubated with rotation at 5 °C for 60 min, and centrifuged at 10000*g* for 30 min. The supernate fraction was mixed with crystalline ammonium sulfate (to 10% w/v), incubated with stirring at 5 °C for 60 min, and then centrifuged again. Precipitated proteins were redissolved in 200 mL of buffer A containing 0.2 M NaCl and loaded on a DEAE-cellulose (DE-52; Whatman) column that had been preequilibrated with the same buffer. The column was washed stepwise with 250 mL of buffer A, followed by 250 mL of buffer A + 0.2 M NaCl, and finally with 250 mL of buffer A + 1 M NaCl. Proteins in the eluate collected from the latter wash were precipitated with ammonium sulfate (20% w/v), pelleted by centrifugation at 10000*g* for 60 min, and redissolved in 10 mL of buffer A + 1 M NaCl. Typical yields were ~60 mg of Gag-related protein/10 g of cell paste. This final solution contained Gag precursor proteins purified to 90−95% homogeneity.

Analysis of Protein−Lipid Binding by Fluorescence Spectroscopy. Large unilamellar vesicles (LUVs) were prepared by rehydrating the negatively charged phospholipid 1-palmitoyl-2-oleoylphosphatidylserine (POPS) in 40 mM HEPES buffer containing 0.5 M NaCl. LUVs were produced by extrusion through polycarbonate filters as described by Hope *et al.* (1985). Sucrose-loaded LUVs were prepared by rehydrating the lipid in buffer containing 0.19 M sucrose prior to extrusion. Fluorescence measurements were performed in an ISS-K2 fluorometer (ISS, Inc., Champaign, IL) using microcuvettes with a path length of 3 mm. Aliquots of POPS LUV solution (from a 200 mM stock) were added to 100 μL of protein (0.1 mg/mL), and the intrinsic fluorescence was monitored by exciting protein−LUV mixtures at 280 nm and scanning the emission from 310 to 420 nm. Spectra were corrected for control samples that contained lipids but no protein, and then for dilution.

Quantitative comparison of membrane affinities was obtained by calculating the concentration of free and bound protein from initial and final values of the emitted fluorescence for each titration. An apparent partition coefficient, K_{app}, was determined from the equation:

$$K_{app} = ([P]_b/[L])/[P]_f$$

where K_{app} is defined as the mole fraction of membrane-bound protein ($[P]_b/[L]$) divided by the concentration of free protein, $[P]_f$. $[P]_b$ is the concentration of membrane-bound protein, and [L] is the lipid concentration. K_{app} values were determined from a hyperbolic fit of the fluorescence data using Sigma Plot (Jandel Scientific Corp.). Membrane association was not reversible over 60 min when monitored by the addition of a large excess of fluorescently-labeled vesicles [see Ehrlich *et al.* (1996)].

Analysis of Protein−Lipid Binding by Sedimentation. Protein (0.2 mg/mL) was mixed with sucrose-loaded LUVs (100 μM) or buffer for 5 min. The mixture was then centrifuged at 30 000 rpm for 45 min at 25 °C in an ultracentrifuge (Beckman, TLA 45 rotor) to pellet LUVs and bound proteins. The supernatant fraction was transferred to a new tube, and the pelleted fraction was resuspended in fresh buffer. Aliquots of each fraction were separated by 15% SDS−PAGE, and electroblotted onto nitrocellulose filter paper. HIV-1 Gag proteins were detected by Western blotting and enhanced chemiluminescence (Amersham), using an anti-CAp24 polyclonal antibody raised in rabbits against purified CA (Ehrlich *et al.*, 1990).

Transfection and Protein Analysis. COS-1 cells were seeded in DMEM supplemented with 5% fetal bovine serum and transfected by the calcium phosphate precipitation method (Graham & van der Eb, 1973). Ten micrograms of each plasmid DNA was added per 100 mm dish. Cells were harvested 48 h after transfection. The medium was removed and cleared by low-speed centrifugation at 1800*g* for 5 min. An equal volume of 15% poly(ethylene glycol) (PEG) was added to the the supernatant fraction, and the mixture was incubated overnight at 4 °C. Precipitable material was collected by centrifugation at 2500*g* for 20 min and the pellet resuspended in phosphate-buffered saline (PBS). To prepare cytoplasmic and membrane-enriched fractions, the cells were washed with PBS, scraped from the plate, resuspended in buffer containing 10 mM Tris, 10 mM NaCl, and 1.5 mM $MgCl_2$, pH 7.4, and subjected to three cycles of freeze−thawing. Following centrifugation at 200*g* for 10 min to remove cell debris and nuclei, the supernatant (cytoplasmic fraction) was separated from the pellet (membrane-enriched fraction) by centrifugation at 30000*g* for 5 min. Membrane-containing samples and cytoplasmic fractions were resuspended in SDS−PAGE buffer and boiled for 3 min. Proteins were separated by 12.5% SDS−PAGE and were detected by Western blotting using anti-p24 polyclonal rabbit antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit antibody.

RESULTS

Membrane Binding of Gag and MHR-Deleted Gag Polyproteins in Transfected COS-1 Cells. The major homology region (MHR) is one of the most conserved regions in retroviral Gag proteins (Wills & Craven, 1991). The region is conserved with respect to both position within the Gag protein and amino acid sequence. Figure 2 (top) shows the conservation of the MHR sequences for several representative subgroups in the Retroviridae family. In HIV-1 Gag, the region extends from residues 285 to 304 and shares ~20−100% identity with the homologous region in other retroviruses. Three residues within the region are absolutely invariant in the family (arrows, Figure 2). Mutation of these amino acids blocks assembly of infectious virions (Craven *et al.*, 1995; Mammano *et al.*, 1994; Strambio de Castillia & Hunter, 1992). The residues in the distal portion of the MHR can be modeled to form an amphipathic helix (Figure 2, bottom; Clish *et al.*, 1996) which presents polar and hydrophobic/aromatic residues on different faces. We have found that peptides encompassing this region bind to model membranes with high affinity (Ebbets-Reed *et al.*, unpublished results).

To determine the role of the MHR in the interaction of Gag precursors with cellular membranes, a Gag polyprotein from which the MHR sequence was deleted was constructed in pgp-RRE-r, a plasmid that expresses the HIV-1 Gag and Gag-Pol polyproteins (Smith *et al.*, 1990). Transient coexpression of pgp-RRE-r and pCMV-*rev* in COS-1 cells results in the synthesis, processing, and assembly of virus-like particles (Mergener *et al.*, 1992; Smith *et al.*, 1990). The

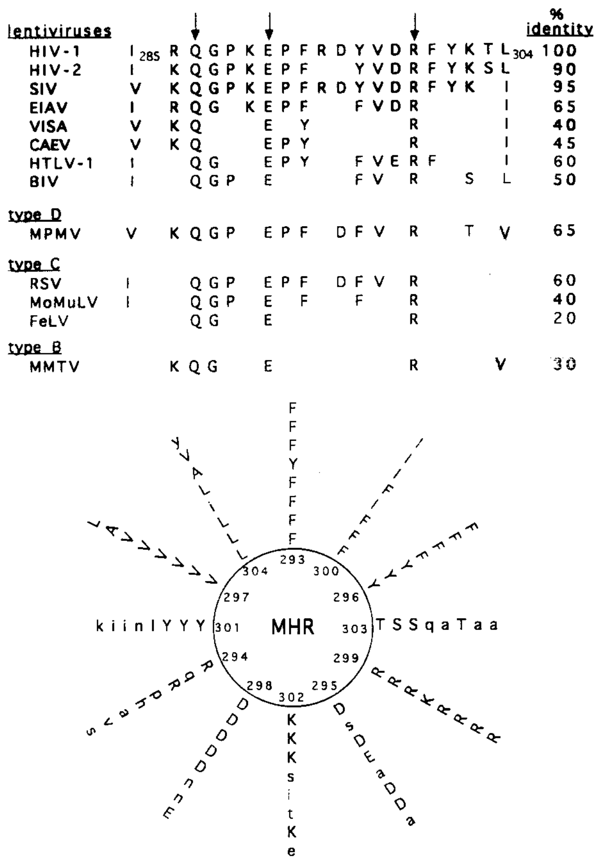

FIGURE 2: (Top) Conservation of the major homology region (MHR) in members of the Retroviridae. Amino acid sequences from the CA proteins of five subgroups (lenti, HTLV-BLV, type D, type C, type B) in the Retroviridae family are aligned. Gaps indicate nonconserved residues. The viruses shown for types B, C, and D are the prototypes. The arrows above the residues denote invariant residues. The degree of identity to the HIV-1 sequence shown is indicated on the right. The HIV-1 sequence shown represents residues 285−304 of pBH10 (Ratner *et al.*, 1985) Gag. SIV, simian immunodeficiency virus (Myers *et al.*, 1990); EIAV, equine infectious anemia virus (ibid.); VISA, visna virus (ibid.); CAEV, caprine arthritis−encephalitis virus (ibid.); HTLV-1, human T-cell leukemia virus, type 1 (Malik *et al.*, 1988); BLV, bovine leukemia virus (Sagata *et al.*, 1985); FeLV, feline leukemia virus (Laprevotte *et al.*, 1984); MMTV, mouse mammary tumor virus (Moore *et al.*, 1987); MPMV, Mason−Pfizer monkey virus (Strambio de Castillia & Hunter, 1992); RSV, Rous sarcoma virus (Schwartz *et al.*, 1983); Mo-MLV, Moloney murine leukemia virus (Shinnick *et al.*, 1981). (Bottom) Representation of the retroviral MHR as an amphipathic helix. The location of HIV-1 residues 293−304 in the predicted structure is indicated. Aligned sequences from eight other members of the family are included, in the following order: HIV-1(at numerical position); HIV-2; SIV; EIAV; HTLV-1; MPMV; RSV; Mo-MLV. Capital letters indicate identical residues or conservative amino acid substitutions.

rev gene encodes Rev, a regulatory viral protein required for cytoplasmic expression of Gag and Gag-Pol messenger RNAs (Smith *et al.*, 1990). Forty-eight hours after transfection of plasmids that express wild-type and MHR-deleted Gag proteins, a cytoplasmic extract, a membrane-enriched fraction, and PEG-precipitable material from the media of comparable cell cultures (∼10^7 cells) were prepared. The protein composition of samples from cells expressing wild-type and mutated polyproteins was analyzed by polyacrylamide gel electrophoresis followed by Western blotting with polyclonal antiserum against CA protein (Figure 3).

No specific signal was detected in cells transfected with *rev* DNA alone (panels A, B, and C, lane 1). Cytoplasmic, membrane-enriched, and media fractions prepared from cells transfected with the wild-type all contained the Pr55gag precursor, a MA-CA processing intermediate (p41), and mature CA protein (p24) (panels A, B, and C, lane 2). Similar results have been reported previously (Kaplan & Swanstrom, 1991; Mergener *et al.*, 1992; Smith *et al.*, 1990). The cytoplasmic fraction prepared from cells transfected with ΔMHR Gag resembled the wild-type pattern (panel A, lane 3); however, the membrane-enriched and the media fractions were different. In contrast to the wild-type, only small amounts of Gag protein processing intermediates were detected in the membrane-enriched fraction (panel B, lane 3), and very little was detected in released particles (panel C, lane 3). A mutant that lacked the N-terminal myristylation signal that facilitates membrane binding (Mergener *et al.*, 1992) was examined in parallel. In the cytoplasm, the Gag polyprotein synthesized by this mutant was proteolytically processed to p41, p25, and p24, as expected (panel A, lane 4), since the HIV PR is active both within cellular compartments and within viral particles (Kaplan & Swanstrom, 1991). Also as expected (Mergener *et al.*, 1992), relatively little Myr$^-$ Gag precursor or cleavage products accumulated in the membrane fraction (panel B, lane 4), and neither immature nor mature particle formation was detected in the media (panel C, lane 4). Thus, the phenotype of the ΔMHR mutant with respect to accumulation of processing intermediates in the membrane-enriched fraction and particle release was very similar to that exhibited by a Gag mutant lacking a known membrane-binding signal.

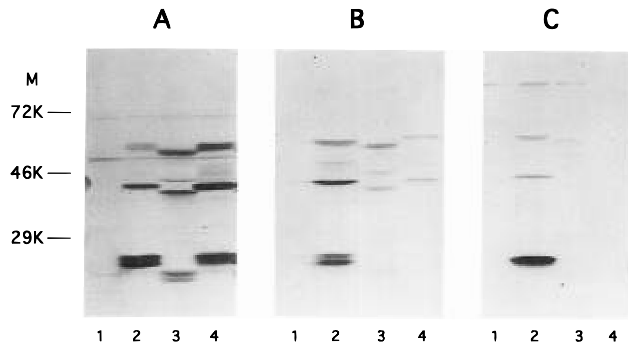

FIGURE 3: Transient expression of wild-type and MHR-deleted Gag proteins in COS-1 cells. Cytoplasmic, crude membrane, and particulate fractions were prepared from transfected cells as described under Materials and Methods and analyzed for viral protein composition by Western blotting and polyacrylamide gel electrophoresis. Molecular size markers are indicated on the left. Panel A, cytoplasmic extract; panel B, crude membrane fraction; panel C, particulate material in media. Lanes 1, transfection with plasmid expressing *rev* alone; lanes 2, transfection with plasmid expressing wild-type Gag/Gag-Pol; lanes 3, ΔMHR Gag; lanes 4, ΔMyr Gag.

Reverse transcriptase (RT) is a virally-encoded enzyme that is required for viral gene expression early in the replication cycle (Dickson *et al.*, 1985). In order to approximate the reduction in viral particle formation due to deletion of the MHR, the particles released from cells expressing the wild-type or the mutated *gag* gene were examined for RT activity. In the experiment shown in Figure 3, the wild-type particles released into the media possessed 60 units of RT activity, while only 21 units of activity were detected in media from cells transfected with ΔMHR *gag* (Table 1). Since comparable amounts of wild-type and mutated Gag-related proteins were detected in the cytoplasm (panel A), the results suggest that deletion of the MHR

Table 1: Particle-Associated Reverse Transcriptase[a]

sample	% control
AMV RT	100 (2.63 units)
wild-type − Rev	0
wild-type + Rev	60
ΔMHR − Rev	0
ΔMHR + Rev	21

[a] The particulate material in the media from comparable cell cultures was precipitated with PEG as described under Materials and Methods and used to assay reverse transcriptase activity. Activity was assayed as described by Susuki *et al.* (1993). AMV RT (avian myoblastosis virus reverse transcriptase, Boehringer Mannheim) is a commercial preparation of enzyme used as a standard.

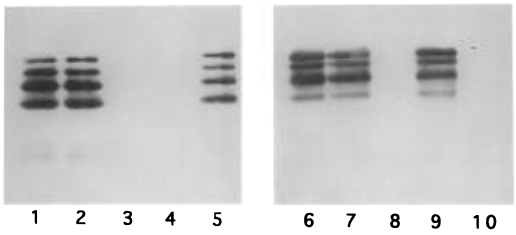

FIGURE 4: Binding of Gag to sucrose-loaded lipid vesicles as measured by sedimentation. Lanes 1−5: Gag. Lanes 6−10: ΔMHR Gag. Gag (lane 1) and ΔMHR Gag (lane 6) were mixed with buffer (lanes 2, 3 and and 7, 8, respectively) or sucrose-loaded POPS LUVs (lanes 4, 5 and lanes 9, 10, respectively). Samples were centrifuged at 30 000 rpm for 45 min in a Beckman TLA 45 rotor and separated into supernatant and pellet fractions. Lanes 2 and 4 are supernatant fractions of Gag samples recovered after mixing with buffer (lane 2) or LUVs (lane 4). Lanes 7 and 9 are supernatant fractions of the ΔMHR Gag obtained after mixing with buffer (lane 7) or LUVs (lane 9). Lanes 3 and 5 are pellet fractions of Gag samples recovered after mixing with buffer (lane 3) or LUVs (lane 5). Lanes 8 and 10 are pellet fractions obtained after mixing ΔMHR Gag with buffer (lane 8) or LUVs (lane 10). The samples were analyzed in SDS−polyacrylamide gels. Proteins were detected by Western blotting using anti-p24 antibody.

reduced the efficiency of productive membrane-associated assembly events.

Membrane Association of Gag Proteins As Determined by Sedimentation. To directly test the hypothesis that deletion of the MHR altered Gag−membrane association, we examined membrane binding in an *in vitro* system. Recombinant Gag proteins were expressed in *E. coli*, purified to ∼95% homogeneity, and tested for membrane binding *in vitro*, using model membranes made with acidic phospholipids as described below. To obtain HIV-1 Gag protein for membrane-binding studies, we constructed plasmid gpV1-ATG (Materials and Methods). This construct provides primarily Gag precursor polyprotein (Pr55) and truncated Gag-Pol precursor protein (Gag-PR), expressed at ∼5% of the level of Gag due to ribosomal frameshifting. Expression of this construct in *E. coli* BL21(DE3) and purification using a nondenaturing protocol produced a protein preparation that contained the full-length Gag precursor polyprotein and three truncated forms of the protein (Figure 4, lane 1). All protein bands were recognized by antibodies directed at the T7 leader sequence present at the N-terminus and at MA and CA sequences (data not shown). The slowest migrating band also was recognized by monoclonal antibody to the C-terminal portion of the p6 domain in Gag, identifying this band as full-length Gag polyprotein. The shorter proteins apparently resulted from C-terminal truncation of the full-length protein. As determined by epitope accessibility, by limited proteolysis with trypsin and the virally-encoded proteinase (PR), or by chemical cross-linking analyses, the recombinant Gag protein appeared to be structurally identical to the native protein (data not shown).

Membrane vesicles were prepared with the 1-palmitoyl-2-oleoyl ester of the negatively charged phospholipid phosphatidylserine (POPS) based on previous demonstrations of efficient Gag binding to acidic phospholipids *in vitro* (Zhou *et al.*, 1994; Ehrlich *et al.*, 1996). Membrane association was assayed using a sedimentation method developed by Rebecchi and co-workers (Rebecchi *et al.*, 1992) in which large unilamellar vesicles (LUVs) loaded with sucrose (0.19 M) were used as the membrane substrate. The high density of the entrapped sucrose permits isolation of the vesicles by sedimentation. Proteins bound to the membrane will sediment with the vesicles and be effectively removed from the supernatant. This assay, in which the membrane-bound proteins are sedimented by ultracentrifugation, is similar to assays previously described by others for analysis of membrane binding of *in vitro* translated proteins (Platt & Haffar, 1994; Zhou *et al.*, 1994). To assess high-affinity binding and to ensure Gag solubility, the binding of Gag was examined using high ionic strength buffer (0.5 M NaCl) which will destabilize weak electrostatic interactions. Under these conditions, Gag membrane binding is principally driven by interactions through the MA and CA domains together with little contribution from the NC region. This was demonstrated (1) by efficient binding under these conditions of MA-CA but not MA or CA alone (Ehrlich *et al* 1996 and (2) by the observation that all of the proteins in the Gag preparation were bound, whether or not the proteins contained the C-terminus (Figure 4, lane 5).

The membrane binding of comparable amounts of the wild-type and ΔMHR Gag proteins was examined. In the absence of added LUVs, the wild-type and ΔMHR Gag proteins remained in the supernatant fractions (Figure 4, lanes 2 and 7, respectively) and did not pellet (lanes 3 and 8). Following addition of the sucrose-loaded LUVs, the wild-type proteins were recovered quantitatively in the pellet fraction (supernatant, lane 4; pellet, lane 5). In contrast, the ΔMHR Gag remained in the supernatant fraction (supernatant, lane 9; pellet, lane 10). These results indicated that deletion of the MHR impaired the intrinsic membrane affinity of the Gag protein, and support the conclusion that the MHR sequence plays a role in membrane association.

Membrane Partitioning of Wild-Type Gag and Mutated Gag Proteins to POPS Bilayers As Determined by Quenching of Intrinsic Fluorescence. Previous studies indicated that the membrane-binding assay based on cosedimentation with sucrose-loaded vesicles is unreliable because (1) leakage of sucrose leads to inefficient pelleting of vesicles, (2) vesicles may rupture upon protein insertion, and (3) antibodies often fail to recognize the membrane-bound protein fraction (Ehrlich *et al.*, 1996). To determine the decrease in membrane affinity caused by mutation of the MHR, membrane partitioning was measured using fluorescence spectroscopy. The K_{app} coefficients were derived in several independent experiments ($n \geq 3$) by relating the increase in fluorescence quenching to the amount of free and bound protein and then fitting the normalized change in fluorescence intensity to a simple binding curve (i.e., a hyperbolic curve), where K_{app} is the midpoint of the curve.

As shown in Figure 5 and Table 2, recombinant unmyristylated HIV-1 Gag exhibited a K_{app} of 3.4 μM when

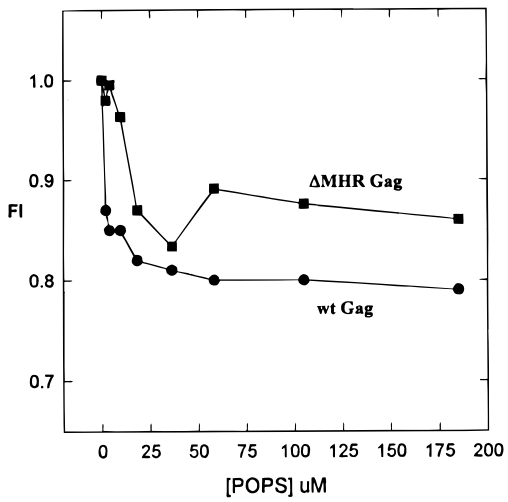

FIGURE 5: Increases in fluorescence quenching of Gag and ΔMHR Gag upon membrane binding. The increase in quenching of the intrinsic fluorescence upon addition of POPS LUVs is expressed as fractional quenching or the change in intensity over its initial value. Samples were excited at 280 nm, and scanned for emissions from 310 to 420 nm. The symbols represent averages of $n > 3$ measurements (closed circles, wild-type Gag; closed squares, ΔMHR Gag).

Table 2: Partition Coefficients of Wild-Type and ΔMHR Gag Precursors

protein	membrane binding affinity (K_{app}, μM)[a]
wild-type Gag	2.3 ± 0.2 3.8 ± 0.26 4.1 ± 1.4
EIAV Gag	3.7 ± 1.1
ΔMHR	12.1 ± 4.3 9.4 ± 7.5 10.5 ± 1.8 8.7 ± 6.0
MAΔ17−32	19.0 ± 4.0
Gln 287Ala	16.0 ± 0.9 22.0 ± 8.0
Glu291Ala	15.0 ± 3.0 15.1 ± 2.3
Arg299Ala	8.8 ± 6.0 12.7 ± 0.8

[a] K_{app} values were determined as described under Materials and Methods.

averaged from at least three independent trials. A comparable value was obtained for EIAV Gag, a protein which is naturally unmyristylated and which was expressed and purified in a similar manner. Both values are within the range of affinities observed for peripheral membrane binding proteins (Parola, 1993; Rebecchi *et al.*, 1992). As expected, deletion of the basic sequence in the MA domain reduced the K_{app} (Table 2). IgG, a protein that is not expected to bind membranes, exhibited no change in fluorescence intensity and did not sediment in the presence of LUVs (data not shown).

The contribution of the MHR sequence to membrane binding was determined by measuring the affinities of mutated HIV-1 Gag proteins. Deletion of the entire 20 amino acid MHR sequence decreased the affinity reproducibly (averaged $K_{app} \sim 10$ μM; Table 2), although the extent of quenching exhibited by ΔMHR Gag samples varied from ~15 to 40% ($n = 3$) due to the presence of nonbinding products in the preparation. In a parallel study, energy transfer from tryptophan residues in the ΔMHR Gag protein to an acceptor probe embedded in the membrane surface [laurodan, 6-dodecanyl-2-(dimethylamino)naphthalene] was detected and gave a binding constant of 12.6 ± 6 μM (energy transfer distance = 22 Å). As this value is consistent with the results obtained by intrinsic fluorescence (shown in Table 2), we conclude that the deletion weakened, but did not completely prevent, membrane association. Single amino acid substitutions of Ala for the Gln287 or Glu291 invariant residues reduced the affinity to a greater extent, while the Arg299Ala mutation had less of an effect than the Glu291Ala or the Gln287Ala mutations. Significantly, these results are directly commensurate with the effect of these mutations on morphogenesis *in vivo* (Mammano *et al.*, 1992), where the Glu291Ala and the Gln287Ala mutations impaired assembly more than deletion of the entire MHR sequence. As expected based on the observed K_{app} values, the Gag mutants that exhibited reduced binding affinities in the fluorescence assay also failed to sediment with sucrose-loaded LUVs in the sedimentation assay (data not shown). The results of these studies indicate that the MHR is a significant determinant of Gag binding activity in this *in vitro* assay, and support the results of the sedimentation and transfection assays.

DISCUSSION

Studies with several retroviruses, using recombinant DNA techniques, have shown that expression of the Gag precursor alone can result in production of budding particles (Delchambre *et al.*, 1989; Hu *et al.*, 1990; Lee & Linial, 1994; Luo *et al.*, 1990; Morikawa *et al.*, 1991). The matrix (MA) domain of Gag plays a critical role in this process (Byrant & Ratner, 1990; Delchambre *et al.*, 1989; Facke *et al.*, 1993; Freed *et al.*, 1994; Gheysen *et al.*, 1989; Hayakawa *et al.*, 1992; Hu *et al.*, 1990; Jacobs *et al.*, 1989; Pal *et al.*, 1990; Rein *et al.*, 1986; Spearman *et al.*, 1994; Wagner *et al.*, 1992; Weaver & Panganiban, 1990; Zhou *et al.*, 1994). In the case of HIV-1, the first 31 residues of Gag can function independently as a membrane-targeting domain when fused to heterologous proteins (Zhou *et al.*, 1994). This domain contains a bipartite motif. One part of the signal consists of the myristylated N-terminus and the following 14 amino acids. Particle formation can occur even in the absence of the entire MA domain if the myristylation signal is maintained (Lee & Linial, 1994). The second part contains a highly basic region which may interact with acidic phospholipids. Residues in the basic domain that have a significant effect on particle release (Freed, 1994) cluster around an extruded loop in MA trimers and could constitute a membrane binding surface (Massiah *et al.*, 1994; Hill *et al.*, 1996). Downstream domains have also been found to influence membrane binding. Assembly of Myr^- MA^- particles at the plasma membrane can occur, although at low efficiency (Lee & Linial, 1994). Binding studies with C-terminally truncated Gag or with Gag proteins containing point mutations in the nucleocapsid (NC) domain identify the seven amino acids located between the two highly conserved Cys-His arrays as necessary for stable membrane association (Platt & Haffar, 1994). Although all of these signals have been shown to play important roles in membrane binding, it is important to note that, unlike the MHR, none of these motifs is absolutely conserved throughout the retrovirus family (Myers *et al.*, 1990). Moreover, none of

these signals alone is sufficient for membrane association (Lee & Linial, 1994; Platt & Haffar, 1994; Zhou *et al.*, 1994).

We have shown that the MHR of HIV-1 Gag directly contributes to membrane binding both in transfected mammalian cells and in *in vitro* assays using LUVs of acidic phospholipids as model membranes. Deletion of the MHR from the Gag precursor significantly reduced membrane affinity *in vitro* and the efficiency of mature particle assembly in transfected mammalian cells. A direct correlation exists between a mutation's impact on particle formation and its effect on membrane binding *in vitro*: We observed that the association of Gag mutants Gln287Ala or Glu291Ala to LUVs was reduced to a greater extent than that of Arg299Ala (Table 2); others have demonstrated that Gag mutants Gln287Ala and Glu291Ala disrupt viral particle assembly to a greater extent than mutation of Arg299 or deletion of the MHR (Mammano *et al.*, 1994). The tight correlation between the phenotype of these mutants in infected cells and their behavior in our *in vitro* assay provides evidence that our experimental system accurately reflects physiologically significant membrane assembly events.

Biophysical measurements performed by Peitzsch and McLaughlin (1993) estimate the membrane binding affinity of myristate to be 10^{-4} M. This is an insignificant contribution to the K_{app} of $\sim 10^{-6}$ M that we estimate for the membrane affinity of our unmodified recombinant Gag protein. If N-terminal myristylation alters the conformation of the Gag protein, as has been suggested (Chazal *et al.*, 1994), membrane binding of the modified protein could be much greater. In our studies with unmyristylated Gag, deletion of the critical basic region in the MA domain reduced the membrane affinity approximately the same extent as mutation of the invariant Gln residue in the MHR. This observation suggests that, in the context of these experiments, the MHR contribution to membrane binding may be as significant as that of the basic motif in MA. This conclusion is supported by the observation that deletion of the MHR sequences from Gag resulted in reduction in membrane partitioning even though the MA domain was intact. Moreover, the observation that deletion of the MHR reduced association of the Gag precursor under high ionic strength conditions (0.5 M NaCl) suggests that the MHR stabilized binding through hydrophobic or very strong electrostatic interactions.

The MHR could influence the membrane association of Gag by directly binding to lipids, possibly by forming an amphipathic helix (Clish *et al.*, 1996). Alternatively, the MHR could influence the membrane association of Gag indirectly. This region may control the tertiary structure of Gag and, thereby, the surface accessibility of one or more membrane binding motifs. The MHR may be a determinant of the quaternary structure of the protein and affect membrane affinity by regulating the oligomerization state of the protein. The latter possibility is supported by the observation that wild-type Gag binding measured at low lipid concentrations that promote protein−protein interactions was consistently weaker than membrane binding assessed under conditions that promote protein−lipid interactions (Ehrlich *et al.*, 1996). This would be explained if higher oligomerization states of Gag bind membranes with lower affinity than Gag monomers or dimers. MHR involvement in Gag−Gag interactions has been suggested by several investigators on the basis of genetic analyses that mapped the location of regions required for particle formation, particle release, and Gag−Gag interactions (Hansen & Barklis, 1995; Hsu *et al.*, 1985; Jones *et al.*, 1990; Luban *et al.*, 1992; Srinivasakumar *et al.*, 1995; Wang *et al.*, 1994). These studies show that while the MA domain is sufficient for Gag release from the cell, efficient formation of viral particles requires an intact CA domain. The studies we report here extend these findings by demonstrating that the MHR contributes to the overall strength of Gag membrane binding. We suggest that this is due to its effect on protein−protein interactions.

In summary, mutations in the MHR of retroviral Gag proteins result in phenotypes that suggest that the region is involved in assembly of viral particles and in postassembly replication events (Craven *et al.*, 1995; Mammano *et al.*, 1994; Strambio de Castillia & Hunter, 1992). As demonstrated in this report, the failure to assemble and release particles can be attributed to defects in assembly-related functions that occur on the membrane surface. Further elucidation of the role of the MHR in Gag−membrane interactions may lead to a clearer understanding of events that are essential for assembly of all retroviruses.

ACKNOWLEDGMENT

We thank S. Fong, L. S. Ehrlich, G. Zybarth, and B. Agresta for advice and for critical reading of the manuscript. We appreciate the excellent technical assistance of I. Jayatilaka, H. McBath, and Theresa Bollinger.

REFERENCES

Byrant, M., & Ratner, L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 523−527.
Chazal, N., Carrieres, C., Gay, B., & Boulanger, P. (1994) *J. Virol. 68*, 111−122.
Clish, C. B., Peyton, D. H., & Barklis, E. (1996) *FEBS Lett. 378*, 43−47.
Craven, R. C., Leure-duPree, A. E., Weldon, R. A., Jr., & Wills, J. W. (1995) *J. Virol. 69*, 4213−4227.
Delchambre, M., Gheysen, D., Thines, C., Thiriart, E., Jacobs, E., Verdin, E., Horth, M., Burny, A., & Bex, F. (1989) *EMBO J. 8*, 2653−2660.
Dickson, C., Eisenman, R., & Fan, H. (1985) in *RNA Tumor Viruses* (Weiss, R., Teich, N., Varmus, H., & Coffin, J., Eds.) pp 512−648, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Ehrlich, L. S., Krausslich, H.-G., Wimmer, E., & Carter, C. A. (1990) *AIDS Res. Hum. Retroviruses 6*, 1169−1175.
Ehrlich, L. S., Agresta, B. E., Gelfand, C. A., Jentoft, J., & Carter, C. A. (1994) *Virology 204*, 515−525.
Ehrlich, L. S., Fong, S., Scarlata, S., & Carter, C. A. (1996) *Biochemistry 35*, 3933−3943.
Facke, M., Janetzko, A., Shoeman, R. L., & Krausslich, H.-G. (1993) *J. Virol. 67*, 4972−4980.
Freed, E. O., Orenstein, J. M., Buckler-White, A. J., & Martin, M. (1994) *J. Virol. 68*, 5311−5320.
Gheysen, D., Jacobs, E., de Foresta, F., Thiriart, C., Francotte, M., Thines, D., & de Wilde, M. (1989) *Cell 59*, 103−112.
Graham, R., & van der Eb, A. (1973) *Virology 52*, 456−467.
Hansen, M. S. T., & Barklis, E. (1995) *J. Virol. 69*, 1150−1159.
Hayakawa, T., Miyazaki, T., Misumi, Y., Kobayashi, M., & Fujisawa, Y. (1992) *Gene 119*, 273−277.
Hill, C. P., Worthylake, D., Bancroft, D. P., Christensen, A. M., & Sundquist, W. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 93*, 3099−3104.
Hope, M., Bally, M., Webb, G., & Cullis, P. (1985) *Biochim. Biophys. Acta 812*, 55−65.
Hsu, H.-W., Schwartzberg, P., & Goff, S. P. (1985) *Virology 142*, 211−214.
Hu, S.-L., Travis, B. M., Garrigues, J., Zarling, J. M., Sridhar, P., Dykers, T., Eichberg, J. W., & Alpers, C. (1990) *Virology 197*, 321−329.

Jacobs, E., Gheysen, D., Thines, D., Francotte, M., & de Wilde, M. (1989) *Gene 79*, 71−81.
Jones, T., Blaug, G., Hansen, M., & Barklis, E. (1990) *J. Virol. 64*, 2265−2279.
Kaplan, A. H., & Swanstrom, R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4528−4532.
Krausslich, H.-G., Schneider, H., Zybarth, G., Carter, C. A., & Wimmer, E. (1988) *J. Virol. 62*, 4393−4397.
Laprevotte, I., Hampe, A., Sherr, C. J., & Gahbert, F. (1984) *J. Virol. 50*, 884−894.
Lee, P., & Linial, M. L. (1994) *J. Virol. 68*, 6644−6654.
Luban, J., Alin, K. B., Bossalt, K. L., Humaran, T., & Goff, S. (1992) *J. Virol. 66*, 5157−5160.
Luo, L., Li, Y., & Kang, C. Y. (1990) *Virology 179*, 874−880.
Malik, K. T. A., Even, J., & Karpas, A. (1988) *J. Gen. Virol. 69*, 1695−1710.
Mammano, F., Ohagen, A., Hoglund, S., & Gottlinger, H.-G. (1994) *J. Virol. 68*, 4927−4936.
Massiah, M. A., Starich, M. R., Paschall, C., Summers, M. F., Christensen, A. M., & Sunquist, W. I. (1994) *J. Mol. Biol. 244*, 198−216.
Matthews, S., Barlow, P., Boyd, J., Barton, G., Russell, R., Mills, H., Cunningham, M., Meyers, N., Burns, N., Clark, N., Kingsman, S., Kingsman, A., & Campbell, I. (1994) *Nature 370*, 666−668.
Mergener, K., Facke, M., Welker, R., Brinkmann, V., Gelderblom, H., & Krausslich, H.-G. (1992) *Virology 186*, 25−39.
Moore, R., Dixon, M., Smith, R., Peters, G., & Dickson, C. (1987) *J. Virol. 61*, 480−490.
Morikawa, S., Booth, T. F., & Bishop, D. H. L. (1991) *Virology 183*, 288−297.
Myers, G., Berzofsky, J., Rabson, A., Smith, T. F., & Wong-Staal, F. (1990) *Human Retroviruses and AIDS.* Los Alamos National Laboratory, Los Alamos, NM.
Pal, R., Reitz, M. S., Jr., Tschachler, E., Gallo, R. C., Sarngadharan, M. G., & Veronese, F. D. (1990) *AIDS Res. Hum. Retroviruses 6*, 21−730.
Parola, A. H. (1993) in *Biomembranes: Physical aspects* (Shinitzky, M., Ed.) pp 159−277, VCH Publishers, New York.
Partin, K., Krausslich, H.-G., Ehrlich, L., Wimmer, E., & Carter, C. A. (1990) *J. Virol. 64*, 3938−3947.
Peitzsch, R. M., & McLaughlin, S. (1993) *Biochemistry 32*, 10436−10443.
Platt, E. J., & Haffar, O. K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4594−4598.
Ratner, L., Haseltine, W., Patarca, R., Livak, K., Starcich, B., Josephs, S., Doran, E., Rafalski, J., Whitehorn, E., Baumeister, K., Ivanoff, L., Petteway, S., Pearson, M., Lautenberger, L., Papas, T., Ghrayeb, J., Chang, N., Gallo, R. C., & Wong-Staal, F. (1985) *Nature (London) 316*, 277−284.
Rebecchi, M., Peterson, A., & McLaughlin, S. (1992) *Biochemistry 31*, 12742−12747.
Rein, A., McClure, M. R., Rice, N., Luftig, R., & Schultz, A. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7246−7250.
Sagata, N., Yasunaga, T., Tsuzuku-Kawamura, J., Ohishi, K., Ogawa, Y., & Ikawa, Y. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 677−682.
Schwartz, D. E., Tizard, R., & Gilbert, D. W. (1983) *Cell 32*, 853−869.
Shinnick, T. M., Lerner, R. A., & Sutcliffe, J. G. (1981) *Nature (London) 293*, 543−548.
Smith, A. J., Cho, M.-I., Hammaarskjold, M.-L., & Rekosh, D. (1990) *J. Virol. 64*, 2743−2750.
Spearman, P., Wang, J. J., Vander Heyden, N., & Ratner, L. (1994) *J. Virol. 68*, 3232−3242.
Srinivasakumar, N., Hammarskjold, M.-L., & Rekosh, D. (1995) *J. Virol. 69*, 6106−6114.
Strambio de Castillia, C., & Hunter, E. (1992) *J. Virol. 66*, 7021−7032.
Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorf, J. W. (1990) *Methods Enzymol. 185*, 60−89.
Wagner, R., Fliessbach, H., Wanner, G., Motz, M., Niedrig, M., Deby, G., von Brunn, A., & Wolf, H. (1992) *Arch. Virol. 127*, 117−137.
Wang, C.-T., Stegeman-Olsen, J., Zhang, Y., & Barklis, E. (1994) *Virology 200*, 520−534.
Weaver, T. A., & Panganiban, A. T. (1990) *J. Virol. 64*, 3995−4001.
Wills, J., & Craven, R. (1991) *AIDS 5*, 639−654.
Zhou, W., Parent, L. J., Wills, J. W., & Resh, M. D. (1994) *J. Virol. 68*, 2556−2569.

BI9606399