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The Carboxyl Terminus of the Human Foamy Virus Gag Protein Contains Separable Nucleic Acid Binding and Nuclear Transport Domains

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The Gag protein of human foamy virus (HFV) lacks Cys-His boxes present in the nucleocapsid (NC) domains of other retroviruses; instead it contains three glycine-arginine-rich motifs (GR boxes). We have expressed the carboxyl end of HFV Gag containing the GR boxes (the NC domain equivalent) and analyzed its nucleic acid binding properties. Our results show that the NC domain of HFV Gag binds with high affinity to both RNA and DNA, in a sequence-independent manner, as determined by filter binding assays. Analysis of a mutant containing a heterologous sequence in place of GR box I indicates that this motif is required for nucleic acid binding and for viral replication. A mutant in GR box II still binds to RNA and DNA in vitro, but virus containing this mutation does not replicate and no nuclear staining of the Gag protein is found in transfected cells. Surprisingly, a revertant from this mutant that completely lacks GR box II and exhibits very little nuclear transport of Gag can readily replicate in tissue culture. This finding thus provides a direct evidence that although the sequences in GR box II can serve as a nuclear transport signal, they are not required for HFV replication and it is unlikely that nuclear localization of Gag protein plays any critical role during viral infection. Taken together, our results suggest that the Gag protein of HFV may be more analogous to the core protein of the hepatitis B virus family than to conventional retroviral Gag protein.

All retroviral genomes contain *gag*, *pol*, and *env* genes that are required for replication. The *gag* gene encodes a polyprotein precursor that is cleaved into smaller proteins by the virally encoded protease after particles are assembled. In most retroviruses, mature virions contain at least three Gag cleavage products called matrix (MA), capsid (CA), and nucleocapsid (NC). MA contains a domain(s) required for proper targeting of the Gag protein to the correct subcellular location for assembly (8, 40). After cleavage, MA may interact with the envelope glycoproteins on the surface of the particles (30, 44). CA composes the major core protein enclosing the nucleoprotein complex (35, 41). The major homology region found in the CA domain is conserved among diverse onco- and lentiviruses (4, 24). NC is involved in RNA packaging through recognition of a specific region of the viral genomic RNA called Ψ (17, 20). After cleavage, NC is found bound nonspecifically along the entire encapsidated RNA molecules (6). Thus, NC possesses specific as well as nonspecific RNA binding activity. Most retroviral NC proteins contain one or two Cys-His boxes that are involved in specific RNA recognition and also may play a role in RNA binding (14, 17, 25). NC is also involved in other functions such as positioning the specific tRNA reverse transcriptase primer on the genomic RNA for priming function (18).

The least studied genus of retroviruses is that of the spumaviruses or foamy viruses. Foamy viruses are prevalent in primates, felines, and bovines but are not pathogenic in these species (38). The prototype virus of this group is human foamy virus (HFV), which is a virus prevalent in chimpanzee populations but not in humans (1, 2). The genome of HFV contains not only the open reading frames (ORFs) corresponding to *gag*, *pol*, and *env* but also several other ORFs at the 3′ end of the genome, located between *env* and 3′ long terminal repeat (11, 12). Only one of these, Bel1, is known to be essential for viral replication; it functions as an activator of transcription (23, 32). A unique feature of foamy viruses is that they contain an internal promoter at the 3′ end of the *env* gene, which is used early in the viral life cycle to express Bel1 and other 3′ ORF proteins (21, 22). The foamy virus *gag* and *pol* genes are overlapping as in other retroviruses, but the *pol* gene is in the +1 reading frame relative to *gag*. Unlike other retroviruses, the enzymatic functions such as reverse transcriptase and integrase encoded within the Pol precursor protein are not expressed as a Gag-Pol fusion protein. Instead, HFV Pol expression is synthesized from a separate spliced mRNA (42).

The Gag protein of foamy viruses also differs from those of other retroviruses. There is little or no cleavage of HFV Gag to the smaller products such as MA, CA, and NC proteins found in mature onco- or lentiviral particles. The only known cleavage of the 78-kDa Gag protein is removal of about 4 kDa from the carboxyl terminus of Gag, generating a 74-kDa protein. This cleavage requires an active HFV protease that is encoded within the Pol protein precursor (19). None of the conventional retroviral Gag landmarks such as the major homology region or Cys-His motifs are present in HFV Gag (11). Instead, foamy virus Gag protein contains three stretches of glycine-arginine-rich motifs (GR boxes) in the carboxyl one-third (the NC domain equivalent) (33). The presence of these highly basic motifs found in foamy virus NC is thus reminiscent of the class of arginine-rich RNA-binding proteins (3) and the C-

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terminal basic domain of hepatitis B virus (HBV) core (C) protein (27).

A unique feature of foamy virus infection is a strong nuclear staining of infected cells reacted with anti-foamy virus antibodies (10, 33). This typical nuclear fluorescence of foamy virus in infected cells is unique among all retroviruses and is regarded as the best serological criterion for foamy virus infection (28). There is a transient transport of Gag precursor protein to the nuclei of infected cells during a single round of viral infection, which is thought to account for the observed nuclear staining. Schliephake et al., using the vaccinia virus expression system, have mapped this nuclear localization signal to GR box II (33). However, it is still unknown if this transient localization of Gag proteins into the nucleus plays any functional role in viral replication.

We were interested in determining whether HFV Gag possesses nucleic acid binding properties analogous to those provided by other retroviral NC proteins and whether the GR boxes are important in viral replication, nucleic acid binding, and/or nuclear transport. In this study, we have expressed and purified the C-terminal region of Gag corresponding in location to the NC domain of murine leukemia virus Gag, using the bacterial system. Our data show that the NC domain of HFV contains a nonspecific binding activity to both RNA and DNA. Using mutants of NC, we have separated the nucleic acid binding activity from the function of nuclear localization of the Gag protein. Our results suggest that while the nucleic acid binding activity is essential for viral replication, the nuclear transport function of NC is dispensable for HFV infectivity in vitro.

MATERIALS AND METHODS

Transfection and infection. BHK-21 and FAB cells were transfected with wild-type (wt) or mutant proviral DNA, using the LipofectAmine reagent (Gibco BRL, Gaithersburg, Md.) as suggested by the manufacturer. Supernatant from transfected cells was filtered and used to infect HEL or FAB cells to determine the viral infectivity as described previously (43). To isolate revertants from the cells harboring the H1 or H3 mutant, transfected cells were subcultured at a ratio of 1:5 twice a week until cytopathic effect (CPE) was detected.

Construction of mutant and expression plasmids. All constructs with mutations in the NC domain of HFV (H1 to H6) were constructed similarly, using a two-step PCR method as described previously (42). Each mutant contains either an 11-amino-acid substitution or insertion at the carboxyl-terminal portion of Gag protein (Fig. 1). The sequence encoding this 11-amino-acid epitope tag (MMYPYDVPDYA) that is derived from the influenza virus hemagglutinin (HA) protein (9) is 5′-ATGATGTACCCATACGATGTTCCAGATTACGCT-3′. The amplified PCR fragments containing the correct mutation were verified by DNA sequencing. Such mutant DNA fragments were subsequently used to replace the wt sequences in the pHFV13 infectious clone (23) to generate proviral constructs. The infectious clone of the H3 revertant, pHFV/H3RR, was constructed by replacing the *Stu*I-*Dra*III fragment (nucleotides [nt] 1846 to 2316) of wt pHFV13 with the corresponding fragment amplified by reverse transcription-PCR from viral RNA of the H3 revertant.

Bacterial expression plasmids for the NC domain were made for both wt and H1, H3, H13, and H5 mutants. A DNA fragment of NC (nt 1844 to 2392) was amplified by PCR using two primers: 5′-GGCG*GGATCCATG*CATCATCATC ATCATCATTTAGGCCTTAATGCCAGAGGAC-3′ and 5′-GGCG*GAATTC*T TAGTCCCTTTGATCTCCGCCG-3′. The first primer contains a *Bam*HI cleavage site (in italics), an ATG initiation codon (bold face), and sequences encoding six histidine residues (underlined), followed by sequences identical to the 5′ end of NC (nt 1844 to 1865). The second primer included sequences complementary to the 3′ end of NC (nt 2371 to 2392) and an additional *Eco*RI cleavage site (in italics). The PCR product was digested with *Bam*HI and *Eco*RI and inserted into a T7 expression vector pGM484. To generate the H13 mutant, a 343-bp *Xba*I fragment from pHFV/H3 was replaced with that from pHFV/H1, which would place both mutations in GR boxes I and II into the same plasmid.

Expression and purification of recombinant proteins. Expression and purification of the recombinant proteins that contained an affinity tag of six histidine residues (6xHis tag) was based on the QIAexpress system as recommended by the manufacture (Qiagen, Chatsworth, Calif.). Expression was induced in *Escherichia coli* BL21(DE3) by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyraonoside) to log-phase bacterial cultures for 3 h. Bacterial pellets were resuspended in 50 mM NaH_2PO_4 (pH 8.0)–300 mM NaCl–20 mM imidazole–1 mg of lysozyme per ml–1 mM phenylmethylsulfonyl fluoride and lysed by sonication. The supernatants collected after sonication were mixed with Ni-nitrilotriacetic acid (NTA) resins. The addition of a 6xHis tag at the amino terminus of NC allowed it bind specifically to Ni-NTA resins. After extensive washing, proteins were eluted with a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 250 mM imidazole. The eluted proteins were further purified through FPLC (fast protein liquid chromatography) Superdex 200 26/60 columns. The purity of each preparation was assessed by Coomassie blue staining after separation on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The protein concentration was measured with a Bio-Rad (Hercules, Calif.) protein assay kit.

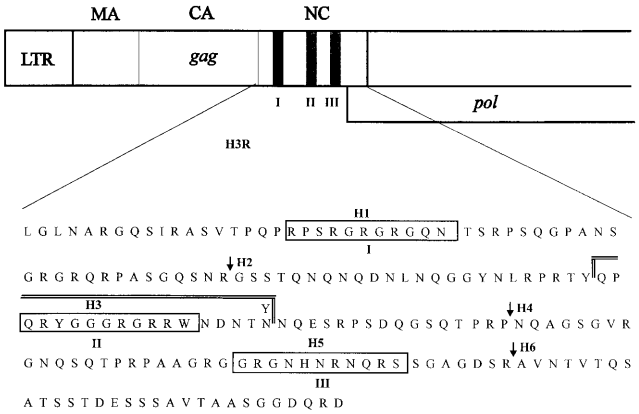

FIG. 1. Locations and amino acid sequences of the NC domain of HFV Gag protein. The three GR boxes are indicated by gray boxes. The boxed amino acids show the sequences of each GR box (I, II, or III) replaced by the HA tag (MMYPYDVPDYA). The arrows show the locations of insertion of the HA tag into the NC domains of H2, H4, and H6. The sequences bracketed by double lines indicate the boundaries of deletion found in the H3RR revertant. The in-frame deletion leads to a change from asparagine (N) to tyrosine (Y). LTR, long terminal repeat.

Preparation of RNA and DNA probes. A ^{32}P-labeled RNA probe was prepared by in vitro transcription from cloned cDNA. Plasmid pR5M, which contained the 5′ end of the HFV genome (nt 1 to 700), was constructed by inserting a 700-bp *Sac*I-*Eco*RI fragment of pHSRV13 into a pBluescript II KS+ vector (Stratagene, La Jolla, Calif.). Plasmid pSKNC contained most of the sequences encoding the NC domain and was constructed by inserting a 470-bp *Stu*I-*Dra*III fragment of pHSRV13 into a pBluescript II SK+ vector (Stratagene). To synthesize virus-specific RNA probes, plasmid pR5M was linearized with either *Eco*RI (at nt 248) or *Eco*RV (at nt 700) before transcription. Plasmid pSKNC was linearized with *Dra*III to generate a 470-bp riboprobe specific to HFV NC sequences. The non-virus-specific RNA probes were derived from sequences encoding either the Rous sarcoma virus Ψ region (381 bp) or the *neo* gene (280 bp).

A ^{32}P-labeled double-stranded DNA (dsDNA) probe was generated by PCR amplification of the HFV NC sequences (nt 1762 to 2422) or sequences encoding the *neo* gene. A single-stranded DNA (ssDNA) probe was prepared by end labeling a virus-specific oligonucleotide (5′-CATAGCTGCTTGGTAACCCGG GTCTACAGCCTGAC-3′, 34-mer) or a non-virus-related primer (5′-CATCCC GGGAAGCTTAGCTGGAGGCCTTGCATCATTTTCTAGAAGCTTCCCG GGATC-3′, 57-mer), using T4 kinase and [^{32}P]ATP.

DNA and RNA binding assays. The nitrocellulose filter binding assay was performed as described elsewhere (36). In brief, standard reaction mixtures (50 μl) contained 10 mM Tris-Cl (pH 8.0), 20 mM NaCl, 1 mM dithiothreitol, 0.01 pmol of ^{32}P-labeled DNA or RNA probe (with a specific activity of 2×10^8 to 5×10^8 cpm/μg), and various amounts of purified NC proteins. After 30 min of incubation on ice, the mixture was diluted with 0.5 ml of ice-cold buffer (10 mM Tris-Cl [pH 8.0], 20 mM NaCl) and immediately filtered through 25-mm 2-pore-size nitrocellulose filters presoaked in the same buffer, using very gentle suction. Filters were then dried under a heat lamp and subjected to scintillation counting. Total input of radiolabeled probe was determined by spotting the same amount of probe on dry filters without filtering or washing. Background counts obtained from incubating bovine serum albumin in the binding assay were subtracted from the values obtained in assays using wt or mutant NC proteins for binding.

Immunoprecipitation and immunofluorescence assays. Two days after transfection, cells were labeled with [^{35}S]methionine for 5 h, and total cytoplasmic extracts were immunoprecipitated with rabbit anti-Gag2 antiserum (33). Samples were analyzed on an SDS–9% polyacrylamide gel and exposed to Kodak XAR-5 film.

Immunofluorescence assays were performed as previously described (33). At different intervals ranging from 24 to 72 h after transfection, cells were fixed for 20 min in cold methanol and incubated with rabbit anti-Gag2 antibody at 37°C for 45 min. Slides were then washed and incubated with the secondary antibody (fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin G;

TABLE 1. Properties of HA-tagged mutants

Virus	Infectivity[a]	RNA and DNA binding[b]	Nuclear transport[c]
wt	++++	++++	++++
H1	−	+	++++
H2	++++	ND[d]	++++
H3	−	+++	−
H4	++++	ND	ND
H5	+++	+++	ND
H6	++	ND	ND
H3RR	+++	ND	+/−

[a] Virus obtained from transfected cells was used to infect FAB cells (43), and blue (β-galactosidase-positive) cells were scored. ++++, titer of ~10^5 infectious units (IU)/ml, +++, titer of 10^4 IU/ml, ++, titer of ~10^3 IU/ml; titer of −, <1 IU/ml.

[b] Measured in the filter binding assay.

[c] Monitored at 24 to 72 h after transfection of BHK cells. The peak of nuclear staining, when it occurred, was observed at 30 to 45 h, as previously reported (33).

[d] ND, not determined.

Dako, Hamburg, Germany). Although only a few stained cells are shown per picture, they are typical of the results of cells transfected with a given plasmid.

RESULTS

Construction of NC mutants. Unlike the case for other retroviruses, the carboxyl terminus of Gag protein (herein designated the NC domain) in foamy virus does not contain Cys-His boxes. Instead, three short stretches of highly conserved GR boxes are found (Fig. 1). To investigate the roles of these GR boxes in viral replication, we substituted each box separately with an HA epitope tag derived from the influenza virus HA protein. Besides the epitope sequences, this 11-amino-acid tag contains two additional methionines at its N terminus to allow the labeling of NC with [^{35}S]methionine, as no methionine or cystine residues are present in this protein domain. Additional mutants were made by inserting this HA tag at the flanking region between each GR box (Fig. 1). Since it has been difficult to obtain a good specific antibody against the NC domain of HFV, one goal of this study was to find a replication-competent virus with an NC domain containing an HA tag that could be recognized by monoclonal antibody 12CA5 (Boehringer Mannheim, Indianapolis, Ind.) in order to examine its biological and biochemical properties.

Biological properties of the NC mutants. To test for replication competence, full-length proviral DNA from wt or NC mutants was transfected into BHK-21 or FAB cells. The efficiency of transfection was roughly estimated by staining the FAB cells 36 to 48 h after transfection. In repeated experiments, transfection efficiencies for the various plasmids were very similar. Virus supernatant from each transfection was filtered and used to infect naive HEL or FAB cells to assay for infectivity (43). As shown on Table 1, substitution of GR box I (H1) or II (H3) with an HA tag was deleterious to virus replication, although replacement of GR box III (H5) reduced virus production only about 10-fold. Virus production was not affected when an HA tag was inserted between GR boxes I and II (H2) or II and III (H4). However, insertion of an HA tag into the C terminus of the NC domain (H6) reduced virus production about 30- to 50-fold, indicating that the carboxyl terminus of NC may be important for viral replication or that the insertion of the HA tag has impaired protein folding of NC for its optimal configuration.

Immunoprecipitation was performed on transfected cells with a rabbit antiserum against the central domain of Gag protein (anti-Gag2 antibody). As shown in Fig. 2, most of the NC mutant-transfected as well as wt-transfected cells produced a 78-kDa Gag precursor protein as well as its 74-kDa cleavage product. The 74-kDa protein is barely detectable in cells transfected with either the H1 or H3 mutant. However, in other experiments using either FAB cells or BHK cells expressing Bel1 protein (data not shown), we have found normal cleavage of the Gag protein. Since Bel1-expressing cells express higher levels of viral products, and FAB cells were selected for high long terminal repeat expression, efficient cleavage seems to depend on the level of viral expression. It is unclear why mutations made at GR box I or II would affect proteolytic cleavage of the Gag precursor protein. In cells transfected with the H6 mutant, which replicated poorly, both Gag-reacting proteins migrated more slowly on SDS-polyacrylamide gels than wt. The difference in gel mobility thus suggests that insertion of the HA tag at the C terminus of NC in the H6 mutant has probably affected the proper protein structure of the NC domain, and this could be responsible for the lowered infectivity.

Bacterial expression and purification of NC proteins. Amino acid substitutions made in GR box I (H1) or II (H3) effectively abolished viral production, suggesting that these highly conserved motifs are important for viral replication. To investigate the biochemical properties of HFV NC and the effects of mutations in GR box I or II, we prepared large quantities of purified proteins by using a bacterial expression system. Plasmids were constructed in a T7 expression vector in which one methionine and six consecutive histidine residues were placed at the amino terminus of NC. The addition of the 6xHis tag to NC allows affinity purification of the protein from Ni-NTA resins. Protein expression and purification in IPTG-induced bacteria were evaluated by SDS-polyacrylamide gel electrophoresis (PAGE). Coomassie-blue stained gels showed that after washing and eluting from the Ni-NTA column, one single major band was found for wt NC, migrating at ~30 kDa (Fig. 3). The minor background bands were then removed by FPLC purification (data not shown). The calculated molecular mass for this 189-amino-acid recombinant NC protein is ca. 21 kDa. The slower mobility of NC on SDS-polyacrylamide gels suggests that the protein may have been subjected to some posttranslational modifications. We also found that the purified NC protein could easily form a dimer in vitro, as it migrated at a position of ~58 kDa on nondenaturing gels (data not shown). All of the purified mutant proteins also migrated at positions similar to that of wt on SDS-polyacrylamide gels, and all formed dimers when assayed on nondenaturing gels (data not shown), indicating no gross change of protein structure in any of these mutants. Because the proteins could be purified under nondenaturing conditions, their native structure should be maintained and therefore could be used in a variety of biochemical analyses.

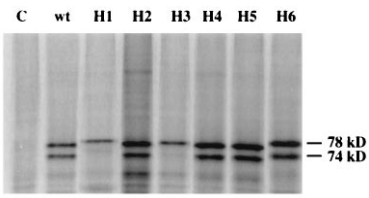

FIG. 2. Analysis of the Gag proteins in wt- or mutant-transfected cells. Two days after transfection of BHK-21 cells with wt or mutant proviral DNA, cells were labeled with [^{35}S]methionine and cells extracts were immunoprecipitated with rabbit anti-Gag2 antiserum. Both 78- and 74-kDa Gag-reacting proteins are indicated at the right. C, control.

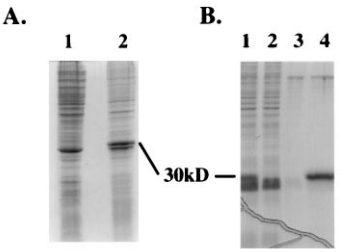

FIG. 3. SDS-PAGE analysis of recombinant 6xHis-tagged HFV NC proteins. Proteins are visualized by Coomassie blue staining. (A) Comparison of cells transformed with control plasmid (vector only; lane 1) and wt NC plasmid (lane 2). The specific band for NC proteins migrates at ~30 kDa. (B) Analysis of the NC proteins after purification on Ni-NTA resin. Lane 1, total lysates from the bacteria transformed with wt NC plasmid; lane 2, flowthrough after binding of the cell extracts to Ni-NTA resins; lane 3, sample after wash; lane 4, protein eluted from Ni-NTA resin.

RNA and DNA binding properties of NC proteins. We first used nitrocellulose filter binding to assay the nucleic acid binding activity of the NC proteins. This method is based on the finding that protein, but not RNA or DNA, binds to nitrocellulose at low to moderate ionic strength (36). Therefore, it is logical to assume that a single protein molecule bound to a radioactive RNA or DNA molecule is sufficient to cause retention of the labeled probe on the membrane. We incubated a set amount of ^{32}P-labeled RNA, dsDNA, or ssDNA probe with increasing amounts of the purified NC protein. The mixtures were then filtered through nitrocellulose membranes, and retention of protein-bound RNA or DNA probe was determined by the radioactive counts left on the filters after subtraction of the background (determined by use of bovine serum albumin). The results from a typical experiment using wt NC are shown in Fig. 4A.

The protein concentration at which half-maximal binding of viral sequence-specific RNA probe to NC occurs is about 15 to 30 ng/50 μl of reaction mixture (ca. 14 to 28 nM). The filter binding assay can be used to obtain an approximate association constant (K_A) for a simple reaction $NC_{free} + RNA_{free} \rightleftharpoons$ NC-RNA. The constant is given by the equation K_A = [NC-RNA]/[NC_{free}] [RNA_{free}]. Since in our assay condition [NC_{total}] is much greater than [RNA_{total}], [NC_{free}] is approximately equal to the total [NC] added to the reaction. Thus, the apparent K_A is simply the reciprocal of the concentration of total protein at which one half of the RNA probe is bound to the filter, i.e., when [RNA_{free}] = [NC-RNA]. According to this calculation, an apparent association constant of NC for the HFV-specific RNA probe is ca. 3.5×10^7 to 7.1×10^7 M^{-1}. Our results also show that the NC protein can bind to dsDNA and ssDNA with an affinity similar to that for RNA (Fig. 4A). No significant difference was found on the binding affinity of NC to non-HFV-derived RNA or DNA probes, either from the *neo* gene or from the Rous sarcoma virus Ψ sequences (data not shown). However, we cannot conclude from these experiments that the NC domain does not preferentially bind to any viral sequence. Such information will require additional assays.

We next tested the nucleic acid binding activity of several of the mutant proteins. As shown in Fig. 4B, the H3 and H5 mutant proteins bind to RNA efficiently, with a binding affinity slightly lower than that of wt NC. Mutation at GR box I (H1) caused a greatly retarded binding activity for both RNA (Fig. 4B) and DNA (data not shown), with an apparent association constant of 2×10^6 M^{-1}. Significant binding was seen only when a large amount of purified H1 protein was included in the assay. With this vast amount of protein in the mixture, binding is probably a reflection of nonspecific association of nucleic acids and proteins. When the protein containing double mutations of GR boxes I and II (H13) was assayed, its apparent association constant was ca. 1.5×10^6 M^{-1} (data not shown), close to that of H1. Therefore, our results indicate that GR box I is responsible for the binding ability of NC to nucleic acids and thus may play an important role in viral replication. Nonetheless, nucleic acid binding activity was not affected when mutations were introduced into GR box II (H3), implying that some other essential function is associated with this domain of NC.

Absence of nuclear transport of Gag in the H3 mutant. Recently, a nuclear localization function has been mapped to the GR box II by using a vaccinia virus expression system (33). This motif was also found to be able to direct heterologous proteins into the nucleus. However, it is not known whether this transient transport of Gag protein to the nucleus is essen-

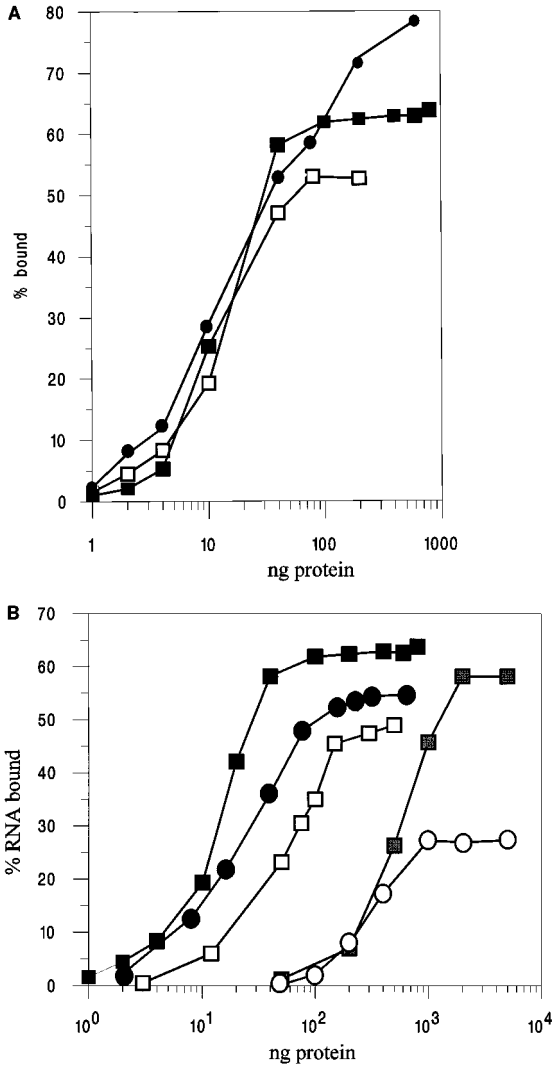

FIG. 4. Nucleic acid binding activity of the HFV NC proteins. Binding was measured by a nitrocellulose filter binding assay using 0.01 pmol of ^{32}P-labeled RNA or DNA probe (with a specific activity of 2×10^8 to 5×10^8 cpm/μg) and various concentrations of purified NC proteins. (A) Binding of wt NC proteins to virus-specific RNA (closed squares), dsDNA (open squares), and ssDNA (circles) probes. (B) RNA binding activity of wt (closed squares), H1 (gray squares), H3 (closed circles), H13 (open circles), and H5 (open squares).

tial for HFV replication. We thus transfected the proviral DNA containing wt and the H3 mutant into BHK-21 cells and examined the distribution of Gag within transfected cells in an immunofluorescence assay. As shown in Fig. 5, while both H1 and H2 mutants translocated their Gag proteins to the cell nucleus 36 to 48 h after transfection, no nuclear staining of the Gag protein was detected in the cells transfected with the H3 mutant. Our results are consistent with those reported previously (33) showing that the nuclear transport function of Gag protein is located in GR box II in NC.

Attempts to isolate revertants from the replication-defective H1 and H3 mutants. No productive viral replication was detected with either the H1 or the H3 mutant, which implies that both functions of nucleic acid binding and nuclear transport activity of NC are important for HFV infection. To directly assess this question, we tried to recover the replication-competent revertants from these mutants by passing the transfected cells extensively to search for emergence of CPE. No CPE was found when H1-transfected cells were subcultured for several months, strongly suggesting an important role of nucleic acid binding activity of NC in HFV replication.

In contrast, revertants were readily recovered from the H3 mutant. Severe CPE was found within 3 weeks of passaging the transfected cells. The filtered supernatant from this revertant culture was able to induce CPE efficiently when used to infect fresh HEL cells. Viral RNA was extracted from the H3 revertant virions, and the DNA fragment encoding the NC domain was amplified by reverse transcription-PCR and sequenced. We found an in-frame deletion (nt 2074 to 2129) (double lines in Fig. 1) flanking the HA tag sequences that were used to replace GR box II in the H3 mutant. This PCR-amplified NC fragment was then used to replace the same region in wt pHFV13 to generate a proviral clone of the H3 revertant, called H3RR. Surprisingly, a relatively high titer of virus was produced after transfection of H3RR DNA (Table 1). The results from immunofluorescence assays show that there is little nuclear staining of Gag protein in cells infected with the H3 revertant viruses (data not shown) or transfected with the infectious clone H3RR (Fig. 6). The revertant does not transport Gag protein into the nucleus efficiently but can still replicate fairly well in tissue culture, which suggests that nuclear localization of Gag protein is not absolutely required for HFV replication.

DISCUSSION

In the case of oncoviruses and lentiviruses, the Gag precursor protein is cleaved by virally encoded protease into at least three smaller proteins, MA (which interacts with the surface glycoproteins), CA (which forms the capsid), and NC (which binds to encapsidated RNA). The Gag protein contains all of the determinants for specific recognition of the packaging signal on the viral RNA (29). Genetic analyses of NC mutants have shown that the Cys-His box(es) and the flanking basic residues are important for the encapsidation of viral genomic RNA (5, 16). However, NC alone is a nonspecific RNA-binding protein, and other domains of the Gag precursor protein may contribute to specific RNA encapsidation. Several in vitro activities have been described for retroviral NC protein. NC binds to both RNA and DNA, with a higher affinity to single-stranded than to double-stranded nucleic acid (13, 34, 37). It has been shown that nucleic acid binding can occur if both Cys-His boxes of human immunodeficiency virus type 1 (HIV-1) are deleted, but basic residues surrounding the boxes are important for in vitro binding (7). The important role of these basic residues for NC's binding to nucleic acids may be due to their positively charged side chain interacting with the phosphate group of the RNA backbone. NC is also involved in positioning primer tRNA and promotes dimerization and DNA strand transfer in vitro (18, 39).

In this study, the region of the HFV Gag protein which corresponds in position to onco- and lentiviral NC was expressed from *E. coli* in a nondenatured form. Using biochemical analyses, we have demonstrated here that as in HIV and the other retroviruses, the C-terminal portion of HFV Gag protein encodes a nucleic acid binding domain. These purified HFV NC protein possesses nonspecific RNA and DNA binding activities in vitro. The binding affinities of HFV NC protein to RNA, ssDNA, and dsDNA are very similar, with an apparent association constant of 10^7 to 10^8 M^{-1}. The striking feature in the carboxyl-terminal portion of HFV Gag protein is the presence of three GR boxes that are highly conserved in all sequenced primate foamy viruses (33). This glycine-arginine-rich motif is similar to the RGG box found in many sequence-nonspecific RNA-binding proteins, although HFV NC does not contain any typical RGG triplets (3). We have mapped the nucleic acid binding domain to GR box I but not box II or III (Fig. 1 and Table 1). In this respect, HFV Gag more closely resembles the C protein of the hepadnaviruses such as HBV. The C terminus of HBV C protein contains four clusters of three to four arginine residues. Such arginine-rich motifs are also found in several RNA-binding proteins such as HIV-1 Rev and Tat (3). In HBV, the proximal repeat is involved in RNA binding, while the distal repeats function in formation of relaxed circle genomic DNA, possibly by binding to the DNA strands (15, 26). Thus far, we have only made one substitution mutant of GR box I whose nucleic acid binding ability has been severely reduced. Further dissection of this region by using smaller substitution or deletion will be required to determine whether the RNA and DNA binding activities are separable.

In tissue culture, cells infected by foamy virus are characterized by a strong nuclear staining when reacted with sera from infected hosts (10, 33). It has been shown that transient nuclear localization of Gag protein may account for the typical nuclear staining of infected cells. By using vaccinia virus expression vectors, nuclear localization has been mapped to GR box II (33). We have extended these studies by using the GR box mutants in the context of the full-length HFV genome. The H3 mutant, which contains an 11-amino-acid substitution of GR box II with an influenza virus HA tag, does not transport Gag into nucleus during infection and does not produce infectious virus. Our data thus confirm that nuclear transport activity is located in GR box II. Several hypotheses have been proposed for the transient nuclear localization of Gag in HFV-infected cells (33). If nuclear transfer of Gag is involved in critical steps such as RNA packaging or viral assembly occurring in the nucleus, or a Gag protein functions in regulation of viral gene expression, such localization would be required for viral infection (31). However, this is not consistent with our results. A spontaneous replication-competent revertant of H3 mutant was isolated and found to lack GR box II. This revertant (H3RR) exhibits little nuclear localization of Gag compared with wt yet replicates fairly efficiently in tissue culture. Our data thus suggest that nuclear localization of the Gag proteins in HFV-infected cells is not completely required for viral replication. The complete loss of infectivity in the H3 mutant is probably due to interruption of the global structure of the Gag protein by replacing GR box II with an HA tag, which disrupts some as yet undetermined function of Gag.

Although the genome structure of foamy virus is similar to that of other retroviruses, replication of HFV is distinct from oncoviruses or lentiviruses. Some aspects of HFV replication

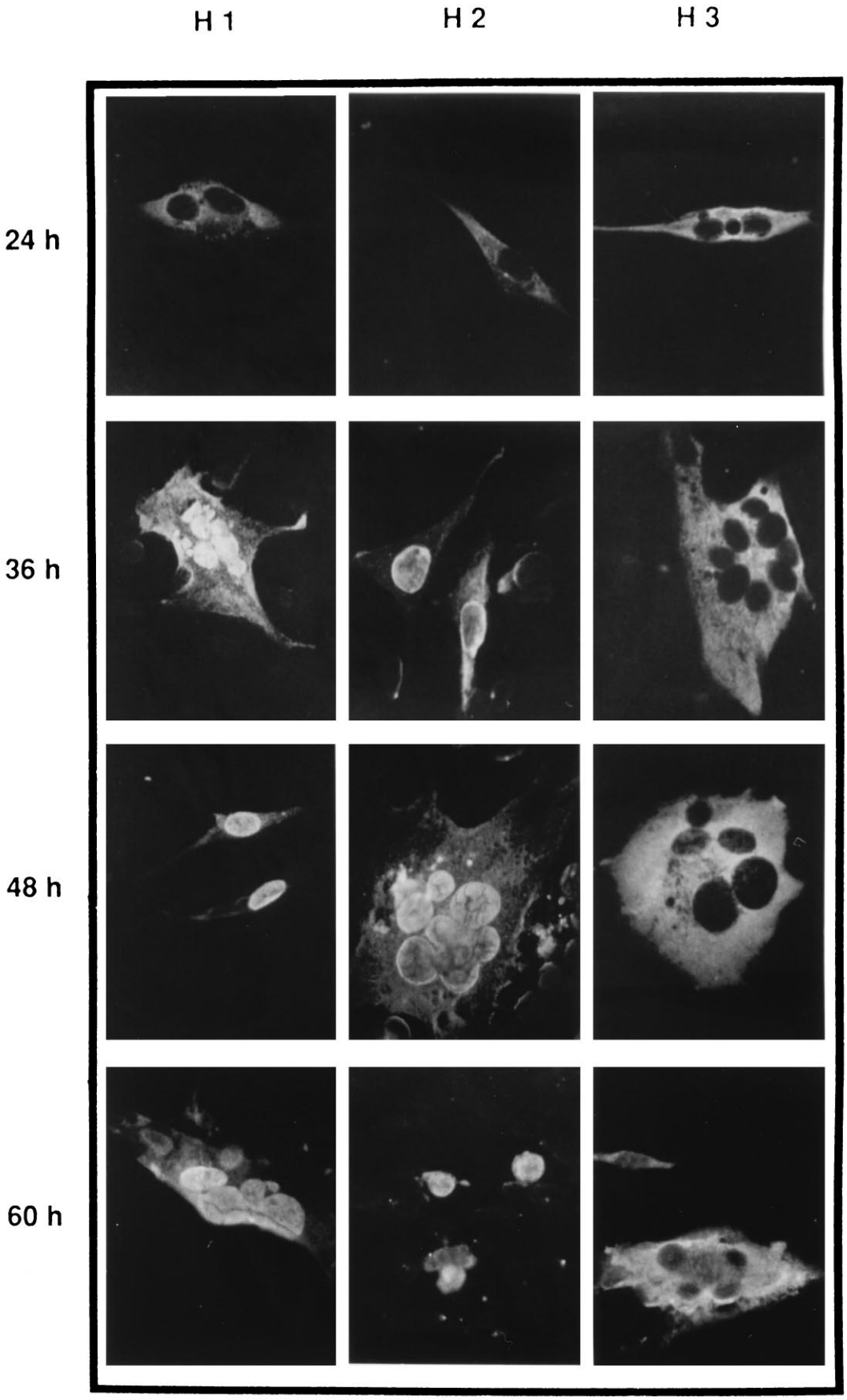

FIG. 5. Subcellular distribution of the HFV Gag protein in BHK-21 cells transfected with H1, H2, or H3 mutant-containing plasmids. After transfection, cells were subjected to immunofluorescence assays using rabbit anti-Gag2 antiserum.

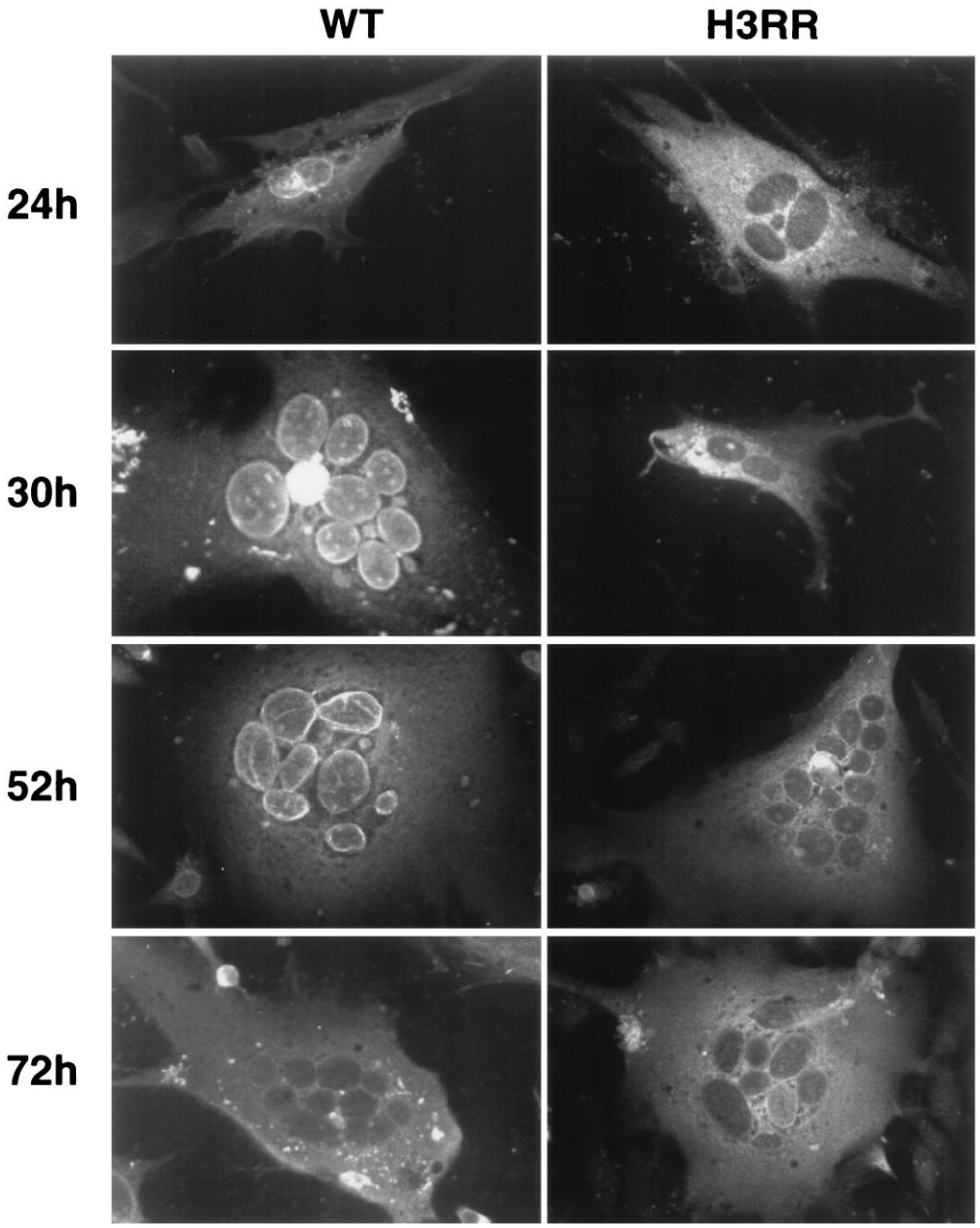

FIG. 6. Subcellular distribution of the HFV Gag protein in BHK-21 cells transfected with plasmid encoding wt virus or the H3 revertant (H3RR). Transfected cells were subjected to immunofluorescence assays using rabbit anti-Gag2 antiserum.

are more reminiscent of the hepadnaviruses than the retroviruses. For example, the Gag precursor protein in the extracellular particles of HFV is not cleaved into mature proteins, Pol is expressed from a spliced mRNA lacking Gag sequences rather than as a Gag-Pol fusion protein, and at least 10% of extracellular HFV particles contain provirus-length dsDNA (42). We now show that like the C protein of HBV, the carboxyl terminus of HFV Gag binds to nucleic acids through an arginine-rich domain. Thus, HFV Gag is probably a multifunctional protein like the C protein of HBV. It is likely that the Gag protein of HFV has domains that interact with the viral glycoprotein akin to MA, form the capsid structure akin to CA, and interact with the viral genome through its carboxyl end akin to NC. The fact that the HFV NC domain interacts equally well with RNA and dsDNA in a sequence-independent manner is consistent with the finding of both RNA and dsDNA in virions. It is possible that the HFV NC domain functions similarly to the HBV C protein and binds to an RNA pregenome and subsequently to the DNA genome which is actively reverse transcribed within particles. However, there is not as yet any evidence that the provirus-length dsDNA in HFV extracellular virions is infectious or required for infection.

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REFERENCES

1. **Achong, B. G., W. A. Mansell, M. A. Epstein, and P. Clifford.** 1971. An unusual virus in cultures from a human nasopharyngeal carcinoma. J. Natl. Cancer Inst. **46:**299–307.
2. **Bieniasz, P. D., A. Rethwilm, R. Pitman, M. D. Daniel, I. Chrystie, and M. O. McClure.** 1995. A comparative study of higher primate foamy viruses, including a new virus from a gorilla. Virology **207:**217–228.
3. **Burd, C. G., and G. Dreyfuss.** 1994. Conserved structures and diversity of functions of RNA-binding proteins. Science **265:**615–621.
4. **Craven, R. C., A. E. Leure-duPree, R. A. Weldon, Jr., and J. W. Wills.** 1995. Genetic analysis of the major homology region of the Rous sarcoma virus Gag protein. J. Virol. **69:**4213–4227.
5. **Dannull, J., A. Surovoy, G. Jung, and K. Moelling.** 1994. Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. EMBO J. **13:**1525–1533.
6. **Davis, N. L., and R. R. Rueckert.** 1972. Properties of a ribonucleoprotein particle isolated from nonidet P-40-treated Rous sarcoma virus. J. Virol. **10:**1010–1020.
7. **de Rocquigny, H., C. Gabus, A. Vincent, M. C. Fournie-Zaluski, B. Roques, and J. L. Darlix.** 1992. Viral RNA annealing activities of human immunodeficiency virus type 1 nucleocapsid protein require only peptide domains outside the zinc fingers. Proc. Natl. Acad. Sci. USA **89:**6472–6476.
8. **Facke, M., A. Janetzo, R. L. Shoeman, and H.-G. Krausslich.** 1993. A large deletion in the matrix domain of the human immunodeficiency virus *gag* gene redirects virus particle assembly from the plasma membrane to the endoplasmic reticulum. J. Virol. **67:**4972–4980.
9. **Field, J., J. I. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler.** 1988. Purification of a *RAS*-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. **8:**2159–2165.
10. **Fleming, W. A., and J. K. Clarke.** 1970. Fluorescence assay of foamy virus. J. Gen. Virol. **6:**277–284.
11. **Flugel, R. M.** 1992. Spumaviruses: a group of complex retroviruses. J. Acquired Immune Defic. Syndr. **4:**739–759.
12. **Flugel, R. M., A. Rethwilm, B. Maurer, and G. Darai.** 1987. Nucleotide sequence analysis of the env gene and its flanking regions of the human spumaretrovirus reveals two novel genes. EMBO J. **6:**2077–2084.
13. **Gelfand, C. A., Q. Wang, S. Randall, and J. E. Jentoft.** 1993. Interactions of avian myeloblastosis virus nucleocapsid protein with nucleic acids. J. Biol. Chem. **268:**18450–18456.
14. **Gorelick, R. J., S. M. Nigida, Jr., J. W. Bess, Jr., L. O. Arthur, L. E. Henderson, and A. Rein.** 1990. Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA. J. Virol. **64:**3207–3211.
15. **Hatton, T., S. Zhou, and D. N. Standring.** 1992. RNA- and DNA-binding activities in hepatitis B virus capsid protein: a model for their roles in virus replication. J. Virol. **66:**5232–5241.
16. **Housset, V., H. de Rocquigny, B. P. Roques, and J. L. Darlix.** 1993. Basic amino acids flanking the zinc finger of Moloney murine leukemia virus nucleocapsid protein NCp10 are critical for virus infectivity. J. Virol. **67:** 2537–2545.
17. **Katz, R. A., and J. E. Jentoft.** 1989. What is the role of the Cys-His motif in retroviral nucleocapsid (NC) proteins? Bioessays **11:**176–181.
18. **Khan, R., and D. P. Giedroc.** 1992. Recombinant human immunodeficiency virus type 1 nucleocapsid (NCp7) protein unwinds tRNA. J. Biol. Chem. **267:**6689–6695.
19. **Konvalinka, J., M. Lochelt, H. Zentgraf, R. M. Flugel, and H.-G. Krausslich.** 1995. Active spumavirus proteinase is essential for virus infectivity but not for formation of the Pol polyprotein. J. Virol. **69:**7264–7268.
20. **Linial, M. L., and A. D. Miller.** 1990. Retroviral RNA packaging: sequence requirements and implications. Curr. Top. Microbiol. Immunol. **157:**125–152.
21. **Lochelt, M., R. M. Flugel, and M. Aboud.** 1994. The human foamy virus internal promoter directs the expression of the functional *bel1* transactivator and Bet protein early after infection. J. Virol. **68:**638–645.
22. **Lochelt, M., W. Muranyi, and R. M. Flugel.** 1993. Human foamy virus genome possesses an internal, bel-1 dependent and functional promoter. Proc. Natl. Acad. Sci. USA **90:**7317–7321.
23. **Lochelt, M., H. Zentgraf, and R. M. Flugel.** 1991. Construction of an infectious DNA clone of the full-length human spumaretrovirus genome and mutagenesis of the bel 1 gene. Virology **184:**43–54.
24. **Mammano, F., A. Ohagen, S. Hoglund, and H. G. Gottlinger.** 1994. Role of the major homology region of human immunodeficiency virus type 1 in virion morphogenesis. J. Virol. **68:**4927–4936.
25. **Meric, C., and S. P. Goff.** 1989. Characterization of Moloney murine leukemia virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein. J. Virol. **63:**1558–1568.
26. **Nassal, M.** 1992. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. J. Virol. **66:**4107–4116.
27. **Nassal, M., and H. Schaller.** 1993. Hepatitis B virus replication. Trends. Microbiol. **1:**221–228.
28. **Neumann-Haefelin, D., U. Fleps, R. Renne, and M. Schweizer.** 1993. Foamy viruses. Intervirology **35:**196–207.
29. **Oertle, S., and P. F. Spahr.** 1990. Role of the Gag polyprotein precursor in packaging and maturation of Rous sarcoma virus genomic RNA. J. Virol. **64:**5757–5763.
30. **Pepinsky, R. B., and V. M. Vogt.** 1984. Fine-structure analyses of lipid-protein and protein-protein interactions of Gag protein p19 of the avian sarcoma and leukemia viruses by cyanogen bromide mapping. J. Virol. **52:** 145–153.
31. **Rethwilm, A.** 1995. Regulation of foamy virus gene expression. Curr. Top. Microbiol. Immunol. **193:**1–24.
32. **Rethwilm, A., O. Erlwein, G. Baunach, B. Maurer, and V. ter Meulen.** 1991. The transcriptional transactivator of human foamy virus maps to the bel 1 genomic region. Proc. Natl. Acad. Sci. USA **88:**941–945.
33. **Schliephake, A. W., and A. Rethwilm.** 1994. Nuclear localization of foamy virus Gag precursor protein. J. Virol. **68:**4946–4954.
34. **Smith, B. J., and J. M. Bailey.** 1979. The binding of an avian myeloblastosis virus basic 12,000 dalton protein to nucleic acids. Nucleic Acids Res. **7:**2055–2072.
35. **Srinivaskumar, N., M.-L. Hammarskjold, and D. Rekosh.** 1995. Characterization of deletion mutants in the capsid region of human immunodeficiency virus type 1 that affect particle formation and Gag-Pol precursor incorporation. J. Virol. **69:**6106–6114.
36. **Steeg, C. M., and V. M. Vogt.** 1990. RNA-binding properties of the matrix protein ($p19^{gag}$) of avian sarcoma and leukemia viruses. J. Virol. **64:**847–855.
37. **Surovoy, A., J. Dannull, K. Moelling, and G. Jung.** 1993. Conformational and nucleic acid binding studies on the synthetic nucleocapsid protein of HIV-1. J. Mol. Biol. **229:**94–104.
38. **Swack, N. S., and G. D. Hsiung.** 1975. Pathogenesis of simian foamy virus infection in natural and experimental hosts. Infect. Immun. **12:**470–474.
39. **Tsuchihashi, Z., and P. O. Brown.** 1994. DNA strand exchange and selective DNA annealing promoted by the human immunodeficiency virus type 1 nucleocapsid protein. J. Virol. **68:**5863–5870.
40. **Weldon, R. A., Jr., and J. W. Wills.** 1993. Characterization of a small (25-kilodalton) derivative of the Rous sarcoma virus Gag protein competent for particle release. J. Virol. **67:**5550–5561.
41. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral Gag proteins. AIDS **5:**639–654.
42. **Yu, S. F., D. N. Baldwin, S. R. Gwynn, S. Yendapalli, and M. L. Linial.** 1996. Human foamy virus replication—a pathway distinct from that of retroviruses and hepadnaviruses. Science **271:**1579–1582.
43. **Yu, S. F., and M. L. Linial.** 1993. Analysis of the role of the *bel* and *bet* open reading frames of human foamy virus by using a new quantitative assay. J. Virol. **67:**6618–6624.
44. **Yu, X., X. Yuan, Z. Matsuda, T.-H. Lee, and M. Essex.** 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J. Virol. **66:**4966–4971.