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Characterization of Moloney Murine Leukemia Virus Mutants with Single-Amino-Acid Substitutions in the Cys-His Box of the Nucleocapsid Protein

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To study the function of the retroviral nucleocapsid protein (NC), we have constructed point mutations in the *gag* gene of Moloney murine leukemia virus (MuLV) that affect a conserved cysteine-histidine motif of NC. The mutants were characterized biologically and biochemically. Cell lines producing the mutant virions were constructed in NIH 3T3 and rat2 cells, and the viral particles released by these cells were characterized for protein and RNA content. The results indicated that most mutations block replication and specifically inhibit the packaging of the MuLV genomic RNA. In some of the mutants, the packaging of the endogenous rat VL30 RNA was not affected as profoundly as was MuLV RNA. NC also seems to have another function distinct from dimer formation and packaging: one mutation reduced viral RNA packaging by only fivefold but completely abolished viral cDNA synthesis, suggesting a defect in reverse transcription.

The *gag* gene of retroviruses encodes proteins necessary for the assembly of the virion particle and for the encapsidation of the viral RNA genome. The gene is expressed in the form of a polyprotein precursor which assembles under the plasma membrane (2) and which is then proteolytically processed to give rise to a number of smaller proteins found in the mature virion. The precursor of Moloney murine leukemia virus (M-MuLV), termed $Pr65^{gag}$, is cleaved to yield four proteins: MA, the matrix-associated protein; p12, a protein of unknown function; CA, the major capsid protein; and NC, the nucleocapsid protein (30). The viral RNA genome is packaged into the virions in a highly condensed form as a 70S dimer (5, 6). Genetic studies have shown that portions of MA and CA are essential for virion particle formation or release; mutations in MA and CA often abolish virion release (22, 42, 46). In contrast, much of the p12 and NC domains seems to be dispensable for assembly, and mutations in these domains generally do not block virion release (10).

M-MuLV NC is a highly basic protein of only 60 amino acids, encoded by the carboxy-terminal domain of the *gag* gene. In the virions, NC is tightly associated with the genomic RNA (13). Purified NC binds nucleic acids in vitro, but this binding shows no clear specificity for particular nucleic acid sequences (11, 40, 45). The phenotype of mutants constructed in Rous sarcoma virus (RSV) NC indicate that this protein is necessary for viral RNA packaging and is involved in the formation of the dimer linkage (37). Recent results obtained in vitro suggest that NC plays a catalytic role in the dimerization of the viral RNA and is necessary for tRNA primer hybridization to the primer binding site (41).

All retroviral NC proteins sequenced so far possess a conserved motif (8) of three cysteine residues and one histidine residue which we refer to as the Cys-His box (see Fig. 1). If the position of the N terminus-proximal cysteine residue is designated n, there is another cysteine residue at $n+3$, a histidine residue at $n+8$, and a third cysteine residue at $n+13$. The Cys-His box pattern can thus be schematized by the formula $CysX_2CysX_4HisX_4Cys$. A glycine residue at $n+7$ is also highly conserved. Most of the Cys-His boxes also contain one or two aromatic amino acids, the first at position $n+1$ or $n+2$ and the second one at $n+9$ (9). In the case of M-MuLV, the latter has been shown to intercalate within the bases of the bound nucleic acid (23). MuLV NC contains only one Cys-His box, while two copies of similar but nonidentical boxes are found in tandem in most of the other retroviral NC proteins. A sequence comparison of the two Cys-His boxes and the phenotypes of RSV NC deletion mutants indicate that the amino terminus-distal box is a degenerated copy of the proximal box (36). The Cys-His box motif is also found in corresponding regions of human immunodeficiency virus, in a *Drosophila* copia clone (38), and in the coat protein of cauliflower mosaic virus (9). A similar pattern also exists in bacteriophage T4 gene 32 product, a single-stranded DNA-binding protein (53).

By analogy to the "zinc fingers" present in many regulatory DNA-binding proteins, it has been proposed that the Cys-His box could bind zinc (1). This has been confirmed for T4 gene 32 product, where zinc has a structural function (15–17). NC affinity for zinc has been demonstrated in an in vitro assay (44), but no significant amounts of metal have been detected in virions (J. Leis, personal communication). Thus, only a subset of the NC proteins in a virion could contain Zn^{2+}, and it remains unclear whether bound metal ions have any role in NC function.

The conservation of the Cys-His box motif in viruses as evolutionarily distant as cauliflower mosaic virus and human immunodeficiency virus strongly suggests that this domain is critical for replication. To probe the functions of this protein in greater detail, we have constructed a series of mutations with single-amino-acid substitutions in a cloned copy of the M-MuLV DNA and have analyzed the effects of the mutations on viral replication. We have constructed point mutations affecting four conserved amino acids of the Cys-His box: the tyrosine residue at $n+2$, the glycine residue at $n+7$, the tryptophan residue at $n+9$, and as a control, the aspartic acid residue at $n+12$. Producer cell lines were constructed in

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NIH 3T3 and rat2 cells, and the phenotypes of the mutants were characterized. The results indicate that most mutations block viral replication and specifically inhibit the packaging of MuLV genomic RNA. NC also seems to have another function distinct from packaging: the replacement of the tyrosine residue at $n+2$ by a serine residue reduced viral RNA packaging by only fivefold but completely abolished viral cDNA synthesis, suggesting a defect in reverse transcription.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 fibroblasts, rat2 cells (50), XC cells (43), and M23 cells (48) were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% calf serum (HyClone Laboratories, Logan, Utah) at 37°C in an atmosphere containing 5% CO_2. NIH 3T3 or rat2 cells transfected with pNCA (7) were the source of wild-type virus. Virus infections were carried out in the presence of 8 μg of Polybrene per ml for 6 h.

Cloned DNAs. pNCA contains a nonpermuted copy of M-MuLV cDNA with two long terminal repeats (7). Plasmid pSV2neo contains the *Escherichia coli* kanamycin resistance gene linked to a simian virus 40 promoter (49). The rat VL30 clone was a gift from Philip Tsichlis (55).

Construction of the mutants. All of the mutations were constructed in a 2,265-base-pair *Pvu*II-*Sal*I subclone of MuLV in M13mp18 (54), kindly provided by Kevin Felsenstein (12).

The following oligonucleotides were synthesized: CM10 (Tyr-2→Ser), 5′ CTTTGCAGGAGGCACAC 3′; CM11 (Gly-7→Val), 5′ CCCAGTGCACCTTTTC 3′; CM12 (Trp-9→Leu), 5′ CTTTAGCCAAGTGCCCC 3′; CM13 (Asp-12→Asn), 5′ GGGACAATTTTTAGCCC 3′; CM17 (Tyr-2→Gly), 5′ CCCTTTTCTTTGCAGCCGGCACACTGGTCG 3′; CM18 (Trp-9→Gly), 5′ GGGACAATCTTTAGCGCCGTG CCCCTTTTCTTTGC 3′.

Three mutations (Tyr-2→Ser, Trp-9→Leu, and Asp-12→Asn) were constructed as described by Zoller and Smith (56); candidate bacterial clones were screened by differential hybridization. The high-efficiency selection method described by Kunkel (28) and commercialized by Bio-Rad Laboratories (Richmond, Calif.) was used for the other mutants according to the manual. The mutagenized regions were sequenced from the *Ava*I site in NC by the Maxam and Gilbert technique (35), and the full-length plasmids were reconstructed in pNCA by using the *Sal*I and *Xho*I sites.

Bacterial cultures. *E. coli* JM101 was grown on minimal medium supplemented with 1 mM thiamine hydrochloride (32). *E. coli* SJ256 (*dut ung thi relA*) was grown according to the instructions of the mutagenesis kit (Bio-Rad). Selective plates and media contained 50 μg of ampicillin per ml. Plasmids were isolated by the alkaline lysate method, and in the case of small cultures (2 ml), the DNA was used without further purification. In the case of large cultures (250 ml), the DNA was purified by equilibrium density gradient centrifugation in cesium chloride-ethidium bromide (32).

Mammalian cell transfections and transformations. Transfections of mammalian cells by viral DNA to test for infectivity were performed in the presence of DEAE-dextran, using 5×10^5 cells per 10-cm petri dish. At confluency, medium was harvested and the cells were trypsinized and plated at a 1:20 dilution. When the cells were confluent again, 3 to 5 days later, the medium was harvested and the cells were UV treated and overlaid with XC cells (43). Reverse transcriptase activity in the culture medium was measured as described previously (18).

Cotransformation (52) of rat2 cells with viral DNA and pSV2neo DNA was performed in the presence of poly-L-ornithine (Sigma Chemical Co., St. Louis, Mo.) as described elsewhere (3). Threefold more viral DNA than pSV2neo was used for the cotransformation, and the cells were split and diluted 1 day after transfection before selection with G418 (400 μg/ml). NIH 3T3 cells were transformed by the calcium phosphate method as described previously (20).

Virus purification. A total of 100 to 160 ml of medium was harvested every 12 h for each cell line over a period of 2 to 3 days. The medium was collected when the cells were 80 to 100% confluent. Four milliliters of medium per harvest of 12 h was collected from each 100-mm plate and stored on ice during the harvesting period. Cellular debris was eliminated by centrifugation at 13,000 × *g* for 10 min, and a sample of the supernatant was stored at −70°C for reverse transcriptase assay. The virus was pelleted by centrifugation for 2 h at 22,000 rpm and 4°C in a Beckman SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.) (90,000 × *g*). The virus was suspended in 1 ml of TNE (50 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA) and further purified on a 25 to 45% sucrose–TNE step gradient in a Beckman SW41 rotor for 12 h at 35,000 rpm and 4°C (150,000 × *g*). The interface was harvested and diluted with TNE, and the virus was pelleted in an SW41 rotor for 3 h at 35,000 rpm and 4°C. The viral pellet was suspended in 200 μl of TNE, aliquoted, and stored at −70°C.

To prepare labeled viral nucleic acids, one confluent plate of each producer cell line was grown with 175 μCi of [5,6-^{3}H]uridine for 14 h in 4 ml of culture medium. Virions were purified as described above. The nucleic acids were extracted from the viral pellets in lysis buffer (100 mM NaCl, 50 mM Tris [pH 7.5], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS], 100 μg of proteinase K [Boehringer GmbH, Mannheim, Federal Republic of Germany] per ml, 50 μg of yeast or *E. coli* tRNA per ml) for 30 min at 37°C and fractionated on a 5 to 23% sucrose–TNE–1% SDS continuous gradient in an SW41 rotor (Beckman) at 40,000 rpm and 22°C for 1.75 h (200,000 × *g*). Fractions were harvested with a peristaltic pump and counted in a scintillation counter.

Analysis of viral proteins. Proteins were analyzed from virions purified as described above. The proteins were separated by polyacrylamide gel-SDS electrophoresis (29) and either stained with Coomassie blue or detected immunochemically on a Western blot (immunoblot) (4). To detect MuLV NC on a Western blot, the viral proteins size separated on a 13% polyacrylamide gel in the presence of SDS were electrotransferred onto 0.1-μm nitrocellulose (PH79, Schleicher & Schuell, Inc., Keene, N.H.) in 0.19 M glycine–0.025 M Tris base–45% methanol at 100 mA for 14 h in a Bio-Rad Transblot apparatus. Under normal conditions (0.45-μm BA85 nitrocellulose–20% methanol) NC does not seem to be retained on the membrane. The filter was UV irradiated for 2 min at 4 cm from a hand-held shortwave UV lamp (UVP, San Gabriel, Calif.) while wet. The filter was stained with 0.2% Ponceau S in 3% trichloroacetic acid (Corning Medical, Palo Alto, Calif.) NC and CA were detected by immunochemistry, using goat antisera (anti-Rauscher CA 77S-102 and anti-Rauscher NC 75S-725 [National Cancer Institute, Bethesda, Md.]) and a Vectastain Elite kit (Vector Laboratories, Burlingame, Calif.).

Analysis of packaged viral RNA. Purified virions were lysed in 100 mM NaCl–50 mM Tris [pH 7.5]–10 mM EDTA–

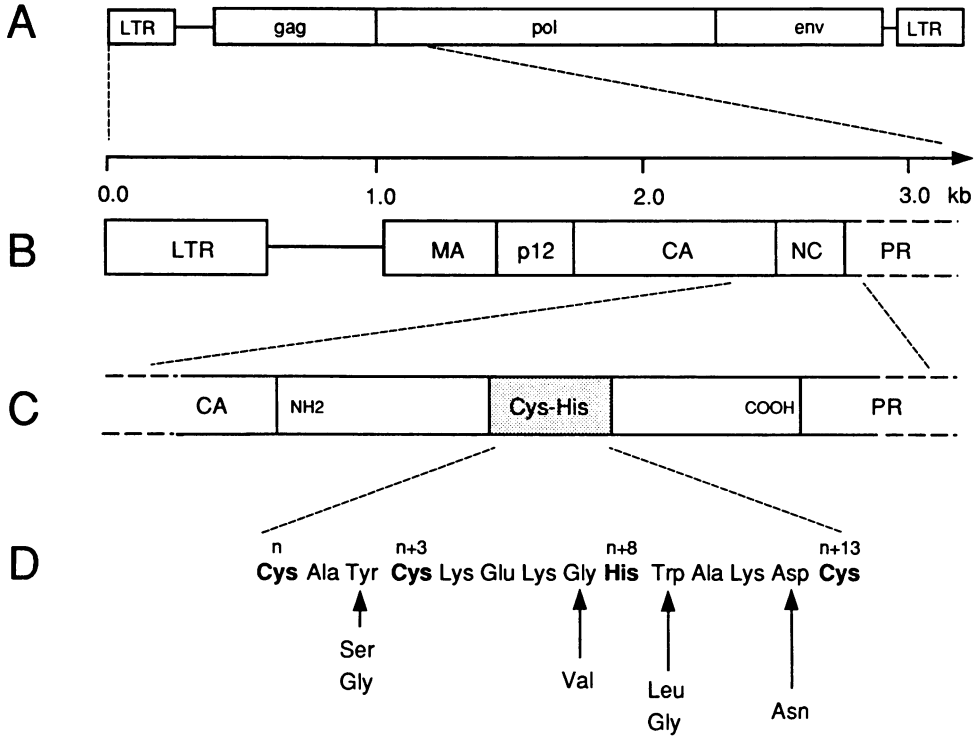

FIG. 1. (A) Complete genome of M-MuLV as a linear provirus. LTR, Long terminal repeat; kb, kilobase. (B) Enlargement of the *gag* gene and 5′ end of the *pol* gene; the new nomenclature for the proteins is used (30): MA, matrix protein (p15); CA, capsid protein (p30); NC, nucleocapsid protein (p10); PR, protease. (C) Schematic representation of MuLV NC (p10); the conserved Cys-His motif is indicated as a stippled box. (D) Amino acid sequence of the Cys-His box with the cysteine and histidine residues in bold face. The mutations are shown below the sequence, and the substituted residues are indicated by an arrow.

1% SDS–100 μg of proteinase K (Boehringer) per ml–50 μg of yeast or *E. coli* tRNA per ml for 30 min at 37°C, and the RNA was purified by two phenol-chloroform extractions, one chloroform extraction, and precipitation with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. The RNA pellet was dissolved in 1% SDS and stored at −70°C. The RNA was fractionated on a nondenaturing 0.8% agarose gel in the presence of 0.5 μg of ethidium bromide per ml and electrotransferred to a ZetaProbe nylon membrane (Bio-Rad) in 10 mM NaOH for 12 to 14 h at 200 mA and 4°C in a Bio-Rad transfer apparatus (modification of reference 51). The membrane was prehybridized for 12 h and hybridized with a nick-translated pNCA or VL30 probe for 12 h as described previously (26). The filter was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS at room temperature for 10 min and for 1 h at 65°C in 2× SSC–1% SDS. The RNA was detected by autoradiography.

To reprobe the filters, the labeled nucleic acid was removed by heating for 1 h at 90°C in 0.5× Denhardt solution–20 mM Tris (pH 7.5) –1% SDS. No remaining signal could be detected after an overnight exposure. The filter was then prehybridized again before hybridization.

Analysis of DNA synthesized in infected cells. Culture medium was harvested from rat2 producer cell lines; the virus concentration was estimated by the quick dot reverse transcriptase assay (18). The medium was then used immediately to infect confluent rat2 cells in the presence of 8 μg of Polybrene per ml.

Low-molecular-weight DNA was isolated from infected cells by the Hirt extraction procedure 36 h after infection (21). The DNA was purified by two phenol extractions, followed by two chloroform extractions, and was fractionated by electrophoresis on an 0.8% agarose gel. The DNA was blotted to nitrocellulose (BA85, Schleicher & Schuell) in 20× SSC and hybridized successively with nick-translated MuLV and VL30 probes as described above. The DNA was detected by autoradiography in the presence of an intensifying screen.

RESULTS

Construction of viral DNAs. To study the function of M-MuLV NC, we have mutated four amino acids of its single Cys-His box region: the two aromatic residues of the box, a tyrosine and a tryptophan residue; the conserved glycine residue; and the aspartic acid residue (Fig. 1). The tyrosine residue at $n+2$ was changed to either a serine (Tyr-2→Ser) or a glycine (Tyr-2→Gly) residue, and the tryptophan residue at $n+9$ was changed to either a leucine (Trp-9→Leu) or a glycine (Trp-9→Gly) residue. The highly conserved glycine residue at $n+7$ was replaced by a valine residue (Gly-7→Val), and the aspartic acid residue at $n+12$ was replaced by an asparagine residue (Asp-12→Asn). The mutations were chosen in order to preserve the potential metal-binding structure formed by the three cysteine residues and the histidine residue.

The mutations were constructed by oligonucleotide-mediated site-directed mutagenesis of an M13 subclone (see Materials and Methods). The mutagenized domain was sequenced, and when the presence of the expected mutation was confirmed, a fragment containing the mutation was isolated and used to replace the corresponding region of the plasmid pNCA containing a complete copy of the wild-type M-MuLV genome (7).

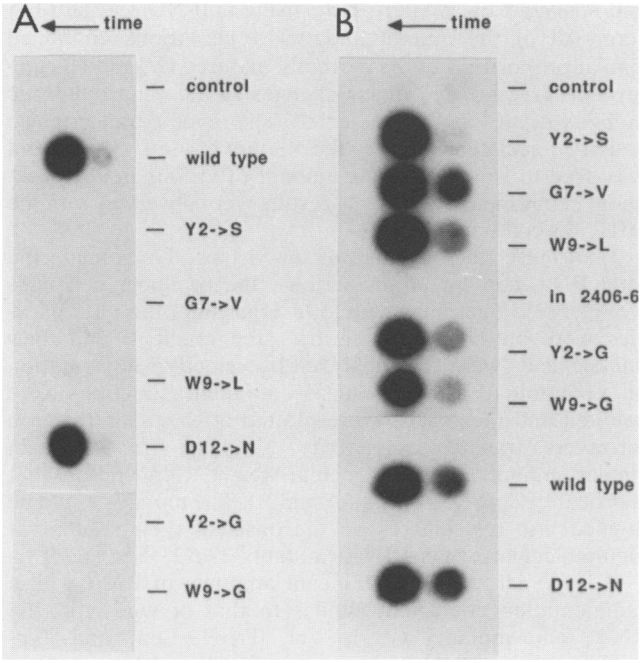

FIG. 2. Biological properties of the mutants. (A) Infectivity assay. Cloned viral DNA was transfected into rat2 cells in the presence of DEAE-dextran as described in Materials and Methods. At 3 days after transfection, the cells were split to allow multiple rounds of infection. After 7 days the reverse transcriptase activity was measured in the supernatant medium (18). The nomenclature used to designate the mutants is the following: the mutated residue is indicated by the one-letter code for the original amino acid, followed by its position in the Cys-His box, relative to the first cysteine residue (position 0); the replacement amino acid residue is indicated after the arrow. (B) Complementation assay. M23 cells were transfected with the mutant clones as described above, and the cells were split. The presence of virus in the culture medium was detected by measuring the reverse transcriptase activity in the culture medium.

Biological activity of the mutant DNAs. (i) Infectivity. To test the mutants for replication competence, full-length viral DNA was transfected into NIH 3T3 and rat2 cells in the presence of DEAE-dextran, and after two passages the reverse transcriptase activity present in the supernatant medium was measured. Figure 2A shows the results obtained when rat2 cells were transfected; the same result was obtained with NIH 3T3 cells (data not shown). With the exception of one mutant, Asp-12→Asn, which showed a phenotype similar to that of the wild-type virus, all of the clones were found to be replication defective. One mutant, Tyr-2→Ser, was weakly positive on NIH 3T3 cells in some assays, transiently inducing a reverse transcriptase activity slightly above the background, but the virus was never able to spread to the whole culture. The XC cell plaque assay was performed on the wild type and mutants Tyr-2→Ser, Trp-9→Leu, and Asp-12→Asn and gave results in agreement with those of the reverse transcriptase assay (data not shown).

We conclude that the tyrosine, the tryptophan, and the glycine residues indeed appear to be critical for virus replication, while the aspartic acid residue in position $n+13$ does not seem to be important for the function of the Cys-His box.

(ii) M23 complementation. To characterize further the biological properties of the mutants, we have used the M23 cell complementation assay. M23 cells contain a defective provirus expressing $Pr65^{gag}$ but no *pol* or *env* gene products, and thus they constitutively release uninfectious particles lacking both reverse transcriptase and envelope glycoprotein (48).

Mutant viruses that provide the *pol* and *env* functions can replicate in M23 cells by complementation. Since the *pol* function is provided as a *gag-pol* polypeptide precursor, the domains of the *gag* precursor involved in membrane targeting and virion assembly of *gag-pol* must also be functional for a mutant to complement. Mutations in the CA domain, for example, block complementation with the M23 provirus, probably because the mutated *gag-pol* precursor is unable to form mixed virions with M23 *gag* precursors (22). Another requirement is that sequences needed in *cis* for replication by the transfected mutant must be present so that *pol* and *env* genetic information can spread in the culture. We have verified that a deletion mutant lacking the Ψ region (34), deficient in RNA packaging but producing wild-type viral proteins, is negative for M23 cell complementation (data not shown).

To assay for complementation, the mutant cloned DNAs were transfected into M23 cells in the presence of DEAE-dextran. When confluent, the cells were replated and the production of virus in the culture medium was measured at confluency by reverse transcriptase assay (18). A linker insertion mutant in CA, *in*2406-6, was used as a negative control. All of the point mutants in MuLV NC described above scored positive in the M23 cell complementation assay (Fig. 2B). This result indicates that these mutants are able to provide *pol* function, that they do not block the formation of M23 viral particles, and that the *cis*-acting RNA packaging signal on their genomic RNA is functional.

Construction of producer cell lines in rat2 cells. To analyze the mutants biochemically, producer cell lines were constructed in NIH 3T3 and rat2 cells. The cell lines were obtained by cotransformation of the mutated pNCA derivatives with plasmid pSV2neo and selection with the antibiotic G418. NIH 3T3 producer clones were isolated for mutants Tyr-2→Ser, Trp-9→Leu, and Asp-12→Asn. The use of NIH 3T3 cells was abandoned when spontaneous reversions of point mutations were observed after multiple passages or during the selection of the cell lines. Producer cell lines were constructed in rat2 cells for all of the mutants; the viruses produced by these cell lines were tested repeatedly, and no reversion events could be detected. All of the mutant viral genomes were able to direct the assembly and release of virion particles. On the basis of reverse transcriptase activity, the rat2 cell lines that we obtained produced between 2- and 10-fold less virus than did rat2 cells infected by the wild-type virus. Some variation in virus production was observed after multiple passages.

The expression of viral sequences in the rat2 cell lines was analyzed by Northern (RNA) blotting of total cellular RNA, hybridized with a full-length MuLV probe. The steady-state amounts of viral RNAs were found to vary widely from one cell line to another but correlated well with the production of virions. In one clone among four expressing mutant Trp-9→Gly (Fig. 3, lane i), the cells expressed deleted genomic and subgenomic RNAs. The occasional recovery of such an aberrant producer is attributable to rearrangements of the viral DNA during transformation.

Virion release and protein composition of the virion particles. To test for effects of the mutations on the production and maturation of the viral proteins, viral particles released by the rat2 cell lines were purified on a sucrose step gradient

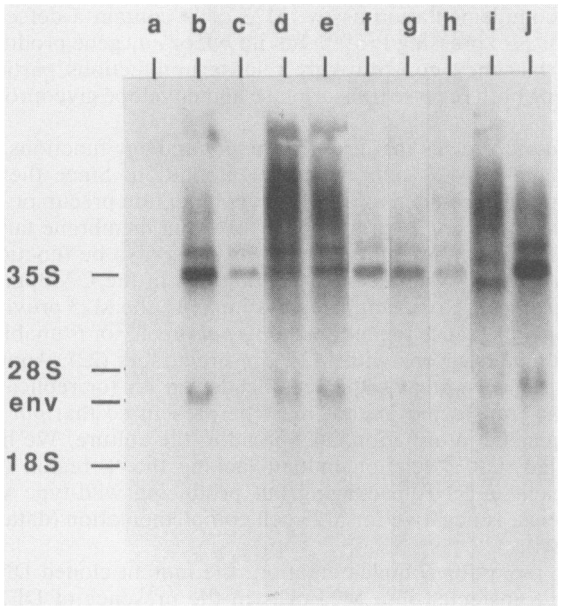

FIG. 3. Expression of viral RNAs in transformed cell lines. Total cellular RNA was extracted from rat2 producer cells as described in Materials and Methods. The RNA was analyzed by Northern blotting with a nick-translated pNCA probe. The filter was submitted to autoradiography for 10 h (lanes a, b, c, f, and h) or 4 days (lanes d, e, g, i, and j). Lanes: a, untransfected cells; b, wild type; c, Tyr-2→Ser; d, Gly-7→Val; e, Trp-9→Leu; f, Tyr-2→Gly (clone 17A3); g, Tyr-2→Gly (clone 17B2); h, Trp-9→Gly (clone 18B4); i, Trp-9→Gly (clone 18C3); j, Trp-9→Gly (clone 18D2). The position of migration of the following RNA species are indicated: 35S, full-length viral transcript; *env*, messenger for the viral envelope glycoproteins; 28S and 18S, rRNAs detected by ethidium bromide staining of the filter.

and analyzed by Western blot, using anti-NC and anti-CA sera. All of the mutant and wild-type virions contained similar proportions of the correctly matured CA and NC *gag* proteins (Fig. 4). No major changes in the electrophoretic mobility of NC could be detected. The reverse transcriptase activity correlated well with the amount of viral proteins and was used to standardize the amount of virions in all subsequent experiments. The same results were observed with the NIH 3T3 cell lines tested.

Total nucleic acid content of the virions. To estimate the total RNA content of the virions, the producer cell lines were labeled overnight with [5,6-^{3}H]uridine, the viral particles were purified on a sucrose step gradient, and their nucleic acids were then analyzed by velocity sedimentation on a continuous 5 to 23% sucrose gradient. Fractions were counted and the results were plotted after standardization for reverse transcriptase activity. All of the viruses contain similar amounts of low-molecular-weight RNA and of what is probably 28S rRNA (Fig. 5A and B). The mutants could be divided into two classes on the basis of their content of high-molecular-weight RNA: mutants Tyr-2→Ser and Tyr-2→Gly, which contain significant amounts of RNA with a sedimentation coefficient similar to that of wild-type 70S RNA, and mutants Gly-7→Val, Trp-9→Leu, and Trp-9→Gly, which appear to be empty of high-molecular-weight RNA.

The nucleic acids were also analyzed on a nondenaturing agarose gel to assay for secondary and tertiary structure. Sufficient amounts of virion RNA were loaded on the gel so that the 70S viral RNA contained in the wild-type virions could be easily detected by ethidium bromide staining. On the basis of that technique, mutants Tyr-2→Ser and Tyr-2→Gly contained significant amounts of nucleic acids, as

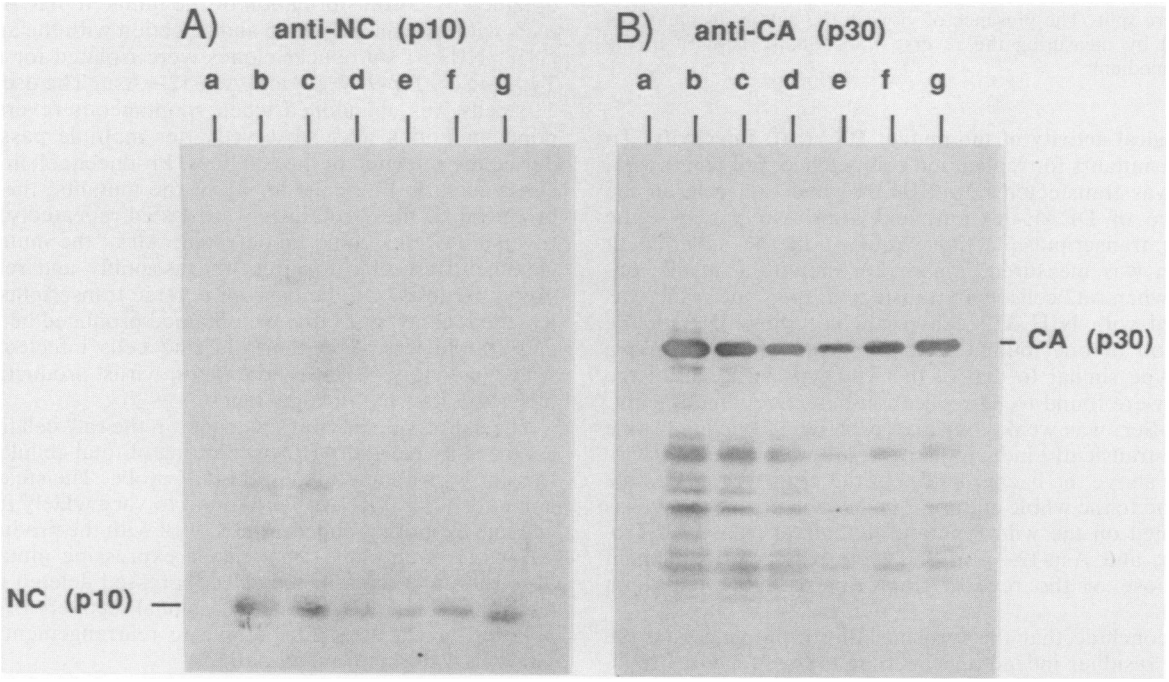

FIG. 4. Analysis of the virion proteins. Viral particles were harvested from the rat2 producer cell lines and purified on a sucrose step gradient. The virions were then analyzed in duplicate on a 13% polyacrylamide gel in the presence of SDS (29); NC (A) and CA (B) were detected immunologically with polyclonal antibodies as described in Materials and Methods. Lanes: a, control; b, wild type; c, Tyr-2→Ser; d, Gly-7→Val; e, Trp-9→Leu; f, Tyr-2→Gly; g, Trp-9→Gly.

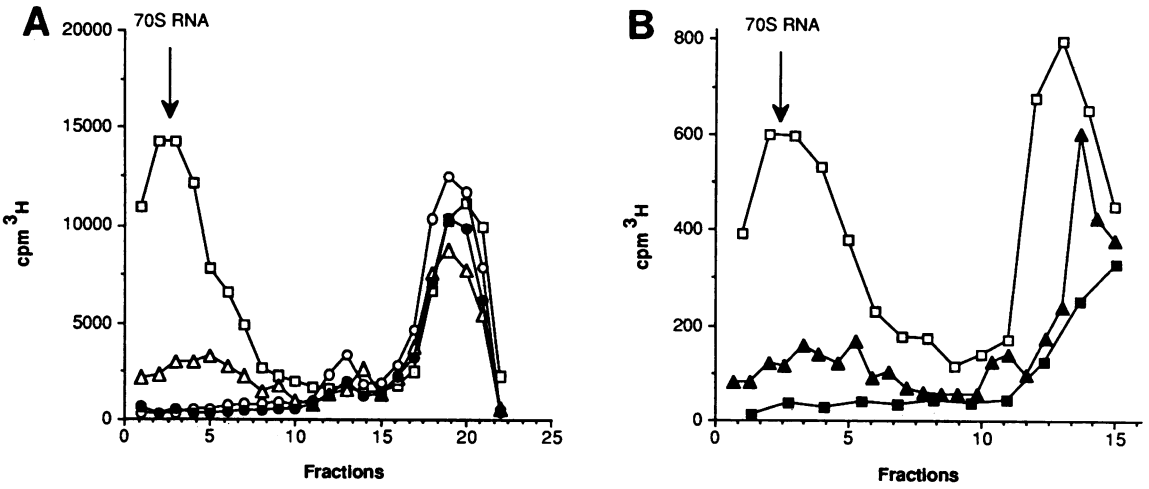

FIG. 5. Total nucleic acid content of the virions. The producer cell lines were labeled overnight with [5,6-^{3}H]uridine, and the virion particles were then purified on a sucrose step gradient; their nucleic acids were extracted and fractionated by velocity sedimentation on a continuous 5 to 23% sucrose gradient. Fractions were harvested and counted, and the results were plotted after standardization for reverse transcriptase activity. Samples and the level of reverse transcriptase activity in counts applied were as follows: (A) wild type (□; 31,500 cpm), Gly-7→Val (○; 4,500 cpm), Tyr-2→Gly (△; 7,500 cpm), and Trp-9→Gly (●; 4,200 cpm); (B) wild type (□; 13,600 cpm), Tyr-2→Ser (▲; 14,200 cpm), and Trp-9→Leu (■; 6,300 cpm). Position of the wild-type dimeric 70S RNA is indicated.

was observed after [5,6-^{3}H]uridine labeling. Upon heating, the migration of the RNA from mutant Tyr-2→Ser shifted from the position of a dimer to that of a monomer, as did RNA isolated from the wild-type virus. The shift was only partial in the case of Tyr-2→Gly (data not shown). Alkaline hydrolysis and RNase A treatment of the agarose gel after size fractionation of the nucleic acids showed that the particles do indeed contain RNA. Two clones, 17A3 and 17B2, also contained a significant amount of low-molecular-weight DNA visible as a smear (data not shown). This DNA does not appear to be of viral origin since it does not hybridize with the total MuLV probe in the blots (Fig. 6A and B, lane f). It is thus unlikely to be the result of an early reverse transcription. With the exception of two bands

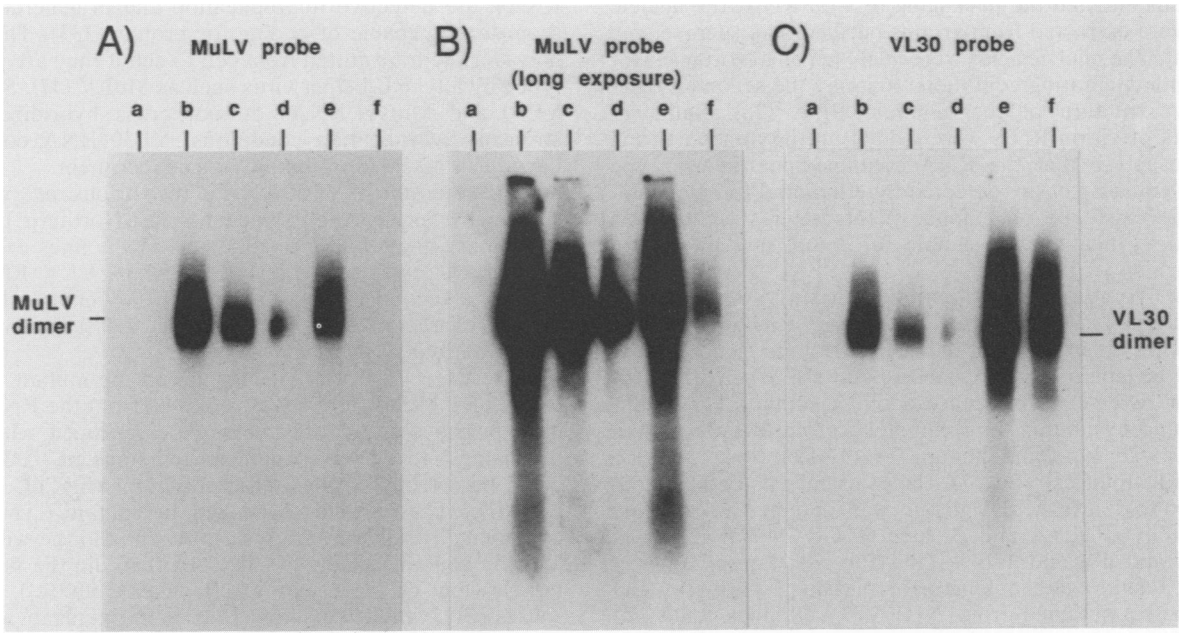

FIG. 6. Viral RNA content of the virions. Culture medium was harvested from the rat2 producer cell lines every 12 h and stored on ice. The virions were concentrated by centrifugation and purified on a sucrose step gradient. The nucleic acids contained in the virions were extracted, fractionated by electrophoresis on a nondenaturing 1% agarose gel, and electroblotted onto a nylon membrane. The filter was probed with a nick-translated pNCA clone to detect MuLV RNA (A and B) and with a VL30 clone to detect VL30 RNA (C). (A) Samples and relative reverse transcriptase (RT) levels loaded were as follows: lanes—a, nontransfected cell line, RT = 0; b, wild type, RT = 100; c, wild-type, RT = 20; d, wild type RT = 4; e, Tyr-2→Ser, RT = 100; f, Tyr-2→Gly, RT = 200. The film was exposed for 1 day at −70°C in the presence of an intensifying screen. (B) Same filter autoradiographed for 6 days under the same conditions. (C) Twenty-four-hour exposure.

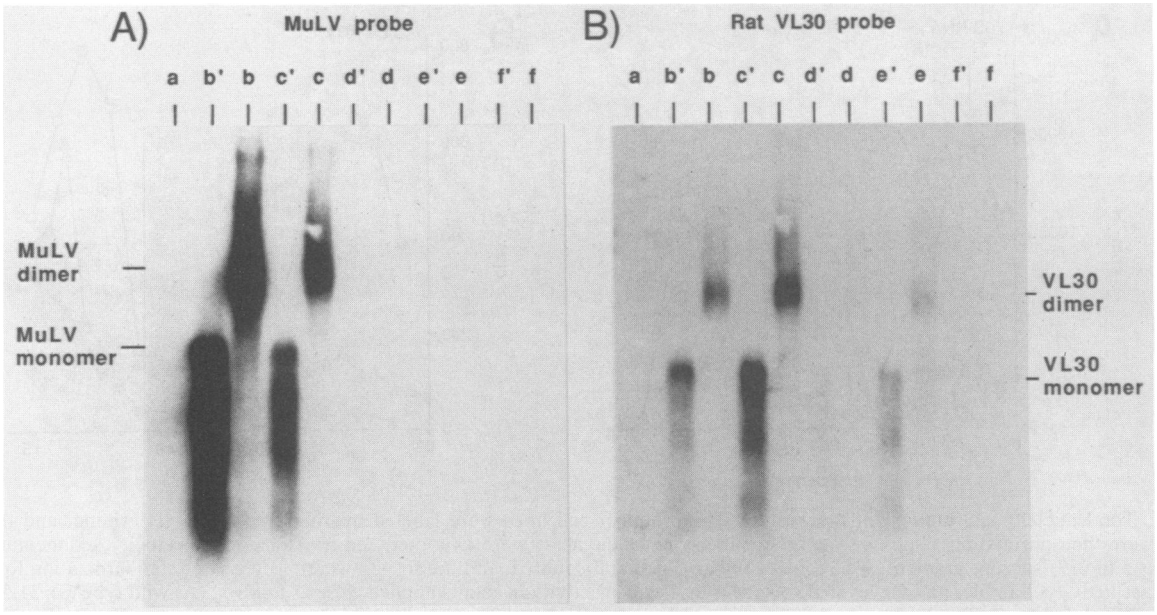

FIG. 7. Viral RNA content of the virions. Analysis of the wild type and mutants Tyr-2→Ser, Gly-7→Val, Try-9→Leu, and Trp-9→Gly. The amounts loaded were standardized for reverse transcriptase activity of the harvested culture medium before purification of the virions. Lanes: a and a′, nontransfected rat2 cells; b and b′, wild type; c and c′, Tyr-2→Ser; d and d′, Gly-7→Val; e and e′, Trp-9→Leu; f and f′, Trp-9→Gly. In each pair of lanes, the first sample was denatured for 3 min at 70°C before loading while the second sample was loaded in the native form. The filter was probed with nick-translated pNCA DNA to detect MuLV RNA (A) and, after removal of the probe, with VL30 DNA (B). The exposure times were 9 h (A) and 36 h (B).

corresponding to rRNA, mutants Gly-7→Val, Trp-9→Leu, and Trp-9→Gly appeared to be empty of nucleic acids by the staining procedure (data not shown).

Viral RNA content of the virions. To determine whether the mutations affected the packaging of viral RNA, the nucleic acids were extracted from virions purified on a sucrose step gradient. The nucleic acids were analyzed on a Northern blot under nondenaturing conditions to assay the secondary and tertiary structure of the genomic RNA (26). Sufficient amounts of virion RNA were loaded on the nondenaturing agarose gel so that the RNA contained in the wild-type virions could be easily detected by ethidium bromide staining. The existence of a noncovalent dimer structure was assayed by heating the sample for 3 min at 70°C before loading.

Upon hybridization with a full-length MuLV DNA probe, only mutant Tyr-2→Ser showed a strong signal migrating at a position similar to that of the wild-type dimer genome (Fig. 6A and B, lane e; Fig. 7A, lanes c and c′). When standardized for reverse transcriptase activity, mutant Tyr-2→Ser was found to contain approximately 5-fold less RNA than did the wild type and mutant Trp-9→Leu was found to contain 25-fold less (Fig. 7A, lanes e and e′). Between 50- and 100-fold less MuLV RNA was detected in mutants Tyr-2→Gly (Fig. 6A and B, lane f), Gly-7→Val (Fig. 7A, lanes d and d′), and Trp-9→Gly (Fig. 7A, lanes f and f′). Similar results were obtained for mutants Tyr-2→Ser and Trp-9→Leu obtained from NIH 3T3 cell lines (data not shown).

The virtual absence of MuLV RNA in mutant Tyr-2→Gly was surprising since other techniques showed that this mutant did contain, in addition to some DNA, significant amounts of RNA. The shift of the signal upon heat denaturation suggested a dimeric or tightly folded structure and a possible retroviral origin for this RNA.

Packaging of VL30 RNA in the mutant virions. Many mouse and rat cells express defective endogenous retroviral sequences as RNAs with a sedimentation coefficient of approximately 30S. These viruslike RNAs, termed VL30 RNAs, are defective for replication and in general do not seem to be capable of coding for proteins (33). They can, however, be transmitted from cell to cell if they are pseudotyped by a type C helper virus such as MuLV (47). Since rat VL30 and MuLV RNAs do not cross hybridize under stringent hybridization conditions, VL30 RNA could not have been detected in the previous experiment.

The expression of VL30 RNA in the various rat2 producer cell lines was analyzed by denaturing Northern blotting, using a specific VL30 probe (55); all of the lines expressed comparable levels of RNA (Fig. 8). Thus, VL30 RNA was used as a second marker besides MuLV genomic RNA to further characterize the RNA packaging function in the mutant virions.

To test for VL30 RNA packaging in the mutant virions, the labeled MuLV probe was removed from the RNA blots used previously and the filters were reprobed with nick-translated VL30 DNA. Significant amounts of VL30 RNA could be detected in the wild-type virions (Fig. 6C, lane b; Fig. 7B). The particles produced by mutant Tyr-2→Ser, containing fivefold less MuLV RNA than did the wild type, actually contained more VL30 RNA than did the wild-type virions (Fig. 6C, lane e; Fig. 7B, lanes c and c′). Mutant Tyr-2→Gly, which was shown to contain significant amounts of non-MuLV RNA and DNA, appeared to package the same amount of VL30 RNA as the wild type (Fig. 6C, lane f). Mutant Trp-9→Leu contained some VL30 RNA, while the other mutant virions seemed to contain approximately 10-fold less VL30 RNA than the wild type (Fig. 7B, lanes e and e′). Mutant Trp-9→Gly did not package either MuLV or VL30 RNA (Fig. 7A and B, lanes f and f′). Among the four

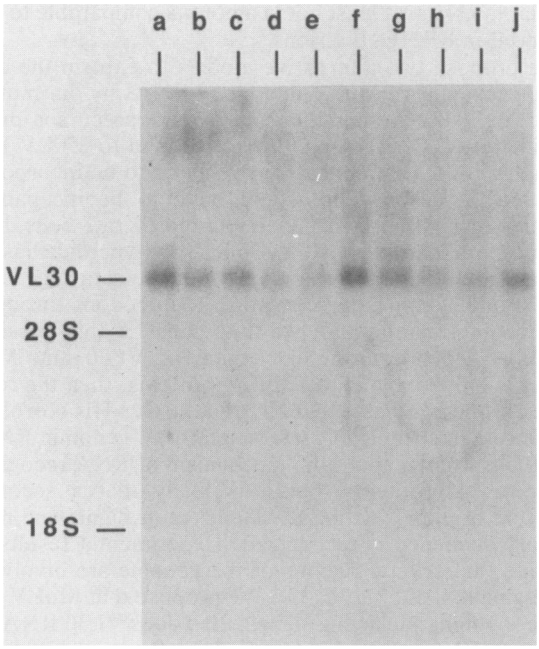

FIG. 8. Expression of VL30 RNA in the rat2 producer cell lines. Total cellular RNA was extracted from the rat2 cells and analyzed by the denaturing Northern blot procedure. The filter was hybridized with a nick-translated VL30 probe (55), washed in 2× SSC for 60 min at 65°C, and autoradiographed for 7 h in the presence of an intensifying screen. Lanes: a, untransfected cells; b, wild type; c, Tyr-2→Ser; d, Gly-7→Val; e, Trp-9→Leu; f, Tyr-2→Gly (clone 17A3); g, Tyr-2→Gly (clone 17B2); h, Trp-9→Gly (clone 18B4); i, Trp-9→Gly (clone 18C3); j, Trp-9→Gly (clone 18D2). VL30, VL30 transcript; 28S and 18S, rRNAs detected by ethidium bromide staining of the filter.

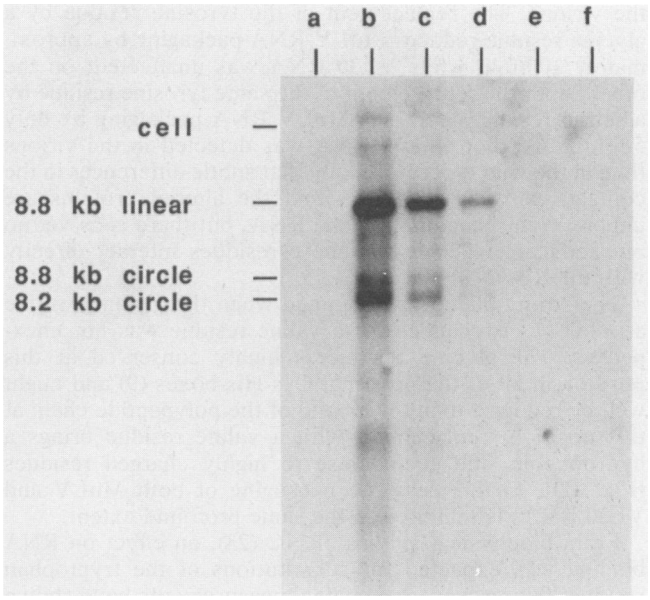

FIG. 9. Viral DNA synthesis after infection of rat2 cells by wild type and mutants Tyr-2→Ser and Tyr-2→Gly. Cell culture medium harvested from the rat2 producer cell lines was used to infect confluent rat2 cells. The viral DNA was extracted 24 h later by the method of Hirt (21) and analyzed by Southern blotting with a nick-translated pNCA probe. The concentration of virus used for infection was estimated by measuring the reverse transcriptase in the culture medium (RT). The amounts of cell extract loaded on the gel were standardized according to these values. Lanes: a, mock infection; b, wild type, RT = 100; c, wild type, RT = 10; d, wild type, RT = 1; e, Tyr-2→Ser, RT = 150; f, Tyr-2→Gly, RT = 65. The film was exposed for 4 days. kb, Kilobase.

cell lines tested for this mutant, one produced abnormally large amounts of virions containing no MuLV RNA but VL30 RNA in amounts similar to those of the wild type (data not shown). We believe that the provirus in this aberrant producer line has suffered additional mutations, and that this is not the true phenotype of the Trp-9→Gly mutant.

These results indicate that mutations in the MuLV NC Cys-His box affect the packaging of MuLV and VL30 RNAs in a different way: VL30 RNA appears to have a stronger and more easily recognized packaging signal than does MuLV RNA.

Synthesis of viral cDNA upon infection. To test whether mutant Tyr-2→Ser can synthesize viral cDNA in vivo, low-molecular-weight DNA was extracted from infected cells (21) and the unintegrated viral DNA was analyzed by Southern blotting. Figure 9 shows that no viral cDNA could be detected, even after a long exposure of the film. When the amount of virions used for the infection and measured by reverse transcriptase assay is taken into account, the mutant appears to synthesize at least 100-fold less cDNA than does the wild type. The same experiment was also carried out on the corresponding producer NIH 3T3 cell lines with the same result (data not shown).

DISCUSSION

Most of the mutations that we have constructed in the Cys-His box blocked virus replication. They interfered neither with the production of virions nor with the maturation of the protein precursors, but they affected the packaging of viral genomic RNA. These results confirm those obtained in the system of RSV (36, 37): they show that NC is necessary for viral RNA packaging and suggest that the Cys-His box is directly involved in RNA binding.

The observation that one mutant, Tyr-2→Ser, only moderately affected at the packaging level, is also unable to replicate indicates that retroviral NC has an additional function distinct from RNA packaging. Such a phenotype was also observed in the avian system when a linker was inserted in the proximal Cys-His box of RSV NC (37). We have not yet analyzed mutant Tyr-2→Ser to determine whether, as reported for the similar RSV mutant (41), the tRNA primer is missing on the genomic RNA.

The rat2 cell line was chosen as the parent for the construction of producer cell lines because they do not express endogenous proviruses closely related to MuLV. These cells, however, do express VL30 RNA, a retrovirus-like RNA which is known to be pseudotyped by type C viruses (47). VL30 sequences appear to be either too defective or not similar enough to MuLV to generate replication-competent recombinants since no spontaneous revertants of point mutations were observed in rat2 cells. The expression of VL30 RNA by rat2 cells could be used in parallel with MuLV RNA to characterize the RNA packaging phenotype of the mutants.

Among all of the mutations, those affecting the tyrosine residue at $n+2$ in the Cys-His box are probably the most interesting. Their phenotype suggests that the tyrosine residue plays a role in the choice of the RNA to be packaged in

the virions. The replacement of the tyrosine residue by a glycine residue reduces MuLV RNA packaging by approximately 100-fold while VL30 RNA was unaffected; on the other hand, the replacement of the same tyrosine residue by a serine residue decreases MuLV RNA packaging by only 5-fold while more VL30 RNA was detected in the virions than in the wild type. It is likely that subtle differences in the contacts between the RNA and the altered proteins are important in recognition of the RNA, but there is as yet no biochemical evidence that these residues interact directly with the RNA.

The strong phenotype obtained when the glycine residue at $n+7$ was exchanged for a valine residue was not unexpected. The glycine residue is highly conserved at this position in all of the proximal Cys-His boxes (9) and might well be required to allow a bend of the polypeptide chain at this position; replacement with a valine residue brings a hydrophobic side chain close to highly charged residues (Fig. 1D). In this case the packaging of both MuLV and VL30 RNAs is inhibited to the same profound extent.

From biophysical studies of NC (23), an effect on RNA binding was expected for substitutions of the tryptophan residue; fluorescence quenching measurements have shown that the tryptophan intercalates between the bases of the bound nucleic acid. This interaction is not possible if the aromatic residue is replaced by a leucine residue and especially by a glycine residue; as a consequence the binding of the RNA is likely to be affected. It is interesting that while no virion RNA can be detected in mutant Trp-9→Gly, small amounts are visible in mutant Trp-9→Leu. As with mutant Gly-7→Val, the packaging of MuLV and VL30 RNAs in this case is decreased compared with that of the wild type, but, overall, the effect on MuLV seems stronger than that on VL30.

The wild-type phenotype obtained when the aspartic acid residue at $n+12$ was replaced by an asparagine residue indicates that the negative charge is not critical for replication.

RSV mutants have been found that contain 50% of the genomic RNA as a monomer, compared with approximately 5% in the case of the wild-type virions. Such an effect was observed with a valine-proline insertion in the proximal Cys-His box (37) and when one of the two boxes was deleted (36). It is thus surprising that the mutations tested here affect only the amount of viral RNA packaged in the particles but never its dimer structure. The RSV and MuLV NC proteins, however, are very different; for example, RSV NC has two boxes in tandem while MuLV NC has only one, and it is possible that other MuLV mutants may ultimately induce similar phenotypes.

One of the most challenging questions about genomic viral RNA packaging in retroviruses is the mechanism of its specificity. The most interesting mutant with a defect at the RNA packaging level described so far is the quail cell line SE21Q1b (31). This cell line produces virions which contain less than 1% of the normal complement of viral RNA and large amounts of poly(A)$^+$ cellular RNAs (14, 31). If NC is responsible for the selective packaging of genomic RNA, then the mutations described here could have resulted in virions similar to those produced by the SE21Q1b cell line. The phenotype of mutants such as Tyr-2→Ser, Tyr-2→Gly, and even Trp-9→Leu suggest that, indeed, MuLV NC is involved in packaging specificity: the mutations decrease the packaging of MuLV RNA but do not affect or even increase the packaging of VL30 RNA. However, these mutations do not seem to allow the packaging of significant amounts of cellular mRNAs, at least not in amounts comparable to those observed in SE21Q1b virions.

We propose two alternative models to explain the differential packaging of MuLV and VL30 RNAs by our mutants. First, VL30 RNA might encode a *gag* precursor protein specific for VL30 RNA and unable to bind to MuLV RNA. Such a VL30 *gag* precursor would have to be incorporated into MuLV virions and would have to be incapable of forming virions by itself, since uninfected rat2 cells do not produce viral particles. No VL30 RNA polypeptide has been reported in the literature, and all sequence data suggest that most VL30 genomes have multiple stop codons throughout their coding regions (33); we thus consider this possibility unlikely. Second, if the packaging of VL30 and MuLV RNAs is only mediated by MuLV proteins, then the results presented here indicate that NC and the Cys-His box play an active role in specifically selecting MuLV genomic RNA.

Little is known about the mechanism of RNA recognition by retroviral proteins, but it is likely that a secondary structure of the *cis*-acting RNA packaging signal and not its primary sequence is recognized. Experimental results also indicate that several regions of the genome are involved in packaging (24, 25, 27, 34, 39). We propose that MuLV RNA has less potent packaging signals than does VL30 RNA, that wild-type NC is necessary to recognize them, and that VL30 RNA has very strong, or very easily recognized, packaging signals. Since VL30 RNA can replicate only by pseudotyping, low packaging requirements are expected to have been selected so that a variety of retroviruses can package and reverse transcribe VL30 RNA.

The absence of significant amounts of cellular RNAs in the virions and the relative resistance of VL30 RNA packaging toward mutations in the conserved cysteine-histidine motif suggest that while the Cys-His box is required for MuLV RNA packaging, another domain of the *gag* precursor or of NC could be involved in the discrimination against packaging of cellular RNAs. The identification of these domains will require further mutagenesis.

In conclusion, we should note that similar mutations in the Cys-His box domain of M-MuLV NC have been characterized recently (19); the results of these studies are fully compatible with ours.

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