Four Moloney Murine Leukemia Virus-Infected Rat Cell Clones Producing Replication-Defective Particles: Protein and Nucleic Acid Analyses

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Four cloned rat cell lines (NX-1 to -4) infected with Moloney murine leukemia virus and defective in virus replication were found to be all different by viral protein and nucleic acid analyses. All four clones produced noninfectious particles and, except for NX-2, at about the same level as wild type. Compared with wild-type virions these defective particles contained larger amounts of *gag* precursor proteins and very little or no p30 or p15. Analysis of intracellular precursor proteins revealed that NX-2 to -4 synthesized normal Pr65^{gag}, whereas NX-1 produced a slightly smaller precursor. Both NX-1 and NX-4 synthesized an intracellular polyprotein with a size similar to that of wild-type Pr180^{gag-pol}. Restriction endonuclease analysis of NX-1 to -4 cellular DNA showed that each clone contained a single integrated provirus which possessed large terminal repeat sequences at both the 5' and 3' ends. The proviruses of NX-1 to -3 appeared normal by restriction endonuclease analysis, but NX-4 provirus had a deletion of 1,700 base pairs comprising part of the polymerase region. The noninfectious particles produced by all four clones packaged Moloney viral RNAs and rat RNAs of two different sizes.

Numerous mutants of avian retroviruses that are defective in replication have been described and have proven instrumental in understanding the genome organization and life cycle of these viruses (15). Fewer conditional or nonconditional replication mutants of mammalian retroviruses have been studied in detail. Of the mammalian mutants in replication the most frequent defectiveness has been the inability to normally process the *gag* precursor polyprotein, Pr65^{gag}, which in wild-type virus is cleaved to the internal virion proteins p15, p12, p30, and p10 (2, 24, 29, 33, 34). A few of these mutants of murine leukemia virus (MuLV), e.g., Rauscher MuLV *ts24* (29) and Moloney MuLV (M-MuLV) *ts3* (34), also accumulate the polymerase precursor polyprotein Pr180^{gag-pol}. Detailed analysis of these two mutants demonstrated that the cleavage of Pr180^{gag-pol} to p85, the active form of reverse transcriptase, occurs after virion release (33).

Other murine replication mutants include those which are defective in reverse transcriptase. An M-MuLV variant that packages a smaller polymerase in its virions has been reported by Gerwin et al. (10). Still other murine mutants in reverse transcriptase include those which produce no Pr180^{gag-pol} precursor and hence no reverse transcriptase in viral particles (24). A third class of replication-defective mu-

tants includes those with defects in the envelope protein. Shields et al. (24) have reported a few M-MuLV mutants with either no detectable or low levels of Pr80^{env}, the precursor to the envelope proteins gp70 and p15E.

The genomes of a few of these avian and mammalian retrovirus nonconditional mutants have been analyzed to identify the types of lesions involved (4, 15, 19, 24). For the mammalian system the analysis of the genomes of nonconditional mutants has revealed that a few of these mutants have smaller virion RNAs, indicating that deletions are responsible for their defectiveness. These mutants include mouse cell lines infected with M-MuLV which only make Pr80^{env} (4) or Pr65^{gag} (24) and none of the other intracellular precursor proteins. In comparison to mammalian retroviruses, deletion mutants in the avian system are numerous, being generated at high frequency, and they have been studied in greater detail (15). Other types of avian nonconditional mutants include those with insertions (19, 20a) or substitutions (9) in the wild-type genome which account for the mutant phenotype. Other genomes, however, such as a few Rous sarcoma virus proviruses of nonproducer quail cells, resemble wild-type virus at least in size and by restriction endonuclease analysis (19).

NX-1 to -4 are four nonconditional replica-

tion-defective clones of rat (NRK) cells infected with M-MuLV which produce noninfectious particles lacking in reverse transcriptase activity and negative in the XC plaque assay (24). This report deals with the further characterization of all four clones with respect to their intracellular and virion proteins and genome structure and expression. All four clones synthesized wild-type intracellular Pr65^{gag}, except for NX-1, which made a slightly smaller Pr65^{gag}. Only NX-1 and NX-4 synthesized a protein similar in size to Pr180^{gag-pol} which immunoprecipitated with anti-reverse transcriptase serum. The particles produced by NX-1 to -4 contained detectable levels of *gag* precursor proteins and viral RNAs. The proviruses of NX-1 to -3 resembled the wild type by restriction endonuclease analysis, but NX-4 provirus contained a large deletion in the polymerase region of the genome.

MATERIALS AND METHODS

Cell lines. Cloned cell lines (NX-1 to -4) and wild-type CP-1 were obtained from E. Rothenberg, Salk Institute, and D. Baltimore, Massachusetts Institute of Technology. These clones were isolated from a mass infection of rat (NRK) cells by M-MuLV (24). A cloned NIH/3T3 cell line productively infected with M-MuLV (Cl 3A) was obtained from R. Weinberg, Massachusetts Institute of Technology. Cell lines were maintained at 37°C in minimal essential medium (MEM; GIBCO Laboratories) with 10% heat-inactivated fetal calf serum.

Immune sera. Goat antisera to purified M-MuLV p30, p15, and gp70 were received from the Virology Laboratory, National Cancer Institute. Rabbit antiserum to M-MuLV reverse transcriptase that also contained cross-reactivity with p15 was a kind gift of O. N. Witte, University of California at Los Angeles.

Virion protein labeling and determination of amounts of viral particles produced by NX-1 to -4. Subconfluent cell monolayers on 10-cm Corning plates were labeled with either 200 μ Ci of [³⁵S]methionine (generously provided by R. Eisenman, Fred Hutchinson Cancer Research Center) or 500 μ Ci of [³H]leucine in 2.5 ml of methionine- or leucine-free MEM per plate at 37°C overnight. Supernatants were collected, and plates were refed with 2 ml of normal MEM for 3 h. Both supernatants were combined and clarified at 3,000 (3K) rpm in an IEC centrifuge for 10 min. Unlabeled Rous sarcoma virus was added, and supernatants were placed on a step gradient of 45 and 25% sucrose in TNE buffer (10 mM Tris-hydrochloride, [pH 7.4], 100 mM NaCl, 1 mM EDTA). Gradients were centrifuged in an SW27 rotor at 23 K rpm at 4°C for 17 h. Fractions (1 ml) were collected, and the peak fractions were pooled and diluted with TNE buffer. Particles were pelleted and resuspended in TNE. Samples (100 μ l) of viral particles were layered on 5-ml 20 to 60% sucrose gradients in TNE and centrifuged in an SW50.1 rotor for 135 min at 36K rpm at 4°C. Fractions were collected by pumping from the bottom of the gradients and assayed for radioactivity (counted

in 5 ml of Biofluor; New England Nuclear Corp.) and sucrose density (by refractive index).

Metabolic labeling. Subconfluent 10-cm plates were each labeled with 125 μ Ci of [³⁵S]methionine in 2.5 ml of methionine-free MEM for 30 min at 37°C. After rinsing with phosphate buffered saline at 0 to 4°C cells were lysed with 0.5 ml of antibody buffer (20 mM Tris [pH 7.4], 50 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 1% aprotinin) per plate. Cell lysates were blended in a Vortex mixer, incubated on ice for 10 min, and clarified in a Brinkman centrifuge for 30 min.

Immunoprecipitations. Protein samples were made 0.1% sodium dodecyl sulfate (SDS) and left on ice for 10 min. The remaining steps followed the procedure of Witte and Baltimore (33). Briefly, samples were precleared with normal goat serum and Formalin-fixed *Staphylococcus aureus*. They were reclarified, and immune serum was added with a sample of concentrated, uninfected NRK-0 cell lysate. Samples were again clarified by centrifugation, and *S. aureus* was added for 2 h. Pellets were suspended in 0.5 ml of RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1% aprotinin) and blended thoroughly, followed by exhaustive washings. Sample buffer (80 mM Tris [pH 6.8], 5% SDS, 10% glycerol) was added to the final pellets, heated at 100°C for 3 min, and centrifuged once to clarify.

SDS-polyacrylamide gel electrophoresis. Polyacrylamide gels of various percentages were poured and electrophoresed by the method of Laemmli (17) as modified by Blattler et al. (5). Gels were fixed and stained with 25% Coomassie blue dye in 7% acetic acid-50% methanol solution. After destaining, gels were fluorographed with Enhance (New England Nuclear). Gels were dried and exposed to preflashed Kodak X-Omat R film at -70°C (6).

Preparation of cellular DNA. Cells grown on plastic Corning roller bottles were rinsed with phosphate-buffered saline, 10 ml of TNE buffer made 0.5% in SDS was added to each roller bottle (ca. 10⁹ cells), and proteinase K (Boehringer Mannheim) was added to a final concentration of 0.2 mg/ml. Cell lysates were collected and incubated at 37°C for 2 h. DNA samples were extracted twice with an equal volume of distilled phenol (pH 8) followed by two extractions of CHCl₃-isoamyl alcohol (24:1). Aqueous DNA samples were dialyzed against 10:1 buffer (10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA) at 4°C overnight.

DNA samples were treated with RNase A (Sigma Chemical Co.) at a concentration of 0.05 mg/ml at 37°C for 1 h. Proteinase K was added to this reaction mix, and digestion was carried out as described above. Samples were again extracted with phenol and CHCl₃-isoamyl alcohol and dialyzed against 10 mM Tris (pH 7.4)-300 mM NaCl-5 mM EDTA followed by 10:1 buffer at 4°C.

Restriction endonuclease digestions. Cell DNA (10 to 30 μ g) was digested with *Eco*RI, *Sal*I, or *Xba*I restriction endonuclease according to the supplier's specifications (Bethesda Research Laboratories, Inc.). To control for completeness of digestion 1 μ g of λ DNA (Bethesda Research Laboratories) was added to 3 μ g of cell DNA and digested under identical condi-

tions. Generation of the expected λ DNA fragments without any partial fragments was an indication of complete digestion. Digested DNA samples were usually ethanol precipitated at -20°C .

Agarose gel electrophoresis, transfer to nitrocellulose, and hybridization of DNA. Digested DNA samples of 15 to 30 μg were subjected to electrophoresis through horizontal 1% agarose (SeaKem) slab gels (13.5 by 22 by 0.3 cm). Electrophoresis conditions were as reported earlier (36). Transfer of DNA from agarose gels to nitrocellulose filters (Schleicher and Schuell) was based on the method of Southern (27) and described in a previous report (36), except that M-MuLV-cloned DNA (kindly given by S. Goff, E. Gilboa, and D. Baltimore, Massachusetts Institute of Technology) was ^{32}P -labeled by nick translation (18) and used as hybridization probe.

Electrophoresis, transfer, and hybridization of RNA. RNA samples were denatured in a mixture of 1 M glyoxal and 50% dimethyl sulfoxide as described by McMaster and Carmichael (20) for 1 h at 50°C . Samples were then electrophoresed through 1% agarose horizontal slab gels in 10 mM sodium phosphate (pH 7.0) running buffer at 40 to 45 mA for 5 h.

Gels were treated with 50 mM NaOH for 30 min at room temperature and then rinsed twice with 20 mM sodium phosphate (pH 6.5) solution. RNA was transferred to diazobenzoyloxymethyl paper prepared and activated by the procedure of Alwine et al. (1).

The diazobenzoyloxymethyl paper was pretreated with a solution of 50 mM sodium phosphate (pH 6.8)–5 $\times$ Denhardt–5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.4)–50% formamide–1% glycine–0.1 mg of herring sperm DNA per ml–0.1% SDS at 41°C for 16 to 18 h. RNA was hybridized to 0.02 to 0.1 μg of ^{32}P -labeled M-MuLV DNA per ml in 20 mM sodium phosphate (pH 6.8)–1 $\times$ Denhardt–5 $\times$ SSC (pH 7.4)–50% formamide–10% dextran sulfate–0.1 mg of herring sperm DNA per ml–0.1% SDS at 41°C for 24 to 48 h. Post-hybridization rinses consisted of one with 2 $\times$ SSC (pH 7.4)–1 $\times$ Denhardt for 30 min at room temperature, two with 0.1 $\times$ SSC–0.1% SDS for 30 min each at 50°C , and finally one with 0.1 $\times$ SSC at room temperature. Papers were exposed to X-ray film with DuPont Cronex Lightning Plus intensifying screens at -70°C for 1 to 14 days (31).

^{32}P labeling of RNA in viral particles. Cells grown to subconfluency were labeled with 260 μCi of $^{32}\text{P}_4$ per ml in phosphate-free MEM for 2 h. The supernatant was then discarded, and normal medium was added for 2.5 h. Virus was collected, and cells were refed and reharvested. Pooled supernatants were processed as described previously (24). Viral RNA was extracted from isolated virus lysed in 1% SDS, deproteinized, and ethanol precipitated.

RESULTS

Production of defective virus particles by cloned rat cell lines NX-1 to -4. Previous characterization of the NX-1 to -4 clones indicated that they had only low levels of reverse transcriptase activity in their supernatants (which might have possibly resulted from the

release of rat endogenous retrovirus) and that they were all unable to generate syncytia in the XC plaque assay (24). These observations have been confirmed in this laboratory (data not shown). The four cell lines did, however, synthesize intracellular viral proteins as previously reported (24). The phenotype of these clones is consistent with either nonproducer cells which are incapable of synthesizing any virus, or cells which can produce viral particles that are lacking in reverse transcriptase and therefore are defective for replication.

To distinguish between these two possibilities particle production by NX-1 to -4 was determined by labeling cells and analyzing their supernatants. A 10-cm plate of 75 to 80% confluency was labeled with [^{35}S]methionine for each of the cell lines. A preparation of particles from the supernatant of each clone (10% by volume) was analyzed by centrifugation on a 20 to 60% linear sucrose gradient. For an internal marker wild-type virus from cloned CP-1 cells (rat NRK cell productively infected with M-MuLV) labeled with [^3H]leucine was centrifuged with NX-1 to -4 particles on each of the gradients. Fractions (330 μl) were collected from each gradient and assayed by counting aliquots in scintillation fluid. The sum of the fractions corresponding to the virion peak of each gradient was calculated and is presented in Table 1. All four clones were able to produce viral particles, and all except NX-2 produced particles at the same level as wild-type rat CP-1. NX-2 synthesized one-tenth the amount of particles as the other rat clones.

Intracellular proteins. Since the lesion(s) responsible for the defectiveness in replication for NX-1 to -4 must not involve particle formation as discussed above, we decided to further characterize the defectiveness of these clones by analyzing their intracellular viral proteins. Examination of the presence of the precursor polyprotein to reverse transcriptase, Pr180^{gag-pol},

TABLE 1. Amounts of viral particles produced by cell lines^a

Cell line	Sum of cpm (10^{-3}) in peak fractions
CP-1	2.8
NX-1	3.2
NX-2	0.3
NX-3	2.8
NX-4	3.5

^a A 10% (by volume) preparation of [^{35}S]methionine-labeled viral particles harvested from a 75 to 80% confluent 10-cm plate for each cell line was centrifuged on a 20 to 60% linear sucrose gradient. Sums of counts per minute (cpm) in fractions corresponding to the peaks were calculated.

was performed by metabolic labeling of cells with [35 S]methionine and immunoprecipitation with a serum that recognized both M-MuLV reverse transcriptase and the internal structural protein p15 (kindly given by O. N. Witte). In Fig. 1 the electrophoretic analysis of reverse transcriptase-related intracellular proteins demonstrated that two of the clones, NX-1 and NX-4 (Fig. 1, lanes D and G), synthesized a polyprotein that comigrated with Pr180^{gag-pol} produced by wild-type clone CP-1 (Fig. 1, lane C). The protein products from an in vitro translation of 38S genomic M-MuLV RNA were immunoprecipitated with the same antiserum and electrophoresed through the same gel as a marker (Fig. 1, lane B). The other two clones, NX-2 and NX-3 (Fig. 1, lanes E and F), did not appear to make any Pr180^{gag-pol}-related intracellular proteins. A cellular protein larger than Pr180^{gag-pol} was immunoprecipitated from all cell lines including uninfected NRK-0 (Fig. 1). This normal cellular component has been observed by other investigators (13, 33).

Since the antiserum used to detect Pr180^{gag-pol} also contained cross-reactivity with p15, which

is a component of Pr180^{gag-pol}, lysed M-MuLV virions were added to immunoprecipitations to eliminate this cross-reactivity. The high-molecular-weight proteins comigrating with Pr180^{gag-pol} were again detected in NX-1 and NX-4 cells, indicating that these proteins were related to reverse transcriptase (data not shown).

Analysis of the intracellular precursor Pr65^{gag} revealed that clones NX-2 to -4 (Fig. 2, lanes D to F) synthesized wild-type-sized Pr65^{gag} (Fig. 2, lane B), but NX-1 produced a slightly smaller gag precursor protein (Fig. 2, lane C). This small difference was reproducible and can also be seen on the polyacrylamide gel displaying both Pr180^{gag-pol} and Pr65^{gag} (Fig. 1, lane D). Other gag intermediate proteins (Pr50-55^{gag} and Pr40^{gag}) were also seen in various amounts for all of the cell lines including wild-type CP-1. Pr76^{gag}, intracellular precursor to the cell surface protein gpPr95^{gag}, was also detected in small amounts in NX-1, -2, and -4 (Fig. 2, lanes C, D, and F). Pr76^{gag} appeared as a prominent band, however, in CP-1 cells (Fig. 2, lane B). The presence of reverse transcriptase-related proteins comigrating with Pr180^{gag-pol} in NX-1 and NX-4 cells was also detected by immunoprecipitation with anti-p30 serum (Fig. 2, lanes C and F).

The third major intracellular protein Pr80^{env}, which is processed to the virion envelope proteins gp70 and p15E, was characterized in each of the cloned cell lines by immunoprecipitation

FIG. 1. Detection of reverse transcriptase intracellular precursor Pr180^{gag-pol}. Monolayers of mutant NX-1 to -4 or wild-type CP-1 cell lines were labeled with 50 μ Ci of [35 S]methionine per ml of methionine-free MEM at 37°C for 30 min. Cell lysates were immunoprecipitated with antiserum to reverse transcriptase (p85) containing some anti-p15 activity as detailed in the text. Samples were electrophoresed through a 7.5% SDS-polyacrylamide gel (17). Gel was fixed, fluorographed with Enhance, and after drying exposed to Kodak X-Omat R film at -70°C for 21 days. Lanes: A, dansylated marker proteins; B, immunoprecipitated in vitro translated products of M-MuLV 38S RNA; C, wild-type CP-1; D, NX-1; E, NX-2; F, NX-3; G, NX-4; H, uninfected NRK-0. Marker proteins were electrophoresed through every gel, and their sizes in kilodaltons are indicated (---). Marker proteins used were phosphorylase A (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), and α -globin (14.4K).

FIG. 2. Intracellular precursor Pr65^{gag}. Cell monolayers were labeled, and cell lysates were processed as described in the legend to Fig. 1, except that anti-p30 was used for the immunoprecipitations. A 10% SDS-polyacrylamide gel was used for electrophoresis, and this gel was treated as described in the legend to Fig. 1 with a 9-day exposure. Lanes: A, marker proteins as in Fig. 1; B, wild-type CP-1; C, NX-1; D, NX-2; E, NX-3; F, NX-4; G, uninfected NRK-0. Marker protein sizes are indicated (---).

with anti-gp70 serum. Envelope precursor proteins with the same mobility as wild-type Pr80^{env} were produced by all four clones (data not shown), confirming the earlier report by Shields et al. (24).

Virion proteins. Since NX-1 to -4 cells produced viral particles and synthesized intracellular Pr65^{gag}, it was of interest to identify which *gag* proteins were actually present in these particles. It is not known what *gag*-related proteins are required for particle production and RNA packaging, which all four clones are capable of doing as discussed below.

Viral proteins in the defective particles were labeled with [³⁵S]methionine and immunoprecipitated with anti-p30 serum. Radiolabeled proteins which precipitated with anti-p30 serum and were analyzed by electrophoresis varied in size and amounts for each of the four clones (Fig. 3). Unlike wild-type virus which contained predominantly p30 and only trace amounts of Pr65^{gag} and Pr40^{gag} (Fig. 3, lane B), the defective particles produced by the NX clones in general packaged larger amounts of different precursor proteins (Fig. 3, lanes C to F). Particles of NX-1, -3, and -4 were found to also package low levels of p30 (Fig. 3, lanes C, E, and F). The p30 bands of these clones appear very faintly in Fig. 3, but were clearly visible on the original autoradiograph. NX-4 contained larger amounts of p30 compared with the other clones. A *gag*-related protein of unknown origin migrating slightly ahead of p30 was also detected for NX-3 and NX-4 (Fig. 3, lanes E and F). For the SDS-polyacrylamide gel system used in these studies, p30 and Pr40^{gag} migrated slightly slower compared with their respective marker proteins, carbonic anhydrase (30K) and ovalbumin (43K).

Analysis of a second major virion internal structural protein p15 by labeling with [³H]leucine and immunoprecipitating with anti-p15 serum indicated that either all four mutants were missing p15 completely or the levels of this protein were below the limits of detection in these experiments (Fig. 4, lanes C to F). This protein, however, was easily detected in wild-type virions synthesized by CP-1 (Fig. 4, lane B), which produced virions at similar levels to the particles of all clones except NX-2. The only detectable protein that was precipitated with anti-p15 serum for NX-1, -3, and -4 was Pr65^{gag}, which contains p15 (Fig. 4, lanes C, E, and F). NX-2 particles lacked this *gag*-precursor protein (Fig. 3, lane D).

Analysis of integrated proviruses of NX-1 to -4 by restriction endonuclease digestion. The protein analysis of clones NX-1 to -4 indicated that all of them were different in their expression of virion and intracellular proteins.

FIG. 3. Analysis of internal structural protein p30 in mutant particles. Subconfluent cell monolayers were labeled with 80 μ Ci of [³⁵S]methionine per ml of methionine-free MEM at 37°C overnight. Collected supernatants were processed as detailed in the text. After lysis viral particles were immunoprecipitated with anti-p30 as described in the text. Samples were electrophoresed through a 17.5% SDS-polyacrylamide gel which was exposed to Kodak X-Omat R film at -70°C for 30 days after fluorography. Lanes: A, marker proteins as in Fig. 1; B, wild-type CP-1; C, NX-1; D, NX-2; E, NX-3; F, NX-4. Sizes of marker proteins are indicated (---).

FIG. 4. Detection of internal structural protein p15 in mutant particles. Subconfluent cell monolayers were labeled with 200 μ Ci of [³H]leucine per ml of leucine-free MEM at 37°C overnight and supernatants were processed as described in the legend to Fig. 3. Immunoprecipitation of particle lysates was performed with anti-p15. Electrophoresis was performed as described in the legend to Fig. 3, and the gel was exposed for 26 days. Lanes: A, marker proteins as in Fig. 1; B, wild-type CP-1; C, NX-1; D, NX-2; E, NX-3; F, NX-4. Sizes of marker proteins electrophoresed in an adjacent lane are indicated (---).

We hoped to establish possible explanations for the phenotypic defects of these clones by studying their viral genomic structure. We first ana-

lyzed the structure of the integrated proviral DNAs of NX-1 to -4 to determine whether any differences from the wild-type genome could be detected.

Previous analysis of the number of integrated proviruses in clones NX-1, -2, and -3 revealed that there was a single provirus copy of the M-MuLV genome in these three clones (28). We performed a similar analysis for the fourth replication-defective clone, NX-4, by digestion of cellular DNA with *EcoRI* endonuclease, which does not cleave the M-MuLV genome (11). With the data shown in Fig. 5 we have confirmed the existence of a single M-MuLV provirus copy in NX-1, -2, and -3 (lanes B, C, and D) and have shown that NX-4 (lane E) also contained one copy. The sizes of each of the DNA fragments generated by *EcoRI* digestion of cellular DNA were larger than M-MuLV genome size (8.8 kilobase pairs [kbp]) (12) based on the size of *EcoRI*-digested λ DNA markers electrophoresed through the same agarose gel. Uninfected rat cell (NRK-0) DNA was routinely digested and electrophoresed through an adjacent lane (Fig. 5, lane A) on the same gel with the NX DNAs. Restriction endonuclease mapping experiments of proviral DNA were performed by the Southern transfer technique (27).

Analysis of provirus DNAs for other retrovirus systems has shown that the majority of them possesses large terminal repeat (LTR) sequences at their 5' and 3' ends (14, 21). To determine the number of LTRs that are present in the integrated proviruses of NX-1 to -4 we digested

cellular DNA with *EcoRI* and *SacI* restriction endonucleases. *SacI* cleaves unintegrated M-MuLV linear DNA in the LTR sequences and at 2.95 kbp from the 5' end of the genome (Fig. 6C) (12), generating two large DNA fragments of 5.65 and 2.5 kbp (Fig. 6A, lane F). Two additional small fragments (0.4 and 0.2 kbp) produced from the ends of the linear DNA were not detectable. The fainter bands of 8.2, 4.5, and 3.7 kbp in the marker lane of M-MuLV (Fig. 6A, lane F) represented *SacI* fragments of the smaller and more abundant relaxed circular DNA missing one LTR (36), which often copurifies with the linear form during Hirt extraction. The purpose for doing a double digestion with *EcoRI* was to reduce the background hybridization from high-molecular-weight cell DNA with partial homology to the M-MuLV probe (data not shown). *EcoRI* was observed to cleave rat genomic DNA more frequently than the other restriction endonucleases used in this study (F. Yoshimura, personal observation). Unintegrated M-MuLV linear DNA digested by appropriate enzymes was routinely electrophoresed in an adjacent lane with digested NX-1 to -4 cellular DNAs for a direct comparison of fragment sizes.

Figure 6A shows that two large DNA fragments of 5.65 and 2.5 kbp were generated by *EcoRI* and *SacI* digestions of NX-1, -2, and -3 cellular DNAs (lanes B, C, and D, respectively). Digestion of NX-4, however, generated two DNA fragments of 3.9 and 2.5 kbp (Fig. 6A, lane E), suggesting a deletion of about 1.700 kbp from the larger 5.65-kbp fragment of the wild-type M-MuLV genome.

Generation of the internal 5.65- and 2.5-kbp DNA fragments by *EcoRI* and *SacI* digestions from each of the proviruses of NX-1, -2, and -3 with identical sizes to the two fragments from unintegrated M-MuLV linear DNA suggests that LTRs which are cut by *SacI* are present at both ends of the proviral DNAs. It was difficult to unequivocally identify junction fragments of each provirus because of the small amount of virus-specific sequences (400 or 200 bases) that would exist in each of these fragments.

The appearance of the 2.5-kbp fragment from NX-4 proviral DNA indicated that the leftmost and internal *SacI* sites were present, and that any deletion in this proviral DNA must come from the region to the right of the 2.95-kbp *SacI* site. Since the NX-4 right fragment differed in size from normal M-MuLV DNA, we were unable from this analysis alone to say whether the right LTR existed in NX-4 proviral DNA. Further analysis as discussed below of NX-4, however, indicated that this clone also contained 5' and 3' LTRs.

FIG. 5. Agarose gel electrophoresis of cell DNA digested with restriction endonuclease *EcoRI* from NX-1 to -4 and uninfected NRK-0 cells. Cellular DNA (15 μ g) was digested with *EcoRI* endonuclease and electrophoresed through a 1% agarose horizontal slab gel. DNA was transferred to a nitrocellulose filter and hybridized to 32 P-labeled M-MuLV DNA, and virus-specific bands were detected by autoradiography. The sizes of marker DNAs (*EcoRI*-digested λ DNA) are indicated in kbp. Lanes: A, NRK-0; B, NX-1; C, NX-2; D, NX-3; E, NX-4.

FIG. 6. Double digestions of NRK-0 and NX-1 to -4 cell DNA with either *SacI* and *EcoRI* in A or *XbaI* and *EcoRI* in B. Cellular DNA (20 μ g) was sequentially digested with *SacI* or *XbaI* followed by *EcoRI* restriction endonucleases. Agarose gel electrophoresis and subsequent treatment of the DNAs were as described in the legend to Fig. 5. Lanes (for both A and B): A, NRK-0; B, NX-1; C, NX-2; D, NX-3; E, NX-4; and F, Hirt-extracted linear M-MuLV DNA. Numbers refer to sizes in kbp of major virus-specific bands. C, Cleavage map of the 8.8-kbp linear DNA of M-MuLV taken from Gilboa et al. (12). Numbers refer to cleavage sites in kbp of *SacI* or *XbaI* restriction endonuclease. Bar indicates region where the deletion in NX-4 proviral DNA has been localized.

Since the sizes of the DNA fragments generated from NX-1, -2, and -3 were identical to the fragments obtained from wild-type unintegrated linear M-MuLV DNA, we can conclude that there are no large deletions or insertions detectable in the proviral DNAs of these three clones. In this size range we have been able to resolve differences of 75 to 100 nucleotides with the gel system used in these experiments (36). We attempted to further map the deletion in NX-4 with double digestion by *EcoRI* and *XbaI* endonucleases. *XbaI* cleaves the M-MuLV genome in the LTR sequences and makes two additional cuts generating two large fragments of 5.57 and 2.35 kbp (Fig. 6B, lane F, and Fig. 6C) (12). Three additional small fragments of 0.28, 0.3, and 0.3 kbp were not detected in these experiments.

As seen in Fig. 6B, the two virus-specific DNA fragments generated by *EcoRI* and *XbaI* double digestions of NX-1, -2, and -3 (lanes B, C, and D) migrated with the same mobility as the fragments generated from digestion of wild-type M-

MuLV unintegrated linear DNA (lane F). Besides the M-MuLV-specific bands, additional faint bands were also detected for NX-3 (Fig. 6B, lane D), which has been observed for some preparations of uninfected NRK-0 DNA (unpublished results). It was not clear what the faint band migrating slightly slower than the 3.9-kbp band in the marker M-MuLV lane was (Fig. 6B, lane F). These results support the previous conclusions from the *SacI* digestions that there are no large deletions detectable in the provirus DNAs of these three clones and that they possess LTRs at the 5' and 3' ends. Although the smaller 2.35-kbp DNA fragment generated by *XbaI* digestion of NX-4 DNA had the same size as the smaller fragment from the other clones, the larger DNA fragment from NX-4 was reduced by about 1,700 nucleotides as compared to the larger fragment generated by the other clones (Fig. 6B, lane E).

This deletion is the same size as that seen in the 5.65-kbp *SacI* DNA fragment discussed above. The common region shared by the *SacI*

and *Xba*I DNA fragments which suffer deletions in NX-4 is that area between 2.95 and 5.85 kbp of wild-type DNA (Fig. 6C). From these data we can only conclude that the deletion maps somewhere within this region but comprises a part of the polymerase region of the genome, which presumably extends from about 2.0 to 5.0 kilobases, roughly corresponding to the ends of Pr65^{gag} and Pr180^{gag-pol}.

Characterization of RNA species in non-infectious particles. Since we have determined that all four proviruses have LTR sequences at their 5' ends which appear to be the site of initiation for transcription of viral RNA (3), presumably all four proviruses have the potential for transcription and packaging of viral RNA. To determine whether the noninfectious particles produced by NX-1 to -4 package M-MuLV RNA, we labeled cells with ³²PO₄, recovered the particles released into the supernatant, and looked for ³²P-labeled RNA molecules. As shown in Fig. 7A the four clones had packaged a number of differently sized RNA molecules. Particles produced by NX-1 (lane B) contained RNAs of 38 and 33S, particles produced by NX-2 (lane C) contained RNAs of 38, 33, and 30S, particles produced by NX-3 (lanes D and F) contained RNAs of 38, 33, and 30S, and particles produced by NX-4 (lane E) contained RNAs of 33 and 30S. Virion RNA from a cloned NIH/3T3 cell line productively infected with M-MuLV (24) was electrophoresed in lane A as a

marker for 38S RNA. Small amounts of 28 and 18S ribosomal RNA were also found in particles of all of the clones. Clone NX-3 produced particles with various amounts of 33S RNA (Fig. 7A, lanes D and F) which differed for each particle preparation. As seen in Fig. 7A (lane F) long exposure of ³²P-labeled RNA from one preparation contained small amounts of 33S RNA.

It has been reported that two classes of rat endogenous viral RNA are expressed in rat cells in various amounts (23, 32). To determine which of the multiple RNAs packaged by these defective particles were specific for M-MuLV and which corresponded to possible rat RNAs, we prepared unlabeled RNA from particles produced by NX-1, -3, and -4. The low production of NX-2 particles (Table 1) prevented identification of the RNAs packaged in them by hybridization analysis. M-MuLV RNAs isolated from unlabeled particles were analyzed by the denaturing gel system of McMaster and Carmichael (20). Half of the RNA preparation from particles collected from the supernatant of a 10-cm plate of cells for each clone was electrophoresed through an agarose gel, transferred to activated diazobenzyloxymethyl paper (1), and hybridized to M-MuLV ³²P-labeled DNA.

The results in Fig. 7B indicated that only the RNA species of 38S for NX-1 (lane C), 38 and 33S for NX-3 (lane D), and 33S for NX-4 (lane E) were related to M-MuLV. The other 33 and 30S RNAs present in the particles of these clones

FIG. 7. Viral RNA from noninfectious particles. RNA was metabolically labeled with ³²P, in A and extracted from particles banded on a sucrose step gradient. Total ³²P-labeled RNA from particles collected from the supernatant of about 2×10^6 cells was denatured in 1 M glyoxal and 50% dimethyl sulfoxide and electrophoresed through a 1% agarose gel in 10 mM sodium phosphate (pH 7.0), which was dried and exposed to Kodak X-Omat film with an intensifying screen at -70°C . Sizes of RNA bands were based on the migration of 38S wild-type M-MuLV RNA and ribosomal RNAs. Lanes in panel A: A, wild-type M-MuLV; B, NX-1; C, NX-2; D, NX-3; E, NX-4; F, a different preparation of NX-3. Unlabeled total RNA isolated from particles from the supernatant of one 10-cm plate (ca. 2×10^6 cells) was prepared as described above and shown in B. After electrophoresis RNA was transferred to diazotized paper as detailed in the text and hybridized to nick-translated ³²P-labeled M-MuLV DNA. Lanes in panel B: A, Rous sarcoma virus; B, wild-type M-MuLV; C, NX-1; D, NX-3; E, NX-4.

were most likely to be rat endogenous retrovirus-like RNAs as described by other investigators (23, 32). The 33S ^{32}P -labeled M-MuLV RNA of NX-3 as detected by hybridization to ^{32}P -labeled DNA was also present in this preparation. RNA from wild-type M-MuLV virions was electrophoresed through the same gel as a marker (Fig. 7B, lane B). Since Rous sarcoma virus was added as carrier in the preparation of these noninfectious particles, we tested whether any cross-hybridization might be detected by the techniques used in these experiments, although it had been earlier shown that Rous sarcoma virus and M-MuLV do not share any homology. We could detect no RSV RNA bands that cross-hybridized with the M-MuLV probe (Fig. 7B, lane A) by the transfer technique used to identify these RNA forms.

DISCUSSION

The M-MuLV-infected rat cell clones NX-1 to -4, previously characterized to be defective in the production of infectious virus (24), were shown in this report to produce defective particles at levels comparable to those of wild-type rat cell clone, except for NX-2, which produced only one-tenth as many. By immunoprecipitation analysis we have shown that the absence of intracellular Pr180^{*gag-pol*} in NX-2 and NX-3 cells probably accounts for their lack of reverse transcriptase activity. For NX-1 and NX-4, however, intracellular proteins which immunoprecipitated with anti-reverse transcriptase serum and comigrated with Pr180^{*gag-pol*} were detected. It was previously reported by Shields et al. (24) that intracellular proteins related to Pr180^{*gag-pol*} were not detectable in any of the NX clones. This discrepancy with the observations reported here may be a result of differences in the amounts of cellular lysates used in the immunoprecipitations.

The lack of reverse transcriptase activity in NX-1 particles in spite of the presence of intracellular Pr180^{*gag-pol*} cannot presently be explained. Either NX-1 is defective in the processing of the *gag-pol* precursor or an inactive form of the enzyme is packaged into the defective particles. Attempts to immunoprecipitate reverse transcriptase from NX-1 particles were unsuccessful. The detection in NX-4 cell lysates of a 180K protein immunoprecipitating with anti-reverse transcriptase serum was surprising since we also showed that the provirus of NX-4 has suffered a deletion of 1,700 nucleotides, corresponding to about 68,000 daltons of protein, in the polymerase region of the genome. The detection of a protein of this size may be the result of the deletion of the normal termination signal

for Pr180^{*gag-pol*} and partial readthrough into the envelope region leading to a *gag-env* fusion product. Such a hybrid protein has been observed for an RSV polymerase deletion mutant (8). The lack of cross-reactivity of this 180K protein with anti-gp70 serum (data not shown), however, indicated that if readthrough has occurred, it was not in the same reading frame as that used for gp70.

Further analysis of intracellular viral proteins revealed that the NX clones were competent for synthesis of Pr65^{*gag*} and Pr80^{*env*}. Pr65^{*gag*} of NX-1 migrated slightly faster than the wild-type protein in SDS-polyacrylamide gels (Fig. 2, lane C), which may represent a very small deletion or a difference in phosphorylation of this protein which would also alter its mobility.

Analysis of the *gag*-related proteins in the defective particles produced by NX-1 to -4 revealed that they contained larger amounts of *gag* precursor proteins as compared with wild-type CP-1. The absence of normal amounts of processed *gag* virion proteins (p30 and p15) did not, however, prevent particle formation or packaging of viral RNAs into them. This is in contrast to the avian system in which it appears that processing of the *gag*-precursor protein is a prerequisite for particle formation (R. Eisenman, E. Hunter, H. Fan, and C. Dickson, *in* N. Teich, R. Weiss, H. Varmus, and J. Coffin, ed., *RNA Tumor Virus*, in press). A similar defective clone of an M-MuLV-infected mouse cell which produces particles without any apparent processing of Pr65^{*gag*} has been reported previously (24).

To determine whether any aberrant DNA structures could account for the defect in replication of NX-1 to -4, restriction endonuclease analysis of the proviral DNA of each of the clones was carried out. Analysis of these proviral DNAs was facilitated by the fact that each clone possessed only a single copy of the M-MuLV genome (28). The presence of LTR sequences in the proviral DNAs of NX-1 to -4 was determined since evidence is accumulating that LTRs may have important roles in (i) the integration of retrovirus DNA into host chromosomes via recombination in the LTR region and acceptor sites (7, 16, 25, 26, 30); (ii) the transcription of proviral DNAs by providing promoter sites which can be recognized by the host cell polymerase to transcribe viral sequences (16, 30, 35), and (iii) the potential transcription of cellular genes downstream from these putative viral promoter sites (35). The results of double digestions of cellular DNA with *Sac*I or *Xba*I and *Eco*RI led to the conclusion that LTRs were present at both ends of the proviral DNA of each of the four clones and that at least the nucleotide se-

quences at the *SacI* and *XbaI* sites were like the wild type.

In comparing the sizes of DNA fragments generated from the integrated proviruses with wild-type unintegrated M-MuLV linear DNA we observed that NX-1, -2, and -3 all had wild-type proviral DNAs as determined by restriction endonuclease analysis with either *SacI* or *XbaI* and *EcoRI*. These three mutants appeared to lack any detectable deletions or insertions in their proviral DNAs by this analysis. The existence of either point mutations or deletions smaller than about 75 base pairs cannot, of course, be eliminated by these experiments. We cannot also exclude the possibility of substitutions in the viral genomes which do not alter the size of the fragments generated by the restriction endonuclease used. By similar analyses we found that NX-4 proviral DNA had a deletion between 2.95 and 5.85 kbp from the 5' end of the viral DNA.

Noninfectious particles produced by all four clones packaged both M-MuLV and rat RNA species. The presence of small amounts of rat C type particles cannot, however, be excluded as the reason for the detection of rat 33 and 30S RNAs. It has been shown, however, that at least the rat 30S species can be rescued by heterologous replication-competent viruses (22). The sizes of the M-MuLV-specific RNAs packaged into these defective particles were concordant with their provirus sizes. However, one surprising observation was made for NX-3, which also packaged a smaller 33S RNA besides the full-length genomic-size 38S RNA. The presence of this smaller 33S RNA species suggests a post-transcriptional processing of genome-sized 38S RNA since NX-3 has a single provirus. It is thought that subgenomic-sized retrovirus mRNA's appear to be generated from 38S mRNA rather than from de novo transcription. This smaller molecule, which may correspond to a defective message for Pr180^{gag-pol} translation, may possibly arise by an abnormal splicing event of either the 5' leader sequence or a more internal region of the genome.

It appears that the genomic analysis of these replication-defective rat clones has resulted in an unambiguous explanation for only NX-4. The aberrant protein profiles of the other three clones await further studies of them for explanations of their defects.

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