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# Type C Viruses from Kirsten Sarcoma-Transformed Mink Cells Co-Cultivated with Primate Cells and Expressing p30 Antigens Related to Feline Leukemia Virus

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Two type C viruses with new antigenic and biological properties were isolated by co-cultivating secondary cell strains established from the kidneys of a baboon (*Papio papio*) and a patas monkey (*Erythrocebus patas*) with mink cells nonproductively transformed by Kirsten sarcoma virus. Both new isolates (designated PP-1R and EP-1R) contain major structural proteins (p30) that are immunologically most closely related to the p30 proteins of feline leukemia viruses. The reverse transcriptases of both viruses, although antigenically related to polymerases of murine and rat type C viruses, are distinct from those of previously described type C viral groups. Both PP-1R and EP-1R can be transmitted to canine and feline cells and to sarcoma virus-transformed, but not normal, mink cells. Both viruses contain RNA genomes partially homologous to those of endogenous mouse and rat type C viruses and the Kirsten sarcoma virus. In addition, the RNA of PP-1R contains a portion of the nucleic acid sequences found in a type C virus isolated from the baboon species *P. papio*. We propose that both new isolates are genetic recombinants formed between endogenous primate type C viral genomes and sequences found in Kirsten sarcoma-transformed mink cells.

Tissues of all genera of Old World monkeys and higher apes, including humans, contain multiple copies of viral gene sequences in their cellular DNA related to the RNA genomes of baboon type C viruses (8, 9a, 50). By co-cultivating a wide variety of primate tissues with a battery of indicator cells that are efficient for isolating and propagating baboon type C viruses from tissues of *Papio cynocephalus* (5, 52) and *Papio hamadryas* (18), we have isolated endogenous type C viruses from other species of baboons (*P. anubis* and *P. papio*) as well as from a closely related genus (*Theropithecus gelada*) (51a).

We now report the isolation of two new type C viruses derived by co-cultivation of primate tissues with a mink cell line nonproductively transformed by Kirsten sarcoma virus (KiSV). The experiments involved co-cultivation of cells from a baboon (*P. papio*) and a patas monkey (*Erythrocebus patas*) with the transformed mink cells, followed by induction of the mixed cultures with the halogenated pyrimidine 5-bromodeoxyuridine (BUdR). The new viruses can be transmitted to canine and feline cells, have retained genetically stable antigenic properties and RNA genomic sequences throughout 1 year of in vitro passage, and produce proteins related to those of both rodent and feline type C viruses.

## MATERIALS AND METHODS

**Cells and culture conditions.** Indicator cell lines used to test for replication of type C viruses were: the mink cell line Mv1Lu (CCL 64), the bat cell line Tb1Lu (CCL 88), and the rabbit cell line SIRC (CCL 60) obtained from the American Type Culture Collection (Rockville, Md.); the canine (FCf2Th) and feline (FEC, FFc60WF, FFc9WF) cell lines from the Naval Biomedical Research Laboratories (Oakland, Calif.); rhesus monkey lung cells, DBS-FRhL-1 (55); rat kidney cells, NRK (12); the murine SC-1 cell line (21); and the human rhabdomyosarcoma cell line A204 established in this laboratory (17). The nonproductively transformed mink cell line 64J1 was derived by transformation of Mv1Lu mink cells by KiSV (23). All cells were grown in plastic tissue culture flasks in the Dulbecco modification of Eagle medium supplemented with 10% calf serum and were transferred with 1% trypsin in phosphate-buffered saline.

Kidneys from West African baboon (*P. papio*) and from a patas monkey (*E. patas*) were minced into small fragments and inoculated into plastic tissue culture flasks containing serum-supplemented, complete medium. After 3 to 4 weeks, the primary cultures were transferred by trypsinization and co-cultivated with the host cell lines listed in Table 1.

TABLE 1. *Recovery of viruses from cultures of P. papio and E. patas cells cultivated with indicator host cell lines*[a]

| Indicator cell line co-cultivated | Species (indicator cells) | Supernatant reverse-transcriptase assay (cpm × $10^{-3}$ [$^3$H]TMP incorporated) | | | |
|---|---|---|---|---|---|
| | | 30[b] | 60 | 90 | 120 |
| With *P. papio* | | | | | |
| A204 | Human | 1.5 | 1.3 | 2.5 | 1.3 |
| DBS-FRhL-1 | Rhesus | 3.9 | 2.5 | *328.6* | *274.8* |
| FCf2Th | Dog | 3.8 | 3.9 | 3.2 | 2.7 |
| FEC | Cat | 0.7 | 2.6 | 2.3 | 2.0 |
| 64J1 | Mink (KiSV nonproducer) | 2.8 | 6.2 | *11.1* | *99.8* |
| With *E. patas* | | | | | |
| A204 | Human | 1.4 | 1.3 | 1.8 | 1.6 |
| DBS-FRhL-1 | Rhesus | 1.4 | 1.6 | 2.3 | 2.5 |
| FCf2Th | Dog | 2.4 | 5.6 | 4.1 | 6.1 |
| FEC | Cat | 1.7 | 1.0 | 0.7 | 2.0 |
| 64J1 | Mink (KiSV nonproducer) | 1.2 | 1.5 | *10.2* | *123.1* |

[a] Indicator cells were co-cultivated with cell strains from either *P. papio* or *E. patas*, and the mixed cultures were treated with BUdR. Supernatant reverse-transcriptase assays were performed on the cultures at 3-week intervals for a period of 7 months. Italicized numbers indicate levels of viral polymerase activity significantly above background.

[b] Days after induction.

After 48 h, the co-cultivated cultures were treated for 24 h with 100 μg of BUdR per ml and transferred at approximately 2-week intervals by trypsinization. After several passages, primate cells were no longer observed in co-cultivated cultures.

**Viruses.** The type C viruses used in these studies included: endogenous baboon viruses from *P. cynocephalus* (M7) (5) and *P. papio* (PP-1) (51a); the endogenous feline virus RD-114 (32); the endogenous rat viruses RT21c (46), VNRK and CCL 38 (27); the endogenous porcine virus PK-15 (51); the Rickard (36) and Gardner-Arnstein (16) strains of feline leukemia virus (FeLV); the Kirsten (26) and Rauscher (35) strains of murine leukemia virus; the endogenous NIH/Swiss xenotropic mouse virus AT-124 (49) and BALB/c N-tropic virus S2CL3 (48); and the simian sarcoma-associated virus (SSAV) (47).

**Infection of host cells with viruses.** Approximately $10^6$ host cells were seeded into 250-ml plastic tissue culture flasks in complete medium containing 2 μg of Polybrene per ml 24 h prior to infection. The cells were infected for 1 h with 1.5 ml of undiluted, filtered (0.45-μm pore size, Millipore Corp.) medium from virus-producing cell cultures. The infecting aliquots in undiluted virus stocks contained type C viruses with $10^4$ to $10^5$ cpm of [$^3$H]TMP incorporated into the polydeoxythymidylate [poly(dT)] product in the assay described below. When applicable, foci of transformation were scored on Mv1Lu mink cells (23) or on FFc9WF cat cell monolayers. Cloning of virus stocks was performed by an end-point dilution method (28), using serial dilutions of concentrated stock virus and canine FCf2Th cells. Cultures were assayed for supernatant reverse transcriptase 3, 6, and 12 weeks after infection.

**Supernatant reverse-transcriptase assay.** A 15-ml volume of medium from each tissue culture flask was clarified by centrifugation at 12,000 × *g* for 10 min. The supernatants were pelleted by centrifugation through 20% glycerol, and the pellets were suspended in buffer, disrupted, and assayed by using a polyriboadenylate template and oligodeoxythymidylate$_{12-18}$ primer as described previously (29). The results are expressed as counts per minute of [$^3$H]TMP incorporated into the radioactive poly(dT) product in a 60-min linear reaction.

**Antisera.** Antisera to p30 proteins and viral polymerases were raised in groups of New Zealand white rabbits; the reactivities of these sera to group-specific and interspecies antigenic determinants of type C viral proteins were described previously (43). Goat sera to the p30 protein of FeLV were also obtained from the Office of Resources and Logistics, Virus Cancer Program, Bethesda, Md., and as a generous gift of R. V. Gilden (Flow Laboratories, Rockville, Md.).

**Polymerase inhibition studies.** Polymerase assays were initiated with a mixture of template, primer, and substrate as described above. Detergent-disrupted viruses (0.01 ml) were used as sources of enzymes. Immunoglobulin G (IgG) was added prior to the addition of enzyme. At least 50,000 cpm of [$^3$H]TMP were incorporated into the acid-precipitable poly(dT) product after 60 min at 37°C, and incorporation was linear in the absence of immune IgG. Inhibition curves were developed by using serial dilutions of immune IgG prepared in the assay buffer (43). Control experiments performed with equivalent quantities of nonimmune IgG showed no inhibition of any enzyme studied in the range of protein concentrations tested. The quantities of immune IgG required for 30% inhibition of

enzyme activity were calculated from at least triplicate inhibition curves generated for each enzyme-antiserum combination (standard deviation, <10%).

**Purification of viral proteins.** Type C viral p30 proteins were purified by gel filtration in Sephadex G-100 and isoelectric focusing (41, 44). All proteins had approximate molecular weights of 30,000 as determined by electrophoresis in 7 and 13% polyacrylamide gels containing sodium dodecyl sulfate, and all were greater than 95% homogeneous as judged by multiple electrophoretic criteria (43, 44). Viral reverse transcriptases were partially purified (37) prior to their use for immunization of rabbits.

**Radioiodination.** Purified p30 proteins (10 $\mu$g) were radiolabeled with $^{125}$I by the chloramine-T method to approximately equivalent specific activities (5 $\mu$Ci/$\mu$g) (19). After separation of unbound iodine, greater than 95% of each labeled protein could be precipitated with 15% trichloroacetic acid, and greater than 90% could be precipitated with an excess of specific immune serum. Labeled test antigens were stored in 0.05 M phosphate buffer (pH 7.5) containing 1% bovine serum albumin and were used within 8 days after labeling. Titration with antisera showed no loss of antigenicity of the labeled preparations during this interval.

**Radioimmunoassays.** Antiserum titrations and competitive radioimmunoassays were performed by using a "double-antibody" method for precipitating immune complexes (41). Competition assays were initiated with 50% of the iodinated test antigen bound; 10,000 cpm of test antigen were employed per assay tube. Ether extracts of cultured cells infected with type C viruses or detergent-disrupted, banded viruses were used as competing antigens, and immune complexes were precipitated with a titered excess of anti-rabbit 7*S* globulin raised in sheep (Pocono Rabbit Farm and Laboratories, Canadensis, Pa.). Radioactivity in precipitates was quantitated in a Beckman 300 gamma counter (±3% error) at a counting efficiency of approximately 65%. The data are plotted as percent displacement of the labeled test antigen from immune complexes (linear ordinate) versus micrograms of competing protein (log scale abscissa). Points on the competition curves represent an average of three to six determinations for each competing antigen in each assay (standard deviation, <8%). By standardization with purified type C viral p30 proteins, a 20% displacement in these systems is obtained with from 0.5 to 1.5 ng of competing protein identical to the labeled test antigen (43, 44). For the assays shown, no significant antigenic differences were observed between the p30 proteins of the same type C virus propagated in cells from different species, or between different isolates from the same type C viral group (43).

**Protein determinations.** The concentrations of purified rabbit IgG were determined from absorbance measurements at 280 nm ($E^{1\%}_{1cm} = 14$). All other proteins were quantitated by the method of Lowry et al. (31), with bovine serum albumin as a standard.

**Preparation of type C viral $^3$H-labeled DNA probes.** The supernatant medium from the various cell lines producing type C viruses was collected at 3-h intervals for 1 day and stored at 2°C. The supernatant was clarified of cellular debris, and the virus was banded on sucrose gradients, pelleted, and used immediately in an endogenous reverse-transcriptase reaction. Reaction mixtures were incubated at 37°C for 2 h and contained: 0.04 M Tris (pH 7.8); 0.06 M KCl; $6 \times 10^{-3}$ M magnesium acetate; $2 \times 10^{-3}$ M dithiothreitol; 0.02% (vol/vol) Triton X-100; 30 $\mu$g of actinomycin D per ml; $5 \times 10^{-5}$ M [$^3$H]TTP (50 Ci/mmol); and dATP, dCTP, and dGTP, each at $2 \times 10^{-3}$ M. These conditions have been shown to result in the synthesis of long, representative DNA copies of the viral genome (11, 25, 38). The $^3$H-labeled DNA probes were deproteinized and purified as described previously (4). The specific activity of the $^3$H-labeled DNA was $1.8 \times 10^7$ cpm/$\mu$g. After hybridization of equimolar amounts of $^3$H-labeled DNA probes and $^{32}$P-labeled 70*S* viral RNA, 62 to 85% of the $^{32}$P-labeled RNA was resistant to digestion by RNase (4; unpublished data). The probes, therefore, contain most of the sequences present in viral RNA in proportions similar to those in the viral genome.

Several of the probes (M7, FeLV, RD-114, and SSAV) were purified further by hybridizing them to viral RNA extracted from the homologous virus grown in cells of a mammalian species different from the one in which the virus had been propagated originally. The nonhybridized portion of the $^3$H-labeled DNA probe was removed by digesting with $S_1$ nuclease (2, 4); probes were then treated with alkali to hydrolyze the RNA, and deproteinized and purified as described previously (4). Viral probes treated in this manner hybridized to 100% of the homologous viral RNA.

A probe containing sequences present in KiSV was prepared as follows. Mink cells nonproductively transformed by KiSV (23) were infected with RD-114 virus, and a $^3$H-labeled DNA probe prepared from the pseudotype virions was hybridized to the RNA of a rat cell line nonproductively transformed by KiSV (KNRK) (1). The nucleic acid sequences that did not hybridize (RD-114 and any mink sequences present in the probe) were removed by $S_1$ nuclease digestion, and the probe was alkali treated and deproteinized. The $^3$H-labeled DNA was subsequently hybridized to the RNA of Kirsten murine leukemia virus (Ki-MuLV) grown in NIH/3T3 cells to remove mouse type C viral sequences, and the portion of the probe that did not hybridize was purified on hydroxyapatite (8). This twice-cycled KiSV probe was used in the hybridization studies described in Table 5.

**Nucleic acid hybridization.** Cellular and viral RNAs were extracted and hybridizations of $^3$H-labeled DNA and RNA were performed as described previously (4); the extent of hybridization was determined with the single-strand-specific nuclease $S_1$ (2, 4).

## RESULTS

Cell strains derived from kidneys of a West African baboon (*P. papio*) and a patas monkey (*E. patas*) were co-cultivated with several cell lines derived from heterologous species in attempts to isolate endogenous primate type C viruses. After 48 h of co-cultivation of primate

cells with a number of heterologous cell lines, the mixed cultures were treated for 24 h with BUdR. Cultures were then serially passaged and tested for the presence of type C viruses using a supernatant reverse-transcriptase assay.

Table 1 shows the results of experiments performed with the *P. papio* (PP) and *E. patas* (EP) cells. In experiments with PP cells, supernatant reverse-transcriptase activity was detected in rhesus monkey lung cells 90 days after BUdR treatment. Low levels of polymerase activity were also detected in co-cultivated cultures of KiSV-transformed, nonproducer mink cells (64J1). Higher levels of supernatant polymerase activity were detected in both cultures 120 days after induction. Supernatant reverse transcriptase activity was also detected in co-cultivated cultures established with EP cells; in the latter case, enzyme activity was only seen in cultures containing 64J1 mink cells. In parallel experiments in which cell strains from *P. papio* or *E. patas* were co-cultivated without BUdR treatment, no supernatant polymerase activity was observed during 7 months of testing. Similarly, no viruses were detected in co-cultivations established with fresh, minced kidney *tissues* from these same animals. A number of other primate tissues, identically treated, have not yielded virus by this method (51a).

The West African baboon type C virus isolated in rhesus cells has been characterized extensively. This isolate, designated PP-1, contains a viral polymerase and a p30 protein that are antigenically indistinguishable from those of endogenous type C viruses isolated previously from several other species of baboons (*P. cynocephalus, P. hamadryas,* and *P. anubis*); however, the PP-1 virus can be distinguished from other baboon type C isolates by nucleic acid hybridization criteria and has a narrower host range since it replicates only in rhesus cells (51a). In contrast, the data presented below show that the viruses isolated from mink cells are new and can be readily distinguished from PP-1.

**Host range of the viruses isolated in mink cells.** The first indication that the virus isolated in rhesus cells (PP-1) differed from the viruses isolated in KiSV-transformed mink cells was derived from comparisons of the host ranges of the new isolates. The cell lines listed in Table 2 were infected with filtered supernatants from the cultures shown in Table 1, and viral replication was monitored by the supernatant reverse-transcriptase assay. The PP-1 virus replicated only in rhesus DBS-FRhL-1 cells and could not be transmitted to the other indicator cell lines tested, including the nonproductively transformed mink cell line 64J1. In contrast, the viruses (designated PP-1R and EP-1R) isolated in 64J1 cells co-cultivated with baboon or patas cells, respectively, replicated in canine, feline, and KiSV-transformed mink cells but not in normal mink (Mv1Lu) or rhesus cells. The PP-1R and EP-1R viruses passaged in either canine, feline, or KiSV-transformed mink cells showed the same host ranges as the original isolates when retested for replication on the same battery of host cell lines, indicating that the host range markers were genetically stable.

**Nontransforming viruses can be derived from PP-1R and EP-1R by end-point dilution.** The mink cell line Mv1Lu, which is not permissive for replication of the PP-1R or EP-1R viruses (Table 2), was used previously as an indicator cell line in assays for focus formation mediated by murine and feline sarcoma viruses (23). When the PP-1R virus growing in KiSV-transformed mink cells was used to infect Mv1Lu cells, several obvious foci of transformed cells were observed after a period of 4 to 6 weeks (3 to 10 foci per T75 flask). Supernatants from cultures of cloned, transformed colonies lacked detectable viral polymerase activity, and no transmissible infectious particles could be isolated. In contrast, when monolayer cultures of feline FFc9WF cells were infected with the PP-1R virus, numerous foci of transformed cells (50 to 75/T75 flask) were observed 10 to 14 days later, and both viral reverse-

TABLE 2. *Host range of viral isolates*[a]

| Host cell line | Species | Uninfected control | Supernatant reverse-transcriptase assay (cpm × $10^{-3}$ [$^3$H]TMP incorporated) | | |
|---|---|---|---|---|---|
| | | | PP-1[b] | PP-1R | EP-1R |
| A204 | Human | 2.5 | 1.8 | 1.3 | 1.4 |
| DBS-FRhL-1 | Rhesus | 2.5 | *551.9* | 4.1 | 2.1 |
| FCf2Th | Canine | 2.0 | 6.6 | *199.6* | *113.5* |
| FEC | Feline | 2.5 | 2.0 | *163.2* | *45.3* |
| FFc9WF | Feline | 1.8 | 0.8 | *217.3* | *34.6* |
| MvlLu | Mink | 1.8 | 2.7 | 4.1 | 1.4 |
| 64J1 | Mink (KiSV transformed) | 3.0 | 5.2 | *133.6* | *28.8* |
| NRK | Rat | 2.5 | 2.8 | 3.1 | 3.4 |
| SC-1 | Mouse | 0.8 | 2.1 | 1.7 | 1.8 |
| SIRC | Rabbit | 1.4 | 2.3 | 1.7 | 1.4 |

[a] Filtered supernatants from cultures producing type C virus (Table 1) were used to infect the various indicator cell lines shown. Supernatant reverse-transcriptase assays were performed 3 and 6 weeks after infection; radioactivity in [$^3$H]TMP incorporated in assays performed at 6 weeks is shown. Italicized numbers indicate levels of viral polymerase activity significantly above background.

[b] Infecting virus.

transcriptase activity and infectious type C virus were detected in the culture supernatants. The ability to readily score foci and isolate infectious type C virus from cells in which the PP-1R virus can grow (FFc9WF cells) and the development of nonproducer foci in cells that restrict its replication (Mv1Lu cells) suggested that the PP-1R virus could rescue the KiSV genome from 64J1 cells (pseudotype formation).

Serial dilutions of PP-1R virus were used to infect canine FCf2Th cells in an attempt to derive "cloned" helper virus lacking the ability to transform indicator cells in vitro. After three passages, virus was obtained by end-point dilution, which did not transform either Mv1Lu mink cells or FFc9WF feline cells. Canine cells infected with undiluted stocks of PP-1R virus continued to produce virus capable of transforming both indicator cell lines. The nontransforming isolates obtained from canine cells were still capable of rescuing sarcoma virus from nonproducer mink cells. Both the cloned helper virus and the uncloned pseudotype stock had the same host ranges and antigenic characteristics (see below). Comparable stocks of nontransforming viruses have been obtained by end-point dilution of EP-1R.

**Immunological comparisons of the reverse transcriptases of PP-1R and EP-1R with enzymes of known type C viruses.** The polymerases of PP-1, PP-1R, and EP-1R were studied by using antisera developed against polymerases of several groups of mammalian type C viruses. The reverse transcriptase of the PP-1 virus was inhibited markedly by immune IgG directed against the polymerase of the M7 baboon virus, whereas the polymerases of the PP-1R and EP-1R viruses were not inhibited (Fig. 1A). These results show that the polymerases of PP-1R and PP-1 differ antigenically from each other.

The PP-1R and EP-1R viral enzymes could be partially inhibited by an antiserum raised

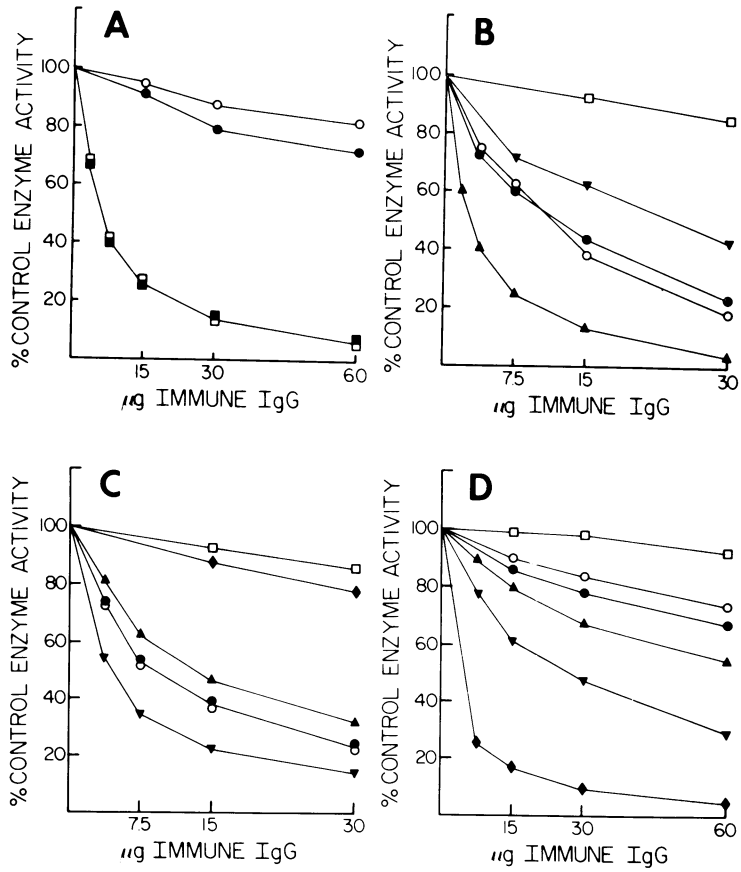


FIG. 1. *Inhibition of type C viral polymerases with antisera to type C viral reverse transcriptases. (A) Anti-baboon type C virus; (B) anti-MuLV (Rauscher strain); (C) anti-RT21c (rat); (D) anti-FeLV (Rickard strain). Symbols: ●, PP-1R; ○, EP-1R; □, PP-1 baboon virus; ■, M7 baboon virus; ▲, MuLV (Kirsten strain); ▼, endogenous rat virus, VNRK; ◆, FeLV (Rickard strain).*

against the reverse transcriptase of MuLV (Rauscher strain) but not to the same extent as the polymerase of KiMuLV (Fig. 1B). The reverse transcriptase of the endogenous rat virus VNRK was even less inhibited, whereas the enzyme of PP-1 was not inhibited. An antiserum raised to the reverse transcriptase of the endogenous rat virus RT21c readily inhibited the enzymes of endogenous rat viruses and cross-reacted strongly with several murine type C viral enzymes (Fig. 1C); with this serum, the enzymes of PP-1R and EP-1R were inhibited strongly but could again be distinguished from murine and endogenous rat type C viruses. An antiserum raised to the polymerase of FeLV also cross-reacted strongly with enzymes of rat type C viruses and less well with enzymes of mouse viruses (cf. reference 6); nevertheless, this antiserum did not significantly inhibit the enzymes of PP-1R and EP-1R (Fig. 1D). Studies performed with antisera to the polymerases of other known classes of mammalian type C viruses (porcine, endogenous feline, and infectious primate) (43) showed no antigenic similarities between the EP-1R and PP-1R enzymes and viral reverse transcriptases from these latter groups.

Table 3 summarizes results obtained with different antisera to type C viral reverse transcriptases. Taken together, the data show that the polymerases of PP-1R and EP-1R are antigenically related to, but can be distinguished from, those of known murine and rat type C viruses. The reverse transcriptases of the new isolates, then, appear to be more closely related to type C viral polymerases of rodent than of primate origin, and possess antigenic properties different from those found in other type C viral groups described.

TABLE 3. *Inhibition of type C viral reverse transcriptases with specific antisera*[a]

| Virus | Immune IgG required (μg) for 30% inhibition | | | |
|---|---|---|---|---|
| | Anti-M7 (baboon) | Anti-MuLV (mouse) | Anti-RT21c (rat) | Anti-FeLV (feline) |
| New isolates | | | | |
| PP-1R | >60 | 4.5 | 3.6 | 54 |
| EP-1R | >60 | 5.4 | 3.6 | >60 |
| Baboon type C virus | | | | |
| PP-1 | 3.0 | >60 | >60 | >60 |
| M7 | 3.0 | >60 | >60 | >60 |
| Mouse type C virus | | | | |
| Kirsten strain | 54 | 1.2 | 6.0 | 26 |
| Rauscher strain | 52 | 1.3 | 5.2 | 22 |
| Moloney strain | 54 | 1.6 | 5.8 | 27 |
| S2CL3 (BALB/c) | 55 | 1.3 | 5.4 | 28 |
| AT-124 (NIH/Swiss) | 48 | 1.4 | 7.0 | 23 |
| Rat type C virus | | | | |
| RT21c | >60 | 10 | 2.1 | 8.5 |
| VNRK | >60 | 9.0 | 2.2 | 11 |
| CCL 38 | >60 | 13 | 2.2 | 11 |
| Feline type C virus | | | | |
| FeLV (Rickard) | >60 | 37 | 48 | 1.0 |
| FeLV (Gardner-Arnstein) | >60 | 41 | NT | 1.3 |

[a] Purified IgG from the various antisera shown were used to inhibit type C viral polymerases. The antiserum to MuLV polymerase was prepared against the Rauscher strain, and the antiserum to FeLV was prepared against the Rickard strain. The amount of IgG required for 30% inhibition was calculated from inhibition curves developed with serial dilutions of IgG. NT, Not tested.

**PP-1R and EP-1R contain p30 antigens related to those of FeLV's.** Analyses of the antigenic characteristics of the major group-specific proteins (p30) of PP-1R and EP-1R were performed by using competitive radioimmunoassays (Fig. 2). In these assays, the amount of viral protein required to initiate competition is a measure of the titer and purity of the competing virus preparation; the slope of the competition curve and final extent of the reaction reflect the degree of relatedness of the competing antigen and radiolabeled p30 protein (43, 44). In an assay for the p30 protein of the M7 baboon type C virus (Fig. 2A), the PP-1 virus competed with the same slope and extent as the M7 baboon virus, whereas no competition was observed with PP-1R or EP-1R. In an assay for the p30 protein of the endogenous feline RD-114 virus (Fig. 2B), both the M7 and PP-1 virus cross-reacted with the labeled RD-114 protein, as indicated by the reduced slopes of the competition curves, whereas, again, the PP-1R and EP-1R antigens were not detected. Thus, the p30 antigens of PP-1R and EP-1R are unrelated to those of endogenous baboon type C viruses, whereas the PP-1 virus contains a typical baboon type C viral p30 antigen (44, 51a).

In an "interspecies" assay (Fig. 2C), which detects the p30 proteins of certain murine (*M. musculus*), rat, and feline type C viruses (18, 34, 43), the PP-1R and EP-1R viruses competed for the labeled test antigen. Assays for the p30 proteins of MuLV's (Fig. 2D) and rat type C viruses (Fig. 2E), however, failed to detect the PP-1R and EP-1R p30 antigens. Unexpectedly, the p30 antigens of both new isolates could be detected in an assay for the p30 protein of FeLV (Fig. 2F). A comparison of the interspecies as-

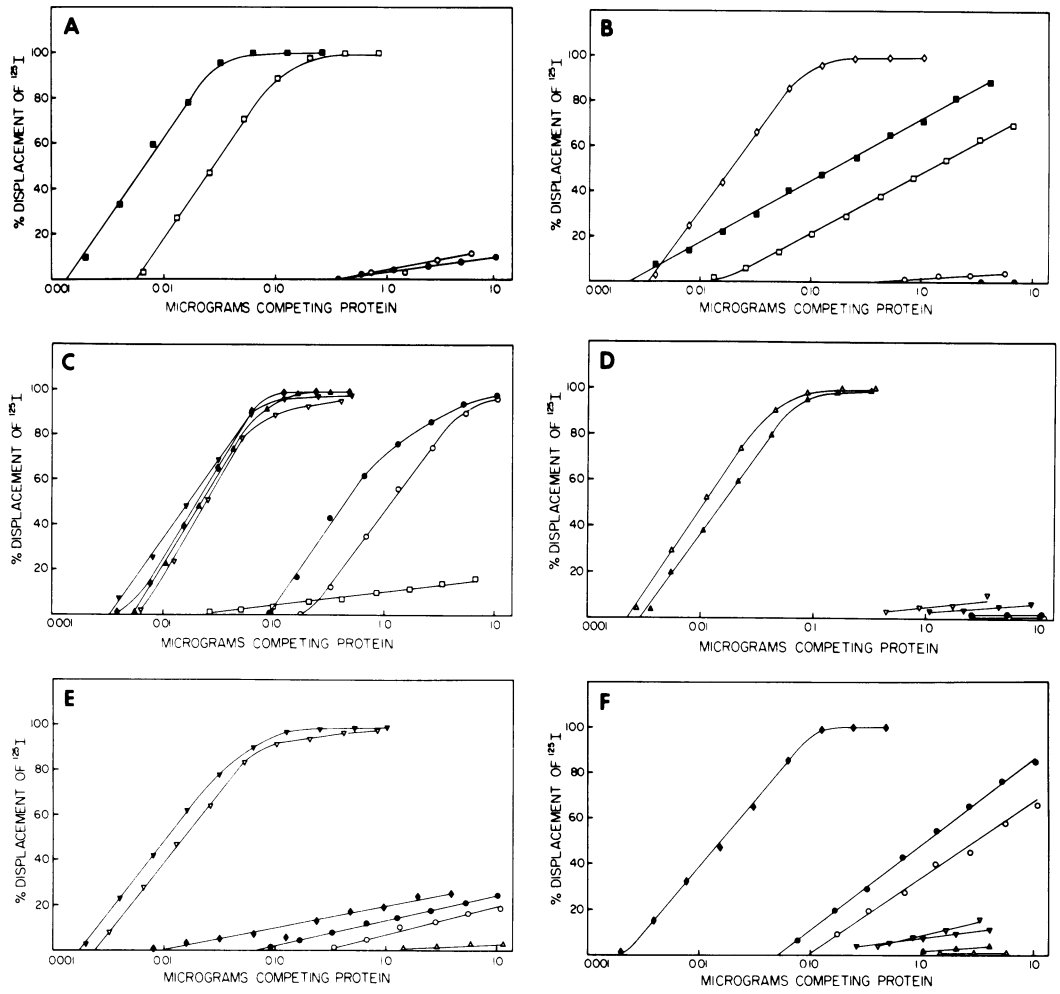


FIG. 2. *Radioimmunoassays for the p30 proteins of various type C viruses. Assay for p30 protein of (A) M7 baboon virus, (B) RD-114 virus, (D) MuLV, (E) endogenous rat virus (RT21c), and (F) FeLV. The "interspecies" assay shown in (C) uses antiserum to FeLV and $^{125}$I-labeled MuLV p30 protein. Symbols: ●, PP-1R; ○, EP-1R; □, PP-1 baboon virus; ■, M7 baboon virus; ▲, MuLV (Kirsten strain); △, MuLV (Rauscher strain); ▼, endogenous rat virus (VNRK); ▽, endogenous rat virus (RT21c); ◆, FeLV (Rickard strain); ◇, endogenous cat virus (RD-114).*

say (Fig. 2C) and the group-specific assays (Fig. 2D through F) shows that all of the PP-1R and EP-1R p30 antigen detected in the interspecies assay is of the FeLV-related type. The slightly reduced slopes of the competition curves obtained with PP-1R and EP-1R in the FeLV assay as compared with the slope obtained with FeLV (Fig. 2F) suggest that both new viruses contain p30 antigens which, although closely related, are not identical to the FeLV p30 protein.

Table 4 quantitates the amounts of p30 antigen found in different cell lines infected with various viral stocks. With extracts of virus-infected cells rather than disrupted virus as competing proteins in radioimmunoassays, FeLV-related p30 antigens were readily detected in extracts of canine, feline, or transformed mink cells infected with either PP-1R or EP-1R. Consistent data have been obtained regardless of cell type or the presence or absence of in vitro transformation. In contrast, KiSV-transformed mink cells, as well as the canine and feline cells used in these studies, did not contain detectable p30 antigens (<3.0 ng/mg of cellular protein). Furthermore, neither the rescue of the KiSV genome using several different helper viruses, nor induction of cells with halogenated pyrimidines, has led to the production of detectable FeLV-related p30 antigen in 64J1

TABLE 4. *Properties of new viral isolates*[a]

| Virus stock | Host cell | Focus-forming activity | Virus titer in host cell (ng of p30 antigen/mg of cell protein)[b] | Antigenic determinants | |
|---|---|---|---|---|---|
| | | | | p30 protein | Reverse transcriptase |
| PP-1 | Rhesus (DBS-FRhL-1) | No | 4,000 | Baboon | Baboon |
| PP-1R | Mink (64J1) | Yes | 98 | FeLV related | Rodent |
| | Canine (FCf2TH) | Yes | 380 | FeLV related | Rodent |
| | Canine (FCf2Th) endpoint diluted | No | 196 | FeLV related | Rodent |
| | Feline (FFc9WF) | Yes | 460 | FeLV related | Rodent |
| EP-1R | Mink (64J1) | Yes | 60 | FeLV related | Rodent |
| | Canine (FCf2Th) | Yes | 95 | FeLV related | Rodent |
| | Canine (FCf2Th) endpoint diluted | No | 84 | FeLV related | Rodent |
| M7[c] | Mink (64J1) | Yes | 700 | Baboon | Baboon |
| SSAV[c] | Mink (64J1) | Yes | 5,000 | Woolly monkey | Woolly monkey |
| AT-124[c] | Mink (64J1) | Yes | 2,750 | Mouse | Mouse |
| FeLV[c] | Mink (64J1) | Yes | 420 | FeLV | FeLV |
| None | Mink (64J1) | No | <2.5 | ND[d] | ND |
| None | Canine (FCf2Th) | No | <3.0 | ND | ND |
| None | Feline (FFc9WF)[e] | No | <3.0 | ND | ND |
| None | Mink 64J1 (BUdR)[f] | No | <2.0 | ND | ND |

[a] Assays for viral reverse transcriptase were performed on pelleted virus from the culture supernatants. Assays for p30 proteins were performed both with pelleted supernatant fluids and with ether extracts of cells.

[b] Cells infected with virus were tested 14 days after infection.

[c] Infection of 64J1 mink cells with M7, SSAV, AT-124, or FeLV results in the rescue of KiSV and the production of pseudotype virions in which the KiSV genome is packaged in the coat of the helper type C virus (23).

[d] ND, Not detected.

[e] The feline cell line FFc9WF is noninducible by halogenated pyrimidines for viruses related to either RD-114 or FeLV; extensive co-cultivations utilizing this line have not led to the production of antigens related to either virus of domestic cats.

[f] Cultures of 64J1 mink cells were treated with 100 $\mu$g of BUdR, per ml, and extracts of cells were tested for p30 antigens and supernatant polymerase at various times (4 and 8 h; 1, 2, 4, 6, 8, 10, 14, and 21 days) after induction.

mink cells. Thus, the antigenic properties of the PP-1R and EP-1R viruses are genetically stable and are independent of the host cell in which the viruses are grown.

**PP-1R contains nucleic acid sequences related to baboon, mouse, and rat type C viruses.** Using $^3$H-labeled DNA transcripts of the RNA genomes of known groups of type C viruses, we attempted to characterize the RNA genomes of PP-1R and EP-1R. Table 5 summarizes the results of experiments in which $^3$H-labeled DNA transcripts prepared from representative members of all known groups of mammalian type C viruses were annealed to the RNA of canine cells producing PP-1R. As shown, PP-1R contains nucleic acid sequences that hybridize, to varying extents, to mouse (KiMuLV) and rat (VNRK) type C viral probes. In addition, PP-1R contains nucleic acid sequences that hybridize to a probe prepared from an endogenous baboon virus (M7) isolated from *P. cynocephalus*. The final extent of homology detected with the M7 $^3$H-labeled DNA probe may be an underestimate since the endogenous virus isolated from *P. papio* (PP-1) is approximately 85% related to type C viruses isolated from *P. cynocephalus* (51a).

An analysis of the rate of hybridization of these probes to the cellular RNA of canine cells producing PP-1R reveals that the $C_rt_{1/2}$ (10) for the hybridization of RNA sequences related to mouse, rat, and baboon type C viral probes was 2,500 to 2,800. These $C_rt_{1/2}$ values are approximately 1/15 of the values obtained for a canine cell line producing baboon type C virus (52) and agree well with the relatively low levels of p30 antigen detected in PP-1R-infected canine cells (Table 4). The similarity of the $C_rt_{1/2}$ values for each of the three probes suggested that sequences related to mouse, rat, and baboon type C viruses were transcribed to a comparable extent in canine cells infected with PP-1R, consistent with the covalent linkage of these sequences in a recombinant virus.

TABLE 5. *Detection of nucleic acid sequences related to several type C viruses in PP-1R and EP-1R*[a]

| Cellular RNA[b] | $^3$H DNA probe (% hybridization) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mouse (KiMuLV) | Rat[c] (VNRK) | Baboon[d] (M7) | Cat (FeLV) | Cat (RD-114) | Pig (PK-15) | Woolly monkey (SSAV) | Rat (RT21c) | Rat (cycled KiSV) |
| PP-1R (FCf2Th cells) | 19 | 15 | 12 | 2 | 3 | <2 | 3 | 2 | 34 |
| EP-1R (FEC cells) | 35 | 15 | <2 | 2 | <2 | <2 | 3 | 4 | 37 |

[a] $^3$H-labeled DNA probes were prepared as described in the text. KiMuLV virus was grown in NIH cells, M7 (baboon) and RD-114 viruses were grown in a bat lung cell line (TblLu), the Rickard strain of FeLV was grown in a cat cell line (FEC), and woolly virus (SSAV) was grown in a human rhabdomyosarcoma cell line (A204). RT21c and VNRK viruses were released spontaneously from rat cells (27, 46), and PK-15 (51) was released spontaneously from the pig kidney cell line (CCL 33, American Type Culture Collection). The preparation of the probe containing Kirsten sarcoma nucleic acid sequences (cycled KiSV) is described in the text; sequences related to KiMuLV have been removed. The percent hybridization values shown are the saturating normalized values obtained from at least three separate experiments after digestion of the hybrids with $S_1$ nuclease. The actual final percent hybridization to the homologous RNAs varied from 76 to 100%. Background values obtained with heterologous RNAs have been subtracted; these varied from 0.5 to 5%. The low levels of hybridization obtained with RD-114 and SSAV $^3$H-labeled DNA probes can be explained by the known homologies of these viruses with endogenous baboon (9, 52) and endogenous mouse (7, 30) type C viruses, respectively.

[b] Cellular RNA was extracted as described previously (4) from canine thymus cells (FCf2Th) infected with PP-1R and from feline cells (FEC) infected with EP-1R.

[c] Hybrids formed with VNRK $^3$H-labeled DNA and RNA of rat VNRK cells have $T_m$ values 2.5°C higher (88.2°C $T_m$) than hybrids formed with PP-1R RNA.

[d] Hybrids formed with M7 $^3$H-labeled DNA and the RNA of dog cells producing M7 virus have $T_m$ values 1.0°C higher (87°C $T_m$) than hybrids formed with PP-1R RNA.

Although canine cells producing EP-1R also contained RNA related to rat (VNRK) and mouse (KiMuLV) type C viruses (Table 5), they do not contain sequences that can be detected with the baboon viral $^3$H-labeled DNA probe. The cellular DNA of *E. patas* contains endogenous type C virogenes that are only partially related to the RNA genome of endogenous viruses isolated from baboon cells (4, 8). Thus, if a low level of endogenous patas type C viral nucleic acid sequences was indeed present in the RNA genome of EP-1R, these might not be detected with the baboon viral $^3$H-labeled DNA probe. The low level of hybridization obtained with the FeLV $^3$H-labeled DNA probe to the RNA of cells producing PP-1R or EP-1R (hybridized to a $C_rt$ of $4 \times 10^4$) shows that the detection of p30 antigen related to FeLV is not due to low levels of FeLV replicating in these cells. These data are consistent with the failure to detect viral polymerase related to that of FeLV in any PP-1R or EP-1R virus stocks.

Scolnick and co-workers (39, 42) showed that the genome of KiSV contains sequences related to mouse and rat type C viruses. Using $^3$H-labeled DNA transcripts prepared from the spontaneously produced, endogenous rat viruses VNRK and RT21c, these investigators reported that $^3$H-labeled DNA probes prepared from the VNRK virus hybridize to the RNA of non-rat cells containing KiSV, whereas probes prepared from RT21c do not detect these sequences (39, 40). Similar results were obtained in our studies with the RNA of cells infected with PP-1R and EP-1R. Since the type C virions spontaneously released by VNRK cells are mixtures of two different rat viruses, and because the RNA of only one of these viruses is homologous to the KiSV genome (39, 40, 42), the amount of rat type C viral information in PP-1R and EP-1R was probably underestimated. To directly test the possibility that PP-1R and EP-1R contain rat viral sequences derived from KiSV, a $^3$H-labeled DNA probe was prepared to a pseudotype of KiSV, and portions of the transcript homologous to the helper virus and KiMuLV were removed (see Materials and Methods). The data (Table 5) show that this probe hybridizes extensively to the RNA of cells producing either PP-1R or EP-1R. Like the $C_rt_{1/2}$ values obtained with the VNRK and KiMuLV $^3$H-labeled DNA probes, the $C_rt_{1/2}$ for this reaction was 2,800.

The results suggest that PP-1R contains sequences related to endogenous type C viruses of at least three different species. Furthermore,

the genome of PP-1R contains additional endogenous viral or other cellular sequences of mink cell origin. $^3$H-labeled DNA transcripts prepared from the PP-1R virus grown in canine cells hybridize not only to the cellular DNA of baboon, rat, and mouse cells but also to mink cellular DNA (unpublished data). Since the probes employed in these studies were not uniform, representative transcripts of the viral genomes, we cannot as yet quantitate the contribution from each of these species. The hybridization data, however, support the conclusion that PP-1R was derived by recombinational events occurring between a type C virus of baboon origin and sequences present in KiSV-transformed mink cells. The similar antigenic and biological properties of PP-1R and EP-1R, and the ability to detect both mouse- and rat-derived sequences in the latter isolate as well, suggest that EP-1R was derived by a similar mechanism.

## DISCUSSION

In the present report, we describe the isolation of a new type C virus (PP-1R) obtained by co-cultivating baboon cells from the species *P. papio* with mink cells nonproductively transformed by KiSV. A similar virus (EP-1R) was obtained from cultures of patas monkey cells cocultivated with the same mink cell line. Unlike the baboon type C virus PP-1, the PP-1R and EP-1R viruses have a broader host range and contain p30 proteins antigenically most closely related to those of FeLV's and polymerases that are immunologically similar to those of murine and rat type C viruses. The antigenic and biological properties of both new isolates do not depend on the host cells in which they are grown, or whether the virus stocks are capable of transforming cells in culture, and have remained genetically stable for longer than 1 year of in vitro passage.

The limited final extent of hybridization of PP-1R RNA to the M7 $^3$H-labeled DNA probe shows that complete copies of the baboon type C viral genome are not present. Thermal melting experiments performed with hybrids between M7 $^3$H-labeled DNA and the RNA of PP-1R indicate that such hybrids have a high $T_m$ (86°C) consistent with a *P. papio* viral origin of the primate sequences (footnotes, Table 5). Thus, PP-1R is not a baboon type C virus that has rescued the KiSV genome from transformed mink cells. Were this the case, complete copies of baboon type C viral RNA would have been detected and, by analogy with other baboon viral pseudotypes of KiSV (Table 4), the p30 protein and reverse transcriptase would be specified by the helper baboon type C virus.

Single-stranded, $^3$H-labeled DNA transcripts of KiMuLV and the endogenous rat virus VNRK also hybridize partially to the RNA of cells producing PP-1R and EP-1R. Only background levels of hybridization were seen with probes prepared from the endogenous rat virus RT21c. Although we have thus far been unable to account for all of the RNA sequences in PP-1R by using these techniques, the methods employed necessitate the preparation of representative and uniform transcripts from at least three classes of type C viruses. In addition, preliminary data show that mink host cell sequences are present in both PP-1R and EP-1R and that both viruses contain the same sets of mink sequences.

Taken together, our data suggest that PP-1R and EP-1R are recombinant type C viruses containing viral genetic elements of at least mouse, rat, and primate origin. If this interpretation is correct, then a minimum of two recombinational events must have occurred. The first of these is amply documented and involves the generation of KiSV that contains mouse and rat type C viral sequences, and additional rat sequences presumably responsible for transformation (39, 40, 42). The mouse and rat sequences in KiSV appear to be covalently linked (40) and sediment together as a defective 30S genome (53). These sequences are integrated into the DNA of KiSV-transformed mink cells (39, 40), the cell line from which PP-1R and EP-1R were isolated. We propose that a second recombinational event occurred in the mink cells and involved primate viral sequences.

Our experiments show, quite unexpectedly, that the p30 proteins of PP-1R and EP-1R are antigenically closely related to those of FeLV's despite the fact that stocks of PP-1R and EP-1R were never passaged in cat cells and sequences related to FeLV $^3$H-labeled DNA were not detected. Thus, viruses of nonfeline origin can code for p30 proteins closely related antigenically to those of FeLV's. Based on nucleic acid hybridization and immunological data, we previously proposed that the viruses of the FeLV group were derived from rodent, and probably rat, type C viruses that infected ancestors of domestic cats and certain other closely related *Felis* species (6). Since rat viral sequences can be detected in the RNA of both PP-1R and EP-1R, one possibility is that the genes coding for the FeLV-related p30 protein originated in rats but are not identical to genes coding for the p30 proteins of previously described rat type C viruses. Alternatively, the p30 proteins of the new isolates could be encoded by genetic ele-

ments of mink cell origin. Were this the case, the results would imply that a similar set of mink type C virogene sequences was incorporated into the genomes of both PP-1R and EP-1R.

The new isolates can be readily distinguished from spontaneously produced, endogenous type C viruses of rats (VNRK, RT21c, and CCL 38) which have not been transmitted to heterologous cells in culture, or from PP-1 and KiMuLV which are restricted from replicating in canine and feline cells and which differ substantially from PP-1R and EP-1R in their antigenic properties and RNA genomes. If the p30 proteins and polymerases of PP-1R and EP-1R are indeed of rodent viral origin, the data would suggest that the recombined rodent portions of their genomes have retained the capacity to code for at least two viral proteins.

Recombination between type C viruses has been reported previously in several different systems. Nondefective avian sarcoma viruses have been shown to undergo genetic recombination with avian leukosis viruses (20, 22, 54, 56). Electrophoretic analysis has demonstrated that cloned avian sarcoma virus recombinants contain only class *a* RNA subunits, which have "fingerprint" patterns of RNase T1-resistant oligonucleotides that differ from those of parental sarcoma virus genomes (3, 13, 14, 24). Thus, recombinants are generated by genetic crossovers rather than by simple reassortment of markers situated on different type C viral genomic subunits. This interpretation is also supported by studies of recombination between temperature-sensitive mutant avian type C viruses (58). Mammalian type C viruses grown in animals or cultured cells of heterologous species can also recombine with host cell genes to form virions with altered biological properties. Murine leukemia viruses, selected for growth in mouse or human cells, may in certain cases represent recombinants between exogenous and endogenous murine type C viruses (45, 57). The recent report of a cloned murine type C virus with dual eco- and xenotropic properties (15) could also be explained by recombinational mechanisms. Studies with the Harvey sarcoma virus have provided additional evidence that a mouse leukemia virus (Moloney strain) propagated in rat cells may recombine with host cell genes to generate viruses with sarcomagenic properties (33, 39, 40).

The PP-1 baboon virus cannot replicate in 64J1 mink cells that contain defective KiSV genomes. The conditions employed in these cocultivation experiments, then, may have strongly selected for the appearance of recombinant particles. Recent experiments in our laboratory have indicated that other classes of type C viruses that replicate poorly or not at all in transformed mink cells may also generate new classes of infectious type C viruses, presumably by a similar mechanism. Selection for recombinant type C viruses may provide one means for isolating portions of endogenous virogenes as integral segments of replicating viral RNA. If endogenous virogenes of certain species have been altered sufficiently, expression of complete, infectious particles may be rare or even nonexistent; experiments like those described here offer one approach for supplying deleted functions and for recovering portions of virogenes from such species.

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