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A new class of genetically transmitted retravirus isolated from *Mus cervicolor*

(endogenous RNA tumor virus/viral gene evolution/nucleic acid hybridization/mammary tumor virus)

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ABSTRACT **The cocultivation of spleen cells from the Southeast Asian mouse, *Mus cervicolor*, with heterologous cell lines has permitted the isolation of a new retravirus (designated M432) that can be transmitted to tissue culture cells of the laboratory mouse, *M. musculus*. Cells infected with M432 contain cytoplasmic type A particles and budding forms with compact, spherical nucleoids; extracellular virions lack surface spikes and have a condensed, central core surrounded by an intermediate line. Like other retraviruses, M432 bands isopycnically in sucrose at 1.16–1.17 g/cm^3 and contains a 70S RNA genome composed of 35S subunits and an RNA-dependent DNA polymerase (RNA-dependent DNA nucleotidyltransferase). The viral reverse transcriptase requires magnesium as a cofactor and transcribes the synthetic template:primer poly(rC)·oligo(dG) more efficiently than poly(rA)·oligo(dT). [^{3}H]DNA transcripts of the viral RNA genome detect multiple copies of endogenous virogene sequences in the cellular DNA of normal *M. cervicolor*, and fewer copies in heterologous cells infected with M432. Partially related nucleic acid sequences are also detected in the DNA of *M. caroli* and *M. musculus* as well as in more distantly related species (rat and hamster), reflecting the evolutionary conservation of these gene sequences in rodents. Although the virus from *M. cervicolor* shares certain morphologic and biochemical properties with murine type B viruses, the new isolate is unrelated by nucleic acid hybridization criteria to the mouse mammary tumor virus, the bovine leukemia virus, the Mason-Pfizer monkey virus, or known murine type C viruses, including endogenous type C viruses isolated from *M. cervicolor*.**

The Retraviridae are enveloped RNA viruses that contain a 70S viral genome composed of 35S subunits and an RNA-dependent DNA polymerase (RNA-dependent DNA nucleotidyltransferase), and mature by budding from the plasma membrane of cells (1). In addition to the type C leukemia and sarcoma viruses which have been isolated from a wide variety of vertebrate species, the Retraviridae include the type B viruses [mouse mammary tumor virus (MMTV) and the endogenous guinea pig virus], the Mason-Pfizer monkey virus, foamy viruses, the bovine leukemia virus, and the visna and progressive pneumonia viruses of sheep (reviewed in ref. 1). Many of these have been shown to produce neoplasia in animals, particularly leukemia, lymphomas, sarcomas, and mammary adenocarcinomas. We now report the isolation of a new genetically transmitted retravirus from the Asian mouse, *Mus cervicolor*. This virus is infectious and replicates well in tissue culture cells of the laboratory mouse *M. musculus*. By certain morphologic and biochemical criteria, the new isolate appears to be related in some of its properties to MMTV isolated from laboratory strains of mice.

Abbreviations: MMTV, mouse mammary tumor virus; C_0t is the concentration of DNA in moles of nucleotide per liter multiplied by time in seconds.

MATERIALS AND METHODS

Viruses and Cell Cultures. Cell lines and viruses were as described (2–5). A cell line established in this laboratory from the lung of a *M. cervicolor* mouse has been continuously passaged for over 1 year. Radiolabeled nonrepetitive DNA prepared from these cells hybridizes completely to DNA extracted from tissues of *M. cervicolor* mice.

Cocultivation with Indicator Cells. A spleen from a *M. cervicolor* mouse was minced and cocultivated with various indicator cell lines derived from heterologous species, including *M. musculus* (NIH/3T3 and SC-1), rabbit (SIRC), rhesus (DBS-FRhL-1), and cat (FFc60WF). Indicator cell lines at 50% confluency were treated with 2 μg/ml of Polybrene for 24 hr prior to cocultivation. Cells were transferred by trypsinization at approximately 1-week intervals; after several passages, the indicator cells overgrew the *M. cervicolor* spleen cells. Supernatants were assayed for the presence of viral polymerase activity at 2-week intervals for 4 months.

Electron Microscopy. Tissues and cell pellets were fixed in 2.5% glutaraldehyde, rinsed with several changes of 0.01 M phosphate-buffered saline, treated with Dalton's chrome-osmium, and rinsed again with several changes of distilled, deionized water (6). The specimens were stained overnight with 1% uranyl-acetate in 50% ethanol, dehydrated with increasing concentrations of ethanol, followed by propylene oxide, and embedded in Luft Epon. Thin sections were stained with lead citrate. Negative stains were prepared by mixing isopycnically banded virus in 0.05 M sodium citrate (pH 6.0) with 2% potassium phosphotungstate (pH 4.0). The mixture was deposited on a carbon-coated grid, drained, and air-dried just prior to use.

Viral Polymerase Assays. Virus from culture supernatants was concentrated and assayed for viral reverse transcriptase as described (2, 3). Reactions were carried out in a total volume of 0.1 ml using 0.5 μl of virus (10 μg of protein) and contained 50 mM Tris·HCl (pH 7.8), 4 mM dithiothreitol, 60 mM KCl, 0.03% Triton X-100, and $MnCl_2$ or $MgCl_2$ as indicated. Poly(rA)·oligo(dT)$_{12-18}$ (10 μg/ml) and 5 μCi of [^{3}H]dTTP (60 Ci/mmole; New England Nuclear) or poly(rC)·oligo(dG)$_{12-18}$ (10 μg/ml) and 5 μCi of [^{3}H]dGTP (37.2 Ci/mmol; New England Nuclear) were used as template:primer and labeled nucleotides in the exogenous reactions. For the endogenous reaction, 1 mM each of dATP, dCTP, and dGTP and 5 μCi of [^{3}H]dTTP (60 Ci/mmol; New England Nuclear) were included in the reaction mixture.

Determination of Genome Size. NIH/3T3 cells infected with the *M. cervicolor* virus were labeled with [^{32}P]phosphoric acid. Virus from culture supernatants was concentrated and

isopycnically banded, and 70S RNA was purified (7). The RNA was dissolved in 0.01 M Tris·HCl (pH 7.5), containing 0.1 M NaCl and 10^{-4} M EDTA, and heated at 95° for 3 min. Denatured RNA was immediately applied to 0.5% agarose–1.5% polyacrylamide cylindrical gels (8) and electrophoresed at 5 mA per tube for 45 min at 4°. Gels were fractionated into 1-mm slices, and radioactive material was eluted and determined by liquid scintillation (9).

Preparation of Viral Reverse Transcript. A 2-hr endogenous reverse transcriptase reaction with detergent-disrupted virus was used to synthesize [^{3}H]thymidine-labeled DNA in the presence of actinomycin D (30 μg/ml) and 10 mM $MgOAc_2$ (10). The labeled product was deproteinized and further purified (7). The [^{3}H]DNA had a sedimentation coefficient of 7 S in alkaline sucrose and a specific activity of 2×10^7 cpm/μg. Protection experiments using ^{32}P-labeled 70S viral RNA were performed as described (7).

Nonrepetitive Cellular DNA Preparation. The cellular DNA of a *M. cervicolor* lung cell line was labeled with [^{3}H] thymidine for 72 hr and extracted (7). Nonrepetitive cellular DNA was isolated by removing the highly reiterated DNA sequences that anneal by a C_0t of 200 (34% of the total DNA) by fractionation on hydroxyapatite (11). The specific activity of the DNA was 1.5×10^4 cpm/μg.

Hybridization. Nuclear DNA was extracted and sonicated to a size of 6–7 S (7). Reaction mixtures contained 0.01 M Tris·HCl (pH 7.4), 0.7 M NaCl, 2×10^{-3} M EDTA, 0.05% sodium dodecyl sulfate, 10,000–20,000 cpm of [^{3}H]DNA/ml, and 2–4 mg/ml of nuclear DNA. Hybridizations were initiated by heating the reaction mixtures to 98° for 10 min, cooling on ice, and incubating at 65°; unhybridized [^{3}H]DNA was digested with the single-strand-specific nuclease, S_1 (7). C_0t values [C_0 is the concentration of DNA in moles of nucleotides per liter and t is the time in seconds (12)] were corrected to a monovalent cation concentration of 0.18 M (13).

RESULTS

Spleen cells from *M. cervicolor* were cocultivated with heterologous cell lines, and supernatants from cultures were assayed for manganese-or magnesium-dependent reverse transcriptase activity. Approximately 12 weeks after the initiation of cocultivation, magnesium-dependent reverse transcriptase activity was detected in cultures containing *M. musculus* cells (SC-1 and NIH/3T3). When filtered supernatant fluids from these cultures were transmitted to other mammalian cell lines, reverse transcriptase activity was detected in cultures of *M. musculus* cells 4 weeks after infection but not in the other cell lines tested, including a cell line derived from the lung of *M. cervicolor* (Table 1). Thus, the virus isolated from *M. cervicolor* could be transmitted to cells of a heterologous murine species but was restricted from replicating in at least one cell line derived from the same species of *Mus*.

NIH/3T3 cells infected with the *M. cervicolor* virus (designated M432) were examined by electron microscopy and compared with RIII tumor cells producing MMTV (Fig. 1). Both infected NIH/3T3 cells and RIII tumor cells contained cytoplasmic A particles as well as virions budding from the plasma membrane. As compared to the particles seen in RIII cells, the cytoplasmic A particles and forms budding from cells infected with the M432 virus were smaller in size (83 nm), and budding particles containing spherical nucleoids lacked the surface spikes characteristic of MMTV. In addition, the extracellular M432 virions were smaller in size (110 nm), lacked surface spikes, and contained centrally located nucleoids surrounded by an intermediate line. Thus, by morphologic criteria, the M432 virus shares certain features in common with MMTV (A particles, doughnut-shaped nucleoids in budding virions, intermediate line) but is distinguishable by other properties (smaller size, central nucleoids, no MMTV-like surface spikes).

Table 1. Host range of *M. cervicolor* virus (M432)

Host cell line	Species	Supernatant reverse transcriptase assay (cpm × 10^{-3}, [^{3}H]dTMP incorporated)
Cervicolor lung	*M. cervicolor*	2.0
NIH/3T3	*M. musculus*	*336.3*
SC-1	*M. musculus*	*89.1*
C3H/MCA-5	*M. musculus*	*40.0*
BALB/KA31	*M. musculus*	*36.8*
NRK	Rat	1.1
SIRC	Rabbit	1.0
FCf2Th	Dog	1.4
FEC	Cat	1.4
DBS-FRhL-1	Rhesus	2.4
A204	Human	1.9

Medium from NIH/3T3 cells infected with M432 virus was filtered and used to infect the host cell lines listed. Medium from infected cultures was tested for viral polymerase activity at 2-week intervals after infection. The results shown are the average values obtained for duplicate experiments 4 weeks after infection. Magnesium (10 mM) was used as the divalent cation. Values in *italics* indicate levels of incorporation significantly above background.

The *M. cervicolor* virus was concentrated from culture supernatants and banded isopycnically in sucrose (ρ = 1.16–1.17 g/cm^3). The viral polymerase activity of detergent-disrupted particles is dependent upon the presence of magnesium (Fig. 2). Manganese cannot efficiently replace magnesium as a cofactor in the endogenous polymerase reaction nor in reactions using synthetic template:primer mixtures. Fig. 2 shows that the M432 enzyme, like enzymes from other retraviruses, transcribes the synthetic template:primer poly(rC)·oligo(dG) more efficiently than poly(rA)·oligo(dT). Thus, M432 contains a virion-associated reverse transcriptase activity similar to that of type B viruses (14).

The reverse transcriptase of the M432 virus was not inhibited by antisera that inhibit polymerases of the known type C viral groups. Radioimmunoassays for the p30 proteins of mammalian type C viruses also failed to detect antigens (<5 ng/mg of cell protein) in cultures of NIH/3T3 and SC-1 cells producing M432. By these criteria, then, the M432 virus is unrelated to mammalian type C viruses, including two recent type C isolates (our unpublished data) from *M. cervicolor*.

Cells producing the *M. cervicolor* virus werre radiolabeled with [^{32}P]phosphoric acid and the RNA genome was extracted. Fig. 3 (inset) shows that the virus contains a 70S RNA genome. When the 70S RNA was heat-denatured and run on an agarose-acrylamide gel, it migrated as subunits of smaller size (Fig. 3). Although the RNA moved as a heterodisperse band, probably as a result of nucleolytic digestion, the major radioactive species had a molecular weight of 3×10^6 when compared to RNA standards.

To determine whether the new viral isolate was endogenous to *M. cervicolor*, we prepared a [^{3}H]DNA transcript of the viral genome using the endogenous reverse transcriptase reaction. Single-stranded [^{3}H]DNA probes were shown by protection experiments performed with ^{32}P-labeled 70S viral RNA to

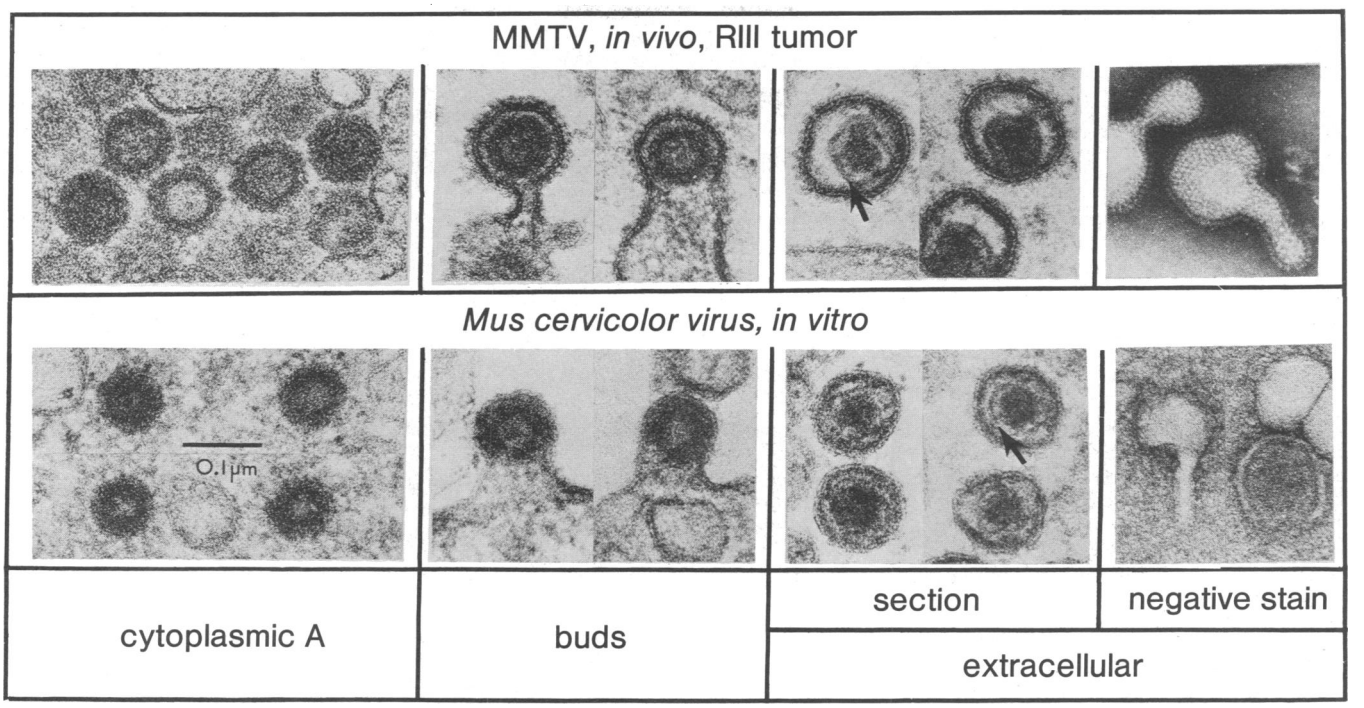

FIG. 1. Electron micrographs of virus particles from NIH/3T3 cells infected with M432 and MMTV from RIII tumor cells. The intermediate line is indicated by the arrows. The marker bar equals 0.1 μm.

represent 40% of the viral genome at a DNA-to-RNA molar ratio of 1.0 and 70% of the genome at a molar ratio of 5.0. Fig. 4 shows that sequences related to the viral genome were detected in the cellular DNA of *M. cervicolor* mice. A comparison of the kinetics of hybridization for this reaction ($C_0t_{1/2} = 6 \times 10^1$) with that seen for the self-annealing of *M. cervicolor* nonrepetitive DNA ($C_0t_{1/2} = 1.5 \times 10^3$) is consistent with the presence of approximately 25 copies of M432 viral-related sequences per haploid genome in the cellular DNA of normal *M. cervicolor* mice. Similar results have been obtained when different tissues were used as a source of DNA or when different mice were examined, indicating that the new virus isolate is endogenous to *M. cervicolor* and, like other endogenous viruses, can be detected in multiple copies in the cellular DNA of normal animals of the species (15). In contrast, when the [^{3}H]DNA probe was annealed to the DNA of NIH/3T3 cells infected with

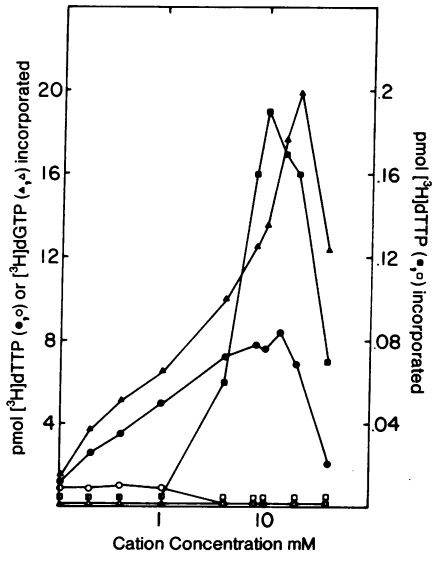

FIG. 2. M432 viral-associated reverse transcriptase activity using magnesium (solid symbols) or manganese (open symbols) as divalent cations. Reactions were performed for 1 hr at 37°, using the synthetic template:primer poly(rA)-oligo(dT) (●, ○), poly(rC)-oligo(dG) (▲, △), or detergent-disrupted virions (endogenous reaction) (■, □).

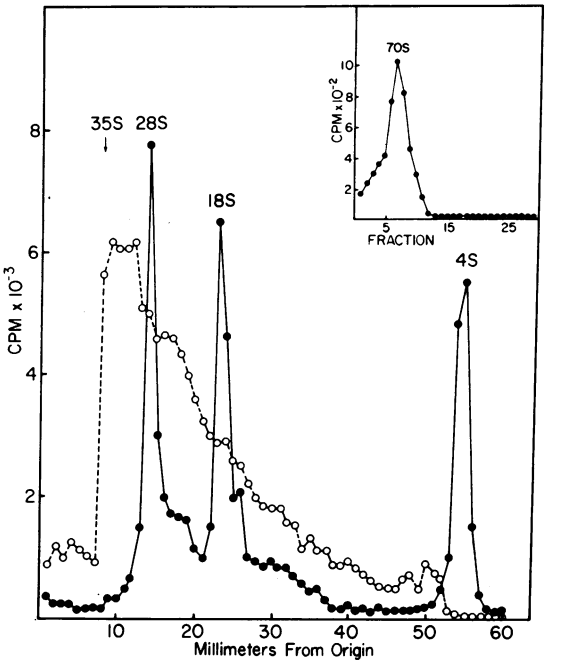

FIG. 3. Determination of M432 genome size using velocity sedimentation in neutral sucrose (inset) and agarose-polyacrylamide gels. ^{32}P-Labeled M432 viral RNA (70 S) from the gradient was heat-denatured and run on a 0.5% agarose–1.5% polyacrylamide gel (○); a mixture of ^{3}H-labeled RNAs (28 S, 18 S, and 4 S; Schwartz/Mann) was run as standards on a parallel gel (●).

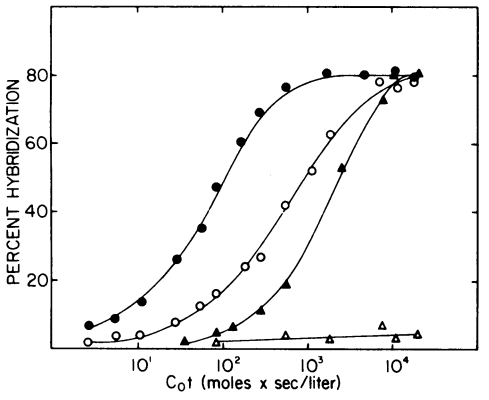

FIG. 4. Hybridization of M432 viral [^{3}H]DNA probe to DNA extracted from pooled *M. cervicolor* tissues (●), NIH/3T3 cells infected with M432 (○), and calf thymus (△). The self-annealing of *M. cervicolor* nonrepetitive DNA is also shown (▲). Hybridization reactions contained 1000 cpm of [^{3}H]DNA probe per 0.05-ml sample; the ratio of unlabeled nuclear DNA to labeled viral DNA or to nonrepeated cellular DNA was 4×10^6 and 1.5×10^3, respectively.

the M432 virus, approximately three proviral copies per haploid genome were detected ($C_0t_{1/2} = 5 \times 10^2$).

It is estimated that *M. cervicolor* diverged from *M. musculus* approximately 5 million years ago and from *M. caroli* somewhat more recently (4 million years) (16). Thus, if virogene sequences of *M. cervicolor* evolved in parallel with other cellular genes, sequences related to the M432 virus might be detected in the cellular DNA of other *Mus* species and possibly in more distantly related rodents (rat and hamster). To test this possibility, we annealed M432 [^{3}H]DNA probes to the cellular DNAs of other rodent species. The final extents of hybridization (at $C_0t = 2 \times 10^4$) and the thermal stabilities of the hybrids are shown in Table 2. As predicted from results obtained with nonrepetitive cellular DNA (16), the M432 viral DNA probe anneals best to the DNA of *M. cervicolor* and to a llesser extent to the DNA of *M. caroli* and *M. musculus*. Only a low degree of hybridization was seen with other rodent DNAs (rat and hamster) consistent with their greater evolutionary distance from present-day murine species. The decrease in the thermal stability of heterologous as compared to homologous hybrids parallels the final extents of hybridization, and indicates that divergent sequences are being detected in heterologous hybridization reactions.

The *M. cervicolor* [^{3}H]DNA probe was hybridized to the RNA of cells producing various murine type C viruses to determine whether the detection of related sequences in the DNA of mice reflects homology with their respective endogenous type C viral genes. The RNA of cells producing various prototype murine type C viruses (BALB/c N-tropic virus, BALB/c xenotropic virus, NIH Swiss xenotropic virus, *M. caroli* xenotropic virus), including cells infected with type C viruses recently isolated from *M. cervicolor* (our unpublished data), did not hybridize to the [^{3}H]DNA probe. The M432 probe also did not hybridize to the RNA extracted from several other retraviruses, including MMTV (RIII milk), Mason-Pfizer monkey virus, and bovine leukemia virus. Taken together, these results show that the M432 virus is not related by the above criteria to these other groups of retraviruses.

DISCUSSION

Two classes of retraviruses have been isolated from murine species. These include the type C (leukemia and sarcoma) viruses obtained from *M. musculus*, *M. caroli*, and *M. cervicolor* and the type B (mammary tumor) viruses isolated to date only from *M. musculus*. The new *M. cervicolor* virus (M432) has properties common to the retravirus family but cannot be assigned to one of the known viral genera. Cells producing the M432 virus contain cytoplasmic A particles and budding virions with doughnut-shaped nucleoids, which distinguish the new isolate from type C viruses, foamy viruses, and bovine leukemia virus, and are most similar to viruses of the type B and Mason-Pfizer monkey virus groups. However, extracellular virions lack eccentric cores and the surface spikes characteristic of type B viruses. By immunologic and nucleic acid hybridization criteria, M432 is unrelated to the known type C viral groups, and no sequence homologies have been detected between the genomes of M432 and other Retraviridae. Since antigenic crossreactions between homologous proteins of different type C viruses can be demonstrated even in the absence of significant nucleic acid sequence homology (17–19), a more definitive classification of the new viral isolate may emerge from studies of the M432 viral structural proteins and their potential antigenic crossreactivities with proteins of other retraviruses.

The M432 virus can be transmitted to cell lines derived from *M. musculus* in which it replicates efficiently but, like xenotropic viruses, it appears to be restricted from replicating in cells of the same species. These properties also distinguish the M432 virus from MMTV, which replicates poorly in heterologous host cell lines and is not released in high titers from infected cells (20, 21).

The germ line of the genus *Mus* contains genetic elements capable of coding for the production of complete type B and type C viruses. For example, using probes prepared from MMTV isolated from *M. musculus*, multiple virogene copies have been detected in the cellular DNA of normal *M. musculus* (22–24) and more divergent copies in the DNA of *M. cervicolor*

Table 2. Nucleic acid homology and thermal stability between the *M. cervicolor* viral genome and DNA from various species

Species	M. cervicolor viral [^{3}H]DNA		
	% Hybrid	t_m	Δt_m
Rodent			
Mouse			
M. cervicolor	100.0	90.5	0
M. caroli	31.0	82.3	8.2
M. musculus	24.0	81.0	9.5
Rat	11.0	<75	>15
Chinese hamster	4.7	NT	—
Guinea pig	0.5	NT	—
Non-rodent			
Cow	1.6	NT	—
Human	1.8	NT	—
Bat	0.4	NT	—
M432-infected NIH/3T3 cell line	100.0	90.5	0

A [^{3}H]DNA probe prepared from the M432 virus was hybridized to nuclear DNA extracted from tissues of the species listed. The % hybrid is the normalized final saturating value (at $C_0t = 2 \times 10^4$); the actual final extent of hybridization of the probe to the cellular DNA of *M. cervicolor* was approximately 80%. The thermal stabilities of the hybrids were determined as described (7, 10). The temperature at which 50% of the hybrids are dissociated (t_m) is listed; the Δt_m is the difference in t_m between the homologous and heterologous hybrids. NT, not tested.

and *M. caroli* (23). By use of [^{3}H]DNA transcripts of the RNA genome of the M432 virus, sequences closely related to those in the probe can be detected in the DNA of *M. cervicolor* while more divergent DNA sequences are found in other *Mus* species. Our data show that the M432 virus is endogenous to *M. cervicolor* and suggest that cells from *M. caroli* and *M. musculus* also have the genetic potential to code for viruses related to M432.

Rodents have been taxonomically classified using anatomic criteria, by studies of homologous proteins, and by nucleic acid hybridization experiments using cellular DNA. Hamsters diverged evolutionarily from the ancestor of mice and rats approximately 20 million years ago, while mice and rats diverged from each other about 10 million years ago (16). The split between the various species of *Mus* has been dated at 5 million years (25), although more recent data suggest that *M. cervicolor* and *M. caroli* had a more recent common ancestor (4 million years) than either species had with *M. musculus* (16). If virogenes coding for the *M. cervicolor* virus have been genetically transmitted in rodents as normal cellular genes for many millions of years, we would expect that other species of *Mus* would contain closely related virogenes, while the other rodents (rat and hamster) would contain more distantly related virogene sequences. [^{3}H]DNA probes prepared from the M432 virus hybridize to rodent cellular DNA in a manner that closely parallels the degree of relatedness of the nonrepeated DNAs of these species. The detection of distantly related sequences in the DNA of rats and hamsters shows that these virogene sequences have been evolutionarily conserved and leads to the conclusion that gene sequences related to the RNA genome of the new *M. cervicolor* isolate have been genetically transmitted in rodents for at least 10–20 million years.

Retraviruses from a given group may be etiologically associated with various forms of neoplasia in animals. For example, MMTV has been shown to be a natural etiologic agent of mammary tumors in *M. musculus* (26, 27). By contrast, an endogenous type B virus isolated from guinea pigs is associated with a high incidence of leukemia rather than mammary adenocarcinoma (28, 29). The ability of the M432 virus to productively infect tissue culture cell lines and presumably animals of murine species other than *M. cervicolor* should facilitate a better understanding of its potential pathogenicity as well as the genetic and physiologic elements that control its infection and expression.

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