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Genetic Individuality of Intracisternal A-Particles of *Mus musculus*

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The nucleic acid sequence relationship between mouse intracisternal type A-particles and type C and B viruses was examined by reciprocal complementary DNA-RNA hybridization; complementary DNAs prepared from the RNAs of intracisternal A-particles were hybridized with high-molecular-weight RNAs from a variety of murine tumor viruses, and complementary DNAs representing a variety of RNA tumor virus genomes were hybridized with the high-molecular-weight RNAs from A-particles. The criterion for homology between two types of virus was that the heterologous hybridization reaction occurs over the same RNA concentration range as the homologous reaction. The results of these hybridizations indicate that there are no major sequence homologies between the RNA of intracisternal A-particles and the RNA of representative members of type B and C viruses of *Mus musculus*.

Intracisternal A-particles from mouse tumor cells share many properties with type B and C extracellular retroviruses, including the presence of high-molecular-weight polyadenylated RNA (11, 16, 22, 34, 35) and DNA polymerase (11, 22, 31, 34, 36). It is important to establish whether intracisternal A-particles have a genetic relationship with any of the RNA tumor viruses. However, tests for sequence homology between A-particle RNA and the RNAs of other viruses have given somewhat conflicting results (11, 17, 23, 34, 37). A major problem in many of these studies has been the uncertainty that the RNAs and the complementary DNAs (cDNAs) used were specific for and representative of the A-particle genome.

We have now isolated samples of purified A-particle RNA that are able to direct the synthesis of the major A-particle structural protein in a cell-free translation system (20). Furthermore, cDNAs that are broadly representative of the purified RNA molecules have been generated by the technique of random priming with calf thymus DNA fragments (28). Using these reagents, we have reexamined the question of nucleic acid sequence relationships between intracisternal A-particles and some recognized RNA tumor viruses of *Mus musculus*.

MATERIALS AND METHODS

Cells and viruses. All cells were grown in Dulbecco-modified Eagle medium containing 10% fetal calf serum. Intracisternal A-particles were prepared from solid MOPC-104E myelomas grown subcutaneously in BALB/c mice, and from neuroblastoma N4 tissue culture cells which originated from a tumor in A/Jax mice as previously described (17). Rauscher murine leukemia virus (RMuLV) was grown in JLS-V9 cells; AKR, L-1, strain murine leukemia virus (AKR-MuLV), Gross passage A murine leukemia virus (Gross MuLV), and Kirsten murine leukemia virus (KiMuLV) were grown in NIH/3T3 cells; and Kirsten murine sarcoma leukemia virus (KiMSV) was grown in NRK cells; all were obtained from Electronucleonics, Inc., Bethesda, Md. The KiMSV contained a 3:1 ratio of sarcoma virus to helper. Mouse mammary tumor virus (MMTV) from the cell line MM5MT/C1 was from the Frederick Cancer Research Center Virus Resources Laboratory. Moloney murine sarcoma leukemia virus (MoMSV) and Moloney murine leukemia virus (MoMuLV) were grown in 3T3 FL cells (7).

Preparation of RNA. High-molecular-weight RNA was prepared from the viruses from Electronucleonics and the MMTV by lysis of banded virus directly on linear gradients of 5 to 25% (wt/vol) sucrose, 0.01 M Tris-hydrochloride (pH 7.5), 50 mM NaCl, and 0.5% sodium dodecyl sulfate. Virus was lysed in gradient buffer containing 1 mM dithiothreitol. After centrifugation in a Beckman SW50.1 rotor at 50,000 rpm for 45 min at 20°C, the 70S peak was pooled, extracted with phenol-chloroform-isoamyl alcohol, and alcohol precipitated as previously described (17). High-molecular-weight A-particle RNA was prepared by centrifugation of heat-denatured A-particle polyadenylated RNA on isokinetic gradients (20). Total RNA from KiMuLV and 70S RNAs from MoMSV and MoMuLV were provided by A. E. Frankel, National Cancer Institute, Bethesda, Md., and were prepared as previously described (7). Cellular RNAs from spleen focus-forming virus (SFFV)-infected NRK cells (30), Abelson virus-infected NRK cells (24, 25), V-

NRK cells (15), Friend MuLV-infected SC1 cells (30), and 70S RNA of MoMuLV from a TK clone of NIH/3T3 cells (10) were a gift from E. M. Scolnick, National Cancer Institute. Cellular RNA from BALB-2-infected A673 cells (7) was provided by A. E. Frankel.

Preparation of cDNAs. A-particle cDNA, earlier designated the "abundant class," was selected from the products of an exogenous reaction with avian myeloblastosis virus RNA-dependent DNA polymerase directed by the oligodeoxythymidylic acid-primed polyadenylic acid RNA of myeloma A-particles (17). Abundant class cDNA protected 40% of ^{125}I-labeled A-particle polyadenylic acid RNA at a cDNA-to-RNA ratio of 1:1 and 42% at a ratio of 3:1 (17). cDNAs representing A-particles 35S, 32S, and 29S RNS (designated 35S cDNA, 32S cDNA, and 29S cDNA) were prepared in exogenous reactions primed with calf thymus DNA fragments (17, 28). This type of random priming has been shown to produce cDNAs uniformly representative of the RNA templates (28). To determine the degree to which the A-particle cDNAs were representative of the A-particle RNAs, two types of protection experiments were done. The ^{3}H-labeled cDNAs were reacted with their template RNAs at RNA-to-cDNA ratios of 1:1, 2:1, and 4:1 for times sufficient to assure that the reactions had gone to completion, and the amount of cDNA protected by the RNA was assayed by S1 endonuclease resistance (17). cDNAs that are uniformly representative of the RNA should show appreciable protection at low RNA-to-cDNA ratios. Levels of S1 resistance, expressed as percentage of the maximum levels of hybridization for each cDNA (see Fig. 1), at the RNA-to-cDNA ratio shown in parenthesis were as follows: 29S cDNA—42% (1:1), 61% (2:1), 69% (4:1); 32S cDNA—60% (1:1), 73% (2:1), 84% (4:1). Comparable data are not available for 35S cDNA, but we have shown that the 35S cDNA is very similar to 32S cDNA in sequence content (14). The 29S cDNA was also reacted with ^{125}I-labeled template RNA, and the RNase resistance of the RNA was determined as previously described (17). The A-particle 29S cDNA protected 58 and 70% of the 29S RNA at cDNA-to-RNA ratios of 1:1 and 2:1, respectively. The NIH 30S cDNA was also prepared in an exogenous calf thymus DNA-primed reaction with the isolated 30S subunit RNA from MoMuLV, and protected 50% of ^{32}P-labeled 30S RNA at a cDNA-to-RNA ratio of 1:1 and 88% at a ratio of 5:1 (10); KiMuLV cDNA was similarly prepared with 70S viral RNA (27). The NIH 30S and KiMuLV cDNAs were provided by E. M. Scolnick.

The following cDNAs were prepared in the endogenous reverse transcriptase reaction primed by calf thymus DNA fragments (27): AT-124 cDNA prepared with virus grown on mink cells (8), RMuLV cDNA from virus grown in NIH/3T3 cells, BALB-2 cDNA, and Gross MuLV cDNA (all provided by R. J. Goldberg, National Cancer Institute); Friend MuLV and SFFV cDNAs (30) and the MMTV cDNA (8) (provided by E. M. Scolnick). The cDNAs were labeled with [^{3}H]dCTP to specific activities of about 2×10^{7} cpm/μg and protected more than 70% of the input homologous viral 70S ^{32}P-labeled RNA at low cDNA-to-RNA ratios (27). KiMSV cDNA and $cDNA_{common}$, provided by A. E. Frankel, were prepared in endogenous reactions (7) with [^{3}H]TTP as the label. Selection and characterization of $cDNA_{common}$, representing sequences common to a variety of murine type C viruses (MoMuLV, MoMSV, RMuLV, KiMuLV, KiMSV, Harvey MSV, and Abelson MSV), have been described by Frankel et al. (7).

Hybridization. ^{3}H-labeled cDNAs (1,000 to 3,000 cpm) and RNAs in 10^{2}-to 10^{7}-fold excess were denatured by heating at 100°C and then incubated at 68°C in 25-μl reactions containing 0.75 M NaCl, 50 mM Tris-hydrochloride (pH 7.5), 0.1 mM EDTA, 0.5% sodium dodecyl sulfate, and 10 μg of *E. coli* tRNA; hybrid formation was assayed with S1 endonuclease (17). C_rt values (concentration of RNA in moles of nucleotide per liter × time in seconds) were determined from reaction rates corrected to 0.24 M sodium phosphate (3).

RESULTS

A-particles from neuroblastoma contain two main species of polyadenylated RNA, as revealed by electrophoresis in agarose gels under fully denaturing conditions; these are designated 32S and 35S RNA. MOPC-104E myeloma A-particles contain one major species, designated 29S RNA, but RNAs of other sizes, including minor amounts of 35S RNA, are also present (20). The major RNA species were purified by centrifugation on isokinetic sucrose density gradients (20), and cDNAs were prepared from each of them in exogenous DNA polymerase reactions primed with calf thymus DNA fragments (28). Reciprocal cross-hybridizations have shown that the 32S and 35S molecules have a very similar sequence content, which includes information contained in the 29S component. All three of these RNAs contain the previously characterized subset of A-particle sequences, originally defined by virtue of their high concentration in the polyadenylic acid RNA of A-particles from myeloma cells and referred to as the "abundant class" of A-particle sequence (17). This set of sequences was shown to be about 1,000-fold reiterated in the mouse genome (16). Data in support of these sequence interrelationships will be presented in detail elsewhere (14; Lueders and Kuff, manuscript in preparation).

Table 1 shows the results of hybridizing the four A-particle cDNAs with the A-particle RNAs and the high-molecular-weight ("70S") RNAs from a variety of extracellular viruses. Results from hybridizing the A-particle RNAs with viral cDNAs are reported in Table 2. Each RNA examined was first hybridized with its homologous cDNA in order to assess the relative concentration of viral sequences. The RNA was then tested against heterologous cDNAs over a wide C_rt range, and the levels of hybridization at 1×, 10×, and 100× the $C_rt_{1/2}$ of the homolo-

TABLE 1. *Hybridization of A-particle cDNAs with high-molecular-weight viral and A-particle RNAs*

RNA	$C_rt_{1/2}$ of homologous reaction[a] (mol·s/liter)	% Hybridized[b] at 1, 10, and 100 × $C_rt_{1/2}$, respectively, of homologous RNA reaction			
		A-particle cDNA			
		35S	32S	29S	Abundant class
Viral[c]					
RMuLV	0.025	0, 4, 19[d]	0, 4, 16[d]	0, 3, 16[d]	2, 10, 30[e]
MoMSV	0.014	0, 0, 1	0, 0, 1	0, 0, 2	3, 0, 0
MoMuLV[f]	0.020	0, 0, 1	0, 0, 4	0, 0, 1	0, 0, 0
KiMSV	0.036	0, 1, 1	0, 0, 5	0, 1, 1	0, 0, 0
KiMuLV	0.045	0, 1, 3	0, 4, 8	0, 1, 2	0, 0, 0
Gross MuLV					0, 0, 0
AKR-MuLV	0.110	1, 3, 10			0, 0, 0
MMTV	0.040	0, 0, 3		0, 0, 2	
A-particle					
35S	0.020	50, 96, 100			33, 89, 93
32S	0.013		50, 96, 100		
29S	0.007			50, 98, 100	49, 90, 100

[a] The viral RNAs were reacted with their corresponding cDNAs in every case except MoMSV and MoMuLV RNAs which were reacted with $cDNA_{common}$ and AKR-MuLV RNA which was reacted with Gross MuLV cDNA. Plateau hybridization levels in these homologous reactions varied between 70% (MoMuLV) and 92% of input counts.

[b] Values are given as percent of maximum levels of hybridization achieved in the reaction of each A-particle cDNA with its homologous RNA. These were as follows: 35S cDNA versus 35S RNA, 74%; 32S cDNA versus 32S RNA, 75%; 29S cDNA versus 29S RNA, 90%; abundant class cDNA versus A-particle polyadenylated RNA, 86% (17).

[c] All 70S viral RNAs were prepared as described in the text.

[d] Values of 50% were reached at 3,000 × $C_rt_{1/2}$.

[e] Plateau value of 50% was reached at 3,000 × $C_rt_{1/2}$.

[f] Two preparations of 70S RNA, one from MoMuLV isolated from 3T3 FL cells and the other from a TK^- clone of 3T3 cells, gave identical results.

gous reaction were determined. Our criterion for homology between two types of virus was that the heterologous hybridization reaction occur within the same C_rt range as the homologous reaction. Hybridization that was apparent only at relatively higher C_rt values was ascribed to RNA species other than the principal viral component.

Most of the 70S viral RNAs (Table 1) showed little or no hybridization with the A-particle cDNAs over a wide C_rt range. Although RMuLV RNA gave partial protection of all A-particle cDNAs, these reactions were observed only at high C_rt values, indicating that the A-particle-related sequences were relatively dilute in the RMuLV RNA preparation. The low reciprocal hybridization of the RMuLV cDNA with A-particle RNAs (Table 2) supports this conclusion. The data in Tables 1 and 2 reveal no significant homologies between A-particle RNA and the major common and distinctive RNA sequences in a variety of exogenous ecotropic type C viruses. This group includes several viruses (MoMuLV, RMuLV, KiMuLV, and KiMSV) which have been reported to share homology with some preparations of myeloma A-particle RNAs (11, 23).

A-particle RNAs were tested for their ability to hybridize a variety of viral cDNAs (Table 2) representing ecotropic (RMuLV, Gross MuLV, and Friend MuLV) as well as xenotropic (BALB-2 and AT-124) classes of viruses. In addition, we also tested NIH 30S cDNA, representing the sarcoma virus-related endogenous 30S subunit of mouse (6, 10). These 30S subunit sequences were of interest because they share certain properties with the A-particle sequences; these sequences are associated with RNA molecules which have a size similar to one of the A-particle RNAs (10, 20), are present in cellular RNA in cases in which transmissible virus is not released (26), and are unrelated by hybridization analysis to known murine retroviruses (26). SFFV cDNA represents a recombinant between mouse type C ecotropic viral sequences and sequences related to xenotropic virus (30). The A-particle 32S and 35S RNAs hybridized very low levels of all these cDNAs; partial reactions were seen with the 29S A-particle RNA in three cases (RMuLV, Gross MuLV, and NIH 30S) at 100× the RNA concentrations required for the homologous reactions. In no case did we see significant hybridization in the same C_rt range as for the homologous reaction.

TABLE 2. *Hybridization of viral cDNAs with high-molecular-weight A-particle RNAs*

Viral cDNA	Maximum viral cDNA hybridized by homologous RNA[a] (%)	% Viral cDNA hybridized[b] by A-particle RNA at 10 and 100 × $C_rt_{1/2}$, respectively, of homologous A-particle reaction[c]		
		35S	32S	29S
RMuLV	82	—, 2	—, 5	2, 15
Gross MuLV	92	0, 4	0, 4	0, 17
Friend MuLV	70		1, 3	
SFFV	70		0, 1	
BALB-2	90	0, 2	0, 1	—, 2
AT-124	69	—, 4	—, 10	—, 1
NIH 30S	76		1, 3	8, 25

[a] Maximum cDNA hybridized in the homologous reaction was determined by hybridization of each cDNA with the corresponding purified viral 70S RNA (RMuLV and Gross MuLV), or with cellular RNAs from virus-infected cells (SFFV, Friend MuLV, BALB-2). AT-124 cDNA hybridization was determined with RMuLV 70S RNA, with which it is expected to react only partially, and that of the NIH 30S cDNA was determined with MoMuLV 70S RNA.

[b] Hybridization of each viral cDNA with the A-particle RNAs is expressed as percent of the maximum hybridization of that cDNA with its homologous RNA. See footnote *a*.

[c] $C_rt_{1/2}$ of the reaction of each A-particle RNA with its homologous cDNA is shown in Table 1.

Abelson MuLV was of particular interest because it has been shown to reduce the latent period for development of mouse myeloma (21), a type of tumor that is characteristically rich in intracisternal A-particles. Isolated viral RNA and cDNA were not available to us in this case, and rat cells infected with Abelson virus were used as a source of RNA for hybridization with A-particle cDNA. Table 3 shows data for the reactions of A-particle 35S cDNAs and abundant class cDNAs with RNAs from Abelson-infected (Abelson-NRK) and uninfected rat cells (V-NRK). The same low level of hybridization was seen in both cases, indicating that A-particles do not have sequence homology with the Abelson virus. It is interesting that a minor portion of the A-particle sequences may be related to sequences expressed in rat cells. Similar low levels of hybridization of the A-particle abundant class cDNA were also seen with RNA from SFFV-infected rat cells (SFFV-NRK), confirming the lack of hybridization of SFFV cDNA with A-particle RNA (Table 2).

RNA from human cells infected with the xenotropic BALB-2 virus was also tested for A-particle sequences by hybridization with A-particle 35S cDNA (Table 3). The lack of protection of the cDNA was consistent with the data in Table 2 in showing that there was no homology between intracisternal A-particles and this endogenous virus.

High-molecular-weight RNA from one cell-derived MMTV was tested with a similarly negative result (Table 1). This finding has general implications, since all cell-derived MMTVs are known to be homologous and to share 75% of their sequences with the milk-transmitted virus (19). In addition, MMTV and their intracytoplasmic type A-particle precursors share major homology (18). The lack of reaction between MMTV RNA and the A-particle cDNAs is consistent with earlier evidence that the structural proteins of MMTV and intracytoplasmic A-particles differ from those of the intracisternal A-particles (12, 32).

In studies of nucleic acid sequence relationships between A-particles and tumor viruses derived from mouse cells, purified high-molecular-weight RNAs rather than total viral RNAs must be used. The importance of this distinction is demonstrated in Fig. 1, which shows the hybridization of A-particle cDNA and KiMuLV cDNA with 70S and total viral RNAs from KiMuLV. Total KiMuLV RNA hybridized 50% of the A-particle cDNA over a C_rt range which indicated that the A-particle sequences were only 10-fold less concentrated than the KiMuLV sequences. Krueger (11) reported a similar extent of reaction with cDNA generated in the endogenous reaction with A-particles from another myeloma, and concluded that A-particles share appreciable sequence homology with C type viruses. When the high-molecular-weight fraction of the viral RNA is used for the hybridization, the reaction with A-particle cDNA is virtually

TABLE 3. *Hybridization of A-particle cDNAs with cellular RNAs*

Cellular RNA	% maximum hybridization at C_rt values of 10^2, 10^3, and 10^4 mol·s/liter, respectively	
	35S[a]	Abundant class[a]
Abelson-NRK[b]	1, 4, 11	7, 14, 16
V-NRK	0, 4, 12	7, 15, 17
SFFV-NRK		—, 13, 15
BALB-2 human[c]	0, 0, —	

[a] A-particle cDNAs.

[b] $C_rt_{1/2}$ of reaction with $cDNA_{common}$ was 10^3 mol·s/liter.

[c] $C_rt_{1/2}$ of reaction with BALB-2 cDNA was 10 mol·s/liter.

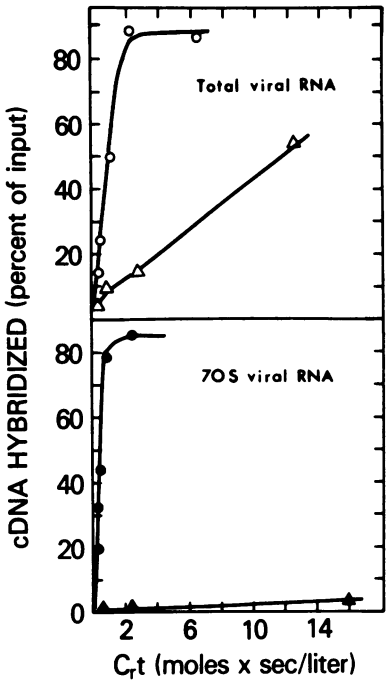

FIG. 1. *Hybridization of KiMuLV 70S and total viral RNAs with KiMuLV and A-particle cDNAs. Hybridization of KiMuLV total RNA from virus grown in NIH 3T3 cells with KiMuLV cDNA* (○) *and with A-particle 35S cDNA* (△); *hybridization of KiMuLV 70S RNA with KiMuLV cDNA* (●) *and with A-particle 35S cDNA* (▲).

eliminated, whereas reaction of KiMuLV 70S RNA with its homologous cDNA shows that the viral sequences are 10× more concentrated than in the total viral RNA. KiMuLV 70S RNA hybridized no more than 3% of the A-particle cDNA even at C_rt values 1,000× the C_rt of the homologous reaction. If A-particles and KiMuLV shared sequences, purification of the viral RNA should have lead to concentration of the A-particle sequences also, and this clearly was not the case.

DISCUSSION

We have conducted a survey for sequence homologies between A-particle RNA and the RNA of a number of mouse viruses. Although not comprehensive, a reasonable range of viral sequences has been included. Thus, the leukemia viruses represented NB-tropic as well as N-tropic viruses (9); the sarcoma viruses included three distinct sets of sarcoma virus-specific sequences (15, 25); the mouse endogenous xenotropic viruses tested were of two distinct classes (1, 29); the MMTV shares sufficient homology with other viruses of this class to be representative of type B viruses. None of the RNAs or cDNAs from these viruses gave significant levels of hybridization with A-particle RNAs or cDNAs. We therefore conclude that none of these representative members of type B and C viruses shares sequence homology with intracisternal A-particles. We have not tested the amphotropic group of viruses, which differ from the ecotropic and xenotropic groups in many respects. However, 85% of the sequences specific for this group are found in MoMuLV (5).

Our results emphasize the critical importance of using high-molecular-weight viral RNAs in sequence homology studies. Thus, A-particle sequences could be detected in the total RNA of KiMuLV produced by NIH/3T3 cells (Fig. 1), at first suggesting a partial homology between the A-particle and viral genomes (11). However, this was not the case, since purified 70S RNA from the KiMuLV preparation subsequently failed to react with A-particle cDNA. A-particle sequences can be expressed at low levels in mouse cells that are free of particles as judged by electron microscopy (14, 17), and apparently these sequences can find their way into the total RNA of C-type virus produced by such cells. Whether this association represents nonspecific contamination or a more specific process (e.g., phenotypic mixing) is not known; even higher levels might be anticipated in the case of extracellular virus produced by A-particle-rich cells such as myeloma, where A-particle sequences constitute a significant fraction of the cytoplasmic polyadenylated RNA (17).

The present findings confirm and extend our earlier studies (17) as well as those of Wong-Staal et al. (34) and Yang and Wivel (37) who found that cDNAs generated from MOPC-104E myeloma and neuroblastoma particles hybridized poorly with RNAs from a variety of type-C viruses. It is important to note that in all of these cases the A-particle-producing cells have been free of extracellular type C viruses (13, 33). On the other hand, significant sequence homologies between the nucleic acids of intracisternal A-particles, myeloma extracellular particles, MoMuLV, KiMuLV, and KiMSV have been reported by Robertson et al. (23) and Krueger (11), respectively. In these studies the cDNAs used were generated by endogenous reactions with A-particle preparations from myelomas which were also producing extracellular viruses (11, 23), and in one case intracytoplasmic type A-particles as well (23). Two possibilities must be considered in evaluating these reports of apparent homology: (i) that intracellular detergent-resistant forms of other retroviruses were included in the A-particle preparation and contributed to the generation of the "A-particle" cDNA; and (ii) that A-particle RNA was asso-

ciated with the extracellular virus preparations in the manner mentioned above. More rigorous analysis is required to establish whether A-particle sequences are in fact incorporated in the genomic RNA of myeloma extracellular virus.

In searching for possible homologies between A-particle RNA and the genomes of other viruses, we have found one case in which our criterion for homology has been met (reciprocal hybridization reactions over the same C_rt range as those for the homologous reactions). The intracisternal A-particles showed partial sequence homology (30%) with an endogenous retrovirus (M432) derived from the Asian mouse *Mus cervicolor* (14). The M432 virus (4) has no sequence homology with mammary tumor virus, with known type C viruses of *M. musculus*, or with two type C viruses of *M. cervicolor* (2). Only a portion of the A-particle sequences was related to those in the high-molecular-weight RNA of M432 virus, and we have suggested that the two types of particles may be related through a recombination event in the evolutionary past. It is possible that other types of viruses could acquire portions of the endogenous A-particle genome through a similar mechanism.

In conclusion, we have found instances in which limited portions of the A-particle sequences were at times associated with extracellular viruses, at very low levels, but in no case were A-particle sequences present in the 70S RNA of any of the viruses from *M. musculus* at levels which would indicate that recombination between the two viral genomes had occurred. Thus, intracisternal A-particles do not appear to share major genetic homology with *M. musculus* oncornaviruses in general, and may represent a genetically unique class of endogenous virus in this species.

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