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Nucleic Acid Homology of Murine Xenotropic Type C Viruses

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Two major subclasses of xenotropic (X-tropic) murine type C viruses can be distinguished by nucleic acid hybridization. The most frequently encountered subclass (MuLV-X^{α}) includes isolates from BALB/c, C57BL/6J, C58/J, AKR/J, CBA/J, and DBA/2J inbred strains and from the Asian feral mouse subspecies *Mus musculus molossinus*. The other subclass (MuLV-X^{β}) consists of viruses isolated from the NIH Swiss and NZB/BINJ strains. Thus, significant polymorphism exists among the endogenous type C virogenes of a single species, *Mus musculus*. MuLV-X^{α} genes are found in strains that also have endogenous mouse-tropic viruses (either N-tropic, B-tropic, or both), whereas the MuLV-X^{β} subclass is restricted to mouse strains from which mouse-tropic viruses have not yet been isolated. The results are consistent with a model which proposes that mouse-tropic endogenous viruses are derived from the MuLV-X^{α} subclass.

The genomes of many mammalian species including mice, rats, hamsters, pigs, baboons, and certain cats have been shown to contain multiple copies of nucleic acid sequences related to the genomes of type C viruses isolated from each of these species (5). The mouse species *Mus musculus* is particularly useful for studying the relationships among these endogenous type C virogenes since there are numerous well-characterized inbred strains of laboratory animals, and since murine endogenous type C virogenes code for the production of viruses with a range of biological properties (1, 3). Two major host range classes of endogenous murine type C viruses have been identified: mouse-tropic viruses which replicate well in murine cells (12) and S-tropic (3) or xenotropic (X-tropic) (14) viruses which are restricted from growing in mouse cells but which replicate in cells from various other species. X-tropic type C viruses can be readily induced in vitro from murine cells by treatment with halogenated pyrimidines (1, 3) and by mixed splenocyte cultures (20), but also can be recovered from tissues of normal laboratory mice (14, 15, 17, 22) and from the Asian feral mouse subspecies *M. musculus molossinus* and *M. musculus castaneus* (16).

In the present report we compare the homology of BALB/c, NZB/BINJ, and *M. m. molossinus* X-tropic viral genomes with those of X-tropic viruses recovered from other strains of mice. It is shown that there are at least two related but clearly distinguishable subclasses of X-tropic viruses. One subclass, constituting the majority of the viruses tested, is highly related to the BALB/c and *M. m. molossinus* viruses. The other class is represented by the NZB/BINJ and NIH Swiss X-tropic viruses. Stephenson et al. came to a similar conclusion after studying the p12 proteins of BALB/c and NIH Swiss X-tropic viruses (21).

MATERIALS AND METHODS

Viruses. X-tropic murine type C viruses were isolated from the spleens of normal animals of the DBA/2J, NZB/BINJ, C58/J, C57BL/6J, and CBA/J inbred strains and from the Japanese feral mouse *M. m. molossinus* as previously described (16, 17). X-tropic BALB/c and AKR/J viruses were induced from cultured cell lines (3). The AT-124 X-tropic NIH Swiss virus was recovered by passaging human RD tumor cells in vivo through imminosuppressed NIH Swiss mice (22). All these viruses were unable to replicate on mouse cells (except for the C58/J X-tropic isolate, see [16]). Viruses were grown in vitro in the rabbit corneal cell line SIRC (CCL 60), obtained from the American Type Culture Collection, Rockville, Md., except for the BALB/c and *M. m. molossinus* isolates which were grown in the canine cell line FCf2Th from the Naval Biomedical Research Laboratory, Oakland, Calif. Cells were grown in Dulbeccos modification of Eagle minimal essential medium containing 10% calf serum (Colorado Serum Co., Denver, Col.). Viruses were banded twice in sucrose density gradients before use.

Preparation of [^{3}H]DNA. [^{3}H]thymidine-labeled DNA was synthesized in an endogenous reverse transcriptase reaction with detergent-disrupted type C virus in the presence of actinomycin D (30 μg/ml), as previously described (4). The [^{3}H]DNA product was deproteinized after 16 h of incubation at 37 C. Further purification was accomplished by fractionation on hydroxylapatite and chromatography on G-50 Sepha-

dex as described previously (7). The specific activity of the [^{3}H]DNA was 2×10^7 counts/min per μg. Less than 5% of the [^{3}H]DNA was resistant to S_1 nuclease. The proportion of 70S viral RNA that was represented in the [^{3}H]DNA was determined by hybridizing the [^{3}H]DNA to the homologous ^{32}P-labeled viral RNA (specific activity 5×10^6 counts/min per μg). The protection of the viral RNA from pancreatic RNase digestion was assayed as described previously (2, 9). The [^{3}H]DNA probes used in these experiments contained 60 to 70% of the respective 70S viral RNA sequences at a [^{3}H]DNA to [^{32}P]RNA molar ratio of 2, and 80% of the sequences at a molar ratio of 5.

Hybridization. Cellular RNA (extracted as previously described [2]) and [^{3}H]DNA were incubated for various times at 65 C in reaction mixtures containing 0.01 M Tris, pH 7.4, 0.4 M NaCl, 2×10^{-3} M EDTA, 0.05% sodium dodecyl sulfate, 20,000 to 40,000 counts/min of [^{3}H]DNA per ml (1 to 2 ng), and 0.5 to 4 mg/ml of cytoplasmic RNA. Thus, the RNA to [^{3}H]DNA ratio in these experiments was 5×10^5 to 4×10^6; at higher ratios the level of hybridization does not significantly increase. All reaction mixtures were overlayed with mineral oil to prevent evaporation. Hybridizations were initiated by heating the reaction mixtures to 98 C for 5 min, cooling on ice, and incubating at 65 C. Unhybridized [^{3}H]DNA was digested with the single-strand-specific nuclease S_1 and residual radioactivity determined as described (2). C_0t values (C_0 is the concentration of cellular RNA in moles of nucleotide per liter and t is the time in seconds) were calculated as the product of the absorbancy at 260 nm (A_{260}) and time (h) divided by 2 and corrected to a monovalent cation concentration of 0.18 M (7, 8). Data were plotted as percent hybridization versus C_0t to determine the final extent of hybridization. Background counts corresponding to 5% or less of the input counts per minute were subtracted from each time point. Points which were within the linear part of the curve were used to calculate Scatchard plots (fraction [^{3}H]DNA hybridized/C_0t versus fraction [^{3}H]DNA hybridized) as suggested by Marsh and McCarthy (19).

RESULTS

Nucleic acid hybridization has been used to compare the viral genomes of X-tropic viruses recovered from various mouse strains. These include AKR/J, CBA/J, C58/J, C57BL/J, DBA/2J, BALB/c, NIH Swiss, NZB/BINJ, and the Asian feral subspecies *M. m. molossinus*. A single-stranded [^{3}H]DNA transcript of BALB/c X-tropic viral RNA was annealed to cytoplasmic RNA from cells producing other X-tropic viruses; the final extents of hybridization (determined with S_1 nuclease) are used as a measure of the degree of relatedness of the hybridized nucleic acid sequences.

The results of these experiments are presented in Fig. 1A and are summarized in Table 1. The final extent of hybridization of the BALB/c [^{3}H]DNA probe with RNA from six of eight other X-tropic virus-infected cells (C57BL/6J, AKR/J, C58/J, DBA/2J, CBA/J and *molossinus*) was approximately 90% of the level obtained in the homologous reaction. In contrast, the final extents of hybridization obtained with cytoplasmic RNA from cells producing the NZB/BINJ and NIH/Swiss X-tropic viruses were significantly lower (66% and 33%, respectively). The saturating hybridization values obtained by C_0t analysis were compared with those derived from Scatchard plots (18) of the same data and are in close agreement (Table 1). These data suggest that the BALB/c X-tropic [^{3}H]DNA probe can distinguish at least two major subclasses of murine X-tropic viruses.

To determine the distribution of the nonhomologous sequences which are represented in the [^{3}H]DNA probe, the thermal stability of the hybrids formed with the BALB/c X-tropic [^{3}H]DNA probe was measured. An inverse relationship exists between the thermal stability of a hybrid and the degree of base pair mismatching (6, 18). The thermal melting profiles are shown in part in Fig. 2A and are summarized in Table 1. The temperature at which 50% of the hybrid is dissociated is designated the T_m. The highest T_m, 87 C, is obtained with the homologous hybrid. Lower T_m values were obtained with heterologous hybrids shown to be less related by final extent of hybridization. The hybrids formed with viral sequences of the six isolates most highly related to the BALB/c X-tropic [^{3}H]DNA probe by final extents of hybridization have T_ms ranging from 85 to 85.5 C. In contrast to this group, the T_m for the hybrid formed with the NZB/BINJ viral RNA is 82.5 C, and with NIH Swiss viral RNA, 79.5 C. The melting data suggest that the measured differences in homology between the genomes of these viruses reflect the existence of varying degrees of base pair mismatching throughout their genomes rather than the conservation of long segments of identical or nearly identical base sequences.

The feral Asian mouse subspecies *M. musculus molossinus* is believed to be evolutionarily separated from the mouse stocks which yielded laboratory strains by thousands of generations. Further, it is known that this subspecies differs from laboratory strains at numerous genetic loci (16). The final extent of hybridization obtained with a [^{3}H]DNA probe prepared from the *M. m. molossinus* X-tropic virus and cytoplasmic RNA extracted from cells producing the other murine X-tropic viruses is shown in part in Fig.

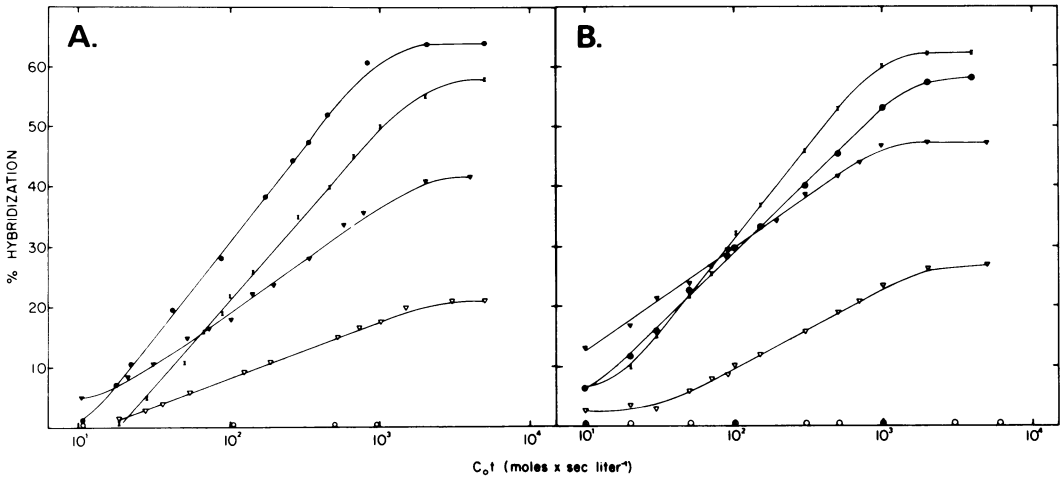

FIG. 1. *Hybridization of BALB/c and M. m. molossinus X-tropic viral [^{3}H]DNA to cytoplasmic RNA extracted from cell lines producing various murine X-tropic viruses. Hybridization reactions contained 1,000 counts/min of [^{3}H]DNA product added per 0.025 ml. The RNA concentration (0.2 to 4 mg/ml) as well as the time of incubation were varied. (A) [^{3}H]DNA prepared from BALB/c X-tropic virus was hybridized to cytoplasmic RNA extracted from cells propagating X-tropic virus isolates from: BALB/c (●); M. m. molossinus (×); NZB/BINJ (▼); NIH Swiss (▽); and the uninfected dog cell line FCf2Th (○). (B) [^{3}H]DNA was prepared from M. m. molossinus X-tropic virus. The symbols are the same as in (A).*

TABLE 1. *Nucleic acid homology by using BALB/c X-tropic [^{3}H]DNA probe*

X-tropic type C virus strain of origin[a]	Final hybridization (%)[b]		T_m[c]
	C_0t analysis	Scatchard plot	
BALB/c	64 (100)	58.5 (100)	87.0
CBA/J	60 (94)	58.5 (100)	85.5
C57BL/6J	60 (94)	57.5 (98)	85.5
AKR/J	56 (88)	56.5 (96)	85.5
DBA/2J	57 (89)	52.5 (89)	85.0
C58/J	57 (89)	51.0 (87)	85.0
M. m. molossinus	58 (90)	50.5 (86)	85.0
NZB/BINJ	42 (66)	32.5 (55)	82.5
NIH Swiss	21 (33)	16.0 (27)	79.5

[a] RNA was extracted from cells that are infected with X-tropic virus isolated from the indicated strain of mice (Materials and Methods).

[b] The actual values are given with normalized values in parentheses. Complete C_0t curves were done to determine the final extent of hybridization by C_0t analysis. Similar saturation values have been obtained in the homologous hybridization with viral RNA (9). These values were also used to determine the final extent of hybridization by Scatchard plot (Materials and Methods). The conditions for hybridization are given in the legend to Fig. 1 and Materials and Methods.

[c] T_m, the temperature at which 50% of the hybrid has dissociated, was determined as described in the Legend to Fig. 2.

1B and is summarized in Table 2. These data show that [^{3}H]DNA prepared from *M. m. molossinus* X-tropic virus is related (final extents of hybridization are 83 to 100%) to the same group of X-tropic isolates from inbred mouse strains which are related to the BALB/c X-tropic [^{3}H]DNA probe. Similarly, major differences are detected between the *M. m. molossinus* probe and NZB/BINJ (66 to 76%) and NIH Swiss (44%) X-tropic viral nucleic acid sequences. These observations are substantiated by the thermal stability of the hybrids as shown in Fig. 2B and summarized in Table 2. The T_ms of all the heterologous hybrids are 1 to 2 C below that of the homologous hybrid, except those formed with NZB/BINJ (ΔT_m 4.5 C) and NIH Swiss (ΔT_m 6 C) viral sequences. Thus, despite the apparent evolutionary divergence of *M. m. molossinus* mice and laboratory strains, the X-tropic isolates from many of these animals are, in general, highly related.

The data obtained with the BALB/c and *M. m. molossinus* X-tropic [^{3}H]DNA probes suggest that two discrete subclasses of murine X-tropic type C isolates exist. To investigate this possibility further, a [^{3}H]DNA probe was generated using the NZB/BINJ X-tropic isolate. The final extents of hybridization obtained with this probe and RNAs from cells supporting the replication of the NIH Swiss, BALB/c and *M. m. molossinus* X-tropic isolates are pre-

sented in Fig. 3 and Table 3. The NZB probe is highly homologous to NIH Swiss RNA sequences (91%) but is less related to *M. m. molossinus* (65%) or BALB/c (54%) viral sequences. This experiment confirms the existence of two subclasses of murine X-tropic isolates.

DISCUSSION

Previous characterization of various murine X-tropic isolates in this laboratory has demonstrated that these viruses are indistinguishable from one another by interference tests or by the currently used assays for viral p30 or reverse transcriptase antigens (3, 13, 16, 17). A recent report by Stephenson et al. (21), however, indicates that BALB/c X-tropic virus can be differentiated from various NIH Swiss X-tropic isolates by a competitive radioimmunoassay developed with the p12 protein from Rauscher MuLV. The present study shows that two major subclasses of murine X-tropic virus can be distinguished by nucleic acid hybridization homology criteria. The most prevalent class is closely related to the BALB/c and *M. m. molossinus* X-tropic virus isolates and includes viruses from AKR/J, CBA/J, DBA/2J, C58/J, and C57BL/6J animals (subclass "MuLV-X^{α}"). The X-tropic viruses from NZB/BINJ and NIH Swiss mice are distinctly different from those of the X^{α} subclass and are also highly related to one another (subclass "MuLV-X^{β}"). However, due to the apparent incomplete representation of the viral genome in the reverse transcript

TABLE 2. *Nucleic acid homology by using M. m. molossinus X-tropic [^{3}H]DNA probe*[a]

X-tropic type C virus strain of origin	Final hybridization (%)		T_m
	C_0t analysis	Scatchard plot	
M. m. molossinus	62 (100)	54.5 (100)	87.0
C58/J	60 (97)	54.5 (100)	85.5
C57BL/6J	60 (97)	53.0 (98)	86.0
DBA/2J	62 (100)	53.0 (98)	86.0
AKR/J	58 (95)	48.5 (90)	85.5
CBA/J	58 (95)	47.0 (87)	85.0
BALB/c	58 (95)	45.0 (83)	85.0
NZB/BINJ	47 (76)	35.5 (66)	82.5
NIH Swiss	27 (44)	24.0 (44)	81.0

[a] See the Legend to Table 1.

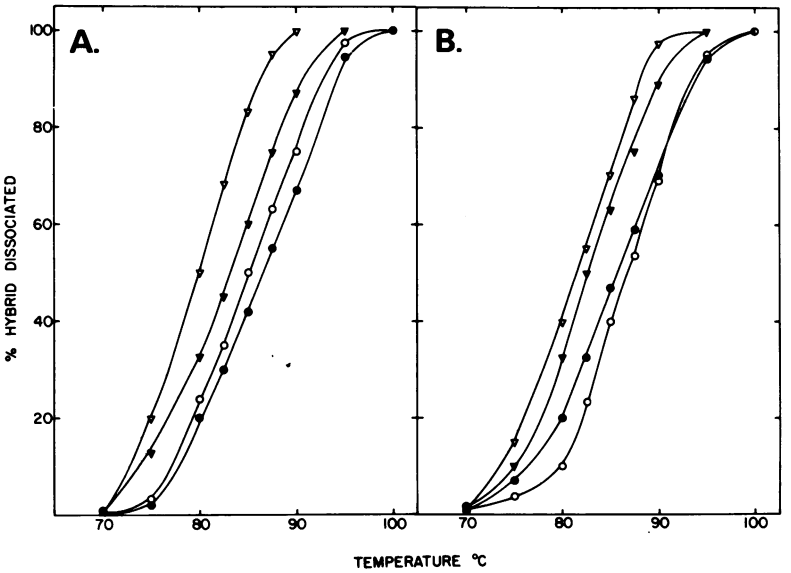

FIG. 2. *Thermal stability of hybrids formed between [^{3}H]DNA probes prepared from BALB/c and M. m. molossinus X-tropic viruses and cytoplasmic RNA from cell lines producing various murine X-tropic viruses. Hybridizations were carried out to a C_0t of 2 × 10^3 at 65 C as described in the legend to Fig. 1. Samples (0.025 ml) were diluted 10-fold in 0.4 M NaCl, heated at the indicated temperature for 5 min, and then digested with S_1 nuclease. These values represent the average of at least three experiments in which duplicate samples were taken at each temperature. The deviation for the T_m is 0.5 to 1 C. (A) The hybrids tested are between [^{3}H]DNA from BALB/c X-tropic virus and cytoplasmic RNA from cells propagating X-tropic virus from: BALB/c (●); M. m. molossinus (○); NZB/BINJ (▼); and NIH Swiss (▽). (B) The [^{3}H]DNA was prepared from M. m. molossinus X-tropic virus. The symbols are the same as in (A).*

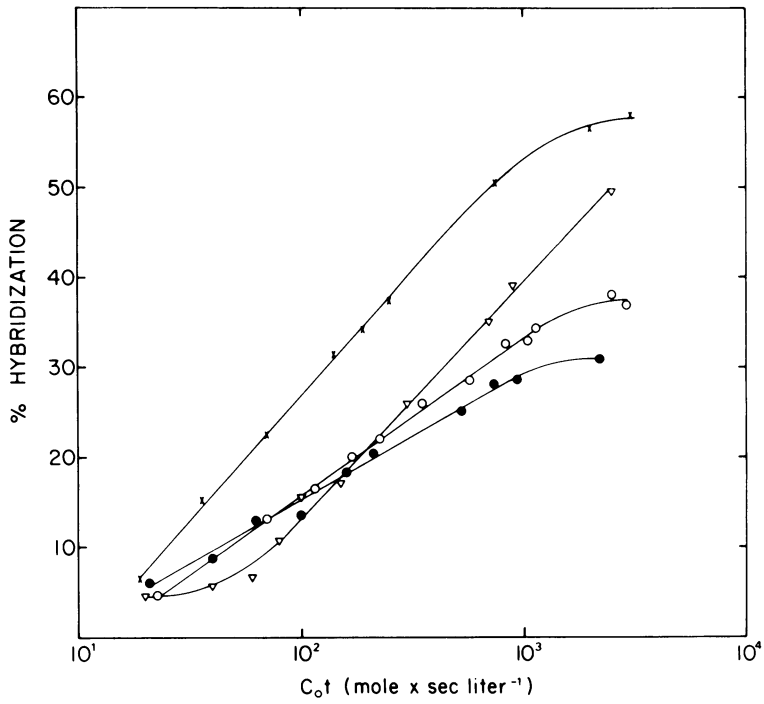

FIG. 3. *Hybridization of NZB/BINJ X-tropic [^{3}H]DNA probe to cytoplasmic RNA extracted from cells propagating various murine X-tropic viruses. The conditions for hybridization are the same as described in the Legend to Fig. 1. The [^{3}H]DNA was hybridized to cytoplasmic RNA extracted from cells producing X-tropic virus from: NZB/BINJ* (×); *NIH Swiss* (▽); *M. m. molossinus* (○); *and BALB/c* (●).

TABLE 3. *Nucleic acid homology by using NZB/BINJ X-tropic [^{3}H]DNA probe*[a]

X-tropic type C virus strain of origin	Final hybridization (%)	
	C_0t analysis	Scatchard plot
NZB/BINJ	58 (100)	57.5 (100)
NIH Swiss[b]		52.5 (91)
M. m. molossinus	37 (64)	38.0 (66)
BALB/c	31 (53)	32.0 (55)

[a] See the legend to Table 1.

[b] Saturation of the [^{3}H]DNA was not reached by a C_0t of 3×10^3.

probes, it is not possible to completely describe in a quanitative manner the genomic differences between these viruses. Host range differences also probably differentiate the X^α subclass of viruses, all of which replicate preferentially in rat NRK and rabbit SIRC cells relative to their ability to replicate in human and other primate cells, from the X^β viruses which preferentially replicate in various primate cells (Stephenson et al. [21]; M. Lieber, unpublished data).

The emphasis on the differences between endogenous virogene sequences should not obscure the fact that all murine type C viruses tested so far are all related to one another by nucleic acid hybridization. This partial relatedness could be the result of retention during evolution of a long segment of identical base sequences common to all the murine type C viral genomes. However, the decreased thermal stability of heterologous hybrids suggests that this is not the case since a long segment of identical sequences would have a high T_m. Rather, the decreased T_ms, being commensurate with the decreasing final extents of hybridization, suggest that the partial homologies measured reflect the general accumulation of base pair differences between virogenes during evolutionary divergence.

Previous hybridization studies have shown that the N-tropic and B-tropic endogenous mouse-tropic viruses from BALB/c cells are highly related (9). Chattopadhyay and associates have performed a series of hybridization experiments by using a [^{3}H]DNA probe made from an endogenous mouse-tropic virus isolated from AKR/J cells (10, 11). Reassociation kinetics of this probe with nuclear DNAs from a large number of mice strains, summarized in

reference 11, indicate that all eight mouse strains (including BALB/c) from which mouse-tropic viruses have been isolated contain virogenes which are very highly related to the sequences in the AKR mouse-tropic probe. Thus, the endogenous mouse-tropic viruses form a relatively homogeneous group (subclass "MuLV-M") different from the X^{α} and X^{β} xenotropic virus subclasses described here.

A classification of endogenous murine type C viruses and the sets of genes that give rise to them into three subclasses based on their nucleic acid homology and on their general host range properties may prove useful for further studies. Previously, murine type C viral isolates have been generally identified by their strain of origin. The present data indicate that viruses with similar host range properties from a variety of strains are highly related by nucleic acid hybridization. For example, the BALB/c X-tropic viruses are much more highly related by genome homology to X^{α} subclass isolates from various other strains of mice than they are to the endogenous N-tropic and B-tropic mouse-tropic viruses present in the same BALB/c cells (9).

Two findings suggest that mouse-tropic (MuLV-M) virogenes may have been derived from xenotropic MuLV-X^{α} virogenes. First, all strains tested from which mouse-tropic viruses have been recovered possess xenotropic viruses of the X^{α} subclass (11). Those strains (NZB and NIH Swiss) from which X^{β} subclass viruses have been obtained have not yielded mouse-tropic viruses despite extensive attempts to isolate them (14, 22). Second, by nucleic acid hybridization criteria, the M class and the X^{α} subclass viruses are more closely related to one another, despite the major difference in host range properties, than either is to viruses of the X^{β} subclass (9). This suggests that M and X^{α} virogenes may have arisen from a common ancestor.

A model which accommodates the presently available data for the major subclasses of endogenous murine type C viruses is presented in Fig. 4. This model proposes that all murine endogenous type C virogenes developed from a common ancestor (V_0) through gene duplication and subsequent mutational events. A common ancestor accounts for the partial homology of all murine type C viral genomes. The branch leading to the MuLV-M virogenes diverged from the MuLV-X^{α} stem sometime after the divergence of the MuLV-X^{α} and MuLV-X^{β} virogenes. The MuLV-M, MuLV-X^{α}, and MuLV-X^{β} subclasses are not completely homogeneous within themselves, but consist of a family of more or less highly related genomes. This model does not apply to laboratory strain murine leukemic virus stocks which have been extensively passaged, with resulting opportunities for very rapid genome mutation and recombination.

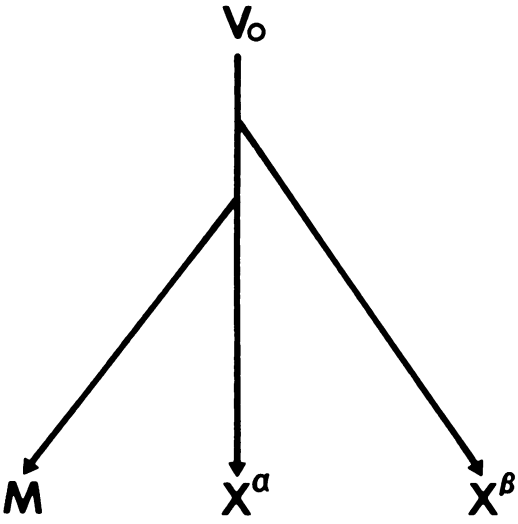

FIG. 4. *A model accounting for the relationships between different groups of endogenous murine type C viruses. All share a common ancestor (V_0). MuLV-M: endogenous mouse-tropic viruses; MuLV-X^{α}: xenotropic viruses from BALB/c, AKR, etc.; MuLV-X^{β}: xenotropic viruses from NIH Swiss, NZB/BINJ.*

Finally, the same strains of mice which possess the common mouse-tropic (class MuLV-M) virogene sequences (AKR/J, C58/J, BALB/c, DBA/2, C57BL/6J [11]) also have the X^{α} subclass of X-tropic virogenes, while the NIH Swiss and NZB strain mice which lack the common mouse-tropic sequences have X^{β} subclass of X-tropic viruses. This interrelationship suggests that MuLV-M and MuLV-X^{α} may be inherited in a coordinate fashion. The possibility that MuLV-X^{α} and MuLV-M are linked is testable by genetic methods, as is the possibility that MuLV-X^{α} and MuLV-X^{β} are still allelic.

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