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Genetics

Evolution of type C viral genes: Preservation of ancestral murine type C viral sequences in pig cellular DNA*

(porcine type C virus/trans-species gene transfer/"fossil" genes/DNA·DNA hybridization)

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ABSTRACT **Domestic pigs (*Sus scrofa*) and other members of the family Suidae have multiple copies of type C viral gene sequences in the cellular DNA of all their tissues. Partially homologous viral gene sequences are also found in cellular DNA of rodents, particularly Muridae. The results lead to the conclusion that type C viral genes were introduced into the Suidae lineage as a result of trans-species infection by an ancestral xenotropic murine virus. The rate of evolution of the virogene sequences in the pig appears to be much slower than that of genes that have remained in the rodent lineage; this may be a consequence of transfer from a shorter-lived animal (the rodent) to a longer-lived one (the pig). We estimate the time of gene transmission as 5–10 million years ago and conclude that the present-day porcine type C virogenes most closely approximate the viral genes as they were several million years ago in the rodent lineage.**

The chromosomal DNA of various mammalian species contains multiple copies of nucleic acid sequences that can code for the production of endogenous type C viral particles (1, 2). With DNA transcripts prepared from the endogenous baboon type C viruses (3, 4), partially homologous sequences have been detected in the cellular DNA of baboons, other Old World monkeys, apes, and man (5, 6). These virogenes have evolved for at least 30 million years (Myr) in the anthropoid primates in a fashion that correlates closely with the taxonomic relatedness of these species, although the rate of evolution of the virogene sequences is faster than that of the nonrepetitive cellular DNA (6).

Type C viruses have also, under natural conditions, been transferred between species that are only remotely related phylogenetically. The one example that has been documented thus far involves the transfer of an endogenous primate type C virus into the germ line of the ancestor of the domestic cat (7). In this report, we describe a second example of horizontal transmission and subsequent integration of endogenous type C viral genomes between distantly related species. In this case, a set of type C virogenes can be shown to have been transferred from an ancestor of the present-day mouse (*Mus spp.*) to the pig (*Sus spp.*).

The available evidence suggests that endogenous type C viruses have been genetically transmitted in rodents for at least 20 Myr; however, rodents have diverged considerably from one another so that virogene sequences, even in the same family, such as mouse and rat, show very little reciprocal nucleic acid sequence homology. By contrast, when the virogene sequences of pigs are examined the results are quite different. Other ungulates, including close relatives of the pig like the South American peccary (*Tayassu tajacu*), do not have detectable virogene sequences related to that of the pig type C viral probe. Extensive homology, however, is found with normal mouse cellular DNA, and detectable but clearly lower levels are also seen with rat and hamster cellular DNA.

Abbreviation: Myr, million years.

* This is paper III of a series; paper II is ref. 7.

MATERIALS AND METHODS

Molecular Hybridization. The [^{3}H]thymidine-labeled DNA probes prepared from endogenous porcine, murine, and rat type C viruses were synthesized from detergent-disrupted virus in the presence of actinomycin D (8). The specific activity of the [^{3}H]DNA was 1.8×10^7 cpm/μg. The [^{3}H]DNA probes contained 60–66% of their respective 70S viral RNA sequences at a [^{3}H]DNA:viral[^{32}P]RNA molar ratio of 1.5, indicating that they contain most of the sequences present in viral RNA and that these sequences are in proportions similar to their content in 70S RNA (5). The source of the viruses used to prepare the DNA probes are listed in the legend to Table 1. Cellular DNA was extracted from tissues and cell lines as described (5). All DNAs were sonically treated to a mean size of 5–7 S (the size of the [^{3}H]DNA probes) as determined by centrifugation on alkaline sucrose gradients (5). DNA·DNA hybridizations were incubated at 65° in reaction mixtures containing 0.01 M Tris·HCl, pH 7.4, 0.75 M NaCl, 2×10^{-3} M EDTA, 0.05% sodium dodecyl sulfate, 30,000–40,000 cpm of [^{3}H]DNA and 2–4 mg of cellular DNA per ml. Hybridizations were

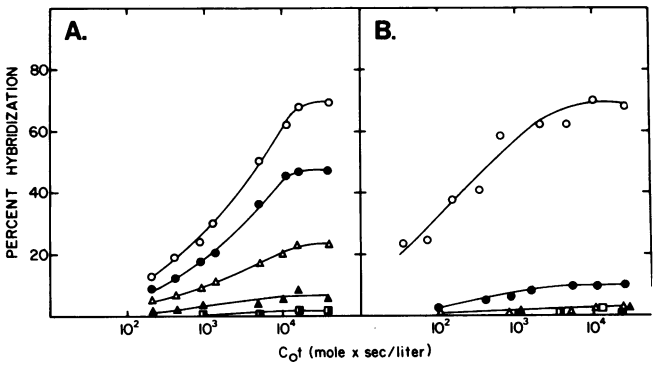

FIG. 1. Hybridization of nonrepetitive BALB/c cellular [^{3}H]DNA and [^{3}H]DNA probe prepared from an endogenous BALB/c virus (S2CL3) to the cellular DNA of various rodents, and to pig DNA. (A) BALB/c/3T3 cell line DNA was labeled with [^{3}H]thymidine and extracted (5). Nonrepetitive cellular DNA was isolated by removing the highly reiterated sequences (approximately 40% of the total DNA) that anneal by a C_0t of 200 by fractionation on hydroxyapatite (5). This [^{3}H]thymidine-labeled DNA was then hybridized to DNA extracted from *Mus musculus* (BALB) liver (○), *Mus caroli* liver or cell culture (●), rat liver (△), hamster liver (▲), pig, peccary, hippopotamus, sheep, or cow liver (□) or capybara liver (■). (B) Annealing of [^{3}H]DNA probe prepared from a BALB N-tropic virus (S2CL3) to the DNA of various rodents, and to pig DNA. Symbols are the same as in (A).

started by heating the mixtures to 100° for 10 min, cooling on ice to 4°, and incubating at 65°. At various times, 0.025 ml portions were removed and frozen at −80°C until digested with the single-strand-specific nuclease, S_1, as described (8). C_0t values (C_0 is the concentration of cellular DNA in mol liter^{-1} and t is the time in seconds) were calculated as suggested by Britten and Kohne (9) as (A_{260} ml^{-1})/2 × hr, and corrected to a monovalent cation concentration of 0.18 M (10).

RESULTS

Detection of nucleic acid sequences related to mouse and pig type C viruses in the cellular DNA of various species

Radioactively labeled nonrepetitive *Mus musculus* cellular DNA (laboratory strain BALB/c) was hybridized to the cellular DNA of various rodents as shown in Fig. 1A. Under the conditions used, *Mus caroli* DNA is 60–70% related to *M. musculus* DNA, rat nonrepetitive cellular DNA is approximately 30% related, and hamster cellular DNA considerably less, though still detectably, related. The relative final extents of hybridization agree well with data previously obtained using somewhat different conditions for determining the evolutionary relationships among rodents (11).

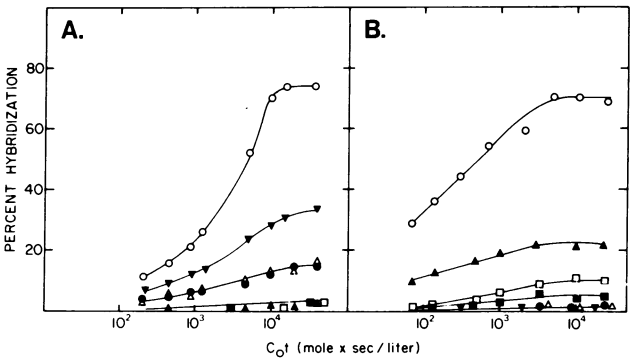

FIG. 2. Hybridization of nonrepetitive pig cellular [^{3}H]DNA and [^{3}H]DNA probe from pig PK-15 type C virus to the cellular DNA of various rodents and artiodactyls. (A) Annealing of nonrepetitive pig cellular [^{3}H]DNA to DNA extracted from domestic pig kidney (○), peccary liver (▼), sheep liver (●), calf thymus (△), mouse liver (▲), rat liver (□), and hamster liver (■). (B) Annealing of [^{3}H]DNA probe prepared from the pig type C virus PK-15 to various DNAs. Symbols are the same as in (A).

Fig. 1B shows a DNA reassociation experiment performed with a [^{3}H]DNA transcript of an endogenous mouse (BALB/c) type C virus and DNAs extracted from several rodent species. Related virogene sequences are barely detected in

Table 1. Nucleic acid homology between mouse, rat, and pig type C viral DNA and the DNA from various species

Species*	Percent hybridization†			
	Pig viral [^{3}H]DNA	Mouse viral [^{3}H]DNA		Rat viral [^{3}H]DNA
		M. musculus (BALB/c)	*M. caroli*	
Rodents				
Mouse (*Mus musculus*)	23.0	100.0	17.5	6.5
(*Mus caroli*)	26.0	13.0	100.0	4.8
(*Mus cervicolor*)	25.0	10.0	12.5	N.T.
Rat (*Rattus norvegicus*)	13.0	2.5	1.5	100.0
(*Rattus rattus*)	11.0	N.T.	N.T.	69.0
Hamster (*Cricetulus griseus*)	5.0	2.0	0.6	3.6
Guinea pig (*Cavia porcellus*)	1.0	1.0	1.0	2.5
Capybara (*Hydrochoerus* sp.)	0.5	2.0	N.T.	2.0
Artiodactyls				
Pig (*Sus scrofa*)	100.0	1.5	2.6	2.1
Wild boar (*Sus scrofa*)	100.0	1.8	N.T.	N.T.
Bush pig (*Potamochoerus porcus*)	86.0	1.6	1.5	1.0
Warthog (*Phacochoerus aethiopicus*)	71.0	1.9	1.7	2.0
Peccary (*Tayassu tajacu*)	1.5	2.0	2.1	1.5
Hippopotamus (*Hippopotamus amphibius*)	2.0	1.5	1.6	1.5
Sheep (*Ovis* sp.)	1.3	1.8	1.5	1.9
Cow (*Bos taurus*)	2.1	2.0	2.0	2.0
Carnivores				
Domestic cat (*Felis catus*)	2.2	4.0	1.5	4.5
Ocelot (*Felis pardalis*)	1.8	N.T.	N.T.	1.9
Lion (*Panthera leo*)	N.T.	2.0	1.9	1.7
Primates				
Woolly monkey (*Lagothrix* sp.)	1.3	1.5	0.8	0.0
Baboon (*Papio cynocephalus*)	2.0	1.5	1.2	1.0
Man (*Homo sapiens*)	1.3	1.8	1.7	0.5

N.T. = not tested.

* Cellular DNA was extracted from various tissues of the species listed (5).

† The percent hybrid is the saturating normalized value obtained after digestion of the hybrids with S_1 nuclease. The [^{3}H]DNA probes were prepared from the porcine PK-15 virus (14); from an endogenous BALB/c mouse virus, S2CL3 (22); from the virus isolated from a *Mus caroli* cell line and propagated in cat embryo cells (16); and from an endogenous rat virus, RT21c (23). The actual final extents of hybridization to cellular DNA from their species of origin were 71%, 76%, 69%, and 89%, respectively.

Table 2. Thermal stability of hybrids formed between nonrepetitive pig and mouse cellular DNA and the DNA from various artiodactyls and rodents

Species†	Nonrepetitive cellular DNA*			
	Pig		Mouse	
	% Hybrid	ΔT_m	% Hybrid	ΔT_m
Artiodactyls				
Domestic pig	100	0.0	2	—
Wild boar	100	0.0	2	—
Bush pig	93	1.3	1	—
Warthog	85	1.8	3	—
Peccary	42	7.7	2	—
Hippopotamus	18	>12	2	—
Cow	15	>12	2	—
Sheep	15	>12	2	—
Rodents				
Mouse				
(*M. musculus*)	2	—	100	0.0
(*M. caroli*)	2	—	61	4.0
Rat	1	—	21	12.0
Hamster	2	—	11	>12
Guinea pig	2	—	3	>12
Capybara	2	—	2	>12

* [^{3}H]Thymidine-labeled nonrepetitive domestic pig and mouse (*M. musculus*) cellular DNA was isolated (5) and hybridized to the cellular DNA of the various species. The percent hybrid is the saturating normalized value obtained after digestion of the hybrids with S_1 nuclease. The actual final extent of domestic pig and mouse cellular DNA hybridization to their cellular [^{3}H]-DNAs was 78% and 74%, respectively. The temperature at which 50% of the hybrids are dissociated (T_m) was 85° and 86° for the homologous domestic pig:domestic pig hybrid, and mouse:mouse hybrid, respectively. The ΔT_m is the difference in T_m between the other DNA·DNA hybrids and the T_m of the homologous hybrid. The T_m values were derived from triplicate experiments in which hybrids were melted (6) at 1° intervals over a range of ±5° from the T_m.

† Cellular DNA was extracted from various tissues of the species listed (5). The scientific names of the various species are listed in Table 1.

Mus caroli and cannot be detected in the rat or hamster cellular genome despite the fact that these rodents also contain multiple copies of endogenous type C virogenes (2, 12).

Fig. 2A shows similar hybridization studies performed with nonrepetitive pig cellular DNA. Other artiodactyls, such as the peccary, cow, and sheep, contain related unique sequence DNA, while, as expected, rodent cellular DNA does not hybridize to pig nonrepetitive DNA under the conditions used. The pig virus PK-15 (13) is an endogenous vertically transmitted type C virus (14); closely related isolates have been detected in six different porcine cell cultures (15). When a pig type C viral probe is hybridized to the cellular DNA of other animals (Fig. 2B) an extensive homology is found with mouse cellular DNA and detectable but clearly lower levels are also seen with rat and hamster cellular DNA. In contrast, members of the same order (peccary, hippopotamus, sheep, and cow) do not contain virogene sequences related to the pig type C virus.

The partial homology observed between the pig type C viral probe and mouse cellular DNA could be the result of a portion of the genomes being highly related or to overall base-pair mismatching. To distinguish between these possibilities, the thermal stability of the hybrids was examined. The hybrid formed between the pig viral probe and DNA extracted from mouse (*M. musculus*) cell cultures or tissues melts 10° lower than the homologous hybrid, which has a T_m (midpoint of melting curve) of 85°. This result indicates considerable base-pair mismatching, and is consistent with the accumulation of mutations throughout the genome rather than the strict preservation of a common virogene segment in both mouse and pig cellular DNA.

Origin of pig type C virus

The nucleic acid homology between the endogenous pig type C viral RNA and murine cellular DNA suggests that the endogenous viruses of these two species have had a common origin. The origin can be clarified by examining the cellular DNAs of related rodents and Suidae for virogene sequences partially homologous to each virus. Table 1 summarizes the nucleic acid homology obtained using mouse, rat, and pig type C viral DNAs and the cellular DNAs from various species of rodents, artiodactyls, carnivores, and primates. The virus from *Mus musculus* barely detects virogene sequences in *Mus caroli* despite the fact that *Mus caroli* has its own endogenous type C virus which can be induced from *M. caroli* cell cultures (16). Similarly, DNA probes prepared from the endogenous virus isolated from *Mus caroli* barely detect homologous sequences in *Mus musculus* cellular DNA. A comparable extent of virogene nucleic acid homology can also be detected in a third species of the same genus, *M. cervicolor*, from which an infectious virus has not yet been isolated. The virogenes of rat, hamster, and more distantly related rodents such as guinea pig and capybara are not detected using these hybridization conditions. Pig cellular DNA and the DNA of other artiodactyls, primates and carnivores also contain no related sequences. Similar results are obtained with an endogenous rat type C viral probe; sequences related to this virus are found only in the cellular DNA of the laboratory rat (*Rattus norvegicus*) and in a closely related species, *Rattus rattus*.

Hybridization studies performed with the pig type C viral probe reveal multiple copies of endogenous virogene sequences in domestic pig and wild boar cell DNA. Related sequences are also found in the bush pig (*Potamochoerus porcus*) and the warthog (*Phacochoerus aethiopicus*), animals that are closely related phylogenetically to the domestic pig. As was shown in Fig. 2B, no related sequences are found in any other artiodactyls tested. However, high levels of hybridization (23–26%) are found with the cellular DNA of three species of *Mus* (*M. musculus*, *M. caroli*, and *M. cervicolor*). The next highest levels are found with the cellular DNA extracted from two species of rats (11–13%). Chinese hamster DNA also has virogene sequences related to the pig virus that are significantly higher (5%) than background values (1–2%). The capybara, a South American rodent in a different suborder than the mouse, rat, and hamster, and the guinea pig, which is also distantly related to the mouse, do not show a detectable level of sequence homology to the pig viral probe.

Table 2 lists the relationships among the nonrepetitive cellular DNAs of artiodactyls and rodents. Domestic pig and wild boar cellular DNA are too closely related to be distinguished by these methods. However, the hybrids formed with the DNA from two African Suidae, the bush pig and the warthog, melt 1.3° and 1.8° lower, respectively, than the homologous domestic pig hybrid. The South American peccary, which belongs to a different family (Tayassuidae), and the still more distantly related hippopotamus, cow, and sheep give correspondingly lower T_ms. Among the rodents,

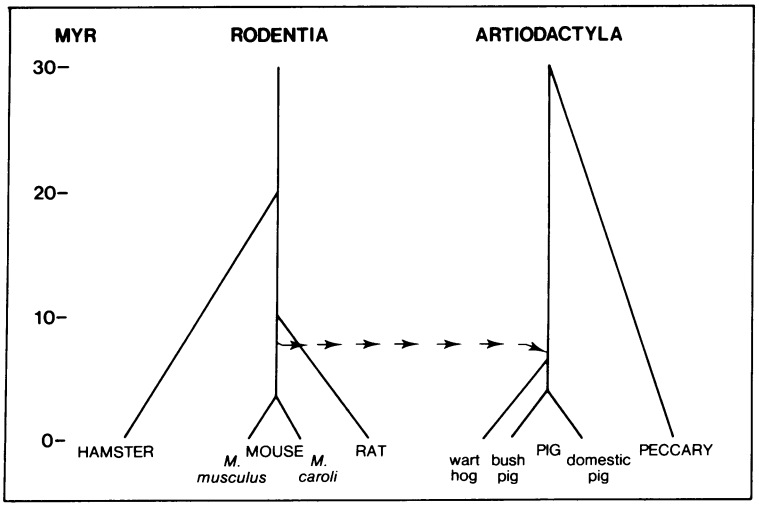

FIG. 3. Transmission of murine virogenes to the porcine germ line. The approximate divergence times for the various rodent species are taken from Rice (11). The estimates for the divergence time of the artiodactyls are derived from the data in Table 2, assuming that the average porcine generation time is 3–4 years, and that a 1° difference in the T_m of nonrepetitive DNA corresponds to 1×10^6 generations. This value has previously been shown to relate nucleotide changes to evolutionary distance based on the fossil record of primates and other mammals (2, 6, 24).

the two species belonging to the same genus (*Mus*) are 61% related (ΔT_m 4°), and the rat, which belongs to a different genus of the same family (Muridae) is more distantly related. The hamster (family Cricetidae), and the guinea pig and capybara are even less related to mouse cellular DNA.

When the virogenes of two species are more closely related to each other than are the unique sequence cellular genes, one must suspect horizontal transmission of virus from one species to another (6). Fig. 3 is a schematic representation of rodent and artiodactyl evolution. Based on the degree of relatedness of unique sequence cellular DNA, as shown in Table 2, and correcting for the generation time of feral rodents (about 0.3–0.5/year) and pigs (3–4 years) (17), an approximate evolutionary tree can be drawn depicting the relationships among rodents and artiodactyls. Among the rodents, *M. musculus* and *M. caroli* are believed to have had a common ancestor approximately 3 Myr ago, rats and mice 10 Myr ago, and hamsters and mice 20 Myr ago (11). Correcting the data obtained with the unique sequence DNA of various artiodactyls for the longer generation time of these animals [assuming that the accumulation of base-pair mutations is primarily a function of the number of generations since the last common ancestor (18)], the divergence times shown in Fig. 3 are obtained. The warthog, bush pig, and domestic pig are all African members of the family Suidae and are more closely related by DNA sequence homology to each other than they are to the peccary.

Since sequences related to the domestic pig type C virus can be detected to the same final extent in three different species of *Mus* and to a considerably lesser extent in rat cellular DNA, we propose that the infection of an ancestor of the domestic pig occurred *after* the rat and mouse had diverged from their common ancestor but *prior* to the *Mus musculus* and *Mus caroli* divergence. This would place the time of infection of pigs with a murine-like virus as occurring between 3 and 10 Myr ago. An analysis of Suidae which contain these virogene sequences likewise reveals that the viral infection must have occurred after the peccary-domestic pig split (about 30 Myr ago) and before the warthog-domestic pig divergence (about 5–7 Myr ago) since both of these latter Suidae contain related virogene sequences. Therefore, by considering the data obtained from both the rodent and artiodactyl orders, the time of infection of an ancestor of the domestic pig with a murine virus is presumed to have occurred between 5 and 10 Myr ago.

DISCUSSION

From the extent of hybridization of the pig type C viral probes to rodent cellular DNA we conclude that the type C virogenes in the pig were transmitted from members of the family Muridae at some point *after* the mouse had separated from the rat, but *before* the different species of mice, *Mus musculus* and *Mus caroli*, had diverged from each other. Rodent viral genes thus gave rise to infectious particles that became incorporated into the porcine germ line. A previous example of transmission of type C viral genes between distantly related species involved the transfer, in the Pliocene, of an Old World monkey type C virus to an ancestor of the domestic cat (6). The two examples parallel each other closely. Primate cell DNA contains nucleic acid sequences related to the RD-114/CCC group of endogenous cat viruses (6); rodent cell DNA likewise contains nucleic acid sequences related to the endogenous pig viruses. The endogenous primate (baboon) and domestic cat (RD-114) type C viral proteins (p30 and reverse transcriptase) are closely related to one another (19, 20), as are the mouse and pig type C viral proteins (16, 21). Wild species related to the domestic cat also have the virogene sequences; similarly, wild relatives of the domestic pig contain sequences related to the pig type C virus. The demonstration of closely related gene sequences in species that have been genetically distinct for millions of years rules out the possibility that these represent contemporary gene transmission events.

The one significant difference between the two examples of interspecies gene transfer is that while probes prepared from the contemporary primate viruses still hyridize to cat cellular DNA, the probes prepared from contemporary mouse viruses *do not* hybridize to pig cellular DNA (see Table 1). Probes prepared from pig viruses also do not hybridize to the RNA of known murine type C viruses (14). Taken together, these results suggest that the sequences in mouse DNA which hybridize to the pig probe may not nor-

Table 3. Number of generations that separate present-day species from the trans-species virus infection event

Species	Generations/ year*	Millions of generations from the time of postulated type C viral gene transfer†
Mouse	2–3	14–21
Rat	2–3	26–39
Hamster	2–3	66–99
Pig	0.25–0.33	1.8–2.3

* The approximate generation time of the species is listed (17).

† The number of generations since the transfer of a type C virus from a rodent to a pig ancestor. We assume this event occurred approximately 7 Myr ago (see *text*). The mouse viral genes have evolved in the *Mus* lineage for 7 Myr since the transfer, a total of 14 to 21 $\times 10^6$ generations, assuming 2–3 generations a year. The rat is separated from the transmission event by the 10 Myr it has evolved since the mouse–rat divergence plus the 3 Myr after that divergence that the viral genes were in the *Mus* lineage, or a total of 26 to 39 $\times 10^6$ generations, assuming again that there have been 2–3 generations per year. Similar calculations for the hamster and pig yield the values shown.

mally be expressed. The mouse viral genes have been transmitted from a relatively short-lived animal, with a rapid generation time (2–3 generations per year), to a considerably longer-lived animal, the pig, where the average generation time is 3–4 years (17). As a result, the ancestral mouse virus genes "trapped" in the pig genome would have evolved for considerably fewer generations in porcine cell DNA than have the same genes that have evolved as part of the rodent germ line. As two species diverge from one another, the common gene sequences they once possessed accumulate base-pair substitutions. If this is a function primarily of the number of generations since divergence, the rate of sequence change should be less in animals with long generation times than in those with short generation times (18). The results described here support that conclusion and show that the viral genes, once integrated, adopt the evolutionary rate of the recipient species. The pig type C viral genome would thus represent the closest present-day representative of an ancient mouse type C viral genome; the viral genes of the pig are, in a sense, "living fossils."

In Table 3 we estimate the distance in terms of number of generations by which the contemporary viral genes are separated from the ancestral rodent virus genes. Assuming the transmission event occurred 7 Myr ago, the modern pig type C virus, having accumulated only two million generations, would correspond in evolutionary age to the murine virus genes of approximately 6 Myr ago. Because of this, the pig type C viral probe detects related sequences in more distant rodents (such as the rat and hamster) than do the present-day mouse viruses, which have been rapidly evolving away from each other and from their common ancestor.

Studies of rates of divergence of specific genes or groups of genes have been limited by the difficulty of their purification. The type C viral genes offer a distinct advantage in that they are produced by and secreted from cells as discrete sets of genes. From the data presented here, we conclude that an endogenous virus of the mouse was transmitted into the germ line of the pig, where it can now be found in multiple copies in the cellular DNA. The present results, along with those described previously, show that these genes have a propensity for trans-species infection and subsequent integration into the germ cells of evolutionarily distant species.

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