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Segregation of RD-114 and FeLV-related sequences in crosses between domestic cat and leopard cat

TYPE C viruses of the RD-114 (ref. 1) group have been isolated, either spontaneously or after chemical induction, from cell cultures of the domestic cat (*Felis catus*)[2–4]. Nucleic acid sequences related to the RD-114 genome are in the DNA of all domestic cats[5–8]. Thus these viral genomes are transmitted vertically from parent to offspring as integral components of cat cellular DNA. Although the family Felidae consists of closely related animals, only four *Felis* species have been found to contain RD-114-related sequences. These include the domestic cat, the European wildcat (*F. sylvestris*), the sand cat (*F. margarita*), and the jungle cat (*F. chaus*); other members of the Felidae lack nucleic acid sequences related to RD-114 (ref. 9). The observation that RD-114 is partially related to the endogenous baboon type C viruses[10–12] and that sequences related to RD-114 are found in the cellular DNA of all Old World monkeys led to the postulate that this group of viruses originated from an endogenous primate type C virus[13] transmitted horizontally to the germ line of ancestors of certain *Felis* species during the Pliocene or early Pleistocene somewhere in the region of the Mediterranean basin[9].

A second distinct group of type C viruses, the feline leukaemia viruses (FeLV), also has been isolated from domestic cats[14]. Although FeLVs are horizontally transmitted among domestic cats, genes partially related to the RNA genome of FeLV are found in *F. catus* DNA[15] and in the DNA of the other species of Felidae which contain RD-114 related nucleic acid sequences[16]. Viruses of the FeLV group are also postulated to have been transmitted to an ancestor of these *Felis* species, but to have originated from a rodent rather than a primate source[16].

The leopard cat (*F. bengalensis*) is a spotted wildcat found throughout South-east Asia which lacks RD-114 and FeLV-related DNA sequences (RD^-, FL^-)[9]. Since leopard cats produce viable offspring when bred with domestic cats (RD^+, FL^+), we studied the segregation of both sets of virogenes in F_1 hybrids and in the progeny of a backcross to the RD^-, FL^- parent. The cellular DNA of the F_1 hybrids contains half the number of copies of each set of sequences. The RD and FL virogenes segregate together in the backcrossed animals in a manner consistent with their localisation at a single chromosomal site.

The reassociation kinetics obtained by hybridising ^{3}H-DNA transcripts of viral RNA to cellular DNA can be used to estimate relative gene frequencies by determination of half C_0t values (the midpoint of the renaturation curve)[17]. The number of gene copies can also be estimated by plotting reassociation kinetics as the reciprocal of the fraction of unhybridised ^{3}H-DNA against C_0t (Wetmur–Davidson plot)[18]. In such plots, the slope is proportional to the number of copies of those sequences measured. Using RD-114 ^{3}H-DNA probes, multiple copies of virus-related sequences can be detected in the cellular DNA of stray domestic cats, domestic cats reared in a germ-free environment (Merck, Sharp and Dohme, West Point, Pennsylvania) and in European wildcats (*F. sylvestris*) (Fig. 1*a* and Table 1). The $C_0t_{\frac{1}{2}}$ values ranged from 120 to 170. In contrast, the cellular DNA of the leopard cat completely lacks RD-114 related sequences. The $C_0t_{\frac{1}{2}}$ values for the self-annealing of non-repetitive domestic cat cellular DNA, and for the hybridisation of the ^{3}H-DNA RD-114 probe to the DNA of a canine thymus cell line infected with RD-114, ranged from 1,800 to 2,000 (Fig. 1, Table 1). Given that $C_0t_{\frac{1}{2}}$ values of 1,800–2,000 are obtained with genes present in a single copy per haploid genome, domestic cat and European wildcat cellular DNAs contain 10–13 copies of RD-114-related sequences per haploid genome. Since these copies represent a family of diverging gene sequences only partially related to one another[19], the calculated number of copies may be an underestimate[20,21].

Leopard cat males were mated to domestic cat females and the F_1 hybrids studied. These DNAs contain a complete complement of sequences related to the RD-114 probe, but only half the number of copies present in the domestic cat parent (Fig. 1*a*). The $C_0t_{\frac{1}{2}}$ values (275–350) represent, as a minimum estimate, five to seven virogene copies per haploid genome. Two kittens obtained from an F_1 hybrid female backcrossed to the leopard cat (Fig. 2) were also studied. Figure 1*a* shows that kitten No. 1 contains all the RD-114-related information, but only half the number of copies ($C_0t_{\frac{1}{2}}$ 280), like the F_1 parent, whereas kitten No. 2 (from the same litter) lacks RD-114-related DNA sequences like its leopard cat parent. These results suggest that the multiple copies of RD-114 re-

Table 1 Segregation of RD-114 and FeLV type C viral sequences in various cats

Cats*		RD-114 $C_0t_{\frac{1}{2}}$†	RD-114 Average no. viral copies‡	FeLV $C_0t_{\frac{1}{2}}$	FeLV Average no. of viral copies
Domestic cat	No. 1	130	12–13	230	8–9
	No. 2	120	12–13	260	7–8
	No. 3	170	10–11	280	7–8
CCC clone 6§		150	10–11	250	7–8
European wildcat		140	11–12	210	8–9
F_1 hybrid	No. 1	350	5–6	550	3–4
	No. 2	330	5–6	400	4–5
	No. 3	275	6–7	500	3–4
	No. 4	300	6–7	450	3–4
Backcross	No. 1	280	6–7	520	3–4
	No. 2	—	0	—	0
Leopard cat	No. 1	—	0	—	0
	No. 2	—	0	—	0
Cell lines					
RD-114/FCf2Th clone 10§		1,800	1	—	0
FeLV/FCf2Th§		—	—	1,900	1

* Cellular DNA was extracted from various organs (spleen, liver, kidney, lung) and hybridised to RD-114 and FeLV ^{3}H-DNA as described in Fig. 1. Domestic cats Nos 2 and 3 were from the germ-free colony of cats at Merck, Sharp and Dohme; animals from this colony have never been found to be positive for infectious feline leukaemia virus. F_1 hybrid cats Nos 1, 2 and 3 and 4 belong to three separate litters.

† $C_0t_{\frac{1}{2}}$ values represent the midpoint of the reannealing curves[17].

‡ The approximate number of copies per haploid genome of sequences related to either RD-114 or FeLV were estimated from reciprocal plots (Fig. 1). The number of copies is determined by the ratio of the slope of each line to the slope of the line described by the reassociation of non-repetitive domestic cat cellular DNA ($C_0t_{\frac{1}{2}}$ = 1,800–2,000; see also ref. 19). In the case of RD-114, where two sets of viral sequences can be detected in cellular DNA, the number of copies listed is the average of the two populations.

§ CCC clone 6 is from a continuous line of domestic cat kidney fibroblasts and is not releasing type C virus[2,3], and RD-114/FCf2Th and FeLV/FCf2Th are a dog thymus cell line infected, respectively, with RD-114 (ref. 1) or with the helper virus from the Gardner–Arnstein[23] strain of feline sarcoma virus.

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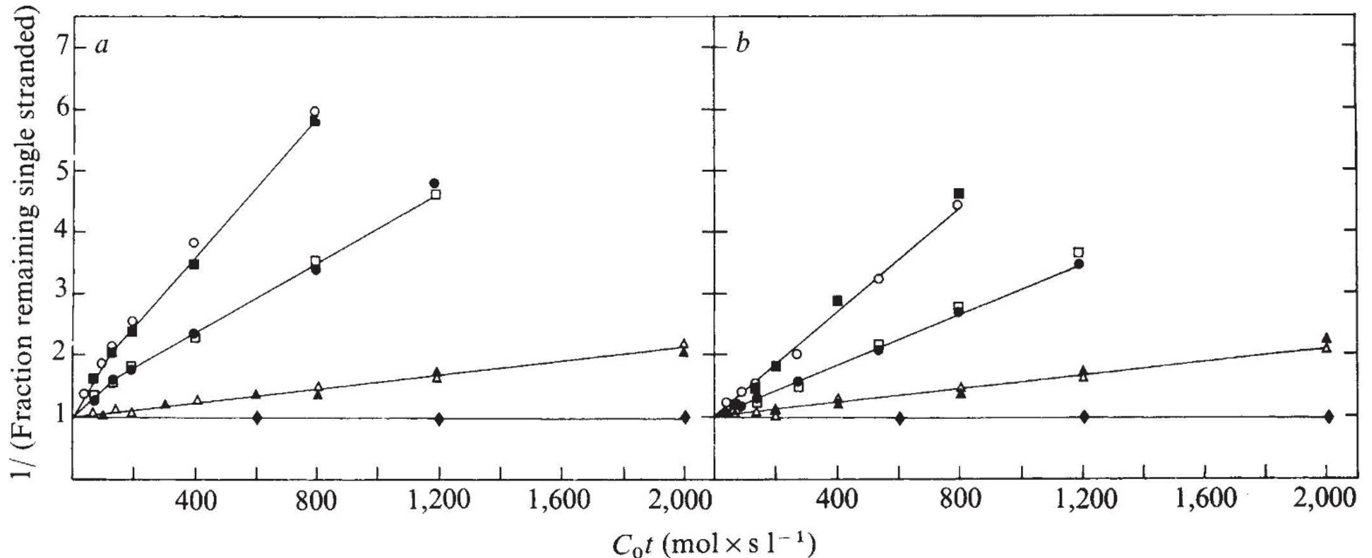

Fig. 1 Analysis of reassociation kinetics by the method of Wetmur and Davidson[18] of RD-114 and FeLV ^{3}H-DNA probes to cat cellular DNAs, and of domestic cat unique sequence cell DNA self-association. The ^{3}H-thymidine-labelled DNA probes were synthesised from detergent-disrupted type C virus in the presence of actinomycin D as described[27]. The specific activity of the ^{3}H-DNA was 1.7×10^7 c.p.m. μg^{-1}. The ^{3}H-DNA probes contained 60–66% of their respective 70S viral RNA sequences at a ^{3}H-DNA:^{32}P viral RNA molar ratio[10] of 2.0. Cellular DNA was extracted from tissues and cell lines as described[10]. All DNAs were treated sonically so as to yield a mean size of 6–8S (the size of the ^{3}H-DNA probes)[10]. DNA : DNA hybridisations were incubated at 65 °C in reaction mixtures containing 0.01 M Tris, *p*H 7.4, 0.75 M NaCl, 2×10^{-3} M EDTA, 0.05% sodium dodecyl sulphate, 30,000 to 40,000 c.p.m. of ^{3}H-DNA and 2–4 mg of cellular DNA per ml. Hybridisations were started by heating the mixtures to 100 °C for 10 min, cooling on ice to 4 °C and incubating at 65 °C. 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[27]. C_0t values (C_0 is the concentration of cellular DNA in mol l^{-1} and t is the time in s) were calculated as suggested by Britten and Kohne[28] as (A_{260} ml^{-1})/2 × h, and corrected to a monovalent cation concentration of 0.18 M (ref. 29). *a*, Annealing of RD-114 ^{3}H-DNA probe to DNA extracted from: ○, domestic cat No. 3; ■, domestic cat No. 1; ●, F_1 hybrid No. 4; □, backcrossed kitten No. 1; ▲, a dog thymus cell line infected with RD-114, and ◆, leopard cat and backcrossed kitten No. 2. Comparable data were obtained with DNA probes prepared from the domestic cat type C virus, CCC[2,3], and with the FS-1 virus isolated from European wildcat cells[30]. △, Self-association of non-repetitive domestic cat cellular DNA. ^{3}H-thymidine-labelled domestic cat cellular DNA was isolated by removing the highly reiterated sequences that anneal by a C_0t of 800 (approximately 40% of the total DNA) by fractionation on hydroxyapatite[10]. This ^{3}H-thymidine-labelled DNA (5×10^5 c.p.m. μg^{-1}) was then hybridised to total domestic cat cellular DNA. b, Annealing of FeLV ^{3}H-DNA probes (Rickard strain)[22] to DNA extracted from: ○, domestic cat No. 2; ■, domestic cat No. 1; ●, F_1 hybrid No. 3; □, backcrossed kitten No. 1; ▲, a dog thymus cell line infected with FeLV, and ◆, leopard cat and backcrossed kitten No. 2. Comparable data were obtained with the Gardner–Arnstein[23] strain of FeLV grown in a canine thymus cell line (FCf2Th). △, Self-association of unique sequence domestic cat cellular DNA. Domestic cat cellular DNA contains at least two distinct sets of RD-114-related sequences which are present in different reiteration frequencies. The F_1 hybrid and backcrossed kitten No. 1 have approximately half the number of copies of each set of related sequences. While only one set of virogene sequences can be detected with the FeLV probe (Fig. 1*b*), the F_1 hybrid and backcrossed kitten No. 1 again are shown to contain half the number of copies as that found in the domestic cat parent.

lated sequences are located together at one (or relatively few) chromosomal sites.

The same cats were examined for FeLV-related genes using ^{3}H-DNA probes prepared from various strains of FeLV[22,23]. No cross-hybridisation between these probes and RD-114 is detectable[15,24]. The results parallel exactly the data obtained with transcripts of RD-114 RNA. The domestic cat parent contains multiple copies of FeLV-related virogenes whereas the leopard cat parent lacks these sequences. The F_1 hybrids contain half the number of copies. Backcrossed kitten No. 1 has the same number of virogene copies as its parent (the F_1 hybrid) although its littermate has no detectable sequences related to FeLV.

Table 1 summarises the hybridisation data; four clear classes of DNAs are evident. The first consists of animals that contain full complements of RD-114 and FeLV-related gene sequences. These cats contain both sets of virogene sequences reiterated a comparable number of times, although there may be fewer FeLV-related copies. The animals in the second class, including all the F_1 hybrids and one of the backcrossed kittens, contain half the virogene complement of the RD$^+$, FL$^+$ parents. A third class contains sequences that anneal to the viral probe with reassociation kinetics comparable with that seen for the association of the most slowly reannealing cellular DNA sequences and therefore probably contains one viral copy. This includes cloned heterologous cell lines producing high titres of either RD-114 or FeLV viruses. The fourth class completely lacks sequences related to either RD-114 or FeLV and includes the leopard cats and one of the backcrossed kittens.

If the multiple copies of RD-114 and FeLV-related sequences present in cat DNA had become physically separated from one another after their entry into the cat germ line[9] they would not all have segregated together. The data rule out the possibility

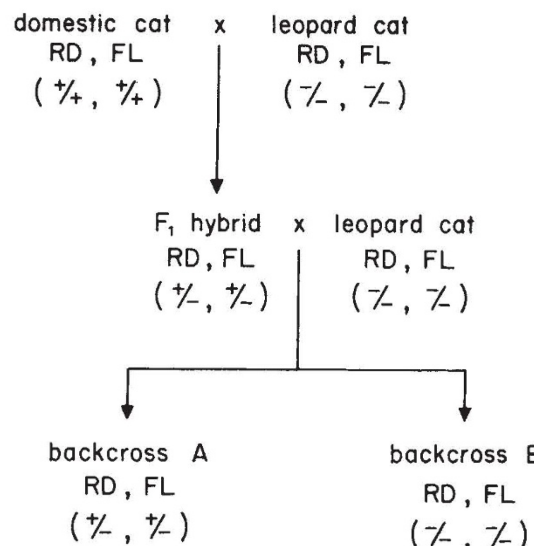

Fig. 2 RD-114 and FeLV type C viral genotypes of the various cats studied.

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that each of the multiple copies of RD-114 or FeLV-related virogenes occurs on a different linkage group, as well as models of non-chromosomal inheritance of the multiple viral copies.

Since only certain *Felis* species contain RD-114 and FeLV-related sequences in their DNA, we propose that both classes of viruses were acquired by cats subsequent to their major radiation, most likely in the Pliocene, and that both sets of sequences have been perpetuated in the germ line[9,16]. The multiple virogene copies presumably arose as a result of gene duplication and/or unequal crossing-over after infection. The presence of multiple copies of both sets of virogenes in cat cellular DNA seems to be a general property of endogenous mammalian type C viruses; mouse, rat, hamster, pig and baboon DNAs also contain similar numbers of copies of their respective endogenous viruses[19].

The genetic crosses described here provide a new approach to the study of the evolution of multiple gene systems. The virogene sequences can be considered as one of the group of moderately repetitive sequences such as the genes for 5S RNA[25], histones[26] and feather keratin[20]. The existence of natural populations of animals that either lack or contain DNA sequences related to both RD-114 and FeLV, and the ability of these cats to interbreed permits the study of the physiological and potentially pathological role of each of these genetically transmitted gene sequences. Hybrid animals containing half the number of virogene copies and virogene-negative cats should allow the study of the effects of gene dose on susceptibility and resistance to diseases mediated by both groups of type C viruses.

We thank J. Koci and G. L. Wilson for assistance, and R. Callahan and C. Sherr for comments and discussion. Supported in part by the Virus Cancer Program of the National Institutes of Health.

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Received June 30; accepted August 26, 1975.

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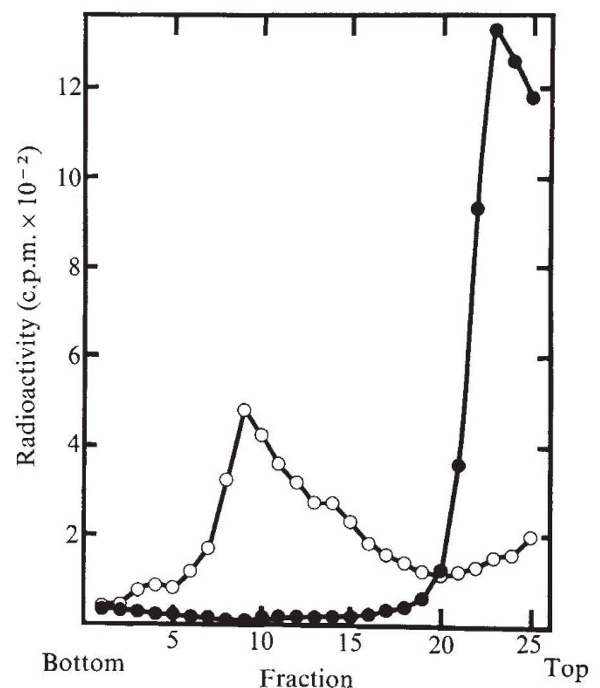
Identification of heat-dissociable RNA complexes in two porcine coronaviruses

THE coronavirus genome has been shown to comprise single-stranded RNA[1,2]. Examination of the viral nucleic acid synthesised by pig kidney cells infected with transmissible gastroenteritis virus (TGEV) suggested that several molecular species, ranging in size between 18 and 28S, were involved in the viral replicative cycle[3]; similarly Tannock found a wide variation in the size of RNA molecules extracted from avian infectious bronchitis virus (IBV) by a phenol–sodium dodecyl sulphate (SDS) method[2]. Extraction of IBV RNA by 1% SDS at 60 °C has, however, revealed a single component of molecular weight 9×10^6 corresponding to 60S by electrophoresis through 2.2% polyacrylamide gels[4].

We have examined the RNA extracted from purified preparations of TGEV and a second porcine coronavirus —haemagglutinating encephalomyelitis virus (HEV)—and have found a 60–70S RNA component which dissociates into 35S and 4S material on heating above 60 °C in a way that closely resembles the genome of the oncogenic RNA viruses.

We had observed that treatment of purified TGEV with 1% SDS at 20 °C disrupted the virions and liberated a high molecular weight complex containing the RNA. On the assumption that this complex might comprise the hitherto undetected ribonucleoprotein, we extracted the material from TGEV preparations radioactively labelled with ^{3}H-uridine or with ^{3}H-leucine to determine which structural polypeptide was associated with the complex. As is shown in Fig. 1, however, the fast moving RNA complex has no detectable protein associated with it, while polyacrylamide gel analysis of the radioactivity remaining near the top of

Fig. 1 Rate zonal sedimentation of TGEV after treatment with 1% SDS at 20 °C. TGEV was grown in secondary pig thyroid cell (APT/2) cultures in the presence of 5-^{3}H-uridine (○) or 4,5-^{3}H-leucine (●) and purified by sucrose gradient centrifugation as described previously[5]. After treatment with 1% SDS at 20 °C for 15 min, each preparation was layered over a 6-ml 15–30% (w/w) sucrose gradient and centrifuged at 250,000*g* for 2 h in a swing-out rotor. The gradients were fractionated by siphon and total radioactivity was determined for each fraction.



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