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Determination of Natural Host Taxonomy of RNA Tumor Viruses by Molecular Hybridization: Application to RD-114, a Candidate Human Virus*

(origin/feline/DNA·RNA/oncornavirus)

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ABSTRACT **The lysogenic bacteriophages and the RNA tumor viruses have in common the ability to add their genetic information to the genome of their host cells. The biological similarity extends further; data summarized here indicate that they both possess homology to the DNA of their uninfected indigenous hosts. Sharing of common sequences with normal host DNA has been established with avian, murine, feline, and primate oncornaviruses. This finding provides a method for determining the taxonomic position of the natural host of any new RNA tumor virus isolated. Application of this approach to RD-114 revealed extensive hybridization to normal cat DNA and little, if any, hybridization to human DNA. We conclude that the data assign a feline origin rather than a human origin to RD-114.**

The present intense search for RNA tumor viruses of human origin has generated an urgent need for new and supplementary methods for identifying the natural hosts of recent isolates. Thus far, investigators have depended principally on serology to resolve such issues. Antisera against the viral group-specific (gs) antigens or against the viral DNA polymerase have been used to establish host relations among the RNA tumor viruses. If a new agent fails to react with antisera against a known virus indigenous to a particular animal, it is concluded that the unknown virus cannot be native to the same host. While the assumption that two viruses indigenous to the same host must share gs and DNA polymerase antigens holds in many instances, its universality is far from established. One obvious exception is the mouse mammary tumor virus and the murine leukemia virus. Others are the two avian viruses, the reticuloendotheliosis virus and the avian myeloblastosis virus. Reticuloendotheliosis virus does not contain any of the gs antigens of avian leukosis virus (1–3). Further, a monospecific antiserum (4) prepared against purified avian myeloblastosis virus-DNA polymerase (5) fails to inactivate the DNA polymerase of reticuloendotheliosis virus, although it does react with the DNA polymerase of the avian Rous sarcoma virus (6).

It would obviously be advantageous to identify the natural host of any new RNA tumor virus by a method that does not depend on the extent of our virological information or the completeness of the catalogue of available antisera. A possible approach stems from previous observations made with bacterial viruses. There exists a group of temperate and transducing bacteriophages (e.g., λ, φ80, 434, P22, etc.) that can integrate their genome into the one of their hosts and thus permanently insert new genetic information. It has been shown (7) that the DNAs of these phages possess homology with the DNA of their uninfected hosts, a relation that is confined to their natural hosts or to bacteria closely related to them. Interestingly, the nonintegrating lytic DNA (7) and RNA (8) bacteriophages show no such homology with host DNA, either before or after infection.

The ability to add viral-specific information to the host genome is a striking biological feature shared by the lysogenic bacteriophages and the oncogenic RNA viruses. It would not be surprising to find that the biological parallelism extends further to encompass homology with the DNA of their natural hosts. That this is in fact the case is supported by a series of isolated observations that were reported without noting their potential taxonomic implications for the host of origin. The data are summarized in Table 1 and involve various avian, murine, and feline RNA tumor viruses. In all cases hybridizations occurred with normal cellular DNA from the natural host, whereas no complexes were observed with DNA from nonindigenous species.

We recently reported (13) a similar situation with a primate sarcoma virus, SSV-1, and noted that homology with normal cellular DNA could be used as a tool for determining the species of origin of a newly isolated RNA tumor virus. It is the purpose of the present paper to apply this methodology to RD-114, a candidate human oncornavirus, isolated from a brain tumor propagated in tissue culture (14). This brain tumor appeared subsequent to prenatal inoculation of cultured human rhabdosarcoma cells into kittens (15). The cells of the tumor were of human karyotype and produced a virus (RD-114) with all the characteristics of a C-type RNA tumor virus. Immunological studies failed to show any relation between RD-114 and the feline leukemia virus, FeLV (16–19).

We report here hybridizations with human and normal cat DNA. The data indicate that RD-114 nucleic acid is extensively related to cat DNA and possesses little or no homology with human DNA. On this basis we conclude that RD-114 is feline, and not human, in origin.

MATERIALS AND METHODS

Viruses. Concentrates of RD-114 virus were obtained from Pfizer, Inc. The virus was banded twice in sucrose gradients,

Abbreviation: gs, group-specific[antigen].

* These results were communicated at the annual meeting of the Special Virus Cancer Program of the National Cancer Institute, at Hershey, Pa., October 30, 1972.

TABLE 1. *Summary of hybridization reactions with viral probes and cellular DNA*

Viral probe	Hybridization with DNA from indigenous or related species	Reaction	Reference	Hybridization with DNA from nonindigenous species	Reaction	Reference
(SR)RSV	Quail embryo	+	9*, 10	HeLa	−	10
	Chicken embryo [COFAL(−) gs(−)]	+	10	Salmon sperm	−	10
				Calf thymus	−	9*
				BHK-21	−	9*
RAV-(0)	Quail embryo	+	10	HeLa	−	10
	Chicken embryo [COFAL(−) gs(−)]	+	10	Salmon sperm	−	10
RAV-1 and	Quail	+	9*	Calf thymus	−	9*
RAV-60	CEF K-813	+	9*	BHK-21	−	9*
AMV	Chicken	+	11*	Mouse embryo	−	11*
				Rat embryo fibroblast	−	11*
MMTV	C-57 black	+	12	Salmon sperm	−	12
				(RSV)-Transformed rat cells	−	12
FeLV	Cat liver	+	Ruprecht, R.M., unpublished results	Marmoset monkey	−	13

A literature review of hybridization reactions with viral DNA probes or radioactively labeled 70S viral RNAs and cellular DNA is given. With one exception (Rous sarcoma virus-transformed rat cells), all DNAs were prepared from cells not infected with RNA tumor viruses. COFAL(−), negative complement-fixing avian leukosis test; gs(−), not containing group-specific antigens; BHK, baby hamster kidney cells; CEF, chicken embryo fibroblast; *, study done with labeled 70S viral RNA; RSV, Rous sarcoma virus (SR = Schmidt–Ruppin strain); RAV, Rous-associated virus; AMV, avian myeloblastosis virus; MMTV, mouse mammary-tumor virus; FeLV, feline leukemia virus.

recovered by pelleting, resuspended in TNE buffer (0.01 M Tris·HCl–0.1 M NaCl–1 mM EDTA), and then used for RNA extraction.

Avian myeloblastosis virus was obtained from tissue culture supernatant fluids, kindly supplied by Dr. J. W. Beard (Duke University), and purified as described (20).

Extraction of 70S Viral RNA. The virus suspension in TNE buffer was incubated for 15 min at 37° with 1 mg/ml of nuclease-free Pronase and 1% sodium dodecyl sulfate. Extraction followed with a 1:1 mixture of phenol–cresol and chloroform containing 4% isoamylalcohol. After ethanol precipitation, the sample was layered onto a 10–30% glycerol gradient in 0.01 M Tris·HCl, (pH 7.5)–0.1 M NaCl–0.01 M EDTA and spun for 3.5 hr at 40,000 rpm at 4° in a SW-41 rotor (Spinco). The 70S region was pooled and precipitated with ethanol.

Tissues. Normal cat tissues were obtained from the animal care center of Columbia University. The spleen mastocytomas F 6020 and F 7070, as well as the bone-marrow mastocytoma F 8200, were obtained from Dr. J. E. Post through Flow Laboratories. All three tissues were negative for virus particles as judged by electron microscopy; however, F 7070 became positive after propagation in tissue culture.

Extraction of DNA from Tissues. The tissue was finely minced, suspended in 5% sucrose in 0.01 M Tris·HCl (pH 7.5)–0.15 M NaCl–0.01 M EDTA buffer, and disrupted with a Potter–Elvejhem homogenizer at 15,000 rpm at 4°. The suspension was centrifuged at 5000 × *g* for 10 min at 4°. DNA was extracted from the nuclear pellet, which was resuspended in 0.01 M Tris·HCl (pH 7.5)–0.15 M NaCl–0.01 M EDTA buffer. DNA was purified as described (13), except that sonication was performed under a nitrogen atmosphere.

Product Synthesis with Avian Myeloblastosis Virus Polymerase and RD-114 70S RNA. A standard incubation mixture contained the following concentration of reagents: 50 mM Tris·HCl (pH 8.3), 8 mM $MgCl_2$, 80 mM NaCl, 0.8 mM dithiothreitol, 200 μM unlabeled deoxyribonucleoside triphosphates, 20 μM [^{3}H]TTP (25,000 cpm/pmol), and 30 μg/ml of RD-114 70S RNA.

Avian myeloblastosis virus polymerase was a kind gift of Dr. D. L. Kacian.

Actinomycin D and distamycin A were used at concentrations of 100 μg/ml and 50 μg/ml, respectively (21). The reaction was terminated by adding Na dodecyl SO_4 to make a 0.1% solution.

Purification of Product [^{3}H]DNA. In addition to the purification steps outlined earlier (21), the [^{3}H]DNA products were incubated for 12 hr with 0.2 M NaOH at 37°, neutralized, and self-annealed in 0.4 M NaCl, 5 mM EDTA, and 0.1% Na dodecyl SO_4 at 65°. After a C_ot value of 0.5 mol × sec per liter (22) had been obtained, the single and double strands were separated by hydroxyapatite column chromatography (23). The single strands (98%) were pooled, passed through Sephadex G-50 to remove phosphate, precipitated with ethanol, and dissolved in a small volume of 5 mM EDTA.

DNA–DNA Hybridizations. Annealing was performed as follows and contained the components indicated: 50 mM

EDTA, 0.1% Na dodecyl SO_4, 7–10 mg/ml of sonicated cellular DNA, and about 100 cpm/μl of single-stranded [^{3}H]DNA product. After denaturation of the DNA at 100° for 3 min, NaCl was added to give a 0.4 M solution. The total volume was 1–2 ml. The reaction mixture was incubated at 65°, and the rate of annealing was monitored by withdrawal of aliquots that were then subjected to hydroxyapatite column chromatography.

Hydroxyapatite Column Chromatography. The sample was taken up in 0.01 M sodium phosphate buffer (pH 7.0) and applied to a column that had been equilibrated with the same buffer at 60° (1 mg of DNA per 1 cm^3 of packed hydroxyapatite).

Single strands were eluted from the column with 0.15 M phosphate buffer at 60°, weakly-bound duplexes at 80°, and stable, well-matched duplexes at 96° with the same buffer. 4-ml Fractions were collected directly in scintillation vials and counted after addition of 10 ml of Aquasol. Recycling of the RD-114 [^{3}H]DNA after exhaustive hybridization to excess normal cat DNA was done similarly. Eluates were collected in 1-ml fractions, of which a small aliquot was counted. The material eluting at 60° in 0.15 M phosphate buffer was pooled, passed over a 25-ml bed volume of Sephadex G-50, precipitated with ethanol, dissolved in a small volume of 5 mM EDTA, and used in subsequent hybridization experiments.

RESULTS

[^{3}H]DNA probes were synthesized with RD-114 70S RNA and purified avian myeloblastosis virus polymerase in the presence of actinomycin D and distamycin A. To insure the reliability of the [^{3}H]DNA, annealing was performed with RD-114 RNA and avian myeloblastosis virus RNA after hybridization with poly(U) to eliminate complexing with poly(A) sequences. The results described in Fig. 1 clearly demonstrate the specificity with which the RD-114 [^{3}H]DNA distinguishes between its own and an unrelated viral RNA. Virtually no complex formation is observed with avian myeloblastosis virus RNA (Fig. 1*B*), whereas 95% of the [^{3}H]DNA is shifted from the DNA region when the annealing is performed with RD-114 RNA (Fig. 1*B*). It should be noted that if the hybridization step with poly(U) is omitted, some 3% of hybrid formation occurs with avian myeloblastosis virus RNA.

The sequence homology of the RD-114 [^{3}H]DNA probe to normal cat and to human DNA was examined by hydroxy-

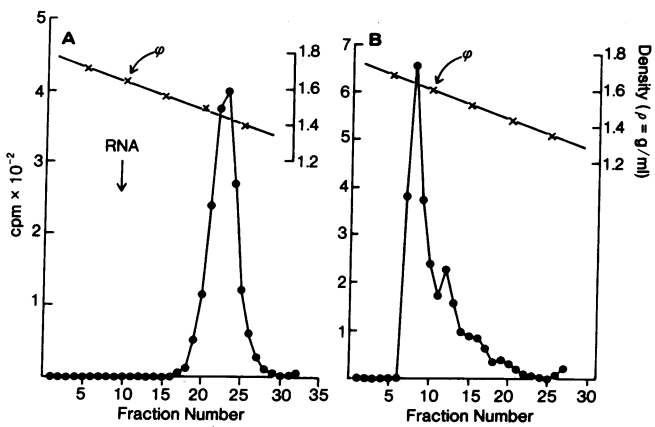

FIG. 1. Cs_2SO_4 equilibrium density gradient centrifugation of RD-114 [^{3}H]DNA after annealing to poly(U)-treated 70S avian myeloblastosis where RNA [*A*] and poly(U)-treated 70S RD-114 RNA [*B*]. 70S viral RNA was incubated at 37° at a concentration of 28 μg/ml in 5 mM EDTA–0.01 M Tris·HCl (pH 7.5) with a 12-fold excess of poly(U) for 10 min. Then, 2000–3000 cpm of single-stranded product was added, and the hybridization mixture was brought to 0.4 M NaCl, 50% formamide, 0.05 M EDTA (pH 7.5), 0.1% Na dodecyl SO_4, 0.12 mg/ml of poly(U), and 10 μg/ml of 70S RNA. The total volume was 100 μl, and the incubation temperature was 37°. The reactions were run to a C_0t value of 2 mol × sec per liter, added to 5.5 ml of 5 mM EDTA, mixed with an equal volume of saturated Cs_2SO_4, and centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 15°. Fractions were collected and assayed for Cl_3CCOOH-precipitable radioactivity.

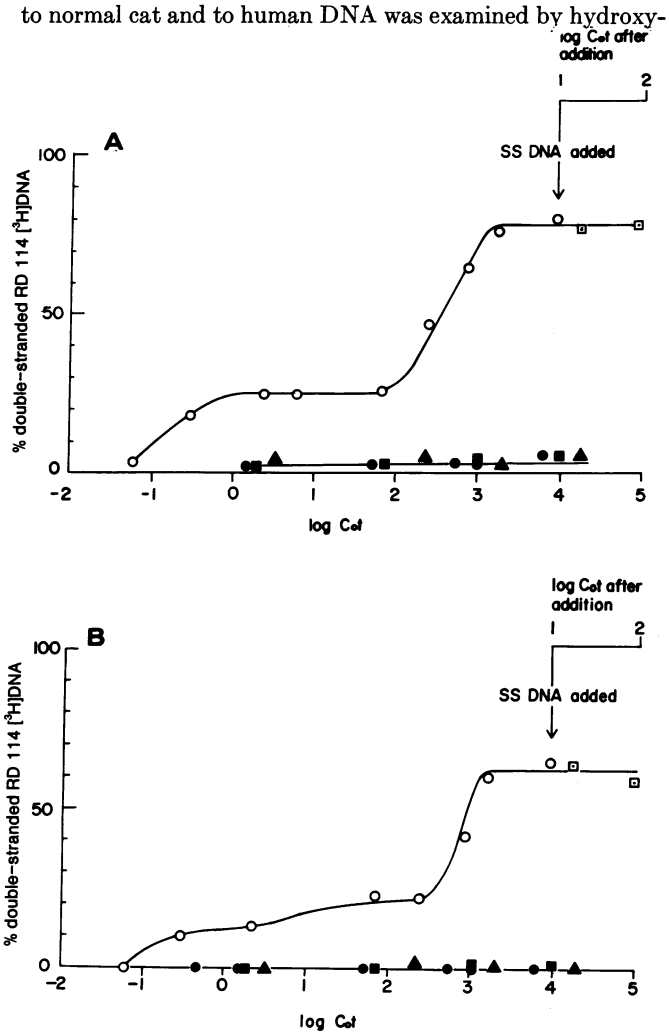

FIG. 2. C_0t curves of DNA–DNA hybridizations using RD-114 [^{3}H]DNA and various cellular DNAs, ○, ⊡, normal cat-liver DNA; ●, avian myeloblast DNA; ▲, normal human spleen DNA; ■, human rhabdomyosarcoma DNA. About 1 mg of cellular DNA was used per 10,000–15,000 cpm of RD-114 [^{3}H]DNA. At various times, aliquots were withdrawn. The amount and thermostability of double-stranded DNA were determined by hydroxyapatite column chromatography. The percent of duplexes is plotted against the logarithm of C_0t (mol × sec per liter). When the reaction leveled off, freshly denatured cat DNA was added (⊡) in 0.4 M NaCl–0.1% Na dodecyl SO_4–0.05 M EDTA. (*A*) C_0t curve of the total duplexes (eluting at 80° and 96°) formed between RD-114 [^{3}H]DNA and various cellular DNAs. (*B*) C_0t curve of the well-matched duplexes (melting above 80°) formed between RD-114 [^{3}H]DNA and various cellular DNAs.

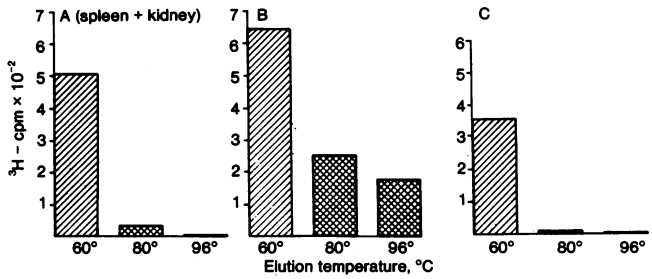

FIG. 3. Hydroxyapatite elution profiles of hybridization reactions between recycled RD-114 [^{3}H]DNA and cellular DNAs, normal cat (spleen and kidney) DNA [*A*], DNA extracted from three cat mastocytomas (*Methods*) [*B*], and normal human-liver DNA [*C*] are given. The DNA used for [*A*] was from a different animal than the DNA used for the previous hybridization (Fig. 2). All hybridizations were run to a C_0t value of 8000 mol × sec per liter.

apatite chromatography (23). This method identifies unpaired single strands that elute with 0.15 M phosphate buffer at 60° and imperfectly paired duplexes that dissociate at 80°. Those that elute between 80° and 96° are counted as properly paired duplexes. Fig. 2 shows typical C_0t curves (22), as determined by hydroxyapatite chromatography. Fig. 2*A* contains the elutions at both 80° and 96° and includes, therefore, the imperfect and perfect duplexes, whereas Fig. 2*B* depicts only the perfect duplexes that melt above 80°. The hybridizations were performed in all cases with the cellular DNA in vast excess (3×10^6 to 1). Under these conditions, annealing to the normal feline DNA occurs with biphasic kinetics, in which 25% of the [^{3}H]DNA anneals rapidly, with a $C_0t_{0.5}$ value of 0.12 mol × sec per liter, and corresponds therefore to highly repeated sequences. The remaining 75% of the [^{3}H]DNA anneals with a $C_0t_{0.5}$ value of 400–700 mol × sec per liter and represents, therefore, sequences of low redundancy. Note that a plateau is reached at a C_0t value of about 1000 mol × sec per liter, and that the further addition of normal feline DNA leads to no additional complex formation. The plateaus achieved indicate that 62% (Fig. 2*B*) of the RD-114 [^{3}H]DNA can form perfectly paired duplexes with normal cat DNA, and if the imperfect duplexes are included (Fig. 2*A*), 78% of the RD-114 DNA is involved. The extensive hybridization with the feline DNA is in sharp contrast with the lack of detectable response with the avian and human DNAs.

We have shown (24) that [^{3}H]DNA synthesized with particles prepared from human leukemic cells could be fractionated by prior exhaustive hybridization with normal human DNA to yield a [^{3}H]DNA that would specifically hybridize to the DNA of human leukemic cells (25). It was of interest to make a similar examination of the 20–30% of RD-114 [^{3}H]DNA that failed (Fig. 2) to hybridize with normal cat DNA. These single-stranded molecules were isolated by hydroxyapatite column chromatography and shown to be still highly specific for 70S RD-114 RNA. 95% Hybridization was obtained by Cs_2SO_4 gradient analysis, whereas no hybridization to 70S avian myeloblastosis virus RNA could be observed. Fig. 3 shows that the recycled DNA forms little, if any, stable duplexes when rechallenged with normal feline DNA (Fig. 3*A*), but does hybridize quite well (Fig. 3*B*) with the pooled DNA isolated from three feline mastocytomas, a result consistent with our findings with human leukemias (25). Note further that the recycled RD-114 [^{3}H]DNA does not hybridize to human DNA (Fig. 3*C*), thus making it unlikely that this fraction contains an enriched component homologous to normal human DNA that may have been missed in the original hybridizations with the unfractionated DNA.

DISCUSSION

The principal aim of the present investigation was to use molecular hybridization to provide information relevant to the probable origin of the RD-114 virus. The results described in Figs. 2 and 3 show clearly that this virus shares sequences with the DNA of normal cat tissues and has very little, if any, detectable homology with human DNA, including DNA from a human rhabdomyosarcoma. Human tumors (26–30) have been shown to contain RNA with some homology to the RNA of corresponding murine oncornaviruses. One might then expect that the DNA of human sarcomas could include some sequences homologous to portions of RD-114 RNA. However, their molar frequency in the DNA would be very low and their detection would require the use of recycled RD-114 DNA enriched for neoplasia-specific sequences.

In any event, it is clear that the hybridization results of Figs. 2 and 3 and the data summarized in Table 1 assign a feline origin to RD-114. At present, the only virus-like particles possessing a 70S RNA and an RNA-instructed DNA polymerase, and reported to contain sequences homologous to normal human DNA, are those that have been identified (24, 25) in human leukemic cells.

The use of molecular hybridization to relate an RNA tumor virus to its host has obvious advantages over serological or similar procedures. No prior knowledge of relatedness or homology to known RNA tumor viruses is required, and, indeed, as we have noted above, this information may be irrelevant to the question at issue. All one needs is to prepare a radioactively labeled nucleic acid homologous to that of the virus and challenge this probe with normal cellular DNAs from an appropriate collection of animals.

Note that in a rigorous sense molecular hybridization does not identify the natural host, but rather its taxonomic position. As may be seen from Table 1, there is no difficulty in assigning murine leukemia virus to mice rather than to rats, but without more quantitative information Rous sarcoma virus would be identified either with chickens or with quail. Such ambiguities are unlikely to complicate the search for an indigenous human virus since only the higher apes would presumably be close enough to share these sequences with man. It is of obvious interest to explore these evolutionary questions with molecular probes made from various oncornaviruses, as well as from the virus-like particles found in human neoplastic tissues (24, 25, 31, 32). The resulting information will not only be of practical value, but it should also illuminate the significance of the indigenous viral-related sequence shared between normal and cancer cells (25). The fact that these sequences are not necessary for transformation (13) suggests that they may tell us more about the evolutionary history of the oncornaviruses than about the mechanism of their pathogenesis.

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