Molecular Relatedness of Mammalian RNA Tumor Viruses as Determined by DNA · RNA Hybridization

Abstract. *Each of six mammalian C-type viruses—including two feline leukemia viruses, three murine leukemia viruses, and the human "candidate" virus RD-114—can be distinguished from each other by hybridizing DNA synthesized by viral reverse transcriptase with viral RNA. Characterization of the DNA · RNA hybrids by hydroxylapatite chromatography revealed nucleotide sequence diversity among the viruses, detectable both by the amount of cross-hybridization and by the decreased thermal stability of heterologous hybrids.*

The presence of RNA-dependent DNA polymerase in RNA tumor virus particles facilitates the preparation of labeled DNA complementary to 60 to 80 percent of the 65*S* to 70*S* viral RNA molecule (*1*) (illustrated in Table 2). We have employed labeled DNA prepared in vitro by this method in conjunction with hydroxylapatite (HAP) chromatography to do quantitative and qualitative studies of DNA·RNA hybrids formed between various RNA tumor viruses. We now report that six oncornaviruses, including the human "candidate" virus RD-114 (*2*), can be distinguished from each other. Even immunologically similar viruses from the same species can be identified as to type. This technique permits rapid identification of new virus isolates by comparison with known strains.

We first determined the capacity of HAP chromatography to discriminate between various nucleic acids. Isotopically labeled nucleic acids were adsorbed to a packed column of HAP (1 cm high by 1 cm diameter) at 50°C in 0.12*M* phosphate buffer (PB) and then eluted by increasing either the ionic strength of the buffer to give a salt elution profile or the column temperature to obtain a thermal elution profile. These studies (Table 1) showed that cellular RNA, double-stranded reovirus RNA, murine leukemia virus RNA, and poly(A)·poly(dT) (*3*) can all be distinguished from single-stranded and double-stranded DNA. When thermal elution profiles were compared with optical melting profiles, similar results were obtained, as noted previously by Britten and Kohne (*4*) and Brenner *et al.* (*5*). Although the same lot of HAP was used throughout these studies, equivalent results were also obtained with two different lots.

When purified viral DNA (DNA copied from viral RNA by the reverse transcriptase enzyme and labeled with [^{3}H]dTTP) was chromatographed on HAP, approximately 80 percent of the DNA eluted in the range of 0.18*M* to 0.22*M* PB; this indicated that most of the product was in the form of a DNA·RNA hybrid. The remaining acid-precipitable material either failed to bind to HAP in 0.12*M* PB or eluted in the region of double-stranded DNA. Thermal elution studies revealed that the native DNA·RNA hybrids had an elution midpoint of ~85°C. Each DNA preparation used in hybridization studies was first characterized by these two methods.

The preparations were then subjected to alkaline hydrolysis to remove the endogenous RNA, after which the DNA was recovered and concentrated as described by Kohne (*6*). The capacity of this single-stranded DNA to reassociate with purified virus RNA was tested (Table 2A). The viral DNA did bind to HAP after it was annealed with homologous viral RNA. The fact that the reassociation reaction is sensitive to ribonuclease treatment demonstrates that viral RNA is required and not DNA present in virion preparations (*7*).

Lai and Duesberg demonstrated the presence of poly(A) sequences in 65*S* viral RNA (*8*). We therefore determined whether poly(A) or other homopolymer sequences were of consequence in our hybridization studies. Although viral RNA was capable of binding poly(dT) synthesized by partially purified virus enzyme with poly(A)·oligo(dT) as template (Table 2B), there was no evidence for significant amounts of poly(dT), poly(dA), poly(dG), or poly(dC) sequences in the viral DNA (Table 2C).

The capacity of DNA from each virus to reassociate with RNA from heterologous viruses is presented in Table 3. These data show that whereas the two feline viruses have closely related nucleotide sequences, as do the murine viruses, little similarity exists between the murine and feline viruses. The conditions chosen are somewhat arbitrary since the values for cross-reactions depend on the conditions of chromatography (*9*). For example, with 0.14*M* PB eluent, a column temperature of 50°C gave cross-reactions roughly equivalent to those seen with 0.12*M* PB at 60°C. This indicates that some highly mismatched (partially helical) molecules fail to bind to HAP in the higher salt concentration and are eluted in a hydrogen-bonded state. When the column temperature was raised to 65°C and 0.12*M* PB was used, cross-reactions between the murine and feline viruses were reduced to background levels without appreciably reducing the extent of binding to homologous RNA (Fig. 1A). The RD-114 virus has little nucleotide sequence similarity to either the feline or murine leukemia viruses by the less stringent criteria (50°C, 0.14*M* PB) used for Table 3.

The thermal stability of interspecific DNA·DNA and DNA·RNA structures has been widely used in prokaryotic and eukaryotic systems as a measure of mismatching between partially complementary genomes (*5*, *10*). The rela-

Table 1. Chromatography of nucleic acids on hydroxylapatite (HAP). Purified ^{3}H-labeled nucleic acids were absorbed to HAP (Bio-Rad lot 6901) in 0.12*M* phosphate buffer (PB) at 50°C. Salt elution was performed in a stepwise manner in 0.02*M* increments from 0.12*M* to 0.3*M* PB. Three 2.0-ml washes were performed at each step. The ranges given are those in which ≧ 80 percent of the labeled nucleic acids eluted. Thermal elution profiles were obtained in 0.12*M* PB, with three 2.0-ml washes at each 5°C increment. By using ethylene glycol in the bath along with a pressurized column, a temperature of 105°C was attained. The radioactivity in each fraction was determined by coprecipitation with 10 μg of calf thymus DNA per milliliter in 10 percent trichloroacetic acid. Precipitates were collected on membrane filters, washed with 10 percent trichloroacetic acid and 70 percent ethanol, dried for 10 minutes at 100°C, and counted in a solution of butyl-1,3,4-phenylbiphenylyloxadiazole in toluene. All samples were corrected for background (~ 30 count/min) determined with blank filters. Reovirus type 3 RNA was a gift from A. Shatkin; MuLV, murine leukemia virus; EM, elution midpoint.

Nucleic acid	Salt elution (*M*)	EM (°C)
18*S* feline embryo fibroblast RNA	0.16–0.18	87
28*S* feline embryo fibroblast RNA	.18– .20	91
65*S* MuLV RNA	.18– .22	92
Reovirus 3 RNA	.18– .20	99
Poly(A)·poly(dT)	.18– .22	69
Cat liver DNA, native	.24– .28	89
Cat liver DNA, denatured	< .12	
Lambda phage DNA, native	.26– .28	
Lambda phage DNA, denatured	< .12	

Table 2. Specificity of viral DNA · RNA hybridization measured by hydroxylapatite chromatography. Murine and feline virus preparations were obtained from Electro-Nucleonics Laboratories, Inc., Bethesda, Maryland. Viral RNA was prepared from particles disrupted with 1 percent sodium lauryl sulfate at 37°C for 1 minute in the presence of diethyl pyrocarbonate (6 percent, by volume) (*17*). An equal volume of chloroform : isoamyl alcohol (24 : 1) was then added, and the preparation was shaken gently. After the preparation was centrifuged for 10 minutes at 10,000*g*, the aqueous phase was again extracted with chloroform and dialyzed against 0.2*M* PB, *p*H 6.8. The RNA prepared in this manner gave absorbance ratios (absorbance at 230 and 280 nm divided by that at 260 nm) of 0.45 to 0.50. Yields ranged from 5 to 10 μg of RNA per milliliter of Electro-Nucleonics purified virus ($\sim 10^{11}$ virus particles per milliliter). Purified virus RNA was used in subsequent hybridization without fractionation with respect to molecular weight. Viral DNA was prepared in the polymerase system essentially as described by Baltimore (*18*). The system contained 0.05*M* tris(hydroxymethyl)aminomethane hydrochloride, *p*H 8.3; 6 m*M* magnesium acetate; 60 m*M* sodium chloride; 20 m*M* dithiotheitol; 2.5×10^{-4}*M* dATP, dGTP, and dCTP; and 0.005 to 0.02 percent Nonidet P-40 detergent, adjusted for each virus preparation to give optimal DNA synthesis in 60 minutes at 37°C. Nascent DNA was labeled with $\sim 10^{-5}$*M* [methyl-^{3}H]dTTP, 7800 count/min per picomole. After 60 minutes the preparation was made 0.01*M* with respect to ethylenediaminetetraacetic acid and 0.2*M* with respect to PB and extracted as described for viral RNA. Endogenous RNA was digested in 0.5*N* sodium hydroxide by placing the solution in a boiling water bath for 10 minutes, after which the single-stranded DNA was absorbed to HAP, freed of ribonucleotides, and concentrated as described by Kohne (*6*). This single-stranded DNA was found to represent a minimum of 50 percent of the murine virus genome as determined by the capacity of the DNA to protect 65*S* viral RNA from ribonuclease digestion (*19*). Reassociation reactions were performed by incubating DNA ($\sim$ 2000 count/min) with 0.6 μg of unfractionated viral RNA in 0.2 ml of 0.2*M* PB at 60°C. Although reassociation was essentially complete after 3 hours under these conditions, the reactions were carried out overnight (12 to 18 hours) for convenience. In separate experiments, hybrids were found to be stable for up to 100 hours at 60°C. Reassociated DNA was detected by passing the solution through a 1-ml (packed volume) HAP column after diluting the sample to 0.14*M* PB. The column was washed with five 2-ml portions of 0.14*M* PB at 60°C to remove the single-stranded DNA, and the reassociated DNA was then eluted with four 2-ml portions of 0.3*M* PB. Radioactivity in each of the fractions was determined by coprecipitation with carrier DNA in 10 percent trichloroacetic acid and counted as in Table 1. Each column was checked for residual radioactivity by dissolving the HAP in 0.3*N* hydrochloric acid, and each experiment was done in duplicate. Where indicated, RNA was first incubated for 30 minutes at 37°C with bovine pancreatic ribonuclease (10 μg/ml) (Worthington Biochemicals, Freehold, New Jersey), determined to be free of deoxyribonuclease activity; FeLV, Rickard feline leukemia virus; RNAse, ribonuclease.

Labeled DNA	RNA source	DNA hybridized (%)
A. FeLV	FeLV	84.6 ± 2
	FeLV + RNAse	4.4 ± 1
	Yeast	1.25 ± 0.5
B. Poly(dT)	FeLV	84.1 ± 2
	FeLV + RNAse	24.1 ± 1
	Poly(A)	94.0 ± 2
	Poly(U)	1.25 ± 0.5
	Poly(G)	16.0 ± 3
	Poly(C)	10.2 ± 2
	None	9.2 ± 1
C. FeLV	FeLV	100*
	Poly(A)	2.2 ± 1.5*
	Poly(U)	0.6 ± 6*
	Poly(G)	0*
	Poly(C)	0*
	None	0*

* Percentage of homology relative to Rickard FeLV RNA. The extent of homologous binding was 68.6 percent, and the background absorption to HAP in the absence of RNA (9.8 percent) has been subtracted from all values.

tionship between temperature and the extent of interviral hybridization led us to examine the thermal stability of viral DNA·RNA hybrids in more detail. In reciprocal studies with Rauscher virus DNA hybridized with Rickard virus RNA (Fig. 1A) or feline virus DNA hybridized with murine virus RNA (not shown), a significant cross-reaction occurred at 60°C in 0.12*M* PB, but this cross-reaction was reduced to background levels ($\sim$ 10 percent) at 65°C. The difference in the elution midpoint was approximately −20°C for cross-species hybrids as compared to the homologous hybrids. A calibration study by Britten and Waring (*11*) indicated that 1 percent mismatching (due to uracil dimers) results in a 1°C decrease in thermal stability in poly(A) · poly(U) helices (200-nucleotide chains). Although we are not certain about the applicability of this relationship to the DNA · RNA hybrids studied here, it is clear that mismatching also occurs in the cross-species hybrids, since essentially none of them have thermal stability approaching that of homologous hybrids. The majority of nucleotide sequences of RNA tumor virus genomes are therefore not conserved from one species to another, and the cross-reaction seen apparently represents less highly mismatched fragments of "randomly evolving" genomes (*12*).

Significant mismatching also occurs between the murine leukemia viruses, as evidenced by a 4° to 5°C decrease in thermal stability of heterologous hybrids (Fig. 1, B to D). That these results are reciprocal again rules out artifacts resulting from RNA or DNA degradation. This technique is thus a sensitive indicator of base sequence differences and permits the differentiation of viruses closely related by the criterion of quantitative hybridization and by immunological methods.

The somewhat surprising molecular divergence among mammalian leukemia virus genomes has a direct bearing on certain biological observations. For example, feline as well as murine leukemia viruses can serve as helper viruses for defective murine sarcoma virus (*13*). The apparent lack of close genetic relatedness between these leukemia viruses suggests that the helper effect is nonspecific and perhaps analogous to nonspecific transduction observed with bacteriophage.

The thermal elution technique described above enables one to positively

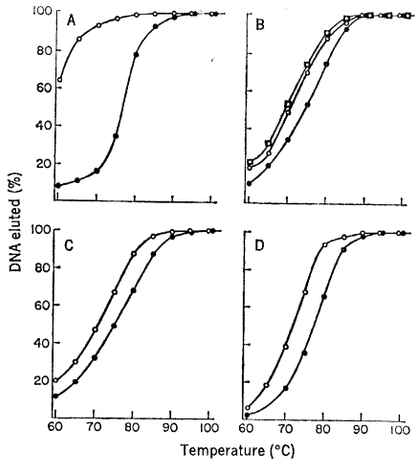
Table 3. Cross-hybridization of various RNA tumor viruses. Hybridizations were carried out as described in legend to Table 2 except that HAP chromatography was performed at 50°C in 0.14*M* PB. All reciprocal hybridizations were done, so that each viral RNA and DNA preparation was shown to hybridize efficiently. Data are results of a single experiment and have been converted to percentage of homology relative to hybridization with homologous RNA (shown in parentheses). Background (binding of viral DNA to HAP in the presence of yeast RNA) ranged from 5 to 10 percent and was subtracted. These experiments were repeated several times, and the cross-reactions were reproducible to within 5 percent with the same viral preparation as well as with different lots of the same virus type obtained from Electro-Nucleonics Laboratories. Two other lots of RD-114 virus (lots 3296-40 and 3296-43, Pfizer, Inc., Maywood, New Jersy) also gave similar results; MuLV, murine leukemia virus; FeLV, feline leukemia virus.

DNA	Viral RNA				
	MuLV Moloney	MuLV Rauscher	FeLV Rickard	FeLV Gardner	RD-114
Moloney	100 (91.2)	91.3	36.0	30.5	
Rauscher	93.1	100 (88.8)	14.9	22.6	
Rickard	36.6	16.8	100 (85.2)	95.1	
Gardner	35.6	15.5	95.2	100 (79.4)	
RD-114	0			16.7	100 (51.4)
Moloney	100 (81.2)				3
Gardner				100 (88.5)	19.1

Fig. 1. Thermal stability of homologous and heterologous viral RNA · DNA hybrids. Reassociation reactions were performed as described in legend to Table 2 with DNA (3000 to 5000 count/min) and 0.6 μg of viral RNA. The mixtures were then diluted to a PB concentration of 0.12*M* and passed through a HAP column at 60°C. Thermal elution profiles were obtained by raising the column temperature in 5°C increments and washing with three 2-ml portions of 0.12*M* PB at each temperature. Radioactivity in each fraction was determined as before. (A) DNA prepared from Rauscher murine leukemia virus RNA was hybridized with Rauscher virus RNA (closed circles) and Rickard feline virus RNA (open circles). (B) DNA prepared from Moloney murine leukemia virus RNA was hybridized with Moloney virus RNA (closed circles), Rauscher virus RNA (open circles), and AKR murine leukemia virus RNA (squares). (C) DNA prepared from Rauscher virus RNA was hybridized with Rauscher virus RNA (closed circles) and Moloney virus RNA (open circles). (D) DNA prepared from AKR murine virus RNA was hybridized with AKR virus RNA (closed circles) and Moloney virus RNA (open circles).

identify each virus type and distinguish it from other closely related viruses. This relatively simple and rapid procedure should enable more sophisticated molecular experimentation on the nature of "endogenous" mammalian viruses (*14*) and putative molecular recombination between virus strains as an adjunct to genetic experiments. Other hybridization experiments in our laboratory have shown that murine virus DNA prepared from viruses grown in cat cells (*13*) hybridizes efficiently with RNA from murine viruses grown in mouse cells and has an equal thermal stability to that of the homologous hybrid (*15*). These experiments show that the nucleic acids characterized in these hybridization experiments are virus-specific, since the same RNA is incorporated into virus particles (and copied by the reverse transcriptase enzyme) regardless of the cell species in which the virus is grown.

We report here that the human "candidate" virus RD-114 is genetically distinct from the strains of feline and murine viruses studied. The RD-114 virus was obtained after the inoculation of human rhabdomyosarcoma cells into the brain of a kitten in utero (*2*). After these human cells were recovered from the resulting tumors, they were producing high titers of nontransforming C-type virus particles. The fact that these particles are not closely related to the two feline viruses examined in-



dicates that they are either (i) a new feline virus unrelated to the Rickard and Gardner strains or (ii) a virus of human origin. We feel the first possibility is correct because we have been able to chemically induce a C-type virus from a cat cell line which is indistinguishable from RD-114 virus (*16*).

The studies reported here form the basis for a taxonomic scheme based on the molecular relatedness between various mammalian RNA tumor viruses. These studies can be expanded by including other laboratory strains of viruses whereupon any new isolate, regardless of the species of cell in which it is grown, can be identified by comparing its genome to those of other viruses. This technique should aid the identification of viruses from human neoplasias.

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References and Notes

1. P. Duesberg and E. Cananni, *Virology* **42**, 783 (1970); J. Stephenson and S. Aaronson, *ibid.* **46**, 480 (1971).
2. R. McAllister, W. Nelson-Rees, E. Johnson, R. Rongey, M. Gardner, *J. Nat. Cancer Inst.* **47**, 603 (1971); R. McAllister *et al.*, *Nature New Biol.* **235**, 3 (1972).
3. Abbreviations: The center dot indicates hybridization between chains; poly(A), polyadenylate; poly(dT), polydeoxythymidylate; oligo(dT), oligodeoxythymidylate; poly(dA), polydeoxyadenylate; poly(dG), polydeoxyguanylate; poly(dC), polydeoxycytidylate; poly(U), polyuridylate; poly(G), polyguanylate; poly(C), polycytidylate; dTTP, deoxythymidine triphosphate; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dCTP, deoxycytidine triphosphate.
4. R. Britten and D. Kohne, *Carnegie Inst. Washington Yearb.* **65**, 92 (1967).
5. D. Brenner, G. Fanning, K. Johnson, R. Citarella, S. Falkow, *J. Bacteriol.* **98**, 637 (1969).
6. D. Kohne, *Biophys. J.* **8**, 1104 (1968).
7. W. Levinson, J. Bishop, N. Quintrell, J. Jackson, *Nature* **227**, 1023 (1970); J. Riman and G. Beaudreau, *ibid.* **228**, 427 (1970); N. Biswal, B. McCain, M. Benyish-Melnick, *Virology* **45**, 697 (1971).
8. M. Lai and P. Duesberg, *Nature* **235**, 383 (1972).
9. R. Britten, *Carnegie Inst. Washington Yearb.* **66**, 69 (1968).
10. M. Martin and B. Hoyer, *Biochemistry* **5**, 2706 (1966); J. Johnson and E. Ordal, *J. Bacteriol.* **95**, 893 (1968); D. Haapala, M. Rogul, L. Evans, A. Alexander, *ibid.* **98**, 421 (1969); A. Bendich and B. McCarthy, *Proc. Nat. Acad. Sci. U.S.A.* **65**, 349 (1970).
11. R. Britten and M. Waring, *Carnegie Inst. Washington Yearb.* **69**, 322 (1965).
12. In the strictest sense, a genome cannot evolve in a completely random fashion because point mutations in certain loci will be lethal. However, these "conserved" regions apparently represent only a small fraction of the RNA tumor virus genome and were not detected at the level of sensitivity achieved in these studies.
13. P. Fischinger and D. Haapala, *J. Gen. Virol.* **13**, 203 (1971).
14. D. Lowy, W. Rowe, N. Teich, J. Hartley, *Science* **174**, 155 (1971).
15. D. Haapala, R. Bassin, P. Fischinger, in preparation.
16. P. Fischinger, P. Peebles, S. Nomura, D. Haapala, *J. Virol.*, in press.
17. F. Solymosy, I. Fedorcsak, A. Gulyas, G. Farkas, L. Ehrenberg, *Eur. J. Biochem.* **5**, 520 (1968).
18. D. Baltimore, *Nature* **226**, 1209 (1970).
19. L. Phillips, D. Haapala, P. Fischinger, in preparation.
20. We thank Arthur O. Thornton for technical assistance.

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Drug-Induced Change in the Distribution of Sulfonamides in the Mother Rat and Its Fetus

Abstract. *The distribution of a highly bound antibacterial sulfonamide was markedly altered in both the mother rat and its fetus by interfering with the binding of this drug to plasma protein in the mother. This effect was due to binding displacement, since the displacing agent had little or no effect on the distribution of another sulfonamide with very low binding to plasma protein.*

Binding to plasma protein not only can interfere with the biological activity of drugs (and endogenous substances), but also it is one of the major factors that influences the distribution of a drug to other body compartments (*1*). It has been shown that the antibacterial activity in vitro and the tissue levels of highly bound sulfonamides in the rat could be significantly increased by intentionally displacing them from binding to protein with another highly bound drug (*2*). Since then, several case reports have