Specificity of the RNA Product of RNA-Dependent DNA Polymerase in
Type C Viruses: II. Quantitative Analysis*

(DNA-RNA hybridization/murine leukemia viruses/feline leukemia virus/primate viruses/nucleic acid homology)

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ABSTRACT A number of mammalian Type C viruses were analyzed for relatedness by the technique of DNA-RNA hybridization. Viral DNAs were prepared in single-stranded form from complexes with 70S viral RNA formed during endogenous polymerase reactions. Extent of hybridization was assayed with the single-strand nuclease (S-1) from Aspergillus oryzae. Results obtained indicated a high degree of viral specificity, with significant cross-reactions being observed only with viruses obtained from within a species, as in the case of mouse and cat viruses, or in the special case of woolly monkey-gibbon comparisons. Comparisons of RD-114 virus, recently determined to be of feline origin, and conventional feline Type C viruses (FeLV), revealed minimal relatedness, especially when feline virus was grown on human cells, thus indicating the possibility of coexistence of greatly disparate Type C viruses within one species. A rat-specific virus, recovered from tumors induced by murine sarcoma virus, was found to contain genetic material common to both the original mouse virus and viruses indigenous to the rat, even though only rat-specific proteins have been detected during infection by this virus.

The discovery of the RNA-dependent DNA polymerase (reverse transcriptase) in RNA tumor viruses (1, 2), and subsequent findings of base sequence complementarity between template viral RNA and product DNA (3–5), have made possible studies of viral interrelationships (6) and have shown presence of viral-specific RNA in infected (7, 8) and apparently normal cells of several species (9–11). In previous studies utilizing cesium sulfate gradient centrifugation to detect RNA-DNA hybrids, mouse, hamster, cat, and viper Type C viruses were found to be completely distinguishable, with no evidence of interviral hybridization (6). Several mouse viruses did, however, show clear evidence of cross-hybridization (6), as did chicken viruses (12). Because of recent findings of crosshybridization between mouse Type C virus DNA products and RNA from human malignant cells (13, 14) and availability of more viruses for comparison, the question of viral interrelationship was re-examined by the use of sensitive and quantitative techniques employing the single-strand specific nuclease (S-1) from Aspergillus oryzae.

MATERIALS AND METHODS

The following viruses, all obtained from supernatant fluid of chronically infected cultures, were used: four strains of virus from mice, Rauscher leukemia virus (RLV), AKR (isolated in NIH Swiss embryo cells, obtained from J. Hartley, NIH), New Zealand black (NZB—strain SCRF 60a) (15), and wild mouse—strain 292 (16); three rat virus strains, the virus originally termed MSV(O) (17), now MSV(RaLV) (18), and two endogenous rat viruses generously provided by Dr. V. Klement (Children’s Hospital, Los Angeles, ref. 19); three cat strains, the Thielien feline lymphoma virus (20), a helper virus derived from the Gardner strain of feline sarcoma virus (21, 22), and the RD-114 virus (23), now known to be of cat origin; two primate viruses obtained from a woolly monkey (24) and a gibbon (25). The mouse viruses were all grown in mouse cells, the rat viruses in rat cells, the cat viruses in cat and human cells, and the primate viruses in human cells; the latter were generously provided by Drs. W. P. Parks and E. M. Scolnick of the National Cancer Institute (26). The above mouse and cat viruses were used interchangeably with Rauscher and feline virus obtained from Electronucleonics Laboratories, Inc. (Bethesda, Md.), and RD-114 and gibbon virus obtained through the courtesy of Dr. J. Gruber (National Cancer Institute) from the John L. Smith Memorial Institute, Pfizer, Inc. (Maywood, N. J.). The gibbon virus was prepared under Contract NOI-CP-3-3247 from the Virus Cancer Program.

The viral 70S RNA and the [3H]DNA products of the RNA-dependent DNA polymerase reaction used for hybridization experiments were purified by sucrose gradient centrifugation (10). To insure specificity, the DNA used was obtained from hybrid complexes with 70S RNA. In kinetic analyses such products gave results similar to those obtained using single-stranded probes prepared in the presence of actinomycin D (H. Okabe, submitted for publication). The latter preparations represented (in our experiments) at least 60% of the viral genome, based on protection of homologous 70S RNA from RNase digestion after annealing. Specific activity of the DNA products averaged 85 × 10⁴ dpm/ng. RNA and DNA (after digestion of RNA) were dissolved in one tenth standard saline-citrate (SSC) and stored frozen at −80°. Hybridization was done in 0.1 ml of 0.02 M Tris-HCl buffer, pH 7.2, containing 0.3 M NaCl, 0.001 M EDTA, and 0.1% sodium dodecyl sulfate at 67°. Hybrid formation was assayed using S-1 nuclease from Aspergillus oryzae (27), and in some comparative experiments the Neurospora crassa nuclease (28), hydroxyapatite fractionation, and Cs₂SO₄ gradient centrifugation.

Abbreviations: RLV, Rauscher leukemia virus; MuLV, murine leukemia virus; SSC, standard saline-citrate, 0.15 M NaCl–0.0015 M sodium citrate buffer, pH 7.2; FeLV, feline leukemia virus; FeSV, feline sarcoma virus; MSV, murine sarcoma virus; WML, wild mouse leukemia virus; RaLV, rat leukemia virus; Cr-β, the product of concentration of RNA hybridized and time of incubation; Tₚ, the temperature at which half of a double-stranded nucleic acid is denatured.

* Paper I in this series is ref. 6.
FeLV (B), gibbon virus (C), were hybridized with increasing amounts of various 70S RNA preparations in 0.3 M NaCl, 0.02 M Tris-HCl, pH 7.2, 0.001 M EDTA, 0.1% sodium dodecyl sulfate (total 100 µl) at 67° for 16-20 hr. The radioactivity in the hybrids formed was determined by precipitation with 10% trichloroacetic acid after S-nuclease digestion, and plotted relative to the hybridization attained with each homologous DNA probe at saturation. The homologous ratios of hybridized to input counts were: RLV 713/880 (0.81); FeLV 977/1349 (0.72); gibbon 1390/1638 (0.84); RD-114 1077/1256 (0.86). The background obtained without added RNA was subtracted before each calculation (54, 54, 60, and 94 cpm respectively). The 70S RNAs used were: RLV, O—O; FeLV, Δ—Δ; gibbon, X—X; RD-114, ■—■; NZB, ■—■; WML, Δ—Δ; AKR, ■—■; FeSV, Δ—Δ; and woolly monkey, O—O.

RESULTS

As shown in Fig. 1, DNA probes prepared from RLV, feline leukemia virus (FeLV), gibbon virus, and RD-114 viruses appeared capable of preferentially reacting with their homologous viral RNAs. The extent of reaction ranged from 72 to 85% of the initial input activity and the figures are adjusted to give 100% values for the maximal hybridization obtained with the homologous RNA. The one-half saturation RNA concentrations averaged approximately 50 ng/ml, corresponding to one-half Cr·t values of 10^−2 in accord with previously reported values (7). The Tm's of the homologous reactions were similar, with an average value of 52° (Fig. 2).

The specificity shown by the S-1 nuclease method was maintained when other methods were employed, including N. crassa single-strand nuclease and hydroxyapatite chromatography. Representative data from several experiments are given in Table 1.

Heterologous Reactions. The availability of multiple murine leukemia virus (MuLV) strains permitted evaluation of interspecies variation. Fig. 1A shows that extensive crosshybridization was obtained when the Rauscher probe was annealed with viral RNA from the NZB virus and a virus isolated from a wild mouse. These viruses could be distinguished from Rauscher on the basis of initial rates of hybridization (approximately one-half that of the homologous reaction) and final extent of hybridization (80-90%) within the concentration range of RNA shown. The AKR virus also showed significant crosshybridization with Rauscher DNA probes, with a saturation value somewhat lower than the other two mouse viruses. When DNA probes were prepared from the other mouse viruses, essentially reciprocal results were obtained; i.e., in each case the homologous virus was differentiated from the other strains isolated from the same species. At the same time, minimal and variable reactions were obtained with non-mouse viruses, with one significant exception (described below). On the basis of the well-known proportionality between hybridization rate and concentration of homologous base sequences (determined on the linear portion of the saturation curves), we estimate that there is less than 1% relationship between either of the two feline virus families (RD-114 and FeLV), and the gibbon virus. One somewhat surprising but reproducible result was the relatively high degree of hybridization seen between mouse virus DNA probes and RNA from the rat-specific virus, MSV(RaLV) (Table 2). Examination of several mouse DNA probes showed that the Moloney strain of murine sarcoma virus (MSV) gave the greatest reaction with RNA from MSV(RaLV) (Okabe et al., in preparation). Since the tumor from which MSV(RaLV) was derived was induced by the Moloney strain of MSV (17), this result could be explained by retention of mouse virus-specific nucleic acid by this pseudotype virus. Support for this conclusion derives from experiments with two recent indigenous rat viruses isolated (19), which showed only minimal (<5%) at maximum RNA concentrations) crosshybridization with mouse viruses. DNA products from these viruses, designated NRK-9 and RPL (19), did, however, hybridize extensively with RNA from MSV(RaLV); thus, the latter virus population contains nucleic-acid sequences common to both mouse and rat viruses (Table 2). This provides the most definitive evidence yet available for the appropriateness of the suggested nomenclature of this virus.

A second possibility for intraspecies comparison was provided by two feline Type C strains, Gardner and Thielen. Using the Thielen DNA probe, comparable hybridization kinetics were obtained with both RNAs. Again, crossspecies reactions were minimal (mouse, gibbon). One comparison of critical importance was between FeLV and RD-114, both of which now appear to be of cat origin (10, 29-32). Previous studies (23, 33-35) have indicated that RD-114 was distinct from FeLV in multiple properties; Fig. 1B and the reciprocal experiment shown in Fig. 1D are consistent.
with the immunological results in showing a lack of crosshybridization at equivalent RNA concentrations. Further, no evident relationship between RD-114 and the primate viruses is seen. In certain instances, especially when the concentration of FeLV RNA was increased, the RD-114 DNA probe did show definite hybridization. Because all cat cells tested contain RD-114 RNA, the possibility of contamination was raised. To circumvent this problem, FeLV was also grown on the RD cells (36) and then used to obtain 70S RNA. This did not affect hybridization with FeLV DNA probes, but did result in a virtual elimination of reaction with RD-114 DNA probe (Table 3). These experiments thus continue to emphasize the discrete nature of "conventional" feline Type C viruses and RD-114.

Another striking result (Fig. 1C) was the high extent of crosshybridization between the two primate viruses, woolly monkey and gibbon. The level of crosshybridization attained at saturating RNA concentrations was approximately 75%, while initial rate estimation allows a calculation of 60% relatedness. This is a magnitude similar to that seen between strains derived from a single species (e.g., AKR vs. Rauscher). When other, non-primate, viruses were tested at equivalent RNA concentrations, no significant crosshybridization was seen.

While the results described above indicate clear levels of specificity, one requirement for interpretation is the availability of reciprocal reagents at equivalent concentrations. Thus, in certain instances with elevated concentrations of RNA, increasing levels of crosshybridization were seen (e.g., the reaction of RLV RNA with gibbon DNA, Fig. 1C). These hybrids are evidently "real," judged on the basis of thermal stability after hydroxyapatite purification, but still at most indicate a small fraction of sequence homology.

**DISCUSSION**

The results presented generally confirm, and also extend, our previous observations (6) based on a limited number of inter-viral comparisons and use of cesium sulfate gradients for analysis of hybrids. The present results indicate a high degree of viral specificity for the DNA probes; for example, even

<table>
<thead>
<tr>
<th>TABLE 1.</th>
<th><strong>Hybridization levels as assayed by several techniques</strong></th>
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<tbody>
<tr>
<td>[3H]DNA</td>
<td>RNA</td>
</tr>
<tr>
<td>-----------</td>
<td>-----</td>
</tr>
<tr>
<td>[3H]DNA</td>
<td>RNA</td>
</tr>
<tr>
<td>RD-114</td>
<td>RD-114</td>
</tr>
<tr>
<td>RD-114</td>
<td>MS-2</td>
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<tr>
<td>GA-FeLV</td>
<td>FeLV</td>
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<tr>
<td>GA-FeLV</td>
<td>MS-2</td>
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<tr>
<td>RLV</td>
<td>RLV</td>
</tr>
<tr>
<td>RLV</td>
<td>MS-2</td>
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</table>

* [3H]DNA probes with activities ranging from 1000 to 1200 cpm were annealed with saturating amounts of homologous RNA or MS-2 RNA as described in Methods. Results with the three viruses appear generally comparable, with the two enzyme assays showing lower backgrounds than the column procedure. The bacteriophage MS-2 RNA was used as a specificity control.

Saturating amounts of purified 70S RNA (about 0.2 μg) from viruses grown in human (RD) or cat (FL-74) cells were annealed with DNA probes and assayed as described in Methods. S-1 nuclease-resistant counts in the absence of RNA (range, 32-60) were subtracted before calculations were made.

**TABLE 2. Presence of mouse and rat type C virus nucleic acid sequences in M-MSV(RaLV)* preparations**

<table>
<thead>
<tr>
<th>Source of DNA transcript</th>
<th>Viral 70S RNA</th>
<th>NRK-9</th>
<th>M-MSV</th>
<th>M-MSV(RaLV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR</td>
<td>2.5†</td>
<td>45</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>NRK-9</td>
<td>100</td>
<td>1.2</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>M-MSV</td>
<td>2.5</td>
<td>100</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>M-MSV(RaLV)</td>
<td>80</td>
<td>84</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* M-MSV(RaLV) and M-MSV indicate that the viruses are from the Moloney strain (see text).

† Percent hybridization of [3H]DNA transcript at saturating concentrations of RNA (>0.5 μg/ml) normalized to 100% for homologous reactants. Input activity ranged from 1100 to 1300 cpm and actual % hybridization in homologous reactions was 82-90% of the input. DNA products of the endogenous rat virus, NRK-9, show minimal hybridization to mouse Type C virus RNAs and mouse virus DNA products likewise do not show significant hybridization with RNA from endogenous rat Type C viruses. M-MSV(RaLV) contains both rat and mouse viral RNA sequences and transcribes them in an approximate equal ratio (62% rat, 38% mouse). The M-MSV preparations were obtained from Electronucleonics Laboratories. DNA transcripts of these preparations readily distinguish M-MSV from AKR and Rauscher viruses within a species, individual strains may be distinguished. The present results obtained with mouse viruses are in general agreement with results from other laboratories (37, 38). This is not an absolute, since two FeLV preparations were not distinguishable. In this case, the feline strains are known not to be pure, but mixtures of several envelope types (39). One striking example of interspecies relatedness was seen, namely in woolly monkey–gibbon comparisons. This relationship was predictable from the base of the known immunologic cross-reactivity of polymerase (34) and gs protein (26, 40) antigenic determinants. In spite of the sharing of one antigenic determinant (gs-3), and of the similar NH2-terminal sequence of at least one of the virion structural proteins (41), indicating a large degree of structural homology, certain pairs, such as mouse–cat, show minimal crosshybridization. This is perhaps related to inherent technical limitations of the hybridization reaction or preferential representation of highly specific sequences in the DNA probes that do not correlate in a 1:1 fashion with structural genes. One striking departure from intraspecies crosshybridization was obtained

**TABLE 3. Influence of cell substrate on RD-114 to FeLV interviral hybridization**

<table>
<thead>
<tr>
<th>Viral 70S RNA</th>
<th>[3H]DNA product cpm hybridized (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD-114(RD)</td>
<td>1,177 (100)</td>
</tr>
<tr>
<td>FeLV(RD)</td>
<td>22 (1.9)</td>
</tr>
<tr>
<td>FeLV(FL-74)</td>
<td>349 (29.7)</td>
</tr>
</tbody>
</table>

Saturating amounts of purified 70S RNA (about 0.2 μg) from viruses grown in human (RD) or cat (FL-74) cells were annealed with DNA probes and assayed as described in Methods.
with RD-114, now known to be of feline origin (10, 29–32); however, this virus shows no evidence of relationship to "conventional" feline Type C viruses. RD-114 could represent a second locus of cat Type C information, which has evolved independently of conventional feline Type C viruses, thus indicating the long-term residency in vertebrates of such genes. A multiple locus argument is consistent with current data on inheritance of mouse viruses (42, 43); however, in the mouse the viruses are evidently quite similar.

The high degree of interspecies specificity described in these studies has made possible the demonstration of both mouse and rat Type C virus genetic information in the virus originally designated MSV(O) (17), now designated MSV(RaLV) (18), based on antigenic analysis. This finding may have implications for studies of the subunit structure of viral 70S RNA.

The question of viral specificity is obviously important for attempts to detect Type C information in human cells (13, 14). One would suspect a virus from a species close to man to provide the best tool in such experiments; however, in a limited number of trials gibbon DNA–human cell RNA hybridizations have been negative (Okabe and Hatanaka, unpublished results). As we observe here, there were certain comparisons in which increasing amounts of heterologous RNA gave some degree of interviral hybridization. Thus, certain DNA probes may contain shared sequence information which may be present in human cells (13, 14) in similar fashion to that RNA found in "normal" mouse (11), chicken (9), and cat (for RD-114) (10) cells, and which may be amplified in the case of malignancies. Such shared information should not be represented in 100% of a heterologous probe (based on interviral comparisons) and would only be considered significant in carefully controlled experiments.

NOTE ADDED IN PROOF

Scolnick et al. (1973) J. Virol. 12, 458, have recently shown that the Kirsten strain of murine sarcoma virus, which was isolated from a rat tumor induced by the Kirsten leukemia virus, contains both rat and mouse Type C virus nucleic acid sequences. The striking extensive hybridization between woolly monkey and gibbon viruses along with supportive immunological data could suggest that these viruses originated from a common source. Recent hybridization data failing to show any homology between these viruses and DNA from their supposed natural hosts supports this hypothesis (E. M. Scolnick, W. P. Parks, T. Kawakami, D. Kohne, H. Okabe, R. V. Gilden, and M. Hatanaka, J. Virol., in press).

We thank Mrs. E. Twiddy for preparation of the viral RNA and [3H]DNA probes, and Drs. S. Mayyas, K. Traul, and K. Larsen for generous cooperation in preparing virus. This work was supported by Contract NIH NO-CP-3-3247 of the Virus Cancer Program of the National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014.


32. Livingston, D. M. & Todaro, G. J. (1973) “Endogenous Type C virus from a cat cell clone with properties distinct from previously described feline Type C virus,” Virology 53, 142–151.


