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Specificity of the DNA Product of RNA-Dependent DNA Polymerase in Type C Viruses: III. Analysis of Viruses Derived from Syrian Hamsters*

(nucleic acid hybridization/hamster–mouse pseudotypes/viral sequences in cellular DNA)

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ABSTRACT **DNA transcripts were prepared from three related viruses of hamster and analyzed by interviral hybridization and by reaction with cellular DNA. A virus (G-HaLV), isolated from a dimethylbenzanthracene-induced tumor cell in Graffi hamsters, contained nucleic acid sequences highly specific for hamster cell DNA and did not react with mouse cell DNA nor did its transcript show homology (<5%) with mouse or rat viral RNAs. The hamster-specific sarcoma virus, B-34, isolated by Bassin and coworkers from tumors induced by the Harvey strain of murine sarcoma virus, contained mouse-, hamster-, and possibly rat-specific sequences. B-34 transcripts were predominantly mouse-specific. GLOH$^-$, a lymphomagenic virus derived by dilution beyond the transforming endpoint of a hamster-specific sarcoma virus obtained from tumors induced by the Gross pseudotype of murine sarcoma virus, also contained hamster- and mouse-specific sequences. Only a portion of its hamster sequence (about 50%) was shared with B-34 and G-HaLV viruses. As expected, transcripts of GLOH$^-$ virus were reactive with mouse and hamster cellular DNA.**

The number of species from which type-C viruses have been isolated has increased rapidly over the past few years. In most cases, these viruses can be distinguished by immunological techniques; however, the technique of nucleic acid hybridization has proven increasingly critical in establishing species of origin of new isolates. This has been possible because of the high degree of specificity observed in interviral hydridization experiments (1–3). Thus, because of the lack of crosshybridization (about 2–3%) between mouse and rat endogenous viruses, it was possible to show mouse and rat sequences in two distinct pseudotypes. In one case (1), in the virus designated originally MSV(O), now MSV(RaLV), this was a predictable result based on rat-specific structural proteins being found in a sarcoma virus isolated from a tumor induced by M-MSV. The second case, the Kirsten sarcoma virus (Ki-SV), resulted from rat passage of Ki-MuLV and, thus, since the recovered virus had mouse virus structural antigens, the presence of rat information was not suspected. This virus is now known to have derived its sarcomagenic information from the rat (4), in contrast to MSV(RaLV), whose sarcoma sequences were derived from the mouse. In both cases, the interviral reactions were duplicated with cellular nucleic acids, e.g., DNA transcripts of endogenous rat virus hybridize to rat and not mouse DNA, while MSV(RaLV) or Ki-SV transcripts show homology with DNA of both species. Recent data of Tsuchida *et al.* suggest that the pseudotype viruses are produced by reassortment of subunits, since in each case the RNA carrying the sarcomagenic information is a 30S subunit, while helper virus subunits are 35 S (5, 6).

This report deals with type-C viruses of the hamster, including a pseudotype produced by rescue *in vivo*, a lymphomagenic virus derived by endpoint dilution from a second distinct pseudotype, and a true endogenous virus of hamsters with no previous history of mouse contact.

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; WML, wild mouse leukemia virus; RLV, murine Rauscher leukemia virus; GLOH$^-$, a hamster virus; G-HaLV, Graffi strain of hamster leukemia virus; B-34, a hamster sarcoma virus; RD 114, an endogenous feline virus; RPL, an endogenous rat virus.

* Ref. 1 is the second paper in this series.

MATERIALS AND METHODS

Viruses. The Graffi strain of hamster leukemia virus, G-HaLV, was obtained from an explant of a tumor induced by dimethylbenzanthracene in a Graffi hamster (ref. 7; R, J. Huebner, unpublished data). The tumor was explanted at the third passage *in vivo* and maintained in tissue culture by Dr. Paul Price (Microbiological Associates) before receipt in this laboratory.

The B-34 virus (8) (courtesy of Dr. R. Bassin, NCI), also referred to as HaSV by some investigators, was derived from a Syrian hamster tumor cell line established from a tumor induced in a newborn hamster by the Harvey strain of murine sarcoma virus, H-MSV, but the virus recovered while sarcomagenic, contains structural proteins characteristic of hamster type-C viruses (9).

The GLOH$^-$ virus was derived by dilution beyond the transforming endpoint of a sarcomagenic virus obtained from hamster tumors induced by the Gross pseudotype of M-MSV (10). This virus is now known to induce lymphomas in suitable hamster strains after long latent periods (R. J. Huebner, unpublished data), and has been referred to by us as HaLV, since we thought it to represent the endogenous virus of hamsters based on antigenic analysis. We should emphasize that with regard to structural proteins, G-HaLV, B-34, and GLOH$^-$ are very similar and easily differentiated from murine viruses. This has been established in several laboratories and has been confirmed by exchange of reagents among those laboratories (9, 11, 12).

The mouse viruses used were the Rauscher strain (RLV) grown in BALB/c cells designated JLSV-9 (13), and the wild mouse 1504E (WML) strain grown in NIH Swiss embryo cells (14).

The RD 114 virus (endogenous feline virus) was grown in RD cells (15), and the RPL virus (endogenous rat virus) was grown in the Lewis rat cell line from which it originated (16).

Viral RNA. High molecular weight viral RNA (70S) from the various viruses was prepared from virus purified by double

gradient centrifugation by lysing with 1% sodium dodecyl sulfate ($NaDodSO_4$) and sedimenting through a 15–30% (w/v) linear sucrose gradient as described (17). The concentration of RNA was estimated from the absorbance at 260 nm.

Cellular DNA. Confluent cell monolayers were washed by decantation four times with Tris-saline, and lysed at room temperature by gently shaking with $NaDodSO_4$ mixture (0.5% $NaDodSO_4$, 0.15 M NaCl, 1.5 mM sodium citrate, 5 mM EDTA, 0.1 M Tris·HCl, pH 8.0). The lysate was collected in a centrifuge tube, 0.25 volume of 2.5 M sodium trichloroacetate (pH 7.6) was added, and the mixture was extracted with an equal volume of chloroform–isoamylalcohol (24:1 mixture) at room temperature for 5 min. The chloroform extraction was repeated three times. To the final aqueous phase, two volumes of chilled ethanol were added, and DNA was spooled out onto a glass rod. The precipitates on the glass rod were rinsed with 70% ethanol containing 0.1 M NaCl and suspended in 15 mM NaCl–1.5 mM sodium citrate (pH 7.2) at a concentration of less than 2 mg/ml. The suspension, maintained in an ice bath, was sheared by sonication five times for 30 sec each by a Branson sonifier (W140D) with a micro-tip at the highest setting, and filtered through a Gelman GA-6 membrane filter. The filtrates were treated with 10 μg/ml of boiled RNase A for 30 min at 37°, and then with 50 μg/ml of self-digested Pronase B for 60 min at 37° in the presence of 0.1 M NaCl and 0.5% $NaDodSO_4$. DNA was again extracted twice with phenol–chloroform, and precipitated with two volumes of ethanol at −20°. The DNA precipitates were dissolved in 15 mM NaCl–1.5 mM sodium citrate and denatured at 80° for 20 min in 0.2 N NaOH. After neutralization with HCl, the solution was extensively dialyzed against 15 mM NaCl–1.5 mM sodium citrate precipitated with ethanol, and dissolved in 15 mM NaCl–1.5 mM sodium citrate.

Freshly excised livers were washed with Tris-saline, minced with scissors, and homogenized in isotonic buffer [0.25 M sucrose–10 mM Tris·HCl (pH 7.4)–10 mM KCl–10 mM $MgCl_2$] with Polytron PT-10 (Brinkman Instruments), at setting 3 for 30 sec in ice. The homogenates were centrifuged at 3000 rpm for 10 min at 4°, and DNA was extracted from the pellets as described above.

The concentration of DNA was estimated by dividing the absorbance at 260 nm by 24. The DNA thus obtained has an average sedimentation coefficient of 5S in alkaline sucrose gradients.

Viral DNA Probe. Single-stranded [^{3}H]DNA transcripts of viral genomes were prepared in the endogenous reaction and purified from viral 70S RNA hybrids as described (17). The specific activity of the probes was about 10^5 dpm/ng of DNA, and they represent at least 60% of the 70S viral RNA genome (1).

Hybridization. [^{3}H]DNA was hybridized with viral 70S RNA with increasing amounts of RNA in 100 μl of 0.3 M NaCl, 20 mM Tris·HCl (pH 7.2), 1 mM EDTA, and 0.1% $NaDodSO_4$ at 67° for 18 hr, as described (17). For [^{3}H]DNA·DNA hybridization, the mixture of [^{3}H]DNA and sonicated denatured cellular DNA in 0.3 M NaCl was divided into 100-μl portions and incubated at 67° for various time intervals. The C_0t values (the product of the initial nucleotide concentration and the time of incubation, mole·sec·liter^{-1}) were calculated from the absorbance and the time of incubation as described (18). The $C_0t_{1/2}$ of the unique sequences in cellular DNA is about 1×10^3 under the hybridization conditions used; the figures give actual data without correction for salt effects. Hybrid formation was assayed by S-1 nuclease as described (17); S-1 nuclease was prepared from α-amylase (Sigma) by a modification (19) of the method of Sutton (20).

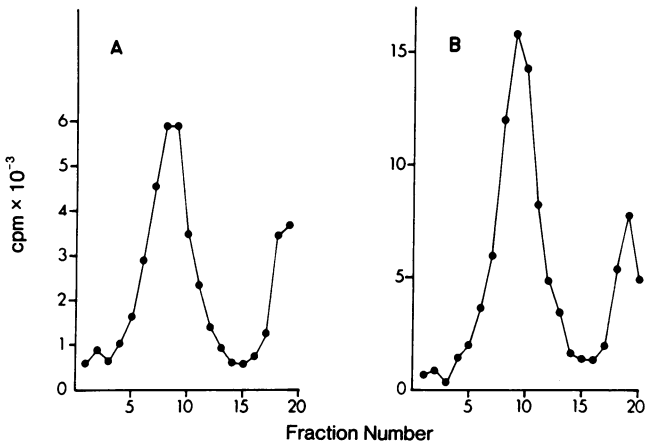

FIG. 1. Sedimentation profiles of [^{3}H]DNA products of the endogenous polymerase reaction of G-HaLV (*A*) and GLOH$^-$ (*B*) viruses. The reaction mixtures of 1 ml, incubated for 60 min at 37° with [^{3}H]dTTP (40 Ci/mmole), were treated with 1% $NaDodSO_4$, layered on 11 ml of 15–30% (w/v) sucrose gradients in 0.1 M NaCl–10 mM Tris·HCl (pH 7.4)–1 mM EDTA containing 0.01% $NaDodSO_4$, and centrifuged at 40,000 rpm for 2.5 hr at 10° in a SW-41 rotor. Sedimentation was from *right* to *left*. Portions of each fraction (25 μl) were precipitated with cold 6% trichloroacetic acid in the presence of 50 μg of calf-thymus DNA. The viral 70S RNA showed peak activity in fraction 9.

RESULTS

Viral [^{3}H]DNA Probe. Previous experience has indicated that DNA transcripts prepared from complexes with 70S RNA formed during the endogenous RNA-dependent DNA polymerase reaction possess the specificity and sensitivity required to detect viral nucleic acids in appropriate cells (1, 17). This method, generally referred to as the simultaneous detection procedure of RNA oncornaviruses (21), was used to prepare probes for the three related viruses of hamster. Characteristic DNA transcripts of GLOH$^-$ and G-HaLV can be detected following the procedures outlined in *Materials and Methods* (Fig. 1). The [^{3}H]DNA that sedimented with viral 70S RNA could be isolated from the complex after alkaline digestion of RNA, and rehybridized specifically to appropriate viral and cellular nucleic acids as described below.

Interviral Hybridization. The G-HaLV DNA transcript proved highly specific for the hamster viruses, forming hybrids with B-34 and GLOH$^-$ viral RNAs (Table 1) and reacting minimally with mouse, rat, or cat viruses. The concentration of RNA used in these experiments (≥2 μg/ml) was above the saturation value required for each DNA transcript regardless of whether homologous or heterologous RNAs were used. An example of this is given in Fig. 2 for the GLOH$^-$ transcript, and similar results were obtained for the other transcripts (not shown). In reciprocal assays with G-HaLV RNA and [^{3}H]DNA transcripts of mouse viruses, a similar minimal degree of hybridization was observed. This clear distinction between mouse and hamster viruses, as in the rat-mouse comparison, makes it possible to deduce the "hybrid" nature of B-34 and GLOH$^-$ viruses. B-34 forms hybrids with G-HaLV

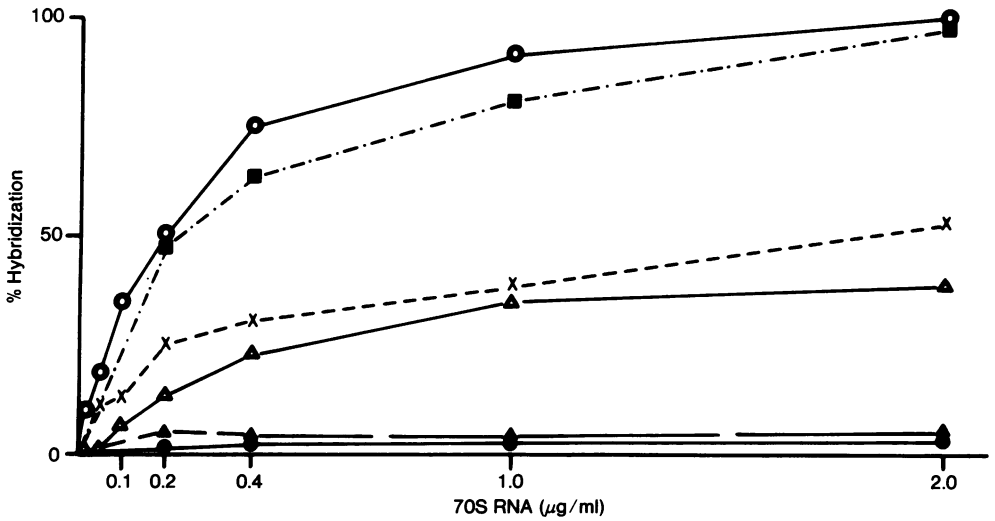

FIG. 2. Hybridization of [^{3}H]DNA probe of GLOH$^-$ virus with increasing amount of various viral 70S RNAs. Fixed amounts of [^{3}H]DNA (880 cpm) were incubated with varying concentrations of RNA in 100 μl of 0.3 M NaCl, 20 mM Tris·HCl (pH 7.2), 1 mM EDTA, 0.1% $NaDodSO_4$ at 67° for 18 hr. Hybridized [^{3}H]DNA was precipitated with 10% trichloroacetic acid after S-1 nuclease digestion and radioactivity was then determined. The values were normalized to that obtained with the homologous GLOH$^-$ 70S RNA (583 cpm) after subtraction of the background of 56 cpm obtained without added RNA. O, GLOH$^-$; ■, B-34; △, G-HaLV; ×, RLV; ▲, RPL; ●, RD-114.

DNA to 85% of the homologous reaction and is also 40–75% protective of two mouse DNA transcripts. Thus, this virus clearly contains both mouse and hamster sequences. The reciprocal experiment with B-34 transcript likewise shows similar evidence of both sequences; however, it has some interesting features. The majority of the transcript is homologous to mouse viral RNA, while only 12% was reactive with G-HaLV RNA. There is apparently preferential transcription of mouse sequences even though B-34 appears to contain the majority of sequences found in G-HaLV (86%). We do not known whether this preferential transcription of mouse sequence *in vitro* is an invariant characteristic of B-34 virus or is subject to variability. At present, the main conclusion is that B-34 virus contains extensive nucleic acid sequences of viruses derived from both the mouse and hamster. Another point of interest is the reaction of B-34 DNA with RPL RNA; B-34 transcript shows a greater reactivity with this RNA than does either of the other two hamster viruses. This point will be examined in the *Discussion*. Reciprocal assays with GLOH$^-$

TABLE 1. *Cross-hybridization between [^{3}H]DNA probes and 70S RNA of various type-C viruses of mouse and hamster origin*

Viral 70S RNA	Hamster [^{3}H]DNA probe			Mouse [^{3}H]DNA probe	
	G-HaLV	B-34 (H-MSV induced)	GLOH$^-$ (M-MSV induced)	WML	RLV
G-HaLV	100	13	40	3.3	
B-34	86	100	98	74	40
GLOH$^-$	40	61	100	67	
WML	1.5	76	51	100	83
RLV	1.1	62	53	84	100
RPL	1.9	8.5	5.5	2.9	
RD-114	5.4	0	3.1	1.9	4.4

Fixed amounts of each [^{3}H]DNA probe (700–1200 cpm) were hybridized for 18 hr at 67° with saturating amounts of 70S RNA (>2 μg/ml) and assayed with S-1 nuclease as described in *Materials and Methods*. The results are presented as the percent of the value obtained with the homologous viral RNA after subtraction of the background counts obtained without added RNA (ranging from 30 to 113 cpm).

TABLE 2. *Rehybridization of [^{3}H]DNA probes of hamster viruses purified by a first cycle hybridization with viral RNA*

A. [^{3}H]DNA transcripts of GLOH$^-$ virus

70S RNA	70S RNA used for first cycle hybridization			
	GLOH$^-$		WML	
	cpm	%	cpm	%
GLOH$^-$	1396	100	876	90.1
G-HaLV	989	46.2	238	12.3
WML	1086	51.7	957	100
RD-114	175	0	137	0

B. [^{3}H]DNA transcripts of G-HaLV

70S RNA	70S RNA used for first cycle hybridization			
	B-34		GLOH$^-$	
	cpm	%	cpm	%
G-HaLV	455	90.0	320	90.4
GLOH$^-$	307	55.5	347	100
B-34	498	100	356	103
WML	69	0	66	0

[^{3}H]DNA probes of GLOH$^-$ (*A*) and G-HaLV (*B*) were first hybridized to the respective viral 70S RNA shown in the column at saturating concentrations. The mixtures were digested with S-1 nuclease, and the nuclease-resistant [^{3}H]DNA in the hybrid form was purified by phenol–chloroform extraction, alkali–heat denaturation, dialysis against 15 mM NaCl–1.5 mN sodium citrate, and ethanol precipitation. The [^{3}H]DNA thus obtained was again hybridized with viral 70S RNAs as described in Table 1.

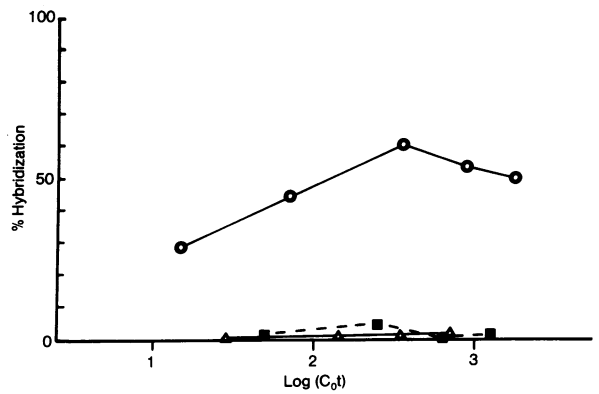

FIG. 3. Hybridization kinetics of G-HaLV [^{3}H]DNA probe with various cellular DNAs. The hybridization was done as described in the *legend of* Fig. 4. The hybridized radioactivity, after subtraction of the background (65 cpm), was plotted relative to the input (740 cpm) against C_0t. The concentrations of cellular DNA were as follows: O, hamster embryo fibroblasts, 2.96 mg/ml; △, C57 mouse liver, 1.17 mg/ml; ■, brown Norway rat liver, 2.08 mg/ml.

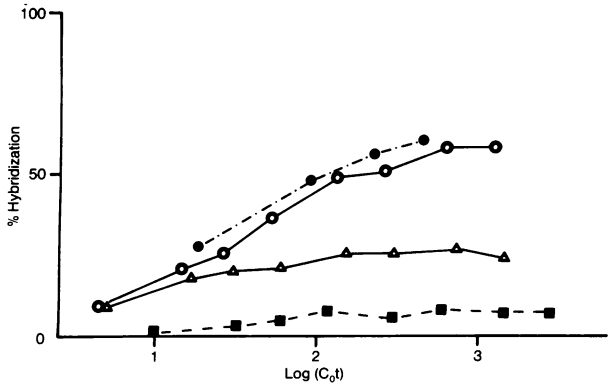

FIG. 4. Hybridization kinetics of GLOH$^-$ [^{3}H]DNA probe with various cellular DNAs. Fixed amounts of cellular DNA and GLOH$^-$ [^{3}H]DNA in 100 μl of 0.3 M NaCl, 20 mM Tris·HCl (pH 7.2), 1 mM EDTA, 0.1% $NaDodSO_4$ were incubated at 67° for various time intervals, and hybridized [^{3}H]DNA was assayed as described in the *legend* of Fig. 2. The values obtained after subtraction of the background without incubation (74 cpm) were plotted relative to the input activity without S-1 nuclease digestion (1500 cpm) against C_0t. No correction for the salt concentration is made in the figures. The concentrations of cellular DNA were as follows: O, hamster embryo fibroblasts, 2.19 mg/ml; ●, GLOH$^-$ cells, 0.75 mg/ml; △, C57 mouse liver, 2.50 mg/ml; ■, brown Norway rat liver, 5.47 mg/ml.

nucleic acids also indicate the presence of both mouse and hamster sequences, as is indicated in Table 1 and Fig. 2. Of interest is the partial protection of GLOH$^-$ DNA by G-HaLV RNA at saturating concentrations, while with this probe B-34 RNA is virtually indistinguishable from GLOH$^-$ RNA. This is to be expected based on the presence of mouse sequences in the GLOH$^-$ transcript and in B-34, but not in the G-HaLV RNA. Not expected was the relatively low extent of hybridization (40%) between G-HaLV DNA and GLOH$^-$ RNA at saturating RNA concentrations. This result indicates either a marked strain difference or lack of a substantial portion of the endogenous hamster genome from GLOH$^-$.

To extend the results of Table 1, a recycling experiment was preformed. GLOH$^-$ DNA was first hybridized to GLOH$^-$ or wild mouse viral RNAs. After S-1 nuclease digestion, the RNA was digested and the DNA was again extracted. Table 2*A* shows that a single cycle absorption on WML RNA reduces the hamster-specific portion of the probe from 46 to 12% of the maximal reaction, while hybridization to GLOH$^-$ RNA does not significantly change the mouse:hamster ratio. The ratio of mouse to hamster (G-HaLV) reactivity was changed from about 1:1 to 8:1 by absorption on mouse virus RNA.

When similar absorptions of G-HaLV were made with B-34 and GLOH$^-$ RNAs (Table 2*B*), confirmation of the results given in Table 1 was obtained. Thus, no reaction with mouse RNA was obtained, while GLOH$^-$ RNA appears to contain only about half of the sequences shared between G-HaLV and B-34.

Hybridization to Cellular DNA. Viral DNA transcripts have been used successfully to detect homologous viral sequences in cellular DNA by the DNA·DNA hybridization technique. With the notable exception of viruses from the woolly monkey and gibbon ape (22), type-C viruses are known to be represented in cellular DNA of their natural host species. This is true of mouse (22–24), cat (25), rat (N. Tsuchida, R. V. Gilden, M. Hatanaka, A. E. Freeman, and R. J. Huebner, *Int. J. Cancer*, submitted), avian (26), and, as we show here, hamster viruses as well. Our own observations indicate that despite long-term passage in cells of heterologous species, pickup of new genetic information is not a common event. For example, RD 114, gibbon ape, or mouse viruses grown in human cells have not acquired nucleic acid sequences homologous to human DNA (H. Okabe, unpublished data). In the present case the results obtained in interviral hybridizations are parallel to those obtained with cellular DNA. G-HaLV DNA shows hybridization only with hamster cell DNA and not with mouse or rat DNA (Fig. 3). In contrast, GLOH$^-$ DNA hybridizes to both hamster and mouse cell DNA and not to rat DNA (Fig. 4). The extent of hybridization to hamster DNA is similar to that observed with G-HaLV DNA. This result appears to resolve the question of whether GLOH$^-$ is missing hamster sequences or is showing a strain disparity from G-HaLV (Table 1). The results suggest that the portion of the sequences not shared with G-HaLV are nevertheless present in hamster cell DNA, thus suggesting strain differences as the most likely explanation.

DISCUSSION

The current data present several new points of information. First, hamster type-C viruses are represented in cellular DNA in a highly specific fashion. Secondly, like the mouse–rat pseudotypes (1, 4), hamster–mouse pseudotypes contain nucleic acid sequences for viruses of both species. The reaction of B-34 probe with RPL RNA suggests that this virus may contain mouse, hamster, and rat sequences. This would not be surprising since the Harvey strain of MSV was obtained by rat passage of Moloney-MuLV (27). Like Ki-SV (4), this virus is expected to contain rat sequences. There was considerable asymmetry in the results obtained in detection of RNA sequences with heterologous probes and the use of B-34 DNA probe in interviral crosses. While both mouse and hamster viral RNAs were easily detected in B-34 RNA, the DNA transcript of B-34 was mainly representative of mouse sequences. This appears to be evidence of differential transcription, but before this can be decided quantitative estimation of mouse

and hamster viral nucleic acid sequences must be done. We note that without G-HaLV reagents, hybridization would have suggested B-34 to be a mouse virus while immunological data indicate it to be of hamster origin. Finally, GLOH$^-$, which we previously thought to be an endogenous HaLV, is in fact a mouse–hamster hybrid. The origin of the oncogenic information in this virus is an interesting question. The low reactivity of GLOH$^-$ RNA with G-HaLV DNA is possibly suggestive of a recombinant virus in which significant amounts of HaLV RNA are missing.

The alternative of a strain difference seems more likely in view of the hybridization of GLOH$^-$ to hamster cell DNA, essentially to the same value as obtained with G-HaLV and hamster cell DNA. We note that some workers have found difficulty in demonstrating endogenous reactivity with GLOH$^-$ (28); whether this is based on technical considerations or a loss of activity in certain cultures is unclear. However, analysis of GLOH$^-$ RNA gave evidence of significant cross-reaction with mouse virus DNA (28). As we show here, this is not based on an intrinsic hamster–mouse relationship, but rather on the fact that GLOH$^-$ is a "hybrid" virus.

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