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Species and Interspecies Radioimmunoassays for Rat Type C Virus p30: Interviral Comparisons and Assay of Human Tumor Extracts

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The major internal protein, p30, of rat type C virus (RaLV) was purified and utilized to establish intra- and interspecies radioimmunoassays. Three rat viruses were compared in homologous and heterologous intraspecies assays with no evidence of type specificity. The only heterologous viruses to give inhibition in these species assays were the feline (FeLV) and hamster (HaLV) type C viruses; these reactions were incomplete and required high virus concentrations. An interspecies assay using a goat antiserum prepared after sequentially immunizing with FeLV, RD 114, and woolly monkey virus p30's and labeled RaLV p30 was inhibited by all mammalian type C viruses, although preferentially by RaLV, FeLV, and HaLV. Thus, as in a previously reported assay developed with HaLV p30, rat, hamster, and cat p30's seem more closely related to each other than to mouse type C virus p30. High levels of specific antigen were found in all cell lines producing rat virus, whereas embryonic tissues from several rat strains and cell lines considered virus-free based on other tests were negative for p30. Rats bearing tumors containing Moloney murine sarcoma virus (RaLV) did not contain free circulating antibody to RaLV p30. Fifty-one human tumor extracts (including two tumor cell lines) were tested for activity in the RaLV species and 47 in the interspecies assays after Sephadex gel filtration and pooling of material in the 15,000- to 40,000-molecular-weight range. At a sensitivity level of 7 ng/ml (0.7 ng/assay) in the interspecies assay, all human tissues, with one exception, were negative. The one positive result is considered nonspecific based on proteolysis of the labeled antigen. Input tissue protein of the purified tumor extracts averaged 1.9 mg/ml with a range of <0.025 to 22 mg/ml. Tissues from NIH Swiss mice processed in the same manner were positive in the interspecies assay but negative in the intraspecies RaLV assay.

The radioimmunoassay (RIA) procedure has been widely used in recent years as a measure of interviral relatedness and in the search for viral gene products in cell lines and tissues. In the case of mammalian type C virus p30, use of this technique gave the first evidence of heterogeneity of interspecies determinants (29), which, along with species-specific determinants, are associated with this major internal virion protein (14). Heterogeneity of determinants of both classes on individual molecules (7, 8) and between p30's of different species is now a well recognized phenomenon (4, 15, 29, 31, 35, 38, 40). Among the murine leukemia viruses, p30 type-specific differences have also been reported (27, 38); these can be correlated with differences in primary structure (27).

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We previously reported RIA for hamster (HaLV) type C virus p30 which indicated a relationship among feline (FeLV) and rat (RaLV) p30's which appeared closer than seen with murine (MuLV) virus p30 (4). To continue these studies and evaluate possible type-specific differences among rat viruses, including an interspecies pseudotype, homologous and heterologous intraspecies and interspecies radioimmune assays have been developed. These assays were also utilized to test a variety of tissue culture cell lines and embryonic rat tissues for presence of p30.

In addition, these assays were utilized to test a number of human tumor extracts for p30 activity. Postive findings have previously been reported using other species-specific and interspecies RIA (36, 39). The current procedures incorporated an inhibitor of proteolytic enzyme

activity; this activity is a potential technical problem in tissue assays leading to false positive results.

MATERIALS AND METHODS

Virus. The purification of banded virus and the isolation of the major structural protein, p30, by isoelectric focusing have been previously reported (24, 26).

Radioimmunoprecipitation assays. Radioimmunoprecipitation assays were performed using a salt precipitation procedure as previously reported (5) and recently modified (6). Competition RIA were performed by the salt technique and also with comparable results (except for tissue extracts) by a double antibody system similar to the technique developed by Parks and Scolnick (29). The salt technique, although suitable for purified protein or whole disrupted virus, has certain inherent technical limitations when used with tissue extracts. Phenylmethanesulfonyl fluoride (Pierce Chemicals) was added to incubation mixtures at a final concentration of 300 μg/ml to inhibit proteolytic digestion of the radioactive p30 during the incubation period (9). The conditions of the competition assay were: 100 μl of labeled RaLV p30 diluted in 0.01 M Tris-hydrochloride, pH 7.4, with 0.15 M NaCl containing 2% bovine serum albumin (BSA); 100 μl of inhibiting proteins in the same buffer containing 0.2% BSA and 100 μl of specific immune serum diluted in 1% normal carrier serum at a dilution sufficient to bind about 50% of input radioactivity were incubated for 3 h at 37 C and overnight at 4 C. Anti-species antibody (either goat anti-guinea pig or rabbit anti-goat gamma globulin) (100 μl) was then added at a dilution sufficient to maximally precipitate the initial complex. Tubes were incubated at 37 C for 2 h and at 4 C for 3 h and then centrifuged 30 min at 4,000 rpm in a Sorvall RC 2B centrifuge equipped with HS4 swinging bucket rotors. Tubes were inverted on absorbent paper, residual fluid was removed from the mouth of each tube with a cotton-tipped swab, and precipitate radioactivities were determined in a Nuclear model 1185 automatic gamma counter with a calculated efficiency for a simulated ^{125}I standard (Nuclear-Chicago) of 79 to 80%.

Labeled antigens. Purified RPL-RaLV p30 was labeled with ^{125}I using the chloramine T procedure (16). Free and protein-bound radioactivity were separated by initial overnight dialysis at 4 C. At this point the specific activities were about 90,000 counts/min per μg. RaLV p30 was further purified by G-100 superfine Sephadex gel filtration to remove traces of free iodine and to separate p30 from higher-molecular-weight aggregates.

Antisera. RIA were made using the following antisera. A homologous serum to the RPL strain of RaLV (RPL-RaLV) was obtained by hyperimmunizing a rabbit with disrupted virus. Several guinea pig sera to Moloney murine sarcoma virus (M-MSV) (RaLV) p30 were screened and a high-titered serum with avid antibodies was selected for a heterologous intraspecies assay. An interspecies assay employed a serum prepared by sequentially immunizing a goat with FeLV, RD 114, woolly monkey (WoLV), and gibbon ape (GaLV) virus p30's. This serum reacts with interspecies specificity in immunodiffusion and complement fixation (CF) tests, detecting all known mammalian type C virus p30, but does not react with avian type C viruses or B type mammalian viruses, even at high antigen concentrations. The details of preparation and characterization of this serum will be published elsewhere.

Cell lines. The MSB-1 cell line shedding M-MSV (RaLV) (41) was originally obtained from Robert Ting and has been maintained for over 5 years in this laboratory. The NRK-9 cell line (20) shedding a spontaneously activated RaLV and the RPL line, a Rous sarcoma virus-transformed rat cell originally produced by Veselý et al. (43), were obtained from V. Klement (20). The origin and properties of the B-34 cell, a transformed hamster cell shedding the HaLV pseudotype of Harvey sarcoma virus, have been discussed (1, 4, 42). Harvey sarcoma virus was derived from rat passage of Moloney MuLV (17), and this is reflected in the presence of RaLV sequences in addition to MuLV sequences in the viral genome (33). A partial analysis of the immunologic properties of HaLV p30 has been previously reported (4). The 78A1 cell line is a rat cell productively transformed with M-MSV (3).

Protein estimations. The protein content of 20% tissue extracts was estimated by the method of Lowry et al. (21) using crystalline BSA as standard (Armou).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using precast 12% gels (Biophor, Bio-Rad) according to the method of Weber and Osborne (44). Molecular weights were estimated by linear regression analysis of the $\log_{10}$ molecular weights of the indicated standards versus mobility relative to the low-molecular-weight marker bromophenol blue: BSA, 68,000; ovalbumin, 43,000; carbonic annhydrase, 29,000; lysozyme, 14,300.

Preparation of rat embryo extracts. Eighteen-day embryos were obtained from six strains of pregnant rats purchased from Microbiological Associates, Bethesda, Md. Twenty percent (wt/vol) aqueous extracts were made from individual embryos as previously published (4). Phenylmethanesulfonyl fluoride (PMSF), obtained from Pierce Chemical Co., was incorporated in the diluent buffer and was prepared as follows: crystalline PMSF was dissolved in dimethyl sulfoxide (ME_2SO) at a concentration of 200 mg/ml, diluted in 0.01 M Tris-hydrochloride (pH 7.4) containing 0.15 NaCl to a final concentration of 300 μg/ml, and then incubated at 37 C for 1 h with frequent vigorous mixing to redissolve the PMSF which precipitates initially in the aqueous buffer. Once dissolved, the PMSF solution remains soluble indefinitely at 4 C.

Human tumors. Frozen human tumors or normal tissues were obtained from Murry B. Gardner, University of Southern California School of Medicine, from the Sloan-Kettering Memorial Institute for Cancer Research, or from HEM Research, Inc., Rockville, Md. Peripheral blood cells from three cases of acute myelocytic leukemia were obtained from Robert Gallo, National Cancer Institute. Five grams (wet

weight) of tissue was homogenized in a Waring blender with 15 ml of 0.05 M Tris-hydrochloride (pH 7.8) and 0.15 M NaCl containing 0.5% Triton X-100 and sonicated at full power for a total of 3 min in a Branson sonifier with alternate 30 s of sonication and 30 s cooling. Sodium deoxycholate was then added to a final concentration of 0.5%, and extracts were incubated for 30 min at room temperature. Samples were centrifuged for 15 min at 2,500 rpm in an IEC PR-6 centrifuge to remove particulate debris and then extracted twice with an equal volume of Genetron. When necessary, samples were frozen at −20 C at this point. Otherwise extracts were centrifuged at 100,000 × *g* for 40 min in a 40.3 rotor and dialyzed for 18 h at 4 C against 0.005 M Tris-hydrochloride (pH 7.8) containing 0.005% Triton X-100. After concentration by lyophilization, samples were resuspended in 2 to 4 ml of sterile distilled water, and any insoluble protein was removed by centrifugation for 15 min at 20,000 rpm in a Beckman J-21 centrifuge using a JA 20 rotor. Samples were treated with 2.5 μg of DNase (Worthington) per mg of protein for 4 to 8 h at 4 C and then chromatographed on standardized G-100 medium Sephadex columns equilibrated and eluted with 0.05 M Tris-hydrochloride (pH 7.8) containing 0.01% Triton X-100. Fractions in the 15,000- to 50,000-molecular-weight range were concentrated by pressure dialysis on an Amicon PM-10 filter. This procedure is essentially that of Sherr and Todaro (36) with the use of Genetron as described by Huebner et al. (19).

Several precautions were explicitly taken to eliminate the possibility of cross-contamination. Vials, high-speed centrifugation tubes, and flasks were considered disposable and were used only for one tumor. Where this was not possible, anything coming in contact with the tumor extract was thoroughly washed in germicidal detergents and autoclaved prior to use, e.g., Waring blenders fraction collector tips. Extracts were chromatographed on siliconized (Siliclad, Clay-Adams) glass columns (1.4 by 75 cm). Inlet and outlet connections consisted of an appropriate sized stopper with a hole bored through the center large enough to accept the tip of a 1.0-ml disposable plastic pipette. A small piece of coarse filter paper prevented the efflux of Sephadex. A new column including tubing and connections with fresh Sephadex was used for each tumor.

Inoculation of rats with M-MSV (RaLV)-producing rat cells. Twenty weanling Brown-Norway rats were inoculated with a single 0.3-ml subcutaneous dose of 0.3 ml of MSV-1 cells shedding M-MSV (RaLV) preinoculation sera, and sera obtained after 1 and 2 weeks were titrated for anti-RaLV p30 antibodies.

RESULTS

Purity of RaLV p30. The ^{125}I-labeled RPL-RaLV p30 preparation migrated as a single radioactive zone when electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gels. (Fig. 1). Neither higher- nor lower-molecular-weight contaminants were observed, even with input radioactivities of 300,000 to 700,000 counts/min per gel.

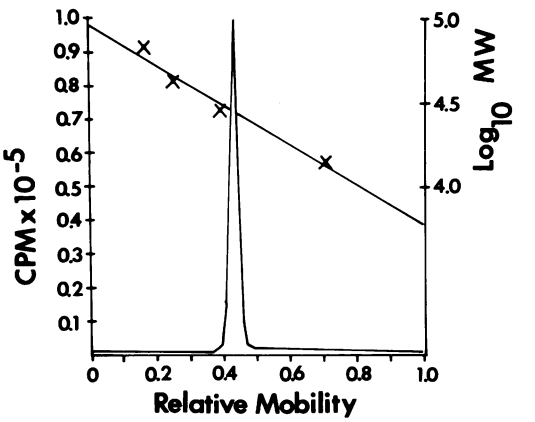

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electropherograms of ^{125}I-labeled RaLV p30. ^{125}I-labeled RaLV p30's (120 μl) (~3 × 10^5 counts/min) were electrophoresed according to the text and references 4 and 44. After staining and destaining, gels were sliced into 80 1-mm slices with a mechanical razor blade device (Bio-Rad), and the radioactivity of individual slices was determined. The molecular weight (MW) of 28,900 ± 2,700 was obtained by linear regression analysis of the $\log_{10}$ molecular weight of the following internal standards versus mobility relative to the tracking dye: BSA, 68,000; ovalbumin, 43,000; carbonic annhydrase, 29,000; lysozyme, 14,300. This value is in general agreement with the reported value for M-MSV (RaLV) of 27,000 (24). Even though the apparent molecular weight is less than 30,000, this protein will be referred to as p30 to indicate homology among this class of proteins in various mammalian type C viruses.*

Antibody precipitability. Greater than 80% of input radioactivity was specifically precipitated at a 1:1,000 dilution of each hyperimmune serum. Binding titers (50%) were 1:5,000 for the homologous rabbit anti RPL-RaLV serum, 1:50,000 to 200,000 for the guinea pig anti M-MSV (RaLV) serum, and 1:50,000 for the interspecies goat serum.

Since we had titrated the guinea pig serum against the homologous M-MSV (RaLV) p30 1 year previous to the current experiments, it is noteworthy that 50% binding titers against the heterologous RPL p30 differed by less than a factor of 2 from the original results, indicating a high degree of similarity between the p30's from these virus strains.

Competition RIA. Figure 2A shows the dose response curves of the homologous intraspecies RIA employing ^{125}I-labeled RPL p30 and the homologous antiserum raised in a rabbit. RPL, M-MSV (RaLV), and NRK-9 virus p30's inhibited with comparable slopes and to a comparable extent, indicating equivalent affinity for antibody. Only very high concentrations of HaLV and FeLV p30's inhibited in this assay.

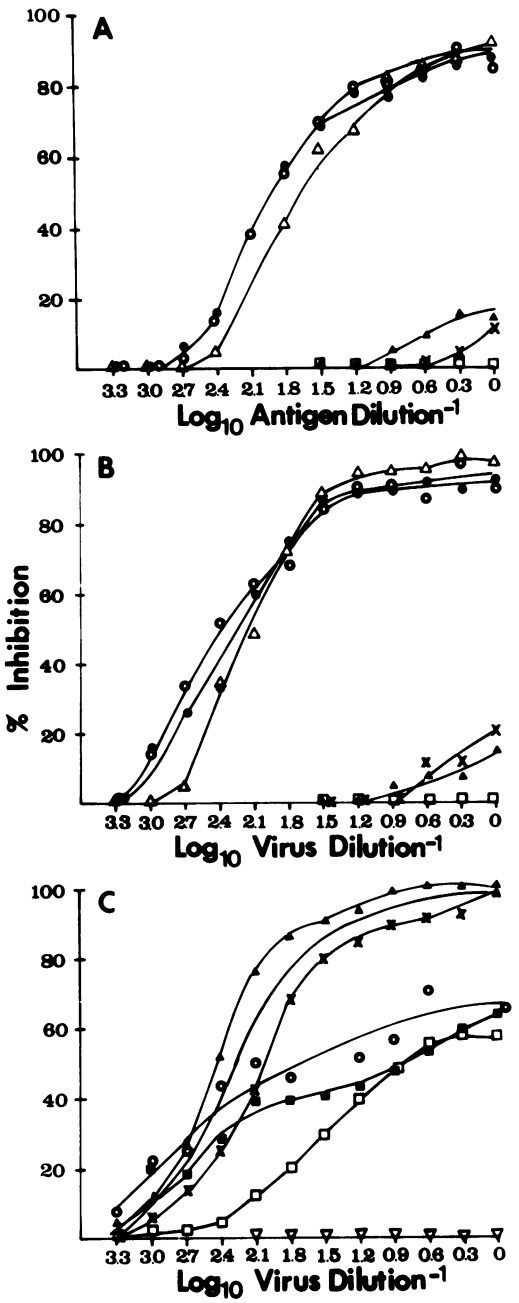

FIG. 2. *Homologous intraspecies (A), heterologous intraspecies (B), and heterologous interspecies (C) assays. ^{125}I-labeled RPL-RaLV p30 (100 μl) in 0.01 M Tris-hydrochloride (pH 7.4), 2.0% BSA, 0.01% Triton-S-100, 0.01 M EDTA and 100 μl of competing antigen diluted in 0.01 M Tris-hydrochloride, 0.2% BSA, and 100 μl of anti-p30 antibody diluted in 10% normal rabbit serum were incubated for 3 h at 37 C and overnight at 4 C. An equal volume (0.3 ml) of cold (4 C) 79% saturated ammonium sulfate was then added, and tubes were mixed vigorously and allowed to stand 12 h at 4 C. Tubes were then handled as stated in the text. The p30 concentrations (micrograms per milliliter) of the initial dilutions were: NRK-RaLV, 1.5; M-MSV-RaLV p30, 5; RPL-RaLV p30, 5; FeLV, 9; HaLV, 10; MuLV, 4; RD 114, 17; WoLV, 9; MP-MV, 4. (A) Homologous intraspecies assay employing ^{125}I-labeled RPL-RaLV and p30 anti-RPL; (B) heterologous intraspecies assay employing ^{125}I-labeled RPL-RaLV p30 and anti M-MSV (RaLV) p30; (C) heterologous interspecies assay employing ^{125}I-labeled RPL-RaLV p30 and a polyvalent serum obtained by sequential immunization of a goat with FeLV, RD 114, WoLV, and GaLV p30's. Symbols (A and B): ●, M-MSV (RaLV) p30; ●, NRK-RaLV; △, RPL-RaLV p30; ▲, FeLV; ✕, HaLV; □, MuLV, WoLV, and MP-MV. (C) ◐, RD 114; ■, WoLV; ▽, MP-MVj —, three strains of RaLV; ▼, FeLV; ✕ HaLV; □, MuLV.*

Comparable levels (4 to 17 μg/ml) of MuLV, RD 114, WoLV, and the non-type C virus Mason-Pfizer monkey virus (MP-MV) did not compete. The sensitivity of this assay for a purified RaLV p30 standard was 20 ng/ml based on a 10% inhibition end point.

Figure 2B shows the inhibition curves obtained when the heterologous intraspecies RIA employing ^{125}I-labeled RPL RaLV p30 and a guinea pig antiserum to M-MSV (RaLV) was inhibited with various type C viruses. The results with this serum were similar to those with the homologous serum in that the three rat viruses produced similar inhibition curves, and in that only FeLV and HaLV p30's produced low-level cross-reactions. This assay, however, was reproducibly four to five times more sensitive than the homologous assay for detection of RaLV p30. Also, as noted for antibody titers, the sensitivity (2 to 7 ng/ml) was similar to that obtained in the past using the homologous labeled antigen. The difference in sensitivity is attributed to the quality of antibody in this particular serum.

Figure 2C shows the competition patterns obtained with three strains of RaLV and other type C viruses in the heterologous interspecies RIA employing serum from the sequentially immunized goat. Again, all rat p30's competed with similar slopes and to the same extent. We were particularly interested in the interspecies reactivities of this serum. FeLV and HaLV competed identically to RaLV, whereas RD 114 and WoLV competed less efficiently. Additionally, the partial reaction with MuLV is noteworthy. The failure of MuLV to compete for more than 60% of RaLV p30 determinants, as compared with FeLV, RaLV, and HaLV, indicates significant differences in the interspecies

p30 determinants of MuLV relative to these three viruses.

The homologous RIA was 16- to 50-fold and both heterologous assays roughly 100-fold more sensitive than complement fixation (CF) tests on the same preparation.

p30 in rat and other cells. Once the sensitivity and specificity of the assays had been established, a variety of virus-producing and control cultures were tested for RaLV p30. p30 was generally detected in high levels only in those cells known to be shedding RaLV (Table 1). Uninfected rat cells, or cells producing other non-rat type C viruses, did not cross-react in the intraspecies assays. Thus, an XC subline producing low levels of RaLV (detected initially in the CF test) contained 120 ng of p30 per mg of cell protein. The RPL cell line used for virus production had the highest observed levels, 6,400 ng of p30/mg of cell protein. Negative cultures contained less than 1 ng/ml.

Hybridization experiments have indicated that passage of heterologous type C viruses in rats and rat cells frequently leads to the incorporation of rat-derived nucleic acid sequences into virions (23, 24). Interspecies pseudotypes and nonproducer cells containing such rat-derived sequences did not contain detectable RaLV p30 (Table 1). Neither K-234, a Kirsten sarcoma virus-transformed Balb/c embryo cell (37), nor four cell lines derived from mutagenized clones of the parent K-234 cell line (18) reacted in the RaLV p30 assay. Two of these clones produced MuLV in high titer. A human cell line derived from an osteogenic sarcoma (22) and a clonal derivative transformed by Kirsten sarcoma virus (30) also did not contain detectable RaLV p30. The transformed human cell does contain the rat-specific nucleic acid sequence associated with the Kirsten sarcoma virus.

Rat embryo extracts. Extracts from several late gestation rat embryos were examined for RaLV p30. With one possible exception, all were completely negative (Table 2) at input protein levels similar to those assayed from cultured RaLV-negative cells.

Lack of RaLV p30 antibodies in rats bearing M-MSV (RaLV) tumors. Geering et al. (10) obtained antisera to MuLV p30 from rats bearing MuLV-induced leukemia. These sera were found to cross-react with FeLV and subsequent type C virus isolates, including RaLV p30 (11). Antisera to MuLV p30 were also found in rats bearing transplant sarcomas induced by MSV (19). We made a limited study of the response of Brown-Norway rats inoculated with tumor transplants of the homologous MSB-1 sarcoma cell producing M-MSV (RaLV). As shown in Table 3, all 20 preinoculation sera obtained at 1 week and 13 of 13 sera obtained 2 weeks after inoculation, when the average tumor size was 46 mm, lacked detectable RaLV p30 antibodies. Thus, in contrast to rats bearing tumors induced by MuLV and MSV, rats with tumor transplants induced by a RaLV pseudo-

TABLE 1. *Assay for RaLV p30 in various established cell lines*[a]

Cell line	Description	RaLV p30 (ng/mg of cell protein)
RPL	Rous sarcoma virus-transformed Lewis rat cell shedding endogenous RaLV.	6,400
MSB-1	BN rat cell shedding M-MSV (RaLV).	500
XC	Wistar rat cell transformed by Rous sarcoma virus shedding endogenous RaLV.	120
NRK-9	Osborne Mendel rat embryo cells shedding endogenous RaLV.	700
SPD-1	Sprague Dawley rat embryo cells, passage 6 (S. Rasheed, unpublished data).	<1.4
SPD-1	Sprague Dawley embryo cells shedding RaLV, passage 56.	800
78A-1	Rat cell productively transformed by M-MSV (MuLV).	<1.1
B-34	Hamster cell productively transformed by Harvey MSV (HaLV).	<0.7
HT-1	Hamster cell nonproductively transformed by M-MSV.	<0.4
K-234	Balb/c mouse embryo cell nonproductively transformed by Kirsten sarcoma virus.	<0.4
43-2	Mutant of K-234, nonproducer.	<0.4
58-2	Mutant of K-234, producing MuLV and transforming virus.	<0.4
M-50	Mutant of K-234, nonproducer.	<0.4
57-1	Mutant of K-234, producing MuLV and transforming virus.	<0.5
HOS	Human osteosarcoma cell.	<0.4
KHOS	HOS nonproductively transformed by Kirsten sarcoma virus.	<0.7

[a] 20% (vol/vol) extracts were diluted serially and tested in the heterologous intraspecies assay. Intraspecies double antibody RIA employing ^{125}I-labeled RPL-RaLV p30 and M-MSV and M-MSV(RaLV) guinea pig antibody.

TABLE 2. *Rat extracts screened and negative for RaLV p30*[a]

Strain	Description	No. of embryos or tissues examined	Range of negativity (ng/mg of protein)
Maxx	Embryos	3	<0.6–0.9
F-344	Embryos	1	2.5[b]
AC-1	Embryos	3	<0.8–1.1
Lewis	Embryos	4	<0.4–0.7
Buffalo	Embryos	3	<0.3–0.7
Wistar	Embryos	3	<0.5–0.6
Fisher	Embryos	6	<0.6–1.8
	Adult female liver	2	<0.5
	Adult female spleen	2	<0.3
	Adult female muscle	1	<1.0

[a] 20% (wt/vol) extracts of 18-day rat embryos from seven strains and adult female liver, spleen, and muscle were assayed for RaLV p30 in a heterologous intraspecies double antibody RIA employing ^{125}I-labeled RPL RaLV p30 anti M-MSV (RaLV).

[b] 20% inhibition in undiluted extract, negative at 1:2 dilution. This result is considered marginal.

TABLE 3. *Lack of humoral antibody to RaLV p30 in Brown-Norway rats bearing M-MSV (RaLV)-induced sarcoma transplants*

Week of study	Tumor incidence (no./no. tested)	Mean tumor diameter (mm)	Anti-RaLV p30 antibodies[a] (no. positive/no. tested)
0	0/20	0	0/20
1	11/20	15 ± 12	0/20
2	13/13	46 ± 6	0/13

[a] Sera were screened at dilutions of 1:20 to 1:320. Four complete titrations of a positive control hyperimmune guinea pig anti-M-MSV (RaLV) p30 serum gave: 50% binding titers of 1:80, 800 ± 6,000 and 10% binding 1:630,000 ± 150,000. Pre- and postinoculation sera bound less than 10% of input radioactivity at 1:20.

type of MSV do not form RaLV p30 antibodies.

Human tumors. Tissue extracts were purified an average of 44-fold after G-100 chromatography and concentration by ultrafiltration. All samples were negative in highly specific CF tests for RaLV, FeLV, MuLV, RD 114, HaLV, and GaLV p30's and negative in a broadly reacting CF test recognizing all known mammalian virus p30's. All but one of the concentrates were negative in both intra- and interspecies RaLV p30 assays at levels of 0.6 to 0.8 ng of RaLV p30 per mg of cellular protein after consideration of purification factors. The single sample producing positive inhibition was an apparently normal autopsy spleen from a patient with pancreatic carcinoma. This concentrate gave positive reactions in both the homologous intraspecies (10% inhibition titer = 1:2, maximal inhibition = 40%) and heterologous interspecies assay (titer = 1:2, 23% inhibition). This same extract gave comparable or higher inhibitory activity in highly specific FeLV, GaLV, and MuLV p30 RIAs and produced complete inhibition with an end point still not reached (~80% inhibition) at 1:128 dilution in an interspecies assay with ^{125}I-labeled RD 114 p30. The latter result was striking since the known sensitivity of the interspecies RIA relative to the CF test would have demanded a CF titer of at least 1:8 if the RIA results were immunologically specific. This was not observed.

Therefore, we suspected false positive results related to degradation of RaLV p30 by tissue proteases co-purified in the 30,000-dalton fractions. This was confirmed by demonstrating the liberation of trichloroacetic acid-soluble fragments when ^{125}I-labeled RaLV p30 was incubated with the concentrate at 37 C according to standard RIA procedure, but not when all incubations were done at 4 C (Table 4). We have previously found this technique to be a

TABLE 4. *Degradation of ^{125}I-labeled RaLV p30 by a partially purified spleen extract from a patient with pancreatic carcinoma*[a]

Determinants	TCA precipitability after incubation at:	
	37 C (routine)	4 C (control)
^{125}I-labeled RaLV p30 + antibody + sample diluent	87.8 ± 0.2	89.1 ± 0
^{125}I-labeled RaLV p30 + antibody + extract	67.7 ± 1	87.2 ± 0.1

[a] Tissue extract or diluent alone was incubated at 37 C for a total of 5 h (3-h primary and 2-h secondary incubation) according to the text. Before the usual centrifugation step, 1.0 ml of 10% normal rabbit serum was added to each tube followed by 1.0 ml of 30% trichloroacetic acid (TCA). Tubes were vigorously mixed, allowed to stand 30 min at 4 C, and centrifuged as usual. The radioactivity of precipitates and supernatants was determined, and TCA precipitability was calculated as the percentage of precipitate counts to the combined supernatant and precipitate counts. Control assays were done identically except that all incubations were at 4 C. Results are expressed as the mean ±1 standard deviation of duplicate tubes.

precise but minimal estimate of the digestion of MuLV p30 (7).

The NIH Swiss spleen extracts gave positive results in the interspecies assay (Table 5) and not the RaLV species assays. This verifies that the purification procedure is suitable for tissue extracts which, based upon extensive studies (19, 28), were known to be p30 positive. The specificity of these tissue concentrates was as predicted by studies of type C viruses from mice and rats.

Full details of attempts to detect p30 determinants of various specificities in human tissues will be presented elsewhere.

DISCUSSION

The RIA procedure first gave evidence of the heterogeneity of p30 interspecies determinants when RD 114 and WoLV-GaLV were observed to give only partial inhibition of an assay using labeled mouse p30 and anti-FeLV p30 (29). Based on data from a variety of studies, including reverse transcriptase inhibition by antibody, molecular hybridization, and p30 amino terminal sequence comparisons, there seem to be three clear subgroups of mammalian type C viruses (12, 13, 25). These are (i) WoLV-GaLV, (ii) RD 114-baboon, and (iii) MuLV, RaLV, FeLV, HaLV. We previously noted that an interspecies assay using labeled HaLV p30 was only partially inhibited by MuLV, whereas FeLV and RaLV gave complete inhibition (4). Similarly, our colleague (S. Oroszlan) has noted that an interspecies reactive serum prepared against FeLV, after specific purification on an HaLV immunoabsorbent, produced identity reactions between HaLV, RaLV, and FeLV but clear partial identity with MuLV compared to these antigens. The current data show that the species-specific assay for RaLV is inhibited to a slight degree by FeLV and HaLV and not MuLV and also that the interspecies assay with labeled RaLV is incompletely inhibited by MuLV. Thus, MuLV p30 appears to be more distantly related to the other members of its subgroup than they are to one another.

We have previously noted that serologic data do not support a simple concept of coevolution of RNA tumor viruses and host genes (12, 13). Recent evidence obtained by nucleic acid hybridization techniques (2) has suggested that horizontal transmission has occurred in the distant past (millions of years ago) in at least one circumstance, namely old world monkey ancestor to cat ancestor to explain the baboon-RD 114 relationship, and thus the possibility of other transmissions is raised. One should not forget however that all mammalian virus p30's

TABLE 5. *Human and animal tissues examined for RaLV p30*

Determinants	Homologous intraspecies	Heterologous interspecies
Normal fetal liver	0/2[a]	0/2
Carcinomas		
Breast	0/11	0/11
Lung	0/8	0/7
Other	0/7	0/6
Hematologic neoplasma[b]		
Hodgkin's disease, normal spleen	0/1	0/1
Hodgkin's disease, involved spleen	0/1	0/1
AML, bone marrow and peripheral white blood cells	0/4	0/3
CML, bone marrow	0/1	NT
ALL, bone marrow	0/1	0/1
CLL, bone marrow	0/1	0/1
Sarcomas	0/4	0/4
Neural		
Glioblastoma	0/2	0/2
Malignant schwannoma	0/1	0/1
Nontumored tissues		
Spleen from patient with CA pancreas	1/1	1/1
Muscle	0/1	0/1
Colon	0/1	0/1
Kidney	0/1	0/1
Breast	0/3	0/3
Animal tissues		
Turkey liver, leucotic	<0.1	<0.1
Normal NIH Swiss liver pool	<0.1	0.1
Normal NIH Swiss spleen pool	<0.2	1.4
FeLV-producing feline lymphocytes	<1.1	150

[a] Tissue extracts were solubilized with detergents once chromatographed on G-100 Sephadex, and fractions in the 15,000- to 40,000-dalton range were pooled, concentrated by pressure filtration, and tested in the homologous intraspecies and heterologous interspecies RaLV p30 RIAs, as detailed in the text. All concentrates were negative in highly specific CF tests for RaLV, FeLV, MuLV, GaLV, and RD 114 p30, and for Mason Pfizer p27. Concentrates were also negative in a broadly reacting CF assay employing serum from a goat sequentially immunized with several p30's. Results are expressed either as the number of samples positive/number tested or, in the case of animal tissues, as the nanograms of RaLV p30/milligrams of cell protein after consideration of purification factors.

[b] AML, Acute myelogenous leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; Cll, chronic lymphocytic leukemia.

are products of homologous genes based on both immunologic and sequence data (25), and that ancestral genes of contemporary viruses must have existed in mammals prior to the times suggested for the horizontal transmission events.

The current p30 RIA also failed to demonstrate any type-specific differences among the RaLV p30's as also recently noted by others (32). Certain of these viruses are interspecies pseudotypes based on nucleic acid sequence relationships; however, only the rat p30 determinants are detected as previously seen in gel diffusion and CF tests. In these viruses p30 species type is correlated with the intracellular presence of a 35*S* subunit RNA corresponding to the helper virus. Similar findings were found with interspecies pseudotypes expressing hamster p30 (42).

In contrast to results obtained in the mouse (19, 28) and hamster (4), we thus far have not found evidence of RaLV p30 in embryo extracts of several rat strains. Since the respective assays were of similar sensitivity, there are evidently quantitative or qualitative differences in levels of expression in different species.

As part of a continuing effort to detect type C viral gene products in human tumors, we have applied the RaLV p30 species and interspecies assays to human tumor extracts nonspecifically purified by gel filtration. With care taken to avoid proteolysis of the labeled probe, these assays gave clear negative results with all but one of a large series of human tumors under conditions where NIH Swiss mouse tissues and an FeLV-producing cell line gave positive results in the interspecies assay. The single exception also gave positive inhibition in the rat and other species-specific assays. This sample degraded labeled p30 under assay conditions; thus, the apparent positive results are explained by proteolysis. Use of a single inhibitor, although extremely useful, would not be expected to control for the multiplicity of proteolytic enzymes present in tissue extracts.

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LITERATURE CITED

1. Bassin, R. H., P. J. Simons, F. C. Chesterman, and J. J. Harvey. 1968. Murine sarcoma virus (Harvey): characteristics of focus formation in mouse embryo cell cultures, and virus production by hamster tumor cells. Int. J. Cancer **3**:265–272.
2. Beneveniste, R. E., and G. J. Todaro. 1974. Evolution of C-type viral genes: inheritance of exogenously acquired viral genes. Nature (London) **252**:456–459.
3. Bernard, C., M. Boiron, and J. Lasneret. 1967. Transformation et infection chronique de cellules embryonnaires de rat par le virus du sarcome de Moloney. C. R. Acad. Sci. **264**:2120–2133.
4. Charman, H. P., N. Kim, and R. V. Gilden. 1974. Radioimmunoassay for the major structural protein of hamster type C viruses. J. Virol. **14**:910–917.
5. Charman, H. P., N. Kim, M. White, and R. V. Gilden. 1974. Failure to detect in human sera, antibodies cross-reactive with group-specific antigens or murine leukemia virus. J. Natl. Cancer Inst. **52**:1409–1413.
6. Charman, H. P., N. Kim, M. White, H. Marquardt, R. V. Gilden, and T. Kawakami. 1975. Natural and experimentally induced antibodies to defined mammalian type C viral proteins in primates. J. Natl. Cancer Inst. Vol. 55.
7. Davis, J., H. P. Charman, S. Oroszlan, and R. V. Gilden. 1973. Multiple distinct antigenic determinants associated with murine type C virus group-specific antigen. Intervirology **2**:326–336.
8. Davis, J., R. V. Gilden, and S. Oroszlan. 1974. Multiple species-specific and interspecific antigenic determinants of a mammalian type C RNA virus internal protein. Immunochemistry **12**:67–72.
9. Fahrney, D. E., and A. M. Gold. 1963. Sulfonyl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, chymotrypsin, and trypsin. J. Am. Chem. Soc. **85**:997–1000.
10. Geering, G., L. H. Old, and E. A. Boyse. 1966. Antigens of leukemias induced by naturally occurring murine leukemia virus: their relation to the antigens of Gross virus and other murine leukemia viruses. J. Exp. Med. **124**:753–772.
11. Geering, G., L. J. Old, and E. A. Boyse. 1970. Shared viral antigen of mammalian leukaemia viruses. Nature (London) **226**:265–266.
12. Gilden, R. V., and S. Oroszlan. 1974. Coevolution of RNA tumor virus genome and vertebrate host genes, p. 460–476. *In* Molecular studies in viral neoplasia. The Williams and Wilkins Company, Baltimore.
13. Gilden, R. V., S. Oroszlan, and M. Hatanaka. 1973. Comparison and evolution of RNA tumor virus components, p. 235–257. *In* E. Kurstak and K. Maramorosch (ed.), Viruses, evolution and cancer. Academic Press Inc., New York.
14. Gilden, R. V., S. Oroszlan, and R. J. Huebner. 1971. Co-existence of intraspecies and interspecies specific antigenic determinants on the major structural polypeptide of mammalian C-type viruses. Nature (London) New Biol. **231**:107–108.
15. Goldberg, R. J., E. M. Scolnick, W. P. Parks, L. A. Yakovleva, and B. A. Lapin. 1974. Isolation of a primate type-C virus from a lymphomatous baboon. Int. J. Cancer **14**:722–730.
16. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. **89**:114–123.
17. Harvey, J. J. 1964. An unidentified virus which causes the rapid production of tumors in mice. Nature (London) **204**:1104–1106.
18. Hatanaka, M., R. Klein, R. Toni, J. Walker, and R. Gilden. 1973. Mutants of nonproducer cell lines transformed by murine sarcoma viruses. I. Induction, isolation, particle production and tumorigenicity. J. Exp. Med. **138**:356–363.
19. Huebner, R. J., G. J. Kelloff, P. S. Sarma, W. T. Lane, H. C. Turner, R. V. Gilden, S. Oroszlan, H. Meier, D. D. Myers, and R. L. Peters. 1970. Group-specific antigen expression during embryogenesis of the genome of the C-type RNA tumor virus: implications for ontogenesis

and oncogenesis. Proc. Natl. Acad. Sci. U.S.A. **67**:366-376.
20. Klement, V., M. O. Nicolson, W. Nelson-Rees, R. V. Gilden, S. Oroszlan, R. W. Rongey, and M. B. Gardner. 1973. Spontaneous production of a C-type RNA virus in rat tissue culture lines. Int. J. Cancer. **12**:366-376.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
22. McAllister, R. M., M. B. Gardner, A. E. Grene, C. Brodt, W. W. Nichols, and B. H. Landing. 1971. Cultivation *in vitro* of cells derived from a human osteosarcoma. Cancer **27**:397-402.
23. Okabe, H., R. V. Gilden, and M. Hatanaka. 1973. Specificity of the DNA product of RNA-dependent DNA polymerase in type C viruses. II. Quantitative analysis. Proc. Natl. Acad. Sci. U.S.A. **70**:3923-3927.
24. Oroszlan, S., D. Bova, R. J. Huebner, and R. V. Gilden. 1972. Major group-specific protein of rat type C viruses. J. Virol. **10**:746-750.
25. Oroszlan, S., T. Copeland, M. R. Summers, G. Snythers, and R. V. Gilden. 1974. Amino acid sequence homology of mammalian type C RNA virus major internal proteins. J. Biol. Chem. **235**:6232-6239.
26. Oroszlan, S., C. L. Fisher, T. B. Stanley, and R. V. Gilden. 1970. Proteins of the murine C-type RNA tumor viruses: isolation of a group-specific antigen by isoelectric focusing. J. Gen. Virol. **8**:1-10.
27. Oroszlan, S., M. R. Summers, C. Foreman, and R. V. Gilden. 1974. Murine type C-virus group-specific antigens: interstrain immunochemical, biophysical, and amino acid sequence differences. J. Virol. **14**:1559-1574.
28. Parks, W. P., D. M. Livingston, G. J. Todaro, R. E. Benveniste, and E. M. Scolnick. 1973. Radioimmunoassay of mammalian type C viral proteins. III. Detection of viral antigen in normal murine cells and tissues. J. Exp. Med. **137**:622-635.
29. Parks, W. P., and E. M. Scolnick. 1972. Radioimmunoassay of mammalian type C viral proteins: interspecies antigenic reactivities of the major internal polypeptide. Proc. Natl. Acad. Sci. U.S.A. **69**:1766-1770.
30. Rhim, J. S., H. Y. Cho, and R. J. Huebner. 1975. Nonproducer human cells induced by murine sarcoma virus. Int. J. Cancer **15**:23-29.
31. Schafter, W., L. Pister, G. Hunsmann, and V. Moennig. 1973. Comparative serological studies on type C viruses of various mammals. Nature (London) New Biol. **245**:75-76.
32. Scolnick, E. M., J. M. Maryak, and W. P. Parks. 1974. Levels of rat cellular RNA homologous to either Kirsten sarcoma virus or rat type C virus in cell lines derived from Osborne-Mendel rats. J. Virol. **14**:1435-1444.
33. Scolnick, E. M., and W. P. Parks. 1974. Harvey sarcoma virus: a second murine type C sarcoma virus with rat genetic information. J. Virol. **13**:1211-1219.
34. Scolnick, E. M., E. Rands, D. Williams, and W. P. Parks. 1973. Studies on the nucleic acid sequences of Kirsten sarcoma virus: a model for formation of a mammalian RNA-containing sarcoma virus. J. Virol. **12**:458-463.
35. Sherr, C. J., L. A. Fedele, R. E. Beneveniste, and G. J. Todaro. 1975. Interspecies antigenic determinants of the reverse transcriptases and p30 proteins of mammalian type C viruses. J. Virol. **15**:1440-1448.
36. Sherr, C. J., and G. J. Todaro. 1975. Primate type C virus p30 antigen in cells from humans with acute leukemia. Science 187:855-857.
37. Stephenson, J. R., and S. A. Aaronson. 1972. Antigenic properties of murine sarcoma virus-transformed BALB/3T3 nonproducer cells. J. Exp. Med. **135**:503.
38. Strand, M., and J. T. August. 1974. Structural proteins of mammalian oncogenic RNA viruses: multiple antigenic determinants of the major internal protein and envelope glycoprotein. J. Virol. **13**:171-180.
39. Strand, M., and J. T. August. 1974. Type-C RNA virus gene expression in human tissue. J. Virol. **14**:1584-1596.
40. Strand, M., and J. T. August. 1975. Structural proteins of mammalian RNA tumor viruses: relatedness of the interspecies antigenic determinants of the major internal protein. J. Virol. **15**:1332-1341.
41. Ting, R. C. 1968. Biological and serological properties of viral particles from a nonproducer rat neoplasm induced by murine sarcoma virus (Moloney). J. Virol. **2**:865-868.
42. Tsuchida, N., R. V. Gilden, and M. Hatanaka. 1975. The size of virus-specific RNA in B-34, a hamster tumor cell producing nucleic acid of type C viruses from three species. J. Virol. **16**:832-837.
43. Veselý, P., L. Donner, J. Činátl, and V. Sovová. 1968. Interaction of Rous sarcoma virus with rat embryo fibroblasts of inbred Lewis strain *in vitro*. Folia Biol. (Prague) **14**:457-465.
44. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.
45. Yalow, R. S., and S. A. Berson. 1968. General principles of radioimmunoassay, p. 7-41. *In* R. L. Hayes, F. A. Goswitz, and B. E. P. Murphy (ed.), Radioisotopes in medicine: in vitro studies. USAEC Division of Technical Information Extension, Oak Ridge. Tenn.