JOURNAL OF VIROLOGY, Mar. 1979, p. 1221-1225
0022-538X/79/03-1221/05$02.00/0

Vol. 29, No. 3

NOTES

Reticuloendotheliosis Virus: Detection of Immunological Relationship to Mammalian Type C Retroviruses

HOWARD P. CHARMAN, RAYMOND V. GILDEN,* AND STEPHEN OROSZLAN

Biological Carcinogenesis Program, Frederick Cancer Research Center, Frederick, Maryland 21701

Received for publication 14 September 1978

Reticuloendotheliosis virus (REV) p30 shares cross-reactive determinants and a common NH_2-terminal tripeptide with mammalian type C viral p30's. An interspecies competition radioimmunoassay was developed, using iodinated REV p30 and a broadly reactive antiserum to mammalian virus p30's. The avian leukosis-sarcoma viruses and mammalian non-type C retroviruses did not compete in this assay. Previous data indicating that the REV group is not represented completely in normal avian cell DNA lead us to speculate that this may be the first example of interclass transmission, albeit in the remote past, among the Retroviridae.

Avian species have been shown to harbor two categories of tumor viruses. The first category is comprised of viruses of the avian leukosis-sarcoma virus complex. These agents are responsible for a diverse spectrum of neoplastic and nonneoplastic avian diseases including forms of leukemia, sarcoma, and carcinoma, as well as osteopetrosis. Members of the avian leukosis-sarcoma virus complex, including nononcogenic endogenous viruses, are present widely in avian species such as chickens and pheasants.

In the second category of tumor viruses are the reticuloendotheliosis viruses (REV). The prototype virus was isolated from turkeys with reticuloendothelial neoplasms (22). However, related viruses are found in chickens and ducks (20). REVs share common antigenic determinants on their reverse transcriptases (15) and on their major structural protein (14), but they are not related to the members of the avian leukosis-sarcoma virus complex (11). REVs appear to be transmitted primarily by extrachromosomal mechanisms, although low levels (<10%) of viral nucleic acid sequences were found in uninfected chicken, pheasant, quail, and turkey cell DNAs, whereas no sequences were detected in duck DNA (12).

Based on morphological considerations and on the preferential activity of the REV reverse transcriptase in the presence of Mn^{2+}, REV (16) was thought to be more closely related to mammalian type C viruses than to the avian leukosis-sarcoma virus complex. Immunological relationships, however, could not be demonstrated with antisera recognizing murine leukemia virus or simian sarcoma virus type 1 p30's (16). We isolated REV p30 and established a sensitive radioimmunoassay for its detection and quantitation. This assay easily demonstrates cross-reactions of REV p30 with antisera to several mammalian type C retroviruses. This has allowed the development of a broadly reactive interspecies assay which detects all mammalian type C viral p30's as well as REV p30. Type D primate viruses did not compete in this assay. Thus, these data are consistent with the hypothesis that REV progenitors originated from an ancestor of mammalian type C viruses (10), based on sequence homology of REV p30 to mammalian type C virus p30's.

REV type T was obtained from Henry Bose, University of Texas, Austin. This virus grows in a continuous line of mycoplasma-negative chicken bone marrow cells (5, 6). Virus was isolated from tissue culture fluids by standard methods (18). Isoenzyme analysis and cytogenic examination, kindly performed by Ward Peterson, The Child Research Center of Michigan, demonstrated that the cells were indeed chicken cells.

Isopycnically purified virus (~20 mg) was disrupted with Triton X-100 and subjected to ion-exchange chromatography on phosphocellulose by the method of Strand and August (21) as modified by Barbacid et al. (1). Fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 5 to 20% gradient slab gels and the Laemmli buffer system

(13). Fractions containing p30 and p15 were pooled, concentrated, and chromatographed on Ultragel ACA-44 to separate these two proteins (1).

Portions of p30-containing fractions (1 to 5 μg of purified p30) were iodinated by the method of Greenwood et al. (8) as modified by Barbacid et al. (2). After removal of free iodine on a small Bio-Gel P-10 column, the iodinated protein was examined for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were stained, the locations of known molecular weight markers were identified, and the gels were cut into 1-mm slices. The activities of individual fractions were then determined in a Nuclear-Chicago model 1185 autogamma counter with an efficiency for ^{125}I of about 80%. The molecular weight of the major REV internal protein was estimated to be 30,400 based on mobilities of iodinated purified proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). Antisera to REV precipitated >95% of this preparation when tested in antibody excess.

Antisera to Tween-ether- or guanidine hydrochloride-disrupted retroviruses, including Rous sarcoma virus, Prague strain Rous sarcoma virus, Rous-associated virus type 0, avian myeloblastosis virus (AMV), AMV p27, bovine leukemia virus, and gibbon ape leukemia virus, and high-titered precipitating antibody to goat immunoglobulin G, were kindly provided by Roger Wilsnak, Huntingdon Research Laboratories, Brooklandville, Md. Comparable goat antisera to RD-114, Rauscher murine leukemia virus, feline leukemia virus, mouse mammary tumor virus, Mason-Pfizer monkey virus, equine infectious anemia virus, squirrel monkey retrovirus, and simian sarcoma-associated virus p27 were produced at the Frederick Cancer Research Center. Purified visna virus p27 and goat antiserum to it were a gift of Ashley Haase, San Francisco Veterans Administration Hospital. Reference antisera to bovine leukemia virus and bovine syncytial virus were provided by Janice Miller and Martin Van der Maaten, U.S. Department of Agriculture, Ames, Iowa. The broadly reactive interspecies antiserum from goats sequentially immunized with several mammalian virus p30's has been previously described (7). Standard double-antibody assays were as published previously (2), with phenylmethylsulfonyl fluoride in all buffers (3). Salt precipitation assays were performed as published previously (3).

A variety of other antisera in addition to anti-REV were tested for the ability to precipitate ^{125}I-labeled REV p30 in both salt precipitation and double-antibody assays. The sera used all had been previously assayed by radioimmunoassays with homologous antigens and were high-titered. Antisera to mouse mammary tumor virus, Mason-Pfizer monkey virus, visna virus, bovine leukemia virus, squirrel monkey retrovirus, and equine infectious anemia virus were negative, as were antisera to Prague strain Rous sarcoma virus, Rous-associated virus type 0, and AMV. In striking contrast, several antisera to mammalian type C viruses, including one prepared by sequential immunization with several distinct p30's (7), completely precipitated REV p30. In addition, the serum designated goat anti-AMV p27 also precipitated REV p30; however, as noted below, we speculate that REV p30 must have been present in the AMV inoculum. Among the type C viral reagents, the highest precipitation titers were with antisera to Rauscher murine leukemia virus, simian sarcoma-associated virus, and gibbon ape leukemia virus, although positive results were also obtained with anti-feline leukemia virus and anti-RD-114 goat sera. Since the latter two antisera were as potent as

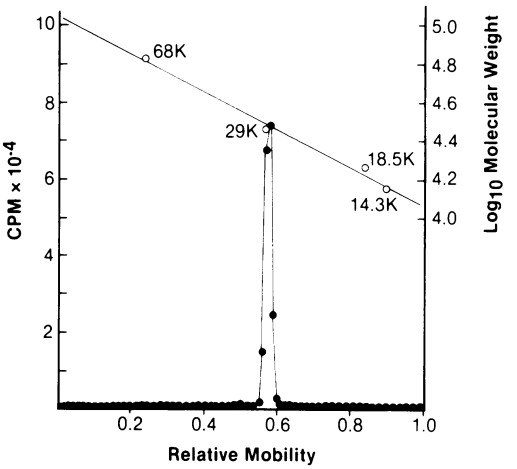

FIG. 1. *Five microliters (3 × 10⁵ cpm) of ^{125}I-labeled REV p30 was mixed with 20 μl of a mixture containing 5 μg each of standard proteins of the indicated molecular weights: bovine serum albumin, 68,000; carbonic anhydrase, 29,000; beta-lactoglobulin, 18,500; and lysoenzyme, 14,300. The proteins were diluted with an equal volume of 2X sample buffer, heated in a boiling-water bath for 5 min, subjected to electrophoresis at 1 mA per gel for 18 h, stained with Coomassie brilliant blue, and destained; the positions of marker proteins were then noted. After the gels were cut into 1-mm sections with a razor blade device, the radioactivity of each fraction was determined in an autogamma counter. The radioactivities were then plotted as a function of mobility relative to the low-molecular-weight tracking dye and known molecular weight marker proteins. The calculated molecular weight was 30,400.*

the former in homologous assays, this may indicate a closer relationship between REV and the former trio of viruses. The dilution of serum giving 50% binding of labeled antigen was lower by a factor of 10^3 in these cross-reacting assays than in homologous assays.

To establish the specificity of these binding assays, competition radioimmunoassays were developed using the various antisera and REV p30. In the case of the single positive AMV serum, tissue culture sources of Rous sarcoma virus did not compete, whereas a single plasma source of AMV did. Thus, we take the reaction of this serum to be directed at REV present in the original inoculum. This may, in fact, account for an earlier report of cross-reactions between AMV and antisera to mammalian type C viruses (4). The extent of REV contamination of AMV plasma stocks is not known to us, but the above results indicate the need for appropriate quality control.

The radioimmunoassay established with a broadly reactive interspecies mammalian type C p30 antiserum (7) and REV p30 was only inhibited by mammalian type C viruses and REV (Fig. 2). Rauscher murine leukemia virus, hamster leukemia virus, rat leukemia virus, and feline leukemia virus all gave equivalent steep slopes, as did REV when diluted appropriately (data not shown). Gibbon ape leukemia virus and simian sarcoma-associated virus also competed efficiently but in several experiments with this particular antiserum did not give complete competition. This observation is currently being reevaluated with purified p30's. RD-114 and baboon viruses gave complete competition but, in comparison to the murine leukemia virus group, had slightly reduced slopes. The existence of three subgroups of mammalian p30's based on sequence data has previously been shown (19); thus, these results are consistent with such a sub-classification. Since the serum used was prepared by sequential immunization with several p30's, no inference can be made regarding degree of relationship to REV p30. Non-type C retroviruses were negative in these assays.

Any biological classification scheme has the common ancestry of contemporary members of designated groups as its unifying thread. While variations in morphology and polypeptide pattern occur among members of the Retroviridae, the similarities in physical structure of the genome, order and number of the structural genes, and mode of replication argue for the reality of the classification. Immunological methods obviously provide a relatively simple means for verifying relationships, whereas primary-structure analysis at the protein or nucleic acid level

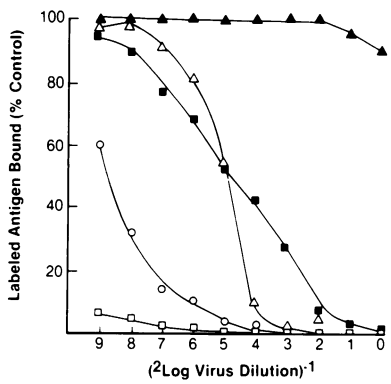

FIG. 2. *All reactants were diluted in radioimmunoassay buffer (0.05 M Tris [pH 7.8] containing 0.15 M NaCl, 0.5% bovine serum albumin, 0.1% sodium azide, 0.4% Triton X-100, and 1.7 mM [300 μg/ml] phenylmethyl sulfonyl fluoride). Competition assays were established with broadly reactive goat antiserum prepared by sequential immunization with several mammalian p30's (7) diluted to give 50% precipitation (1:400 dilution) of labeled REV p30 (7.5 × 10^3 cpm input). Mixtures containing 50 μl of antiserum, 50 μl of iodinated p30, and 100 μl of disrupted virus were incubated for 1 h at 37°C and then overnight at 4°C. Undiluted pig anti-goat immunoglobulin (20 μl) was then added, followed by 0.4 ml of buffer (0.01 M Tris [pH 7.8], 0.1% Triton X-100, 0.1 M NaCl, 0.001 M EDTA), and the tubes were incubated for an additional hour at 37°C and for 4 h at 4°C. After centrifugation for 15 min at 2,500 rpm in a Sorval RC 2B centrifuge with HS4 rotors, supernatant fluids were aspirated to waste, and precipitate radioactivity was determined in a Searle model 1285 autogamma counter. Results are expressed as the percentage of binding relative to that of the control containing trace label and antibody but lacking competing antigen. Symbols: ▲, Mason-Pfizer monkey virus (similar negative results obtained with bovine leukemia virus, squirrel monkey retrovirus, visna virus, and progressive pneumonia viruses); △, feline leukemia virus (comparable results were obtained with rat leukemia virus and hamster leukemia virus); ○, Rauscher murine leukemia virus; □, REV (2 mg/ml). Because of the variability in actual viral protein relative to extraneous proteins in these preparations, no attempt was made to normalize the data.*

offers more sensitive and compelling proof. Even where homology cannot be demonstrated, the presence of conserved features such as cleavage sites in polypeptein precursors of viral structural proteins is presumptive evidence of common ancestry. For example, a protein of ca. 30 × 10^3 molecular weight that is initiated with proline followed by one or two hydrophobic residues (Table 1) is a common feature of all the retroviruses analyzed (Oroszlan and Gilden, *Comprehensive Virology*, in press). The specific tripeptide prolyl-leucyl-arginine is found at the amino

TABLE 1. *NH_2-terminal sequences of the major internal proteins (p24 to p30) of several retroviruses*[a]

Virus[b]	Sequence
Type C avian	Pro-Val-Val-
Type C mammalian	Pro-Leu-Arg-
MMTV	Pro-Val-Val-
MPMV	Pro-Val-Thr-
BLV	Pro-Ile-Ile
Visna	Pro-Ile-Val-
REV	Pro-Leu-Arg-

[a] Sequence data, except for REV, are those given by Oroszlan and Gilden (in press).

[b] Abbreviations: MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; BLV, bovine leukemia virus.

terminus of all mammalian type C viruses (19; Oroszlan and Gilden, in press) and thus is a marker sequence for this group of viruses. Consistent with our immunological data are the data of Hunter et al. (10), which we have now confirmed as showing precisely this tripeptide sequence in REV p30 and, in addition, ca. 40% homology over ~30 residues with mouse or cat type C viruses. Hunter et al. (personal communication) have also shown immunological cross-reactivity between REV and mammalian type C viral p30's. This provides compelling evidence for the conclusion that the REV group should be closely aligned with the mammalian type C viruses, since the avian leukosis-sarcoma virus p30 homolog (p27) shows no significant sequence homology (10, 17) or cross-reactivity with REV or mammalian type C virus p30's. We note that only one serum to AMV p27, but not other reference sera to members of the avian leukosis-sarcoma virus group, precipitated REV p30. The preimmune serum from this goat lacked antibodies, whereas all postimmune sera had high titers. Although intercurrent infection with REV is possible, our interpretation is that the immunizing preparation obtained by agarose gel filtration of plasma AMV contained REV p30. Additionally, the chicken bone marrow cell line producing REV has been repeatedly checked for avian leukosis-sarcoma virus and endogenous leukosis viruses (6).

Since REV is not represented in normal avian cell DNA, it may well represent a mammalian type C virus reintroduced in Aves. If so, this would be the first case of an interclass transmission, although this is clearly speculation at present. An equally plausible hypothesis would have mammalian type C viruses originate from REV (or a progenitor virus). This situation is strikingly similar to horizontally transmitted retroviruses in mammals which derive from another species (7) (e.g., gibbon virus from *Mus* progenitors) or have an unknown germ line origin (e.g., visna virus, bovine leukemia virus, equine infectious anemia virus). Despite strong emphasis on inheritance in the germ line as the "usual" means of retrovirus survival, these viruses bring to mind the concept of "obligate communicability" described by Gross (9).

These speculations aside, the REV interspecies p30 competition assay using antibody to mammalian p30 provides a broadly cross-reactive system which may be useful in studying species that have not yet unequivocally yielded type C viruses or gene products.

This work was supported by Public Health Service contract N01-CO-75380 from the Virus Cancer Program, National Cancer Institute.

We thank Sharon Bladen and James Dobbs for excellent technical assistance, and Terry Copeland for sequence analyses.

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