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Distribution and Expression in Mammals of Genes Related to an Endogenous Type C RNA Virus of *Odocoileus hemionus*

STEVEN R. TRONICK,* MARJORIE M. GOLUB, JOHN R. STEPHENSON, AND STUART A. AARONSON

Laboratory of RNA Tumor Viruses, National Cancer Institute, Bethesda, Maryland 20014

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An endogenous type C virus recently isolated from the Columbian black-tailed deer (*Odocoileus hemionus*) was used as a molecular probe to study the distribution of virus-related nucleotide sequences in cellular DNAs of mammalian species. By DNA-DNA hybridization, the most extensive homology was demonstrated between the viral complementary DNA and cellular DNA isolated from *Odocoileus* species. DNAs of representatives of other genera within the same family, *Cervidae*, were partially related to the virus, consistent with the phylogenetic relationship of these species to *Odocoileus*. *O. hemionus* viral sequences were also detected within cellular DNAs of members of a more distantly related artiodactyl family, *Bovidae*. These findings suggest the genetic transmission of type C viral genes within cervids and bovids for a period of at least 25 to 30 million years. There was no detectable nucleotide sequence homology between *O. hemionus* virus and representatives of other major groups of mammalian type C viruses. These results indicate that despite the known antigenic relatedness of mammalian type C viruses, the *O. hemionus* virus has diverged sufficiently to be considered the prototype of a separate group. By radioimmunological techniques, it was possible to detect and partially purify, from normal tissues of cervid species, antigens related to the major structural protein of the *O. hemionus* virus. The present findings, that *O. hemionus* virus has been genetically transmitted for millions of years and yet has maintained the ability to be expressed as infectious virus, argue for positive evolutionary selective pressures for the maintenance of type C viral genes.

Species representing several classes of vertebrates are known to contain genetic information for type C RNA viruses (reviewed in reference 1). Endogenous viruses so far isolated from mammals have been found to share antigenic determinants among one or more (5, 20, 40, 43, 46) of their structural components, suggesting an evolutionary relatedness of these viruses. Most mammalian endogenous viruses appear to comprise two major groups, more closely related to prototype endogenous viruses of either rodents or primates (9, 11, 49). Recently, an endogenous type C virus has been isolated from cells of the Columbian black-tailed deer, *Odocoileus hemionus* (2). The present studies were undertaken to study the genetic relatedness of this new virus with the known mammalian endogenous type C virus groups. The distribution of genetic sequences and expression of gene products related to the *O. hemionus* virus has been investigated in species phylogenetically related to deer representing different families within the order *Artiodactyla*.

MATERIALS AND METHODS

Viruses. *O. hemionus* type C virus was propagated in a human tumor cell line (A673) or a horse cell line (CCL57) obtained from the American Type Culture Collection, Rockville, Md. (2). Virus was purified from tissue culture fluids (harvested at 24-h intervals) by isopycnic banding. Other viruses were obtained as sucrose gradient-purified preparations through the courtesy of J. Gruber, Office of Resources and Logistics, National Cancer Institute, Bethesda, Md. Mammalian type C viruses included a virus of AKR mice, AKR-murine leukemia virus (MuLV) (18); a virus of Wistar-Furth rats (12); a virus of pigs (50); two viruses of cats, feline leukemia virus (FeLV) (33) and RD114 (28); a virus of baboons (*Papio cynocephalus*) (7); and viruses isolated from gibbon apes (22) and a woolly monkey (48). Other viruses included mouse mammary tumor virus (26), Mason-Pfizer monkey virus (17), and avian myeloblastosis virus (6).

Tissues. Frozen tissues were kindly supplied by B. O'Gara, University of Montana, Bozeman; R. Dieterich, University of Alaska, Fairbanks; R. Hugie, University of Maine, Orono; G. Esra, Los Angeles Zoo; R. Amity, Fairfax County, Va., Ani-

mal Shelter; L. Stuart, National Institutes of Health Animal Center, Poolesville, Md.; and W. Boever, St. Louis Zoo.

Preparation of ^{3}H-labeled viral cDNA. Viral ^{3}H-labeled complementary DNA (cDNA) was prepared from sucrose gradient-purified virus in reaction mixtures containing 20 mM Tris-hydrochloride, pH 7.8; 60 mM KCl; 1 mM dithiothreitol; 5 mM $MgCl_2$; 0.2 mM each dATP, dCTP, and dGTP; 0.01 mM [^{3}H]TTP (50 Ci/mmol; New England Nuclear); 50 μg of actinomycin D per ml (Calbiochem); 0.014% (vol/vol) Triton X-100; and 50 μg of viral protein per ml. The reaction mixture also contained as primer 0.8 mg of DNase I-digested salmon sperm DNA per ml (47), the addition of which resulted in a 6- to 10-fold increase in the amount of cDNA synthesized. After incubation at 37°C for 4 h, the reaction was terminated by addition of an equal volume of 0.2 M Tris-hydrochloride (pH 7.5), 0.2 M NaCl, 0.05 M EDTA, 0.8% sodium dodecyl sulfate, 25 μg of *Escherichia coli* DNA per ml, and 1 mg of self-digested Pronase per ml. ^{3}H-labeled viral cDNA was purified as previously described (37). The specific activity of viral cDNA's used in these studies was around 2×10^7 cpm/μg. In most instances, it was possible to estimate genetic complexities of viral cDNA's by their ability to protect the ^{32}P-labeled homologous 70S RNA from RNase digestion; generally, at least 40 to 60% of the viral RNA was hybridized at a DNA:RNA molar ratio of 2, and 60 to 80% of the RNAs could be hybridized by the homologous cDNA at a ratio of 10. With viruses that grew to only low titer, including *O. hemionus* virus, it was not possible to prepare sufficient quantities of ^{32}P-labeled viral RNA for accurate complexity determinations.

Preparation of radioactive unique-sequence cell DNA. Cultured cells (*O. hemionus* and A673) were incubated in the presence of 10 μCi of [^{3}H]thymidine per ml for 48 h. The cells were harvested, and DNA was isolated and sheared as described below. To isolate the slowly reannealing fraction (14), the [^{3}H]DNA was denatured by boiling and then allowed to reassociate to a C_0t value of 200 (see below) at 65°C in the presence of 0.01 M Tris-hydrochloride (pH 7.5), 0.6 M NaCl, and 0.1 mM EDTA. Single-stranded DNA was separated from reassociated strands by hydroxyapatite chromatography as described by Britten et al. (13). The specific activity of the [^{3}H]DNAs was 2×10^5 to 3×10^5 cpm/μg. The [^{3}H]DNA representing the unique-sequence fraction was less than 5% resistant to S1 nuclease (8, 24).

Preparation of RNA and DNA. Cellular RNA was extracted by the hot-phenol method of Scherrer (36), and virion RNA was prepared as described by Schincariol and Joklik (37). RNA was determined both by absorbance at 260 nm and by the orcinol method (38). Cellular DNA was purified by a modified Marmur procedure (27) as described by Britten et al. (13). DNA was fragmented to an average size of 500 nucleotides, using a VirTis model 60K homogenizer. DNA concentration was determined by absorbance at 260 nm and diphenylamine assay.

Molecular hybridization. DNA-RNA hybridization was performed by incubating varying amounts of RNA from virions or cultured cells with 500 to 1,000 cpm of ^{3}H-labeled viral cDNA for 18 to 48 h at 68°C in 0.05 ml of 0.6 M NaCl, 10 mM Tris-hydrochloride (pH 7.5), and 0.1 mM EDTA. Hybrid formation was assayed by the S1 nuclease method (8, 24). For DNA-DNA hybridization, about 0.8 mg of cell DNA and 0.5 ng of [^{3}H]cDNA in a volume of 0.2 ml were heated at 100°C for 10 min and cooled quickly, and the reaction was carried out at 62°C (T_m of homologous hybrid minus 25°C) in the solution described above for DNA-RNA hybridization. Samples were removed at various times, frozen in a dry ice-acetone bath, and then assayed for hybrid formation by the S1 nuclease method. Hybridization data are expressed in terms of C_0t and C_rt (moles · second per liter) for reactions involving DNA-DNA and DNA-RNA, respectively (14). These values have been corrected to a monovalent cation concentration of 0.18 M based on the data of Britten et al. (13).

Radioimmunoassays. A double-antibody competition immunoassay that detects *O. hemionus* virus has been described (2). Briefly, reaction mixtures containing a limiting amount of rabbit antiserum to the *O. hemionus* virus and unlabeled competing antigen in 0.01 M Tris-hydrochloride, 0.1 M NaCl, 0.001 M EDTA, 0.1% Triton X-100, and 1% bovine serum albumin, pH 7.8, were incubated at 37°C for 1 h. About 10,000 cpm of ^{125}I-labeled RD114 viral p30 was then added, and incubation was continued for 3 h at 37°C, followed by 18 h at 4°C. Reaction mixtures were subsequently diluted to 1.0 ml in 0.01 M Tris-hydrochloride, pH 7.8, mixed with 0.025 ml of goat anti-rabbit immunoglobulin G, incubated for 3 h at 4°C, and centrifuged at 2,500 rpm for 15 min, and the resulting precipitate was measured for ^{125}I radioactivity. Immunoassays for the major structural proteins of Rauscher MuLV and FeLV have been reported in detail (40).

Isolation of *O. hemionus* virus-related proteins from normal tissues. Extracts were prepared by tissue homogenization in an equal volume of 0.01 M Tris-hydrochloride, 0.01 M EDTA, and 0.5% Triton X-100, pH 7.8. Homogenates were clarified by centrifugation for 60 min at 30,000 rpm in a Beckman type 30 rotor, dialyzed overnight against 0.01 M Tris-hydrochloride, 0.001 M EDTA, and 0.1% Triton X-100, pH 7.8, and applied to a Whatman DE-52 column (1.5 by 25 cm) equilibrated with the same buffer. The column was washed with 100 ml of the same buffer, and bound proteins were eluted with 200 ml of a 0.0 to 0.5 M KCl linear gradient. Individual fractions were dialyzed against 0.01 M Tris-hydrochloride and 0.001 M EDTA, pH 7.8, and assayed for immunological reactivity as described above. Fractions with the highest levels of reactivity were pooled and subjected to further purification by agarose gel filtration in the presence of 6 M guanidine hydrochloride as previously described (45).

RESULTS

Analysis of *O. hemionus* virus for nucleotide sequence homology with other mammalian type C viruses. Previous studies have indicated that known mammalian type C viruses share cross-reactive antigenic determinants

among several of their structural proteins (5, 20, 40, 43, 46). These findings have suggested a common progenitor in their evolution (5). Nonetheless, type C viruses endogenous to some mammalian species exhibit greater nucleotide sequence homology than would be expected on the basis of the degree of evolutionary divergence of the species from which these viruses originate (9, 11, 29, 32, 49, 52). In the first series of investigations, the type C virus isolated from *O. hemionus* cells was analyzed by molecular hybridization for genetic sequence homology with representatives of these known mammalian endogenous virus groups.

About 85% of the *O. hemionus* viral cDNA formed hybrids with its homologous virion RNA, whereas the same probe did not detectably anneal to RNA of any of the other viruses tested (Table 1). cDNA's were also prepared from representative rodent and primate type C viruses and tested with these same RNA preparations. AKR-MuLV cDNA hybridized significantly to woolly monkey viral RNA, and rat viral cDNA annealed to FeLV RNA. Neither cDNA hybridized to *O. hemionus* viral RNA or to RNA of a representative endogenous primate virus. As previously reported (49), viral cDNA prepared from the baboon endogenous virus hybridized significantly to RD114 viral RNA. However, this probe lacked detectable homology with RNA of *O. hemionus* virus or any of the rodent viruses tested. None of the type C viral cDNA probes hybridized with RNA of mouse mammary tumor virus or Mason-Pfizer monkey virus (Table 1). These results indicate that *O. hemionus* virus lacks detectable nucleotide sequence homology with representative rodent and primate endogenous type C viruses.

Distribution of *O. hemionus* viral nucleotide sequences in cellular DNAs of species phylogenetically related to *O. hemionus*. Deer species belong to the family *Cervidae*, within the order *Artiodactyla* (even-toed ungulates). The distribution of nucleotide sequences homologous to *O. hemionus* viral cDNA was determined in cellular DNAs of several cervid species. Cellular DNAs of *O. hemionus* and *O. virginianus* (white-tailed or Virginia deer) exhibited nearly identical hybridization kinetics with the *O. hemionus* viral cDNA (Fig. 1). The maximum extent of hybridization with each was around 85% at a C_0t of 2×10^4. DNAs isolated from species of other cervid genera, including *C. canadensis* (American elk or wapiti), *C. elaphus* (European red deer), *Rangifer arcticus* (caribou), *Dama dama* (fallow deer), *Alces alces* (moose), and *Muntiacus muntjack* (muntjac), also hybridized significantly to *O. hemionus* viral cDNA. The kinetics of the reaction of each were very similar (Fig. 1), and the

TABLE 1. *Analysis of O. hemionus type C virus for nucleic acid homology with other reverse transcriptase-containing RNA viruses*

Viral RNA source	Maximum hybridization values (%) with cDNA prepared from the following type C viruses[a]			
	O. hemionus	*Mus musculus* (AKR)	*Rattus norvegicus* (Wistar/Furth)	*Papio cynocephalus*
Type C				
O. hemionus	100	<3	<3	<3
AKR-MuLV	<3	100	6	<3
Rat	<3	5	100	<3
Woolly monkey	<3	10	<3	<3
Gibbon ape	<3	9	<3	<3
Pig	<3	<3	<3	<3
FeLV	<3	<3	10	<3
Baboon	<3	<3	<3	100
RD114	<3	<3	<3	29
Avian myeloblastosis virus	<3	<3	<3	<3
Non-type C				
Mouse mammary tumor virus	<3	<3	<3	<3
Mason-Pfizer monkey virus	<3	<3	<3	<3

[a] Hybridization reactions were performed as described in the text. Viral RNA was extracted from either virions or virus-infected cells. Reactions were carried out to C_rt (initial nucleotide concentration × time) values 10- to 100-fold in excess of the C_rt value at which maximum hybridization was observed with the appropriate homologous system. The hybridization values obtained for each cDNA were normalized to the maximum percent hybridization obtained with the respective homologous viral RNA. Those values were as follows: *O. hemionus*, 85%; *M. musculus*, 90%; *R. norvegicus*, 92%; and *P. cynocephalus*, 92%.

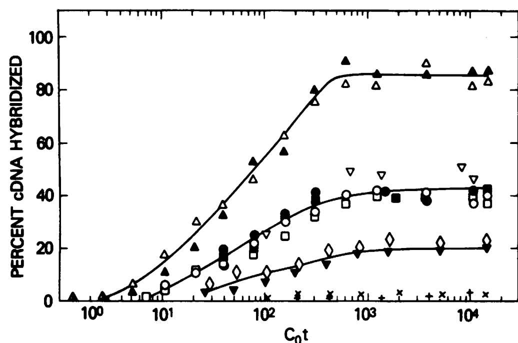

FIG. 1. *Hybridization of O. hemionus viral cDNA to cellular DNAs of representative artiodactyl species. DNA-DNA hybridization was performed as described in the text. [³H]cDNA, prepared from O. hemionus virus grown in human A673 cells, was annealed to cellular DNAs extracted from normal liver tissues of the following species: black-tailed deer, O. hemionus (△); white-tailed deer, O. virginianus (▲); caribou, Rangifer arcticus (□); elk, Cervus canadensis (○); red deer, C. elaphus (●); fallow deer, Dama dama (■); moose, Alces alces (▽); mountain sheep, Ovis canadensis (◇); blackbuck, Antelope cervicapra (▼); llama, Llama peruana (×); and pig, Sus scrofa (+). The results represent mean values of at least two experiments.*

final extents of hybridization achieved were about 40% at C_0t values of 2×10^4.

The distribution of sequences related to *O. hemionus* viral cDNA was also investigated in DNAs of species representing other families of the order *Artiodactyla*. Cellular DNAs tested included those of *Suidae* (domestic pig), *Camelidae* (llama), and *Bovidae* (antelope and sheep). The extent of hybridization achieved with representative bovid species was around 20% (Fig. 1), whereas annealing of the *O. hemionus* viral cDNA by suid and camelid DNAs was not significantly above the background levels of around 1 to 5% achieved with other cellular DNAs (Fig. 1 and Table 2). The genetic complexity of viral cDNA was not possible to determine. Thus, the probe may not uniformly represent the viral genome. If those sequences represented in the probe have diverged at a rate that differs from that of the total viral genome, the hybridization values obtained here could differ somewhat from those of a perfectly representative probe.

Thermal stability of hybrids formed between *O. hemionus* viral cDNA and cellular DNAs of various artiodactyl species. The decreased genetic sequence homology between *O. hemionus* viral cDNA and artiodactyl species other than *Odocoileus* could be explained either by deletion of specific viral nucleotide sequences from those cellular DNAs or by base-pair mismatching due to evolutionary divergence of a common endogenous viral genome in the course of the evolution of these species. To investigate these possibilities, the thermal stabilities of hybrids formed between various cellular DNAs and the *O. hemionus* viral cDNA were analyzed. Hybrids formed at a C_0t of 2×10^4 were heated from 60 to 100°C, and the thermal dissociation of the hybrids was assayed by S1 nuclease (Fig. 2 and Table 2). The T_m for the homologous hybrid of *O. hemionus* viral and cellular DNA was 86.5°C, indicating a well-matched hybrid. A similar T_m was observed for hybrids formed between the viral probe and *O. virginianus* DNA. The T_m values of hybrids of the viral probe and cellular DNAs of other cervid genera were significantly lower, with ΔT_m values ranging from 5.5 to 10.5°C. The ΔT_m values of hybrids between the *O. hemionus* virus and cellular DNAs of the bovid species tested were even lower (ΔT_m = 12 to 13°C). These results imply that the differences in extent of genetic sequence homology between *O. hemionus* virus cDNA and cellular DNAs of other artiodactyl species were due to evolutionary divergence of related endogenous viral genomes rather than to deletion of a portion of an otherwise highly conserved viral genome.

The nucleotide sequence divergence of virus-related genes in the DNA of artiodactyl species was compared with the divergence of genetic sequences of the cellular DNAs of some of these same species (Table 2). For these experiments, radioactive unique-sequence DNA was prepared from *O. hemionus* cells and hybridized with a vast excess of nonradioactive cell DNA of other mammals. The DNAs of cervid species displayed substantial homology (>80%) with *O. hemionus* unique-sequence DNA. There was decreasing sequence relatedness with DNAs of bovid and camelid species. The T_m values of these hybrids were also decreased as might be expected for such phylogenetically divergent species. These results suggest that cell DNA sequences of artiodactyls have diverged less rapidly than their endogenous viral sequences.

Reiteration frequency of *O. hemionus* viral sequences in *O. hemionus* cellular DNA. The reiteration frequency of *O. hemionus* viral nucleotide sequences within *O. hemionus* cellular DNA was next determined. The $C_0t_{1/2}$ of the reaction involving viral cDNA with *O. hemionus* liver DNA was 5.5×10^1 (Fig. 3). The $C_0t_{1/2}$ for the reassociation of *O. hemionus* unique-sequence cell DNA was 2×10^3. These findings indicate the presence of at least 30 to

TABLE 2. *Nucleotide sequence homology and thermal stabilities of hybrids formed between O. hemionus viral cDNA and O. hemionus unique-sequence cell DNA and the cellular DNA of mammalian species*[a]

Source of DNA	*O. hemionus* viral cDNA		*O. hemionus* unique-sequence cell DNA	
	Percent hybridized	ΔT_m[b]	Percent hybridized	ΔT_m[b]
Deer (*Cervidae*)				
Black-tailed (*O. hemionus*)	100	0	100	0
White-tailed (*O. virginianus*)	100	0	NT[c]	
Fallow deer (*Dama dama*)	48	6.5	NT	
Caribou (*Rangifer arcticus*)	46	7.5	83	4
Elk (*Cervus canadensis*)	47	6.5	NT	
Red deer (*C. elaphus*)	47	6.5	86	4.5
Muntjac (*Muntiacus muntjack*)	45	5.5	86	3
Moose (*Alces alces*)	47	10.5	69	8
Other artiodactyls				
Blackbuck (*Antelope cervicapra*)	23.5	12	70	8
Mountain sheep (*Ovis canadensis*)	23.5	13	65	8.5
Llama (*Llama peruana*)	<3	–[d]	27	14
Pig (*Sus scrofa*)	<3	–	NT	
Other mammals				
Mouse (*Mus musculus*)	<3	–	3	–
Rat (*Rattus norvegicus*)	<3	–	NT	
Cat (*Felis catus*)	<3	–	NT	
Dog (*Canis familiarus*)	<3	–	NT	
Baboon (*Papio cynocephalus*)	<3	–	NT	
Rhesus monkey (*Macaca mulatta*)	<3	–	NT	
Orangutan (*Pongo pygmaeus*)	<3	–	NT	
Human (*Homo sapiens*)	<3	–	NT	
Nonmammalian				
Salmon (*Onchorhynchus* spp.)	<3	–	NT	
Chicken (*Gallus gallus*)	<3	–	NT	

[a] Hybridizations were carried out to a C_0t value of 2×10^4 (see text and Fig. 1). [^{3}H]thymidine-labeled *O. hemionus* unique-sequence cell DNA was prepared as described in the text. Reactions with this probe contained a 1,000-fold excess of the appropriate unlabeled liver DNA. Hybridization values for reactions with cDNA and unique-sequence DNA have been normalized to the respective maximum hybridization values observed with *O. hemionus* DNA.

[b] The T_m value for hybrids between *O. hemionus* cDNA-*O. hemionus* liver DNA was 86.5°C, and the T_m for *O. hemionus* unique-sequence DNA-*O. hemionus* liver DNA was 83.5°C.

[c] NT, Not tested.

[d] –, Hybridization too low to determine T_m.

40 copies of *O. hemionus* viral genome within the host cell genome. The $C_0t_{1/2}$ values for hybridization of the viral cDNA with cellular DNAs from other cervid families (Fig. 1) also indicate the presence of multiple viral copies within the genomes of these species. For comparison, the number of copies of virus-related nucleotide sequences present in exogenously infected cells was determined. The $C_0t_{1/2}$ value for the hybridization reaction involving *O. hemionus* viral cDNA and DNA isolated from human A673 cells infected with the *O. hemionus* virus was 0.8×10^3, whereas the $C_0t_{1/2}$ value for the reassociation of human cell unique-sequence DNA was 2×10^3. This indicates the presence of about two to three copies of viral genetic information in exogenously infected cells.

Endogenous type C viral antigen expression in cervid species. The above results indicated the preservation over a prolonged evolutionary period of multiple copies of viral genetic sequences related to *O. hemionus* virus among two families, *Cervidae* and *Bovidae*, of the order *Artiodactyla*. It was of interest to determine whether endogenous viral genes were detectably expressed in normal tissues of representative artiodactyl species. Immunological analysis involved a competition radioimmunoassay in which limiting antiserum to *O. hemionus* virus was used to precipitate ^{125}I-labeled p30 of RD114 virus. This assay sensitively detects the major structural antigen of *O. hemionus* virus. Liver cell extracts, from *R. arcticus* and *O. virginianus*, showed significant reactivity in this immunoassay. In contrast,

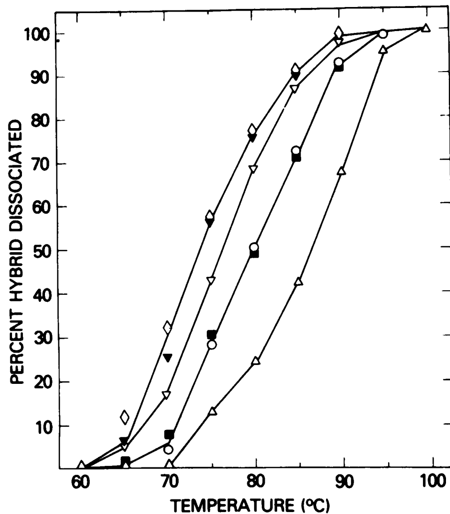

FIG. 2. *Thermal dissociation of hybrids formed between O. hemionus viral cDNA and DNAs of representative artiodactyl species. Reactions were carried out to a C_0t value of 2×10^4. Samples were cooled quickly, and aliquots were incubated for 5 min at 5-degree intervals from 60 to 100°C in 0.6 M NaCl. The amount of hybrid remaining at each temperature was determined by the S1 nuclease method. Data have been normalized to the actual number of S1-resistant ^3H counts after incubation at 60°C. The melting curves for each hybrid are designated by the same symbols used in Fig. 1.*

the same extracts did not react in immunoassays for the major structural proteins (p30) of Rauscher MuLV or FeLV.

To further establish the specificity of the immunological reactions, the tissue-associated antigens were partially purified by DEAE ion-exchange chromatography and then subjected to molecular size analysis by agarose gel filtration chromatography in the presence of 6 M guanidine hydrochloride. The antigenic reactivity partially purified from *R. arcticus* liver co-chromatographed with a ^{125}I-labeled p30 marker (Fig. 4). Similar results were obtained with antigen from *O. virginianus* liver (data not shown). These procedures resulted in 50- to 100-fold increases in the antigenic activity per milligram of cell protein. The immunological specificity of the partially purified antigens after sequential purification by ion-exchange chromatography and agarose gel filtration is indicated in Fig. 5. Each competed efficiently and to a final extent of at least 80% in the assay that detected *O. hemionus* virus p30; however, detectable reactivity was not observed in control immunoassays. These results indicate that *O. hemionus* virus-related genetic sequences are detectably expressed in normal tissues of species representing at least two cervid genera.

DISCUSSION

The distribution of genetic sequences related to an endogenous type C virus of the Columbian black-tailed deer (*O. hemionus*) has been studied in the cellular DNAs of phylogenetically related species. By molecular hybridization, cDNA prepared from *O. hemionus* virus demonstrated the most extensive nucleotide sequence homology with DNAs of species within the genus *Odocoileus*. DNAs of species of other genera within the cervid family hybridized to about 50% of the level obtained with *Odocoileus* cellular DNA. The lower T_m values of hybrids formed between the viral cDNA and DNAs of less phylogenetically related species indicated random base-pair mismatching, as might be expected to accumulate during the course of evolution (23).

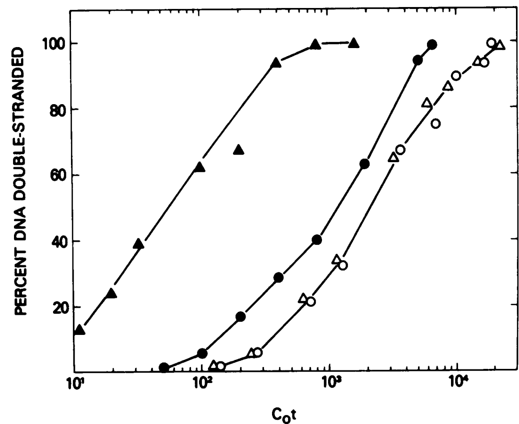

FIG. 3. *Comparison of the reiteration frequency of O. hemionus viral sequences in O. hemionus cellular DNA and DNA of O. hemionus virus-infected human cells. O. hemionus viral cDNA was hybridized with O. hemionus liver DNA (▲), and the kinetics of this reaction were compared to the rate of hybridization of O. hemionus ^3H-labeled unique-sequence cell DNA with a 1,000-fold excess of O. hemionus liver DNA (△). The O. hemionus viral cDNA was also hybridized with DNA isolated from human cells (A673) infected with the O. hemionus virus (●), and the rate of this reaction was compared with that for the reannealing of human cell ^3H-labeled unique-sequence DNA with a 1,000-fold excess of DNA isolated from uninfected A673 cells (○). The conditions for hybridization reactions were as described in the text. A clonal line of O. hemionus virus-infected A673 cells was obtained by a previously described microtiter procedure (44). Data were normalized to the maximum percent hybridization obtained for each reaction. These values were 85%, viral cDNA versus O. hemionus liver DNA; 80%, viral cDNA versus infected A673 cell DNA; 74%, O. hemionus unique-sequence DNA; 76%, human unique-sequence DNA.*

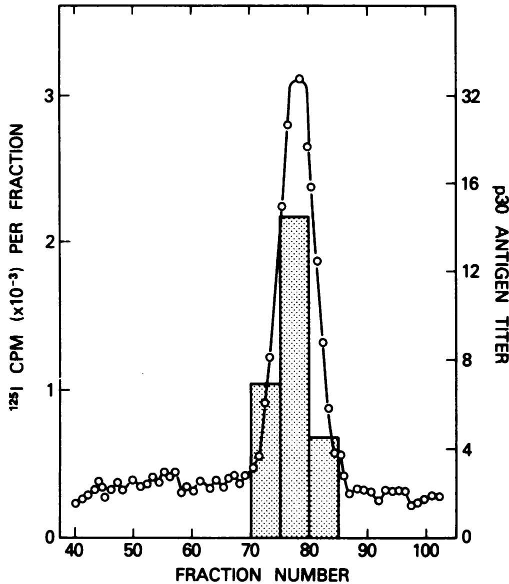

FIG. 4. *Molecular size analysis of O. hemionus virus-related antigen partially purified from liver extracts of the cervid species R. arcticus. Extracts were prepared and partially purified by DEAE-cellulose chromatography as described in the text. Fractions containing antigenic activity were pooled, concentrated, mixed with tracer amounts of ^{125}I-labeled RD114 p30, and subjected to agarose gel filtration chromatography in the presence of 6 M guanidine hydrochloride as previously described (45). Individual fractions (1 ml) were analyzed for radioactivity, pooled in groups of five, dialyzed, concentrated, and assayed for antigenic activity (see text). Immunoassay results are expressed as the reciprocal of the antigen dilution at which 50% displacement of ^{125}I-labeled p30 was achieved. Symbols: Radioactivity, ○; antigenic activity, stippled bars.*

The degree of nucleotide sequence divergence demonstrated between *O. hemionus* viral cDNA and cellular DNAs of representative species within the cervid family is consistent with postulated phylogenetic relationships between these species (34). The fossil record indicates that ancestors of present-day *Odocoileus* species arose during the early Pliocene and migrated to the New World during the middle or late Pliocene. This genus has been separated from other cervids for at least 5 to 10 million years (3, 34). Our findings that type C viral genetic sequences are also present in related Old World cervid genera argue that this endogenous virus became associated with the ancestors of *Odocoileus* before their migration to the New World. Present day *Odocoileus* species are the only vertebrates of New World origin so far shown to contain endogenous type C viral genes. Thus, the present results indicate that type C viruses have existed in the New World for at least 5 to 10 million years.

Genetic sequences partially related to *O. hemionus* virus were also demonstrated in cellular DNAs of artiodactyls belonging to the more distantly related bovid family. This is the first evidence for the presence of endogenous type C viral genes in this family. Cervid and bovid families are believed to have shared their most recent common ancestor about 25 to 30 million years ago (34). The existence of related endogenous viral information in representatives of each family indicates that type C virus must have become genetically associated with the progenitor of these families over 25 to 30 million years ago. The lack of detectable hybridization of the *O. hemionus* viral cDNA to cellular DNAs of species representing more distantly related artiodactyl families, *Tylopoda* and *Suidae*, argues that this type C virus may have entered the cervid-bovid lines after their divergence from other artiodactyl families around 50 to 55 million years ago (34). Alternatively, the genes of a common progenitor virus may have diverged sufficiently so as to no longer be detectable in cellular DNAs of more distantly related artiodactyls, using *O. hemionus* viral cDNA as a molecular probe.

Analogous molecular hybridization techniques have indicated that endogenous type C viruses have existed within primate and rodent orders for at least 30 million years (11, 49). The evidence also suggests the interspecies transmission of primate and rodent viruses to species

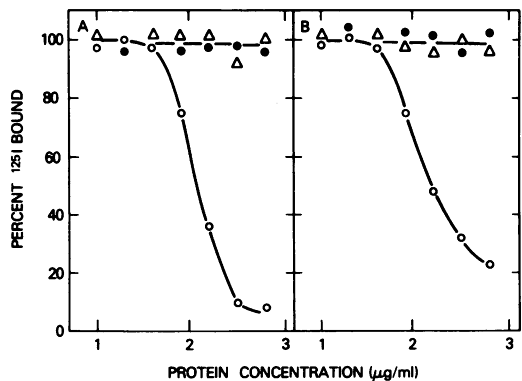

FIG. 5. *Immunological analysis of 30,000-molecular-weight O. hemionus virus-related antigen partially purified from normal livers of cervid species. Antigen preparations were analyzed in competition radioimmunoassays for the p30 antigens of the O. hemionus type C virus (○), Rauscher murine leukemia virus (●), and FeLV (△), as described in the text. Antigenic reactivity of partially purified antigens prepared from (A) O. virginianus and (B) R. arcticus.*

representing other mammalian orders during more recent periods of evolution (5 to 10 million years ago) (11, 49). In the present report, analysis of cellular DNAs of a few species representing mammalian orders other than *Artiodactyla* failed to reveal evidence of genetic sequence homology with *O. hemionus* virus. A more comprehensive analysis would be necessary to exclude the possibility of interspecies transmission of the *O. hemionus* virus or its evolutionary precursor to or from other mammalian species.

Recent studies have demonstrated the immunological relatedness of several of the respective structural proteins of mammalian type C viruses (5). This evidence strongly argues that mammalian type C viruses have had a common origin. By molecular hybridization, *O. hemionus* virus lacked detectable nucleotide sequence homology with other mammalian type C viruses. Thus, endogenous viruses of cervid and bovid species appear to have diverged sufficiently from other mammalian endogenous viruses to be considered a separate group.

In the present report, nucleotide sequences related to *O. hemionus* viral cDNA were found to be reiterated 30 to 40 times within the genome of *O. hemionus*. After the initial demonstration of multiple copies of endogenous viruses within mouse (16, 19) and avian (4, 35, 51) cellular DNAs, the reiteration of endogenous viral genes was also documented in other species (10). Another common characteristic of endogenous viruses is the expression by normal cells of viral antigens in the absence of complete virus release (21, 30, 41, 42, 45). It was possible to detect and partially purify viral antigens related to *O. hemionus* virus from normal tissues of two cervid species. This antigen was shown to cross-react with mammalian type C viral p30 and to co-chromatograph with p30 by ion exchange and gel filtration. Thus, in many respects, the endogenous virus of *O. hemionus* cells has been shown to be analogous to endogenous viruses of other mammalian species. That type C viruses have been genetically transmitted for millions of years and yet have maintained their ability to be partially or even completely expressed argues strongly for positive evolutionary selective pressures for the preservation of type C viral genes.

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