Physical and Chemical Properties of an Oncornavirus Associated with a Murine Adrenal Carcinoma Cell Line

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A type C oncornavirus has been isolated from a continuous cell line of murine adrenal carcinoma in culture. The particles have a buoyant density of 1.165 g/cm³, exhibit typical type C morphology by electron microscopy, possess an RNA-dependent DNA polymerase, and have a high molecular weight RNA (6.1×10^6) which can be denatured to a homogeneous lower molecular weight species (3.2×10^6) when extracted from rapidly harvested "immature" virions. The virus is related antigenically to other mammalian oncornaviruses and exhibits a similar, although much more complex, sodium dodecyl sulfate gel electrophoretic profile of virion proteins when compared to the profiles of other type C RNA tumor viruses.

Virus particles with typical type C morphology and the properties of murine leukemia oncornaviruses were found to be associated with the cloned cell line Y-1 from a functional adrenal carcinoma (16). The original tumor arose as a result of exposure of LAF₁ mice to the ionizing radiation of a nuclear fission bomb test explosion (23) and, although it is not directly possible to link the etiology of the tumor with the expression of this particle, the expression of virus in radiation-induced tumors is a well-established phenomenon (9, 14). It has been postulated (11) that exposure to ionizing radiation or chemical carcinogens, or the advent of old age, triggers some stochastic cellular process leading to the derepression of a vertically transmitted "oncogene." This then results in tumor formation and perhaps, concomitantly, virus expression. Y-1 virus is one of the few such type C oncornaviruses found to be associated with cultured tumor cells of epithelial origin (i.e., a carcinoma) and lends universality to the oncogene concept originally postulated from data obtained for mesenchymal neoplasms (i.e., sarcomas, lymphomas, and leukemias).

The studies reported in this communication further characterize the physical and chemical properties of this carcinoma-associated virion. A later report will deal with its *in vivo* characteristics.

MATERIALS AND METHODS

Solutions and radioactive isotopes. Standard virus buffer contained 0.1 M NaCl, 1 mM EDTA, and 10 mM Tris-hydrochloride, pH 7.2 (NET buffer). Except as otherwise indicated, gradient solutions

were prepared on a weight/weight basis. [*methyl*³H]-thymidine-5'-triphosphate (47.5 Ci/mmol) was obtained from New England Nuclear Corp. [³H]uridine (27 Ci/mmol), and [³²P] as orthophosphate (carrier free, 1 mCi/ml) were purchased from Amersham/Searle Corp.

Cell cultures. Murine adrenal carcinoma cells (clone Y-1, ATCC no. CCL-79) were maintained in Ham's F-10 (GIBCO). Moloney leukemia virus was propagated in high-passage-level Swiss mouse embryo cells (courtesy of A. Hackett, Special Virus Cancer contract no. E-73-2001-101) maintained in Eagle minimal essential medium.

Virus harvest and purification. Y-1- and Moloney virus-containing culture fluids were removed from the cells every 24 h, or at times otherwise specified, and cleared of cellular debris by low speed centrifugation. Virus was harvested by passage through the Spinco CF-32Ti continuous-flow zonal rotor over a 25 to 55% discontinuous sucrose gradient. Purification was by rate-zonal centrifugation through 25 to 55% continuous sucrose gradients in the Spinco SW 27 rotor. Virus-containing fractions were diluted in NET buffer and pelleted in the Spinco 60Ti rotor. These pellets were either used immediately for the operations described below or were resuspended in NET buffer containing 10% glycerol and 0.02 M dithiothreitol (DTT) and stored at -70 C until used.

Preparation of radiolabeled virus and cells. For the various operations described below requiring radiolabeled virus, subconfluent monolayers of virus-infected cells in plastic flasks (Falcon Plastics) were cultivated in culture medium with [³H]uridine, 25 μ Ci/ml, or [³²P], 25 μ Ci/ml. The tissue culture fluids were then collected after 16 to 24 h of incubation, except as otherwise indicated, and the radiolabeled virus was harvested and purified as described above. HeLa cells were incubated in the presence of carrier-free [³²P], 25 μ Ci/ml, for 16 h, and the cells were removed for RNA extraction.

RNA extraction and analysis. Y-1 cells and Moloney virus-infected mouse embryo cells were incubated for 16 h in the presence of either [^3H]uridine or [^{32}P], and the culture fluids were collected. Thereafter, fluids were replaced and collected at 5- or 15-min intervals. The 16-h harvests contain a population of radiolabeled virus representing an age range from newly synthesized species ("immature" virions) to those with a 16-h extracellular life ("mature" virions), whereas the 5- and 15-min harvests ("rapid harvests") contain radiolabeled virions which are predominantly immature. These various viral preparations were gradient density purified, and high speed pellets were subjected to disruption and electrophoresis in 2.4% polyacrylamide gels as previously described (C. H. Riggan, M. C. Bondurant, and W. M. Mitchell, *Intervirology*, in press). In some instances the virus material was first heat-denatured at 80 C for 3 min before electrophoresis. After electrophoresis at 7 mA/gel for 2.5 h the gels were frozen and sliced, and the slices were analyzed for radioactivity by liquid scintillation. An internal gel migration marker of ribosomal RNA was prepared by the extraction of [^{32}P]-labeled HeLa cell RNA by the method of Scherrer and Darnell (20).

Analysis of viral DNA polymerase. Except as otherwise noted, viral DNA polymerase was assayed in a solution containing 0.015% (vol/vol) Nonidet P-40, 40 mM KCl, 20 mM DTT, 12 mM MgCl_2 , [*methyl* ^3H]thymidine-5'-triphosphate (47.5 Ci/mmol) at 12.5 $\mu\text{Ci/ml}$, poly (rA)-oligo (dT) $_{10}$ (P-L Biochemicals, Inc.) at 10 $\mu\text{g/ml}$, and 50 mM Tris-hydrochloride, pH 8.3. Incubation was for 60 min at 37 C. Trichloroacetic acid-precipitable radioactivity was determined by liquid scintillation counting. A gradient analysis of virion-associated polymerase activity was performed on samples of fractions from a 25 to 55% sucrose gradient of virus centrifuged to equilibrium in the Spinco SW 50.1 rotor.

Electrophoretic analysis of viral proteins. Gradient purified virus was subjected to electrophoresis in 10% polyacrylamide SDS disc gels as previously described (18). The gels were fixed and stained as described by Dunker and Rueckert (5) and scanned at 600 nm in the Gilford model 2410 linear transport gel scanner.

RESULTS

Sedimentation behavior and morphology.

Isopycnic gradient centrifugation of [^3H]uridine-labeled Y-1 particles harvested from cell culture fluids demonstrates a viral buoyant density of 1.165 g/cm^3 . Electron microscopy of material from this density band reveals particles of the typical type C morphology with an outer membrane diameter of 112 ± 8 nm containing a centrally located internal nucleoid with a diameter of 78 ± 5 nm.

Analysis of high molecular weight viral RNA. The RNA extracted from Y-1 virus labeled for 16 h with [^3H]uridine (16-h virus) comigrates with RNA from 16-h [^{32}P]-labeled

Moloney leukemia virus (Fig. 1A). This RNA species, analogous to the 60 to 70S RNA found by velocity gradient centrifugation, has a molecular weight of about 6.1×10^6 relative to HeLa cell 28 and 18S RNA (Fig. 1B) with molecular weights taken as 1.9×10^6 and 0.71×10^6 , respectively (15). The values obtained from a logarithmic plot of molecular weight versus migration (Fig. 1B inset) are not precisely those of mass, however, since the mobility of RNA in polyacrylamide gels is believed to be conformation dependent and, hence, a function of molecular volume rather than of molecular weight (4).

Further demonstrating the striking similarity between the Y-1 and Moloney viruses is the electrophoretic pattern obtained when the RNA from rapid harvest (5- and 15-min) virus is heat-denatured prior to electrophoresis (Fig. 1C). The RNAs migrate primarily as a single peak, analogous to the 35S RNA of velocity sedimentation, with a molecular weight approximately 3.2×10^6 (Fig. 1C inset). In contradistinction, it is observed that when mature (16-h) virus is heat-denatured prior to electrophoresis, the gel pattern obtained is one of considerable heterogeneity (Fig. 1D) from the 3.2×10^6 region to the bottom of the gel.

Analysis of viral DNA polymerase. The Y-1 virus has an RNA-dependent DNA polymerase of relatively low activity associated with it when assayed with endogenous template. The activity only becomes significantly detectable (Fig. 2A) when the assay is supplied with the exogenous synthetic template-primer poly (rA)-oligo (dT) $_{10}$. This activity cosediments with the virus in isopycnic sucrose gradients to the viral density of about 1.16 g/cm^3 (Fig. 2B). The polymerase shows peak activity when the virus is assayed in very low concentrations of Nonidet P-40 detergent (Fig. 3A). This low and narrow detergent range around 0.015% Nonidet P-40 is consistent with previous observations for mammalian oncornavirus polymerase activity (1, 21). A monovalent cation concentration of about 30 to 100 mM KCl (Fig. 3B) and a divalent cation concentration of about 6 to 18 mM MgCl_2 (Fig. 3C) were also observed to be optimal for Y-1 polymerase activity.

Antigenic reactivity and electrophoretic analysis of viral proteins. The Y-1 adrenal cell carcinoma virion has been shown to be indistinguishable from the endogenous N-tropic viruses of BALB/c, C58, and AKR mouse cells by competition radioimmunoassay against purified 12,000 molecular weight viral polypeptide (J. R. Stephenson, personal communication). In addition, Y-1 virus exhibited the murine species-specific reactivity (8) by cross-reaction in im-

FIG. 1. Electrophoretic analysis of native and heat-denatured viral RNA. Viral and cellular RNA was prepared, as described in Materials and Methods, and [^3H] Y-1 virus RNA (●) was coelectrophoresed with either [^{32}P]HeLa cell RNA or [^{32}P] Moloney virus RNA (▲). (A) Coelectrophoresis of 16-h native Y-1 and Moloney virus RNA. (B) Electrophoresis of 16-h native Y-1 virus RNA with HeLa cell RNA marker. Inset is a plot of log of molecular weight versus migration showing a molecular weight of 6.1×10^6 for 70S Y-1 RNA with HeLa cell 28 and 18S RNA taken as 1.9×10^6 and 0.71×10^6 , respectively (see text). (C) Coelectrophoresis of heat-denatured rapid harvest Y-1 (5-min) and Moloney (15-min) virus RNA. Inset is a molecular weight plot showing the molecular weight of Y-1 heat-denatured 35S RNA species to be 3.2×10^6 relative to HeLa cell RNA migration. (D) Electrophoresis of heat-denatured 15-min Moloney virus with heat-denatured 16-h Y-1 virus RNA illustrating the age-acquired lability of the latter species.

FIG. 2. Analysis of Y-1 virus for RNA-dependent DNA polymerase activity. (A) Dilutions of gradient purified virus were assayed for polymerase by incorporation of [^3H]TMP into the trichloroacetic acid-precipitable product of a poly(rA)·oligo(dT) $_{10}$ template-primed reaction as described in Materials and Methods. (B) Cosedimentation of polymerase activity with Y-1 virus in an equilibrium sucrose gradient. [^3H]TMP incorporated (●); density (▲).

munodiffusion with both the Moloney leukemia and AKR viruses against either goat anti-Moloney or anti-AKR sera.

To compare the major virion polypeptides of

the Y-1 virus with those of other oncornaviruses, Y-1, AKR, Moloney, mouse mammary tumor, and avian myeloblastosis viruses were disrupted and electrophoresed in parallel SDS-polyacrylamide gels. Comparison of scans of stained gels (Fig. 4) demonstrates that Y-1 virus exhibits a number of polypeptides which comigrate with those of AKR and Moloney virus, including what has been termed the p30 or 30,000 molecular weight group-specific protein—the major internal protein constituent and antigenic determinant of the type C viruses (8). The electrophoretic pattern of these virus proteins is dissimilar to those for both the type C avian myeloblastosis virus and the type B murine mammary tumor virus.

DISCUSSION

For a particle isolated from the cell line of a functional endocrine carcinoma, the Y-1 virus surprisingly fulfills a large number of chemical and physical criteria which characterize the oncornaviruses associated with, and indeed

FIG. 3. Reaction conditions for optimal polymerase activity. Polymerase assays were performed as described in Materials and Methods except that the concentration of detergent, KCl, and $MgCl_2$ were varied as indicated. (A) Dependence of polymerase activity on Nonidet P-40 concentration. (B) $[K^+]$, and (C) $[Mg^{2+}]$ dependence.

often pathognomonic for, certain mammalian mesenchymal neoplasms. Grossly, Y-1 mirrors other oncornaviruses in its type C morphology, equilibrium density of 1.165 g/cm^3 (17), and a sedimentation velocity approaching 700S (unpublished observation) (2). In addition, the RNAs of Y-1 and Moloney leukemia virus strikingly resemble each other by the proximate mobility of both their high molecular weight and rapid-harvest denatured species in polyacrylamide gels. The heterogeneous gel pattern of 16-h mature virus has also been observed for both of these oncornaviruses (C. H. Riggin, M. C. Bondurant, and W. M. Mitchell, *Inter-virology*, in press). These results imply that the RNA of murine oncornaviruses begins its extracellular life enclosed in the viral coat in the high molecular weight "aggregate" form (figure not shown) which, upon heating, denatures to configurationally stable 35S "subunits." We have found no evidence of the lower molecular weight precursors described for murine sarcoma virus (6) and for Rous sarcoma virus (3). Sixteen-hour harvests of Y-1 virus also have the high molecular weight species, but the heat-denatured subunits are heterogeneous in size. The antecedents to this age-acquired subunit heterogeneity are not understood, but the end result may be one of increased lability to virion-associated or culture medium-associated nucleases (19).

The DNA polymerase of the Y-1 virus has a low endogenous activity which can be increased several fold by the addition of a synthetic template and, relative to avian myeloblastosis virus, the purified enzyme has a remarkably low specific activity (unpublished observation). This is perhaps not surprising in light of the observed low infectivity of indigenous murine oncornaviruses (10) and the fact that polymerase activity is a direct correlative of infectivity (12).

The reaction optima for Y-1 polymerase activity are consistent with those of other murine oncornaviruses. In particular, the value of 0.015% Nonidet P-40 agrees with previous reports (1, 21) that even a slight excess of detergent will produce a marked inhibition in murine virus polymerase activity. In contradistinction, although the murine virus activity is virtually eliminated at detergent concentrations above 0.05%, we and others (1, 7) have observed that avian oncornaviruses exhibit maximal polymerase activity over a wide range of Nonidet P-40 concentrations from approximately 0.1 to 0.2%.

The electrophoretic profile of the Y-1 virus proteins, particularly the p30, and the antigenic reactivity of disrupted virus clearly demonstrates the consanguinity of this particle and other murine leukemia viruses. Such a relationship may extend the oncogene hypothesis (11, 22) to the formation of epithelioid tumors by the derepression of a previously covert viral genome either possessing oncogenic potential or closely linked so as to be coexpressed with some other genomic oncogenic determinant. However, the qualification must be made that, because of the long passage of this tumor through laboratory mice (23) and consequently the long passage of the cloned cells in culture (16, 24), a pathognomonic link cannot be directly established between the radiation-induced murine carcinoma and the Y-1 particle. Further, the ubiquitous nature of oncornaviruses in cultures of mammalian cells (13) can be used as an argument for an adventitious or passenger status for this virus as well as for the oncogene concept.

Although the origins of the Y-1 virus are unknown, and may likely remain so, it should be remembered that tumors arising from mesoderm-derived tissues are more the exception than the rule, especially in human populations. If it can be demonstrated unequivocally, at

FIG. 4. Comparison of the electrophoretic mobilities of the proteins of various oncornaviruses to Y-1 virus. Viruses were disrupted and electrophoresed in 10% polyacrylamide SDS disc gels as described in *Material and Methods*. The gels were stained with Coomassie blue and scanned at 600 nm. The markers of molecular weight were: (1) human IgG, 160,000; (2) bovine albumin, 67,000; (3) ovalbumin, 45,000; (4) chymotrypsinogen A, 25,000; (5) myoglobin, 17,800; (6) cytochrome c, 12,800.

least in experimental animals, that leukemia viruses are etiologically involved in neoplasias of this type, then a case should be made for the involvement of similar viruses in neoplastic events centered in epithelial tissues from which arise the more prevalent carcinomata.

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