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Mason-Pfizer Virus Characterization: a Similar Virus in a Human Amniotic Cell Line

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Mason-Pfizer monkey virus (MP-MV) is a RNA virus with an RNA-instructed DNA polymerase first isolated from a rhesus monkey mammary adenocarcinoma in 1970. Until recently, there have been no other isolates. A continuous human amnion cell line, AO, was found to be producing a virus indistinguishable or closely related to the Mason-Pfizer virus as measured by morphological, immunological, and biochemical methods. By thin-section electron microscopy, the extracellular virus particle in AO line is 115 to 130 nm in diameter and has a preformed nucleoid (80 to 90 nm) before budding, properties which are also characteristic of MP-MV. Two proteins of the virus from the AO line were studied. By immunodiffusion, sera which react specifically with MP-MV give a line of identity with virus from the AO line. The AO viral RNA-instructed DNA polymerase purified by phosphocellulose chromatography was specifically inhibited by anti-MP-MV polymerase sera, and the AO cells contained both DNA and RNA sequences related to MP-MV ^{3}H-DNA. Viruses thus far indistinguishable from MP-MV have also recently been found by others in different human lines, raising again the question of the species of origin of MP-MV. Because the virus in the AO cells cannot be differentiated from MP-MV, we attempted to determine the origin of MP-MV virus by measuring DNA sequences related to MP-MV ^{3}H-DNA in uninfected human and rhesus monkey cells. The quantity of MP-MV-like DNA sequences in uninfected primate tissues was found to be much lower than the amount of DNA sequences of murine type-B or type-C viruses in uninfected murine tissues. Thus, it was not possible to determine whether the virus produced by AO cells or MP-MV was of human or monkey origin, or both.

Virus particles with morphological similarities to RNA viruses possessing RNA-instructed DNA polymerase (retraviruses) have been reported in transformed human cell lines (1, 8, 16, 12, 17, 32, 33; H. Bauer, personal communication). Despite morphological similarities by electron microscopy, the usual appearance of these virus-like particles did not entirely satisfy the criteria which define the ultrastructural appearance of established type-C or type-B retravirus particles (6, 10). Cultures containing these particles have shown no cytopathic effects, and these virus-like particles have not yet been adequately characterized by biochemical techniques. This report confirms the presence of virus particles in five different human cell lines (investigation of several human cell lines and viruses is being conducted as part of a joint agreement between cancer virologists in the U.S.A. and the U.S.S.R.). The virus from one of the lines, a human amniotic cell line, was selected for further study by immunological and biochemical techniques. It was found to be closely related to the Mason-Pfizer monkey virus (MP-MV), first described in 1970 in biopsy material from a rhesus mammary adenocarcinoma (9) and subsequently propagated in primate tissue culture cells.

MATERIALS AND METHODS

Cells and viruses. Five virus particle-positive human cell lines were examined in the present study. The normal human amniotic cell, AO, was studied most extensively. Characterized by Bykovsky and Miller, AO has been continuously propagated in cell culture for over 10 years (17).

The other cell lines studied were J96, isolated in 1954 from the peripheral blood of a patient with leukemia (32); CaOV, derived from a patient with an

ovarian adenocarcinoma (16); DAPT, derived from a patient with an astrocytoma (8); and T9, a human cell strain, WI-38, "transformed" by human leukemic leukocytes (1). These five lines were contaminated with *Mycoplasma*. Cell lines derived from human tumor tissue from patients with a rhabdomyosarcoma, A204, and an epidermoid carcinoma, A172, are nonvirus-releasing human cell cultures established in collaboration with George Todaro, National Cancer Institute (NCI). Details of their isolation and cultivation will be described elsewhere (D. Giard et al., in press).

Uninfected human lymphocytes, NC-37, were grown as suspension cultures. All cells were grown in the Dulbecco-Vogt modification of Eagle medium supplemented with serum. AO, J96, CaOV, DAPT, and T9 were supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.). All other lines were supplemented with 10% fetal calf serum heated at 56 C for 30 min from the same source.

The MP-MV was grown in monkey embryo fibroblasts and was obtained from M. Ahmed, Pfizer Laboratories, Maywood, N. J., through J. Gruber and D. Howell, NCI. NC-37 lymphocytes infected with MP-MV were from the same source. MP-MV from infected A204 cell cultures were prepared as twice sucrose density gradient-purified virus.

Fluids from the AO cell cultures grown in roller bottles (Bellco, Vineland, N.J.) were collected, and virus-like particles were partially purified by repeated banding in sucrose density gradients monitored by electron microscopy. The work was carried out under strict isolation at Pfizer Laboratories, Maywood, N.J. in a special unit for this purpose under the direction of K. Traul. The AO cell line was the only line in the incubator, safety cabinet, and centrifuges that were used. At all other NCI-supported facilities, strict attention was given to biohazard containment procedures designed to reduce possible contamination of either the culture or the environment. Rhesus monkey liver was obtained from animals used for the preparation of primary monkey kidney cell cultures. The tissue was frozen at −80 C until used for DNA extraction.

Preparation of ^{3}H-DNA. MP-MV preparations were incubated at 37 C in reaction mixtures of from 5.0 to 10.0 ml and contained: 0.02 M Tris-hydrochloride, pH 7.8; 0.06 M KCl; 0.006 M magnesium acetate; 2×10^{-3} M dithiothreitol (DDT); 0.02% (vol/vol) Triton X-100; 5×10^{-4} M dTTP, dATP, dGTP, and 1×10^{-5} M ^{3}H-dCTP, 20 Ci/mmol; and 25 μg of actinomycin D per ml. Reactions were incubated either for 16 to 18 h or for 30 to 60 min, and then were deproteinized with phenol-chloroform as described (5). After deproteinization, the reactions incubated for the longer time period were processed through a Sephadex G-50 column to remove phenol and unreacted isotope, treated with 0.5 N NaOH to remove RNA, and dialyzed extensively against 0.01 M Tris-hydrochloride, pH 7.2, and 10^{-5} M EDTA (5). After deproteinization, the ^{3}H-DNA from the 30 to 60 min reactions were processed by glycerol gradient centrifugation to obtain the ^{3}H-DNA complexed to 60 to 70*S* MP-MV RNA as described by Manly (19) and Schlom and Spiegelman (27). This ^{3}H-DNA was then treated with alkali and dialyzed as described above. It is important to note that all hybridization reactions reported were carried out with ^{3}H-DNA prepared in both ways to ensure that the RNA sequences detected in hybridization represented sequences included in 60 to 70*S* RNA. No attempt was made to prepare product from viral RNA prepared under denaturing conditions sufficiently stringent to remove any 4*S* RNA associated with 70*S* viral RNA or to exclude potential aggregation of cellular RNA which might cause it to migrate at 60 to 70*S* in a glycerol gradient. Two factors prevented the preparation of adequate amounts of ^{3}H-DNA product from the virus in AO cells. First, only low levels of virus are prepared in culture (~10% of MP-MV NC-37), and second, the culture is contaminated with *Mycoplasma sp.*

Preparation of cellular RNA. Cytoplasmic RNA was obtained by homogenizing trypsinized cell suspensions at 4 C in 0.01 M Tris-hydrochloride, pH 7.8, 0.0015 M magnesium acetate, and 0.01 M KCl in a glass, tightly fitting Dounce homogenizer. The extracts were then spun at 10,000 × *g* for 10 min at 4 C to remove nuclei, cellular membranes, and undisrupted cells. Sodium dodecyl sulfate (SDS) and Tris-hydrochloride, pH 9.0, were added to the clarified supernatant fluid to achieve final concentrations of 1% and 0.1 M, respectively, and then were extracted with phenol-chloroform (5). RNA was precipitated with ethanol and dialyzed as described above before use.

Preparation of cellular DNA. DNA was extracted from cells by the procedure of Marmur (20) and treated with 20 μg of RNase A per ml (previously heated for 15 min at 90 C) in 0.01 M Tris-hydrochloride, pH 7.2, and 0.01 M EDTA for 60 min at 37 C. The DNA was re-extracted twice with phenol-chloroform and dialyzed against 0.01 M Tris-hydrochloride, pH 7.2, 0.15 M NaCl, and 0.001 M EDTA. The dialyzed DNA was sonically treated by procedures described elsewhere (23) to reduce its size to 4 to 5*S* as judged by alkaline sucrose sedimentation.

Hybridization. RNA·^{3}H-DNA and DNA·^{3}H-DNA hybridization was carried out as previously described and analyzed with the use of S1 nuclease (5, 18).

Purification of AO polymerase. The concentrated (1,000-fold) virus from 5 liters of AO culture fluid was disrupted with 2% Triton X-100, 1.0 M KCl, 6×10^{-3} M DTT, and 0.02 M Tris-hydrochloride, pH 8.0, for 30 min at 4 C. The virus was then diluted with buffer containing 0.05 M imidazole, pH 7.0, 2×10^{-3} M DTT, 20% (vol/vol) glycerol, and 1 mg of bovine serum albumin per ml to a KCl concentration of 0.1 M, and was applied to a P-11 phosphocellulose column (1.5 × 15 cm) equilibrated in 0.05 M imidazole, pH 7.0, 0.10 M KCl, 2×10^{-3} M DTT, 0.10% (vol/vol) Triton X-100, and 20% (vol/vol) glycerol (buffer A). The column was washed with 100 ml of buffer A, and the enzyme was eluted with a 60-ml linear gradient of 0.10 to 0.65 M KCl in buffer A. Fractions of 4.0 ml were collected. Samples of 0.015 ml were assayed for polymerase activity in 0.10-ml reaction mixtures containing 0.02 M Tris-hydrochloride, pH 7.8, 0.04 M KCl, 0.006 M magnesium acetate, 2×10^{-3} M DTT: $0.02A_{260}$ poly rA·oligo $dt_{12\text{-}18}$, and 5

$\times 10^{-6}$ M ^{3}H-TTP (50 Ci/mmol). Reactions were for 60 min at 37 C and were processed for determination of trichloroacetic acid-precipitable material as described (28). A peak of activity was detected about midway through the gradient, following the major DNA polymerase activity of the *Mycoplasma* as previously described (29). Fractions were concentrated on an Amicon (Lexington, Mass.) ultrafiltration device with a P-10 filter and stored in samples at −170 C.

Immunological assays. Polymerase antibody studies were as described (28, 29). Details are given in the figure legends. Radioimmunoassays for mammalian type-C viral antigens were as described (22, 24, 25). Antisera to isolated MP-MV polypeptides were prepared by Roger Wilsnack, Huntingdon Labs., Baltimore, Md., by immunizing goats with pooled fractions from agarose chromatography in 6 M guanidine hydrochloride. Reactivities were analyzed by double-gel diffusion in 1% agarose, complement fixation, and immunofluorescence to exclude reactivities with bovine serum or host cellular components. Because reactivity seems to be specific for virion polypeptides, no absorption was performed. Other goat and rabbit anti-MP-MV sera were prepared by using disrupted viral preparations. They showed reactivities similar to the sera prepared against the purified polypeptides.

Electron microscopy. Cells growing as monolayers were fixed, sectioned, and examined as described (8, 22).

RESULTS

Morphological studies. Several types of particles were seen in thin-section electron microscopy of subconfluent cells from all five virus particle-positive cell lines. Particles with completely formed nucleoids (diameter 80 to 90 nm) during budding are shown in Fig. 1 (the AO cell). The extracellular particles from each of the cultures measured 115 to 130 nm in diameter and did not demonstrate prominent surface projections. Occasional extracellular particles had eccentrically placed nucleoids. Intracellular nucleoids were noted in clusters (not shown) in the cytoplasmic matrix and can be defined as intracytoplasmic A particles (5, 10).

Although over 90% of the budding particles had completely formed nucleoids (Fig. 1), the remaining buds showed varying degrees of incompletely developed nucleoids. These may represent morphological variants of the predominant particle type, sectioning artifacts, or multiple virus types. If there were two distinct virus types present in these cells, the particle with incompletely developed nucleoids would be more closely akin to morphological definitions of type-C particles, and the particle with a completely formed nucleoid is more analogous to the morphological type-B particle. As will be seen from the studies described in the following sections, the multiple morphological forms noted in these lines are most consistent with the morphogenetic pattern of MP-MV (9).

Immunological studies—polymerase. The AO cell line and its associated virus were selected for further investigation. To characterize further the virus particles in the AO culture, the potential viral polymerase was purified by phosphocellulose chromatography as described in Materials and Methods. The peak of activity detected with poly rA·oligo $dT_{12\text{-}18}$ and magnesium as divalent cation (14) was tested for inhibition by antisera to the MP-MV polymerase, and the woolly monkey type-C viral polymerase (Fig. 2). Neither control IgG nor IgG containing woolly polymerase antibody inhibited the AO polymerase activity, even at IgG levels of 150 μg. Antisera to the MP-MV polymerase showed 50% inhibition at 50 μg of IgG, with virtually complete inhibition at higher levels of antisera. MP-MV polymerase was inhibited to a comparable extent by using the same concentration of antiserum (26). The results indicate that the AO virus polymerase is closely related to the MP-MV polymerase.

Other antigens. MP-MV grown in either monkey or human cells contains a major protein species of 27,000 daltons as determined by SDS-polyacrylamide gel electrophoresis or 6 M guanidine-hydrochloride chromatography (GUHCL) (unpublished data). The localization of this polypeptide within the virion is not known. By using goat antisera to either ether-disrupted MP-MV or to an appropriate GuHCl-fractioned polypeptide of MP-MV, the predominant serum reactivity reaction is with this 27,000 dalton polypeptide. Ether-disrupted preparations of the 1.16 to 1.19 density gradient region of AO culture fluid preparations reacted with these sera demonstrate a line of identity by double diffusion in gels with MP-MV grown in either monkey or human cells (Fig. 3). Woolly monkey type-C virus prepared under comparable conditions and tested at a variety of protein concentrations (0.1 to 5 times the AO protein concentration) did not react with any of the anti-MP-MV sera tested (not shown).

Radioimmunoassays for the measurement of mammalian type-C viral interspecies determinant (gs-3) and for the major structural proteins (P30[gs]) of the woolly monkey, cat, RD114, and murine type-C viruses P30(gs) polypeptides were repeatedly negative in tests with either banded virus from the AO and J96 cell lines or 20% (vol/vol) cell extracts from all five cell lines. This confirms our previous experience that MP-MV does not cross-react with mam-

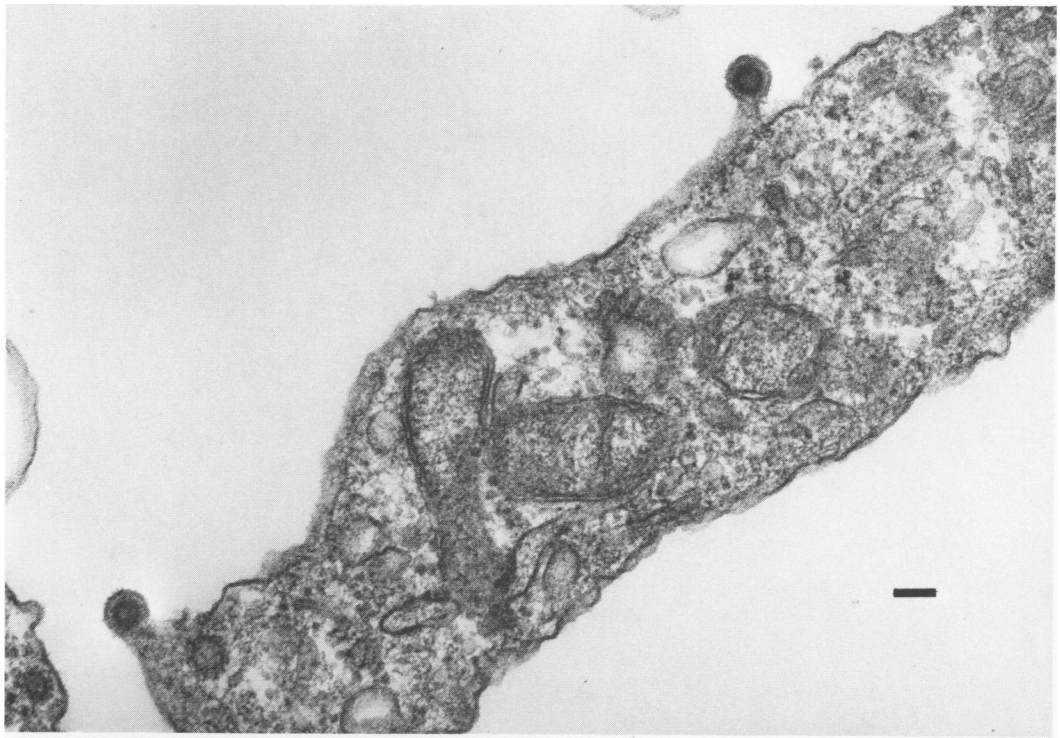

FIG. 1. *Thin-section electron micrograph of AO cell, with two budding particles. Bar represents 100 nm.*

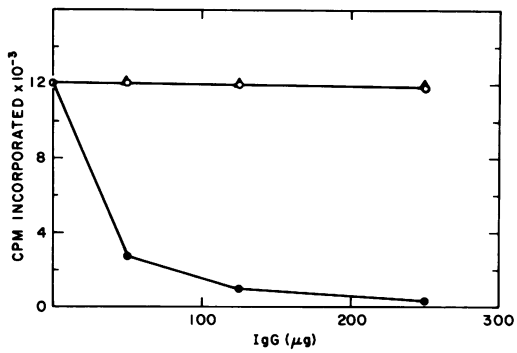

FIG. 2. *Antibody inhibition of AO polymerase. Antibody inhibition studies were performed as previously described (28). The AO polymerase, 10 μg of protein, was incubated in reactions as described in Materials and Methods by employing ^3H-CH_3-TTP as substrate (50 Ci/mmol) and poly rA·oligo dT_{12-18} as template. (▲), Control IgG; (○), anti-woolly polymerase IgG; (●) anti-MP-MV polymerase IgG.*

malian type-C viral proteins (24, 28) and further supports the conclusion that there is only a single virus type in all five cell cultures.

Hybridization. Because proteins of the AO virus reacted immunologically with sera against MP-MV proteins, we next examined the nucleic acid sequences in the RNA and DNA of AO cells. The cytoplasmic RNA from AO cells, NC-37 uninfected cells, and NC-37 cells infected with MP-MV were hybridized to MP-MV ^{3}H-DNA (Fig. 4). Both the MP-MV-infected NC-37 cells and the AO cells contain RNA which readily hybridizes to the MP-MV ^{3}H-DNA product. With approximately 100 μg of MP-MV-infected NC-37 cellular RNA and 600 to 700 μg of AO cellular RNA, respectively, saturation values were achieved. At saturation values, 60–70% of the product was rendered resistant to S1 nuclease digestion. RNA from uninfected NC-37 human cells and two other human tumor cell lines (A204 and A172) gave less than 1 to 2% hybridization with the MP-MV ^{3}H-DNA (data not shown). The results indicate that comparable final levels of RNA·^{3}H-DNA hybridization with MP-MV ^{3}H-DNA can be detected in cultures producing MP-MV or AO virus. Whether the RNA measured in both cases is the same, or simply highly related, will require additional study.

To test for viral DNA sequences in AO cell culture, C_0t studies (7, 11) were performed by utilizing the single-stranded ^{3}H-DNA copy of the MP-MV RNA. The cellular DNAs studies included calf thymus DNA, NC-37 DNA, AO

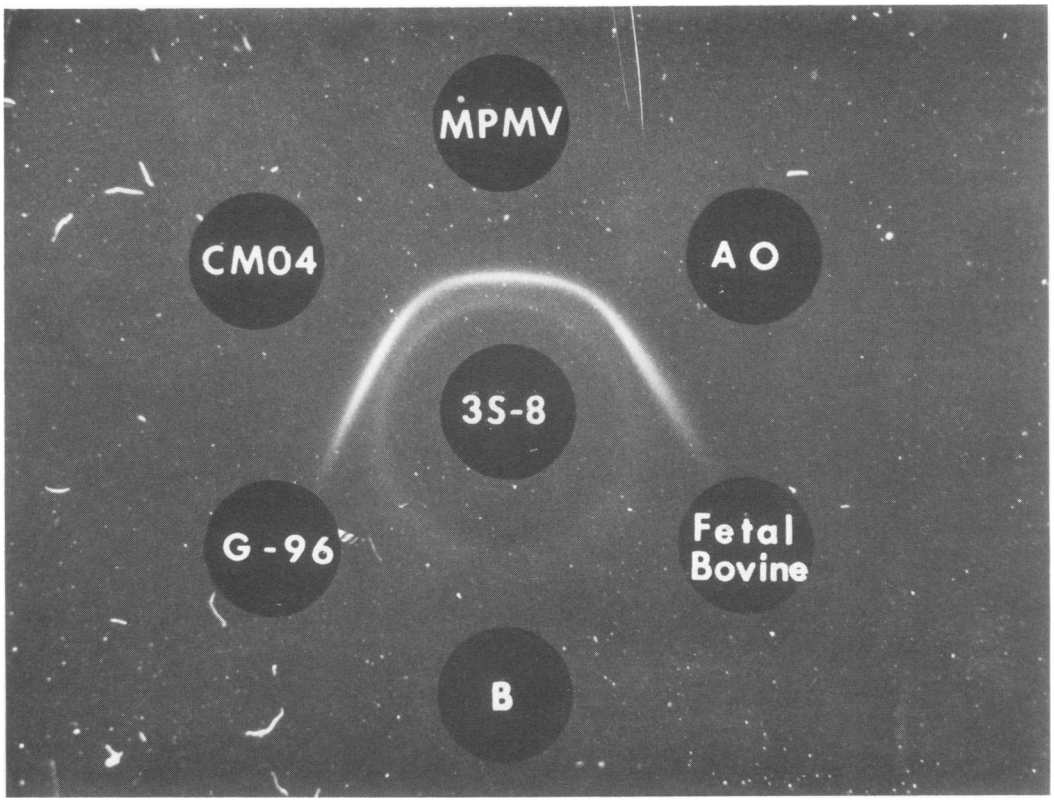

FIG. 3. *Immunodiffusion analysis of MP-MV and AO purified virus with goat anti-MP-MV. Serum (9.2 mg/ml) is contained in the center well (0.02 ml). From the top well in a clockwise direction, the wells were filled with MP-MV from monkey embryo cells (MPMV) (1,500 μg/ml) and virus from AO cells (AO) (850 μg/ml), fetal bovine serum (~6 mg/ml), phosphate-buffered saline (B), G96, J96, (G-96) 20% cell (vol/vol) extract (2 mg/ml), and MP-MV from A204 (CM04) cells (1,250 μg/ml). All preparations except the buffer and fetal bovine serum were extracted twice with 10 volumes of diethyl ether to solubilize the viral constituents. The failure of the G96 cell extract to react reflects the lower viral protein content. Even with banded virus, the AO well had to be filled four times to give the reaction shown.*

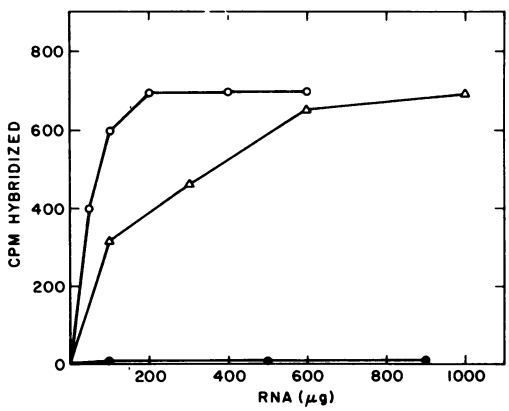

FIG. 4. *RNA·^{3}H-DNA hybridization with AO cellular RNA. Hybridization reactions contained approximately 1,100 counts/min of MP-MV ^{3}H-DNA product made as described in Materials and Methods from an endogenous reaction of the MP-MV. Hybridization and analysis by S1 nuclease were performed as previously described (5). In the absence of RNA, 40 trichloroacetic acid-precipitable counts/min above counter background were retained on membrane filters; counter background was 25 counts/min and was subtracted.* (○), *RNA from MP-MV infected NC-37;* (△), *cellular RNA from AO cells;* (●), *RNA from NC-37, A204, or A172 cells.*

DNA, MP-MV-infected NC-37 cellular DNA, and rhesus monkey liver DNA (Fig. 5). The MP-MV single-stranded ^{3}H-DNA readily reacted with the AO cellular DNA and MP-MV NC-37 cellular DNA. At a C_0t value of 2 to 3 × 10^4, approximately 70 to 80% of the single-stranded DNA had hybridized. In contrast, no hybridization was detected with calf thymus DNA (less than 10 counts/min above a counter background of 25 counts/min). The DNA from

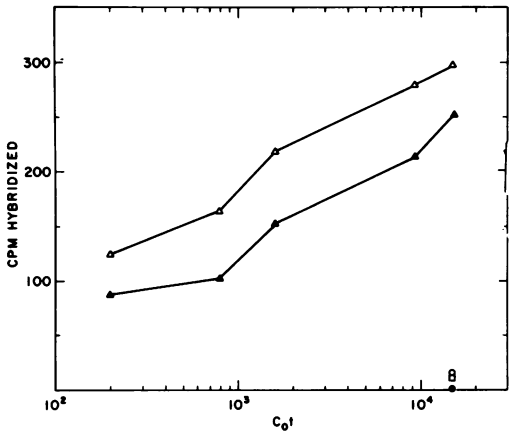

FIG. 5. *C_0t analysis of AO cellular DNA. DNA·^{3}H-DNA hybridization was performed as previously described (23). Counter background was 25 to 30 counts/min and has been subtracted from all values. Each reaction contained 350 trichloroacetic acid counts per min per sample tested. MP-MV ^{3}H-DNA hybridized to DNA from NC-37 cells infected with MP-MV (Δ). MP-MV ^{3}H-DNA hybridized to DNA from AO cells (▲), rhesus monkey liver DNA (○), NC-37 cellular DNA (□), and calf thymus DNA (●).*

uninfected NC-37 cells and rhesus monkey liver consistently (10 separate experiments) gave low levels of hybridization (20 to 30 counts/min over calf thymus background, 2 to 3% of the counts). Because the final values of hybridization were so low with these latter DNAs, no attempt was made to perform a full C_0t analysis on them. However, the results indicate that DNA from both the AO culture and the NC-37 cells infected with MP-MV are readily distinguishable from uninfected NC-37 DNA and rhesus monkey liver DNA and do contain sequences homologous to MP-MV RNA.

DISCUSSION

Morphologically and immunologically the AO virus is indistinguishable from the MP-MV. AO virus producing cells also yielded RNA and DNA which readily hybridized to a single-stranded ^{3}H-DNA copy of MP-MV RNA. The final levels of hybridization in each case closely approximated those obtained with the RNA and DNA from MP-MV-infected cells, indicating identity or a significant degree of homology. Human lymphoid and transformed cell lines employed as controls for the RNA hybridization studies were negative with MP-MV ^{3}H-DNA. Similarily, DNA sequences from three uninfected human cell lines and a rhesus monkey liver reacted only slightly with the MP-MV ^{3}H-DNA. This provided a low background which made it possible to identify the DNA sequences in the AO cell lines as being highly related to the MP-MV.

The results indicate that the AO cell line contains a virus closely related to the MP-MV. Ultrastructural studies suggest similar results with the other four lines, although more studies are necessary to establish this immunological and biochemical identity to MP-MV. Recently, other investigators have reported that certain human cell lines contain viruses also closely related to the MP-MV (12; K. V. Ilyin et al., Vop. Virusol., in press; H. Bauer, personal communication). The culture of Hooks et al. was from the brain of a patient with Jakob-Creutzfeldt disease, and Bauer's studies have been with lines derived from HeLa cells, a human cervical carcinoma cell. The present results on the AO cell line combined with the other information raise the question of the species of origin of these reverse transcriptase-containing viruses (retraviruses) as well as their natural mode of spread and role in human or primate oncogenesis. The human or the monkey "isolates," or both, may represent laboratory contaminants (13). Alternatively, either the human or monkey isolates may reflect inherited, spontaneously activated viral genes. It is not possible to distinguish conclusively between these alternatives with the present information.

As one approach to determine the species of origin, DNA·DNA hybridization was employed (7, 25). This approach has several assumptions important to the interpretation of our present results: that retraviruses are (i) present as DNA in infected cells, (ii) are widespread in the species of origin by either vertical or horizontal transmission, and (iii) that the hybridization procedures using ^{3}H-DNA viral product detects unique viral sequences. There is evidence for the correctness of the first two assumptions in chicken and murine model systems (4, 15, 31); however, in primates these assumptions remain to be validated.

The low level of hybridization of uninfected primate cellular DNA with the MP-MV ^{3}H-DNA is in contrast to high levels of hybridization obtained by using murine mammary tumour virus single-stranded ^{3}H-DNA with mouse cell DNA even from cells not producing MMTV (30, 23). The barely detectable hybridization with MP-MV ^{3}H-DNA and with rhesus monkey liver DNA also are in marked contrast to the high levels observed with low leukemia incidence C57Bl6 mouse liver DNAHy using single-stranded ^{3}H-DNA copies of type-C mouse RNA tumor viruses (unpublished data). Thus, even

though DNA·^{3}H-DNA hybridization was carried out to C_0t values at which over 70% of the unique sequence cellular DNA reassociates, only 2 to 3% of the MP-MV ^{3}H-DNA hybridized with uninfected human and rhesus monkey DNA. In fact, because the ^{3}H-DNA probes were not prepared from viral RNA under denaturing conditions which would insure only viral sequences in the ^{3}H-DNA probe, the low levels of reactivity observed may represent nonviral sequences. With the stated assumptions in the DNA·DNA hybridization approach, it is nevertheless apparent that uninfected rhesus monkey and human cells DNA contain less than one-tenth as many copies of MP-MV sequences than uninfected mouse tissue contains of either mouse type-B or type-C viral DNA sequences.

Although MP-MV-related viruses are present in a human tissue culture cell line, we still do not have definitive proof of their species of origin. In fact it is even possible that the original virus-positive rhesus monkey could have been infected by contact with humans analogous to observations of "measles" in monkeys secondary to human exposure (21). Before it is possible to conclude that these virus isolates represent a new class of human viruses with many of the properties associated with other RNA viruses containing reverse transcriptase it will be necessary to know considerably more about the natural history of these viruses in primates.

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ADDENDUM IN PROOF

Walter Nelson-Rees, Cell Culture Laboratory, University of California, School of Public Health, Naval Biomedical Research Laboratory, Oakland, California, has tested the five cell cultures and found that they each have a type-A glucose-6-phosphate-dehydrogenase (G6PD) mobility pattern and a human karyotype with modal chromosomal numbers ranging from 52 to 61. They all lack a Y chromosome. HeLa (ATCC) cells also have a type-A G6PD mobility pattern and a modal chromosomal number of 82 and are of female origin. Chromosomal banding studies are in progress to determine if the cell lines employed in this paper are of HeLa origin.

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