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Oncornavirus Expression in Human × Mouse Hybrid Cells Segregating Mouse Chromosomes

(B-tropic virus/DNA polymerase/group-specific antigens/type C virus)

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ABSTRACT **Human × mouse hybrid clones obtained by fusing transformed human (VA2) cells with embryonic mouse brain cells were tested for their ability to spontaneously express type C virus particles. It had been previously shown that these hybrid cells preferentially retained human chromosomes while mouse chromosomes were lost. The culture fluid from one cell line was found to contain type C particle markers in abundance, and typical budding C particles were observed in the cells by electron microscopy. In contrast, no particle markers were detected in the culture fluid from parental cells and several other hybrid cell lines. Subclones of the virus-positive cell line continued to lose mouse chromosomes and were found to vary more than 100-fold in their culture fluid DNA polymerase activity. The hybrid cell viruses, termed HMV1, banded in a sucrose gradient between 1.14 and 1.16 g/ml, possessed viral group-specific antigens, and exhibited B-tropic host range for replication in mouse embryo cells, but did not replicate in human cells when directly applied. The virus did not transform mouse cells but was able to rescue the defective murine sarcoma virus from sarcoma-positive, helper-virus-negative cells. Activity of the DNA polymerase associated with HMV1 was similar to the activity of Rauscher murine leukemia virus (MuLV) DNA polymerase in its preference for poly(rA) over poly(dA) as a template, use of endogenous template, detergent requirement, and inhibition by antiserum directed against MuLV-DNA polymerase. The results suggest that human × mouse hybrid cells segregating mouse chromosomes can spontaneously express endogenous type C viruses and that such hybrid cell lines may be used for the isolation of latent mammalian oncornaviruses and analysis of viral gene regulation.**

Information for the expression of mouse oncornaviruses can be vertically inherited and also can exist in unexpressed form in somatic as well as germinal cells (1–10). Crosses between inbred mouse strains have led to the identification of several genes relevant for virus expression (3–8). Similarly, using the techniques of somatic cell hybridization, parental cell lines which differ in their phenotype or genotype may be fused, and the resulting hybrids analyzed for viral markers (11–14) as the hybrid cells undergo chromosome segregation. Human–mouse hybrid cells are useful for this type of genetic analysis, since many of the parental chromosomes and gene products are easily distinguished and because these hybrids preferentially lose chromosomes from one parent (11, 15, 16).

The usual pattern of chromosome segregation in human–mouse hybrid cells is retention of mouse and loss of human chromosomes (15). Recently, however, the opposite result was found in that human chromosomes were retained and mouse chromosomes lost in hybrid cells made by fusing the simian virus 40 (SV40)-transformed human cell, VA2, to brain cells freshly derived from mouse embryos (17, 18). Such human–mouse hybrid cells would appear to be useful for the study of genes important for mouse oncornavirus expression, because the loss of chromosomes containing regulatory genes could result in alteration of the expression of virus, provided that structural and regulatory genes are located on different chromosomes. In this report, oncornavirus expression in a series of human–mouse hybrid cell lines segregating mouse chromosomes was studied by assaying for particulate DNA polymerase activity in culture fluids. A spontaneously derived type C virus was isolated from the culture fluid from one of the hybrid cell lines, and some properties of this virus were investigated.

Abbreviations: MuLV, murine leukemia virus; FeLV, feline leukemia virus; CF, complement fixation; CFU, complement-fixing unit; MEC, mouse embryo cells; gs, viral group-specific antigen; SV40, simian virus 40; HAT, hypoxanthine-aminopterin-thymidine; EDTA, ethylenediaminetetraacetic acid; S^+-H^-, sarcoma-virus-positive, helper-virus-negative.

MATERIALS AND METHODS

Cell Lines. The cells were grown in 95% Dulbecco–Vogt modification of Eagle's medium, 5% fetal bovine serum (D5) and, where indicated, the medium contained 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (D5-HAT). Hybrid cells were generated by fusing human VA2 cells [SV40-transformed human WI-18 cells, deficient in hypoxanthine phosphoribosyltransferase (11)] to freshly dissected normal mouse (C57BL/6, NIH/Swiss-GP) embryo (15–18 days *in utero*) nervous system cells using β-propiolactone-inactivated Sendai virus followed by selection in HAT medium as reported (17, 18). For DNA polymerase and group-specific (gs) antigen tests, cultures of 18 day *in utero* C57BL/6 whole brain and whole embryos were initiated by trypsinization, seeding at high density (10^7 cells per 100-mm plate), and growth in D5HAT. Homogenates of noncultured C57BL/6 18-day embryonic and adult whole brain were also prepared by sonication. The cell lines presented were free of mycoplasma according to bacteriologic examinations performed by Microbiological Associates, Inc., Bethesda, Md.

Preparation of Particulate Fraction from Culture Fluid. Cells were grown to near confluency in 100-mm Falcon petri

TABLE 1. *Particulate DNA polymerase activity in culture fluid from parental and hybrid cells*

Cells cultured	DNA polymerase activity in culture fluid pellet, Δ pmol of [^{3}H]dTMP incorporated/ reaction	Mean number of chromosomes		Number of mouse isozymes present (15 tested)
		Telocentric	Biarmed	
Parent				
VA2	0.0	6	68	0
C57BL/6 embryo brain	0.0	40	0	15
C57BL/6 whole embryo	0.0	40	0	—
Hybrid				
VMPOC18E	0.0	31	50	12
VMPE18F	0.0	27	58	14
VMPOC15M	3.2	26	51	15
VMPTH12	16.0	19	86	9
VME10	0.4	11	63	6
VMPTH28G	0.0	9	64	3
VMSC10	0.0	8	82	1
VMSG5	0.3	5	84	0

Cells were grown in D5HAT except for VA2, VME10, VMSC10, and VMSG5, which were grown in medium D5. Hybrids VME10, VMSC10, and VMSG5 were derived from fusion to nervous system cells of NIH/Swiss mice, while the other lines arose from fusion with C57BL/6 embryonic nervous system tissue (16). VMPTH12 arose from fusion to deep brain stem (thalamus); VMPOC15M arose from fusion to optic cortex. The hybrid cell lines were selected as well-isolated colonies on separate fusion plates, and alphabetic suffixes indicate which lines were recloned. The cell lines were tested 80–160 generations after fusion. Embryo brain (18 days *in utero*) and whole embryos were trypsinized, cultured in D5HAT, and tested for DNA polymerase activity 2, 4, 7, 9, 14, and 31 days later. The radioactive product in DNA polymerase reactions containing the culture fluid pellet from cell line VMPTH12 was found to band at a density of 1.42 g/ml in a Cs_2SO_4 gradient and was indistinguishable from authentic 5′-dTMP after hydrolysis with DNase I and venom phosphodiesterase; it was not digested by treatment with NaOH, trypsin or RNase. Chromosome number was determined on 20–30 metaphases per cell line including cultured embryonic brain. There are an average of six D and G group human chromosomes in VA2 which may be confused with mouse telocentric chromosomes depending on the state of chromosome spreading when chromosome banding techniques are not used. The total chromosome number equals the sum of telocentric and biarmed chromosomes. Fifteen different isozymes in which the human and mouse forms are distinguishable on starch gel electrophoresis were scored on the cell lines 20–40 generations before testing for DNA polymerase activity. Thus the number of mouse isozymes probably is overestimated. Detailed isozyme and chromosome data on the hybrid cell lines will be presented elsewhere (18).

dishes, fresh culture fluid was added, and the cells were allowed to grow for another 24–48 hr. The culture fluid was collected, centrifuged at 5000 × *g* for 10 min, and the supernatant centrifuged at 39,000 × *g* for 30 min in a Sorvall SS34 rotor at 3°. The resulting pellet from 40 ml of culture fluid was resuspended in 0.15 ml of 50 mM Tris·HCl, pH 7.5, 5 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 30% glycerol, quick-frozen in dry ice, and stored in the vapor phase of a liquid nitrogen freezer. This procedure resulted in a 270-fold concentration of particulate material in the culture fluid and in recovery of more than 90% of the virus present as measured by recovery of DNA polymerase activity.

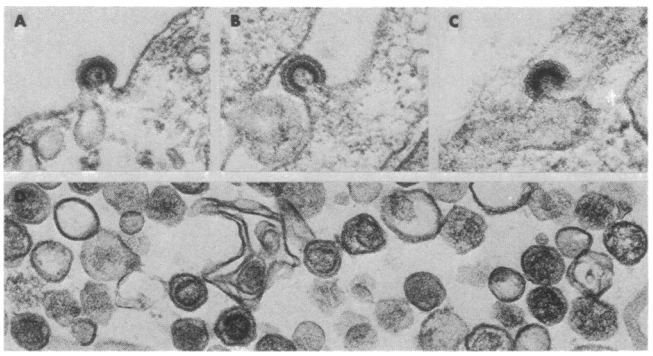

FIG. 1. Electron micrographs of type C virions budding from human × mouse hybrid cell line VMPTH12 80-100 generations after fusion (panel *A*), and two subclones 50–60 generations later (subclones 121 and 1220 are shown in panels *B* and *C*, respectively). Culture fluid particulate fraction from VMPTH12 cells is shown in panel *D*. After fixation in 2.5% glutaraldehyde for 1 hr, cell monolayers were scraped off with a rubber policeman, sedimented into a pellet and postfixed in buffered chrome-osmium tetroxide for 1 hr. The pellets were then rinsed in 2% uranyl acetate for 30 min, dehydrated, and embedded in Epon-araldite. Thin sections were doubly stained in uranyl acetate and lead citrate and examined in an Hitachi HU-11E electron microscope. The magnification of each is 55,000.

DNA Polymerase (EC 2.7.7.7) Assay. The assay was performed as previously described (19). Each reaction in a final volume of 35 μl contained the following components: 50 mM Tris·HCl, pH 7.7; 20% glycerol; 400 μg/ml of bovine-plasma albumin; 0.05% Nonidet P40; 2 mM dithiothreitol; 1 mM *p*-hydroxymercuribenzoate in 20 mM glycylglycine (20); 0.4 mM EDTA; 5 mM magnesium acetate; 40 mM KCl; 40 μg/ml of $(dT)_{12-18}$; 200 μg/ml of poly(rA); 0.5 mM [*methyl*-^{3}H]dTTP, lithium salt (180–300 cpm/pmol); and 5 μl of culture fluid particulate fraction. Incubation was in silicone-treated soft glass tubes at 37° for 60 min. All values for DNA polymerase activity were determined in a range of proportionality between activity and both time of incubation and amount of particulate fraction present in the reaction. The blank value equal to 0.2–0.4 pmol of [^{3}H]dTMP incorporated per reaction, determined in reactions without enzyme, was subtracted from data presented as Δ pmol [^{3}H]dTMP incorporated per 5 μl of culture fluid particulate fraction. Rabbit antiserum directed against DNA polymerase activity of Rauscher murine leukemia virus (MuLV) was generously provided by Drs. E. Scolnick and W. Parks, NIH (21).

Assay for Oncornavirus gs Antigens. Antigens were measured using a quantitative complement fixation (CF) assay in a final reaction volume of 1 ml [1.4–1.8 exact units of complement added/reaction (22)]. Goat antiserum (Lot no. F36024) directed against gs structural protein purified from Rauscher MuLV by isoelectric focusing was generously provided by Dr. R. Gilden, Flow Laboratories. This antiserum (diluted

1/340) was capable of recognizing both interspecies (gs-3) and mouse intraspecies (gs-1) antigens (23). Isopycnically banded Rauscher MuLV (Electro-Nucleonics, Lot. no. 146-4-4) and Rickard feline leukemia virus (FeLV, Electro-Nucleonics, Lot no. 346-7-13) were assayed as reference antigens. Sonicated cell extracts, 2–20 mg/ml of protein in 50 mM KPO_4, pH 6.8, stored in the vapor phase of liquid N_2, were extracted with 5 volumes of ether, and the aqueous phase centrifuged 5000 × *g* for 10 min and then diluted in CF buffer (142 mM NaCl, 5 mM Na diethylbarbiturate, pH 7.5, 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μg/ml of bovine-plasma albumin). Culture fluid particulate fractions were treated sequentially with 0.05% Nonidet P40, 500 mM NaCl, and ether, and were serially diluted in CF buffer. The diluted samples for testing were added to reactions in a volume of 0.2 ml. The dilution in these 0.2-ml aliquots required to give 50% inhibition of complement fixation was taken as the end-point and the reciprocal expressed as complement-fixing units (CFU) per reaction. Detection of antigen in culture fluid pellets was dependent upon Nonidet P40 treatment. Protein was measured by the method of Lowry *et al.* (24).

Electron Microscopy. This was performed in collaboration with Drs. Mathew Daniels (NIH) and William Hall (Electro-Nucleonics, Bethesda, Md.) on glutaraldehyde-fixed cell pellets or cells fixed *in situ*.

Assays for Biologic Activity. Replication in mouse embryo cells (MEC) (secondary cultures prepared from animals at 16 days' gestation) and other cell lines was tested as follows: VMPTH12 culture fluid was concentrated 100-fold and then 1.0 ml was used to infect 2×10^5 cells 24 hr after plating. Culture fluid was changed every 3 days and then tested for particulate DNA polymerase activity 14 days later. Sarcoma virus rescue was assayed using sarcoma-virus-positive, helper-virus-negative (S^+H^-) cells from which the defective sarcoma virus, Gz-MSV, can be rescued by MuLV (25, 26). S^+H^- BALB/c mouse cells (clone A1-2) were co-cultivated with VMPTH12 cells (3×10^5 cells of each type) for 5 days in a 75-cm^2 flask. Supernatant fluid was collected and tested for focus-forming activity by infecting BP cells (BALB/c peritoneal cells, H. Oie and A. Gazdar, unpublished) 24 hr after plating (with or without 25 μg/ml of DEAE-dextran). The dishes were examined 5–6 days later for discrete foci of transformation.

RESULTS

Oncornavirus Markers and Hybrid Cells. Expression of oncornavirus by a variety of human × mouse hybrid cells segregating mouse chromosomes was tested by assay for particulate DNA polymerase activity in the culture fluid. The number of mouse (telocentric) chromosomes and mouse isozymes varied widely in the hybrid cells. No DNA polymerase activity was found in the culture fluids from VA2 cells or cultured C57BL/6 mouse-embryonic brain cells, or from four of the hybrid clones (Table 1). In contrast, significant activity was found in the culture fluid from hybrid clones VMPTH12 and VMPOC15M. At the time of testing, the VMPTH12 cell line contained 86 human (biarmed) chromosomes and all 15 human isozymes tested for, while there were only 19 telocentric chromosomes and 9 of 15 mouse isozymes tested for (18). Sixty generations earlier, this cell line contained 36 telocentric chromosomes and 87 biarmed chromosomes.

TABLE 2. *Expression of gs antigens in parental and hybrid cells*

Cells cultured	gs antigens in culture fluid pellet, CFU/reaction	gs antigens in cell homogenate, CFU/mg of protein
Parental		
VA2	<10	<4
C57BL/6 embryo brain	<10	<1
C57BL/6 whole embryo	<10	<2
Hybrid		
VMPTH12	94	279
VMPOC15M	88	14
VMPE18F	<10	<4
Reference virus		
Rauscher MuLV	>12,800	—
Rickard FeLV	3,100	—

Intra- and interspecies gs antigens were assayed in culture fluid particulate fractions and cell homogenates treated as described in the *Methods*. Isopycnically banded. MuLV and FeLV reference viruses were treated with 0.05% Nonidet P40. Embryo brain and whole embryo homogenates contained no gs antigen detectable by complement fixation when freshly removed from the animals or after 31 days of culture.

Electron microscopic examination of VMPTH12 hybrid cells revealed immature type C particles budding from the plasma membrane and typical type C particles in culture fluid particulate fractions (Fig. 1). In contrast, no type C particles were seen in the VA2 parental cells or in embryo brains (and whole embryos) cultured for 2 weeks.

Parental and hybrid cells were tested for oncornavirus antigens using antiserum that detected both intra- (murine gs-1) and interspecies (gs-3) antigens (23) (Table 2). Homogenates and culture fluid from VA2 and cultured embryonic-brain cells contained no detectable viral antigens by CF assay. The two hybrid cell lines with appreciable DNA polymerase activity in the culture fluid exhibited gs antigen(s) in both the cell homogenates and culture fluid particulate fractions. A polymerase-negative hybrid line (VMPE18F) that contained large numbers of mouse and human chromosomes had no detectable viral gs antigen(s).

The inheritance of oncornavirus was examined by recloning the VMPTH12 cell line and then testing subclones for particulate DNA polymerase activity in their culture fluids. The subclones varied over 100-fold in the amount of DNA polymerase activity detected (Table 3). Two subclones with significant activity still had typical type C particles budding from the plasma membrane 50–60 generations later (Fig. 1).

Characteristics of the culture fluid particulate fraction from the human × mouse hybrid cell VMPTH12

DNA Polymerase Activity (Table 4). The DNA polymerase activity in VMPTH12 culture fluid particulate material was dependent upon Nonidet P40 and magnesium, and poly(dA) did not substitute for poly(rA) as template. A very similar pattern was observed with reactions containing Rauscher MuLV DNA polymerase. When poly(rA) was omitted, significant activity was observed with the hybrid cell particulate

TABLE 3. *Particulate DNA polymerase activity in culture fluid from VMPTH12 subclones*

Cell line	DNA polymerase activity, Δ pmol of [³H]dTMP incorporated/ reaction	Mean chromosome number (range)	
		Telocentric	Biarmed
VMPTH12	16.0	19 (11–29)	86 (71–101)
VMPTH12H	10.4		
Subclones			
1220	30.8	13 (7–18)	84 (80–91)
1215	13.5		
122	11.6		
121	7.5	16 (13–19)	81 (65–90)
1222	2.5		
1214	2.0		
124	1.7		
1218	1.3		
1212	1.3		
1217	1.0		
1216	1.0		
1210	0.8	14 (6–20)	84 (73–93)
127	0.3	15 (11–19)	83 (68–97)
1211	0.2		

Subcloning was performed by plating single VMPTH12 cells in 100-mm petri dishes and removing well-isolated colonies on separate plates with the aid of porcelain pennicylinders. Subclone VMPTH12H was derived 40–60 generations after fusion, while the other subclones were derived approximately 100 generations after fusion. All subclones were tested 30–40 generations after cloning. Chromosome numbers are mean values for 20 cells. Total chromosome number equals the sum of biarmed and telocentric chromosomes.

material, suggesting the presence of an endogenous poly(rA)-containing RNA. Activity with this endogenous template was stimulated when the other three deoxynucleotides were added, and this activity was sensitive to preincubation with RNase. The culture fluid particulate fraction and Rauscher MuLV were inhibited similarly by antiserum directed against Rauscher MuLV DNA polymerase.

Density of the DNA Polymerase Activity and gs Antigens. Equilibrium centrifugation in a sucrose gradient (Fig. 2) resulted in a banding pattern for the DNA polymerase activity that was heterogenous with average density 1.145 g/ml. Complement fixation activity using anti-gs antiserum was present in the fractions corresponding to the peak of polymerase activity. DNA polymerase activity associated with Rauscher MuLV banded at 1.16 g/ml in gradients run in parallel.

Biologic Activity. Particulate material in the culture fluid from VMPTH12 cells (termed HMV1 for human × mouse hybrid cell virus—1) was examined for host range of viral replication by directly applying the particulate fraction to human cells and mouse embryo cells of either the N-type (NIH/Swiss) or B-type (BALB/c and C57BL/6). Two weeks later the culture fluid from these cells was tested for particulate DNA polymerase activity. The results indicate that replication occurred in B-type mouse cells but not in human cells or N-type mouse cells (Table 5). Mouse embryo cells (C57BL/6) and BP cells chronically infected with VMPTH12

TABLE 4. *Comparison of DNA polymerase activities associated with viruses*

Modification	Amount of [³H]dTMP incorporated per reaction (percent)	
	Human × mouse hybrid cell virus (HMV1)	Rauscher MuLV
None	100	100
− Nonidet P40	2.2	5.7
− Mg^{++}	0.9	0.1
− poly(rA)	6.4	0.1
− poly(rA); + poly(dA)	0.7	0.2
− poly(rA); + dATP, dCTP, dGTP	14.2	0.3
− poly(rA); + dATP, dCTP, dGTP + 15-min preincubation, 37°, with 1.3 mg/ml of RNase	0.2	0.0
+ 0.4 μl of MuLV DNA pol antiserum	24	30
+ 0.6 μl of MuLV DNA pol antiserum	32	23
+ 1.0 μl MuLV DNA pol antiserum	4	4

DNA polymerase activity (100%) for HMV1 (culture fluid particulate fraction from VMPTH12 cells) was 13.6 Δ pmol of [³H]dTMP incorporated/reaction; 100% activity for Rauscher MuLV was 136 Δ pmol of [³H]dTMP incorporated/reaction. *MuLV DNA pol antiserum*, rabbit antiserum directed against MuLV DNA polymerase activity.

culture fluid did not show transformation. However, culture fluid from co-cultivated VMPTH12 cells and S^+H^- cells when applied to BP cells yielded foci of transformed cells (titer 150 focus-forming units/ml).

DISCUSSION

Human and mouse cells not expressing oncornavirus markers were fused, yielding hybrid cell lines some of which elaborated typical type C virus particles. Particulate material in the culture fluid from one of the hybrid cell lines, VMPTH12, was biochemically and immunologically related to both the DNA polymerase and structural protein of murine leukemia virus. This particulate material also contained a nontransforming viruses termed HMV1, that replicated after direct infection in mouse but not in human cells. Its replication in BALB/c and C57BL/6 mouse cells and its restriction in NIH/Swiss cells indicate that it is a B-tropic virus (5, 27). HMV1 rescued a defective sarcoma virus from S^+H^- cells (25, 26), and the resultant pseudotype virus Gz-MSV(HMV1) transformed susceptible B-type mouse cells. It will be interesting to compare the biologic activity of HMV1 with other B-tropic viruses such as radiation leukemia virus, RadLV (28), oncornaviruses with neurotropism (29), and to retest its host range after further chromosome segregation.

Information presented suggests but does not prove HMV1 was expressed in hybrid cells as a result of chromosome segregation. Oncornavirus production was not detected in parental cells or in several hybrid lines with either a full or nearly depleted complement of mouse chromosomes. While oncornavirus can be induced from C57BL cells (28) by bromodeoxyuridine, it has not been shown to be produced by cul-

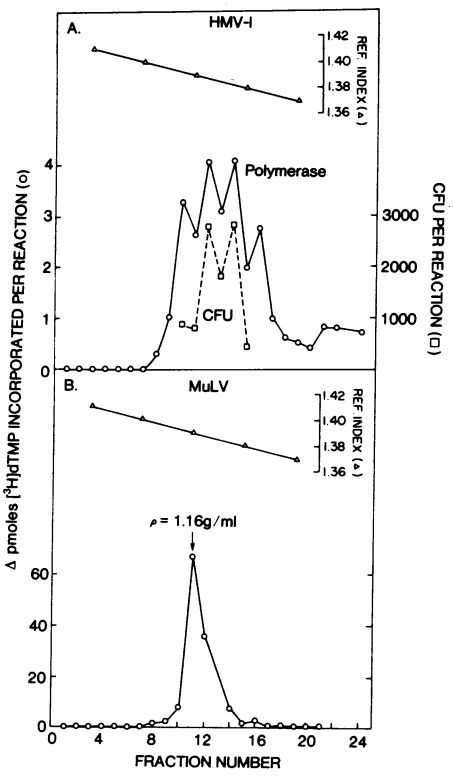

FIG. 2. Isopycnic centrifugation of HMV1 (panel *A*) and Rauscher MuLV (panel *B*). Centrifugation at 50,000 rpm was performed for 3 hr at 2° in a SW50.1 rotor. Linear 5-ml 15–65% sucrose gradients contained 50 mM Tris·HCl, pH 7.9, at 5°; 0.1 mM EDTA; 1 mM dithiothreitol; 80 mM KCl. The amount of DNA polymerase activity in 25-μl portions of each fraction was determined as described in *Methods* except that the final reaction volume was 50 μl and reactions contained 12% glycerol plus the sucrose contributed by the gradient fractions. Recovery of HMV1 DNA polymerase activity during the centrifugation was 82%. The amount of murine gs antigen(s) in 50-μl portions of each fraction was determined as described in *Methods*. Gradient fractions were not treated with Nonidet P40, NaCl, or ether prior to assay.

tured but untreated normal C57BL cells (28, 30), and contamination with an exogenous B-tropic oncornavirus appears unlikely. Analysis of VMPTH12 subclones showed they continued to segregate mouse chromosomes, and some appeared to segregate the ability to express virus. However, documentation of the mode of origin will rely on further studies including demonstration of synteny between the presence of specific mouse chromosome(s) and oncornavirus expression. Evidence of such synteny by somatic cell hybrid analysis will complement germinal genetics linkage data (3, 5–8). The role that the SV40 genome in VA2 plays in such expression is at present unknown, and oncornavirus expression and character could be influenced by the source of human or mouse parental cells and exposure of hybrid cells to oncornavirus-inducing agents (10).

Hybrid cells are biologically unique tools for studying factors related to malignancy (11–14, 31, 32). They have already been used to study the expression of oncornavirus in mouse × mouse and mouse × hamster cells (12–14). However, human × mouse hybrids offer the opportunity to assay for human factors related to the expression of virus and human genes incorporated in such particles. Work with nonreplicating human × mouse heterokaryons suggests human cells contain factors which can restrict the replication of murine oncornaviruses (33). Human × mouse hybrids segregating either mouse or human chromosomes could result in the expression of previously latent xenotropic or human oncornaviruses as mouse or human regulatory genes are lost. However, the potential for biologic hazard in work with human–rodent hybrid cells expressing oncornaviruses must be recognized (34).

TABLE 5. *Infectivity of HMV1 for human and mouse cells*

Cultured cells infected	DNA polymerase activity, Δ pmol of [³H]dTMP incorporated/reaction
Mouse embryo cells	
C57BL/6	22
BALB/c	8
NIH/Swiss	≤0.01
Human cells	
VA2	≤0.02
WI 38	≤0.01

VMPTH12 culture fluid (passages 5-15) was concentrated 100-fold (DNA polymerase activity 13.4 Δ pmol per reaction), and cell lines were infected as described in *Methods*. Culture fluid was changed every 3 days. Seven to 14 days after infection, culture fluid was concentrated 100-fold and tested for DNA polymerase activity as previously described (35) using 15 μl of culture fluid particulate fraction.

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