A MURINE TERATOCARCINOMA STEM CELL LINE CARRIES SUPPRESSED ONCOGENIC VIRUS GENOMES*

By KAY HUEBNER, NOBUO TSUCHIDA, CECELIA GREEN, and CARLO M. CROCE‡

From The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Stem cell and differentiated cell lines derived from transplantable murine teratocarcinomas have been established in tissue culture by several methods (1–6) and have been characterized as nullipotent, pluripotent, and terminally differentiated. Pluripotent stem cells show remarkable similarities to the pluripotent cells of the early embryo and the stem cells of one particularly well studied teratocarcinoma, the OTT6050 tumor established by Stevens (7), have been shown to be totipotent because single stem cells from this tumor have been injected into blastocysts of mice and the offspring derived from injected blastocysts have been shown to be mosaic in most, if not all, tissues, including the germ line (8). Cells derived from stem cell lines carried in tissue culture for many generations are also able to participate in formation of normal tissue in mosaic mice (9, 10, 6).

Thus, pluripotent or totipotent stem cell lines and the differentiated cell lines derived from them have great potential for the study of differences in regulation of gene expression in embryonic versus differentiated cells. It has already been demonstrated that stem and differentiated cell lines do indeed respond differently to infection with oncogenic viruses. Stem cell lines are resistant to infection with DNA tumor viruses (11, 12) and murine C-type viruses (13, 14), and this resistance is not a result of lack of penetration of these viruses (15, 14).

We have established (6) stem cell and differentiated cell lines from a murine strain 129 testicular teratocarcinoma, OTT6050, which had been carried as a solid tumor in strain 129 mice before these studies. The stem and differentiated cell lines have been characterized for their interaction with murine leukemia virus and the stem cells have been found to be nonpermissive for productive infection by Moloney leukemia virus (Mo-MuLV)[1] in accordance with the results of others. However, each of the differentiated lines established from this OTT6050 tumor was found to be producing an N-tropic murine leukemia virus which closely resembles N-tropic AKR MuLV and the stem cell line carries DNA sequences homologous to this N-tropic virus which are not expressed as viral proteins or infectious virus in stem cells.

* Supported in part by grant VC 220 from the American Cancer Society, by U.S. Public Health Service research grants CA 16885, CA 20741, CA 21069, and CA 21124 from the National Cancer Institute, RR05540 from the Division of Research Resources, and GM 20700 from the Institute of General Medical Sciences, and by grant 1-522 from the National Foundation—March of Dimes.

‡ Recipient of Research Career Development Award CA 00143 from the National Cancer Institute.

[1] *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; FBS, fetal bovine serum; FFU, focus-forming units; HMW RNA, high molecular weight RNA, ME, mouse embryo; Mo-MuLV, Moloney leukemia virus; PBS, phosphate-buffered saline; PFU, plaque-forming units; SDS, sodium dodecyl sulfate; SSEA-1, stage specific embryonic antigen; SSC, 0.15 M NaCl, 0.015 M sodium citrate; T_m, thermostability.

Materials and Methods

Cells. The teratocarcinoma-derived cell lines used in this study are listed in Table 1. The teratocarcinoma cell lines established in this laboratory were obtained as follows: 129 mice bearing OTT6050 tumors were received from Dr. Beatrice Mintz of the Institute for Cancer Research (Fox Chase, Pa.). Tumors were excised and observed for out growth in tissue culture in RPMI medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum (FBS). For establishing stem cell lines, cultures containing >50% stem cells (morphological criteria) were split at high ratios (1:50–1:100) until cultures were obtained which consistently retained the stem cell morphology. Such cultures were mutagenized and selected in bromodeoxyuridine (BrdU). When colonies appeared, the concentration of drug was increased and clones were obtained which retained the stem cell morphology and were resistant to 100 μg/ml BrdU. One such clone was designated OTT6050AF1 BrdU. Differentiated cell lines were obtained by selecting stem cell colonies and transferring these cultures at low split ratios (1:5–1:10). All teratocarcinoma-derived cell lines established in this laboratory were carried in RPMI medium with 10% FBS. All other cell lines used in this study were maintained in minimal essential medium supplemented with 10% FBS. Other cell lines used in this study were XC cells, a rat cell line used in the UV-XC plaque test (16), for murine leukemia virus (MuLV); SC-1 cells (17), a feral mouse embryo cell line which is susceptible to replication of N- and B-tropic MuLV, (obtained from Dr. J. Hartley); 129ME, 129 G_{IX}^-ME, and C57BIME cell lines established from embryos of strain 129, 129 G_{IX}^- and C57BL/6 mice, respectively (K. Huebner, unpublished results); primary cultures of BALB/c and NIH mouse embryo cells obtained from Flow laboratories; mink S+L− (18) cells obtained from P. Peebles, National Cancer Institute; mink lung cells obtained from the ATCC; HT1080 human fibrosarcoma cells (19); J1SV9 cells (20); J1SV9 cells producing B-tropic MuLV; BALB-3T3 cells (21); and human tumor cell lines producing either class II or class III xenotropic MuLV (22) which were generously provided by Dr. S. Hino.

Viruses. NB tropic Mo-MuLV and N-, B-, and X-tropic MuLV seed stocks were obtained from Dr. J. Hartley (NIAID). Ecotropic virus stocks were grown in SC-1 cells or secondary cultures of NIH or BALB/c mouse embryo (ME) cells after seeding cells in polybrene (2 μg/ml) (23). Xenotropic BALB virus 2 (Class II virus from J. Hartley) and NZB (class III virus from A. Ishimoto) stocks were grown in mink lung cells or human cells seeded 24 h before infection in medium containing polybrene (2 μg/ml). Ecotropic virus stocks were titered for XC-plaque-forming ability and usually had a titer between 10^6 and 10^7 plaque-forming units (PFU)/ml; X-tropic virus was titered for focus-forming ability on mink S+L− cells (18) and stocks used had titers of 10^4 focus-forming units (FFU)/ml. N-, B-, and X-tropic viruses were used as controls when testing the tropism of the virus isolated from OTT6050 cell lines.

XC Plaque Test. Supernatant fluids of viral stocks to be assayed for XC-plaque-forming ability were filtered and titrated as previously described (23), by the UV-XC procedure of Rowe et al (16). SC-1 cells were routinely used in the plaque test but if it was necessary to determine the tropism of the ecotropic virus, NIH (Fv-1^{nn}) and BALB/c (Fv-1^{bb}) secondary ME cultures were used.

Mink S+L− Focus Assay. Supernates or viral stocks to be titered for focus-forming ability on mink S+L− cells were filtered and 0.5 ml of each sample with appropriate dilutions was inoculated into duplicate Petri dishes which had been seeded 24 h previously with 3×10^5 mink S+L− cells/dish in medium containing 2 μg/ml polybrene (24). After 1 h absorption, inocula were removed and 5 ml medium containing 10% FBS added. Medium was changed every 2 d and on day 8–10, foci were enumerated and FFU/ml calculated.

Reverse Transcriptase Assay. The assay procedure used was that described by Manly (25) with some modifications as described previously (23). Reaction mixtures contained the following components in a 50-μl final volume:Tris-hydrochloride buffer, 50 mM, pH 8.3; NaCl, 60 mM; dithiothreitol, 20 mM; [^{3}H]thymidine triphosphate, 21 μM 1,600 cpm/pmol; polyriboadenlyic acid, 50 μM; oligothymidylic acid, 5 mM; Nonidet P-40, 0.05%; $MnCl_2$, 1 mM, and 10 μl of sample to be tested. The reaction mixtures were incubated at 37°C for 60 min. Incorporation of [^{3}H]thymidine triphosphate was determined by adsorption onto DEAE filters. Supernates of cells to be tested for reverse transcriptase activity were either tested directly or a 100-fold concentrated sample was tested. Polymerase activity was expressed as picomoles [^{3}H]thymidine

triphosphate incorporated per milliliter of supernate.

Indirect Immunofluorescent Staining for Detection of Cellular and Viral Antigens. The following sera were used in indirect immunofluorescence staining of fixed or viable cells to determine if MuLV antigens were being produced: (*a*) Anti-Rauscher MuLV gp69/71 (26), (*b*) Anti-Rauscher MuLV p30 (26), (*c*) Anti-AKR MuLV p30, (*d*) Anti-Gross MuLV gp69/71, (*e*) Anti-Gross MuLV p30. Sera a and b were kindly supplied by Doctors Strand and August: sera c, d, and e were kindly supplied by Dr. Jack Gruber through the National Cancer Institute Viral Oncology Resources and Services. All sera were produced in goats against purified viral proteins. Fluorescein-conjugated rabbit anti-goat serum (Antibodies Inc., Davis, Calif.) was used as the second reagent in the indirect assay. Indirect immunofluorescence on fixed cells was performed as follows: subconfluent cultures on coverslips were fixed in acetone; the anti-viral sera were applied to coverslips, and incubated at 37°C in a humidified atmosphere for 30 min, after which coverslips were washed extensively with phosphate-buffered saline (PBS) and fluorescein-conjugated anti-goat serum applied for 30 min at 37°C. After washing, the coverslips were counterstained with 0.1% solution of Evans blue. For surface immunofluorescence of viral antigens, viable single cell suspensions were stained according to the procedure of Campbell et al. (27). Viable single cell suspensions were also stained for H-2 antigen and for the embryonic antigen of teratocarcinoma stem cells, SSEA-1, which is detected by the monoclonal antibody described by Knowles et al. (28) and Solter and Knowles (29). Mouse alloantisera D33 and D-2 reactive to the H-2 specificities shown in Table II were obtained by B. Knowles from the Transplantation and Immunology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and were used at dilutions of 1:10 with fluorescent rabbit IgG specific for mouse IgG heavy and light chain as the second reagent (used at dilution of 1:10). Anti-SSEA-1 was provided by D. Solter and B. Knowles and used at a dilution of 1:1,000. Fluorescent goat IgG, specific for mouse IgM heavy chain, was used as the second reagent at a dilution of 1:10.

Preparation of MuLV cDNA Probes. cDNA probes were prepared from AKR N-tropic MuLV grown on SC-1 cells and from the virus produced by the differentiated cell line, OTTF12. Virus was harvested at 12-h intervals, concentrated, and purified, and ^{3}H-labeled cDNA was synthesized by the endogenous reaction of the purified virus in the presence of actinomycin D and DNase I-digested calf thymus DNA primer (30) and purified as described previously (31). The ^{3}H-labeled cDNA was hybridized with equal amounts of viral high molecular weight (HMW) RNA and DNA-RNA hybrids (30–40% of the input cDNA) formed fractionated by hydroxylapatite column chromatography and treated with NaOH to destroy RNA as described previously (31). The cDNA thus prepared had a sp act of 2×10^7 cpm/μg.

Preparation of Cellular DNA and RNA. Cellular DNA was extracted from OTT6050AF1 BrdU, PCC4, and OTTF12 cells, AKR liver, and from 129/J embryos. Tissue was rinsed with Dulbecco's PBS, minced with a pair of scissors, suspended in 10 vol of Tris-buffered saline-EDTA buffer (0.01 M Tris HCl, pH 7.0, 0.01 M EDTA, and 0.1 M NaCl), homogenized with a Virtis homogenizer (Virtis, Gardiner, N. Y.) at 0°C, 10,000 rpm for 10 min. After homogenization, sodium dodecylsulfate (SDS) and proteinase K (Merck & Co., Inc., West Point, Pa.) were added to 1% and 100 μg/ml, respectively, and the mixture was incubated at room temperature overnight. Cultured cells were washed with PBS, suspended in 10 vol of STE buffer containing proteinase K (100 μg/ml), disrupted by addition of SDS (final concentration: 1%), and incubated at room temperature overnight. Nucleic acids were extracted from the lysate with 80% phenol and chloroform and isoamyl alcohol (24:1 vol/vol), precipitated with 2 vol of ethanol, and collected by a low speed centrifugation as described previously (32). The precipitated nucleic acid was suspended in V buffer (0.01 M Tris HCl, pH 7.0, 0.01 EDTA, and 0.5 M NaCl). After the addition of 2 vol of glycerol, DNA was sheared with a Virtis homogenizer (33) as described previously (32). Nucleic acids were collected by precipitation with ethanol, resuspended in 0.3 N NaOH, and the solution was incubated at 37°C overnight, and neutralized with acetic acid. After the addition of EDTA (10 mM), proteinase K (100 μg/ml), and SDS (1%), the solution was incubated at 37°C for 1 h. DNA was extracted with phenol and chloroform:isoamyl alcohol as described above. The aqueous phase was treated with 2 vol water-saturated ether, and DNA was precipitated with 2 vol of ethanol. The precipitate was collected by low speed centrifugation, and lyophilized or dried with N_2 gas. The

final precipitate was dissolved in a small volume of 0.1 SSC (0.15 M NaCl, 0.015 M sodium citrate). The DNA solution thus obtained had an absorbancy ratio of >1.8 when absorbancies were compared at 260 and 280 nm. The concentration of DNA was calculated on the basis that 20 U of absorbancy at 260 nm is 1 mg/ml of DNA solution. For isolation of RNA, cells grown as monolayer cultures were treated with pronase (50 μg/ml), washed with PBS, and disrupted with 1% SDS in the presence of proteinase K (500 μg/ml) as described by Hayward (34). Nucleic acid was extracted with phenol and digested with DNase I as described previously, (35). Viral HMW RNA was prepared as described previously (36) except that after lysis of virions with 1% SDS, the lysate was treated with proteinase K (500 μg/ml) at 20–25°C for 30 min.

Hybridization and T_m Analysis. Mixtures of cellular DNA (3–5 mg/ml) and [^{3}H]cDNA (7,500 cpm) were made in 150 μl of 0.55 N (Na^+) sodium phosphate buffer (pH 6.8) containing 0.1% SDS and 1 mM EDTA. 10-μl aliquots were distributed into capillary tubes and incubated for various lengths of time at 66°C as described previously (32). The amount of hybrid formed was assayed by the batch elution method of hydroxylapatite as described previously (32) except (1) that the hybrid formed was eluted with 0.12 M phosphate buffer containing 0.4% SDS at 100°C and (2) that the radioactivities in hybridized and unhybridized fractions were measured in a scintillation counter after the addition of aquasol (New England Nuclear) to each fraction. For [^{3}H]cDNA and cellular or viral RNA hybridization, 5 × 10^4 cpm/ml of [^{3}H]cDNA was mixed with RNA of a saturating concentration in 3 × SSC and 30% formamide. The mixture was incubated at 50°C for 7 d and diluted 20-fold with 1 × SSC after the incubation. An equal portion of aliquots was incubated at 0, 50, 60, 70, 80, 85, 90, 95, 100°C for 1 min and then quickly chilled in ice water. The amount of hybrid was quantitated by the S-1 nuclease method as described previously (36). T_m analysis of hybrids between [^{3}H]DNA and a saturating concentration of cellular DNA was done essentially by the methods described above, except that the solution of [^{3}H]DNA and cellular DNA mixture was dialyzed against 1 × SSC before heat treatment.

Results

Sources and Characterization of Teratocarcinoma-Derived Cell Lines. Teratocarcinoma-derived cell lines established in this laboratory and those obtained from others are described in Materials and Methods and are listed in Table I with a brief description and source of the cell line and/or tumor. The first three cell lines listed in Table I (PCC4, F9, and OTT-Brinster) were established by other investigators from tumors derived from the transplantable strain 129 OTT6050 tumor of Stevens. The fourth cell line listed (FA-25) was established by D. Solter from an AKR embryo. All other lines were established in this laboratory; all OTT6050 lines established in this laboratory were derived from one tumor, OTT6050A. Because, as will be seen in the following experiments, the cell lines established from the OTT6050A tumor had properties which are distinct from the properties of the F9 and PCC4 cell lines, two other tumors (OTT6050B and OTT6050C) were obtained from Dr. Mintz and studied as primary tissue cultures to be sure that the viral expression observed in OTT6050A-derived cell lines was not unique to one tumor. In addition a cell line, OTT-E, established and carried by Dr. Solter from the OTT6050A tumor, was also studied. Because teratocarcinoma stem cells do not express H-2 antigens (37, 38, 39) but do express a stage-specific embryonic antigen (SSEA-1) which is not expressed by differentiated cells (28, 29), and the reverse is true for differentiated cell lines, we have used surface antigens as markers for characterization of our stem and differentiated cell lines. Using monoclonal antibody which detects the specific embryonic antigen (SSEA-1) on murine teratocarcinoma cells (28, 29) and mouse anti-H-$2K^b$ and anti-H-$2D^b$ serum we have stained the surfaces of our stem and differentiated cells and

TABLE I

Origins and Characteristics of Teratocarcinoma-Derived Cell Lines

Cell line	Description	Tumor origin and reference for cell line	Source of cells
Established by other laboratories			
PCC4	Pluripotent stem cell line which remains undifferentiated in tissue culture	OTT6050 (2)	D. Solter
F9	Nullipotent stem cell line which does not differentiate even in vivo	OTT6050 (1)	D. Solter
OTT-Brinster	Mixed culture of stem and differentiated cells established by D. Solter from tumor of R. Brinster	OTT6050	D. Solter
Fa-25	Mixed culture of stem and differentiated cells established by D. Solter	AKR embryo (29)	D. Solter
Established in this laboratory from OTT6050 tumors from ICR*			
OTT6050AF1 BrdU	Pluripotent stem cell clone which remains undifferentiated in tissue culture but is able to participate in formation of mosaic mice	OTT6050A (6) (First tumor from B. Mintz, ICR)	C. Croce
OTTF12 (d)	Pluripotent stem (~90% undifferentiated) cell line from which OTT6050AF1 BrdU was cloned	OTT6050A	C. Croce
OTTS3,OTTS4 293S	Differentiated cell lines all established from one initial flask of primary explanted tumor (flask 1)	OTT6050A	K. Huebner
OTTF7	Differentiated cell line established from Flask 7 of primary tumor explant	OTT6050A	K. Huebner
OTTF12	Differentiated cell line established from OTTF12 (d)	OTT6050A	K. Huebner
OTT-E	Differentiated cell line established by D. Solter from OTT6050A embryoid bodies	OTT6050A	D. Solter
Primary cultures from OTT6050 tumors from ICR			
OTT-B	Primary embryoid body culture	OTT6050B (second tumor from ICR)	M. Dewey and B. Mintz
OTT-C	Primary tissue culture derived from solid tumor	OTT6050C (third tumor from ICR)	C. Croce

* ICR, Institute for Cancer Research.

various control cells as described in Materials and Methods and have obtained the results presented in Table II. From this data it can be concluded that the OTT6050AF1 BrdU cells are 97% stem cells (because they are 97% positive for SSEA-1 antigen and <3% positive for H-2 antigen), whereas the OTTF12 cells are 99% differentiated because they are negative for SSEA-1 antigen and 99% positive for H-2 antigen.

Interaction of Teratocarcinoma-Derived Cell Lines with MuLV. Périès et al., (13) demonstrated that the pluripotent stem cell ine, PCC4, did not produce viral reverse transcriptase activity or infectious virus after infection with Mo-MuLV, Gross MuLV, or Friend MuLV nor did these stem cells undergo morphological changes after infection with Moloney murine sarcoma virus (Mo-MuSV). Teratocarcinoma (embryoid body)-derived differentiated cell lines, on the other hand, were capable of producing each of the above MuLV's and were susceptible to transformation by Mo-MuSV. Teich et al. (14) using another series of teratocarcinoma-derived stem and differentiated cell lines demonstrated that the teratocarcinoma-derived undifferentiated (nulli and pluripotent stem cells) cells were entirely nonpermissive for production of Mo-MuLV although virus adsorption and penetration were not restricted. After infection of the pluripotent stem cell line, proviral DNA sequences were detected but transcription into viral RNA was not detected. Very early in our studies of cell lines established from the OTT6050A tumor, we observed reverse transcriptase activity in supernates of mixed cultures of stem and differentiated cells. Thus, stem

TABLE II

Presence of Serologically Detectable H-2 and SSEA-1 Antigens on Stem and Differentiated Cell Lines

Target cells	Mouse strain and H-2 type	Percentage positive by immunofluorescence		
		Anti-H-2K^b (33)	Anti-H-2D^b (2)	Anti-SSEA-1 monoclonal antibody
F9-stem	129 H-2^{bc}	<1	<1	99
OTT6050AF1 BrdU (stem)	129 H-2^{bc}	<3	<3	97
OTTF12 (differentiated)	129 H-2^{bc}	99	99	0
FA-25 (stem and differentiated)	AKR H-2^k	<3	<3	76
BALB-3T3 fibroblasts	BALB H-2^d	<1	<1	0
129 ME fibroblasts	129 H-2^{bc}	80	85	0

and differentiated cell lines, and mixed cultures of stem and differentiated cells derived from the OTT6050A tumor in this laboratory were tested for spontaneous expression of C-type viral functions. To be certain that spontaneous C-type viral expression was not a result of laboratory contamination of our cell lines, a cell line derived from OTT6050A and maintained by D. Solter in a different laboratory was also tested, as were primary cells from two subsequent tumors, OTT6050B and OTT6050C, obtained from ICR (the same source as for the OTT6050A tumor) which consisted of mixed cultures of stem and differentiated cells. Results of tests for C-type viral expression (spontaneous or after infection by Mo-MuLV) are given in Table III. Culture medium collected from uninfected and infected cells were tested for the presence of reverse transcriptase activity, infectious virus (by XC-plaque assay) and the infected and uninfected cells were tested for production of viral antigens (by indirect immunofluorescence) as described in the legend for Table III. Results of these tests are summarized in Table III. The stem cell lines F9 and PCC4 were resistant to productive infection by all three criteria. The cell lines derived from the OTT6050-ICR tumors gave somewhat different results. The differentiated cell lines derived from the OTT6050A-ICR tumor were spontaneously producing MuLV which formed XC plaques on SC-1 cells. In addition, the stem line (OTTF12 (d)), primary tumor explants, and embryoid body cultures all showed some evidence of production of MuLV, as detected by reverse transcriptase activity or by XC-plaque assay. Only the OTT6050AF1 BrdU cell line which is >97% undifferentiated was completely negative for virus expression by all three criteria. The evidence presented in the summary of results in Table III suggested the possibility that the stem cells of OTT6050 tumors from ICR contain MuLV in a suppressed state which on differentiation is expressed as infectious virus.

Characterization of MuLV Produced by OTT6050-ICR Tumor-Derived Cell Lines. To determine its host range, the virus isolated from differentiated lines derived from the OTT6050A tumor was used to infect cells of Fv-1^{nn} and Fv-1^{bb} genotype and mink cell lines. The results of this experiment are summarized in Table IV. The virus did not replicate in other teratocarcinoma stem cell lines, nor did it produce reverse transcriptase activity after infection of mink S+L− cells or after cocultivation of

TABLE III

MuLV Infection of Teratocarcinoma-Derived Cell Lines

Cell lines	Infected with	Reverse transciptase activity	Titer $\log_{10}$ on SC-1 cells	Expression of		
				gp69/71 surface	gp69/71 cytoplasmic	p30
		pmol/ml of supernate	*PFU/ml*			
From other laboratories						
PCC4	No virus	<0.1	0.0	–	–	–
	+Mo-MuLV	<0.1	0.0	–	–	–
F9	No virus	<0.1	0.0	–	–	–
	+Mo-MuLV	<0.1	0.0	–	–	–
OTT-Brinster	No virus	<0.1	0.0	–	–	–
	+Mo-MuLV	NT	NT	–	NT	NT
From OTT6050 tumors from ICR						
OTT6050AF1 BrdU	No virus	<0.1	0.0	–	–	–
Stem	+Mo-MuLV	<0.1	0.0	NT	–	–
OTTF12 (d)	No virus	0.4	1.3	few +	few +	few +
Mostly stem	+Mo-MuLV	.08	NT	NT	NT	NT
2935	No virus	10.7	4.6	+ + + +	+ + + +	+ + + +
	+Mo-MuLV	5.3	NT	NT	NT	NT
OTT-E Diff.	No virus	17.6	NT	NT	NT	NT
OTTF7	No virus	37.3	4.6	+ + +	+ +	+ +
	+Mo-MuLV	11.0	NT	NT	+ +	+ +
OTTF12	No virus	40.0	4.5	+ + +	+ +	+ +
OTT-B, Primary Stem and OTT-C Diff.	No virus	6.0	3.0	NT	NT	NT
	No virus	2.0	2.3	NT	NT	NT
Controls						
BALB/c ME	No virus	<0.1	0.0	–	–	–
	+Mo-MuLV	76.6	6.0	+ + + +	+ + + +	+ + + +
$129G_{1X}^{-}$ME	No virus	<0.1	0.0	–	–	–
	+Mo-MuLV	54.3	5.6	+ + + +	+ + + +	+ + + +

NT, not tested. ICR, Institute for Cancer Research.

Infection. Cells to be tested were seeded in quadruplicate at a density of 2×10^6 cells/flask (75 cm^2) in medium containing 2 μg/ml polybrene and 24 h later, two flasks (for each cell line) were infected with 1 PFU/cell Mo-MuLV and two flasks were mock infected. Cells were then cultured for up to 2 wk and supernates were collected, filtered, and frozen every 2 d. After the last collection of supernate, infected and uninfected cells were trypsinized and tested for expression of C-type viral antigens as described below. Aliquots of unconcentrated supernates were tested for plaque-forming MuLV on SC-1 cells and the remainders of the supernates were concentrated 100-fold and tested for reverse transcriptase activity. Each cell line was tested at least three times; results shown in the table are from a single experiment but all results were consistent with those shown.

Viral Antigens. After the last collection of supernates, cells were trypsinized and either stained directly (as viable cells) in suspension by indirect immunofluorescence for expression of surface gp69/71 using anti-Rauscher MuLV gp69/71 or were seeded in Petri dishes with coverslips for fixation and tested for cytoplasmic viral antigens by indirect immunofluorescence.

All results in the table were obtained using anti-Rauscher MuLV gp69/71 and p30 (sera a and b described in Materials and Methods) but similar results were obtained using anti-Gross MuLV gp69/71 and p30 (sera c, d, and e, described in Materials and Methods).

TABLE IV

Host Range of MuLV Produced by OTT6050A-Derived Cell Lines

Source of virus	Reverse transcriptase activity			[Titer Log_{10}] XC plaque (PFU/ml) on		[Titer log_{10}] FFU/ml on
	PCC4	F-9	Mink lung	NIH-ME	BALB/c-ME	Mink S+L− cell
	pmol/ml of supernate					
OTTF7	<0.1	<0.1	<0.1	4.6	2.3	0
OTTF12	<0.1	<0.1	<0.1	4.9	2.8	0
OTT-C	NT	NT	NT	1.1	0	NT
OTT-E	NT	NT	NT	3.7	1.7	NT
OTTS4	NT	NT	NT	4.3	2.0	0
Controls						
Mink/NZB (producing X-tropic virus)	NT	NT	11.0	0	0	4.0
BALB/c ME/B-MuLV	NT	NT	<0.1	2.0	4.0	0
NIH ME/N-MuLV	NT	NT	<0.1	6.0	4.0	0

Supernates of cell lines on the left were filtered and inoculated into cultures (listed across the top of the table) which had been seeded previously in medium containing polybrene (2 μg/ml). Each cell line was treated in this way two or more times and results were similar; results given in this table are from a single experiment.

Reverse Transcriptase Assay. Supernates were collected from infected cells every other day for 2 wk, and supernates were pooled, concentrated, and assayed for reverse transcriptase activity.

XC-Plaque Assay. Petri dishes, seeded previously in medium containing 2 μg/ml polybrene, were inoculated with appropriate dilutions of the virus-containing supernates and later irradiated and overlaid with XC cells as described in Materials and Methods. Virus was also assayed on $129G_{IX}^{-}ME$ (Fv-1^{nn}) and on C57BIME (Fv-1^{bb}) cells with similar results.

Focus Assay. To determine if xenotropic virus was present, mink S+L− cells were infected with appropriate dilutions of virus containing supernates and observed for foci of transformed cells.

Infectious Center Assay and Cocultivation Experiments. To increase the sensitivity of the assays for biological activity of virus, the cell lines listed on the left were treated with mitomycin C and then seeded directly onto monolayers of NIHME, BALB/c ME, and mink S+L− cells and developed as for XC-plaque assay or a mink S+L− assay. In every case, the infectious center assay results agreed with those obtained from tests of the virus-containing supernates. From infectious center assays it was possible to determine that >90% of cells in the OTTF7 and OTTF12 cell lines were producing virus. For cocultivation experiments, mitomycin C-treated cells were seeded with mink lung cells and supernates were taken every 2 d for a month; supernates were then pooled, concentrated, and tested for reverse transcriptase activity. The virus derived from the OTT6050A-derived lines did not show xenotropic virus activity by any of these assays.

OTTF7 cells with mink lung cells (Table IV) and thus contains no detectable xenotropic or amphotropic virus. It is ecotropic and grows preferentially in cells of the Fv-1^{nn} genotype. Virus was collected from supernatant medium of the OTTF12 cells and from AKR N-tropic MuLV producing SC-1 cells and [^{3}H]cDNA probes, prepared as described in Materials and Methods. These two cDNA probes were then compared for homology to each of the respective high molecular weight viral RNA's from the purified virus preparations. In addition, these cDNA's were hybridized with cellular RNA extracted from the differentiated cell lines, 293S and OTTF12, and with RNA from HT1080 human cells and thermostabilities (T_m's) of the cDNA/RNA hybrids were determined. Results of the comparison of these two viruses are given in Table V.

TABLE V

Comparison of Nucleotide Sequences of AKR N-Tropic MuLV and N-Tropic MuLV Produced by OTT6050A-Differentiated Cell Lines

Source of RNA	[^{3}H]cDNA from			
	AKR N-tropic MuLV		OTTF12 N-tropic MuLV	
	Hybridization*	T_m (°C)	Hybridization	T_m (°C)
	%		%	
AKR N-tropic MuLV HMW	100*	85	>95	85
OTTF12 MuLV HMW	>95	84.5	100	85
293S cells			>95	85
OTTF12 cells	>95	84		
PCC4 cells	29	79		
129 embryo			29	79
J1SV9 cells producing B-tropic virus	>95	85		
Human cell producing class II xenotropic MuLV	80	81	70	80.5
Human cell producing class III xenotropic MuLV	43	79		
HT1080 cells	<5		<5	

[^{3}H]cDNAs were prepared from the endogenous N-tropic virus of AKR mice and from the virus produced by the OTT6050A-derived differentiated cell line, OTTF12, and hybridized to saturating levels with RNA. The amount of hybrid formed was determined by the S-1 nuclease method (41). T_m determinations were carried out as described in Materials and Methods.

* The level of hybridization between [^{3}H]cDNA and the homologous HMW viral RNA was normalized to 100%; the actual saturation level was >90%.

As can be seen from the data presented in this table the hybridization saturation level of the cDNA's with the HMW viral RNA from the heterologous N-tropic virus or with RNA from cells producing the heterologous N-tropic virus was >95% and the T_m's for the cDNA/HMW viral RNA hybrids were essentially the same whether the cDNA was hybridized to the homologous or heterologous HMW N-tropic viral RNA. In contrast, the hybridization saturation levels of thee cDNA's with RNA from cells producing class II and class III viruses is lower and the T_m's of the latter type of cDNA/RNA hybrids are much lower. We conclude from this comparison that the two viruses, N-tropic virus from AKR mice and N-tropic virus from OTT6050A-derived differentiated cells are indistinguishable on the basis of biological properties and molecular hybridization studies.

Viral DNA Sequences in OTT6050A-Derived Stem and Differentiated Cell Lines. The results presented above suggested that stem cells derived from the OTT6050A tumor may carry suppressed N-tropic MuLV genomes. To test this possibility, cellular DNA's were extracted from OTT6050AF1 BrdU, OTTF12, and PCC4 cells, and from AKR liver tissue, and 129/J embryo tissue, and hybridized with AKR N-tropic MuLV [^{3}H]cDNA. As shown in Fig. 1, OTT6050AF1 BrdU DNA hybridized to as high a saturation level (73%) as did DNAs from AKR liver and OTTF12 cells, both of which carry N-tropic MuLV genomes. On the other hand, the DNA's from 129/J embryo tissue and the PCC4 cells from which no ecotropic virus has been isolated,

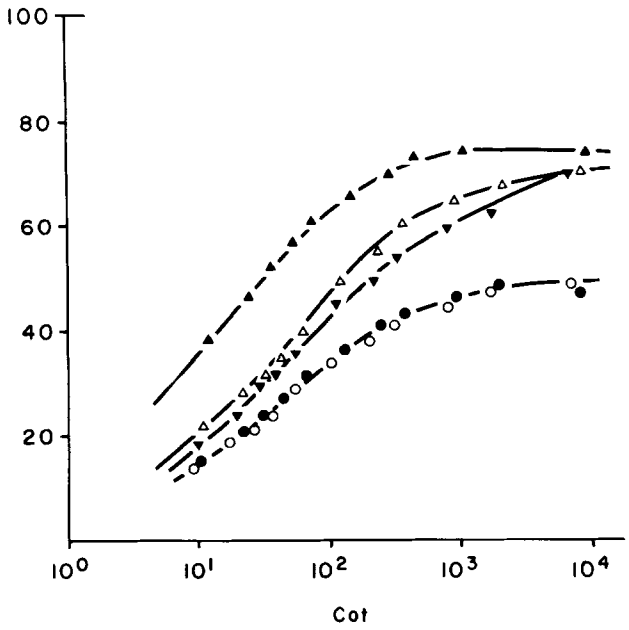

FIG. 1. Hybridization of cellular DNA with AKR N-tropic MuLV [^{3}H]cDNA. [^{3}H]cDNA was hybridized with cellular DNAs from OTT6050AF1 BrdU (5.1 mg/ml, ▲), OTTF12 (4.2 mg/ml, △), PCC4 (4.4 mg/ml, ●), AKR liver tissue (3.8 mg/ml ▼), and 129/J embryo tissue (3.8 mg/ml, ○) in capillary tubes as described in Materials and Methods. The extent of hybridization was determined by the hydroxylapatite batch elution method. C_0t is the product of the initial concentration of DNA (mol/liter) and the incubation time (s) as defined by Britten and Kohne (40).

TABLE VI

Thermostability of Hybrids between Cellular DNAs and cDNAs

DNA source	T_m (°C) of hybrid with AKR N-tropic MuLV cDNA	T_m (°C) of hybrid with OTTF12 MuLV cDNA
OTT6050AF1 BrdU cell	81.5	82
OTTF12 cell	81	81.5
Human cell-producing class II xenotropic virus		77.5
PCC4 cell	77.5	76.5

hybridized only to the 50% level. In addition, as shown in Table VI, the hybrids between the OTT6050A derived cellular DNAs (from both OTT6050AF1 BrdU and OTTF12) and the cDNAs from both AKR N-tropic MuLV or OTTF12 derived MuLV have higher T_m's than do the hybrids between PCC4 cellular DNA and the same two cDNA's. The results of these molecular hybridization and thermal denaturation studies demonstrate that the OTT6050A-derived stem cell line, OTT6050AF1 BrdU, contains N-tropic MuLV-related DNA sequences which are not present in PCC4 or 129/J embryo-derived DNA. It can also be estimated from the $C_0t_{1/2}$ values of Fig. 1 that the DNA of the stem cell line, OTT6050AF1 BrdU, contains more copies of the N-tropic AKR virus related DNA sequences than does the DNA derived from the differentiated cells, OTTF12, 100% of which produce N-tropic MuLV.

Discussion

We have described a set of murine teratocarcinoma derived stem and differentiated cell lines which we believe will be extremely useful in the study of the mechanism of control of viral gene expression in stem cells.

The stem cell line, OTT6050AF1 BrdU, consists of 97% undifferentiated cells and is completely negative for spontaneous expression of ecotropic murine leukemia virus by reverse transcriptase assay and XC-plaque assay of supernatant medium and for expression of viral protein by indirect immunofluorescence. The stem cells do, however, contain DNA sequences homologous to an N-tropic MuLV cDNA probe. Because none of the cells in the stem cell line shows any evidence of virus production, it is very unlikely that the presence of these DNA sequences could be explained by productive infection of a few cells in the culture, especially because the stem cell line carries more copies of these DNA sequences than do the cells of the differentiated cell line OTTF12, 100% of which are positive for viral protein expression and which are producing N-tropic MuLV. Thus, we have the ideal set of cells for the study of regulation of expression of genes in stem versus differentiated cells because the cells contain a set of genes (N-tropic viral genes), present in both the stem and differentiated cells, for which transcription and/or translation is differentially regulated in the two cell types.

These viral DNA sequences are probably not sequences of a virus endogenous to strain 129 mice because the DNA/DNA hybrids between PCC4 cellular DNA and N-tropic MuLV cDNA have a themal stability which is lower than that of DNA/DNA hybrids of OTT605AF1 BrdU cellular DNA with the N-tropic MuLV cDNA. The route of introduction of this viral DNA into the teratocarcinoma tumor cells is not known but we believe that, regardless of the origin of the virus and the route of introduction into the cells, these cell lines will provide a valid model for the study of regulation of expression of ecotropic MuLV in stem versus differentiated cells.

It is not yet known if all differentiated cells express the virus (if only a few did, the infection would spread since the cells of strain 129 mice are permissive for N-tropic MuLV) and when after differentiation the virus is expressed (probably not immediately because the stem cell line is not 100% free of differentiated cells and yet it is not possible to detect infectious virus in these cells even by cocultivation with NIH ME cells).

We are presently testing various agents which are known to induce virus production and/or differentiation in other systems (such as Friend erythroleukemia cells) to determine if and when the N-tropic virus is produced after differentiation of stem cells. In addition, because chimeric mice are being produced with this stem cell line, and hybrids derived from it (6, 42), we will have an opportunity to follow expression of the N-tropic virus in vivo.

Summary

Murine teratocarcinoma stem cells are nonpermissive for productive infection by a variety of DNA (polyoma and SV40 virus) and RNA (murine leukemia and sarcoma virus) tumor viruses whereas differentiated murine cells derived from the stem cells are permissive for productive (or abortive in the case of SV40) infection by these same viruses. The block to productive infection by these oncogenic viruses is at a postpenetration step in the replication cycle of these viruses but the precise level of the block

has not been established for any of these viruses. In this report we describe teratocarcinoma-derived stem and differentiated cell lines which should be especially useful in determining the level of the block to replication of ecotropic murine leukemia virus in murine teratocarcinoma stem cells. The stem cell line, OTT6050AF1 BrdU, which is completely nonpermissive to productive infection by Moloney murine leukemia virus and consists of 97% pluripotent stem cells, contains DNA copies of an RNA tumor virus which is indistinguishable from the N-tropic murine leukemia virus of AKR mice. The stem cells are negative for expression of viral reverse transcriptase, p30 and gp69/71 and no virus is found by XC plaque assay or other biological tests. Differentiated cells established from the same teratocarcinoma tumor are 100% positive for viral gp69/71, p30, and produce large amounts of reverse transcriptase activity and N-tropic virus as detected by biological assay. The virus isolated from the differentiated cells is closely related, if not identical to AKR N-tropic virus by nucleic acid hybridization studies and is thus not an endogenous virus of the 129 strain of mice. The teratocarcinoma tumor from which the cell lines were established had been carried in 129 mice and perhaps at some time in the mouse passage history the tumors were infected (nonproductively) with the N-tropic virus. Regardless of the origin of this viral DNA, the OTT6050A derived stem and differentiated cell lines should be extremely useful in defining in stem cells the step at which ecotropic murine leukemia virus replication is blocked.

We thank Dr. Beatrice Mintz for the OTT6050 tumors and Dr. Michael Dewey for preparation of an embryoid body culture. We thank Dr. Davor Solter for helpful discussions on characterization of our cell lines and D. Solter and B. Knowles for the generous contributions of anti SSEA-1 and anti H-2 sera.

We gratefully acknowledge the technical assistance of Joan Barrick and Matthew Burday.

Received for publication 20 February 1979.

References

1. Bernstine, E. G., M. L. Hooper, S. Grandchamp, and B. Ephrussi. 1973. Alkaline phosphatase activity in mouse teratoma. *Proc. Natl. Acad. Sci. U. S. A.* **70:**3899.
2. Jakob, H., T. Boon, J. Gaillard, J. F. Nicholas, and F. Jacob. 1973. Teratocarcinoma de la souris: isolement, culture et proprietes de cellules a potentialites multiples. *Ann. Microbiol. (Paris).* **124B:**269.
3. Lehman, J. M., W. C. Speers, D. E. Swartzendruber, and G. B. Pierce. 1974. Neoplastic differentiation: characteristics of cell lines derived from a murine teratocarcinoma. *J. Cell. Physiol.* **84:**13.
4. Martin, G. R., and M. J. Evans. 1974. The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture. *Cell.* **2:**163.
5. Martin, G. R., and M. J. Evans. 1975. The differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **72:**1441.
6. Illmensee, K., P. C. Hoppe, and C. M. Croce. 1978. Chimeric mice derived from human-mouse hybrid cells. *Proc. Natl. Acad. Sci. U. S. A.* **75:**1914.
7. Stevens, L. C. 1970. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and post-implantation mouse embryos. *Dev. Biol.* **21:**364.
8. Illmensee, K., and B. Mintz. 1976. Totipotency and normal differentiation of single teratocarcinoma cells cloned by injection into blastocysts. *Proc. Natl. Acad. Sci. U. S. A.* **73:** 549.

9. Papaioannou, V. E., M. W. McBurney, and R. L. Gardner. 1975. Fate of teratocarcinoma cells injected into early mouse embryos. *Nature (Lond.)*. **258:**70.
10. Dewey, M. J., D. W. Martin, G. R. Martin, and B. Mintz. 1977. Mosaic mice with teratocarcinoma-derived mutant cells deficient in hypoxanthine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. U. S. A.* **74:**5564.
11. Swartzendruber, D. E., and J. M. Lehman. 1975. Neoplastic differentiation: interaction of simian virus 40 and polyoma virus with murine teratocarcinoma cells in vitro. *J. Cell. Physiol.* **85:**179.
12. Lehman, J. M., I. B. Klein, and R. M. Hackenberg. 1975. The response of murine teratocarcinoma cells to infection with DNA and RNA viruses. *In* Teratomas and Differentiation. M. I. Sherman and D. Solter, editors. Academic Press, Inc., New York. 289.
13. Périès, J., E. Alves-Cardoso, M. Canivet, M. C. Debons-Guillemin, and J. Lasneret. 1977. Lack of multiplication of ecotropic murine C-type viruses in mouse teratocarcinoma primitive cells. *J. Natl. Cancer Inst.* **59:**463.
14. Teich, N. M., R. Weiss, G. R. Martin, and D. R. Lowy. 1977. Virus infection of murine teratocarcinoma stem cell lines. *Cell.* **12:**973.
15. Swartzendruber, D. E., T. D. Friedrich, and J. M. Lehman. 1977. Resistance of teratocarcinoma stem cells to infection with SV40: early events. *J. Cell. Physiol.* **93:**25.
16. Rowe, W. P., W. E. Pugh, and J. W. Hartley. 1970. Plaque assay techniques for murine leukemia viruses. *Virology.* **42:**1136.
17. Hartley, J. W., and W. P. Rowe. 1975. Clonal cell lines from a feral mouse embryo which lack host-range restrictions for murine leukemia viruses. *Virology.* **65:**128.
18. Peebles, P. T. 1975. An in vitro focus-induction assay for xenotropic murine leukemia virus, feline leukemia virus C, and the feline-primate viruses RD-114/ccc/M-7. *Virology.* **67:**288.
19. Rasheed, S., W. A. Nelson-Rees, E. M. Toth, P. Arnstein, and M. B. Gardner. 1974. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer (Phila.)*. **33:**1027.
20. Wright, B. S., P. A. O'Brien, G. P. Shibley, S. A. Mayyasi, and J. C. Lasfargues. 1967. Infection of an established bone marrow cell line (JLS-V9) with Rauscher and Moloney murine leukemia viruses. *Cancer Res.* **27:**1672.
21. Aaronson, S. A., and G. J. Todaro. 1968. Development of 3T3 like lines from BALB/c mouse embryo cultures: transformation susceptibility to SV40. *J.Cell. Physiol.* **72:**141.
22. Hino, S., J. R. Stephenson, and S. A. Aaronson. 1976. Radioimmunoassays for the 70,000-molecular-weight glyco-proteins of endogenous mouse type C viruses: viral antigen expression in normal mouse tissues and sera. *J. Virol.* **18:**933.
23. Toyoshima, K., and P. K. Vogt. 1969. Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. *Virology.* **38:**414.
24. Huebner, K., and C. M. Croce. 1976. Production of B-tropic murine leukemia virus by somatic cell hybrids between mouse peritoneal macrophages and simian virus 40-transformed human cells. *J. Virol.* **18:**1143.
25. Manly, K. F. 1974. Histones stimulate polyribonucleotide-directed polydeoxyribonucleotide synthesis by murine leukemia virus. *J. Virol.* **13:**305.
26. Strand, M., and J. T. August. 1973. Structural proteins on oncogenic ribonucleic acid viruses. Interspec II, a new interspecies antigen. *J. Biol. Chem.* **248:**5627.
27. Campbell, G., L. T. Goldstein, and B. B. Knowles. 1975. The use of flow microfluorimetry in the analysis of the phenotype expression of mouse histocompatibility antigens. *J.Cell Biol.* **64:**719.
28. Knowles, B. B., D. P. Aden, and D. Solter. 1978. Monoclonal antibody detecting a stage-specific embryonic antigen (SSEA-1) on preimplantation mouse embryos and teratocarcinoma cells. *Cur. Top. Microbiol. Immunol.* **81:**51.
29. Solter, D., and B. B. Knowles. 1978. Monoclonal antibody defining a stage-specific mouse

embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. U. S. A.* **75:**5565.

30. Taylor, J. M., R. Illmensee, and J. Summers. 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. *Biochim. Biophys. Acta.* **442:**324.
31. Tsuchida, N., M. S. Shih, R. V. Gilden, and M. Hatanaka. 1974b. Sarcoma and helper-specific RNA tumor virus subunits in transformed nonproducer mouse cells activated to produce virus by treatment with bromodeoxyuridine. *J. Virol.* **14:**1262.
32. Tsuchida, N., R. V. Gilden, M. Hatanaka, A. E. Freeman, and R. J. Huebner. 1975. Type-C virus-specific nulceic acid sequences in cultured rat cells. *Int. J. Cancer.* **15:**109.
33. Britten, R. J., D. E. Graham, and B. R. Neufield. 1974. Analysis of repeating DNA sequences by reassociation. *Methods Enzymol.* **29**(part E):363.
34. Hayward, W. S. 1977. Size and genetic content of viral RNAs in avian oncovirus-infected cells. *J. Virol.* **24:**47.
35. Tsuchida, N., and M. Green. 1974. Intracellular and virion 35S RNA species of murine sarcoma and leukemia viruses. *Virology.* **59:**258.
36. Tsuchida, N., M. S. Robin, and M. Green. 1972. Viral RNA subunits in cells transformed by RNA tumor viruses. *Science (Wash. D. C.).* **76:**1418.
37. Artzt, K., and F. Jacob. 1974. Absence of serologically detectable H-2 on primitive teratocarcinoma cells in culture. *Transplantation (Baltimore).* **17:**633.
38. Vitetta, E., K. Artzt, D. Bennett, E. A. Boyse, and F. Jacob. 1975. Structural product of the T locus isolated from sperm and murine teratoma cells. *Proc. Natl. Acad. Sci. U. S. A* **72:** 3215.
39. Forman, J., and E. S. Vitetta. 1975. Absence of H-2 antigens capable of reacting with cytotoxic T cells on a teratoma line expressing a T/t locus antigen. *Proc. Natl. Acad. Sci. U. S. A.* **72:**3661.
40. Britten, R. J., and D. E. Kohne. 1968. Repeated sequences in DNA. *Science (Wash. D.C.).* **161:**529.
41. Tsuchida, N., C. Long, and M. Hatanaka. 1974. Viral RNA of murine sarcoma virus produced by a hamster-mouse somatic cell hybrid. *Virology.* **60:**200.
42. Illmensee, K., and C. M. Croce. 1979. Xenogeneic gene expression in chimeric mice derived from rat-mouse hybrid cells. *Proc. Natl. Acad. Sci. U. S. A.* **76:**879.