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Viral Transfer, Transcription, and Rescue of a Selectable Myeloproliferative Sarcoma Virus in Embryonal Cell Lines: Expression of the *mos* Oncogene

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A derivative of the myeloproliferative sarcoma virus (Neo^r-MPSV) carrying the *mos* oncogene and dominant selection marker for neomycin resistance (Neo^r) was introduced into embryonal carcinoma and embryo-derived cell lines by transfection and infection using pseudotypes with Friend helper virus (Friend murine leukemia virus [F-MuLV]). Cells resistant to G418 (a neomycin analog) were cloned and expanded. Transductants retained an undifferentiated phenotype as judged by morphology, tumorigenicity, and cell-surface antigen analyses. Nucleic acid analysis of infectants revealed both Neo^r-MPSV and F-MuLV proviruses, although no virus was released. G418-resistant transductants remained nonpermissive for the expression of other proviruses and for subsequent superinfection. Northern analysis showed expression of full-length Neo^r-MPSV, as well as *mos*-specific subgenomic RNA. *mos* sequences were deleted from Neo^r-MPSV (Neo^r*mos*$^{-1}$), and pseudotypes were used to infect embryonal carcinoma cells. No morphological differences were observed in either *mos*$^+$ or *mos*$^-$ transductants as compared with parental cell lines. However, *mos*$^+$ transductants showed an enhanced anchorage-independent growth compared with that of *mos*$^-$ transductants in agar cloning. PCC4 transductants were induced to differentiate with retinoic acid and superinfected with F-MuLV. Infection with viral supernatant in fibroblasts and in mice confirmed the rescue of biologically active Neo^r-MPSV.

Embryonal carcinoma (EC) and embryo-derived (EK) cells provide a unique means for the introduction of recombinant genes into the mouse (5, 9, 12, 28). The potential for differentiation of EC and EK cell lines is similar to that for the cell mass of the early embryo, and thus they can participate in the normal development of somatic as well as functional germ cell lines after their introduction into early embryos (5, 12, 38). Moreover, transductant EC and EK cell lines can be characterized in vitro before chimera formation to examine integration and expression of the transduced genes.

Retroviruses have been shown to be natural transducing vectors in eucaryotic cells, and as such they provide a vehicle for introducing cloned genes into embryonal cells. They offer the advantage over DNA-mediated gene transfer of stable integration and higher transducing efficiency (15, 20). Previous studies have shown, however, that although the retrovirus efficiently integrates into the genome of the teratocarcinoma cell (29, 35, 42) or early embryo (11), expression of the virus is not detected or is very low. This block is presumably at the level of transcription (8, 17, 21, 39), although a secondary block, subsequent to RNA transcription, may also occur (8, 34).

In this study, we were able to detect expression in EC and EK cell lines of a retroviral recombinant carrying the transposon neomycin resistance (Neo^r) gene and the *mos* oncogene. The myeloproliferative sarcoma virus (MPSV), a member of the Moloney sarcoma virus (Mo-MSV) family, was used as the basis of the two retrovirus vectors used in these experiments. MPSV is a replication-defective sarcoma virus containing Moloney murine leukemia virus (Mo-MuLV) sequences and the *mos* oncogene (15, 36). It induces extensive changes in the hematopoietic system, including spleen foci in mice, and can transform fibroblasts in vitro (15, 27). Infection in fibroblasts and mice has shown that expression of the Neo^r gene within the MPSV genome does not disrupt expression of the *mos* oncogene (W. Ostertag et al., J. Gen. Virol., in press). A second vector was constructed in which the *mos* gene was deleted as a control for *mos* expression.

The efficiency of transfer and expression of Neo^r-MPSV into EC and EK cells is presented in this report. Analyses of G418-resistant EC and EK transductants confirmed integration and expression of the entire Neo^r-MPSV genome. Phenotypic effects of *mos* expression were determined. We demonstrate that retroviral functions were not subject to an EC-specific block, as the provirus was successfully rescued with helper virus after differentiation was induced with retinoic acid (RA). The unaltered biological property of Neo^r-MPSV to transform fibroblasts and hematopoietic cells was confirmed.

MATERIALS AND METHODS

Recombinant plasmid vectors. Construction of Neo^r-MPSV (p663neo) (Ostertag et al., in press) and Neo^r*mos*$^{-1}$ (pC6M$^-$neo) (40) are described elsewhere in detail and are represented schematically in Fig. 1. The 1.5-kilobase (kb) *Bgl*II-*Bam*HI fragment originating from the bacterial transposon Tn*5* contains the coding region of the aminoglycosyl phosphotransferase type II (32), conferring resistance to the antibiotic G418 (a neomycin analog) in eucaryotic cells. Expression of the Neo^r gene in the two vectors is dependent

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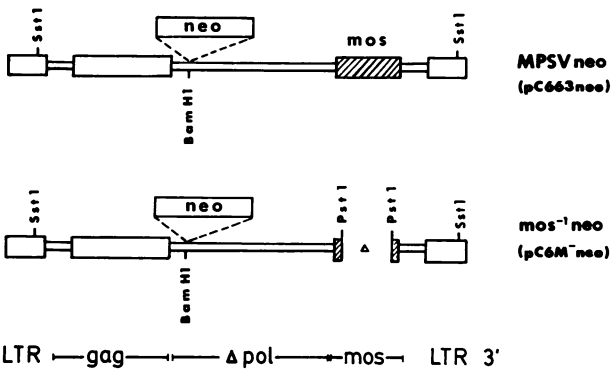

FIG. 1. Diagram of the two retroviral vectors used in transfection and infection experiments. Neo^r-MPSV was constructed by ligating the Tn*5* coding region into the *Bam*HI restriction site in the defective *pol* gene of the molecularly cloned MPSV proviral DNA (Ostertag et al., in press). To construct the *mos*$^-$ vector, three internal *Pst*I fragments of the *mos* coding region were deleted in Neo^r-MPSV (40). The Neo^r gene was oriented in the same translational direction as the MPSV *gag* and *mos* genes.

on viral transcription signals as verified by S1 mapping (unpublished data), and translation presumably occurs by the same mechanism as that of the *pol* gene in Mo-MuLV (44). Previous work in fibroblasts has shown that the expression of the Neo^r gene does not disrupt *mos* expression (Ostertag et al., in press). The transforming activity of the Neo^r*mos*$^{-1}$ vector was tested in fibroblasts and mice; it proved to be negative for its potential to transform both fibroblasts and cells of the hematopoietic system (40).

Cells. PCC4aza3 and PCC4aza1 are 8-azaguanine–resistant EC cell clones of clone PCC4 which was originally derived from tumor OTT6050. PCC4aza1 and PCC4aza3 do not differentiate either spontaneously or after treatment with RA. The clonal derivative of PCC4aza1, PCC4aza1R, is inducible on exposure to RA (13). PCC4 (RA)$^{-1}$ is an RA-resistant subclone of PCC4aza1R which does not differentiate on exposure to RA. The differentiated yolk sac cell clone PYS-2 (16) is also derived from OTT6050 and served as a control. TG12 is a spontaneous mutant of the parental cell line PSA4 isolated from the tumor OTT5568 (37). B2B2 was obtained directly from in vitro cultures of a mouse strain 129 blastocyst (5). Both cell lines were grown on X-ray–inactivated SC1 fibroblast feeder layers (except when noted). For G418 selection experiments, a G418-resistant SC1 cell line which had been transfected with pAG60 (4) was used. All EC cell lines were maintained in modified Eagle medium (27).

RAT 1 cells (43) releasing Neo^r-MPSV (RAT 1-4Mneo+N) (Ostertag et al., in press) or Neo^r*mos*$^{-1}$ (C6M$^-$neo#11) (40) were obtained on transfection with one of the two vectors, followed by infection with biologically cloned Friend murine leukemia virus (F-MuLV; 643/22N) (26). Endpoint dilution virus supernatant of these cell lines was used to obtain secondary nonproducer NRK cell clones. Clones 1-4Mneo#21 or #23 and C6Mneo#11/4, each containing one unaltered copy of the respective proviral genome, were superinfected with F-MuLV to obtain infectious and cloned pseudotypes.

Characterization of the EC and EK phenotype. (i) Cell surface antigen analysis. The monoclonal antibody SSEA-1, which defines a stage-specific mouse embryonic antigen, was kindly provided by P. Goodfellow (London) (3) and was used to define the differentiated state of the uninduced and induced cells. Anti-mouse fluorescein isothiocyanate-labeled antisera were used as a second antibody; cells were grown on cover slips, fixed in acetone for 15 min on ice, washed, and incubated at 37°C with the first and later the second antibody.

(ii) Tumor formation. Subcutaneous tumors were produced in strain 129 mice by inoculating 5×10^5 cells per mouse. TG12 and B2B2 cell lines grown on fibroblast feeder layers were released from feeder layer cells by gentle shaking. Cells were pelleted and suspended in phosphate-buffered saline. Solid tumors developed within 4 to 5 weeks.

Induction of differentiation in EC cells. Differentiation of EC cells was induced by exposing logarithmically growing cells to 10^{-7} M RA maintained as a stock solution of 10^{-2} M in dimethyl sulfoxide. Treatment with RA was performed for 5 days. Controls were exposed to 0.001% dimethyl sulfoxide, the concentration which was used during the induction of RA-treated cells.

Transfection and infection experiments. Plasmid DNA was transfected into cells as described earlier (15). The calcium phosphate precipitate, formed with 10 μg of plasmid DNA and 20 μg of calf thymus DNA, was added to a flask containing 10^6 cells. TG12 cells were plated out without feeder cells in medium containing 10^{-4} M β-mercaptoethanol 24 h before transfection. Thirty-six hours after transfection, TG12 cells were trypsinized and plated on freshly prepared Neo^r-SC1 feeder cells. Cells were fed daily and transferred weekly to a fresh feeder layer.

Virus stock dilutions containing either Neo^r-MPSV or Neo^r*mos*$^-$ and F-MuLV helper were added to 5×10^5 cells in T75 flasks or, alternatively, to 3×10^3 cells in 24-well plates. Twenty-four hours after infection, TG12 and B2B2 cells were trypsinized and plated on freshly prepared Neo^r-SC1 feeder cells.

Cells which were exposed to DNA or to virus were maintained for 36 to 48 h without selection and then subjected to a medium containing 400 μg of G418 per ml. This concentration of G418 kills all cells without an active Neo^r gene within 10 to 14 days. Alternatively, infected PCC4 cell lines were subjected to increasing levels of G418 (100 to 400 μg) over a period of 16 days, starting selection on day 4. Colonies were counted after 20 days. A fraction of the resistant colonies were isolated and expanded for further analysis.

Virus activity. Virus titer was estimated by assaying supernatants for reverse transcriptase (RT) activity (27). The fibroblast-transforming activity was determined by fibroblast focus formation on NRK or RAT 1 cells at endpoint dilution. Leukemia-inducing activity was determined by counting spleen foci of mice 16 days postinfection (intravenously) as described earlier (27).

DNA and RNA analysis of transductant colonies. DNA purification, restriction enzyme digestion, and Southern blot analysis were performed as described earlier (15). Total cellular RNA was extracted (2), glyoxylated, and subjected to agarose gel electrophoresis (20 μg per slot) (18). The RNA was transferred to a GeneScreen Plus, as recommended by the supplier (New England Nuclear Corp.). TG12 and B2B2 cell lines were transferred to G418-sensitive SC1 feeder cells 5 days before DNA and RNA isolation. The G418 concentration was reduced to 200 μg/ml, which could be tolerated by the SC1 cells until harvest. In some cases, G418 selection was removed for economic reasons during large-scale PCC4 transductant cell culture preparations. A Neo^r-specific DNA fragment isolated from plasmid pAG60 (3), a *mos*-specific fragment from plasmid pMS1 (24), and a cloned F-MuLV

proviral DNA from plasmid pFMuLV21A1 (22) were ^{32}P labeled by nick translation and used as probes.

RESULTS

Transfer of Neo^r-MPSV into EC cells. (i) Transfer by transfection. Four EC and EK cell lines, PCC4aza1R, PCC4aza3, TG12, and B2B2, were transfected with Neo^r-MPSV plasmid DNA. After selection with 400 μg of G418 per ml for 2 to 3 weeks, colonies were counted. The results of experiments with PCC4aza3 and PCC4aza1R are summarized in Table 1, along with the results from control transfection experiments with RAT 1 cells.

No G418-resistant TG12 or B2B2 clones were obtained in four separate experiments in which Neo^r-SC1 feeder cells were used (see Materials and Methods). TG12 cells were adapted to feeder-independent growth in β-mercaptoethanol (23) 1 week before transfection. In one of four experiments, G418-resistant cells were obtained after 5 weeks of G418 selection. The resulting nonclonal cell line was named TG12 IB. The frequency of transfection could not be determined accurately, but it was quite low.

(ii) Transfer by infection. Neo^r-MPSV–F-MuLV virus complex used to infect EC and EK cell lines was obtained from F-MuLV–superinfected RAT 1-4Mneo or one of its two subclones (no. 21 and 23). Infected cell lines were challenged with G418 either 36 to 48 h or 96 h after infection. After 14 to 20 days of G418 selection, resistant colonies were counted. The results for experiments with PCC4aza3 and PCC4aza1R are summarized in Table 1. The frequency of Neo^r gene transduction in PCC4 cell lines compared with that in fibroblasts was determined and expressed as a ratio (Table 1). Individual G418-resistant PCC4 colonies were isolated and expanded.

G418-resistant TG12 and B2B2 colonies appeared within 2 weeks after infection. The frequency of transduction could not be accurately determined owing to the disruption of colony formation that occurred when feeder layers were exchanged. Three Neo^r cell lines from separate experiments were established and named TG12 I, TG12 II, and B2B2 I.

Confirmation of EC and EK phenotype in transductant cell lines. To exclude the possibility that the population receptive to the expression of Neo^r was limited to cells that had differentiated spontaneously or that differentiation had occurred after expression of the Neo^r-MPSV genome, the transductant cell lines were characterized in terms of their EC or EK phenotype. Neo^r cell lines obtained after either infection or transfection were injected into 129/J mice. Solid tumors developed in all mice within 4 weeks. Cells from these tumors were heterogeneous and showed extensive differentiation. All of the G418-resistant cell clones, including cell lines derived through tumor passage in mice, were unaltered in expression of the SSEA-1 antigen. The results are summarized in Table 2.

TABLE 1. Transfer of Neo^r-MPSV into PCC4aza3 and PCC4aza1R cells by transfection and infection

Cell line	Cloning efficiency (%)	Expt 1 (transfection)		Expt 2 (infection)	
		No. of clones[a]	Ratio of transduction frequency[b]	No. of clones[c]	Ratio of transduction frequency[b]
RAT 1	37	361	1	10^6	1
PCC4aza3	79	0.9	2.5×10^{-3}	42	4.2×10^{-5}
PCC4aza1R	44	0.6	1.6×10^{-3}	63	6.3×10^{-5}

[a] Number of clones per microgram of Neo^r-MPSV plasmid DNA per 10^6 cells.

[b] Frequency of gene transduction in EC cell lines compared with that in fibroblasts.

[c] Number of clones per FFU of Neo^r-MPSV virus. Neo^r-MPSV was obtained after superinfection of nonproducer clones 1-4Mneo#23. Similar numbers were obtained with virus of clone RAT 1-4Mneo or virus of subclone 1-4Mneo#21. The multiplicity of infection in the experiments with PCC4 cells was 20.

TABLE 2. Characterization of the parental and transductant EC and EK lines[a]

Cell line	Neo^r-MPSV transfer	% cells immunofluorescent for SSEA-1	Tumorigenicity (5×10^6 cells per mouse)
PYS-2[b]		<0.5	–
PCC4aza1R		36	+
pCC4aza1R			
#2	Transfection	28.5	+
#5	Transfection	32	+
#6	Transfection	40	+
#4	Infection	36	+
#5	Infection	41	+
#6	Infection	45	+
TG12		62	+
TG12 IB	Transfection	58	+
TG12 I	Infection	55	±
TG12 II	Infection	57.5	±
B2B2		49	+
B2B2 I	Infection	45	+

[a] Results are from one representative experiment in which 200 cells of each line were screened.

[b] PYS-2, a differentiated cell line derived from the teratocarcinoma cell line OTT6050, was used as a negative control.

G418-resistant transductant cell lines were reexposed to F-MuLV to test an altered permissiveness for viral expression. RT activity was still at background levels in the PCC4 transductants that were analyzed (Table 3). No detectable levels of gp70 were present that may have inhibited virus infection (data not shown).

Stability of Neo^r phenotype in transductant clones. Evidence suggests that a total block to retroviral replication is a multiple-step process which does not occur immediately after virus integration. If selective pressure is relaxed, it is conceivable that a mixed-cell population would emerge, including cells in which a retroviral transcription block had subsequently occurred. Transductant PCC4 cell clones were grown in the absence of G418 for 10 generations. The cloning efficiency in the presence and absence of G418 was determined and compared with that of parallel cell populations in which selective pressure had not been released. The results are shown in Table 4. G418-resistant cells obtained by transfection exhibited a reduced cloning efficiency in the presence of G418 after growth in nonselective conditions. However, infected transductant cell clones demonstrated stability of the Neo^r phenotype. The cloning efficiencies obtained for all six infected clones were comparable whether or not selective pressure had been released.

Analysis of transduced viral genes. Genomic DNA of G418-resistant cell lines was isolated and analyzed by Southern blot hybridization. *Sst*I restriction endonuclease cuts only in the long terminal repeat (LTR) of Neo^r-MPSV and is thus diagnostic of an intact proviral structure. Autoradiographs are shown in Fig. 2 and 3. In transfected cell lines, we detected copies of both full-length and altered sizes that hybridized to both Neo^r and *mos* probes, although we often detected altered fragments that hybridized only to the Neo^r probe (Fig. 2, lanes b, and data not shown). No bands were

TABLE 3. Virus expression dependent on the state of differentiation

Cell clone	Treatment of clones		% cells fluorescent for SSEA-1	Virus release	
	RA	F-MuLV		RT activity (%)	Fibroblast transforming activity of supernatant
F4-6[a]	−	−		100	No
PYS-2	−	−	<0.4	<0.75	ND[b]
PCC4aza(RA)$^{-1}$	−	−	28	ND	ND
	+	−	30	ND	ND
PCC4aza1R	−	−	36	<0.62	No
	−	+	ND	<0.53	ND
	+	−	<0.5	<0.3	No
	+	+	ND	5.5	ND
PCC4aza1R#4	−	−	36	<0.52	No
	−	+	ND	<0.68	ND
	+	−	<0.4	<0.75	No
	+	+	ND	6.2	Yes
PCC5aza1R#5	−	−	41	0.9	No
	−	+	ND	0.56	ND
	+	−	<0.75	0.4	No
	+	+	ND	22.5	Yes
PCC4aza1R#6	−	−	45	<0.2	No
	−	+	ND	<0.41	ND
	+	−	<0.5	<0.7	No
	+	+	ND	15.8	Yes

[a] F4-6 is a high-virus-producing Friend cell line used as a standard for RT activity (30).
[b] ND, Not determined.

detected in transfected PCC4aza3 with either probe; this was probably a result of the release of G418 selection during large-culture preparations and subsequent loss of Neo^r sequences that were not stably integrated (see above).

Infected transductant cell lines contained one or multiple full-length copies of the viral genome that hybridized to both Neo^r and *mos* probes (Fig. 2A and 2B, lanes c to f; Fig. 3A and 3B). In B2B2 I, altered copies with deleted *mos* sequences were also detected (Fig. 2, lanes f). Bands 1 to 2 kb smaller than full-length hybridizing to both *mos* and Neo^r probes were also detected in PCC4aza1R clones (Fig. 3A, 3B, and 3C, lanes b to d). It has been shown by additional Southern analysis with other restriction endonucleases that *gag* sequences have been deleted in these proviral genomes, presumably to facilitate Neo^r expression.

G418-resistant PCC4 clones obtained by infection were also examined for the presence of F-MuLV helper sequences by using an F-MuLV DNA probe. *Sst*I fragments of 6.3 and 2.7 kb are specific for F-MuLV (1). Bands correlating to these sizes were found in all resistant EC cell lines which were tested (Fig. 3C).

Infected PCC4aza1R cells which had not been challenged with G418 were also analyzed for the incorporation of Neo^r-MPSV sequences. Two cultures of 5×10^5 cells were infected with 10^7 focus-forming units (FFU) of Neo^r-MPSV. One culture was subjected to G418 selection, and after 2 weeks 380 colonies were scored. In the parallel, untreated culture, 10 colonies were randomly selected, and dot-blot analysis was performed. Six colonies contained sequences that hybridized to a Neo^r probe (data not shown). None of these six clones was G418 resistant. These numbers can be used to calculate the number of infectious and integrating viral particles required to obtain one cell which expresses the Neo^r gene. If 60% of 5×10^5 cells (3×10^5 cells) are infected by using 10^7 FFU and an average of 380 resistant clones are obtained from 3×10^5 cells, then the fraction of cells expressing the Neo^r gene is less than 1 of 10^3.

Expression of the transduced viral genes. Total cellular RNA of TG12 II was analyzed by Northern blots for the expression of Neo^r-MPSV sequences. Autoradiographs are shown in Fig. 4. Hybridization to a Neo^r probe detected an 8.1-kb transcript in TG12 II cellular RNA, as well as in cellular RNA from the control cell line 1-4Mneo cl 23. Hybridization with a *mos* probe detected the same 8.1-kb transcript, as well as a subgenomic 4.5-kb transcript with TG12 II RNA. In longer exposures with 1-4Mneo cl 23, the same subgenomic 4.5-kb fragment, as well as a 2.6-kb

TABLE 4. In vitro stability of Neo^r phenotype in either transfected or infected EC clones

Cell clone	Cloning efficiency[a]			
	Continuous selection		No selection for 10 generations	
	−G418	+G418	−G418	+G418
PCC4aza1R		$<10^{-8}$	44	$<10^{-8}$
Transfected transductants				
PCC4aza1R#2	38	40	40	8
PCC4aza1R#5	36	35	35	$<2 \times 10^{-5}$
PCC4aza1R#6	32	39	38	$<10^{-6}$
Infected transductants				
PCC4aza1R#1	35	35	36	35
PCC4aza1R#2	30	32	27	30
PCC4aza1R#3	40	38	40	34
PCC4aza1R#4	49	51	48	53
PCC4aza1R#5	50	49	52	46
PCC4aza1R#6	38	38	35	38

[a] The cloning efficiency of transfected or infected PCC4aza1R clones was determined in the presence or absence of G418 by growing the cells continuously under selective conditions or maintaining the cells for 10 generations without selection before cloning.

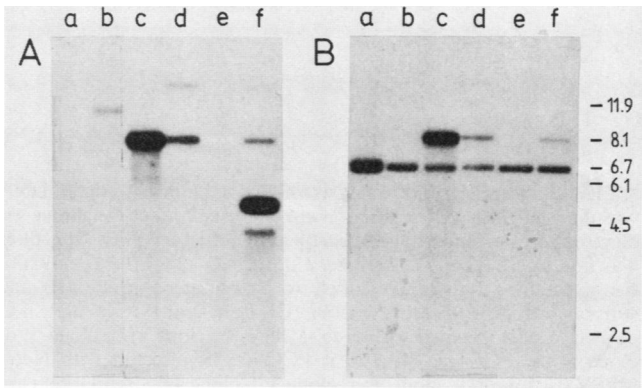

FIG. 2. Southern blot analysis of TG12 and B2B2 clones. Cellular DNA was digested with *Sst*I restriction enzyme, separated by agarose gel electrophoresis, and transferred to a GeneScreen Plus. The filter was hybridized to a Neo^r gene probe (A) and a *mos* gene probe (B). Lanes: a, TG12; b, TG12 IB transfected with Neo^r-MPSV; c to d, TG12 infected with Neo^{r}MPSV (c, TG12 I; d, TG12 II); e, B2B2; f, B2B2 infected with Neo^r-MPSV. *Sst*I cleaved once in each LTR of Neo^r-MPSV, resulting in an 8.1-kilobase-pair fragment.

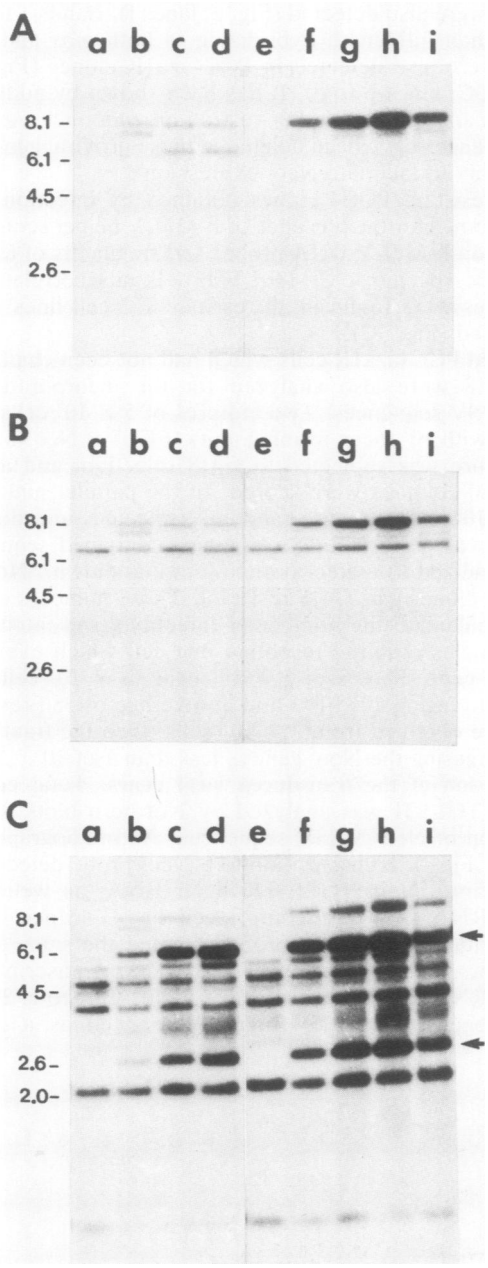

FIG. 3. Southern blot analysis of PCC4 clones. Cellular DNA was digested with *Sst*I restriction enzyme, separated by agarose gel electrophoresis, and transferred to a GeneScreen Plus. The filter was first hybridized to a Neo^r gene probe (A) and, after removal of the hybridized radioactive label, to a *mos* gene probe (B) and subsequently to an F-MuLV probe (C). Lanes: a, PCC4aza1R; b to d, pCC4aza1R infected with Neo^r-MPSV (b, clone 4; c, clone 5; d, clone 6); e, PCC4aza3R; f to i, PCC4aza3R infected with Neo^r-MPSV (f, clone 1; g, clone 2; h, clone 3; i, clone 4). Size markers are given in kilobase pairs. *Sst*I cleaved Neo^r-MPSV once in each LTR, resulting in a fragment of 8.1 kilobase pairs. The 6.3 and 2.7 kilobase pairs are *Sst*I fragments specific for F-MuLV and indicated by arrows (1). In addition to Neo^r-MPSV specific sequences, endogenous *mos* sequences (6.7 kb) were detected with the *mos*-specific probe (panel B).

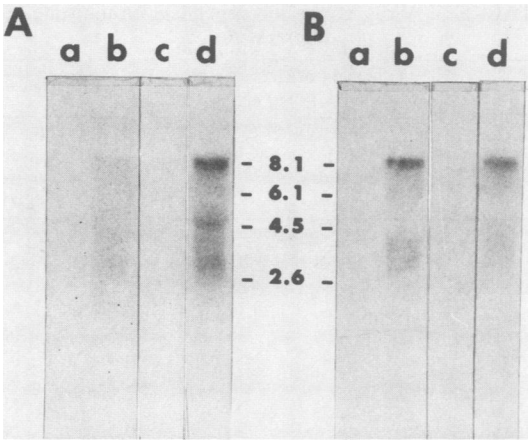

FIG. 4. Northern blot analysis of RNA extracted from Neo^r-MPSV–infected cell lines. Detection of transcripts homologous to *mos*- (A) and Neo^r- (B) specific sequences in NRK cells (lane a), Neo^r-MPSV–infected NRK nonproducer cells (1-4Mneo cl 23) (lane b), TG12 (lane c), and Neo^r-MPSV–infected TG12 cells (TG12 II) (lane d). Size markers are given in kilobase pairs.

fragment, was detected with the *mos* probe, but not with the Neo^r probe (data not shown).

As shown by Southern blot analysis (Fig. 3C), transductant cell clones contained multiple copies of F-MuLV helper. This was to be expected, as high titers of helper virus (10^7 XC U/5 × 10^5 PCC4 cells) were used in infections, and earlier reports showed that EC cells are permissive for retroviral penetration and integration (39, 42). Expression of F-MuLV was assessed by RT activity (Table 3). Neither clones selected by G418 nor clones randomly selected released a detectable quantity of F-MuLV, although less than about 1 in 10^4 cells releasing virus would not have been detected by this assay.

Anchorage independence correlated with *mos* expression. Northern blot analysis indicated that the *mos* gene was transcribed and, hence, possibly translated in transductant cell clones. However, no detectable morphological changes were observed in the Neo^r-MPSV–expressing cell clones. To ascertain whether the expression of the transforming gene resulted in increased anchorage independence of cells, the cloning efficiency in agar was determined. As a control, G418-resistant PCC4aza1R clonal cell lines, obtained by infection with Neo^r*mos*$^-$ pseudotypes from superinfected NRK nonproducer cell line C6M$^-$#11/4, were also tested. In Neo^r*mos*$^-$, the *mos* gene has been partially deleted (Fig. 1) and is functionally inactivated as determined by FFU in fibroblasts and spleen FFU in infected mice (40). The results of agar cloning (Table 5) indicated an enhanced anchorage independence in *mos*-containing transductants.

Release of pseudotypes containing the Neo^r-MPSV genome after induction of differentiation. G418-resistant PCC4aza1R cell lines were exposed to RA to induce differentiation. The parental PCC4aza1R cell clone and the PCC4aza(RA)$^{-1}$ cell clone (which is refractory to RA induction) were used as controls. RA did not induce differentiation in PCC4aza(RA)$^{-1}$ as expected, but converted all other cells tested into epidermislike cells. A diminished growth rate and reduction of the expression of SSEA-1 below detectable levels indicated an almost complete conversion to the differentiated derivative of EC cells (Table 3).

Expression of virus release was tested by assaying RT activity. Even though they contained integrated copies of

TABLE 5. Enhanced anchorage-independent growth of PCC4azalR cells infected with *mos*-containing vector

Virus source	Cell clone	Cloning efficiency in agar (%)[a]
Uninfected	PCC4azalR	6
C6M⁻neo#11/4 + N[b] (*mos*⁻)	cl 2	12
	cl 3	9
	cl 5	10
	cl 6	7
1-4Mneo#23 + N[b] (*mos*⁺)	cl 1	43
	cl 2	29
	cl 3	52
	cl 4	47

[a] Ten, 100, or 500 cells were plated in minimal essential medium–10% fetal calf serum containing 0.3% agar. The clones were counted 10 to 14 days after plating. Each percentage represents the mean of three independent experiments.

[b] N, F-MuLV clone 643/22N.

F-MuLV, none of the cell clones (Fig. 1) released helper virus after exposure to RA. This confirmed earlier results of our group (1, 10) and others (8, 39) indicating that newly introduced genes inactivated during the undifferentiated state cannot be reactivated upon differentiation. Reexposure to F-MuLV, however, confirmed the now permissive state of the differentiating EC cells: all superinfected cell clones released retrovirus (Table 3).

To establish whether released viral particles also carried the Neo^r-MPSV genome that had integrated before differentiation, NRK cells were infected and selected for G418 resistance. Neo^r cell clones were established, indicating release of the Neo^r-MPSV genome. Furthermore, virus from every G418-resistant clone tested transformed fibroblasts (Table 3) and induced leukemia in infected animals (data not shown). These results indicated that infectious and transforming Neo^r-MPSV could be rescued in differentiating G418-resistant PCC4 cells; segregation of the two gene functions had not occurred.

DISCUSSION

Neo^r-MPSV can be used successfully as a gene transfer vector in EC or EK cells, by either transfection or, more efficiently, viral infection. Transductant clones expressing the entire Neo^r-MPSV genome were obtained after G418 selection of infected feeder-independent PCC4 EC cells, as well as two feeder-dependent cell lines that retain their ability to take part in the formation of chimeric animals (5, 38): the aneuploid EC cell line TG12 (33) and the euploid EK cell line B2B2 (5). Infection with the Neo^r-MPSV virus resulted in the isolation of 10- to 100-fold more resistant clones per cell number than in experiments where transfection was used. Furthermore, the expression of the Neo^r phenotype in infected transductants was stable.

Comparisons of gene transfer efficiency in fibroblasts and EC cell lines (Table 1) suggested that in EC cells relative to fibroblasts, transfected DNA is expressed at a higher frequency than DNA introduced by infection. This phenomenon could have been a consequence of the loss of specific sequences upon transfection (e.g., LTR sequences), resulting in the promotion or enhancement of the Neo^r gene by proximal sequences endogenous to the EC genome, or, alternatively, a consequence of the introduction of multiple copies of Neo^r-MPSV into each cell, increasing the chance of expression. Analysis of transfected transductants confirmed loss of Neo^r-MPSV sequences and multiple copy number of the transduced genes. Strong selective pressure possibly favored the selection of a subclass of cells with amplification of the Neo^r gene or deletions in particular sequences (e.g., *mos* sequences) that may be deleterious when highly expressed.

The frequency of expression of the Neo^r-MPSV genome in EC cells is low relative to that in fibroblasts (Table 1). The introduction of virus and recombinant expression vectors into embryos and EC cells has shown that gene regulation in the developing embryo is a complicated process. The chromosomal integration site (11), degree of methylation (8, 21, 39), and tissue-specific transcriptional signals (17) have all been correlated with gene expression, acting singly or in concert. Earlier work with Mo-MuLV indicated that a block to retrovirus replication occurred in EC cells; although the virus was fully integrated within the genome, no virus particles or RNA transcripts were detected. The success of detecting transductants by using our retroviral vector in EC and EK cells, as well as two other Neo^r-transducing retrovirus vectors described recently (34, 41), may in part have lay in the use of G418 selection. We were able to detect transductants occurring at a frequency of 2×10^{-6}; this was much more sensitive than standard RT assays. Furthermore, the possibility that a second block that prevents virus release subsequent to RNA transcription also occurs in the undifferentiated state cannot be ruled out (34).

G418-resistant transductants remained nonpermissive for additional virus expression, suggesting that a *cis*-acting mechanism for expression was involved. The relatively low frequency of expression could thus represent the fraction of integration sites which are permissive for expression. Preliminary work by Sorge et al. indicated that integration into transcriptionally active sites may allow transcription of the retrovirus genome (34). Alternatively, the frequency of expression may represent the frequency of mutated Neo^r-MPSV that permits virus-controlled transcription in EC cells. Studies have indicated that the tissue-specific enhancers of Mo-MuLV are not recognized in F9 cells (17). Mutations possibly occur in these regulatory sequences that allow recognition in EC cells as previously shown in polyoma mutants (14). Indeed, previous work in our laboratory has shown that point mutations in the U3 region of the MPSV LTR as compared with that of Mo-MSV or Mo-MuLV confer an altered target cell specificity to the virus, as demonstrated by its effect on the hematopoietic system (36, 40; C. Stocking et al., submitted). It could be envisaged that only slight alterations in the LTR may be required for the recognition of transcriptional signals in EC cells or that they are already recognized to various degrees in EC lines of different origins.

Earlier work suggested that a total block to retroviral replication does not occur immediately after virus infection and is a multiple-step process. Methylation of the proviral genome has been implicated as one mechanism in which the retroviral genome is silenced (39); its delayed onset, however, suggests that it is a secondary block that fixes the quiescent transcriptional state (8, 21). Studies with azacytidine suggest that this block continues after differentiation is induced (21). Our results showed that the G418-resistant infectants were not subject to a temporal secondary inactivation of retrovirus expression, as evidenced by stability of the Neo^r phenotype after selective pressure was removed. Furthermore, the successful rescue of the integrated Neo^r-MPSV provirus after the induction of differentiation supports these results, as well as demonstrating that no additional block to viral functions contained in the LTR

had occurred. The Neo^r-MPSV remains transcriptionally active from sequences in the LTR.

Equally significant is that the unaltered biological activity of the provirus can be confirmed after rescue. Infection into fibroblasts and mice confirmed its Neo^r-transfer and transforming potential. Segregation of the two functions did not occur. This was supported by Northern analysis of the EC transductants, which revealed that not only did a full-length genomic RNA species hybridize to both Neo^r and *mos* sequences expressed, but also that a subgenomic RNA species hybridized only to the *mos* probe. Attempts have been made in our laboratory to detect the *mos* protein in both fibroblasts and EC cells infected with MPSV by using 37-55 v-*mos* sera obtained from R. Arlinghaus. Our results have thus far proved negative despite the transformed phenotype in fibroblasts. This is not surprising in view of the low levels of *mos* protein needed for transformation (27a).

Although no morphological changes were observed in the transductant cell lines, our studies showed an enhanced anchorage-independent growth in EC clones expressing the *mos* gene. This showed direct evidence of the interaction of the *mos* oncogene with early embryonic cells. Expression of *mos*, however, does not cause differentiation of EC lines as shown for c-*fos* (20). Studies with c-*myc* and c-*src* in F9 cells by M. Karin have also shown that differentiation is not promoted after expression (personal communication), nor have altered growth properties of EC cells with these oncogenes been reported.

Until recently, cellular *mos* (c-*mos*) gene expression had been detected only in a mouse plasmacytoma (31), although expression had been sought in prenatal development (19) and in established murine cell lines (6, 7). Propst and Vande Woude recently detected significant levels of *mos* transcripts in mouse embryos, testes, and ovaries (30). Work with embryonic cells in culture may be able to define the role of *mos* expression in mouse development. The vectors described here, Neo^r-MPSV and Neo^r*mos*$^{-1}$, as well as a similar selectable vector expressing a temperature-sensitive *mos* product (15, 25), can be used in conjunction with EC and EK cell lines to further study *mos* expression. The use of EK cells further extends the utility of this approach due to their efficient ability to contribute to germline chimera formation (3a, 5).

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