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Retroviral mutants efficiently expressed in embryonal carcinoma cells

(myeloproliferative sarcoma virus/tissue-specific enhancer/neomycin-resistance gene/*mos* oncogene/gene transfer)

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ABSTRACT **The myeloproliferative sarcoma virus (MPSV) is a unique member of the Moloney murine sarcoma virus family. Due to mutations in the U3 region of its long terminal repeat, MPSV has an expanded host range that includes cells of the hematopoietic compartment. Using a MPSV recombinant containing the gene for neomycin-resistance (Neo^R-MPSV), we demonstrate that the host range of MPSV also includes undifferentiated F9 embryonal carcinoma cells. Transfer of G418-resistance with Neo^R-MPSV to F9 cells is almost as efficient as G418-resistance transfer to fibroblasts, in contrast to G418-resistance transfer to PCC4 embryonal carcinoma cells, which is at least 3 orders of magnitude lower. To isolate Neo^R-MPSV mutants that are efficiently expressed in PCC4 cells, G418-resistant PCC4 cell lines were induced to differentiate, and the provirus was rescued by superinfection with murine leukemia virus. Viral isolates (PCMV-5 and -6; PCMV = PCC4 cell-passaged Neo^R-MPSV) were obtained and assayed for expression in embryonal carcinoma cells. The efficiency of Neo^R transfer was equally as high in both F9 and PCC4 as in fibroblasts. *mos* oncogene expression was unaltered as judged by transformation capability. No gross alteration in the coding region and in the long terminal repeat was detectable by restriction enzyme analysis. Neo^R-MPSV and its mutants PCMV-5 and -6 can thus be utilized as vectors for the efficient transduction of genes into embryonic cells.**

Embryonal carcinoma (EC) cells provide a unique *in vitro* model for the study of gene expression in early embryonic cells. The use of retroviruses as tools to introduce and transcribe DNA in embryonic or EC cells has been hampered because of inefficient expression of the integrated genome. Replication of retroviruses is undetectable in EC cells (1–4), and the expression of selectable marker genes inserted in the retroviral genome is low (5–7) unless internal promoters are provided (8, 9). The block of expression in the preimplantation embryo and in EC cells is not fully understood, but evidence indicates that the defect is in transcription from the long terminal repeat (LTR) (3, 10–12).

Mutants of polyoma virus have been isolated that escape suppression and express efficiently in EC cells, such as F9 (13–15), PCC4 (16–18), and LT cells (17, 18). The molecular alterations in some of the mutant polyoma genomes have been mapped to the viral enhancer, a region known to respond to tissue-specific factors (19). Replacing the enhancer in the Moloney murine leukemia virus (Mo-MuLV) LTR with the enhancer of F9 polyoma results in expression in F9 cells, normally nonpermissive for Mo-MuLV expression, indicating that the Mo-MuLV enhancer is nonfunctional in EC cells (20).

Spontaneous or induced mutants of murine retroviruses with more efficient expression in EC cells have not been isolated, although attempts have been made (6). An obvious way to isolate such mutants is to passage a retrovirus in EC cells and test for a mutant virus in the rare transduced cell lines that express the viral genome. Use of a selectable marker gene within the retroviral genome and under control of the LTR would facilitate this work.

We used in this study a G418-resistant [neomycin-resistant (Neo^R phenotype)] derivative of the myeloproliferative sarcoma virus (MPSV). MPSV is a member of the Moloney murine sarcoma virus (Mo-MuSV) family containing the *mos* oncogene within a defective Mo-MuLV genome (21, 22). It is unique to other described Mo-MuSV variants in that it not only induces sarcomas but also a myeloproliferative disorder, including spleen focus formation in adult mice (22). MPSV has a broader host range than the classical Mo-MuSV and spleen focus-forming virus (SFFV) variants have (23), expressing efficiently in many different cell types, such as fibroblasts and hematopoietic (22) and epithelial cells (24). Molecular recombinants between Mo-MuSV and MPSV have demonstrated that the expanded host range of pathogenicity of MPSV can be attributed to point mutations in the U3 region of the viral LTR (25).

We show in this study that, in contrast to other Moloney-related retroviruses (1–4, 7, 11), expression of Neo^R-MPSV is relatively high in F9 cells as compared to PCC4 cells. To obtain possible mutants that are efficiently expressed in PCC4 cells, we isolated the provirus genomes from G418-resistant PCC4azalR cell lines obtained after infection with Neo^R-MPSV (5). PCC4azalR cells can be induced to differentiate to epithelial cells with retinoic acid, resulting in removal of the block to viral expression. Unexpressed proviruses introduced prior to differentiation remain blocked by a *cis*-acting mechanism and cannot be rescued by superinfection with helper virus (26); however, a provirus that is expressed before differentiation escapes this block and can be rescued after induction with retinoic acid (5). We report here the properties of two rescued viruses from Neo^R-MPSV-transduced PCC4azalR cell lines.

MATERIALS AND METHODS

Construction of *neo*-transducing MPSV Vector. The construction of the Neo^R-MPSV vector is described in detail

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Abbreviations: cfu, colony forming units; EC, embryonal carcinoma; ffu, focus forming units; Fr-MuLV, Friend murine leukemia virus; LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus; Mo-MuSV, Moloney murine sarcoma virus; MPSV, myeloproliferative sarcoma virus; Neo^R, neomycin resistance/resistant; Neo^R-MPSV, MPSV recombinant containing the gene for Neo^R; PCMV, PCC4 cell-passaged Neo^R-MPSV.

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elsewhere (27). Basically, the region of Tn5 coding for Neo^R (*neo*) (28) was inserted into the *Bam*HI site of the *gag–pol* junction of plasmid pC663 containing the proviral genome of MPSV clone 6-6-3 (29). The transcriptional orientation of the *neo* insert is in the same direction as that of the viral genes, and the transcription of *neo* is promoted from the viral LTR (unpublished data).

Cell Lines. RAT-1 and NRK are rat fibroblasts (29). PCC4 and F9 are two mouse EC cell lines (30). PCC4azaIR is a subclone of PCC4 that can be induced to differentiate *in vitro* in the presence of 10 μM retinoic acid (31). 643/22N is a producer of Friend murine leukemia virus (Fr-MuLV) (22) used as a helper virus to rescue the defective Neo^R-MPSV genome.

1-4Mneo-23 is a nonproducer NRK cell line containing one full-length copy of Neo^R-MPSV (27). It was obtained after infection with limiting dilutions of supernatant from a Fr-MuLV-superinfected G418-resistant cell line containing transfected copies of Neo^R-MPSV. Cells were selected for growth in the presence of G418 (0.4 mg/ml), and clones that did not release reverse transcriptase (22) were established as nonproducer cell lines.

PCC4azaIR clones 5 and 6 are G418-resistant PCC4azaIR cell clones obtained by infection with Neo^R-MPSV from the above-mentioned cell line. Both clones contain two copies each of Neo^R-MPSV (5).

All cells were routinely cultured in minimal essential medium containing 10% fetal calf serum.

Virus Assays. Reverse transcriptase and fibroblast transformation assays with MPSV were carried out as described (22).

Assay for the Efficiency of Transduction of *neo* in RAT-1 and EC Cells. RAT-1, PCC4azaIR, or F9 cells were seeded into 24-well plates (1.5×10^3 cells per well) and inoculated 24 hr later with serial dilutions (4 wells per dilution) of viral supernatant in the presence of 6 μg of Polybrene per ml overnight. After 3 to 4 days, the cells were cultured in selection medium containing 0.4 mg of G418 per ml. The cells were fed without passage every 3 to 4 days, and positive clones at the end-point dilution were counted after 16 days of selection. No cells in the uninfected control had survived at this time point.

Verification of the Undifferentiated Phenotype of EC Cells. Immunofluorescence studies were performed as described (26). SSEA-1, a gift of P. Goodfellow, is a mouse hybridoma line secreting antibodies that react specifically with undifferentiated EC cells (32). Fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (Tago, Burlingame, CA) was used as the second antibody.

Three-month-old syngeneic female (129) mice were injected subcutaneously with 10^7 G418-resistant or uninfected PCC4azaIR cells. After 4 weeks tumors were removed, and frozen sections were examined for the presence of cells indicative of teratocarcinomas.

Molecular Analysis of the Proviral Genome. Preparation of high molecular weight DNA and Southern blot analysis were performed as described (29, 33). Plasmids pAG60 (34) and pmS1 (35) were used as *neo*- and *mos*-specific probes, respectively. Restriction enzymes were purchased from Bethesda Research Laboratories and used under the recommended conditions.

RESULTS

Expression of Neo^R-MPSV Genome in F9 Cells. We have previously shown that Neo^R-MPSV transfers G418 resistance to PCC4azaIR cells with an efficiency 3–5 orders of magnitude lower than to fibroblasts (5). However, in view of results showing that F9 and PCC4 cell lines are differentially responsive to polyoma mutants adapted to growth in EC cells (17), we decided to determine also the efficiency of Neo^R-MPSV expression in F9 cells.

Table 1. Transfer of Neo^R to RAT-1 fibroblasts and F9 EC cells with Neo^R-MPSV and PCC4 cell-passaged Neo^R-MPSV (PCMV)

Virus	Exp.	Neo^R*, cfu/ml RAT-1	Neo^R*, cfu/ml F9	Ratio of Neo^R in fibroblasts/EC cells
Neo^R-MPSV†	1	4.1×10^3	7.8×10^2	5.2
	2	4.7×10^3	4.7×10^2	10
PCMV-5‡	1	4.1×10^3	2.0×10^3	2.1
	2	1.25×10^5	2.3×10^4	5.4
PCMV-6‡	1	1.9×10^3	3.1×10^3	0.6
	2	2.0×10^5	1.5×10^4	13.3

*Neo^R colony forming units (cfu) were determined at end-point dilution of viral supernatants on 1.5×10^3 cells.
†Virus released from 1-4Mneo-23 (27) nonproducer cells after superinfection with Fr-MuLV clone 643/22N.
‡Virus released from NRK nonproducer cell lines carrying one copy of the respective PCMV cloned virus after superinfection with Fr-MuLV clone 643/22N.

Serial dilutions of Neo^R-MPSV obtained from 1-4Mneo-23 cells were used to infect F9 cells and, as controls, PCC4azaIR and RAT-1 cells. After 16 days, G418-resistant colonies were scored and compared (Table 1). The efficiency of Neo^R transfer to F9 cells was lower by a factor of 2–5 than to fibroblasts, but 3 orders of magnitude higher than in PCC4 cells. F9 cells that expressed Neo^R-MPSV retained their undifferentiated phenotype as judged by morphology and SSEA-1 antigen expression (data not shown). Wild-type Neo^R-MPSV is thus a retrovirus with a high efficiency of expression in F9 cells.

Recovery and Biological Properties of Integrated Neo^R-MPSV from Neo^R-PCC4azaIR Cells. If expression of Neo^R-MPSV is generally blocked in PCC4 cells, how does every thousandth virus escape suppression? Two alternative models can be discussed to account for Neo^R-MPSV expression in PCC4 cells: (*i*) integration into a unique site of the PCC4 genome that would allow expression or (*ii*) a mutation arising in the MPSV genome itself that would permit expression in an expanded host range.

To test the latter hypothesis, we took advantage of already established G418-resistant PCC4azaIR cell lines infected

Table 2. Transfer of Neo^R to fibroblasts and PCC4azaIR cells with Neo^R-MPSV and PCMV

Virus	Exp.	Neo^R, cfu/ml RAT-1	Neo^R, cfu/ml PCC4azaIR	Ratio of Neo^R in fibroblasts/EC cells
Neo^R-MPSV‡	1	1.4×10^4	6	2.3×10^3
	2	1.8×10^3	1	1.8×10^3
	3	1.25×10^4	1.5	8.3×10^3
	4	4.7×10^3	1.25	3.8×10^3
PCMV-5‡	1	1.9×10^5	1.8×10^5	1.1
	2	2.5×10^3	2.5×10^2	10
	3	2.1×10^4	2.8×10^3	7.5
	4	4.1×10^3	9.4×10^3	4.3
PCMV-6‡	1	3.1×10^3	3.1×10^2	10
	2	1.9×10^3	6.3×10^2	3
	3	2.3×10^5	1.6×10^5	1.4
	4	1.2×10^3	1.2×10^2	10

*Neo^R cfu were determined at end-point dilution of viral supernatants on 1.5×10^3 cells.
†Virus released from 1-4Mneo-23 (27) nonproducer cells after superinfection with Fr-MuLV clone 643/22N.
‡Virus released from NRK nonproducer cell lines carrying one copy of the respective PCMV cloned virus after superinfection with Fr-MuLV clone 643/22N.

with Neo^R-MPSV (5); Neo^R PCC4aza1R clones 5 and 6 were induced to differentiate with 10 μM retinoic acid for 5 days. Superinfection with Fr-MuLV resulted in the release of the integrated and expressed Neo^R-MPSV provirus. End-point dilutions of supernatants containing the released virus were used for infection of NRK fibroblasts to establish nonproducer clones. Viruses released after superinfection of these cell clones were termed PCMV-5 and PCMV-6 for PCC4 cell-passaged Neo^R-MPSV.

Pseudotypes of these two virus isolates were prepared with Fr-MuLV and tested at end-point dilution for Neo^R transfer on RAT-1, PCC4aza1R, and F9 cells. Both isolates transfer G418 resistance to PCC4aza1R with nearly equal efficiency as to that on RAT-1 and F9 cells (Table 2). The two mutants differ from wild-type Neo^R-MPSV by enhancement of Neo^R transfer by 3 orders of magnitude (Tables 1 and 2).

PCMV-5 and -6 retain their ability to transform fibroblasts. Focus-forming units (ffu) were determined in parallel with Neo^R cfu (data not shown). Often ffu values were up to 1 order of magnitude less than Neo^R cfu, but this was due to the difficulty in judging transformation in the assay conditions. When individually analyzed, every Neo^R RAT-1 colony showed a transformed phenotype when grown to confluency. Furthermore, PCMV and wild-type MPSV induced spleen focus formation in adult mice similarly (data not shown).

Morphology of G418-Resistant EC Cells. Neo^R-PCC4aza1R

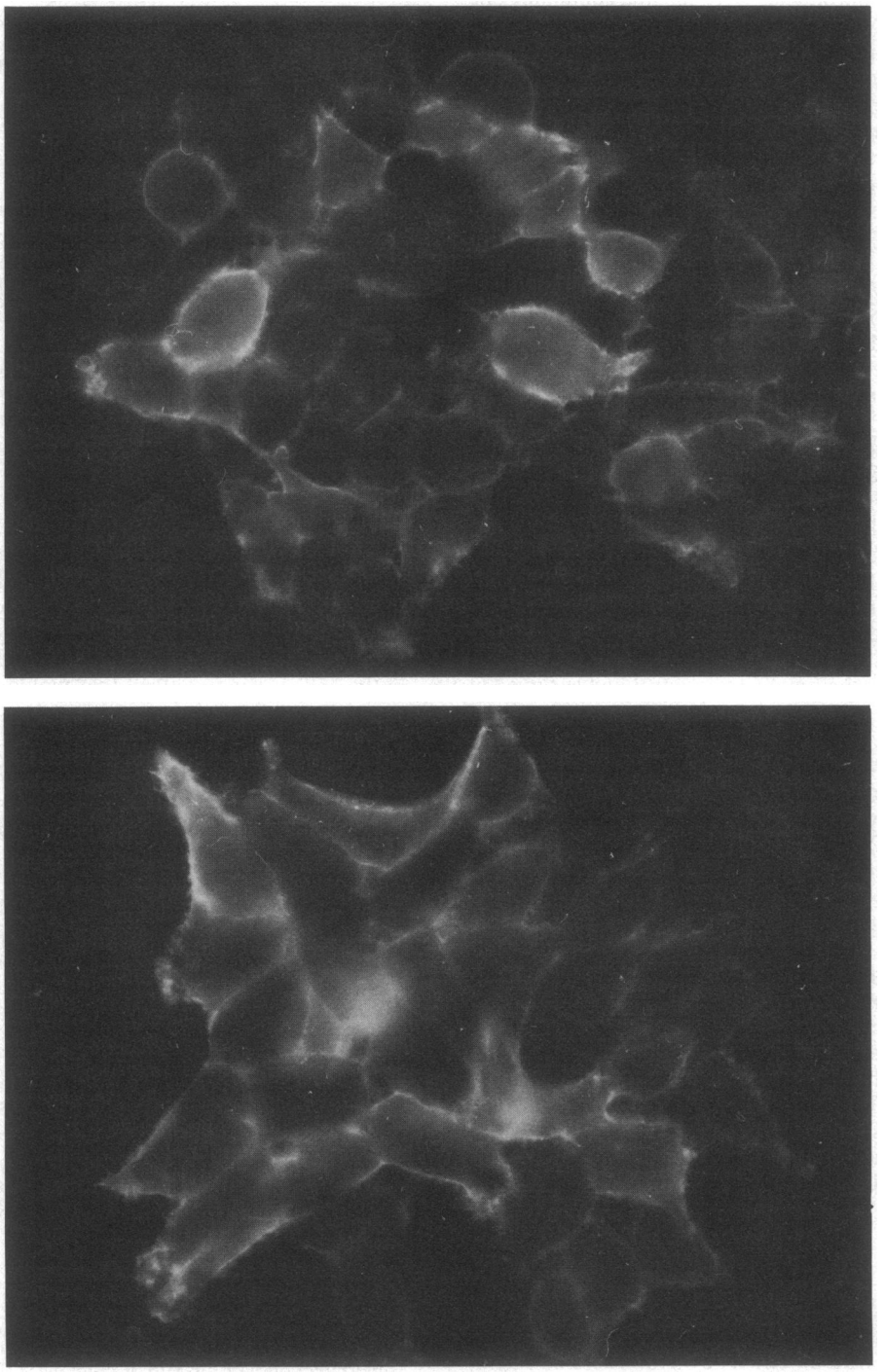

FIG. 1. Immunofluorescence of anti-SSEA antisera to surface antigens of EC and differentiated teratocarcinoma cells. Cells were exposed to anti-SSEA antisera and subsequently to fluorescein isothiocyanate-conjugated anti-mouse Ig. (*Upper*) PCC4aza1R. (*Lower*) Neo^R-PCC4aza1R clone 5.

cells expressing Neo^R-MPSV or PCMV proviruses were morphologically unaltered. SSEA-1 is a monoclonal antibody that distinguishes EC cells from differentiated EC progeny (ref. 32; Fig. 1). The Neo^R cells expressed SSEA-1-specific antigens at a similar level as did uninfected control cells (Table 3). They also formed teratocarcinomas in syngeneic 129 mice that were indistinguishable from tumors derived from uninfected PCC4aza1R cells. Thus, the enhanced expression of PCMV in PCC4aza1R cells does not result from the selection of cells that may have differentiated spontaneously prior to infection.

Molecular Analysis of PCMV. Southern blot analysis of 1-4Mneo-23, PCMV-5 NRK-1, and PCMV-6 NRK-1 was performed by using *mos* and *neo*-specific probes (Fig. 2). *Sst* I is a restriction enzyme that cuts only within the viral LTR and, thus, allows a size estimate of the total genome of MPSV. The genomic size of both mutant PCMVs appears similar to that of the wild-type Neo^R-MPSV and hybridized to both specific probes. Digests with *Eco*RV, *Kpn*I, *Sma* I, and *Xba* I gave no indication of structural alterations in the LTR of the two PCMV isolates; all tested sites were preserved (data not shown).

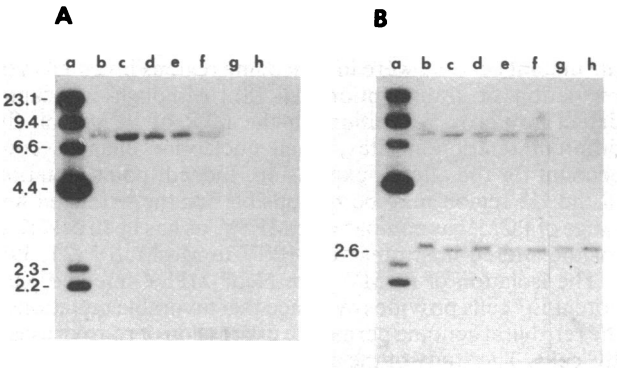

FIG. 2. Southern blot analysis of infected cell lines. Cellular DNA (20 μg per lane) was digested with *Sst* I endonuclease. Filters were hybridized with probes specific for *neo* (*A*) and *mos* (*B*). Lanes: a, λ phage DNA digested with *Hin*dIII and end-labeled; b and f, 1-4Mneo-23; c, 1-4Mneo-21; d, PCMV-6 NRK-1; e, PCMV-5 NRK-1; g, NRK; h, RAT-1. Sizes are shown in kb; the 2.6-kb band is the endogenous c-*mos* fragment.

DISCUSSION

Retroviral vectors are important tools for the transfer of foreign genes into cells (for review, see ref. 37). Retroviruses integrate specifically and are expressed at high levels in most mammalian cells. High titers of retrovirus vectors can be obtained, and these permit infection of virtually any cell of a sensitive population. However, EC cells (1–4) and early embryos (12) are refractory for retroviral expression. Infrequent cases have been reported where a gene is expressed from an intact retroviral LTR in undifferentiated EC cells (5–7), but no conclusive evidence could be shown to explain how the retrovirus escaped transcriptional suppression. One study showed tentative evidence to support the idea that a favorable integration site is necessary for expression (6).

Although the retroviral enhancer region has been implicated as being one possible defect in the retroviral transcription in EC cells (20), no retroviral mutants, such as described for the polyoma virus (13–18), had been isolated that could be expressed efficiently in EC cells. We report here the efficient expression of a Mo-MuSV mutant, MPSV, in F9 cells and, furthermore, the isolation of two mutants of MPSV, PCMV-5 and PCMV-6, that can efficiently transduce and express genes in both F9 and PCC4 cells.

Our success in obtaining viral mutants that are expressed in EC cells was due to the development of a retrovirus recombinant encoding a dominant selectable marker and the selection of a retrovirus with an already altered host range. Use of an intact retrovirus with the gene for G418 resistance offered several advantages: (*i*) the efficient transduction method of infection vs. DNA-mediated transfer, (*ii*) easy and sensitive detection of the expressed proviral genome, and (*iii*) rescue of the intact provirus after superinfection of induced differentiated cells.

Table 3. Characterization of the parental and transductant EC lines

Cell lines	Transduced virus	% cells immunofluorescent for SSEA-1*	Tumorigenicity, 5×10^6 cells per mouse
PYS-2†	—	0.5	−
PCC4aza1R	—	60.9	+
Clone 5	Neo^R-MPSV	62.5	+
Clone 6	Neo^R-MPSV	41.4	+
2 to 5/1‡	PCMV-5	58.1	+
2 to 5/3‡	PCMV-5	53.1	+
2 to 6/1‡	PCMV-6	45.2	+
2 to 6/2‡	PCMV-6	52.7	+

*Results are from one representative experiment, where 200 cells of each line were screened.
†PYS-2, a differentiated cell line derived from the teratocarcinoma cell line OTT6050, was used as a negative control (36).
‡Two colonies of PCMV-5- and PCMV-6-infected PCC4aza1R cells were isolated and expanded.

We have previously shown that MPSV has an extended host range as compared to the related Mo-MuLV variants. MPSV causes not only fibroblast transformation *in vitro* and induction of sarcomas *in vivo* but also a rapid myeloproliferative disease in adult sensitive mouse strains (38). Changes in the U3 region of the viral LTR are responsible for the altered host-range properties of MPSV as compared to the related Mo-MuSV (25). The critical point mutations in the U3 region of MPSV as compared to those of Mo-MuLV are clustered in the direct repeat region of the LTR and/or the putative glucocorticoid hormone receptor binding domain of the LTR (ref. 39; unpublished data). The efficient expression of the wild-type Neo^R-MPSV conferring G418 resistance in the EC cell line F9 (Table 1) would suggest that the same alterations in the U3 region that are responsible for the expansion of the host range to hematopoietic cells are also important for expression in F9 cells.

Our results show that both of the two Neo^R-PCC4 cell lines that permitted efficient rescue of provirus harbored a Neo^R-MPSV mutant. This would suggest that *neo* transduction in PCC4 cells by Neo^R-MPSV requires a mutation in the integrated provirus.

In addition to the *neo* gene, Neo^R-MPSV also includes the *mos* oncogene. Our studies have shown that *neo* expression does not disrupt *mos* expression. Segregation of the two gene functions has not been observed in 1000 individual clones selected either for G418 resistance or *mos*-dependent transformation (5, 27). One could speculate that an alteration in *mos* expression in Neo^R-MPSV may be necessary for efficient expression of the provirus in PCC4 cells. Although *mos* expression in PCC4aza1R cells has been shown to cause an increased anchorage independence in agar cloning (5), no alteration in the undifferentiated phenotype is observed in PCC4aza1R cells infected either with Neo^R-MPSV (5) or PCMV (this paper). PCMV has retained its ability to transform fibroblasts *in vitro* and cause sarcomas *in vivo*. Furthermore, it still causes the myeloproliferative syndrome in adult mice similar to Neo^R-MPSV. Therefore, the different efficiency of *neo* transduction of Neo^R-MPSV and of PCMV cannot be attributed to an altered expression of the *mos* oncogene.

Molecular analysis of papovavirus mutants that are expressed in EC cells has shown that the decisive alterations in the mutant genome were in noncoding regions involved in the regulation of transcription (13–18). Although we cannot detect any gross alterations in the LTR of PCMV on five different restriction sites, small nucleotide changes could account for the altered expression. Indeed, point mutations in the U3 region may be responsible for the extended host range of PCMV as compared to MPSV, as has been described for the altered host range of MPSV to Mo-MuSV (25, 39).

The isolation of PCMV from Neo^R-MPSV-infected pluripotent EC cells provides evidence that favorable mutations in the retroviral genome permit the expression of retroviruses in EC cells. The study of the alterations in the viral genome and its interaction with the environment of the EC cell nucleus should provide clues for understanding the regulation of gene expression in embryonic cells.

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