Analysis of Fv-1 restriction in two murine embryonal carcinoma cell lines and a series of differentiated derivatives

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We have used antibiotic-resistant retrovirus vectors rescued by Fv-1-sensitive murine leukaemia viruses (MuLV) to examine the Fv-1 phenotype of two undifferentiated embryonal carcinoma (EC) cell lines derived from teratocarcinomas of mouse strain 129. In addition, a set of EC cell-derived differentiated cell lines was analysed. Restriction of both B-tropic and endogenous N-tropic virus is characteristic of the N~ type restriction reported in mouse strain 129. However, results indicate that Fv-1 restriction is not expressed in the PCC4.azalR EC cell line. In contrast, the F9 EC cell line showed a strong restriction of the B-tropic pseudotyped vector but failed to restrict endogenous N-tropic pseudotypes. The Fv-1 gene thus seems to be differentially expressed in two EC cell lines derived from the same mouse strain. Furthermore, the selective restriction of B-tropic but not endogenous N-tropic MuLV in F9 cells suggests that these activities function independently of each other. Analysis of PCC4.aza1R-derived differentiated cell lines revealed that three fibroblast cell lines derived by retinoic acid-induced differentiation were also phenotypically silent for Fv-1. However, a pre-adipocyte line established following simultaneous exposure to retinoic acid and 5-azacytidine showed strong restriction of both B-tropic and endogenous N-tropic MuLV. Although additional data suggest that there is no correlation between the differentiated pre-adipocyte phenotype and Fv-1 expression, our results nonetheless show that N~ restriction can be observed in some derivatives of PCC4.aza1R cells, presumably by activating expression of the Fv-1 gene.

Introduction

The mouse Fv-1 gene is responsible for restricting the replication of many murine leukaemia viruses (MuLVs) (Lilly, 1970, 1973; Jolicoeur, 1979). MuLV strains not restricted by the Fv-1b allele carried by BALB/c and certain other mouse strains are designated as B-tropic. Conversely, MuLV strains able to replicate efficiently in mouse cells possessing the Fv-1b allele (e.g. NIH Swiss) are classified as N-tropic (Hartley et al., 1970; Pincus et al., 1971a, b). The Fv-1 host range system of MuLV involves reciprocal sensitivity or insensitivity to these two principal alleles of the Fv-1 locus. A third Fv-1 phenotype, designated N', has been described in the literature as a more restrictive variant of N-type restriction (Steeves & Lilly, 1977; Mayer et al., 1978). It has been suggested that this phenotype is the result of a third allele, Fv-1n'. The mouse strains reported to possess N' restriction (129, RF/J, RFM/Un, NZB and NZW) block B-tropic virus infection, similar to Fv-1n strains, and in addition restrict some N-tropic MuLVs (endogenous ecotropic) but not others (exogenous Gross passage A) (Steeves & Lilly, 1977; Mayer et al., 1978; Tennant et al., 1983). We will refer to the subclass of N-tropic viruses that are not restricted in N'-type hosts as N'-tropic.

The virion target for Fv-1 restriction is the capsid protein CA (or p30) (Schindler et al., 1977; Gautsch et al., 1978). A difference in two adjacent amino acids (residues 109 and 110 of a 263 amino acid protein) distinguishes the prototype N- and B-tropic viruses (DesGrolliers & Jolicoeur, 1983; Ou et al., 1983). The CA proteins from endogenous N-tropic and N'-tropic Gross passage A MuLV have the same predicted sequence of amino acids at residues 109 and 110 but differences elsewhere in the protein are thought to be responsible for the distinct Fv-1-sensitive phenotypes (W. K. Yang et al., personal communication). Certain laboratory strains of MuLV have lost sensitivity to Fv-1 alleles and are termed NB-tropic. The basis of this insensitivity is unknown but

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appears to involve other regions of the p30 protein (Ou et al., 1983).

The step in MuLV replication sensitive to Fv-1 is at or before the integration step which forms the provirus and may involve aspects of reverse transcription and/or integration (Jolicoeur & Rassart, 1980; Yang et al., 1980; Chinsky & Soiero, 1981; Iwamoto et al., 1985). It is not known how the Fv-1 product functions in blocking MuLVs at this step, or what other function in normal cell physiology this product serves. Apparently all normal target cells for retrovirus replication in the mouse host constitutively express the Fv-1 gene because leukemogenesis and other measures of virus replication in vivo reflect the animal's Fv-1 genotype. With the exception of a few cell lines that appear to have spontaneously lost Fv-1 restriction (Gisselbrecht et al., 1974; Hartley & Rowe, 1975; Yoshikura et al., 1982), cells in culture are thought also to express Fv-1 constitutively (Hartley et al., 1970).

We were interested in investigating Fv-1 expression in mouse cells that had not been previously examined for Fv-1 phenotype owing to their inability to support MuLV replication. One such cell type is the embryonal carcinoma (EC) cell. The murine EC cell system has been used extensively as a model to study gene regulation in vivo and in vitro under certain culture conditions and can also be chemically induced to differentiate following treatment with various agents, such as retinoic acid (Jetten et al., 1979; Linder et al., 1981; Sherman et al., 1981). Like normal embryonic stem cells, EC cells are unable to support the replication of most retroviruses or the expression of most retrovirus-derived vectors (Peries et al., 1977; Teich et al., 1977; Gautsch, 1980; Speers et al., 1980). Therefore, it has not been possible to demonstrate Fv-1 gene expression in these cell types by conventional assays. Retrovirus restriction in EC cells appears to be a result of both (i) a transcriptional block due to inefficient recognition of the proviral regulatory sequences (Linney et al., 1984, 1987; Loh et al., 1987; Weicher et al., 1987) as well as (ii) an inhibitory activity attributed to a 5′ proviral sequence which encodes the RNA leader sequence of the genomic transcript and is associated with reduction of steady-state mRNA levels in EC cells (Loh et al., 1987). Changes in the control of gene expression during differentiation allow the provirus to be expressed; differentiated derivatives of EC cells are therefore permissive for retrovirus infection.

Recently, variant retroviruses capable of replicating in EC cells have been described (Barklis et al., 1986; Linney et al., 1987; Weicher et al., 1987). Retrovirus vectors have been constructed using regulatory sequences from Moloney MuLV variants and mutants (Weicher et al., 1987) as well as by using internally placed regulatory sequences to drive antibiotic resistance genes (Linney et al., 1987). In this study we used antibiotic-resistant retrovirus vectors, including one designed for expression in EC cells, to examine the Fv-1 phenotype of two different EC cell lines and a set of differentiated derivatives.

**Methods**

Materials. Growth medium [RPMI 1640, Ham's F12, Eagle's MEM (EEM)], Dulbecco's modified Eagle's medium (DMEM), antibiotics, foetal bovine serum (FBS), calf serum and gentamicin (G418) were obtained from Gibco. Insulin, dexamethasone and 5-azacytidine were purchased from Sigma. Epidermal growth factor (EGF) was obtained from Collaborative Research.

Cell culture. SC-1, NIH/3T3, RFM/3T3, AKR, BALB/3T3 clone A31, PCD1 and XC cells were obtained from Drs W. K. Yang (Oak Ridge, Tenn., U.S.A.) and R. W. Tennant (Research Triangle Park, N.C., U.S.A.). The undifferentiated EC cell lines F9 (Artzi et al., 1973) and PCC4.azalR (Jetten et al., 1979) and the myoblast-derived differentiated PCD1 cell line (Nicolas et al., 1976; Peries et al., 1977) are clonal cell lines derived from teratocarcinomas of mouse strain 129. Attempts to generate stable differentiated cell lines from F9 were unsuccessful, although F9 cells differentiate readily in response to retinoic acid treatment. However, differentiated cell lines were derived from the EC cell line PCC4.azalR as follows. PCC4.azalR cells (plated the previous day in 60 mm plates at a density of 5 × 10^5 to 1 × 10^6 cells/plate) were induced to differentiate into mesenchymal cells following a 6 day exposure to retinoic acid (4 days at 10^-6 M followed by 2 days at 10^-7 M). Cultures were refed with fresh medium supplemented with drug every 2 days during the treatment regimen. These cells were then trypsinized, plated at clonal density and single colonies of differentiated cells were expanded and subcloned. Three fibroblast cell lines (PCC4D2, PCC4D4 and PCC4D7) derived following simultaneous retinoic acid and 5-azacytidine treatment of PCC4.azalR cells were used. Subsequent exposure of PCC4D2, PCC4D4 and PCC4D7 cell cultures to 5-azacytidine induced differentiation of these cells into pre-adipocytes (unpublished data). A pre-adipocyte cell line (PCC4D1) derived following simultaneous retinoic acid and 5-azacytidine treatment of PCC4.azalR cells was also used. In this case, 24 h 5-azacytidine treatment (5 μg/ml) was begun on day 4 of retinoic acid exposure. Cultures were grown to confluence in the presence of insulin (4 μg/ml) and dexamethasone (10^-3 M) and regions of fat-producing cells were trypsinized and expanded.

F9, PCC4.azalR and differentiated PCC4.azalR derivatives were maintained in growth medium consisting of a 1:1 mixture of Ham's F12 and RPMI 1640 supplemented with 10% FBS, 15 ng EGF/ml, 4 μg insulin/ml, 2 mM-l-glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml. Other cell lines were propagated in EEM supplemented with either 5% (SC-1, AKR, XC, RFM/3T3) or 8% (PCD1) FBS or DMEM in the presence of 5% calf serum (NIH/3T3, BALB/3T3). All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ in air.

Viruses and assay techniques. The recombinant clones of WN1802B MuLV (pWB5), WN1802N MuLV (pWN41), Gross leukaemia virus...
Fv-1 regulation of MuLV replication

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Table 1. Properties of helper viruses used to generate Fv-1-sensitive G418-resistant pseudotypes

<table>
<thead>
<tr>
<th>MuLV strain</th>
<th>Molecular clone</th>
<th>Fv-1 tropism</th>
<th>Species tropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFV</td>
<td>pRFV105</td>
<td>N</td>
<td>Ecotropic</td>
</tr>
<tr>
<td>WN1802N</td>
<td>pWN41</td>
<td>N</td>
<td>Ecotropic</td>
</tr>
<tr>
<td>WN1802B/WN1802N chimera</td>
<td>pWB/n-69*</td>
<td>N</td>
<td>Ecotropic</td>
</tr>
<tr>
<td>Gross passage A</td>
<td>pGN104</td>
<td>N†</td>
<td>Ecotropic</td>
</tr>
<tr>
<td>WN1802B/Gross passage A chimera</td>
<td>pWB/gn-2*</td>
<td>N*</td>
<td>Ecotropic</td>
</tr>
<tr>
<td>1504A</td>
<td>–</td>
<td>N*</td>
<td>Amphotropic</td>
</tr>
<tr>
<td>4070A</td>
<td>–</td>
<td>N*</td>
<td>Amphotropic</td>
</tr>
<tr>
<td>WN1802B</td>
<td>pWB5</td>
<td>B</td>
<td>Ecotropic</td>
</tr>
<tr>
<td>Moloney</td>
<td>p63-2</td>
<td>NB</td>
<td>Ecotropic</td>
</tr>
</tbody>
</table>

* pWB/n-69 and pWB/gn-2 were constructed by inserting the 1.3 kbp BamHI-HindIII sequence (the Fv-1 target) from the gag-pol regions of pWN41 and pGN104, respectively, into the homologous region of pWB5.
† Ntropism is defined by the ability of a virus to be restricted in B-type cells but unrestricted in N- as well as Ntropism cells.

passage A (pGN104; Boone et al., 1983), Moloney MuLV (p63-2; Linney et al., 1984) and the endogenous virus from the RFM/Un mouse strain (pRFV105; Liou et al., 1983) were used to generate infectious virus stocks. The p63-2 clone of Moloney MuLV was a gift from Dr H. Fan (Irvine, Ca., U.S.A.). The amphotropic virus isolates 4070A and 1504A (Hartley & Rowe, 1976; Rasheed et al., 1976) were provided by Dr M. Cloyd (Galveston, Tx., U.S.A.). The nomenclature, Fv-1 specificity and species tropism of the viruses used in this study are summarized in Table 1. The Psi-2-MSV-DHFR-neo packaging cell line (Williams et al., 1984) was a gift from Dr R. C. Mulligan (Boston, Mass., U.S.A.); the replication deficient MSV-DHFR-neo retrovirus vector produced by this packaging line will be henceforth referred to as D-neo. The Rous sarcoma virus (RSV) linker-1 retrovirus vector (RV-neo), containing an RSV long terminal repeat as an internal promoter/enhancer (Boone et al., 1989), was constructed specifically for expression in EC cells according to the strategy outlined by Linney et al. (1987).

Routine virus infections were carried out by plating cells at a density of 5.0 x 10^4 to 6.0 x 10^4 cells/35 mm well. The following day, cell cultures were infected for 2 h at 37°C in the presence of 16 μg/ml polybrene/ml. For assays measuring G418-resistant colony formation, cells were selected by exposure to G418 beginning 24 h post-infection. The drug concentration used for selection was 125 μg/ml culture medium for PCC4.azalR and PCC4.azalR-derived cells and 500 μg/ml for all other cell lines. Cultures were fed with fresh drug-containing medium every 3 days and stained after 2 weeks for scoring G418-resistant colonies.

In some assays, virus infection was determined by XC cell plaque formation (Klement et al., 1969) or by a modified infectious centre XC plaque assay as follows. Cells were plated at a density of 7.5 x 10^5 cells/60 mm plate. The following day, cultures were infected at an m.o.i, ranging from 0.01 to 1.0. Cells were incubated for 2 h, refed with fresh growth medium and incubated for an additional 8 h. The infected cell cultures were then trypsinized and transferred at various dilutions onto permissive SC-1 cells (plated the previous day at a density of 6.0 x 10^4 cells/35 mm well). The cocultured cells were then grown to confluent monolayers, u.v.-irradiated (150 J/m2) and overlaid with XC cells. At confluence, XC cell cultures were stained and scored for plaque formation.

Table 2. G418-resistant colony-forming titres of pseudotyped virus stocks*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Fv-1 type</th>
<th>N-tropic†</th>
<th>N-tropic (4070)‡</th>
<th>N-tropic (1504)§</th>
<th>N-tropic‖</th>
<th>B-tropic‖</th>
<th>NB-tropic**</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-neo</td>
<td>D-neo</td>
<td>D-neo</td>
<td>D-neo</td>
<td>D-neo</td>
<td>D-neo</td>
<td>D-neo</td>
<td>D-neo</td>
</tr>
<tr>
<td>RV-neo</td>
<td>RV-neo</td>
<td>RV-neo</td>
<td>RV-neo</td>
<td>RV-neo</td>
<td>RV-neo</td>
<td>RV-neo</td>
<td>RV-neo</td>
</tr>
<tr>
<td>SC-1</td>
<td>3.0 x 10^5</td>
<td>9.0 x 10^5</td>
<td>1.9 x 10^5</td>
<td>1.1 x 10^6</td>
<td>2.4 x 10^4</td>
<td>5.2 x 10^4</td>
<td>3.5 x 10^5</td>
</tr>
<tr>
<td>Akr</td>
<td>ND</td>
<td>1.9 x 10^6</td>
<td>4.1 x 10^5</td>
<td>5.1 x 10^6</td>
<td>8.3 x 10^4</td>
<td>8.7 x 10^4</td>
<td>8.1 x 10^5</td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>6.8 x 10^5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RfM/3T3</td>
<td>6.4 x 10^5</td>
<td>8.5 x 10^5</td>
<td>3.0 x 10^6</td>
<td>1.6 x 10^6</td>
<td>3.4 x 10^4</td>
<td>4.0 x 10^4</td>
<td>2.1 x 10^4</td>
</tr>
<tr>
<td>Balb/3T3</td>
<td>3.2 x 10^6</td>
<td>1.0 x 10^6</td>
<td>6.9 x 10^5</td>
<td>2.9 x 10^6</td>
<td>2.5 x 10^5</td>
<td>2.7 x 10^5</td>
<td>2.4 x 10^5</td>
</tr>
</tbody>
</table>

* Phenotypic mixtures were prepared by rescue of the retrovirus vectors D-neo and RV-neo with Fv-1-sensitive helper viruses as described in Methods.
† N-tropic, ecotropic Gross passage A MuLV.
‡ N-tropic, amphotropic isolate 4070A MuLV.
§ N-tropic, amphotropic isolate 1504A MuLV.
‖ N-tropic, endogenous ecotropic RFV (RFM/Un mouse) MuLV.
¶ B-tropic, ecotropic WN1802B MuLV.
** NB-tropic, ecotropic Moloney MuLV.
†† ND, Not determined.
**Analysis of Fv-1 restriction.** Retrovirus vectors RV-neo (used for EC cells) and D-neo (used for all other cell lines) were transfected into the Psi-2 packaging cell line (Mann et al., 1983) by electroporation. The resulting replication-deficient recombinant viruses were used to introduce RV-neo and D-neo into SC-1 cells and NIH/3T3 cells, respectively. Rescue of neo vectors by high multiplicity infection with MuLVs of each of the known Fv-1 specificities yielded a panel of Fv-1-sensitive virus pseudotypes similar to other rescued replication-defective genomes (Bassin et al., 1975). The D-neo and RV-neo pseudotyped stocks were equivalent in their Fv-1 sensitivity when tested against a panel of cells of known Fv-1 phenotype (Table 2).

**Results**

The two undifferentiated EC cell lines used in this study, PCC4.azalR and F9, have not been examined previously for their Fv-1 phenotype due to their inherent restriction of MuLV replication; however, they would be expected to show the N-type restriction described for mouse strain 129. To study the Fv-1 phenotype of these cells and a series of differentiated derivatives, we used Fv-1-sensitive pseudotyped stocks of two G418-selectable retrovirus vectors (D-neo and RV-neo). As shown in Table 2, both D-neo and RV-neo vector integration in infected cells was subject to Fv-1 restriction as expected for N-, N+, and B-tropic MuLV. In addition, data from direct XC cell plaque assays (not shown) showed the same expected pattern of Fv-1 restriction for the helper virus component of the phenotypic mixtures tested.

An additional finding of interest is the observation that, unlike D-neo virus rescued by endogenous N-tropic MuLV, D-neo virus rescued with the amphotropic virus isolates 4070A and 1504A was not restricted in RFM/3T3 (N-type) cells (Table 2). This pattern was also seen using these pseudotyped amphotropic isolates in N-type PCD1 cells (not shown). Although previously designated N-tropic on the basis of their restriction in Fe-l cells, these amphotropic viruses apparently share the Fv-1 host range of Gross passage A MuLV and we have thus designated them as N-tropic.

**Fv-1 restriction in EC cell lines**

We used the pseudotyped RV-neo vector stocks to infect undifferentiated PCC4.azalR and F9 cells to determine their Fv-1 phenotype. Results (Fig. 1) are expressed as a relative titre (ratio of G418-resistant colony-forming virus titres in experimental cell lines relative to titres in the permissive cell line SC-1, which is phenotypically silent for Fv-1) (Hartley & Rowe, 1975). RFM/3T3 cells and PCD1 cells are included as standards for the N-type restriction phenotype. The G418-resistant colony formation titres were lower in PCC4.azalR and F9 cells than in SC-1 cells for all RV-neo virus stocks examined (Fig. 1), consistent with the finding that retrovirus vectors designed for transcription in EC cells are nonetheless expressed less efficiently in EC cells than in other mouse cells (Linney et al., 1987). In the in vitro studies of Hartley et al. (1970), Fv-1 restriction was typically evident as a virus titre 30- to 1000-fold less than that measured in permissive cells. In the case of EC cells, one must also compensate for the overall decreased infectability of all RV-neo virus pseudotypes. Therefore, a 30-fold or greater decrease in relative titre compared to the NB-tropic pseudotyped RV-neo relative titre is considered to be evidence of Fv-1-specific restriction. Control RFM/3T3 and PCD1 cells (both N-type) clearly showed susceptibility to NB-tropic and N-tropic RV-neo viruses while strongly restricting both endogenous N-tropic and B-tropic RV-neo viruses relative to SC-1 cells. In contrast, PCC4.azalR cells were approximately as sensitive to infection by N- and B-tropic RV-neo virus pseudotypes as they were to NB- and N-tropic RV-neo virus pseudotypes. Results using additional pools of N- (WB/gn-2) and N-tropic (WN41 and WB/n-69) RV-neo viruses confirm these findings. Thus, the relative titres of all RV-neo virus pseudotypes tested in PCC4.azalR cells were within a sevenfold range of each other, suggesting a lack of Fv-1 gene expression in this EC cell line.

F9 cells, although showing a strong restriction of B-tropic RV-neo virus, apparently fail to restrict any of the endogenous N-tropic pseudotypes tested. The titre of B-tropic RV-neo virus was 1000-fold lower in F9 cells than...
in SC-1 cells. More relevant, the relative titre of B-tropic RV-neo virus was at least 60-fold lower than those for unrestricted N\textsuperscript{r} and NB-tropic RV-neo viruses, indicating an \textit{Fv-1}-specific restriction of the B-tropic pseudotyped vector. In contrast, relative titres of the N-tropic pseudotypes tested were within a twofold range of titres of the unrestricted N\textsuperscript{r} and NB-tropic RV-neo viruses in F9 cells. These results are even more striking because the chimeric N-tropic WB/n-69 virus genome is identical to that of B-tropic WB5 except for the insertion of the 1.3 kbp \textit{BamHI}-\textit{HindIII} sequence from the \textit{gag-pol} region of \textit{WN}41 (the \textit{Fv-1} target) into the homologous region of WB5. These results suggest that F9 cells selectively restrict B-tropic but not N-tropic pseudotyped RV-neo viruses, exhibiting the restriction pattern which defines the N- rather than the N\textsuperscript{r}-type phenotype.

\textbf{Fv-1 restriction in differentiated cell lines}

We also examined \textit{Fv-1} expression in a series of differentiated EC cell lines. Three fibroblast cell lines (PCC4D2, PCC4D4 and PCC4D7) derived from retinoic acid-induced differentiation of PCC4.azalR cells were infected with each of the pseudotyped D-neo vectors. Titres of the NB-tropic D-neo vector in all three cell lines were within twofold that measured in SC-1 cells (Fig. 2). In addition, titres of the N- and B-tropic ecotropic as well as N\textsuperscript{r}-tropic amphotropic D-neo vectors were within a fivefold range of that measured in SC-1 cells. These data indicate that, like their progenitor cell line, these PCC4.azalR derivatives do not exhibit \textit{Fv-1}-specific restriction. However, a fourth differentiated derivative (PCC4D1, a pre-adipocyte cell line) exhibited a strong restriction of the B-tropic D-neo vector (a 1000-fold reduction in titre relative to SC-1 cells) as well as endogenous N-tropic D-neo vector (greater than a 100-fold decrease in titre) but was relatively permissive for ecotropic and amphotropic N\textsuperscript{r}-tropic vectors. This pattern is characteristic of the N\textsuperscript{r}-type restriction reportedly expressed in mouse strain 129.

We also measured infectivity of the helper virus components of the pseudotyped D-neo vector stocks by XC cell plaque formation in a modified infectious centre assay. PCC4D1 and PCC4D4 cells were infected with the pseudotyped D-neo vector stocks and then transferred to SC-1 cells to quantify XC cell plaque formation (Table 3). The data confirm that PCC4D1 cells express the N\textsuperscript{r} restriction phenotype, whereas the PCC4D4 cells are fully permissive.

We were interested in determining whether induction of \textit{Fv-1} expression correlates with the differentiated pre-adipocyte phenotype, a property that distinguishes PCC4D1 cells from the other cell lines examined. Therefore, we isolated and examined 21 pre-adipocyte subclones derived from 5-azacytidine treatment of either PCC4D4 or PCC4D7 fibroblasts, which do not express \textit{Fv-1} restriction (Fig. 3). All of these PCC4D4 and PCC4D7 pre-adipocyte sublines were found to be phenotypically silent for \textit{Fv-1} restriction. Two additional series of differentiated cell lines were also examined for their \textit{Fv-1} phenotype: (i) six fibroblast cell lines isolated following exposure of PCC4.azalR to retinoic acid (in the same manner as PCC4D2, PCC4D4 and PCC4D7) and (ii) six cell lines generated following simultaneous rather than sequential exposure of PCC4.azalR cells to retinoic acid and 5-azacytidine (as for PCC4D1). None

Table 3. \textit{Fv-1} expression measured by both \textit{G418}-resistant colony formation and XC cell plaque formation*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>D-neo (N)\textsuperscript{†}</th>
<th>D-neo (N)</th>
<th>D-neo (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G418</td>
<td>XC</td>
<td>G418</td>
</tr>
<tr>
<td>PCC4D1</td>
<td>1.4 × 10\textsuperscript{5}</td>
<td>1.2 × 10\textsuperscript{6}</td>
<td>1.9 × 10\textsuperscript{2}</td>
</tr>
<tr>
<td>PCC4D4</td>
<td>6.2 × 10\textsuperscript{5}</td>
<td>3.2 × 10\textsuperscript{6}</td>
<td>3.6 × 10\textsuperscript{2}</td>
</tr>
</tbody>
</table>

* Vector component of the pseudotyped virus stocks is expressed as G418-resistant c.f.u./ml. Helper virus component is expressed as XC cell p.f.u./ml.

† Parentheses denote \textit{Fv-1} tropism of the helper virus component of the pseudotyped mixtures.
Fig. 3. Determination of Fv-1 restriction in pre-adipocyte sublines of PCC4.azalR-derived fibroblasts. PCC4D4 and PCC4D7 cells, both phenotypically silent for Fv-1, were induced to differentiate into pre-adipocytes following 5-azacytidine treatment. Virus titres (G418-resistant c.f.u./ml) in four pre-adipocyte clonal sublines of PCC4D7 and 17 subclones of PCC4D4 cells are shown compared to their progenitor cell lines, to PCC4D1 cells and to standard N, B and N' cell lines. For the B-tropic D-neo pseudotyped vector, AKR cells were used as the Fv-1 standard, whereas NIH/3T3 cells were used with N- and N'-tropic D-neo viruses.

of these cell lines exhibited Fv-1 restriction when infected with the Fv-1-sensitive D-neo vectors (data not shown). The isolated instance of N' restriction in PCC4D1 cells indicates that although the gene(s) responsible has apparently been induced in these cells, there appears to be no direct correlation between development of the differentiated pre-adipocyte phenotype in particular and Fv-1 expression.

Discussion

Although the Fv-1 gene is the principal genetic locus governing sensitivity of mouse cells to MuLV infection, the mechanisms responsible for its genetic regulation and biological activity remain largely unknown. Studying the expression of Fv-1-sensitive retrovirus vectors in EC cells provides a means of addressing questions regarding the status of Fv-1 gene expression in cells which are not normally permissive targets for MuLV infection. In addition, the behaviour of pluripotential EC cells in culture approximates in many ways that of normal undifferentiated embryonic stem cells of the mouse. Consequently, investigating Fv-1 restriction in an EC cell system is a potentially useful means of examining Fv-1 gene expression in cells representing various developmental stages of the mouse.

While confirming the Fv-1 sensitivity of the pseudotyped vectors used for this study, we noted that the two amphotropic virus isolates used (4070A and 1504A) are N'-tropic in their Fv-1 host range. An earlier report characterized amphotropic viruses as N-tropic based upon their restriction in mouse cells possessing the Fv-1b allele (Hartley & Rowe, 1976). Among the N-type strains shown in that study to be permissive for amphotropic MuLV was NZW, which has since been shown to exhibit N' restriction. We can therefore state that these amphotropic viruses are unlike endogenous ectropic N-tropic viruses in having the ability to infect and replicate in cells of the N' phenotype. This host range is the same as that previously described only for the exogenous Gross passage A MuLV. It would be interesting to determine whether these amphotropic isolates share the same amino acid differences in the CA protein as have been detected in Gross passage A relative to endogenous N-tropic MuLV (W. K. Yang et al., personal communication).

This series of experiments also presented us with a second unusual observation: the existence of three apparently different restriction phenotypes among cell lines derived from the same mouse strain (129). The N'-type Fv-1 phenotype is clearly expressed in the myoblast-derived differentiated PCD1 cell line, which strongly restricts both B-tropic and endogenous N-tropic RV-neo virus (Fig. 1). The undifferentiated F9 cells restrict the B-tropic pseudotyped RV-neo virus but support endogenous N-tropic RV-neo virus infection. Finally, the EC cell line PCC4.aza1R is phenotypically silent for Fv-1 expression. The interpretation of the Fv-1 phenotypes observed in F9 and PCC4.aza1R cells could be complicated by the existence of one or more undefined factors which may contribute to the mild general restriction we noted for all MuLVs in EC cells. Such additional types of restriction in EC cells may or may not interact with or involve the same mechanism(s) as Fv-1 restriction.

Previously published reports have also described cell lines which, like PCC4.aza1R, apparently lack the Fv-1
function. The SC-1 cell line (Hartley & Rowe, 1975), which is now accepted as a fully permissive reference standard in Fv-1 restriction studies, was derived by clonal selection of a feral mouse embryo culture which was characterized as N-type in its early passage. Similarly, Gisselbrecht et al. (1974) reported the loss of Fv-1 gene expression in 3T3FL cells, a clonal subline of Fv-1b 3T3 cells. Yoshikura et al. (1979, 1982) reported the isolation of both typical Fv-1\* cells and cells dually permissive for N- and B-tropic MuLV from the inbred mouse strains DDD and G. It is not clear how each of these cell lines developed equal susceptibility to both N- and B-tropic MuLV but the emergence of these dually permissive cell clones seemed to result from the loss of a pre-existing Fv-1 function. We do not know whether the clonal PCC4.aza1R cell line represents another example of Fv-1 functional loss or whether there are alternative explanations for PCC4.aza1R permissiveness. The fact that Fv-1 is expressed in the F9 EC cell line indicates that lack of Fv-1 expression is not characteristic of EC cell lines in general.

The Fv-1 phenotype exhibited by F9 cells raises some interesting questions about the nature of N-type restriction, which to date has been defined only as a decreased sensitivity to endogenous N-tropic MuLVs in cells from certain strains compared to other Fv-1\* mice. It was proposed that this phenotype indicated the existence of a series of Fv-1\* alleles rather than a single genotype (Mayer et al., 1978). However, preliminary results from cross-breeding experiments between BALB/c (B-type) and RFM/Un (N-type) mice support the idea that the factor responsible for endogenous N-tropic virus resistance in RFM/Un mice segregates independently of the Fv-1 locus (W. K. Yang et al., personal communication). Our observation that restriction of B-tropic MuLV is evident in F9 cells in the absence of restriction of endogenous N-tropic viruses also suggests that the two activities are separable. Undifferentiated F9 cells may represent an 'intermediate' phenotype where the Fv-1 gene restricting B-tropic virus replication is actively expressed but the additional undefined restriction locus is not. Alternatively, the N-type phenotype may be the result of a separate gene(s) which modifies normal Fv-1\* function; this modifying gene may be unexpressed in F9 cells. In either case, the virion target for this additional restriction is most likely to be CA (as it is for Fv-1 restriction) because fragment exchange experiments map the sensitivity to the same region of gag (L. R. Boone, unpublished results).

We were interested in determining whether differentiation of the Fv-1-silent PCC4.aza1R cells into cell types which are known to express Fv-1 (i.e. fibroblasts) would result in expression of Fv-1. Indeed, the N-type phenotype is expressed in the differentiated myoblast PCD1 cell line (Fig. 1). It is not known, however, whether the progenitor EC cell of the PCD1 cell line is similar to the PCC4.aza1R EC cell line in its lack of Fv-1 expression and therefore direct comparison of PCD1- and PCC4.aza1R-derived cells is not possible. We examined a total of 37 PCC4.aza1R-derived fibroblast and pre-adipocyte clonal cell lines (16 of which were established directly from PCC4.aza1R cells) and found that only the pre-adipocyte cell line PCC4D1 expressed Fv-1 restriction (Fig. 1). These results therefore failed to substantiate any direct association between the pre-adipocyte differentiated state in particular and Fv-1 expression. Nonetheless, the data from PCC4D1 cells do indicate that Fv-1 expression can be initiated under certain (albeit undefined) conditions. PCC4D1 cells may represent a somewhat 'more differentiated' state and may possess a genetic constitution slightly altered from that in the other pre-adipocyte cell lines generated for this study. Alternatively, it is possible that a random event unrelated to programmed differentiation could be responsible for expression of the N\* restriction phenotype in these cells. Regardless of the mechanism, the expression of Fv-1 in PCC4D1 cells could be of considerable use in attempting to isolate and characterize further this important gene which regulates MuLV infection and leukemogenesis in mice.

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References


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