Retroviral Vector Gene Expression in F9 Embryonal Carcinoma Cells

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When F9 embryonal carcinoma (EC) cells are infected with retroviral vectors, the efficiency of expression of selectable genes is considerably lower than that in mouse fibroblasts infected with the same retroviral vectors. In this study, several retroviral vectors with regulatory sequences placed immediately 5' to a selectable gene were constructed, packaged, and used to infect mouse fibroblasts and F9 EC cells. With selection as an assay, there was a hierarchy of relative expression in F9 cells compared with that in mouse fibroblasts. These internally placed regulatory sequences are the source of the mRNAs detected in F9 EC cells, while both retroviral long-terminal-repeat promoters and internal promoters are the source of steady-state mRNAs in mouse fibroblasts. This effect was observable with both the internally placed herpes simplex virus thymidine kinase promoter and the Moloney murine leukemia virus promoter.

The study of the interaction of viruses with the stem cell and differentiated derivatives of murine teratocarcinoma cells has led to the possibility that the stem cell, embryonal carcinoma (EC), may regulate its gene expression through transcriptional networks different than those of the differentiated cells which arise from it. Polyomavirus, simian virus 40, and the retrovirus Moloney murine leukemia virus (M-MuLV) all enter the stem cell upon infection, but the expression of their genes is blocked in the EC cell (5, 6, 9, 11, 12, 19, 21, 24, 28-30, 33-35, 37). Mutants of polyomavirus, PyEC mutants, have been isolated by several laboratories (5, 11, 24, 33, 37), and these mutants replicate and express their genes in certain EC cell lines. The mutations are usually located in a region of the polyomavirus genome which has been functionally defined by De Villiers and Schaffner (2) as the enhancement sequence region of the virus. Recombinant DNA constructs containing mutant and wild-type fragments from the polyomavirus genome have been coupled to indicator genes and transferred back into EC cells, differentiated derivatives, and other mouse cell lines. The results suggest that the mutated regions increase the activity or selection response of the indicator gene in EC cells when compared with parallel constructions derived from wild-type polyomavirus fragments (9, 13).

When M-MuLV infects EC cells, it integrates into the genome but does not express its genes (21, 28, 34). The molecular basis(es) for this restriction has not been fully explained. There have been conflicting reports on the possible role of methylation in inactivating or repressing expression from the retroviral long terminal repeats (LTRs) (6, 19, 28). Previously, we reported that when we replaced the M-MuLV enhancer region with a sequence from a PyEC mutant, PyF101, coupled this LTR subfragment to the bacterial chloramphenicol acetyltransferase gene (*cat*), and transferred this construction to F9 EC cells, we could detect expression from this recombinant construction but not from a similar construction which had the M-MuLV enhancer region (12). Our interpretation of these results was that the M-MuLV enhancer region was restricted in F9 EC cells.

One indication that viral sequences may be important to the block in M-MuLV expression comes from the work of

Ostertag and co-workers (4, 25), who used the myeloproliferative sarcoma virus derivative on EC cell lines. By using this virus and derivatives of it, an increased retroviral expression on the F9 EC cell line could be detected. Recently, Barklis et al. (1) and Taketo and Tanaka (32) have shown that in those rare retroviral vector transformants on EC cells, either the position of the integration events or a mutated virus appears to be the cause of expression in EC cells.

Previous reports by others concerning the infection of EC cells with retroviral vectors have indicated that the efficiency of transferring a selectable gene such as the bacterial phosphotransferase gene (*neo*) from the Tn5 transposon gene to EC cells compared with that to mouse fibroblasts is 1,000- to 500,000-fold less (1, 23, 26, 31). Two reports have indicated that the simple introduction of regulatory sequences between the LTRs of retroviral vectors increases this efficiency of transferring selectable genes into EC cells (23, 39). In this study, we compared the efficiencies of promoter sequences plus or minus enhancer sequences introduced between the LTRs of packaged retroviral vectors in transferring and expressing selectable genes in F9 EC cells compared with mouse fibroblasts.

MATERIALS AND METHODS

Cells. Cell culture was performed as previously described for F9 and NIH 3T3 cells (5, 13, 14).

Recombinant DNA constructions. The recombinant DNA constructs were based upon the Moloney sarcoma virus construct pMV (22). The vector pMV was modified by S1 nuclease digestion of the *Pst*I site and ligation of *Bgl*II linkers to this site. A *Bgl*II (5')-to-*Bam*HI (3') fragment of the *neo* gene was isolated from the vector pBRNEO (27) and cloned into this *Bgl*II site. This produced the vector pMVBglNEO-3. To construct pMVMOpNEO, a *Hae*III-to-*Sma*I fragment from a cloned M-MuLV LTR, pMLV-C/R/B (12), was ligated to *Bgl*II linkers, subcloned, and then introduced 5' to the *neo* gene in the pMVBglNEO-3 vector at the *Bgl*II site. To construct the pMVMOpNEO vector, a *Sau*3AI (5')-to-*Sma*I (3') subfragment of the M-MuLV LTR was isolated from the pMLV-C/R/B (12) plasmid with *Bam*HI linkers on the *Sma*I end. This was subcloned into the *Bam*HI site of the pUC12 plasmid and reisolated by *Sau*3AI

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restriction of the plasmid. This fragment was then inserted into pMVBglNEO-3 at the *Bgl*II site just 5' to the *neo* gene. To construct the pMV101TKprNEO vector, a *Hinc*II fragment of a previously described (13) F101TK U-1 construct was isolated which had the PyEC F101 *Pvu*II-4 fragment coupled to the herpes simplex virus thymidine kinase (TK) promoter. This fragment had *Bgl*II linkers ligated onto it, and the fragment was cloned into the *Bgl*II site 5' to the *neo* gene of the pMVBglNEO-3 vector. To construct the pMVTKprNEO vector, a TK promoter (*Bam*HI linker on the 5' *Pvu*II site of the TK promoter) was coupled at its natural *Bgl*II site to the *Bgl*II 5' end of the *neo* gene in the pBRNEO plasmid and the 5' *Bam*HI-TK-*neo*-3' *Bam*HI fragment was cloned into the *Bgl*II site of the pMV vector. All of the recombinant DNA manipulations were performed by using *Escherichia coli* DH-1 as the host.

Packaging retroviral vectors. CsCl-ethidium bromide-purified supercoils (20 μ g per 10-cm plate) were transferred into psi-2 packaging cells (15). Colonies were selected with 400 μ g of G418 per ml (1 mg of 40% G418 per ml). Individual psi-2 clones were isolated and cultured, and fresh medium without G418 was added 24 h before collection of the medium. The medium was centrifuged at $3,000 \times g$ for 5 to 10 min, and the supernatant was aliquoted and frozen at -80°C . Later in this study, we learned that we could improve our titers by transfecting the amphotropic packaging cell line PA12 (18) in the same way, collecting the medium 48 to 72 h after transfection, and then using this medium to infect the ecotropic packaging cell line psi-2 (15). This procedure resulted in psi-2 clones with titers 5- to 20-fold higher than those obtained with direct transfection of psi-2 cells. This observation is consistent with the report (10), indicating that retroviral vectors introduced by infection yield more RNA than do retroviral vectors introduced by transfection. When vectors were packaged this way, there is an AP after the vector name, which designates passage through an amphotropic system to the ecotropic psi-2 system. Infection of the psi-2 cells was done as described below for the F9 EC and mouse NIH 3T3 cells.

Retroviral vector infection. Cells were plated at 4×10^5 to 5×10^5 cells per 10-cm plate the day before infection. The next day the cells were treated with Dulbecco modified Eagle medium minus serum plus 20 μ g of Polybrene per ml for 1 h. One milliliter of retroviral vector medium or retroviral vector medium diluted in normal tissue culture medium was added per plate and adsorbed to the plates on a rocking table in an incubator. After the 2-h adsorption period, 10 ml of culture medium was added to each plate. Twenty-four hours after the end of vector adsorption to the cells, medium with 400 μ g of G418 per ml plus 10^{-4} M beta-mercaptoethanol (20) was added to the plates. Fresh medium with G418 was added every 2 days until cell death was apparent and then every 3 days.

RNA analysis. Total poly(A)⁺ RNA was isolated from cells by the procedure of Vennstrom and Bishop (38). RNAs were analyzed by separation on formaldehyde-agarose gels, capillary blotting to nitrocellulose or GeneScreen Plus membranes (New England Nuclear Corp.), and hybridization to nick-translated probes containing the *neo* gene or the human γ -actin gene (8). The specific activities of the *neo* probes were 5.6×10^8 cpm/ μ g (Fig. 2, lanes A, B, C, a, b, and c) and 1.5×10^8 cpm/ μ g (lanes D, E, and e). The specific activities of the γ -actin probes were 7×10^7 cpm/ μ g (lanes A, B, C, a, b, and c) and 8×10^7 cpm/ μ g (lanes D, E, and e).

Primer extension analysis (3) was done with a 33-mer oligonucleotide complementary to the *neo* gene coding se-

FIG. 1. Structure of retroviral vectors used in this study. (A) pMVBglNEO-3, the basic vector into which various regulatory sequences were introduced. (B) pMVTKprNEO, the herpes simplex virus TK promoter (5' *Pvu*II to 3' *Bgl*II) was coupled to the *neo* gene and then was inserted into the *Bgl*II site of pMV. (C) pMV101TKprNEO, the *Pvu*II-4 fragment of the PyEC mutant PyF101 coupled to the herpes simplex virus promoter (5' *Pvu*II to 3' *Hinc*II) was inserted into the *Bgl*II site of pMVBglNEO-3. (D) pMVMOprNEO, the M-MuLV promoter (5' *Hae*III to 3' *Sma*I) was introduced into the *Bgl*II site of pMVBglNEO-3. (E) pMVMOeprNEO, the M-MuLV enhancer plus promoter (5' *Sau*3A1 to 3' *Sma*I) was introduced into the *Bgl*II site of pMVBglNEO-3. bp, Base pairs.

quence. The 3'-terminal base of the oligonucleotide was complementary to base 16 3' to the ATG for the *neo* gene. Ten picomoles of the oligonucleotide was kinased with [α - 32 P]ATP and passed over a NACS column (Bethesda Research Laboratories, Inc.) to remove unincorporated label. Ten micrograms of poly(A)⁺ RNA was annealed to 2×10^4 to 4×10^4 cpm of the primer in a volume of 10 μ l of annealing buffer (75 mM KCl, 5 mM Tris [pH 7.5]). This mixture was heated for 3 min at 100°C and then transferred to a 60°C bath for 3 h. The annealing reaction mixture was brought to 37°C , and 10 μ l of $5 \times$ avian myeloblastosis virus reverse transcriptase salts (50 mM KCl, 25 mM MgCl_2 , 10 mM dithiothreitol, 240 mM Tris [pH 8]), 5 μ l of 20 mM dXTP, 0.5 μ l of RNasin, 22.5 μ l of diethylpyrocarbonate-treated H_2O , and 20 U (2 μ l) of avian myeloblastosis virus reverse transcriptase were added. The reaction was run at 37°C for 90 min. The product was then hydrolyzed with alkali: 5.5 μ l of 2 M NaOH was added to the reaction mixture, and the mixture was incubated for 2 h at 42°C and then neutralized with 5.5 μ l of 3 M sodium acetate. Five micrograms of carrier yeast tRNA was added, and the product was precipitated with 2.5 volumes of ethanol. The precipitates were dissolved, run on an 8% polyacrylamide sequencing gel (16), and autoradiographed.

RESULTS

The basic vector which we started with was the pMV vector of Perkins et al. (22). This was modified to allow placement of the bacterial *neo* gene at the original *Pst*I site of the vector. Regulatory sequences were inserted just 5' to the *neo* gene (Fig. 1). These sequences were transferred into psi-2 cells to package the vectors. In some instances, the vectors were transferred into the amphotropic packaging line PA12 (18) first and then medium from these cells was used to infect psi-2 cells.

TABLE 1. Titers of retroviral vectors on fibroblasts and EC cells^a

Vector	Titer on:		NIH 3T3/F9	Titer on FOT5	NIH 3T3/FOT5
	NIH 3T3	F9			
pMVBglNEO-3	1.4×10^5	2.6×10^1	5,400		
pMVTkprNEO	4.9×10^5	1.2×10^3	420		
pMV101TKprNEO	2.5×10^6	1.2×10^5	20		
pMVMOPrNEO	2.4×10^5	6.2×10^2	380		
pMVBglNEO-3 (PAP)	1.3×10^6			9.1×10^2	1,428
pMVMOPrNEO (PAP)	4.2×10^5			3.4×10^3	123
pMVMOPrNEO (AP cl C)	9.1×10^5			7.3×10^3	125
pMVMOPrNEO (AP cl D)	7.1×10^5			5.5×10^3	129

^a FOT5 is an ouabain-resistant, hypoxanthine phosphoribosyltransferase-deficient F9 EC cell derivative (36). PAP means the vector was transfected in psi-2 cells, infected into PA12 cells, and then infected into psi-2 cells. AP means the vector was transfected into PA12 cells and then infected into psi-2 cells. Because of the absence of a difference in the ratio of titers of pMVMOPrNEO and pMVMOPrNEO vectors on FOT5 cells and NIH 3T3 cells, we examined two separate psi-2 clones (cl C and cl D) of the pMVMOPrNEO vector. These results are representative of at least four different retroviral vector infections of the cells with the vectors. The medium used was always taken from individual clones of psi-2 cells.

The retroviral vector infection experiments were all performed with medium from clones of the packaging cell line to minimize the possibility of infection with vectors which did not represent the DNA originally transferred to the packaging cell lines. Typical G418 selection data are given in Table 1. The data in the right-hand column indicate the relative ability of the vector to produce selectable colonies on mouse fibroblasts compared with F9 EC cells. When a vector with an internal TK or M-MuLV promoter (pMVTkprNEO or pMVMOPrNEO) was used, there was a modest (approximately 10-fold) increase in the ability of the vector to produce G418-resistant colonies compared with fibroblast colonies. A vector containing an internal M-MuLV promoter-plus-enhancer fragment (pMVMOPrNEO) gave the same relative titer as did a vector containing just the internal M-MuLV promoter fragment (pMVMOPrNEO). This suggests that the internal M-MuLV enhancer has no obvious positive or negative effect on the adjacent internal M-MuLV promoter. In transfection constructs, Gorman et al. (7) have shown that the enhancer appears to have a negative effect on the M-MuLV promoter when transfected into EC cells.

When a vector construct included both an internal TK promoter and the *PvuII*-4 fragment from the polyomavirus PyF101 mutant which productively infects F9 EC cells (5) and which we have shown contains enhancerlike activity (13) on F9 EC cells (pMV101TKprNEO), there was an additional increase in the relative EC-to-fibroblast titer (Table 1). This suggests that with an EC-permissive regulatory sequence, the number of retroviral integration events which allow for G418 selection can be further increased.

To verify that the increased EC/fibroblast relative titers of the vectors with internal promoters (Table 1) were produced by transcription from the internal promoters, poly(A)⁺ RNA was isolated from mixed clones of F9 EC cells, and fibroblasts were infected with these vectors. Southern blot analysis (data not shown) of these clones indicated by that criterion that the vectors were intact. Northern blot (RNA blot) analysis of RNA isolated from infected and selected clones is shown in Fig. 2. The major transcripts from the pMVBglNEO-3 vector (lanes A and a) were of a size that a transcript would have if initiated from the LTR (based on comparison with rRNAs in the gel and also the size of the actin RNA when the blots were rehybridized with a human γ -actin probe [8]). However, the vectors with internal promoters yielded two transcripts in fibroblasts and one major transcript in F9 EC cells. The smaller EC cell transcript was of a size that would be expected for a transcript coming from the internal promoter.

It should be noted that the amount of transcript from F9 cells infected with the pMVTkprNEO vector (Fig. 2, lane D) and the pMV101TKprNEO vector (lane E) was approximately the same even though the relative titer was 10-fold higher on EC cells for the pMV101TKprNEO vector. Our interpretation of this is that a certain level of *neo* transcript is necessary for G418 selection and that the weaker internal regulatory sequence of the pMVTkprNEO vector is more dependent upon integration in sites of the genome which

FIG. 2. Northern blot analysis of poly(A)⁺ RNAs from G418-selected (400 μ g/ml) mixed clones of EC and fibroblast cells. Lanes: A, RNA from pMVBglNEO-infected FOT5 EC cells; a, RNA from pMVBglNEO-infected NIH 3T3 cells; B, RNA from pMVMOPrNEO-infected FOT5 cells; b, RNA from pMVMOPrNEO-infected NIH 3T3 cells; C, RNA from pMVMOPrNEO-infected FOT5 cells; c, RNA from pMVMOPrNEO-infected NIH 3T3 cells; D, RNA from pMVTkprNEO-infected F9 cells; E, RNA from pMV101TKprNEO-infected F9 cells; e, RNA from pMV101TKprNEO-infected NIH 3T3 cells. Lanes A, B, C, a, b, and c had 10 μ g of poly(A)⁺ RNA, and the blot was exposed for 12 h. Lanes D, E, and e had 5 μ g of poly(A)⁺ RNA, and the blot was exposed for 48 h. A human γ -actin probe (8) was nick translated and used to rehybridize the blots to examine RNA concentration and integrity. The relative sizes of the RNAs were estimated from rRNA markers run on the same gels (not shown). The RNAs initiating from the LTR are indicated by arrowheads, and RNAs initiating from the internal promoter are indicated by arrows.

FIG. 3. Primer extension analysis of poly(A)⁺ RNAs from mixed clones of F9 and FOT5 EC cells infected with different retroviral vectors. Lanes: A, RNA from F9 cells infected with pMV101TKprNEO; B, RNA from F9 cells infected with pMVTkprNEO; C, RNA from FOT5 cells infected with pMVMOeprNEO; D, RNA from FOT5 cells infected with pMVMOprNEO. The arrows identify the region of the expected primer extension products. Lane T, T lane of M13 dideoxy sequencing markers. The numbers on the left represent lengths in bases as known from the M13 sequence.

enhance expression of the vector. Similar RNA data were obtained with clones of fibroblasts and F9 EC cells infected with these vectors. However, with individual F9 EC clones infected with pMVTkprNEO, the minor, presumably LTR-initiated transcript observed in lane D was not seen. We presume this was due to an occasional integration event which allowed for LTR expression.

If the RNAs from cells infected with the pMVMOprNEO vector (Fig. 2, lane B, for FOT5 EC cells and Fig. 2, lane b, for NIH 3T3 cells) and the pMVMOeprNEO vector (lane C for FOT5 EC cells and lane c for NIH 3T3 cells) are

compared, it can be seen that fibroblasts produced two transcripts when infected with these vectors. For the internal M-MuLV promoter alone, the major transcript in fibroblasts (lane b) was the larger transcript coming from the LTR. However, for the internal M-MuLV enhancer-plus-promoter construct (lane c), the RNAs were approximately equivalent. We presume that the increased level of the smaller transcript from the pMVMOeprNEO-infected fibroblasts compared with that from the pMVMOprNEO-infected fibroblasts was due to the addition of the internal enhancer in the pMVMOeprNEO vector. For F9 cells, a major, smaller transcript coming from the internal M-MuLV promoter of both constructs was obtained. The concentrations of the RNAs were approximately the same, although as mentioned for the internal TKpr and 101TKpr constructs, this cannot be used as an argument for similar regulatory signal strength since the RNA was from clones which were selected.

To verify that the Northern blot analysis of RNAs from EC cells (Fig. 2) represented transcriptional initiations at the internal promoters, primer extension analysis was performed on the RNAs by using an oligonucleotide complementary to the 5' end of the *neo* gene. Primer extension analysis of RNAs from EC cells selected for expression of pMV101TKprNEO, pMVTkprNEO, pMVMOeprNEO, and pMVMOprNEO indicated correct initiation at the internal promoters (Fig. 3). The F9 RNA gave clear bands at all the predicted sizes for the four internal promoter constructs. The 101TKpr and TKpr constructs showed the stutter previously shown for the TK promoter (17). The small differences in the sizes of these primer-extended RNAs were based on slight differences in the construction of the original vectors (see Materials and Methods).

DISCUSSION

The results presented here support the conclusions of previous investigators (23, 39) that internally placed regulatory sequences increase the relative titer of retroviral vectors on infected EC cells compared with that on infected mouse fibroblasts. In addition, differences were illustrated between the choices of regulatory sequences placed in such vectors. The TK promoter and M-MuLV promoter increased the relative titer approximately 10-fold. The addition of a PyEC enhancer to the TK promoter increased the relative titer approximately another 10-fold (Table 1). However, the addition of the M-MuLV enhancer to the internal M-MuLV promoter had no observable effect upon the relative titer in F9 EC cells when compared with that of a retroviral vector which had the internal M-MuLV promoter alone.

The pMVTkprNEO and pMV101TKprNEO data (Table 1) suggest that the PyEC F101 regulatory sequence, which has been shown to function effectively in F9 EC cells (13), can increase the relative F9/NIH 3T3 titer compared with an internally placed TK promoter-derived vector, pMVTkprNEO. However, we observed no difference between the pMVMOprNEO and pMVMOeprNEO vectors on F9 EC cells. This supports our previous interpretation based on transfection experiments with F9 EC cells (12) that the M-MuLV enhancer is not an effective enhancer in EC cells. If there is a negative effect of the M-MuLV enhancer on adjacent promoters in F9 EC cells (7), then the existing M-MuLV enhancers in the LTRs of the retrovirus may be sufficient for the effect.

Another point can be made with respect to the selection data (Table 1) and RNA data (Fig. 2). A vector such as

pMV101TKprNEO, which transferred selection for the *neo* gene 10-fold better than the pMVTkprNEO vector did in F9 EC cells, still produced selected clones which gave amounts of *neo* transcripts approximately equal to those obtained from EC cells selected for expression of the pMVTkprNEO vector. That is, the selection differences were not represented by steady-state RNA differences. While this may be explained in many ways, one possible explanation is that a smaller subset of integration events is selected with the vector having the internal TKpr construct alone. When these clones are then examined for RNA, they may represent populations in which the sequences adjacent to the sites of the integration events play a larger role in determining the level of RNA produced. When F9 cells were infected with equivalent fibroblast titers of these two vectors and RNA was then examined without selection, the level of RNA detected more closely corresponded to the selection data shown in Table 1 (unpublished data).

The possible importance of retroviral vector integration in F9 EC cells is illustrated by the recent work of Barklis et al. (1) and Taketo and Tanaka (32). Their evidence suggests that when selection is made for expression of a simple retroviral vector in EC cells, two events can be observed: (i) integration adjacent to cellular sequences which function as positive regulatory elements in F9 EC cells (cellular enhancer acting on retroviral vector promoter or a regulatory region which initiates RNA outside of the vector) or (ii) selection of retroviral vector mutants which are now permissive for expression in F9 EC cells. Given these observations, we interpret the data presented in this paper in the following way. The M-MuLV and Moloney sarcoma virus LTRs are not effective regulatory sequences in F9 EC cells. For expression to occur without a mutation in the vector, a rare integration event near a cellular site is needed which could allow for transcription initiation from that cellular site or enhancement of expression from the LTR. As internal regulatory sequences are placed into retroviral vectors of increasing relative strength in F9 EC cells, the expression of the vector becomes less dependent upon its integration site and therefore a larger percentage of the retroviral integration events in F9 EC cells yield selectable EC colonies.

While the nature of the block to expression from regulatory sequences in the LTR is still ill defined, the results of previous studies (23, 39) and this study suggest a potential means of circumventing the LTR expression block in mouse stem cells. The hierarchy illustrated in Table 1 should allow for choices of internal regulatory sequences for retroviral expression vectors and retroviral vectors expressing targeted antisense RNAs (36).

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