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Transfer of genes into embryonal carcinoma cells by retrovirus infection: efficient expression from an internal promoter

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The expression of retroviral genomes after infection of embryonal carcinoma (EC) cells is usually restricted. We have constructed a retroviral vector which has the potential to express efficiently in EC cells an inserted sequence, the selectable neomycin (*neo*) gene, from an internal thymidine kinase promoter. A second retrovirus vector contained in addition a v-*myc* oncogene, which in NIH 3T3 cells could be expressed from the viral long terminal repeat (LTR) through a subgenomic mRNA. 10–100% of the EC cells infected with recombinant virus gave rise to *neo*-resistant colonies following drug selection. The *neo* gene was expressed in both the non-permissive EC and the permissive NIH 3T3 cells at similar levels, and transcription of *neo*-specific RNA was initiated at the proper site in the thymidine kinase promoter. In contrast, v-*myc* mRNA was produced only in the NIH 3T3 cells and not in EC cells, even after induction of differentiation. Thus, genes inserted into retrovirus constructs can easily be introduced into EC cells by virus infection, even without drug selection, and they can be efficiently expressed when transcribed from promoters other than the viral LTRs.

Key words: EC cells/retrovirus vectors/TK promoter/neomycin resistance/v-*myc*

Introduction

Embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, provide a unique opportunity for the introduction of specific recombinant genes into mice. The stem cells have the capacity to participate in normal development to form all somatic as well as functional germ cells after re-introduction into early embryos (Graham, 1977; Mintz and Fleischman, 1981; Stewart, 1984). Integration and expression of genes inserted into EC cells can be examined in undifferentiated and in differentiated cells prior to chimaera formation, thereby providing an advantage over other methods used for introducing foreign genes into mice.

The efficiency of introducing genes into EC cells by DNA-mediated gene transfer is low (Linnenbach *et al.*, 1981; Wagner and Mintz, 1982; Bucchini *et al.*, 1983; Nicolas and Berg, 1983). Infection with retrovirus vectors offers an alternative approach for gene transfer. However, a difficulty with using such vectors with EC cells is that viral gene expression is repressed even though infection with Moloney murine leukemia virus (MLV) results in the integration of proviral DNA into the host cell genome (Stewart *et al.*, 1982; Gautsch and Wilson, 1983; Niwa *et al.*, 1983). The restriction of viral gene expression in EC cells may partly be due to inefficient expression from the MLV long terminal repeat (LTR) (Linney *et al.*, 1984), although additional restriction mechanisms may also exist (Stewart *et al.*, 1982; Gautsch and Wilson, 1983; Niwa *et al.*, 1983).

Here we report the construction of recombinant retroviruses which are used to infect and express specific genes in various established EC cell lines. Similar efficiencies in obtaining neomycin-resistant (*neo*^{R}) colonies were found with the EC-cells and the permissive NIH 3T3 cells. The retroviruses express efficiently the selectable *neo* gene from an internal thymidine kinase (TK) promoter. An additional inserted gene, the v-*myc* oncogene, which could be expressed from the viral LTR in NIH 3T3 cells, remained unexpressed in the EC cells, although it was transmitted along with the *neo* gene.

Results

Generation of recombinant retroviruses

The two different retrovirus vectors are shown in Figure 1. Both contain the thymidine kinase (TK) promoter linked to the dominant selectable *neo* gene, thereby conferring G418 resistance. The MV-4-*neo* vector was constructed by inserting a TK-*neo* fragment into the retrovirus vector pMV-4, which is based on MLV (Vennström *et al.*, 1984). The MMCV-*neo* construct is similar to MV-4-*neo*, except that a v-*myc* oncogene, derived from the avian retrovirus OK10, was inserted upstream from the TK-*neo* element. In NIH 3T3 cells, the MMCV-*neo* virus expresses the *myc* oncogene from a subgenomic mRNA generated by splicing transcripts originating in the MLV LTR (Vennström *et al.*, 1984). Infectious virus was generated by transfecting the vectors into NIH 3T3 cells along with plasmid DNA containing the entire MLV genome. The transfected cells were subjected to G418 selection after initial subcultivation in non-selective medium, and pools of *neo*-resistant (*neo*^{R}) clones were used to generate virus stocks. Stocks had titers of 1–2 x 10^6 *neo*^{R} colony forming units (*neo*-c.f.u.) per ml of culture fluid when assayed on NIH 3T3 cells (Table I). To obtain infectious virus, free of MLV helper virus, the DNA constructs were transfected into ψ2 cells (Mann *et al.*, 1983) which produced virus titers of ~1 x 10^4 *neo*-c.f.u./ml.

Retrovirus infection of EC cells following neo selection

The efficiency of isolating EC cells under selective conditions was determined by infecting three EC cell lines with varying dilutions of virus. Table I shows that all infections gave a high pro-

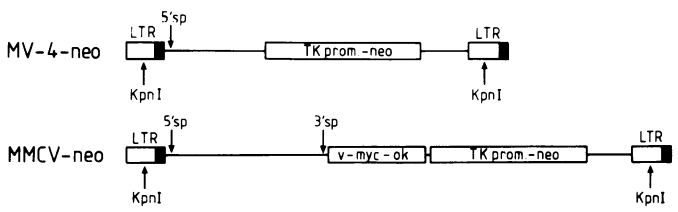

Fig. 1. Structure of the retrovirus vectors. The LTRs and adjacent viral sequences are derived from MLV. Both constructs also contain the neomycin resistance gene (*neo*) driven by the TK promoter from herpes simplex virus. The MMCV-*neo* virus encodes in addition the v-*myc* gene from the avian retrovirus OK10. The details of their construction are given in Materials and methods. 5'sp: 5' splice donor site; 3'sp: 3' splice acceptor site.

Table I. Relative efficiencies of *neo*^{R} colony formation after infection of EC and NIH 3T3 cells with retroviral vectors containing an internal TK promoter

Vector	Relative numbers of stable transformants			
	NIH 3T3	F9	P19	PC13
MV-4-*neo*	1	0.5 – 1	0.05 – 0.1	0.1 – 0.5
MMCV-*neo*	1	0.2 – 0.5	–	–
MMCV*neo* (ψ 2)	1	0.1 – 0.5	–	–

NIH 3T3 and EC cells were infected in parallel and subjected to selection for *neo*^{R}. The number of *neo*^{R} NIH 3T3 and EC colonies (*neo*-c.f.u.) were compared by standardizing the NIH 3T3 colonies to 1. 1 represents 50 – 100 *neo*-c.f.u/ml obtained after infection with 10^{-4} diluted MV-4-*neo* or MMCV-*neo* virus and 10^{-2} dilutions of MMCV-*neo* (ψ2) virus; the range of *neo*-c.f.u. was obtained from three independent experiments.

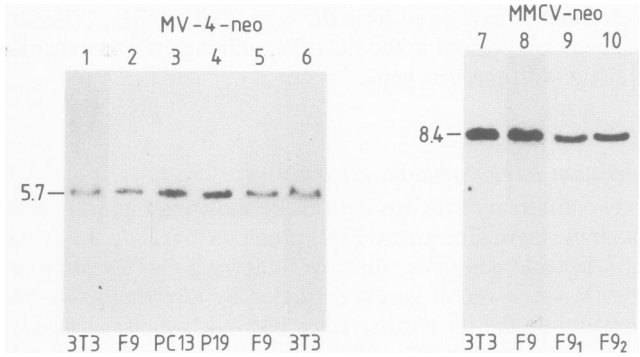

Fig. 2. Analysis of DNA from NIH 3T3 and EC cells infected with MV-4-*neo* or MMCV-*neo* virus. Southern transfer analysis of *Kpn*I-digested genomic DNA from mass cultures of virus-infected *neo*^{R} NIH 3T3 and EC cells (**lanes 1 – 4, 7** and **8**). **Lanes 5 – 6** contain DNA from cells grown for 20 days after infection but not subjected to selection for drug resistance; **lanes 9** and **10** show DNAs from two individual *neo*^{R} F9 cell clones obtained after infection with the helper-independent MMCV-*neo* (ψ2) virus.

portion (10 – 100%) of cells resistant to *neo*, and the number of *neo*^{R} colonies obtained was proportional to the multiplicity of virus infection. Both F9 EC cells and NIH 3T3 cells infected with the MV-4-*neo* virus gave similar numbers of *neo*^{R} clones, whereas fewer but still high numbers of colonies were observed with the P19 and PC13 EC cells. The MMCV-*neo* virus gave 2- to 10-fold fewer colonies with F9 cells than with NIH 3T3 cells. However, the overall efficiency in obtaining *neo*^{R} EC cell clones using these viruses is 100- to 1000-fold higher than that achieved by DNA transfection or with retroviruses expressing the *neo* gene from the viral LTR (unpublished data).

Characterization of proviral DNA

The structure and average copy number of proviral sequences was analyzed by the Southern procedure. High mol. wt. DNA from cultures consisting of >100 individual *neo*^{R} colonies as well as from independently derived *neo*^{R} cell clones of NIH 3T3 and EC cells, was digested with *Kpn*I. This enzyme only cuts in the LTRs, and is therefore diagnostic of an intact proviral structure. The expected fragments of 5.7 kb for the MV-4-*neo*-infected cells and 8.4 kb for the MMCV-*neo* clones were detected (Figure 2). Single-copy integration is suggested by similar band intensities and was demonstrated by cleaving DNA from individual F9 clones with *Eco*RI and hybridization either with a *neo* or a v-*myc* specific probe (data not shown).

Expression of genes introduced by virus infection

To determine whether the levels of *neo* expression in selected NIH 3T3 and EC cells were comparable and could account for

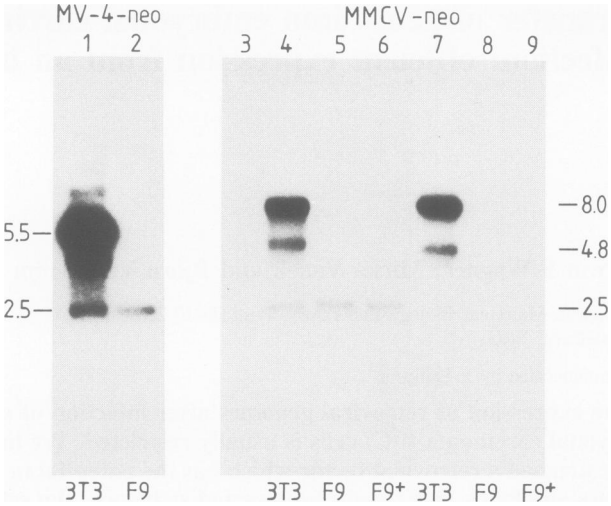

Fig. 3. RNA analysis of virus-infected and selected NIH 3T3 and F9 cells. Expression of virus encoded *neo* and v-*myc* RNAs in pools of *neo*^{R} clones of NIH 3T3 cells (**lanes 1, 4, 7**) and F9 cells before (**lanes 2, 5, 8**) and after (**lanes 6, 9**) retinoic acid induction. **Lanes 1 – 6** were hybridized with a *neo*-specific fragment and **lanes 7 – 9** with a v-*myc* specific probe (Vennström *et al.*, 1981).

the high efficiency of *neo*^{R} colonies in EC cells, total poly(A)$^+$ RNA from mass cultures of MV-4-*neo*-infected cells was analyzed by the Northern procedure (Figure 3). Equal amounts of a 2.5-kb mRNA species which hybridized to the *neo*-specific probe were detected in both cell types. Apparently no RNA transcribed from the viral 5′ LTR was detected in the *neo*^{R} F9 cells, whereas the genomic 5.5-kb RNA was the predominant RNA species in the NIH 3T3 cells. The minor RNA species found in the NIH 3T3 cells probably originate from transcriptional read-through products or are generated from cryptic splice sites, as has been observed with other vectors (unpublished data). Analysis of poly(A)$^+$ RNA from MMCV-*neo*-infected NIH 3T3 cells showed the expected 8-kb genomic RNA, the subgenomic 4.8-kb *myc* mRNA and the 2.5-kb *neo* mRNA. In the F9 cells only the 2.5-kb *neo* mRNA was seen, at a level comparable with that observed in NIH 3T3 cells. When these infected EC cells were treated with retinoic acid to induce differentiation, no change in the pattern of RNA transcription was observed (Figure 3).

Correct RNA start sites in the infected EC cells

S1 nuclease analysis was used to characterize further and to quantitate the RNAs produced by the infected NIH 3T3 and F9 cells. To map the 5′ end of the *neo*-specific RNA, we used a subcloned 658-nucleotide *Pvu*I-*Bgl*II fragment containing the TK promoter and adjacent pBR322 sequences (Figure 4). RNA from both NIH 3T3 and F9 cells protected a 56-nucleotide fragment indicating that the *neo*-specific 2.5-kb mRNA had initiated properly at the TK cap site in both cell types. No additional protected fragments were detected in RNA preparations from undifferentiated or differentiated EC cells (Figure 3, lane 3, 5, 6). In the NIH 3T3 cells an additional protected fragment of 133 nucleotides was observed. The size of this fragment, present in a 50- to 100-fold excess over the 56-nucleotide TK fragment, is what would be expected from protection by genomic or *myc* specific RNAs. These results are in agreement with the data obtained from the Northern blot analysis (Figure 3) and show that the levels of *neo* mRNA originating from the MV-4-*neo* and MMCV-*neo* proviruses are comparable between the permissive NIH 3T3 cells and the non-permissive EC cells.

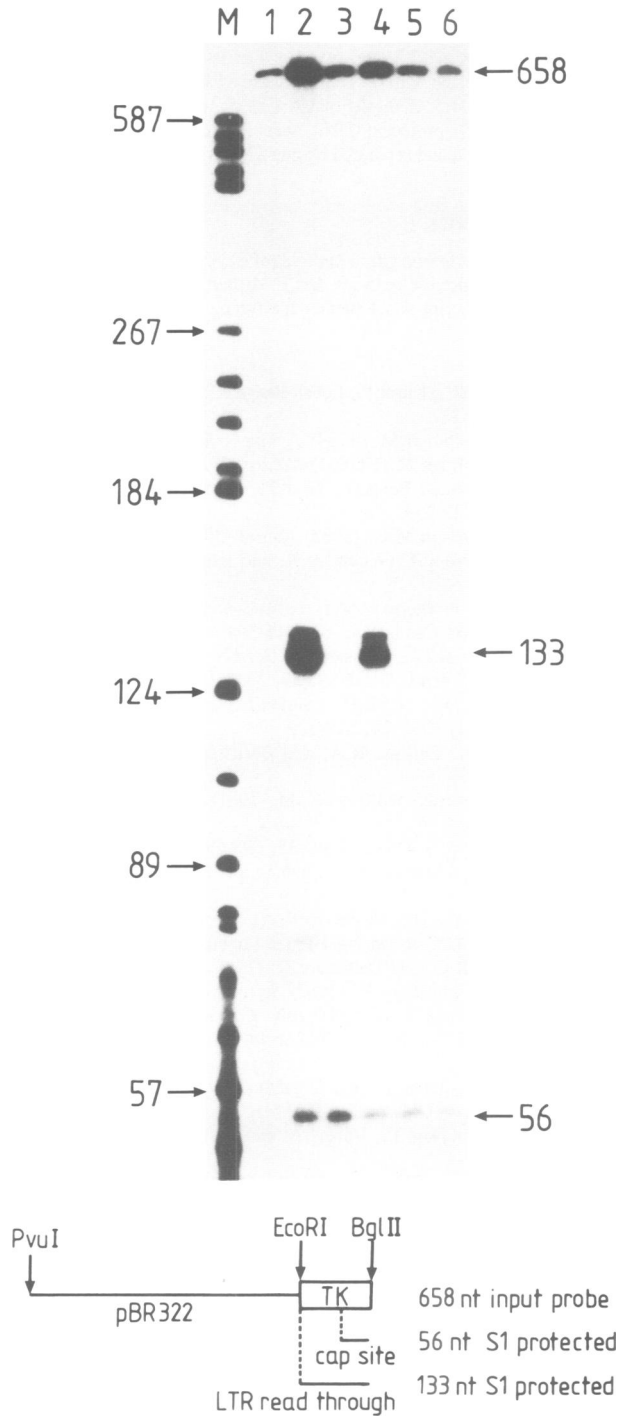

Fig. 4. S1 nuclease analysis of TK-specific mRNA from: uninfected F9 EC cells (**lane 1**); MV-4-*neo* infected NIH 3T3 (**lane 2**) and F9 EC cells (**lane 3**); MMCV-*neo*-infected NIH 3T3 (**lane 4**); and F9 EC cells before (**lane 5**) and after (**lane 6**) retinoic acid induction. **Lane M** contains *Hae*III fragments of pBR322 end-labelled with ^{32}P. The TK-specific probe, subcloned in pBR322, was end-labelled at the *Bgl*II site and excised with *Pvu*I as shown at the bottom of the figure.

Infection of EC cells without selection

To investigate further the usefulness of retroviruses for introducing genes into embryonic cells and for expression studies, we determined the efficiencies of proviral DNA integration in EC and NIH 3T3 cells in the absence of *neo* selection. F9 cells were infected with MV-4-*neo* and grown in unselective medium up to 20 days, to allow integration of proviral copies to occur (Stewart *et al.*, 1982). Analysis of DNA isolated from these cells indicated that the unselected cells contained 1 – 2 copies of proviral DNA and that the integration efficiency in the NIH 3T3 and in the F9 cells was similar (Figure 2, lane 5, 6). Preliminary data on the potential of the proviral genomes to express the *neo* gene without selection suggest that some restriction mechanism is inactivating the *neo* expression in the EC cells. F9 cells were infected with MV-4-*neo* and a small number of cells were plated at various times (10 – 20 days) after infection in the presence and absence of drug selection. It was found that only a small percentage (1 – 5%) of the colony-forming F9 cells were able to grow in the presence of G418, whereas the percentage of neo^R colonies on NIH 3T3 cells was appreciably higher. This observation was confirmed when the phosphotransferase activity of the *neo* gene product was direclty assayed in cell extracts (Reiss *et al.*, 1984). The enzyme activity in extracts prepared from F9 cells, which were infected for 5 or 10 days with MV-4-*neo*, was ~90% reduced as compared with extracts from NIH 3T3 cells (unpublished data). These data may be interpreted that different mechanisms are responsible for the efficient expression of proviral genomes that were selected for expression and for the poor expression of proviruses obtained after infection without selection.

Discussion

Our results demonstrate that retrovirus vectors with an internal TK promoter can be used to integrate and to express foreign genes in EC cells following drug selection. The efficient expression of the *neo* gene from the TK promoter after virus infection and *neo* selection is most likely the reason why a large fraction of cells acquire *neo* resistance. The TK promoter can function in EC cells (Linnenbach *et al.*, 1981; Wagner and Mintz, 1982; Linney and Donerty, 1983) and it is conceivable that the TK-*neo* element confers transcriptional competence to most proviruses when selected for expression. The fact that in the infected EC cells apparently no expression from the 5′ LTR was detected is in agreement with the data reported by Linney *et al.* (1984) who showed that the LTR enhancer sequences are not active in F9 cells. However, with other retrovirus vectors we have found some promoter activity from the LTR which allows a low level of gene expression in a small proportion of infected and selected EC cells (unpublished data).

Infection of EC cells without selection for drug-resistant colonies showed that genes can readily be introduced and that the majority of EC cells can be infected. In addition, preliminary data have shown that a small fraction of EC cells have the potential to express the *neo* gene in the absence of selection. However, the mechanism which inactivates the expression from the provirus remains unknown and is currently being investigated. It appears that the block in expression is at the level of transcription, as was reported earlier for MLV-infected EC cells (Stewart *et al.*, 1982). *De novo* methylation of the incoming retroviral genomes, which was correlated with the lack of expression of MLV in F9 cells (Stewart *et al.*, 1982), does not seem to cause the restriction in expression (unpublished data). It is possible that the site of integration as well as the particular promoter used have a profound influence on the activity of the gene introduced into the EC cells. Experiments are in progress to exchange the TK promoter with other regulatory sequences known to function in EC cells. In addition, studies to express the v-*myc* oncogene in EC cells under the control of the TK promoter in a construction similar to MMCV-*neo* are under way.

Previous reports have indicated that deletions of viral sequences can occur with vectors containing exogenous DNA with pro-

moters (Emerman and Temin, 1984). This was not observed with the vectors we used. It is possible that the stability of the TK-*neo* viruses we constructed can be attributed to the absence of poly(A) addition signals and repetitive sequences in the inserted DNA fragments.

The vectors described here have already been successfully used for infection of EK cells which are derived directly from embryo outgrowth (Evans and Kaufman, 1981). Selected and pre-characterized cell clones were subsequently aggregated with embryos and used to produce mouse chimaeras (Stewart, 1982; Stewart and Wagner, in preparation). In addition, these and other specifically designed retrovirus vectors can be used for introducing genes efficiently into haematopoietic stem cells (Joyner *et al.*, 1983; Williams *et al.*, 1984) as well as directly into embryos as established for MLV infection (Jaenisch *et al.*, 1981), to study the restriction of viral gene expression and the action of developmentally regulated genes and their controlling elements.

Materials and methods

Cells and viruses

The retroviral constructs were transfected into NIH 3T3 TK$^-$ cells (referred to as NIH 3T3) together with a DNA clone containing the entire MLV helper virus genome (Plasmid pZAP, Shoemaker *et al.*, 1981). Confluent cells were subcultured twice in Dulbecco's medium containing 10% fetal calf serum and 2 μg/ml polybrene to allow virus spread, and then subjected to selection for *neo*^{R} cells with 800 μg G418/ml. Dishes containing 50 or more drug-resistant colonies were trypsinized, pooled and further cultivated for production of virus stocks. This procedure gave virus stocks with titers of ~1 x 10^6 *neo*-c.f.u./ml when assayed on NIH 3T3 cells.

To generate a helper-virus free stock of virus, the ψ2 cell line (Mann *et al.*, 1983), which contains a packaging-deficient helper virus genome, was transfected with cloned MMCV-*neo* DNA, and individual clones of *neo*^{R} cells were screened for high virus production by a reverse transcriptase assay (Goff *et al.*, 1982). Virus stocks from high producing clones had titers of ~1 x 10^4 *neo*-c.f.u./ml on NIH 3T3 cells.

EC cells (5 x 10^4) were plated in 60 mm dishes 1 day prior to infection. To infect, the medium was removed and 0.5 ml of filtered, undiluted and $10^{-2}-10^{-4}$ diluted medium from virus-producing cells was added in the presence of 4 μg/ml polybrene. After a 1–2 h incubation with occasional gentle shaking, 4 ml of medium was added. Two days later the medium was replaced with medium containing 1 mg G418/ml. After 10–12 days in selective medium, colonies were stained and counted. At the same time mass cultures consisting of 500–1000 colonies per plate or single colonies were isolated and expanded for further analysis.

Pools of *neo*^{R}F9 cells obtained after infection with MMCV-*neo* were differentiated for 6 days in the presence of 10^{-7} M retinoic acid and 1 mM dibutyryl cAMP (Strickland and Mahdavi, 1980).

Construction of viruses

The MV-4-*neo* vector was constructed by inserting a 2.3-kb *Bam*HI TK-*neo* fragment into the pMV-4 retrovirus vector (Vennström *et al.*, 1984). This *Bam*HI insert contains the 825-nucleotide *Bam*HI-*Bgl*II fragment of the herpes virus TK promoter region (including an *Eco*RI site referred to in Figure 3, McKnight and Kingsbury, 1982), joined to a 1.5-kb *Bgl*II-*Bam*HI fragment from the Tn5 *neo* resistance gene (Rothstein *et al.*, 1980). A plasmid pIPB$_1$, containing this TK-*neo* gene was obtained from Dr. R.Sweet. The MMCV-*neo* vector was constructed by insertion of a 2.5-kb v-*myc* *Bam*HI-*Eco*RI fragment from the avian retrovirus OK10 (Pfeifer *et al.*, 1983) into the MV-4-*neo* construct after addition of a *Bam*HI linker to the *Eco*RI site. The cloning procedures were essentially as described in Maniatis *et al.* (1982). The MV-4-*neo* genome is 5.8 kb and the MMCV-*neo* genome is ~8 kb.

Analysis of DNA and RNA

High mol. wt. DNA was isolated and digested with *Kpn*I, which cuts only in the LTR sequences; DNA was fractionated in a 0.9% agarose gel, transferred to nitrocellulose filter and hybridized with a ^{32}P-labelled *neo* probe.

Poly(A)$^+$ RNA was isolated from pools of >100 clones of virus-infected cells that had been selected in G418-containing medium. 4 μg of RNA per lane was separated in formaldehyde-agarose gels, blotted to nitrocellulose filters and hybridized with a ^{32}P-labelled *neo* or v-*myc* probe as described (Frykberg *et al.*, 1983).

S1 protection analysis with a TK-specific fragment was done after end-labelling at the *Bgl*II site with polynucelotide kinase as illustrated at the bottom of Figure 3. 2.5 μg of poly(A)$^+$ RNA, prepared from *neo*^{R} mass cultures, was hybridized with the probe at 41°C for 16 h in 10 μl of 80% formamide, 40 mM Pipes pH 6.4, 5 mM EDTA pH 7.5, 400 mM NaCl as described (Hynes *et al.*, 1983). The mixture was digested with 60 U of nuclease S1 (PL Biochemicals) in 100 μl of 30 mM NaAc pH 4.5, 250 mM NaCl, 1 mM $ZnSO_4$, 2 μg carrier DNA at 30°C for 40 min. S1-protected DNA was extracted with phenol, ethanol-precipitated, denatured and separated by electrophoresis in a 6% acrylamide gel containing 7 M urea.

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Note added in proof

After this manuscript was submitted, two studies were published reporting the use of selectable retroviruses for EC cell infection (Rubenstein *et al.*, 1984; Sorge *et al.*, 1984).