J. Embryol. exp. Morph. **97 Supplement**, 263–275 (1986)
Printed in Great Britain © *The Company of Biologists Limited 1986*

The expression of retroviral vectors in murine stem cells and transgenic mice

COLIN L. STEWART, ULRICH RÜTHER, CHRISTA GARBER, MIRKA VANEK AND ERWIN F. WAGNER

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, Federal Republic of Germany

I. INTRODUCTION

The introduction of recombinant DNA into mouse embryos has proved to be a useful technique in addressing a number of important developmental questions, e.g. the identification of DNA sequences controlling tissue-specific gene expression and the role of genes in growth control (for reviews see Palmiter & Brinster, 1985; Wagner & Stewart, 1986). Currently the favoured method for gene transfer into mice is by DNA injection into a pronucleus of the fertilized egg. However, an alternative method is to exploit retroviruses as vectors for introducing genes either directly into embryos or into embryonal carcinoma (EC) or embryonic stem (ES) cells which can then be used to form chimaeric mice (Bradley, Evans, Kaufman & Robertson, 1984). There are a number of advantages to using retroviral vectors, the major one being that they can infect a wide variety of cells at an efficiency approaching 100 %. In several instances they are the only means at present for introducing genes into certain cell types, such as haemopoietic precursor cells (Keller, Paige, Gilboa & Wagner, 1985). In the infected cell the vector usually integrates stably into the chromosomal DNA as a single copy at a random site. This integration as a single copy can be exploited for cell lineage analysis, e.g. in the haemopoietic system (Keller *et al.* 1985). The expression from the provirus can also be accurately measured, unlike the situation with DNA-mediated gene transfer where the DNA is often integrated in tandemly repeated copies. In addition, retroviruses seem to be potentially useful as insertional mutagens (Jaenisch *et al.* 1985).

There are, however, disadvantages to using these vectors, some of which are technical and may be overcome in the future. One problem has been the instability of the proviral structure, in that sometimes rearranged vector sequences are found (Joyner & Bernstein, 1983). The size constraint of approximately 8 kb of DNA, that can be incorporated within a vector is clearly a disadvantage, especially for the expression of genomic DNAs. In addition, the proper functioning of regulatory elements that are positioned in the proximity of the viral long terminal repeats

Key words: retroviral vectors, mouse stem cells, transgenic mice, LTR promoter, gene expression, *neo*^{R} gene, DNA methylation.

(LTRs), e.g. for tissue-specific or inducible gene expression has yet to be demonstrated.

The infection of mouse embryos and EC cells with retroviruses was initially used as a model system to study the control of gene expression during mouse development (Jaenisch & Jähner, 1984). However, infection of preimplantation embryos or EC cells with the Moloney-murine Leukaemia virus (MLV) did not result in expression of the virus in either instance (Stewart, Stuhlmann, Jähner & Jaenisch, 1982; Jähner *et al.* 1982). In infected embryos, this inhibition of expression was usually maintained throughout development except for some Mov strains, where activation of the virus occurred in some unidentified cells (Jaenisch *et al.* 1981; Jaenisch & Jähner, 1984).

In this review we will demonstrate that retroviral vectors can be used as a tool for studying gene expression in transgenic mice and embryonic stem cells. We will discuss the unique versatility of these vectors and the various strategies which can be used at present for designing the appropriate expression vector. We will also address the problem of inhibition of expression in early embryonic cells and how this repression can be circumvented *in vitro* and *in vivo*. We will conclude by showing the potential of these vectors to express a variety of genes, e.g. an inducible c-*fos* proto-oncogene, so that the role of these genes in differentiation can be studied both *in vitro* and *in vivo*.

II. INFECTION OF EMBRYONIC STEM CELLS WITH RETROVIRAL VECTORS

(A) *Expression from the proviral LTR*

The first retroviral vectors used in gene transfer studies were relatively simple constructs. They contained a dominant selectable marker gene (see Fig. 1A), consisting of either the Tn5 neomycin resistance gene (*neo*^{R}) or the *Eco*Gpt gene (Gpt) expressed from the 5′ LTR (Mann, Mulligan & Baltimore, 1983; Keller *et al.* 1985). Infection of fibroblasts usually resulted in a cloning efficiency in the order of 100% (Wagner, Vanek & Vennström, 1985*a*). However, infection of various EC cell lines resulted in a cloning efficiency some three to five orders of magnitude lower, depending on the cell line (Sorge, Cutting, Erdman & Gautsch, 1984; Wagner *et al.* 1985*b*). The stem cell clones usually contained a single copy of the vector and expressed the selected gene inefficiently compared to fibroblast clones (Stewart, Vanek & Wagner, 1985; Wagner *et al.* 1985*b*; Fig. 2A).

We have recently shown that infected EC cell clones, selected and characterized *in vitro* for the expression of a *neo*^{R} gene, were able to form viable adult chimaeras when the EC cells were reintroduced into embryos (Stewart *et al.* 1985). In these animals the expression of the gene under the control of the 5′ LTR was maintained in all the chimaeric tissues (and probably throughout development). This result indicated that the LTR could function as a promoter throughout embryogenesis and probably in all adult tissues. Furthermore, it illustrated the potential use of these cells in allowing them to be characterized with respect to the expression of the introduced DNA *in vitro*, before their introduction into embryos for

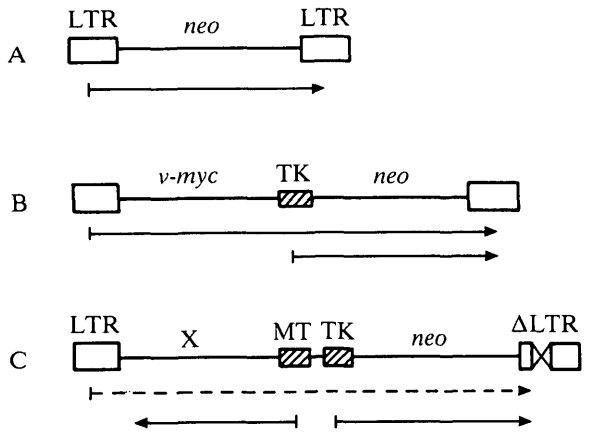

Fig. 1. Schematic presentation of three retroviral vectors described in the text. (A) The selectable *neo*^{R} gene is expressed from the 5′ LTR. (B) Expression of two genes, the v-*myc* oncogene from the 5′ LTR and the selectable *neo*^{R} gene from the internal TK promoter. (C) Self-inactivating (SIN) vector with a deleted 3′ LTR and two internal promoters expressing the genes in opposite orientations. MT, inducible human metallothionein promoter regulating the expression of gene X.

All thin lines are the expected RNA transcripts from the integrated proviral DNA. In the SIN vector the dotted line is the transcript produced from the 5′ LTR during synthesis of the virus. This genomic transcript is not synthesized in the infected target cell when the deleted 3′ LTR replaces the intact 5′ LTR (see section III).

subsequent development *in vivo*. Thus, EC cells selected to express genes *in vitro* could be used to study the effect that the expression of certain genes (e.g. growth control genes) might have on early embryonic development.

(B) *Expression from an internal promoter*

An alternative method for expressing genes from retroviral vectors involves the use of internal promoters, positioned between the two LTRs (Fig. 1B). This allows the expression of genes from two different promoters, the LTR and a constitutive promoter. Such a vector is shown in Fig. 1B and is termed MMCV-neo (Wagner *et al.* 1985*a*); the v-*myc* gene is expressed from the 5′ LTR while the *neo*^{R} gene is under the control of the herpes simplex thymidine kinase promoter (TK). This vector gave very high cloning efficiencies in the order of 10% on infection of EC cells. Virtually all infected EC cells were resistant to G418 and expression of the *neo*^{R} gene from the TK promoter occurred as efficiently as in fibroblasts (Wagner *et al.* 1985*a*). In fibroblasts the v-*myc* oncogene was active but no transcripts were observed in the selected EC cells showing the 5′ LTR was not functioning. In support of these results it has been shown recently that a vector expressing the *neo*^{R} gene from an internal hybrid SV40 TK promoter was also more efficient at expressing the selectable gene in EC cells than the 5′ LTR (Rubenstein, Nicolas & Jacob, 1984).

The MMCV-*neo* vector was tested in chimaeras to see if the internal TK promoter could function in all tissues and whether the LTR can be activated in

some tissue or in differentiated cells. ES cells were infected with the vector, and cell clones that carried a single copy were then used to form chimaeras (Stewart *et al.* 1985). In all chimaeras analysed, only the *neo*^{R} gene of the vector was expressed. The 5′ LTR positioned in front of the v-*myc* gene was not transcribed in several tissues analysed. Even when genomic DNA from one of the ES clones was transfected onto fibroblasts, no LTR expression was restored.

The paradox with LTR activity is that LTRs do function efficiently in fibroblasts and haemopoietic cells; yet they do not function in cell types derived from infected embryos or in EC/ES cells, provided they are not selected for activity. The possible reasons will be discussed in greater detail in the next section. However, several attempts have been made at modifying the LTR so that it can function more efficiently in embryonic cells, either by substituting the LTR's enhancer sequences with an enhancer that can function in the stem cells (Linney *et al.* 1984) or by isolating spontaneous mutants (Franz *et al.* 1986). The enhancer modification in the LTR does not appear to be sufficient for efficient expression in embryos although it may do so in some adult tissues (van der Putten *et al.* 1985). In the case of the mutant LTRs their function in embryos has yet to be shown.

(C) *The inhibition of retrovirus expression: does the methylation status of the proviruses correlate with expression?*

The restriction in retrovirus expression is itself of relevance to understanding general questions concerning the developmental control of gene expression. This inhibition appears to operate at a number of different levels: it has been shown that the LTR can function as an inefficient promoter in the EC cells (Linney *et al.* 1984; Stewart *et al.* 1985) and that trans-acting negative regulatory factors may also be operating in these cells (Gorman, Rigby & Lane, 1985). However, it is not clear how the LTR can function, when it is selected for activity. Furthermore, what is the mechanism that inhibits the expression of retroviruses throughout embryogenesis or during differentiation of EC cells? It has been suggested that the LTR can undergo some mutation that allows it to function as a promoter in EC cells (Franz *et al.* 1986). Alternatively some cis-acting mechanism related to the chromosomal site of integration may also be involved (Sorge *et al.* 1984). Concerning the maintenance of this inactivation, it has been argued that *de novo* methylation of the proviral genome is instrumental in suppressing and maintaining the inhibition of expression (Jaenisch & Jähner, 1984). Thus hypermethylation of the proviral genome correlated with the absence of expression, whereas hypomethylation correlated with expression.

Using various EC cell clones that express a single copy of a retroviral vector, we have addressed the question of the role of methylation. Two EC cell lines, F9 and P19, were each infected with a vector either carrying the *Eco*Gpt or the *neo*^{R} gene and cell clones were isolated by selection. In two representative F9 Gpt clones the vector was expressed at about one quarter of the level found in fibroblasts (Fig. 2A, lanes d, e). Interestingly in one of the clones a second mRNA band of almost equal intensity was also seen (Fig. 2A, lane d). Whether this was due to

either readthrough from a promoter upstream to the vector or readthrough from the vector to some downstream sequences has yet to be ascertained. Nevertheless, it illustrates the fact that the chromosomal site of integration can influence the

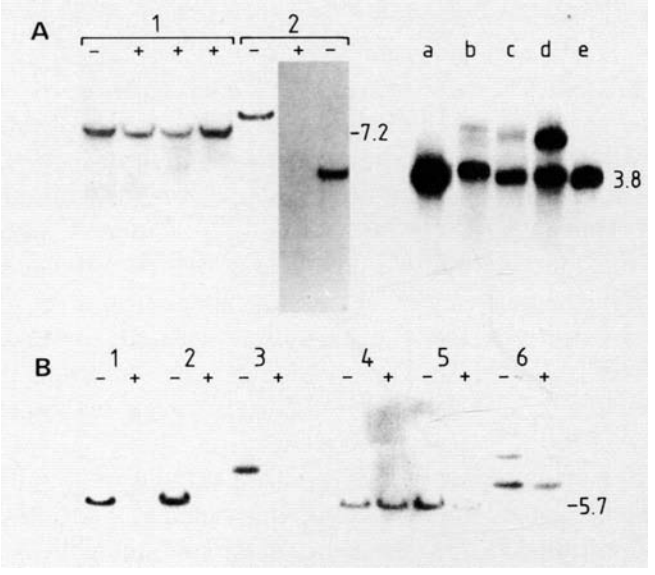

Fig. 2. The expression and methylation of retroviral vectors in selected clones of F9 and P19 EC cells.

(A) In the left panel DNA analysis is shown from a P19 clone containing a single copy of the Gpt vector that expresses the Gpt gene from the 5′ LTR (Mann *et al.* 1983). In lane 1 the DNA was digested with *Hin*dIII alone (−), which does not cut in the vector, or together with three methylation-sensitive enzymes (+) *Cfo*1, *Hpa*II and *Ava*I, respectively. This showed that the vector in this clone at the sites analysed was methylated. In lane 2 one F9 clone containing a single copy of the vector was also digested with *Hin*dIII (−) or *Hin*dIII/*Cfo*1 (+) or *Sac*1 alone (−). In this clone the vector was unmethylated at the sites analysed as shown by its disappearance after digestion with *Cfo*1. The right-hand panel shows expression of a 3·8 kb transcript of the Gpt vector in EC cells. Lane a, control RNA from 3T3 fibroblasts that produce the vector. Lanes b and c show RNA from two P19 clones; one corresponding DNA analysis (from b) is shown in the left-hand panel (1). Lanes d and e show RNA from two F9 clones. That the DNA (from d) is a single copy and of the expected size is shown by the *Sac*1 (−) and *Hin*dIII digests in lane 2.

(B) Lanes 1–3 are three F9 clones, each containing a single copy of the vector and expressing the neo^R gene from the 5′ LTR (Wagner *et al.* 1985*b*). Lanes 1 and 2 show the DNA from two clones that were digested with *Sac*1 (−) or together with *Hpa*II. Lane 3 shows a *Hin*dIII (−) *Hin*dIII+*Hpa*II (+) digestion of the third clone. In all clones the vector sequences were unmethylated at the sites analysed. In lanes 4–6 DNA from three P19 clones infected with the same vector are shown. The same digestions were performed with the DNA in the + lanes being digested with *Hpa*II. The clone in lane 6 contains two copies of the vector. As with the Gpt vector in P19 clones described in Fig. 2A, the neo^R vector in the P19 clones was also methylated at the *Hpa*II sites, as shown by its resistance to digestion.

pattern of transcription, provided that an intact provirus is integrated. DNA digestion of the F9 clones with a set of methylation-sensitive enzymes showed that the vector was unmethylated for the number of sites tested, since the characteristic genomic length fragment disappeared after digestion (Fig. 2A,B).

When clones isolated from the EC cell line P19, that either contained the *Eco*Gpt or *neo*^{R} vector were analysed, expression was similar to that found in the F9 clones (Fig. 2A, lanes b, c). However, DNA analysis of the two *Eco*Gpt clones revealed that the entire vector (including both LTRs) was methylated at the sites analysed as shown by their resistance to digestion with three methylation-sensitive enzymes (Fig. 2A, lane 1). A similar observation was made with three of the *neo*^{R} clones (Fig. 2B, lanes 4, 5, 6). In both sets of experiments the vector had been simultaneously introduced with wild-type helper viruses. All clones contained at least one copy of the helper virus which was not expressed and was also methylated as determined by the resistance to digestion with the methylation-sensitive enzymes (data not shown). Thus expression of the vectors in these cells was probably not due to a cellular mutation resulting in the expression of all integrated retroviruses or vectors.

A second series of experiments was directed to analyse the methylation and expression of the *neo*^{R} gene in infected but unselected F9 and 3T3 cells at various times after infection. In 3T3 fibroblasts the vector containing the *neo*^{R} gene under control of the LTR was efficiently expressed 5 days after infection whereas at the same time point and also after 10 days postinfection no *neo*^{R} expression, as measured by the neomycin phosphotransferase (NPTII) activity, was found in the F9 cells (Fig. 3A). DNA analysis showed that even at 20 days postinfection the vector in both cell types remained unmethylated for the internal *Hpa*II sites (Fig. 3A).

In a comparable set of experiments, using a vector where the *neo*^{R} gene was under the control of an internal promoter, expression was again maintained in the infected 3T3 fibroblasts. In addition, expression in the infected F9 cells at 20 days (postinfection) was still detectable (Fig. 3B). DNA analysis from both populations of infected cells showed again that the vectors remained unmethylated for the internal sites.

These results show that the level of methylation of two different vectors in EC cells, either under selectable or nonselectable conditions and as well for two different types of selection, cannot be easily correlated with the expression of the vectors. It appears that the role of methylation in regulating retroviral gene expression remains unclear, despite the possible importance of methylation regulating gene expression in other systems (for a review see Dörfler, 1985). It can still be argued that there is a crucial site within the LTR that, if methylated, would result in no expression of the virus or vector. It may be that this site is not detectable by the panel of enzymes we have used here, and thus the results presented here might only be clarified by genomic sequencing of the LTRs which should detect the methylation of every cytosine within these regulatory sequences.

III. TRANSGENIC MICE OBTAINED BY INFECTION OF EMBRYOS WITH RETROVIRAL VECTORS

The experiments described in section II(B) showed that the internal TK promoter expressing the *neo*^{R} gene was functional in EC cells as well as in chimaeras obtained from selected ES cell clones. We therefore decided to test its ability to function *in vivo* by directly infecting embryos with the same vector, MMCV-neo (Fig. 1B). In initial experiments, the infected embryos were allowed to develop to the 14th day of gestation. Fifty foetuses were recovered and were explanted into culture under selective conditions. Eleven produced cell lines which grew under these conditions in G418-containing medium. Subsequent analysis showed that

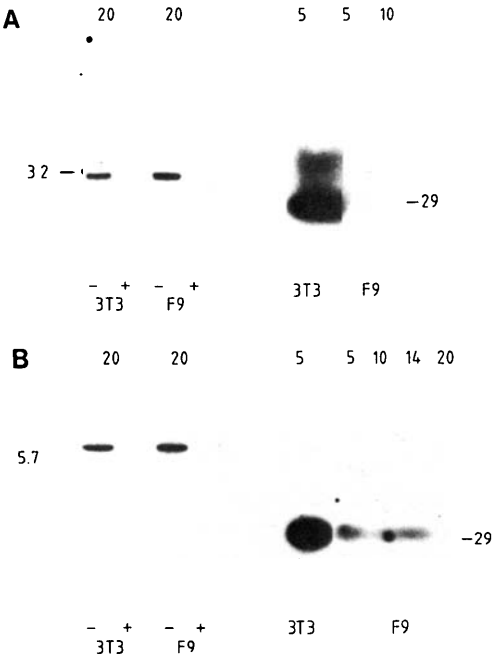

Fig. 3. Expression and methylation of retroviral vectors in an unselected population of F9 and P19 EC cells.

(A) F9 EC cells or 3T3 fibroblasts were infected with the vector shown in Fig. 1A (Keller *et al.* 1985). In the right-hand panel the expression of the *neo*^{R} protein by the NPTII assay is shown in 3T3 fibroblasts, 5 days postinfection (for details of the assay see Stewart *et al.* 1985). In the F9 cells no expression was seen at 5 or 10 days postinfection. In the left-hand panel DNA digestion of 3T3 and F9 cells is shown at 20 days postinfection; with *Sac*1 alone (−), which cuts once in each LTR, or together with *Hpa*II (+). In both populations the vector remained unmethylated at *Hpa*II sites as shown by the disappearance of the *Sac*1 band after digestion.

(B) F9 EC cells or 3T3 fibroblasts were infected with a vector expressing the *neo*^{R} gene from an internal TK promoter (Fig. 1B and Wagner *et al.* 1985*a*). In the right-hand panel, expression of the *neo*^{R} gene by NPTII activity was found in fibroblasts after 5 days of infection and also at a reduced level in the F9 EC cells even 20 days postinfection. In the left-hand panel *Sac*I digests of the DNAs with (+) or without (−) *Hpa*II are shown, which revealed that the vector remained unmethylated at the *Hpa*II sites.

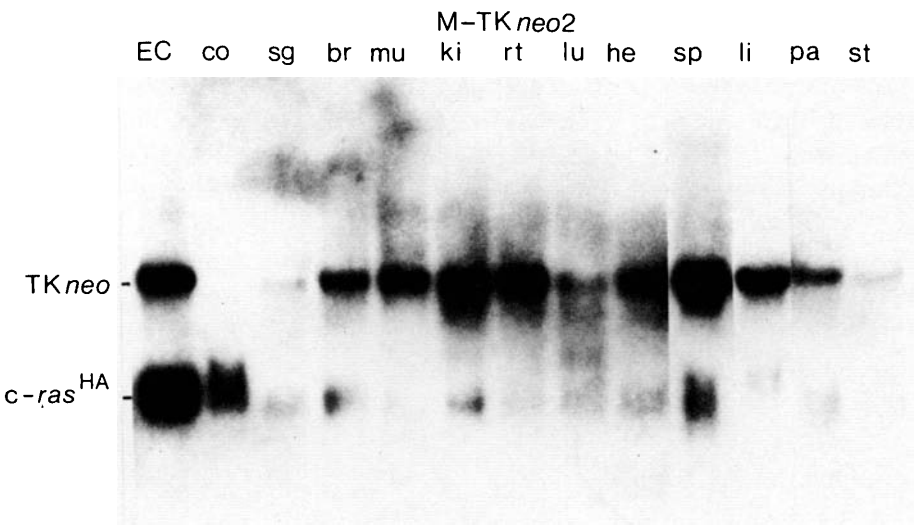

Fig. 4. Expression of the TK*neo* transcripts in an F_1 offspring from the M-TK*neo*2 mouse line. RNA was isolated from the different organs, 20 μg total RNA electrophoresed and hybridized to a *neo* specific ^{32}P-labelled probe. A c-*ras*^{Ha} probe was also included in the hybridization to check for the intactness of the RNA loaded on the gel. Abbreviations: EC, (−) control from an F9 EC clone expressing the same vector; co (−) control liver RNA from a normal mouse. sg, salivary gland; br, brain; mu, muscle; ki, kidney; rt, reproductive tract; lu, lung; he, heart; sp, spleen; li, liver; pa, pancreas; st, stomach and intestine.

these foetuses contained between one to five copies of the vector and, as seen in the chimaeras, only the *neo*^{R} gene of the vector was expressed (Wagner *et al.* 1985*b*). These experiments were repeated and the infected embryos were allowed to develop to term. Out of 17 mice born, 6 were positive for the vector sequences and, of these, 3 transmitted a single copy of the vector to their offspring. The 3 transgenic mouse lines were called M-TK*neo*1-3 (C. Stewart *et al.* in preparation).

It was next of interest to determine if expression of the vector can be found in the adult tissues of these mice that had been infected at the preimplantation stage when usually retroviral gene expression is permanently inhibited. RNA analysis from F_1 offspring showed that the *neo*^{R} gene expressed from the TK promoter was active in all adult tissues in two mouse lines, M-TK*neo*2 and 3 (Fig. 4). In the third transgenic line (M-TK*neo*1), the vector had integrated on the X chromosome as shown by breeding analysis and expression of the *neo*^{R} gene was only found in some organs, notably the muscle, kidney and brain (C. Stewart *et al.* in preparation). No evidence for transcripts from the LTR containing the v-*myc* oncogene was found, which is in agreement with the former discussion of LTR suppression in early embryos.

These results show for the first time that it is possible to produce transgenic mice by embryo infection using retroviral vectors and that the introduced genes are expressed. A number of other groups have reported that embryos can be infected

with retroviral vectors and that the vectors were also transmitted to the offspring (Jähner *et al.* 1985; Huszar *et al.* 1985). However, expression of the vectors has not been reported either when the gene in question was under the control of the 5′ LTR (with the exception of a virus containing the polyoma enhancer; Van der Putten *et al.* 1985) or when the SV40 promoter itself was used as an internal promoter (Huszar *et al.* 1985).

IV. GENE EXPRESSION FROM RETROVIRAL VECTORS CONTAINING TWO INTERNAL PROMOTERS, BUT DEFECTIVE LTRS

One obstacle in using retroviral vectors containing intact LTRs for studying gene regulation is their presence in the proviral DNA. These elements encoding the promoter and enhancer functions can interfere in an unpredictable manner with other regulatory signals, e.g. inducible or tissue-specific promoters. One possible solution is the use of self-inactivating (SIN) retroviral vectors (formerly called suicide vectors; Wagner *et al.* 1985*b*). These vectors contain a deletion in the 3′ LTR, which abolishes the enhancer and promoter functions (Yu *et al.* 1986; Figs 1C, 5). The rationale for such a vector with a deleted 3′ LTR is based on two observations: (i) upon DNA transfection into packaging cell lines, the intact 5′ LTR is used to produce genomic RNA, which can be packaged into an infectious virus particle. (ii) During viral replication and integration in the target cell, the 3′ deletion is duplicated and transferred to the 5′ LTR leading to an inactive provirus. Expression of a selectable or regulatable gene is therefore dependent on the internal signals and is independent of the viral regulatory regions.

Three prototype SIN vectors were constructed that carry two genes: the selectable *neo*^{R} gene expressible from the TK promoter and either the c-*fos* proto-oncogene, the Ad12E1A gene or the CAT gene, all under the control of the internal human metallothionein (hMT) promoter (Yu *et al.* 1986; Fig. 5A). Both *neo*^{R} resistant F9 and 3T3 cell clones were obtained, which were then analysed with respect to their proviral DNA structure and for inducibility of expression using $CdCl_2$ or dexamethasone. As shown by DNA analysis in Fig. 5B, correct transfer of the 3′ deletion to the 5′ LTR was seen in bulk infected and selected cells, and with some clones inducible c-*fos* expression was also observed (Fig. 5C).

A similar finding was obtained with the viruses carrying the E1A gene. Using the Sp6-RNase protection assay, inducible and correct RNA initiation from the hMT promoter was detected (Fig. 6) as was also observed with the SIN virus containing the CAT gene. However, in this latter construct we were unable to detect an enzymatically active protein, although no obvious DNA rearrangements have been detected (data not shown). One possible explanation for this observation was that the insertion of a gene in the opposite transcriptional orientation to the LTR might produce splice sites that can form small internal deletions in the gene during the replication process leading to the synthesis of an inactive protein.

It seems that the different inserts have to be tested thoroughly for each individual construct and that these novel viruses have to await testing in the *in vivo* environment in mice.

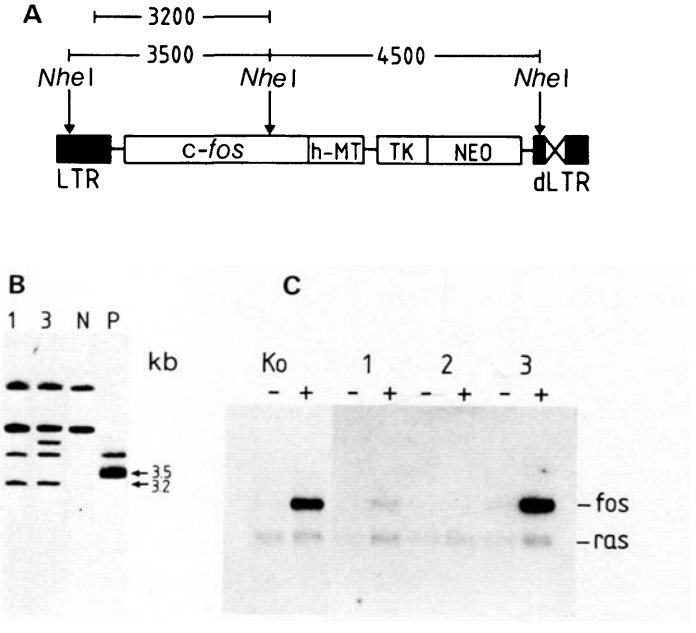

Fig. 5. Expression of the MT-c-*fos* gene in 3T3 fibroblasts introduced by infection with a SIN vector.

(A) The DNA construct consists of the human MT promoter linked to the promoterless mouse c-*fos* gene, having three introns and a polyadenylation signal. The construct also contains the neo^R gene expressed from the TK promoter. The MT-c-*fos* construct was inserted in the SIN vector in the antisense orientation (see also Fig. 1C).

(B) Analysis of proviral DNA in cells infected with virus derived from the SIN vector described in A. Virus derived from independently isolated $\psi 2$ cells was used to infect 3T3 fibroblasts. Cellular DNA from bulk infected 3T3 cells was digested with *Nhe*I which has a recognition site within the viral LTRs (see A) as well as within the c-*fos* gene generating a 3·5 kb and 4·5 kb fragment as shown in A. If the deletion in the 3′ LTR is transferred to the 5′ LTR of the provirus, a 300 bp shorter fragment of 3·2 kb in size will be present in the infected cell DNA. The filter was hybridized with a ^{32}P-labelled c-*fos* containing plasmid. Lanes 1, 3: Two virus infected and selected cell lines; N, uninfected cells; P, MT-c-*fos* SIN plasmid. The 4·5 and 3·5 kb DNA fragments as well as the 3·2 kb DNA fragment in the infected cells are indicated by the arrows. The third band in lane P migrating below the 3·5 kb band represents plasmid sequences. The two upper bands present in all lanes, including the uninfected 3T3 cells, are endogenous c-*fos* sequences. The origin of the additional band in lane 3 which is about 6–7 kb long is not known and can also be detected as faint band in uninfected 3T3 cells.

(C) RNA analysis in cells infected with the MT-c-*fos* SIN vector. Lanes 1, 2 and 3 are 20 μg total RNA from three cell lines derived by infection with viruses. Ko is RNA from a cell line derived by transfection with the MT-c-*fos* hybrid gene alone. Hybridization was performed with a ^{32}P-labelled c-*fos* specific probe and a ^{32}P-labelled *ras* specific probe which serves as an internal control for the amount of RNA loaded in each lane. + and − indicate cells that were or were not incubated for 6 h in the presence of 5×10^{-6} M-$CdCl_2$ before isolation of RNA.

V. CONCLUSIONS

The results reviewed in this article demonstrate the versatility of retroviral vectors in transferring and expressing genes efficiently in EC/ES cells and transgenic mice. These mice can be obtained by embryo infection or *via* chimaera formation from *in vitro* selected and characterized EC/ES cell clones. The vectors also provide us with the potential to introduce genes into a variety of different stem cells either to study the consequences of gene expression on these cells or to use the viral integration site as a molecular marker to analyse cell lineages. We have also outlined the different strategies for the derivation of efficient expression vectors. The genes of interest can be expressed from the viral LTR and from internal promoters or through the use of SIN vectors, that allow expression

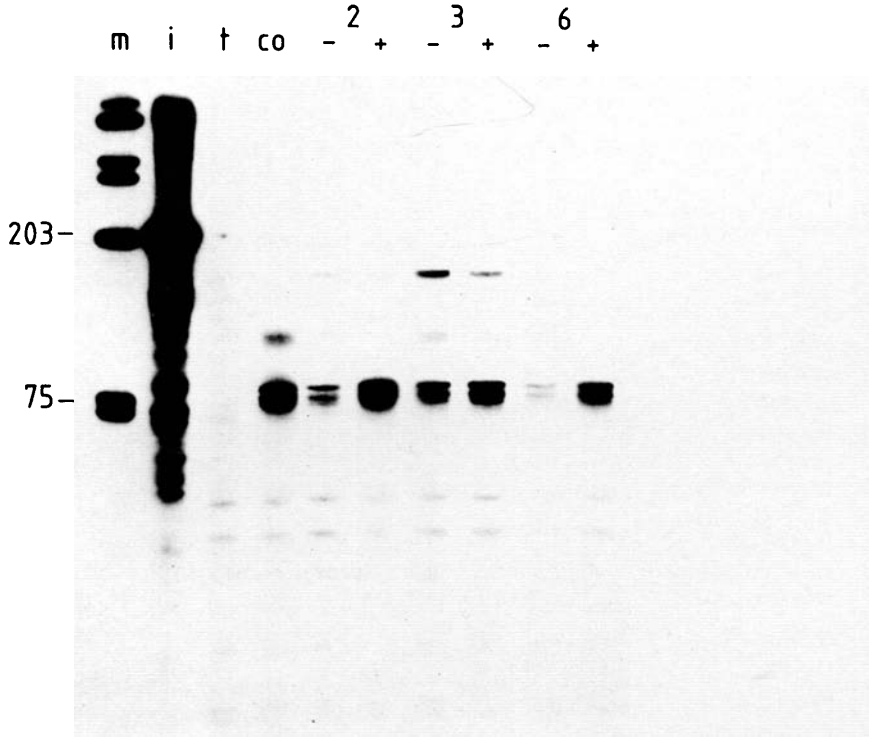

Fig. 6. Sp6-RNase mapping of RNA from three 3T3 cell lines derived from infections with three independently produced SIN vectors containing the MT-Ad12E1A gene. After infection and selection in G418, RNA from bulk colonies was isolated either 4 h after induction with 5×10^{-6} M-$CdCl_2$ or without induction. 20 μg of total RNA was hybridized with an Sp6 *in vitro* transcript of 201 bases length. This transcript hybridizes to the first 76 untranslated bases of the human metallothionein promoter that was fused to the 5′ end of the E1A gene. After overnight hybridization the RNA was digested with RNaseA and T1 under conditions where double-stranded RNA remains intact. The samples were electrophoresed and autoradiographed. Abbreviations: m, markers; i, Sp6 *in vitro* transcript of 201 bases length; t, tRNA negative control; co, positive control RNA from a cell line producing the human metallothionein transcripts. Lanes 2, 3, 6: three independent 3T3 RNAs; −, without induction; +, induction with $CdCl_2$.

without interference from the enhancer/promoter elements in the LTRs. In embryonic stem cells and in mice derived from infected embryos the LTR suppression can be circumvented by using an internal promoter. The molecular basis for this suppression in EC cells is still unknown and in our experiments we were unable to relate the inhibition of expression causally to the methylation status of the integrated proviruses.

We have described the derivation of transgenic mice by infection of preimplantation embryos that express the *neo*^{R} gene from the internal TK promoter in all tissues and which also stably transmit this phenotype over many generations. Such *neo*^{R} mice expressing a dominant selectable marker in any cell may be of benefit to a wide range of research fields, since they can provide the experimenter with a predetermined marked cell type. The demonstration that an internal promoter can function efficiently in mice has been a stimulus to investigate whether inducible or tissue-specific regulatory elements within a retroviral vector can be used. We hope that these elements, in conjunction with the efficiency and versatility offered by these vectors, will allow us to study the function that (proto-)oncogenes have in cell proliferation and differentiation both in an *in vivo* and *in vitro* environment.

The authors wish to thank their colleagues within the department for fruitful discussions. They also thank Ines Benner for help in the preparation of the manuscript.

REFERENCES

BRADLEY, A., EVANS, M., KAUFMAN, M. H. & ROBERTSON, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature, Lond.* **309**, 255–256.

DÖRFLER, W. (1983). DNA methylation and gene activity. *A. Rev. Biochem.* **52**, 93–124.

FRANZ, T., HILBERG, F., SELIGER, B., STOCKING, C. & OSTERTAG, W. (1986). Retroviral mutants efficiently expressed in embryonal carcinoma cells. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3292–3296.

GORMAN, C. M., RIGBY, P. W. J. & LANE, D. P. (1985). Negative regulation of viral enhancers in undifferentiated embryonic stem cells. *Cell* **42**, 519–526.

HUSZAR, D., BALLING, R., KOTHANY, R., MAGLI, M. C., HOZUMI, N., ROSANT, J. & BERNSTEIN, A. (1985). Insertion of a bacterial gene in the mouse germ line using an infectious retrovirus vector. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8587–8591.

JAENISCH, R., JÄHNER, D., NOBIS, P., SIMON, I., LÖHLER, J., HARBERS, K. & GROTKOPP, D. (1981). Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. *Cell* **24**, 519–529.

JAENISCH, E. & JÄHNER, D. (1984). Methylation, expression and chromosomal position of genes in mammals. *Biochim. biophys. Acta* **782**, 1–9.

JAENISCH, R., BREINDL, M., HARBERS, K., JÄHNER, D. & LÖHLER, J. (1985). Retroviruses and insertional mutagenesis. In *CSH Symp. Quant. Biol.*, vol. **50**, *Molecular Biology of Development*, Cold Spring Harbor Laboratory, pp. 439–445.

JÄHNER, D., STUHLMANN, H., STEWART, C. L., HARBERS, K., LÖHLER, J., SIMON, I. & JAENISCH, R. (1982). *De novo* methylation and expression of retroviral genomes during mouse development. *Nature, Lond.* **298**, 623–628.

JÄHNER, D., HAASE, K., MULLIGAN, R. C. & JAENISCH, R. (1985). Insertion of the bacterial *Gpt* gene into the germ line of mice by retroviral infection. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6927–6931.

JOYNER, A. L. & BERNSTEIN, A. (1983). Retrovirus transduction: segregation of the viral transforming function and the Herpes Simplex virus TK gene in infectious Friend spleen focus-forming virus thymidine kinase vectors. *Molec. Cell Biol.* **3**, 2191–2202.

KELLER, G., PAIGE, C., GILBOA, E. & WAGNER, E. F. (1985). Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. *Nature, Lond.* **318**, 149–154.

LINNEY, E., DAVID, B., OVERHAUSER, J., CHAO, E. & FAN, H. (1984). Non-function of a Moloney murine leukaemia virus regulatory sequence in F9 embryonal carcinoma cells. *Nature, Lond.* **308**, 470–472.

MANN, R., MULLIGAN, R. C. & BALTIMORE, D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**, 153–159.

PALMITER, R. D. & BRINSTER, R. L. (1985). Transgenic mice. *Cell* **34**, 343–345.

RUBENSTEIN, J. L. R., NICOLAS, J.-F. & JACOB, F. (1984). Construction of a retrovirus capable of transducing and expressing genes in multipotential embryonic cells. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7137–7140.

SORGE, J., CUTTING, A. E., ERDMAN, V. D. & GAUTSCH, J. W. (1984). Integration-specific retrovirus expression in embryonal carcinoma cells. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6627–6632.

STEWART, C. L., STUHLMANN, H., JÄHNER, D. & JAENISCH, R. (1982). *De novo* methylation, expression and infectivity of retroviral genomes introduced into embryonal carcinoma cells. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4098–4102.

STEWART, C. L., VANEK, M. & WAGNER, E. F. (1985). Expression of foreign genes from retroviral vectors in mouse teratocarcinoma chimaeras. *EMBO J.* **13B**, 3701–3709.

VAN DER PUTTEN, H., BOTTERI, F. M., MILLER, A. D., ROSENFELD, M. G., FAN, H., EVANS, R. M. & VERMA, I. M. (1985). Efficient insertion of genes into the mouse germ line via retroviral vectors. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6148–6152.

WAGNER, E. F. & STEWART, C. L. (1986). Integration and expression of genes introduced into mouse embryos. In *Experimental Approaches to Mammalian Embryonic Development.* (ed. J. Rossant & R. A. Pedersen). Cambridge: Cambridge University Press (in press).

WAGNER, E. F., VANEK, M. & VENNSTRÖM, B. (1985*a*). Transfer of genes into embryonal carcinoma cells by retrovirus infection: efficient expression from an internal promoter. *EMBO J.* **4**, 663–669.

WAGNER, E. F., KELLER, G., GILBOA, E., RÜTHER, U. & STEWART, C. L. (1985*b*). Gene transfer into murine stem cells and mice using retroviral vectors. In *CSH Symp. Quant. Biol.*, vol. **50**, *Molecular Biology of Development*, Cold Spring Harbor Laboratory, pp. 691–700.

YU, S.-F., VON RÜDEN, T., KANTOFF, P., GARBER, C., SIEBERG, M., RÜTHER, U., ANDERSON, W. F., WAGNER, E. F. & GILBOA, E. (1986). Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3194–3198.