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Technical Communication

Insertional mutagenesis of preneoplastic astrocytes by Moloney murine leukemia virus

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Retroviral infection can induce transcriptional activation of genes flanking the sites of proviral integration in target cells. Because integration is essentially random, this phenomenon can be exploited for random mutagenesis of the genome, and analysis of integration sites in tumors may identify potential oncogenes. Here we have investigated this strategy in the context of astrocytoma progression. Neuroectodermal explants from astrocytoma-prone GFAP-v-*src* transgenic mice were infected with the ecotropic Moloney murine leukemia virus (Mo-MuLV). *In situ* hybridization and FACS analysis indicated that astrocytes from E12.5–13.5 embryos were highly susceptible to retroviral infection and expressed viral RNA and proteins both *in vitro* and *in vivo.* In average 80% of neuroectodermal cells were infected *in vitro* with 9–14 proviral integrations per cell. Virus mobility assays confirmed that Mo-MuLV remained transcriptionally active and replicating in neuroectodermal primary cultures even after 45 days of cultivation. Proviral insertion sites were investigated by inverse long-range PCR. Analysis of a limited number of provirus flanking sequences in clones originated from *in vitro* infected GFAP-v-src neuroectodermal cells identified loci of possible relevance to tumorigenesis. Therefore, the approach described here might be suitable for acceleration of tumorigenesis in preneoplastic astrocytes. We expect this method to be useful for identifying genes involved in astrocytoma development/progression in animal models.
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Introduction

Astrocytomas are among the most common tumors of the central nervous system in humans. Several genetic abnormalities are frequently found in astrocytomas (Kleihues and Ohgaki, 1997; Nagane *et al*, 1997), but the molecular mechanisms underlying the malignant progression of these tumors are not completely understood. Some animal models for gliomas have been developed using chemical and viral carcinogens but yielded little information about underlying genetic lesions.

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At present, only few transgenic models for astrocytomas are available. Mice expressing the SV-40 large T antigen under the control of the glial fibrillary acidic protein (GFAP) promoter region develop extremely aggressive brain tumors of presumed astrocytic origin, which lead to death of transgenic animals within 1 month (Danks *et al*, 1995). A strategy to mimic the mechanisms leading to development of glioma-like lesions in mice transgenic for an avian retroviral receptor was recently published (Holland *et al*, 1998). This system is very promising, because it allows for combining different genetic lesions characteristic of human tumors, but it is complex and the events participating in tumor progression are difficult to analyze. A mouse model for astrocytoma was established in our laboratory by expressing the v-*src* oncogene within the context of a modified GFAP transgene (Mucke *et al*, 1991). Pathological astrogliosis is detected in nearly all transgenic animals

2–3 weeks postnatally, but only 14% of GFAP-v-*src* transgenic animals develop astrocytomas within 1 year (Weissenberger *et al*, 1997; Maddalena *et al*, 1999; Theurillat *et al*, 1999). This indicates that expression of v-*src* by itself is not sufficient for tumor induction and that additional genetic hits are required. Thus, this model may provide a tool to analyze the cooperation of genetic steps involved in malignant progression of astrocytoma.

The goal of this research is to identify cellular genes that participate in astrocytoma progression. In the present study, we chose to use retroviral insertional mutagenesis using the replication-competent ecotropic Moloney murine leukemia virus (Mo-MuLV). It is assumed retroviral infection will enhance tumorigenesis in GFAP-v-*src* transgenic mice by random activation of cellular protooncogenes and/or, perhaps, disruption of tumor suppressor genes. If tumorigenesis is accelerated, cellular genes can be identified using the integrated provirus as a sequence tag.

Retroviral insertional mutagenesis has been efficiently used to target different genes in a number of MuLV-induced hematopoietic tumors, MMTV-induced mammary tumors, and others (Jonkers and Berns, 1996). Genes playing an important role during tumor progression are often targeted at late stages in tumorigenesis and occur as a result of superinfection of cells, implying that retroviral mutagenesis and development of malignant disease are a function of continuous viral replication. However, primary retroviral infection usually results in a limited number of proviral integrations per genome in infected cells due to expression of envelope proteins that bind to the Mo-MuLV receptor and block entry of additional viral particles (Wang *et al*, 1992). Because the tumor cells that have been analyzed often contained 10 or more proviruses, virus interference in these cells must be suppressed. This may be attributed to provirus silencing or to high receptor levels on the target cells, as well as to the generation of "mink cell focus-forming virus" (MCFV). MCFV utilizes receptors different from those used by exogenous ecotropic Mo-MuLV, thus enabling superinfection of the target cells (Rein and Schultz, 1984).

For retroviral insertional mutagenesis to be successful for the study of astrocytomas, it must be determined whether superinfection of glial cells will take place, and—if not—which number of target cells are necessary to achieve saturation mutagenesis. In this study, we have addressed the following questions: (1) at what stage of development are primary mouse neuroectodermal cultures most susceptible to retroviral infection; (2) which is the most efficient method of infection of astrocytes derived from GFAP-v-*src* transgenic mice; and (3) whether Mo-MuLV will be able to replicate in these cells *in vitro* and *in vivo.* Because positional cloning of integration sites is very laborious, we have established a reliable PCR protocol for amplifying Mo-MuLV-flanking sequences in neuroectodermal cells. Analysis of a limited number of sequences identified two interesting integration sites, indicating that this system might be useful for discovery of cellular genes cooperating with v-*src* in astrocytoma tumorigenesis.

Results

Efficiency of infection of neuroectodermal cells is dependent on developmental age

Primary neuroectodermal cultures were prepared from GFAP-v-*src* transgenic embryos (embryonic age: E12.5, E13.5, or E17.5), and from postnatal P:1 transgenic mouse brains. Retroviral infection was performed using the standard polybrene-enhanced protocol (8 μg/ml). Efficiency of infection was monitored by FACS analysis for membrane expression of *env*, ensuring that only cells with transcriptionally active provirus are identified. As shown in Figure 1, neuroectodermal cells derived from GFAP-v-*src* transgenic embryos at early stages of development (E12.5–13.5) were more susceptible to MoMuLV infection than those from transgenic embryos at later stages of development (E17.5) or postnatal mice (P:1).

Primary neuroectodermal explants support Mo-MuLV replication

Transcriptional activity of the virus is an important parameter for retroviral insertional carcinogenesis. To study the functional activity of the virus, we performed a mobility assay using a NIH 3T3/*lac*Z cell line expressing a replication-defective retroviral vector carrying the functional *lac*Z gene. Then, 5 to 7 days after infection, supernatant from infected neuroectodermal cells was layered onto *lac*Z cells. Two to 3 days later, supernatant was collected, filtered, and placed onto indicator 3T3 and/or neuroectodermal cells with addition of 8 μg/ml polybrene. After 3 more days, cells were stained for β-galactosidase activity (Figure 2). In contrast to control supernatant from uninfected NEC, which did not produce β-galactosidase-positive cells, supernatant from infected neuroectodermal cultures was able to mobilize the *lac*Z retroviral vector. Therefore, primary mixed neuroectodermal cultures are able to support the replication of MoMuLV. Even after 45 days of culturing neuroectodermal cells *in vitro*, the virus was functionally active and capable of infecting NIH 3T3 and/or neuroectodermal cells. FACS analysis with 83A25 monoclonal antibody showed that approximately 50% of primary NEC could be infected with Mo-MuLV produced by NEC cultured for 45 days *in vitro.*

Parameters affecting the efficiency of retroviral infection of neuroectodermal cells

We investigated various methodologies for preparing viral stocks and for infecting neuroectodermal cells

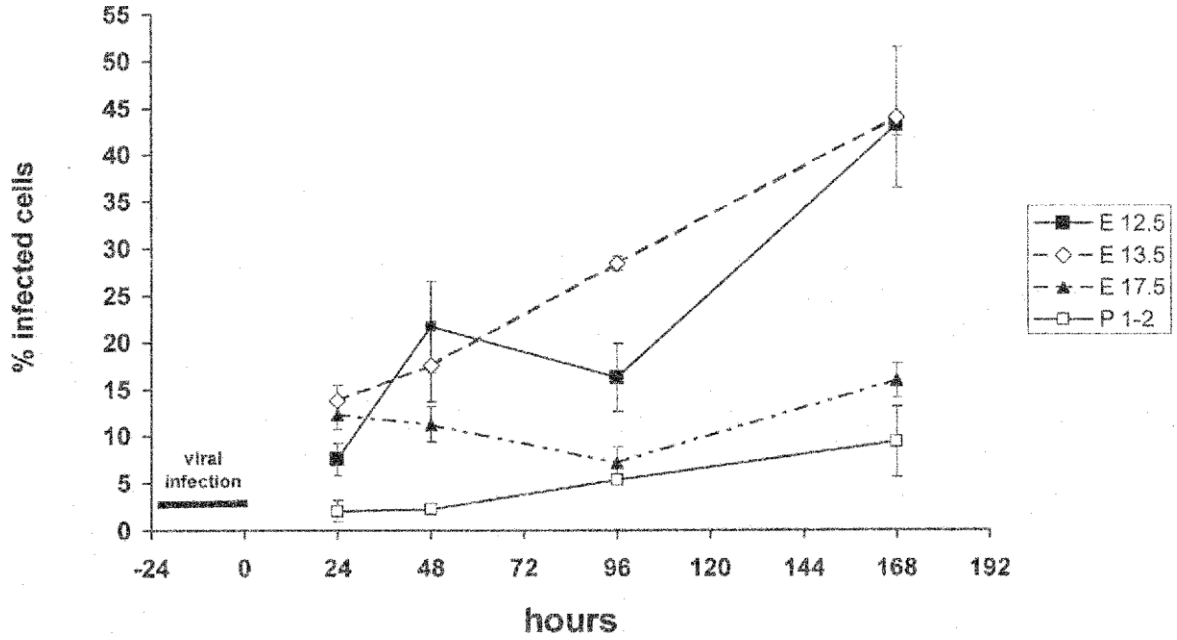

Figure 1 Susceptibility of primary mixed neuroectodermal cultures to Mo-MuLV infection at various stages of development. Cultures were prepared from E12.5; 13.5; 17.5 GFAP-v-*src* transgenic embryos, as well as from postnatal P1 transgenic mice. Efficiency of infection was measured by FACS analysis with monoclonal antibody 83A25 to Mo-MuLV *env*. The highest proportion of infection was evidently achieved with cells from early embryos.

from E12.5–E13.5 GFAP-v-*src* transgenic embryos as well as 3T3 cells as indicators. Reduced content of fetal calf serum can increase the stability of virions. Therefore, wild-type virus-producing cells were grown in medium with either 5% or 10% FCS. However, survival of neuroectodermal cells was found to be significantly reduced in medium with 5% FCS, thus negating any benefit of virus stability. Therefore, 10% FCS was used in further experiments.

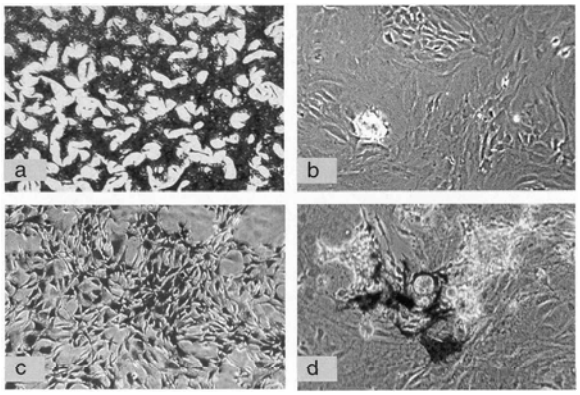

Figure 2 Virus mobility assay. Seven days after infection supernatant from infected or uninfected neuroectodermal cells was layered onto NIH3T3/LacZ cells (**a**, NIH3T3/LacZ cells stained for β-galactosidase, $\times$100). Three days later, supernatants were filtered and placed onto wild-type NIH 3T3 cells. Supernatant from uninfected neuroectodermal cells did not result in transfer of β-galactosidase (**b**, $\times$200), while supernatants from Mo-MuLV-infected neuroectodermal cells transferred the *LacZ* transgene to wild-type NIH 3T3 cells (**c**, $\times$100) and, somewhat less efficiently, also to further primary neuroectodermal cells (**d**, $\times$200).

The half-life of retroviruses is much shorter at 37°C than at 32°C. To prepare virus-containing supernatant, virus-producing cells were grown to approximately 80% confluence. Medium was then discarded and a reduced volume of fresh medium was added at 37°C. After 16 h, virus-containing medium was collected, filtered, and used immediately for cell infection. Alternatively, virus-producing cells were kept at 32°C for 72 h and fresh medium was added 16 h prior collecting viral supernatant. In our hands, incubating virus-producing cells at 32°C before the collecting virus-containing supernatant provided higher efficacy and much better reproducibility of infection (51.2 $\pm$ 22.6% at 37°C versus 73.7 $\pm$ 11.6% at 32°C in three independent experiments).

The effective "infective" volume around cells is small, and most retroviral particles never reach their target cells (Chuck *et al*, 1996). It was reported that calcium-mediated precipitation or "spin infection" could improve the efficacy of retroviral infection by changing local conditions affecting the kinetics of virus absorption to the membranes of target cells (Morling and Russell, 1995). We compared the standard polybrene-enhanced protocol of retroviral infection to calcium-mediated precipitation and centrifugation of cells with viral supernatant. In our system, optimal parameters balancing high efficiency of infection and good survival rates of cells were achieved by using a standard polybrene protocol. FACS analysis with 83A25 monoclonal antibody showed that on average 78 $\pm$ 7% (as determined in three replicas in two independent experiments) of neuroectodermal cells can be infected

in vitro, as compared to 45 ± 10% in spin infection and 62 ± 2% in calcium-mediated infection (data not shown). To confirm that Mo-MuLV-infected neuroectodermal cells can differentiate into astrocytes, we cultured Mo-MuLV-infected as well as uninfected primary neuroectodermal cells *in vitro* during 5–6 weeks under the conditions described. FACS analysis with antibodies to GFAP revealed that 70–90% of primary neuroectodermal cells were astrocytes. To confirm that astrocytes are indeed Mo-MuLV-infected, we performed two-color FACS analyses for Mo-MuLV *env* and GFAP. A significant population of astrocytes (85%) was efficiently infected with Mo-MuLV after 5–6 weeks of culture (Figure 3A–D). This result was confirmed by double immunocytochemical staining (Figure 3E). GFAP antibody gave strong cytoplasmic staining of cells which were also strongly positive for Mo-MuLV *env*. Unspecific binding of antibodies was estimated on uninfected cells and NIH 3T3 cells and was found to be marginal (data not shown). We did not see significant differences in astrocyte numbers judged by GFAP immunoreactivity between infected and uninfected neuroectodermal cultures *in vitro* (see Figure 3), nor in grafts originating from infected or uninfected cells when using an optimized conditions of infection. Therefore, we argue that Mo-MuLV infection of primary neuroectodermal cells did not alter significantly the proportion of cells differentiated into astrocytes.

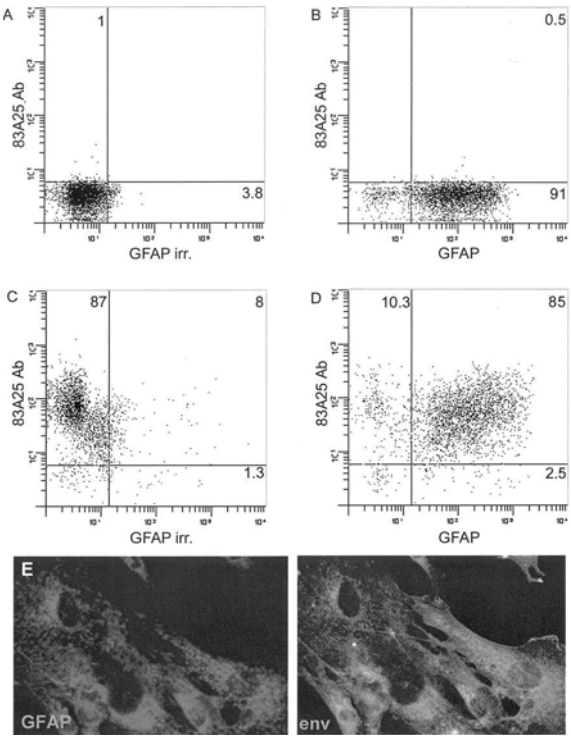

Figure 3 Astrocytes can be efficiently infected with Mo-MuLV. **A–D**: FACS analysis of uninfected (**A, B**) and infected (**C, D**) neuroectodermal cells with 83A25 antibody to Mo-MuLV *env* (vertical axis) and irrelevant pre-immune serum (**A, C**) or anti-GFAP antiserum (**B, D**) (horizontal axis). The vast majority of astrocytes (>80%) were infected with Mo-MuLV. **E**: Two-color immunofluorescent analysis indicating that neuroectodermal cells were positive for both GFAP and *env* markers. While GFAP stains the cytoskeleton of astrocytes, *env* is detectable in a larger fraction of the cytoplasm.

In summary, by optimizing the conditions for virus production and infection, we were able to achieve high efficacy of infection with good survival of neuroectodermal cells by using the standard polybrene-enhanced protocol of retroviral infection. On average, 80% of neuroectodermal cells were infected *in vitro* when using optimized condition of infection. After 5–6 weeks culturing in culture, a highly prevalent population of cells expressed both astrocytic (GFAP) and viral (*env*) markers, confirming that astrocytes can be efficiently and persistently infected with Mo-MuLV.

Ex vivo and in vivo infection of astrocytes by Mo-MuLV

The most common approach in insertional mutagenesis is to infect newborn mice predisposed to certain type of tumors with a replication-competent retrovirus. We have therefore infected newborn transgenic GFAP-v-*src* mice intraperitoneally or intracranially with either virus-containing supernatant or virus-producing NIH 3T3 cells. Three weeks after infection, animals were sacrificed and brains were analyzed by immunohistochemistry and *in situ* hybridization for the presence of the virus. In the case of intraperitoneal infection, only traces of virus could be detected in cells of the frontal telencephalon and, rarely, in the subependymal layer and in the olive. Intracranial infection was much more successful, especially when performed with virus-producing cells. Clusters of positive cells were detected in the frontal telencephalon and the ependyma of lateral ventricle. The whole hippocampal area was strongly positive, as well as some cells in the caudoputamen, the corpus callosum, the cerebellum (granular layer, flocculus, vermis), and the basal part of the pons (Figure 4a).

To determine whether astrocytes were infected, we performed double stains, consisting of *in situ* hybridization with a viral *env* probe for the presence of viral transcripts followed by immunohistochemistry for GFAP (Figure 4b). As negative control, *in situ* hybridization was performed with a sense probe (Figure 4c). A number of astrocytes were found to be double-labeled, especially in the hippocampal area. Therefore, neonatal astrocytes can be infected by Mo-MuLV *in vivo.*

In a further approach, embryonic brain cells derived from E12.5–13.5 GFAP-v-*src* transgenic embryos were infected with Mo-MuLV overnight *in vitro* and transplanted on the next day into the brain of recipient mice. In a first series of experiments we used syngeneic C57BL/6 mice as recipients. Analysis of the graft in 10 days after transplantation showed undifferentiated grafts (Figure 4d) with a prominent inflammatory host reaction that was confirmed

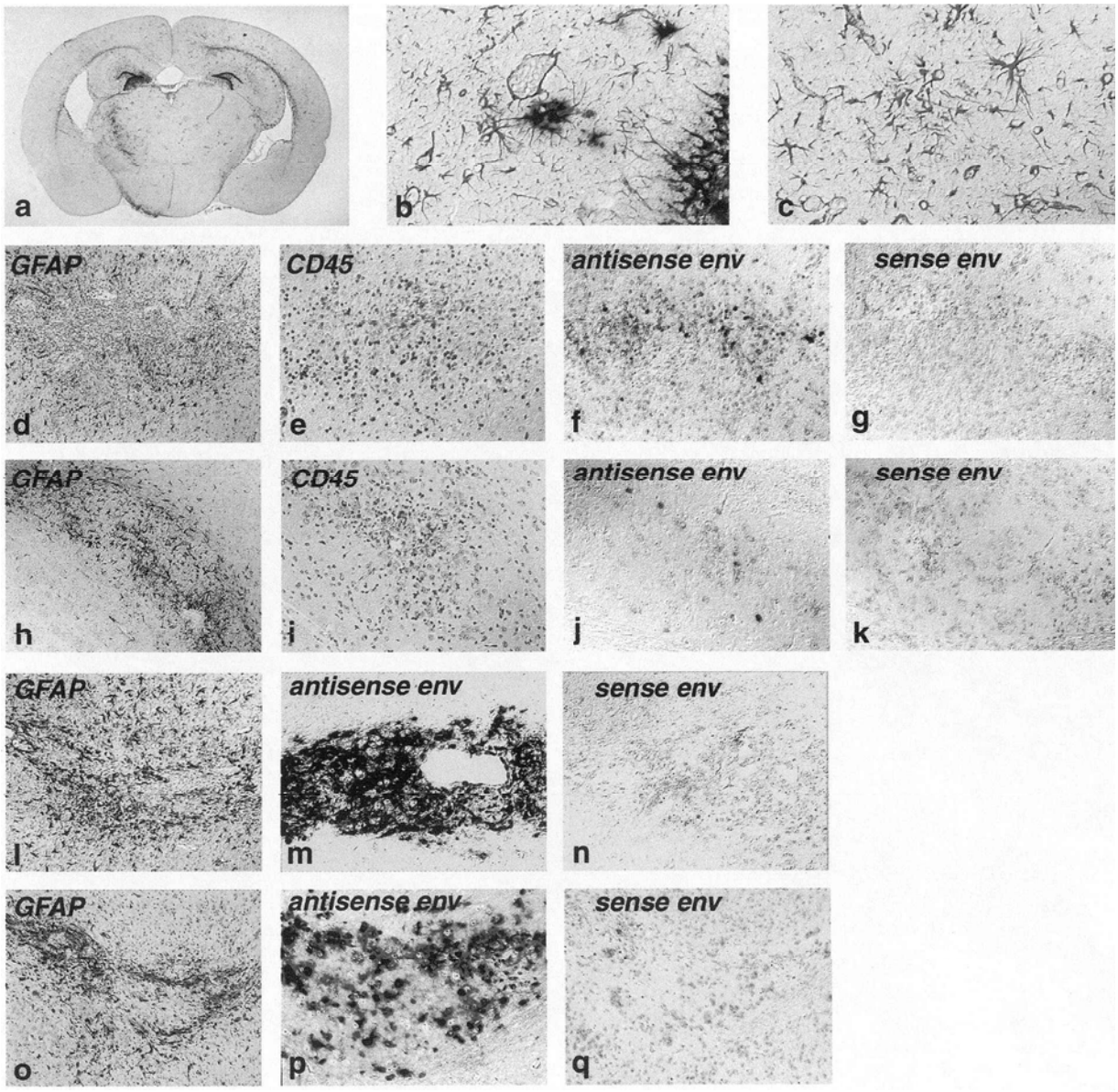

Figure 4 **a, b, c**: Intracranial infection of newborn GFAP-v-*src* mice with virus-producing NIH 3T3 cells. **a**: *In situ* hybridization with *env*-specific probe identifies virus-positive cells in the brain; **b**: hippocampal area, *in situ* hybridization followed by GFAP staining; **c**: negative control, *in situ* hybridization with sense probe and GFAP staining. **d–g**: 10 days after transplantation into the brain of C57BL/6 mice. Undifferentiated graft (**d**, GFAP) with prominent lymphocyte infiltration (**e**, CD45) contains virus-expressing cells (**f**, *in situ* hybridization with antisense probe; **g**: hybridization with sense probe). **h–k**: 30 days after transplantation into the brain of C57BL/6 mice. Mostly differentiated graft (**h**, GFAP) with reduced inflammatory reaction (**i**, CD45) and rare infected cells (**j**: *in situ* hybridzation with antisense probe, **k**: negative control). **l–n**: 10 days after transplantation into the brain of nu/nu mice. Undifferentiated graft (**1**, GFAP) strongly positive for viral transcripts (**m**: *in situ* hybridzation with antisense probe; **n**: negative control). **o–q**: 30 days after transplantation into the brain of nu/nu mice. GFAP-positive graft (**o**) shows many virus-positive cells (**p, q**: *in situ* hybridization with antisense and sense probe, respectively).

by CD45 immunostaining (Figure 4e). Immunohistochemistry with 83A25 monoclonal antibody and *in situ* hybridization for Mo-MuLV showed presence of the virus in infected cells but not in the surrounding brain parenchyma (Figure 4f,g). One month after transplantation, mostly differentiated grafts (Figure 4h) with significantly decreased lymphocyte infiltration were present (Figure 4i) but only very rarely infected cells were detectable (Figure 4j,k). Since the replication status of the virus can be an important issue for insertional mutagenesis, for further experiments we used *nu/nu*/C57B1b/6 mice as a host for transplantation experiments. Ten days after transplantation into nude mice, we could see undifferentiated grafts without any signs of inflammatory reaction (Figure 4l). *In situ* hybridization of the

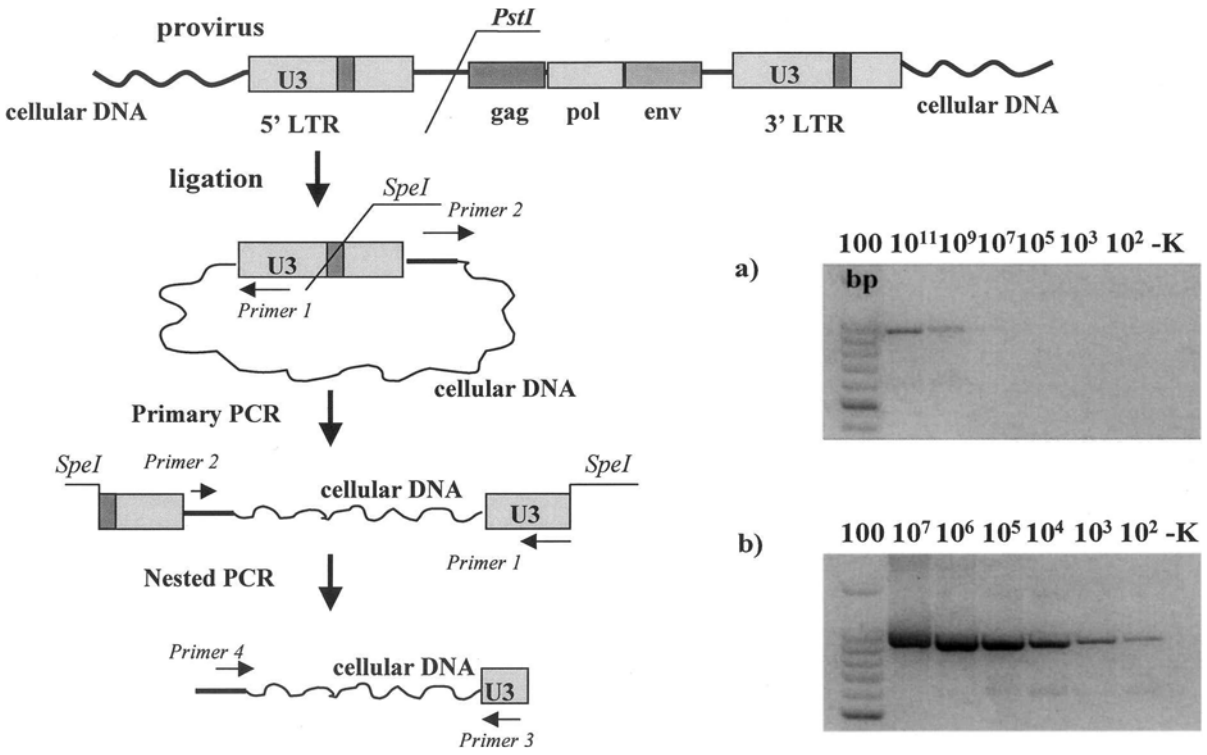

Figure 5 Principle and sensitivity of inverse PCR. To aviod amplification of internal part of provirus *PstI* was selected to cut the proviral sequence immediately downstream of the 5′ LTR. Digested DNA was self-ligated and subjected to primary PCR followed by amplification with nested primers. Sequences present in 100 copies per genome could be amplified from circularized DNA (**a**). Additional *SpeI* digest to relinearize circular DNA before amplification significantly increased the sensitivity of the method: 100 ng of DNA was sufficient to amplify the sequence present as a single copy (**b**). Negative control (−K)—genomic DNA only.

grafts with a Mo-MuLV probe showed numerous cells strongly positive for viral RNA (Figure 4m,n). One month after transplantation (Figure 4o) levels of virus expression tended to be lower than at 10 days, but we could still detect many virus-infected cells in grafts (Figure 4p,q). Thus, using both *in vivo* and *ex vivo* approaches we were able to show that Mo-MuLV can be efficiently delivered to CNS.

Characterization of genomic sites of proviral integration

To identify sequences flanking the provirus, we used the inverse PCR (IPCR) protocol (Silver and Keerikatte, 1989), which enables cloning and amplification of flanking sequences through a ligation step. IPCR conditions were optimized in preliminary experiments using a plasmid containing a Mo-MuLV provirus and flanking sequences (Reik *et al*, 1985). Fragments containing the 5′-LTR and flanking sequences were generated by cutting with *Pst*I, which cuts MoMuLV immediately downstream of the 5′-LTR (Figure 5). After self-ligation, the DNA was subjected to nested PCR as described before. This IPCR protocol resulted in the amplification of a fragment of the predicted molecular weight of 930 bp (Figure 5).

To estimate the sensitivity of the method, logarithmic dilutions of plasmid DNA were prepared and mixed with a constant amount of genomic DNA (1.5 μg). Under these conditions, the sensitivity of IPCR allowed amplification of sequences presents in $\geq$100 copies per genome (Figure 5a), which is not sufficient for reliable detection of single proviral integration sites. To increase the sensitivity, the restriction enzyme *Spe*I was used to cut the ligated DNA between the two primers before amplification. This manipulation relinearized circular self-ligated DNA before PCR amplification, such that the unknown genomic DNA stretches adjacent to the sites of integration become flanked by known retroviral sequences both at the 5′ and at the 3′ ends. This procedure significantly increased the sensitivity of the method: plasmid sequences present as single copies were successfully amplified upon dilution in 100 ng of genomic DNA (Figure 5b).

To simulate virus-induced tumors, MoMuLV-infected NIH3T3 cells were subjected to end-point dilution. Clones originating from single cells were expanded and used as a model for the optimization of IPCR. Southern blot analysis of genomic DNA with Mo-MuLV-specific U3 probe showed that NIH3T3 clones contained 5–7 integration sites of MoMuLV provirus (data not shown). To amplify the proviral flanking sequences, we used the IPCR protocol established for amplification of the Mo-MuLV plasmid, but used the Expand^{TM} Long

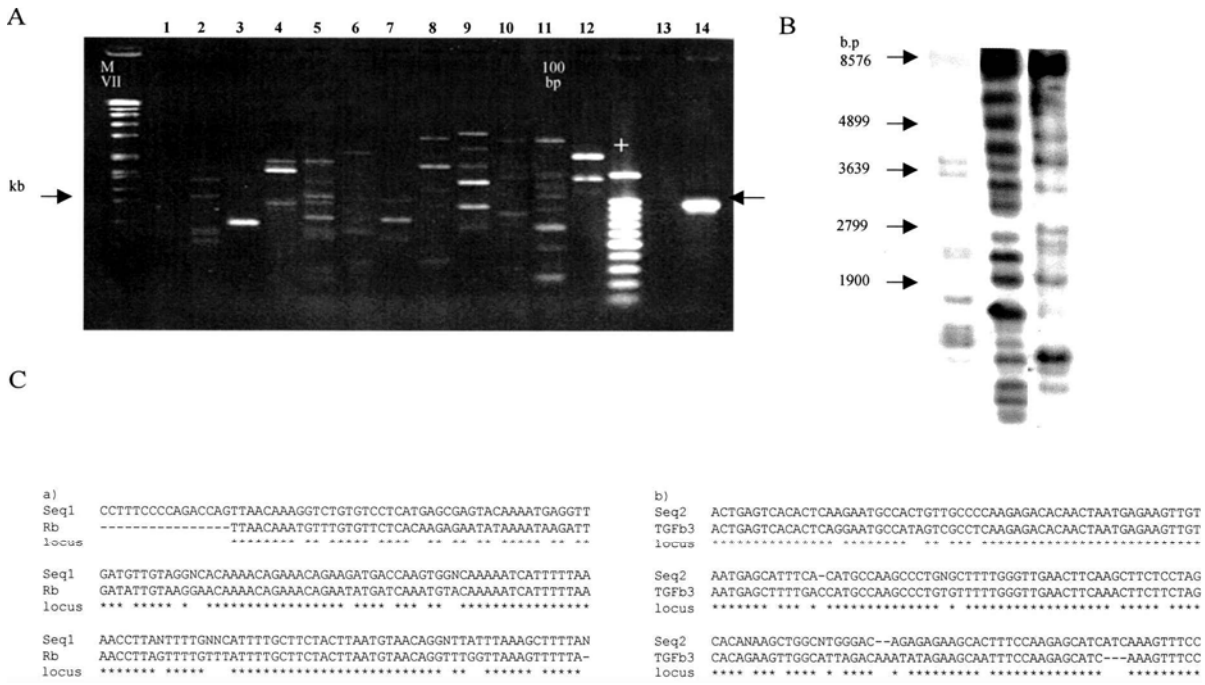

Figure 6 Integration sites of Mo-MuLV in virus-infected cell clones. (**A**) Long-range–inverse PCR for amplification of flanking provirus sequences in Mo-MuLV-infected NIH3T3 clones. Lanes 1–12: DNA from virus-infected clones originated from single cells. Lane 13: negative control (DNA from uninfected cells). Lane 14: positive control (plasmid DNA). (**B**) Southern analysis of virus integration sites in infected neuroectodermal clones with Mo-MuLV-specific U3 probe. (**C**) provirus-flanking sequences amplified by IPCR in Mo-MuLV-infected neuroectodermal clones. (**a**) sequence producing a significant alignment with human retinoblastoma susceptibility gene (Rb); (**b**) with human chromosome 14q24.3 clone Bac2, containing unknown gene and TGFβ (TGFβ3). Asterisk (*): identity of sequences. Dash (-): gap in alignment.

Template PCR System for the amplification step. This system provided reproducible results and we were able to amplify longer PCR products of up to 4 kb (Figure 6a).

We isolated a number of proviral integration sites in Mo-MuLV-infected neuroectodermal clones. Neuroectodermal cells isolated from E:12.5–13.5 GFAP-v-*src* transgenic embryos were infected with Mo-MuLV and propagated *in vitro.* After approximately 3 weeks in culture, cells formed transformed foci. Several of the fastest-growing clones were isolated, expanded, confirmed by immunohistochemistry to be GFAP-positive and used for analysis. Proviral copy numbers were estimated by Southern analysis with a Mo-MuLV-specific U3 probe and determined to range between 9 and 14 (Figure 6b). *Pst*I cuts twice downstream of the 5′LTR at positions 568 and 744; therefore, all fragments smaller than 7.6 kb represent unique cellular flanking segments of the 5'LTR. IPCR was carried out according to the protocol described above. PCR bands were cut from gel, DNA was extracted, directly sequenced and submitted to the GenBank/EMBL/Blast databases. Of 10 amplified PCR fragments subjected to analysis, 2 produced significant matches (Figure 6c). In one case, the provirus was integrated into the 17th intron of the retinoblastoma (Rb) gene. It is interesting that 17th intron of the Rb gene contains an ORF for the purinergic receptor P2Y5 in antisense direction that was disrupted by retroviral insertion as well. In another case, the sequence showed a high similarity with the sequence of human chromosome 14q24.3 clone BAC270M14, which contains TGF-β 3 and other unknown genes.

Discussion

Although several genetic abnormalities have been described in human gliomas (Kleihues and Ohgaki, 1997; Nagane *et al*, 1997), many of the genetic interactions responsible for development and progression of the disease are still uncharacterized. GFAP-v-*src* transgenic mice (Theurillat *et al*, 1999; Weissenberger *et al*, 1997) are predisposed to astrocytomas, and may provide a powerful model for the identification of cellular genes collaborating with the transgene during tumor development and/or progression. In a first attempt to identify such genes, we performed Mendelian crosses of GFAP-v-*src* mice with mice deficient in *p53* or *Rb* and studied the incidence and morphology of astrocytoma in the progeny (Maddalena *et al*, 1999).

The prior studies were designed to test known candidate genes for enhancement of tumorigenesis. To identify completely novel, hitherto unsuspected

genes, we established a random mutagenesis approach. Infection of GFAP-v-*src*-derived neuroectodermal cells with Moloney murine leukemia virus (MoMuLV) may introduce activating mutations, which could potentially collaborate with the transgene in astrocytoma development and progression. In this case, enhanced frequency of tumor formation and shortened latency period would be detected.

Retroviral insertional mutagenesis is a statistical, largely random process: an increase in the number of integration sites results in a higher probability of mutation of a relevant gene. To obtain saturation mutagenesis of the mammalian haploid genome, at least 10^7 integrations are theoretically needed. This number agrees well with *in vitro* insertional mutagenesis studies of hematopoietic progenitor cells, where it has been estimated that 1 in 5×10^6 retroviral integrations lead to altered phenotype (factor-independent growth) (Stocking *et al*, 1993). To obtain large number of integrations, one needs to infect a large number of cells or to obtain multiple integrations per cell.

Many factors can influence the efficiency of retroviral infection. Susceptibility of the developing brain to retroviral infection is dependent on the stage of CNS development (Löhler, 1988). Infection of primary neuroectodermal cultures from E12.5, 13.5, and 17.5 GFAP-v-*src* transgenic embryos, as well as from P1 postnatal transgenic mice, confirmed that susceptibility of primary astrocytes to MoMuLV infection depends on their developmental stage: primary glial cultures derived from E12.5–13.5 were highly susceptible to retroviral infection compared to cells derived from later stages of development. Susceptibility to retroviral infection correlated best with the proliferative potential of the cells since passage through mitosis is required for successful virus integration and establishment of retroviral infection in neuroectodermal cells. Notably, Mo-MuLV-infected neuroectodermal cells appeared morphologically normal. This was especially important, as infection of neuronal cells with neurotropic Mo-MuLV variants was shown to be cytotoxic (Wong *et al*, 1992; Shikova *et al*, 1993).

Viral replication in neuroectodermal cells will not only lead to an increase in the number of infected cells and integration sites but also proves that the integrated provirus is transcriptionally active, an important parameter for retroviral insertional mutagenesis. To test whether neuroectodermal cells undergo productive infection, a virus mobility assay was performed using NIH 3T3/*LacZ* cells, which produce replication-deficient virus harboring an active *Lac*Z gene. This experiment indicated that ecotropic Mo-MuLV is able to productively replicate in neuroectodermal cells derived from E12.5–13.5 GFAP-v-*src* embryos even after 45 days of culture, thus providing a potential possibility for superinfection of the cells. In addition to the presence of appropriate receptors and to the cycling status of the cells of interest, the efficiency of infection is also dependent on virus titer, virion stability, and conditions of retroviral infection. Because Mo-MuLV replication can be cytopathic, a balance between high efficiency of infection and good survival of infected neuroectodermal had to be found. Concentration of polybrene and fetal calf serum in retroviral supernatant has a profound effect on efficiency of infection and on stability of retroviral particles (Andreadis and Palsson, 1997). However, in this system reduced concentration of FCS in viral stocks significantly decreased survival of neuroectodermal cells. High efficiency of infection was achieved by culturing virus-producing cells at 32°C before collecting viral supernatant (Kotani *et al*, 1994), probably because the half-life of retrovirus is significantly shorter at 37°C than at 32°C.

Several methods to improve the efficiency of infection are based on the principle of concentrating viral particles to increase the effective virus concentration around the cells to be infected. Precipitation of retroviral vectors by addition of calcium chloride to phosphate-containing medium (Morling and Russell, 1995) can increase the apparent titer 5–50-fold. The mechanism leading to increased efficiency of spin-infection (Takiyama *et al*, 1998) is unclear: perhaps secondary fluid movements are created during centrifugation and more retroviral particles can reach their cell target during their short life span. However, the standard polybrene-enhanced protocol provided better results than the calcium-precipitation and the spin protocol: approximately 80% of the neuroectodermal cells could be infected *in vitro* with good survival of the cells. FACS and immunocytochemical analysis showed that a high proportion of cells was positive both for astrocytic (GFAP) and viral (*env*) markers, confirming that astrocytes can be efficiently infected with Mo-MuLV.

Interestingly, we could detect 9–14 virus integration sites in individual Mo-MuLV infected neuroectodermal clones. This finding may argue in favor of an oligoclonal nature of the clones analyzed, since it is extremely difficult to grow virus-infected clones from single primary neuroectodermal cells. However, when we subcloned our clones, the same number and pattern of integration sites could be observed (data not shown).

We then compared various methods for delivery of infectivity to the brain. The most common approach in insertional mutagenesis is to infect newborn mice with replication-competent virus. While the majority of animals inoculated as adults are resistant to persistent MuLV infection, neonates inoculated with MuLV show little anti-viral response and become life-long virus carriers, replicating the virus in target cells. We assessed the efficiency of infection of astrocytes of newborn transgenic GFAP-v-*src* mice by the intraperitoneal and the intracranial route. In the case of intraperitoneal inoculation, one might

expect that virus, after reaching the spleen (one of the primary sites for replication), would replicate more efficiently and would eventually achieve higher titers in the brain as well. For intracranial infection, we compared injection of virus-containing supernatant to injection of virus-producing NIH3T3 cells. Intracranial infection with virus-producing NIH 3T3 fibroblasts was clearly superior to virus-containing supernatant and was much more efficient than intraperitoneal infection. Immunohistochemistry and *in situ* hybridization revealed that many astrocytes had been infected with Mo-MuLV.

Transplantation of neuroectodermal cells into the brain of syngeneic mice is a very well-established technique in our laboratory and was successfully used for the study of neurodegenerative diseases and for delivery of gene of interest to CNS (Aguzzi, 1998). The specific advantage of this method is that it allows manipulation of primary neuroectodermal cells at early stages of development (E12.5–13.5), which were most susceptible to infection. Besides, transplantation into the brain of recipient mice results in normal differentiation of the graft, thus imitating the *in vivo* situation. The disadvantage of the model is that a limited number of the cells (approx. 10^6) can be transplanted. Moreover, transplantation into the brain of syngeneic immunocompetent C57BL/6 mice led to strong antiviral immune response and, as a consequence, to down-regulation of retrovirus expression and replication. Although according to some data, Mo-MuLV cannot be eliminated from CNS cells by the immune surveillance and infected cells can serve as a reservoir in CNS (Hein, 1995), it is unlikely that sufficient levels of replicating virus can be maintained under such conditions. Instead, after transplantation of infected cells into nu/nu mice Mo-MuLV could replicate in infected grafts for at least one month after transplantation.

Another possible *in vivo* approach would be to cross-breed of GFAP-v-*src* and Mov-13 (Schnieke *et al*, 1983) transgenic mice, which contain ecotropic Mo-MuLV as a transgene that is activated on day 16 of embryogenesis. Mo-MuLV becomes widely expressed in the CNS of these mice including glial cells (Löhler, 1988). Thus, these mice could provide a useful tool to accelerate tumorigenesis in GFAP-v-*src* transgenic mice.

The ultimate success of this approach depends also on host factors. While slow-transforming retroviruses can infect a wide range of different cell types, only cells of particular types ever develop into tumors, and no slow-transforming viruses until now have been linked to CNS tumors in mice. Although this specificity is determined in part by the proliferation capacity of target cells, viral control sequences linked mostly to U3 region of the long terminal repeats (LTR) also influence the pathogenicity (Lenz *et al*, 1984; DesGroseillers *et al*, 1985; Stocking *et al*, 1985). For a proviral LTR to activate a proto-oncogene, its enhancers must function efficiently in the target cell: this could bias the expression of genes targeted by retroviral insertional mutagenesis. The expression studies reported here suggest that this is the case in the Mo-MuLV-astrocytoma paradigm.

The ultimate goal of this study is to identify mutations introduced by retroviral infection using the integrated provirus as a molecular tag. Our initial choice was to amplify integration sites by PCR with virus-specific primers along with arbitrary primers that will hybridize within a statistically defined range of the provirus-flanking cellular DNA (Sorensen *et al*, 1993). However, this protocol produced a number of unspecific bands that hindered the interpretation of the results. We therefore optimized an inverse PCR (IPCR) protocol (Silver and Keerikatte, 1989), which allows the cloning and amplification of provirus-flanking sequences through a ligation step.

Preliminary experiments with a plasmid containing a Mo-MuLV provirus demonstrated the feasibility of this system. However, the sensitivity of IPCR was not sufficient to allow the amplification of sequences present at <100 copies per genome. Sensitivity was dramatically increased by cutting ligated DNA between the 5′ ends of the primers: single copy of plasmid DNA could be detected in 100 ng of genomic DNA. This improvement in sensitivity is likely to come about because linearization of the circular template releases DNA supercoils and facilitates amplification. The best results were achieved by combining this protocol with long-range PCR, which allowed for amplification of up to 4 kb of flanking sequences.

Using the improved IPCR protocol, we were able to amplify MoMuLV flanking sequences in infected neuroectodermal clones, infected and propagated *in vitro*. Of 10 sequences subjected to a database search, 2 produced significant matches. In one clone, the virus was integrated into the 17th intron of the retinoblastoma gene, which encodes the purinergic receptor P2Y5 as well. Thus, this integration could lead to disruption of both genes. In another clone, integration had occured in a locus containing unknown genes and TGFβ-3. The significance of these findings is not clear at the moment and further analysis is required. It is remarkable, however, that analysis of limited number of sequences showed retroviral integration into loci which could be relevant for tumor development or progression. As a next step, we intend to study whether these clones are able to form colonies in soft agar and give rise to tumors in nude mice, and finally to search for common integration sites in a number of tumors, which may be indicative of major pathways resulting in malignant transformation.

In conclusion, we have established the technical prerequisites needed to accelerate tumorigenesis in astrocytes derived from GFAP-v-*src* transgenic mice. Using an optimized infection protocol, $1–1.5 \times 10^6$ neuroectodermal cells derived from one transgenic

embryo can be infected at 80% efficiency with an average 10 provirus integration sites, giving a total of $8–12 \times 10^6$ independent integration events. According to the theoretical and experimental estimates of saturation mutagenesis (Stocking *et al*, 1993, Jonkers and Berns, 1996), we would expect approximately one malignant clone per one transgenic animal. As the incidence of spontaneous tumors in GFAP-v-src mice is approximately 14% per year, the method described here may accelerate tumorigenesis in this animal model. We anticipate that the usefulness of the approach delineated in this study will increase proportionally to the rapid pace of progress in genomic sequencing of model organisms (Lin *et al*, 1999). Our future experiments will clarify the feasibility of using this approach to accelerate tumorigenesis *in vitro* and *in vivo* in GFAP-v-*src* transgenic mice.

Material and methods

Moloney murine leukemia virus stocks
NIH 3T3 cells (clone 1A, Fan and Paskind, 1974) producing replication-competent ecotropic Moloney murine leukemia virus (Mo-MuLV) were kindly provided by J Allen, The Netherlands Cancer Institute, Amsterdam. Cells were grown in DMEM with 10% FCS (Sigma, USA), 1% pyruvate (Gibco-BRL, Scotland), and 2% glutamine (Gibco). The titer of the viral stock was estimated by mobilization of a defective virus carrying a puromycin resistance marker and was $>10^7$ plaque forming units (pfu)/ml. Virus-containing supernatant was collected, filtered through a 0.45-μm filter (Schleicher and Schuell), and quickly frozen in liquid nitrogen, or used directly to infect target cells.

Primary neuroectodermal cultures
Primary neuroectodermal cultures were prepared from E12.5, E13.5, or E17.5 GFAP-v-*src* transgenic embryos and from P1 GFAP-v-*src* postnatal mice. Telencephalic brain tissue was harvested and transferred to Dulbecco modified Eagle's medium (DMEM) containing 4.5 mg/ml glucose, 10% fetal calf serum (FCS) (Sigma, USA), and 1% penicillin/streptomycin (Gibco-BRL, Scotland) after dissecting the brain from surrounding tissues under a stereomicroscope as previously described (Isenmann *et al*, 1996). Prior to infection, neuroectodermal cells derived from one embryo ($1–1.5 \times 10^6$ cells) were treated with trypsin-DNase I (0.25% trypsin, 0.1 mg/ml DNase I in phosphate-buffered saline (PBS), 10 min at 37°C) until they formed small clusters, and plated into one well of a six-well dish. We found that the conditions of neuroectodermal cell preparation are critical to the efficiency of retroviral infection: large clusters of cells reduced the efficiency of infection, while dissociation into single cells increased infectibility but significantly decreased viability.

Retroviral infection
Several protocols were compared to optimize retroviral infection of neuroectodermal cultures. In a standard protocol, dissociated cells were incubated overnight with 2 ml of virus-containing supernatant with 10% FCS and 8 μg/ml polybrene. Medium was exchanged the next morning. Calcium phosphate precipitation of virus particles (Morling and Russell, 1995) was performed by adding 5 mM $CaCl_2$ to FCS-free virus-containing supernatant and subsequent incubation for 30 min at RT. After collecting precipitated virus by centrifugation (11 600 g, 1 min), precipitate was diluted in 1 ml of FCS-free medium and used for infection. Spin-infection (Takiyama *et al*, 1998) was carried out by overlaying viral supernatant with polybrene (8 μg/ml) and centrifuging the cells in a microtiter rotor at 1800 rpm twice for 45 min at room temperature. Fresh virus was applied before the second round of centrifugation.

Virus mobility assay
NIH 3T3/*lac*Z cells (constructed in the lab of A Berns, The Netherlands Cancer Institute, Amsterdam) produce a replication-deficient ecotropic Mo-MuLV that harbors a functional *lac*Z gene and can be used to detect the presence of wild-type retrovirus in a virus mobility assay. Test solutions containing presumptive replication-competent Mo-MuLV were layered on top of NIH 3T3/*lacZ* cells. After 3 days, the supernatant was collected, filtered through a 0.22-μm filter, and used to infect indicator NIH 3T3 cells. After a few days, NIH 3T3 cells were fixed with 0.5% glutaraldehyde/PBS for 5 min at room temperature, washed 3 times with PBS, and incubated with staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) in ferri-ferrocyanide buffer at 37°C. After 24 h, cells were monitored for acquired β-galactosidase activity.

Antibodies, immunohistochemistry and FACS analysis
Rat hybridoma cells producing monoclonal antibody 83A25 against Mo-MuLV envelope protein (*env*) were kindly provided by Dr F Malik (Rocky Mountain Laboratory, Hamilton, Montana, USA) (Evans *et al*, 1990). To perform FACS analysis, infected cells were detached from the plate using 1–5 mM of EDTA in PBS, incubated with 83A25 antibody-containing supernatant for 1 h on ice, and with secondary anti-rat antibodies conjugated with phycoerytrin (PE) (Caltag, USA) for 45 min on ice. As negative controls, we used uninfected neuroectodermal cells, as well as infected neuroectodermal cells incubated only with secondary antibody. For GFAP FACS analysis, cells were fixed with 4% formaldehyde for 10 min and permeabilized with 0.1% Triton X100 for 10 min at 4°C. Incubation with polyclonal rabbit antibody to GFAP (Dako, Denmark, 1:100) and secondary goat anti-rabbit antibody labelled with FITC (Pharmingen, USA) as well as all washing steps were carried out in

permeabilization buffer (0.1% Triton-X100, 4% FCS, 0.1% NaN_3) at 4°C. Nonspecific binding of GFAP antibody was assessed using irrelevant polyclonal normal rabbit antiserum. For two-color FACS analysis, the cells were incubated first with 83A25 antibody, fixed, permeabilised, and incubated with antibody to GFAP followed by incubation with anti-rabbit and anti-rat secondary antibodies.

For immunocytochemical studies, cultured cells growing on coverslips were fixed with ethanol–acetic acid or 4% paraformaldehyde followed by permeabilization with 0.1% Triton X100 for 10 min at RT. Cells were incubated with monoclonal antibody to GFAP directly labelled with Cy3 (1:400, Sigma, USA) and/or 83A25 monoclonal antibody followed by incubation with secondary rabbit anti-rat antibody labeled with FITC (Dako, Denmark). Immunohistochemical GFAP staining on brain tissue fixed during 4 h with 4% formalin and 4% acetic acid was performed according to standard procedures with polyclonal rabbit anti-GFAP antibody (1:300, Dako, Denmark).

Genomic DNA isolation, Southern blot analysis, and inverse PCR (IPCR)

Genomic DNA was isolated by incubation of cells overnight at 55°C in 50 mM Tris-HCl buffer (pH 8.0) with 100 mM EDTA, 100 mM NaCl, 1% SDS, and 100 μg/ml Proteinase K. DNA was purified by phenol–chloroform extraction, precipitated with isopropanol, and resuspended in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA). For Southern blot analysis, 12 μg of genomic DNA were digested with *Pst*I and hybridized with a P^{32}-labeled U3-specific Mo-MuLV probe (Cuypers *et al*, 1984).

The IPCR protocol was optimized using a plasmid containing the Mo-MuLV provirus clone Mov-3, extending from the 5'-LTR to the *Cla*I site in the *env* gene (Harbers *et al*, 1981; Reik *et al*, 1985). One μg of DNA was digested with *Pst*I followed by heat inactivation (65°C, 15 min). Self-ligation of digested DNA was carried out in 500 μl with 1 unit of T4 DNA ligase overnight at 12°C. After enzyme inactivation (94°C, 30 min), 250 μl of ligation mixture were precipitated with 100 μl 5 M ammonium acetate and 900 μl of ethanol. The pellet was washed twice with 70% ethanol and dissolved in 10 μl of TE buffer, pH 8.0. Ligated DNA was precipitated and dissolved in 10 μl of TE, pH 8.0. DNA (3 μl) was then relinearized with 2.5 units of *Spe*I (which cuts Mo-MuLV at position 283) in a final volume of 5 μl before amplification: we found that this step increased vastly the yield of the reaction. Primary PCR was carried out in the same tube by adding all necessary reagents. Most reproducible results were obtained with the Expand™ Long Template PCR System (Boehringer Mannheim, Germany) (Benkel and Fong, 1996).

The nucleotide sequences of the primers used in IPCR to amplify genomic sequences flanking the 5′ Mo-MuLV LTR were as follows: for primary PCR, 5′-TGG CGT TAC TTA AGC TAG CTT-3′ (nt 7835–7816, numbering as in Shinnick *et al*, 1981) located in the 5′ LTR and 5′-TTA GAG GAG GGA TAT GTG GTT-3′ (nt 549–568) in the region preceding *gag*. For nested PCR, we used 5′-TAC AGG TGG GGT CTT TCA TT-3′ (nt 7864–7844) in the 5′ LTR, and 5′-GCG CGT CTT GTC TGC TGC AG-3′ (nt 450–470) in the region preceding *gag*.

The PCR profile for the primary PCR was as follows: 1 cycle 94°C for 5 min; 40 cycles 94°C, 1 min, 55°C, 1 min and 72°C, 3 min; 1 cycle 72°C, 7 min. Nested PCR was run under the same conditions except that the annealing temperature was increased to 65°. The selected conditions of amplification using Expand™; were the following: 1 cycle, 94°C for 5 min; 12 cycles, 94°C, 30 s, 55°C, 10 s, 68°C, 3 min; 20 cycles, 94°C, 30 s, 55°C, 10 s, 68°C, 3 min + 20s; 1 cycle, 68°C, 7 min. Nested PCR: 94°C, 5 min; 10 cycles, 94°C, 30s, 65°C, 10s, 68°C, 3 min; 20 cycles, 94°C, 30s, 65°C, 10s, 68°C, 3 min + 20s; 1 cycle, 68°C, 7 min.

In situ hybridization

Fifteen μg of plasmid containing Mo-MuLV provirus extending from 5'LTR to the *Cla*I site in the *env* gene from the infectious pMov-3 (Harbers *et al*, 1981; Reik *et al*, 1985) was digested with *SmaI* and *SpeI.* Fragments of 160 bp and 1233 bp corresponding to positions 6075 to 6235 and 6235 to 7468 of the *env* gene were excised from agarose gels, and DNA was extracted with the QIAquick Gel Extraction Kit (Qiagen). Purified DNA was cloned into pBluescript KS+ (Stratagene) and digested with *SmaI* alone for 160 bp fragment or *SmaI* and *SpeI* for longer fragment. Fidelity of constructs was verified by sequencing. The *in vitro* transcriptions using DIG RNA Labelling Kit (Boehringer Mannheim Biochemica, Germany) from T7 or T3 promoters was performed according to the manufacturer to generate sense or antisense riboprobes. Hybridization was carried out at 62°C with digoxigenin-labelled probes at 100–200 ng/ml for 12 h. For subsequent GFAP immunolabelling, sections were heated at 80°C to inactivate alkaline phosphatase, labelled with GFAP antibody and alkaline phosphotase-labelled secondary goat anti-rabbit antibody (1:30, Dako) and visualized by the fuchsin color reaction according to manufacturer's protocol.

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