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Characterization of the xenotropic retrovirus in the Sp2/0 mouse myeloma cell line

Thomas Heinemeyer[#,1], Lorin Schumacher[#,2], Wiebke Hansen[#,3], Hansjörg Hauser, Dagmar Wirth and Manfred Wirth*

Department of Regulation and Differentiation, Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany.

ABSTRACT

Cell lines are used for the production of pharmaceutical proteins such as antibodies, and sophisticated steps are employed in order to clear viral contamination from the final product. The recent occurrence of a new xenotropic retrovirus derived from xenografting has drawn attention to the dissemination of xenotropic mouse (and related) retroviruses. We therefore investigated human and animal cell lines, which are reported to be free of exogenous retroviruses, by using multi-primer ultrasensitive reverse transcription together with the polymerase chain reaction (RT-PCR) to detect murine C-type retroviruses. Several mouse myeloma cell lines were found to release retroviruses. These cell lines all have P3K cells derived from BALB/c tumor MOPC21 as their common ancestor. Successful infection of mink cells, but not of mouse and human cells, indicated a xenotropic host range. Full length sequence analysis of the isolated and cloned viruses revealed distinct homology with known xenotropic mouse retroviruses, except for a domain in the p12/CA boundary of the *Gag* gene. Despite a low virus release rate in tissue culture, an unexpected outburst of viruses was observed in fermentation runs with Sp2/0 myeloma cells. These findings are of relevance for the safety of protein products derived from myeloma cells.

KEYWORDS: myeloma cell line, mouse retrovirus, xenotropic, X-MLV

INTRODUCTION

The murine genome contains a high percentage of sequences related to retroviral or retroid elements [1-3], and thus infectious and noninfectious retroviruses are well-known companions of cell cultures of murine origin. As certain isolates (species) of murine retroviruses (amphotropic, polytropic, and xenotropic) have acquired the ability to infect non-mouse cells, e.g., human cells, these murine retroviruses are regarded as a safety issue in the production of pharmaceuticals. The putative presence of murine retroviruses necessitates a sophisticated purification and viral clearance program to exclude viral contamination of products derived from cell cultures. Exogenous mouse retroviruses are released as infectious particles and they represent a main safety concern. Endogenous retroviruses are widely distributed in rodents, but because of defects in their regulatory region or structural genes, they reside within the host cell or are released as non-functional particles. Such endogenous retroviruses or retroviral elements have been shown to contribute to the formation of active viruses by recombination [4]. Furthermore, silent proviruses can become infectious under certain inducing conditions. In most cases, retroviral infection is not accompanied by a visible

*Corresponding author: MWI@helmholtz-hzi.de

[#]Current addresses: [1]Hoffmann La Roche Diagnostics, Penzberg, Germany. [2]Evolva SA, Basle, Switzerland. [3]Molecular Infection Immunology, University Hospital, Essen, Germany.

cytopathic effect and, therefore, remains undetected, especially if virus replication is slow, and if titers are low. Retroviruses can induce lymphoma in primates [5]. Furthermore, a new human xenotropic retrovirus (XMRV, xenotropic murine leukemia virus related retrovirus) was initially associated with human prostate cancer and chronic fatigue syndrome [6, 7] but later this was identified as contamination by a laboratory-generated recombinant of two endogenous mouse retroviruses [8, 9]. Because of the association of viruses with diseases, the characterization of cells with respect to their viral burden is essential in cell cultures that are used for the production of pharmaceuticals. Several mouse cell lines stored by cell collecting facilities are reported to be free of exogenous retroviruses. However, such data were often based on standard reverse transcriptase assays, which unfortunately exhibit limited sensitivity. A nested PCR assay targeting the *Gag* gene has been used as a more sensitive tool to detect a wide range of mouse C-type retroviruses [10] in a screening of established cell lines for their viral load. The screening has revealed that the Sp2/0-Ag14 cell line, a descendant of a BALB/c mouse tumor, releases a mouse C-type retrovirus. Our aim here was to characterize this retrovirus by isolating and cloning the virus and determining its complete nucleic acid sequence.

MATERIALS AND METHODS

Cell culture and cell lines

If not otherwise stated, cells originated from ATCC (American Type Culture Collection) and were propagated as recommended by the supplier. Sp2/0 cells were cultivated in complete RPMI (Roswell Park Memorial Institute) medium and were derived from ATCC (CRL1581), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, the German Resource Centre for Biological Material) (ACC 146), Novartis AG, Basle, Switzerland, and Hoffman LaRoche Diagnostics, Penzberg. BHK (Baby Hamster Kidney cells) (ATCC CCL10), NIH3T3 (ATCC 1658), and 293 cells (ATCC CRL 1573) were cultivated in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum, streptomycin (100 µg/ml), and ampicillin (100 U/ml). CHO (Chinese Hamster Ovary cells) (DSMZ ACC 126), X63Ag8.653 (DSMZ ACC43), P3/NSI/1-Ag4-1 (DSMZ ACC145), Karpas-299 (DSMZ ACC31), FOX-NY, and MDBK (ACC174) were all derived from DSMZ (Braunschweig, Germany). MN7 is a mouse osteogenic cell line [11].

RT-PCR detection

Virus sampling from supernatants was performed as described previously [10]. The RT-PCR detection of mouse C-type retroviruses was performed by using *Gag*-specific primers as detailed in [10]. For quantitation, serial dilutions of supernatants from unknown samples were generated and processed. As a control, supernatants from Moloney Murine Leukemia virus (MoMuLV)-infected NIH3T3 virus were co-processed. For proviral PCR, the same primer sets were used, but chromosomal DNA served as template, and the RT step was omitted.

Cloning of the virus released from Sp2/0 cells

Chromosomal DNA of mink Mv1-Lu isolated at 5 passages after infection with viral supernatants of Sp2/0 cells (ATCC CRL 1581) was used to amplify overlapping fragments of the proviral DNA. AmpliTaq (Perkin Elmer) was employed according to the manufacturer's protocol to amplify a 1.9-kb LTR-Gag spanning fragment (Primers WW0274 and OPRMu1Nh), a 1.9-kb Env fragment (primers env8 and env9), and a 700-bp LTR fragment (primers GAGspeI and H2IrLTR). The Expand™ Long Template PCR kit (Roche Molecular Biochemicals) was used to amplify a 6.3-kb Gag-Env fragment (primers PsiSpeI and XenvPsEc) and a 370-bp fragment covering the p2e protein (primers P2eXeno and U3Revers). For sequence comparison, the 6.3-kb Gag-Env fragment was also amplified by using chromosomal DNA from Sp2/0 cells.

For sequencing, the PCR fragments were cloned into pUC19. At least two clones were sequenced by using the AutoRead™ sequencing kit (Pharmacia) and the A.L.F. DNA sequencer (Pharmacia) according to the manufacturer's protocols. No differences were determined within the overlapping sequences. Four independent clones of the 4.6-kb Gag-Env fragment obtained from infected Mv1-Lu cells and two independent clones of the same fragment obtained from Sp2/0 cells were sequenced. The error rate was determined to be 0.04%, which corresponds to 1 error in 2300 bp.

Southern blotting

Southern blot analysis was performed in order to determine copy numbers. Samples of 30 µg of cellular DNA digested with the relevant restriction enzyme were run on an agarose gel and blotted onto a Zeta-Probe (BioRad). Hybridization with the 1.9-kb Env PCR fragment was performed at 68 °C in a buffer containing 0.5 M $Na_xH_xPO_4$ (pH 7.2), 7% SDS, and 2 mM EDTA (Ethylendiaminetetra acetic acid).

RESULTS AND DISCUSSION

Screening of cell lines for the presence of mouse C-type retroviruses

Several cell lines of biotechnological relevance and cell lines previously stated as being free of retrovirus by established cell culture collections were screened for the presence of retroviral contamination. For this purpose, we used a previously developed RT-PCR method capable of detecting up to 10 viral particles per reaction [10]. This method allows the detection of exogenous and endogenous retroviruses and provides a significantly higher sensitivity compared with traditional viral detection methods. The RT-PCR employs a set of degenerate primers complementary to the *Gag*-coding region and exhibits stringent specificity for mouse C-type retroviruses such as ecotropic (MoMULV; HoMULV), amphotropic (4070A, 10A1), and xenotropic (NZB, CWD) murine retroviruses, in addition to Friend leukemia virus, spleen-focus-forming virus, radiation leukemia virus, and endogenous AKR retrovirus. Furthermore, XMRV, a xenotropic retrovirus recently detected as a contaminant introduced into prostate tumor cells and falsely associated with chronic fatigue syndrome (CFS) because of its detection in patients with CFS, is also covered by this PCR.

Table 1 depicts results from the RT-PCR and the chromosomal proviral PCR. Several cell lines (BHK, HEK 293, and Mv1Lu) and cellular supernatants derived therefrom tested negative in PCR and RT-PCR when the primers specific for mouse C-type retroviruses were used. Others such as NIH3T3 contained proviral DNA in their genome, but no release of exogenous mouse C-type retroviral particles was detected. Interestingly, our experiments

Table 1. Presence of murine retroviral sequences from chromosomal DNA and viral-particle-derived RNA in the supernatants of various cell lines.

Cell line	Species	Cell type	PCR [1] (chromosomal)	RT-PCR [1] (supernatant)
293	human	epithelial	-	-
Karpas DSMZ ACC31	human	lymphoma	n.d.	-
BHK-21	hamster	fibroblast	-	-
CHO	hamster	epithelial	+	-
MN7	mouse	n.d.	n.d.	-
Sp2/0 ATCC CRL1581	mouse	myeloid	++	+
Sp2/0 DSMZ ACC146	mouse	myeloid	n.d.	+
Sp2/0 source 3	mouse	myeloid	++	+
Sp2/0 source 4	mouse	myeloid	n.d.	+
P3/NSI/1-Ag4-1	mouse	myeloid	n.d.	+
X63-Ag8.653	mouse	myeloid	n.d.	-
FOX-NY	mouse	myeloid	n.d.	+
NIH3T3	mouse	fibroblast	++	-
MDBK	bovine	epithelial	n.d.	-
Mv1LU	mink	epithelial	-	-

[1] n.d.: Not determined, -: no signal, +: positive signal, ++: strong positive signal.

revealed that three closely related myeloma cell lines, namely, Sp2/0, FOX-NY, and NSI/1-Ag4.1 cells, produced retroviruses. Mouse C-type proviral DNA was found in their genome, and retroviral RNA was detected in exogenous particles in the cell supernatants. For confirmation, the experiments were repeated several times with consistent results. To exclude adventitious laboratory infection, Sp2/0 Ag14 cells from three other sources were tested. All samples released particles containing RT-PCR-positive RNA sequences.

All retrovirus-positive myeloma cell lines are descendants of P3K, a cell line established from the BALB/c mouse tumor, MOPC21 [12, 13]. Estimations of viral titers by RT-PCR with serial dilutions of supernatants revealed that the Sp2/0 cell line released 2-3 orders of magnitude fewer viruses compared with MoMuLV-infected NIH3T3 cells (data not shown). To verify results derived from RT-PCR, we used a different assay that is commercially available. In a non-radioactive RT-ELISA (enzyme-linked immunosorbent assay) detection method (Hoffmann La Roche Diagnostics), a weak signal was obtained after the concentration of virus from 1 ml Sp2/0 supernatant by ultracentrifugation. The detection limit of this assay with an increased reaction time from 30 min to 2 h was determined to be around 10^{-2} units reverse transcriptase per assay, which is equivalent to 2×10^4 to 1×10^5 retroviral particles. This suggests that distinct amounts of RT-positive particles are present in Sp2/0 supernatants.

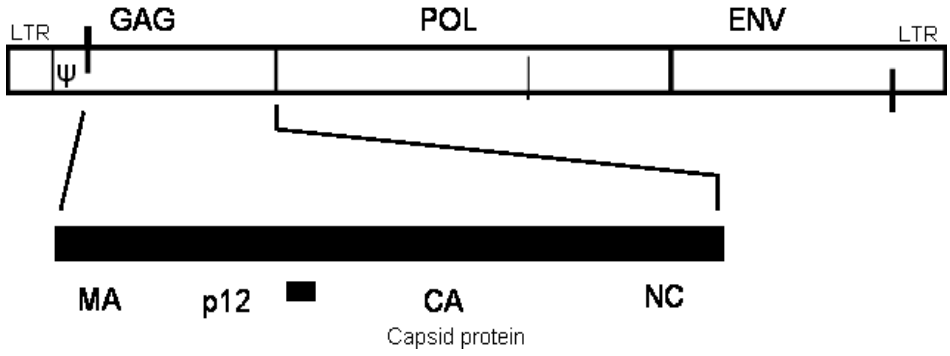
Conventional reverse transcriptase assays are not sensitive enough to detect low-titer murine retroviruses, a finding that originally led to the augmentation of reverse transcriptase assays by more sensitive RT-PCR procedures in facilities engaged in routine virus screening. Most, if not all, of the hybridomas found were reported to be negative in a commonly used reverse transcriptase assay recommended by relevant sources as being suitable for the examination of cell lines for the presence of retroviral particles [14, 15]. The assay traditionally used was based on the activity of reverse transcriptase on a poly(A) RNA template with radioactively labeled nucleotides (^{3}H, ^{32}P) as the substrate [16, 17]. Indeed, we were unable to detect the Sp2/0-derived virus in the conventional tritium thymidine incorporation test [17], modified as described in [18]. Compared with MLV infected NIH3T3 cells, Sp2/0 cells released 2-3 orders of magnitude fewer viruses; this might explain the inability of the conventional reverse transcriptase assays to detect retroviruses in cell lines. Our findings confirm earlier investigations that estimated the retroviral load of rodent myeloma and hybridomas by using immunological methods [19]. Remarkably, in a survey of the ATCC, at least 436 cell lines originate from fusion with Sp2/0 myelomas and 22 from fusion with NS01. Our findings suggest that these descendants are also retrovirus-positive.

Complete nucleotide sequence of the xenotropic Sp2/0 retrovirus

Because of the frequent use of the Sp2/0-Ag14 cells in research and industrial antibody production, we decided to investigate the properties and the primary structure of the contaminating exogenous retrovirus in detail. In order to obtain the primary sequence of the Sp2/0 retrovirus, we tried to amplify RNA from virions in the supernatant. However, we failed to efficiently amplify large fragments by PCR. The genomic DNA of Sp2/0 cells could not be used specifically to amplify the active virus genome, as all murine cells including Sp2/0-Ag14 cells harbor a large number of endogenous C-type retroviruses. Therefore, Mv1 Lu cells, which are free of mouse C-type retroviruses (compare Table 1), were infected with Sp2/0 supernatants. Southern blot analysis indicated a low number of integrated proviral DNA (data not shown). Genomic DNA prepared from cells at 5 passages after infection was used as a template for PCR. PCR primers that bind to regions of high homology as deduced from the comparison of published nucleotide sequences of known mouse C-type retroviruses were selected. Overlapping subfragments were amplified and cloned. The primary structure of the pol region of xenotropic retroviruses is often not known. Sequence analysis of *Gag*- and Env-coding regions allowed the design of primers specific for the isolated retrovirus. With these primers, a 4.6-kb fragment (XbaI-EcoRI) covering the *Pol* region, the 3' region of *Gag*, and the 5' region of Env could be cloned from PCR by using the supernatant of Sp2/0 cells. The full length sequence of the virus was determined. At least two clones of each fragment were sequenced (see Materials and Methods for details).

The primary structure deduced from the full length sequence of the virus reveals features typical of a replication-competent mouse C-type retrovirus (Fig. 1). The complete nucleotide sequence is accessible from GenBank (Accession number X94150). The regulatory regions for transcription, RNA processing, translation, and protein processing and the signals necessary for replication resemble those in the ecotropic MoMuLV retrovirus. However, alignment of Env protein sequences shows extensive homology to the Env region of xenotropic retroviruses (Fig. 2). Based on these sequences, one can expect efficient virus production, given that the provirus(es) is/are located in a favorable chromosomal region.

The Gag-Pol region shows extensive homology to the corresponding region of the spleen focus-forming virus (SFFV), mink cell focus-forming (MCF) viruses, radiation leukemia virus (RadLV), and endogenous mouse retroviruses, e.g., AKV virus. In particular, 96% homology was found to exist in the Gag region in XMRVs found in prostate (LAPC4, VCaP) and in xenotropic viruses detected in small cell lung carcinoma cells (SCLC 417); these viruses presumably entered the cells during xenografting. Furthermore, similar homology exists for a recombinant virus of aged DBA/2 mice. Gag from xenotropic NZB 9-1 exhibits 92% homology to Sp2/0 virus Gag. However, distinct differences were found in the primary structures of the Gag proteins when compared with ecotropic murine leukemia viruses such as MoMuLV (Fig. 3). These alterations are also present in xenotropic mouse retroviruses with published Gag primary structures. Most prominently, an insertion of three bases at positions 1250, 1282, and 1324 in the xenotropic Sp2/0 retrovirus results in a frameshift mutation. The region between the first frameshift and its reversion encompasses 75 nucleotides. The alterations that result from these frameshift mutations are coincident

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                                          <-gag p12 | gag p30-> Capsid Protein (CA)
                          *                                *
     5' GGGAGACGGGAGCCCCCU.GUGGCCGACUCCACUACCUCGCAGGCAUUCC.CCCUCCGCGCAGGAGGAAACGGACAGCUUCAAUACUGG  3'
REMLM    GlyArgArgGluProPro.ValAlaAspSerThrThrSerGlnAlaPheP.roLeuArgAlaGlyGlyAsnGlyGlnLeuGlnTyrTrp
XenoGes  GlyLysArgAspProProArgGlySerPheHisHisLeuSerGlyPheProHisSerValTrpGlyValMetValSerCysSerThrGly
     5' GGCAAAAGAGACCCCCCCCGCGGCAGUUUCCACCACCUCUCGGGCUUUCCGCACUCCGUUUGGGGGGUAAUGGUCAGUUGCAGUACUGG  3'
           1240         *      1260      1270      1280 *    1290      1300      1310      1320

          *
     5' CCG.UUCUCCUCUUCUGACCUUUACAACUGGAAAAAUAAUAACCCUUCUUUUUCUGAAGAUCCAGGUAAACUGACAGCUCUGAUCGAGUC 3'
REMLM    Pro.PheSerSerSerAspLeuTyrAsnTrpLysAsnAsnAsnProSerPheSerGluAspProGlyLysLeuThrAlaLeuIleGlu
XenoGes   ArgPheSerSerSerAspLeuTyrAsnTrpLysAsnAsnAsnProSerPheSerGluAspProGlyLysLeuThrAlaLeuIleGlu
     5' CCGGUUUUCCUCCUCGGAUCUAUAUAACUGGAAAAAUAAUAACCCUUCCUUCUCCGAAGAUCCAGGUAAAUUGACUGCCUUAAUCGAGCU 3'
           *  1330      1340      1350      1360      1370      1380      1390      1400      1410
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Fig. 1. Localization of a 75-b frameshift region in *Gag*. Top: Schematic drawing of the proviral genome of the xenotropic Sp2/0 retrovirus. Middle: Enlarged *Gag*-coding region; the region of divergence is shown as a black bar below *Gag*. Bottom: Comparison of the amino acid sequences of Gag from xenotropic Sp2/0-derived retrovirus (xenogag) (GenBank accession number X94150) and the ecotropic Moloney murine leukemia virus (EMBL: REMLM). The positions of the frameshifts are shown by asterisks. The original reading frame of the capsid protein is restored after the third nucleotide insertion. Note the conserved viral protease cleavage site (Phe-Pro) at the p12/p30 boundary.

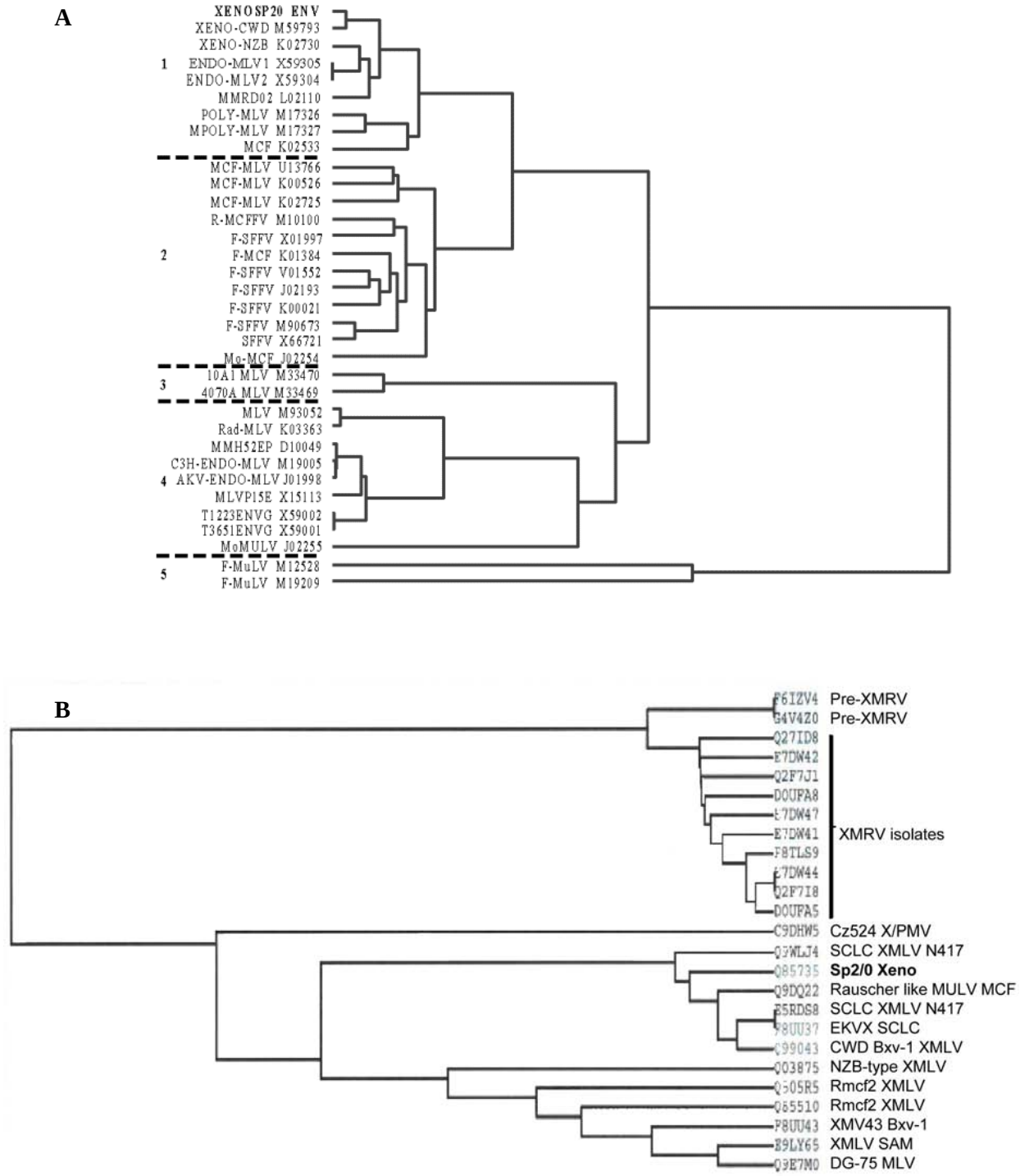

Fig. 2. Homology trees of mouse retroviral Env proteins. A. Amino acid sequences of Env from ecotropic, xenotropic, amphotropic exogenous, and various endogenous retroviruses were aligned by using the GENMON program package (GBF, Braunschweig), and the degree of homologies is depicted. Abbreviations of the retroviral denominations are followed by the GenBank accession numbers of the retroviral genes. **B.** Homology tree derived from the alignment of Env proteins of various xenotropic MRVs identified in the UniProt database (www.uniprot.org) after a BLAST search for similarity to the Env of Sp2/0 XMLV (Q85735). The Align option (CLUSTAL Omega) embedded in UniProt was used.

with substitutions of 21 amino acids in the p12/capsid protein (CA) boundary (Fig. 3). Strikingly, the phenylalanine/proline protease cleavage site for the processing of the Gag-precursor protein into p12 and CA, which lies in the region of divergence, is conserved. This substituted region is totally missing in other xenotropic MRV isolates investigated so far. In the infection cycle of xenotropic mouse retroviruses, p12 functions early as part of the preintegration complex (PIC) and late in virus budding. The C-terminus in p12 is dispensable if substituted by elements mediating chromosome binding. CA is known to form the 'inner core' or capsid of the virus by oligomerization. Earlier investigations involving the use of linker scanning mutagenesis within the CA region of a related retrovirus have shown that the full integrity of CA is a prerequisite for efficient viral particle formation [20]. Thus, the divergence in the Sp2/0 virus CA N-terminus might influence the efficiency of particle formation and might explain the low viral titer found in Sp2/0 cells.

Amino acid substitutions have also been found in the region coding for distinct retroviral enzymes. In contrast to the well-conserved reverse transcriptase of the pol region of mouse retroviruses (with only 20/157 differing amino acids in 10 different virus strains) [21], the integrase shows a higher degree of divergence when compared with the respective MoMuLV proteins. The envelope or SU protein exhibits 99.2% and 96.7% homology to the envelopes of the xenotropic retroviruses of the CWD mouse [22]) and the NZB xenotropic retrovirus [23], respectively (Fig. 2A, Fig. 3) suggesting a xenotropic host range for the Sp2/0 retrovirus. Interestingly, a 95% homology exists in the Env protein of recently described XMRV virus isolates (Fig. 2B). The initial reports of this virus confused the scientific community, as XMRVs were detected in samples from patients suffering from CFS and prostate cancer and were linked to these diseases. However, XMRVs have now been identified as laboratory recombinant viruses introduced by the xenografting of cells in mice, which confirms that these viruses are not responsible for these diseases [9, 24-27]. Deviations from published CWD xenotropic Env have been observed in positions 56 (T->N) and 57 (A->S), which is close to the amino terminus, and in 309 (E->G), which is downstream of a proline-rich region in a part of the protein with unknown function. Alterations in positions 105 (S->T) and 573 (A->G) are conservative. Twenty one substitutions were observed when the sequence was compared with the NZB xenotropic Env. Whereas most of these substitutions are conservative and do not alter the character of the amino acid, substitutions in positions 293 (K->E), 429 (D->G), and 433 (G->D) convert a positively charged or an uncharged amino acid into a negatively charged residue. The substitutions are located in a region close to the gp70/p15 cleavage site or downstream of the proline-rich region. Furthermore, data on recently described xenotropic MRVs have revealed that other retroviruses, with up to 99% identity to the *Env* region of Sp2/0 virus, have appeared in small cell lung cancer cells such as SCLC N417 and EKVX (Fig. 2B) [26-28], and show high similarity in the Env protein to previously reported murine retroviruses [22, 29, 30]. Most alterations in the *Env* gene of the Sp2/0 virus are conservative when compared with the amino acid sequence of published xenotropic *Env*. Non-conservative substitutions reside in a region of the protein not directly involved in receptor binding, suggesting that alterations of the Env amino acid sequence should not considerably influence the putative xenotropic host range of the Sp2/0-derived retrovirus.

Recently, the DNA sequence of a *Pol* fragment has been reported in a MRV residing in several human melanoma cells, which also underwent passaging through mice [31]. The published pol DNA fragment of 517 bp exhibits 100% homology to the corresponding part of the Sp2/0 retrovirus reported here. However, no information on other regions of the viral genome from melanoma mouse retrovirus is available to date. Thus, any speculations on the identity of the virus await confirmation.

Determination of proviral copy numbers of the xenotropic retrovirus in Sp2/0 mouse myeloma cells

The copy number of xenotropic viruses in Sp2/0 cells was estimated by determination of the number of integration sites by means of Southern blot analysis (Fig. 4). For this purpose, the genomic DNA of Sp2/0-Ag14 cells was treated with various endonucleases that cut both within the virus and

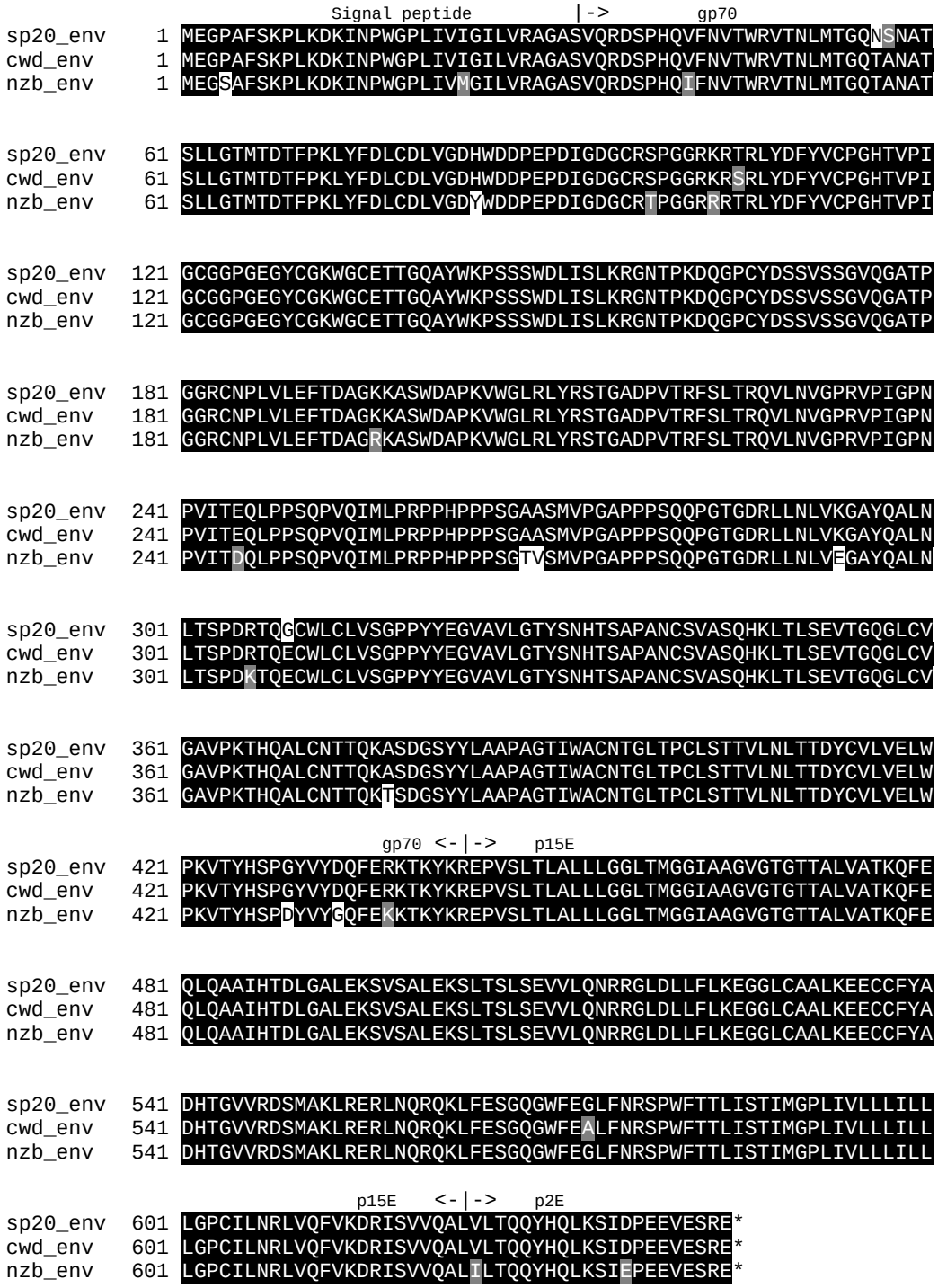

Fig. 3. Comparison of mouse retroviral envelope proteins. The amino acid sequences of various mouse retroviral envelope proteins were aligned with that of Sp2/0-derived retrovirus by using the GCG pileup program [36] and BoxShade software (Kay Hofmann and M. Baron, freeware program accessible via ftp.embl-heidelberg.de). Black boxes indicate identity of amino acids, grey boxes show amino acids with similar properties. Sequences were derived from GenBank, and accession numbers are shown. Abbreviations: NZB-9-1, Env of the xenotropic mouse retrovirus derived from NZB mouse; CWM-S-5X, Env of the xenotropic retrovirus of the CWD mouse; REMLM, Env of ecotropic MoMuLV. Asterisk: stop codon.

the bordering elements of the host genome (as deduced from the sequence). The LTRs of all C-type viruses exhibited a recognition site for NheI. Hybridization with the 1.9-kb xenotropic Env element obtained from the supernatant of Sp2/0 cells by PCR under high stringency conditions revealed a fragment of 8.3 kb, which is expected for full length xenotropic viruses. The high intensity of the signal indicates an elevated number of virus copies per cell. Additional smaller bands suggest non-functional xenotropic viruses. BamHI cuts twice within the pol region, whereas HindIII does not cleave the xenotropic Sp2/0 virus. Accordingly, in the BamHI or BamHI/HindIII double-digest, hybridization products of more than 4.6 kb reflect different integration sites. About 7-10 fragments of this size were obtained for the BamHI digest and 5-8 fragments for the BamHI/HindIII digest (Fig. 4). EcoRI cuts the virus once within the *Env* gene giving rise to two fragments larger than 1.8 kb and 6.7 kb. As expected, the number of different fragments in this digest is roughly doubled. In summary, about 5-10 copies of Sp2/0 provirus per cellular genome can be estimated from this analysis. Since both exogenous and endogenous viruses are detected by this method, the number of active virus copies might be lower.

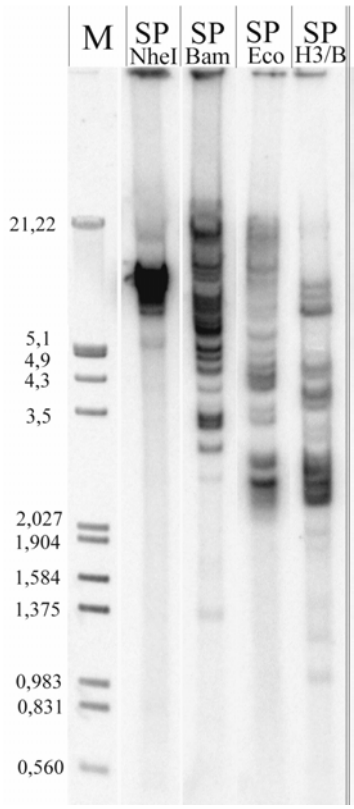

Fig. 4. Xenotropic proviral copies in Sp2/0 chromosomal DNA. Southern blot analysis of Sp2/0 cells. For each lane, 30 µg chromosomal DNA was digested with NheI (lane 1), BamHI (lane 2), EcoRI (lane 3), and HindIII + BamHI (lane 4). The DNA was hybridized with the 1.9-kb xenotropic Env fragment obtained by PCR from infected Mv1-Lu cells. EcoRI cuts the proviral DNA in the *Env* region once; NheI has a recognition sequence in the *LTRs*. HindIII (H) does not cut the provirus. M: S^{35} labeled λ HindIII/EcoRI digest.

Viruses released from Sp2/0 cells are infective and have a xenotropic host range

Infection studies on various cell lines were performed to determine the infectivity of the virus released by Sp2/0 cells and to confirm the tropism suggested by the nucleic acid data (Table 2). Because of their host range, mouse retroviruses can be classified as ecotropic, amphotropic, xenotropic, or polytropic reflecting the different Env proteins and the usage of different cellular receptors. Ecotropic retroviruses infect mouse and rat cells exclusively, amphotropic and polytropic retroviruses have a more extended host range, and xenotropic retroviruses do not infect mouse cells but cells of other species. Mouse NIH3T3 cells, mink Mv 1 Lu cells, and human HEK 293 cells were each challenged three times with the Sp2/0 retrovirus to reveal the virus tropism in presence of 8 µg/ml Polybrene®. At 5 passages after treatment, nested RT-PCR was performed by using the supernatant of infected cells [10]. The expected infection pattern for the viral strains (amphotropic (A), ecotropic (E), polytropic (P), and xenotropic (X)) is indicated in Table 2. As shown, mink Mv 1 Lu cells, but not mouse NIH3T3 cells or human 293 cells, can be infected by the virus. The inability to infect a mouse cell line typically used for the titration of ecotropic and amphotropic retroviruses is indicative of a xenotropic host range. The reason why human 293 cells could not be infected by the virus is unclear. The receptor by which xenotropic retroviruses enter the cell has been identified [32-34] and is expressed in a broad range of human tissues.

Kinetics of virus release

Virus production in tissue culture plates was found to be consistently low. In order to provoke

a variety of physiological conditions and to simulate conditions for the large scale production of a therapeutic protein, we followed the kinetics of virus production in two fermentor runs. Recombinant Sp2/0 cells were used. Sp2/0 cells were propagated over a period of 45 days. A typical course of cell cultivation and virus accumulation is depicted in Fig. 5. Cell numbers (viable/dead) and virus release were determined throughout the experiments. In order to obtain data concerning virus production, RNA in virions from cell-free supernatants was quantitated by using endpoint dilution and the nested RT-PCR test [10]. Except during the very beginning of the fermentation period, the amount of virus released was higher than that from culture plates. An unexpected burst could be found before cell density reached 10^6 cells/ml. Virus production declined definitely before the plateau phase of cell

Table 2. Tropism of the Sp2/0-derived retrovirus.

	Species	Infection pattern expected [1]				Infection pattern observed [2]
		E	P	A	X	
NIH3T3	mouse	+	+	+	-	-
Mv 1 Lu	mink	-	+	+	+	+
293	human	-	+	+	+	-

[1] A: amphotropic; E: ecotropic; P: polytropic: X: xenotropic.
[2] +: PCR detection of the 117-bp fragment.
-: not detected by PCR.

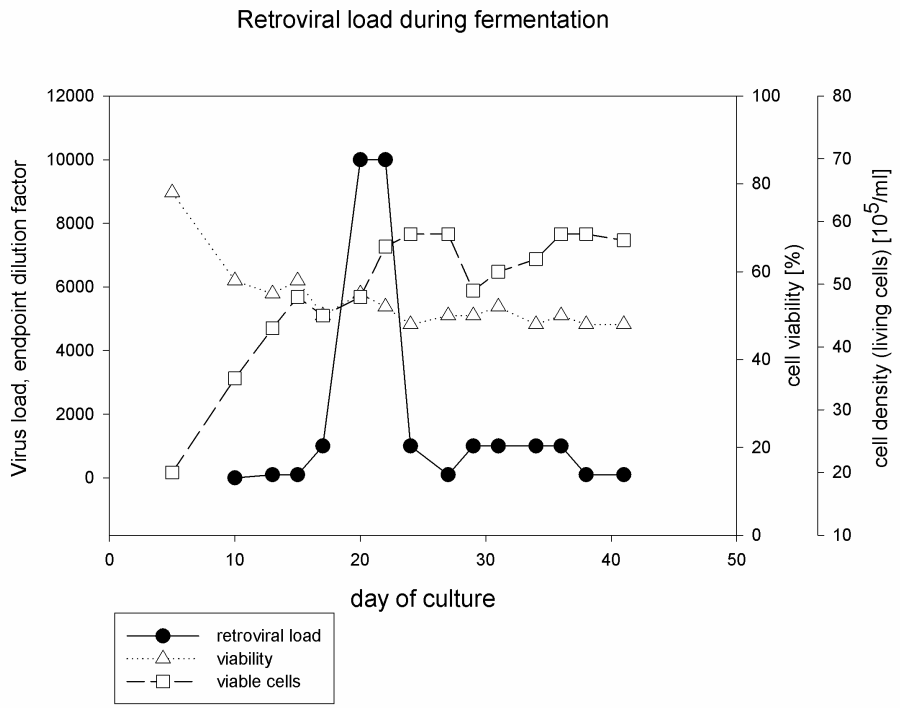

Fig. 5. Virus accumulation in a Sp2/0 bioreactor run. A recombinant Sp2/0 cell line was cultivated in a fermentor in RPMI. Cell numbers (viable/dead) and retrovirus accumulation were monitored. Retrovirus accumulation was determined by RT-PCR quantitation of virion RNA from endpoint dilution of supernatants. Values refer to the highest dilution showing a positive signal in RT-PCR.

growth was reached. A similar phenomenon was encountered in a second fermentation run (data not shown). We do not know the reason for the uncorrelated production of retroviruses in the log phase of host cell growth in the fermentor. Limitations in cellular factors needed for virus production might contribute to such an effect. On the other hand, the accumulation of a retrovirus inhibitor might adversely affect virus production in the late phase of cultivation [35]. Moreover, a cellular regulatory mechanism active under certain conditions might be involved in the virus shut-off. Deeper insight into the mechanism underlying accumulation could allow the control of retrovirus release during pharmaceutical protein production. This might significantly contribute in the handling of Sp2/0 cells.

CONCLUSION

We characterized the xenotropic retrovirus of the Sp2/0 Ag14 cell line by nucleic acid sequencing, copy number determination in the cellular genome, and infectivity tests. Because of the wide distribution and use of these cells in fundamental research and production processes, various safety concerns might arise. Since retroviruses contain a lipid shell, they can be easily inactivated by certain physical treatments (temperatures > 56 °C, low pH) and chemicals (detergents, hypochlorite) with individual inactivation rates ranging from log 3 to log 6. Experiments involving a combination of standard procedures for the purification of antibodies guarantee cumulative inactivation with a sufficient safety margin (maximum achievable log titer/log cumulative inactivation). In the case of the Sp2/0 fermentor runs, we applied standard procedures for the purification of antibodies to inactivate the Sp2/0 retrovirus. Sp2/0 retrovirus RNA was not detectable in the final product, even by RT-PCR technique (data not shown).

An acceptable solution for the problem of virus clearance might consist in the application of gene knock-out strategies to remove active retroviral DNA from the Sp2/0 chromosome. However, the estimated copy numbers of 5-10 (Fig. 4) make it difficult to eradicate the actively transcribed provirus by using classical approaches. New routes, facilitated by the sequencing of the mouse genome, might allow step-wise elimination/inactivation of all virus copies. Alternatively, investigations addressing the efficient shut-off of retrovirus synthesis in Sp2/0 might provide promising strategies.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

REFERENCES

1. Stoye, J. P. and Coffin, J. M. 1987, J. Virol., 61, 2659.
2. Stoye, J. P. and Coffin, J. M. 1988, J. Virol., 62, 168.
3. Coffin, J. M., Stoye, J. P. and Frankel, W. N. 1989, Ann. NY. Acad. Sci., 567, 39.
4. Coffin, J. M. 1992, Curr. Top. Microbiol. Immunol., 176, 143.
5. Donahue, R. E., Kessler, S. W., Bodine, D., McDonagh, K., Dunbar, C., Goodman, S., Agricola, B., Byrne, E., Raffeld, M., Moen, R., Bacher, J., Zsebo, K. M. and Nienhuis, A. W. 1992, J. Exp. Med., 176, 1125.
6. Urisman, A., Molinaro, R. J., Fischer, N., Plummer, S. J., Casey, G., Klein, E. A., Malathi, K., Magi-Galluzzi, C., Tubbs, R. R., Ganem, D., Silverman, R. H. and DeRisi, J. L. 2006, PLoS Path., 2, 211.
7. Lombardi, V. C., Ruscetti, F. W., Gupta, J. D., Pfost, M. A., Hagen, K. S., Peterson, D. L., Ruscetti, S. K., Bagni, R. K., Petrow-Sadowski, C., Gold, B., Dean, M., Silverman, R. H. and Mikovits, J. A. 2009, Science, 326, 585.
8. Kearney, M. F., Spindler, J., Wiegand, A., Shao, W., Anderson, E. M., Maldarelli, F., Ruscetti, F. W., Mellors, J. W., Hughes, S. H., Le Grice, S. F. J. and Coffin, J. M. 2012, PLoS One, 7, e30889.
9. Cingöz, O., Paprotka, T., Delviks-Frankenberry, K. A., Wildt, S., Hu, W. S., Pathak, V. K. and Coffin, J. M. 2012, J. Virol., 86, 328.
10. Heinemeyer, T., Klingenhoff, A., Hansen, W., Schumacher, L., Hauser, H. and Wirth, M. 1997, J. Virol. Meth., 63, 155.
11. Mathieu, E., Schoeters, G., Vander Plaetse, F. and Merregaert, J. 1992, Calc. Tiss. Int., 50, 362.
12. Horibata, K. and Harris, A. W. 1970, Exp. Cell. Res., 60, 61.

13. Potter, M. 1972, Physiol. Rev., 52, 631.
14. CBER. 1993, Points to consider in the characterization of cell lines for the production of biologicals. Centre for Biologics and Research.
15. Onions, D. 1997, Safety evaluation of products derived from mammalian cell lines, de Gruyter, Berlin, New York.
16. Goff, S., Traktman, P. and Baltimore, D. 1981, J. Virol., 38, 239.
17. Sherr, C. J. and Todaro, G. J. 1979, Meth. Enzymol., 58, 412.
18. Artelt, P. 1990, PhD Thesis, Technical University Braunschweig, Braunschweig.
19. Deo, Y., Ghebremariam, H. and Cloyd, M. 1994, Hybridoma, 13, 69.
20. Hansen, M., Jelinek, L., Whiting, S. and Barklis, E. 1990, J. Virol., 64, 5306.
21. Ey, P. L., Freeman, N. L., Bela, B., Haese, P. M., Li, P. and McInnes, J. L. 1999, Arch. Virol., 144, 2185.
22. Massey, A. C., Coppola, M. A. and Thomas, C. Y. 1990, J. Virol., 64, 5491.
23. O'Neill, R. R., Buckler, C. E., Theodore, T. S., Martin, M. A. and Repaske, R. 1985, J. Virol., 53, 100.
24. Paprotka, T., Delviks-Frankenberry, K. A., Cingöz, O., Martinez, A., Kung, H. J., Tepper, C. G., Hu, W. S., Fivash Jr., M. J., Coffin, J. M. and Pathak, V. K. 2011, Science, 333, 97.
25. Hué, S., Gray, E. R., Gall, A., Katzourakis, A., Tan, C. P., Houldcroft, C. J., McLaren, S., Pillay, D., Futreal, A., Garson, J. A., Pybus, O. G., Kellam, P. and Towers, G. J. 2010, Retrovirol., 7, 111.
26. Sfanos, K. S., Aloia, A. L., Hicks, J. L., Esopi, D. M., Steranka, J. P., Shao, W., Sanchez-Martinez, S., Yegnasubramanian, S., Burns, K. H., Rein, A. and De Marzo, A. M. 2011, PloS One, 6, e20874.
27. Zhang, Y. A., Maitra, A., Hsieh, J. T., Rudin, C. M., Peacock, C. D., Karikari, C., Brekken, R. A., Stastny, V., Gao, B., Girard, L., Wistuba, I., Frenkel, E., Minna, J. D. and Gazdar, A. F. 2011, Canc. Biol. Ther., 12, 617.
28. Antoine, M., Wegmann, B. and Kiefer, P. 1998, Virus Genes, 17, 157.
29. Hook, L. M., Jude, B. A., Ter-Grigorov, V. S., Hartley, J. W., Morse, H. C. 3rd, Trainin, Z., Toder, V., Chervonsky, A. V. and Golovkina, T. V. 2002, J. Virol., 76, 12112-12122.
30. Ter-Grigorov, V. S., Krifuks, O., Liubashevsky, E., Nyska, A., Trainin, Z. and Toder, V. 1997, Nat. Med., 3, 37.
31. Deichmann, M., Huder, J. B., Kleist, C., Näher, H., Schüpbach, J. and Böni, J. 2005, Arch. Dermatol. Res., 296, 345.
32. Tailor, C. S., Nouri, A., Lee, C. G., Kozak, C. and Kabat, D. 1999, Proc. Nat. Acad. Sci., 96, 927.
33. Yang, Y. L., Guo, L., Xu, S., Holland, C. A., Kitamura, T., Hunter, K. and Cunningham, J. M. 1999, Nat. Genet., 21, 216.
34. Battini, J. L., Rasko, J. E. J. and Miller, A. D. 1999, Proc. Nat. Acad. Sci., 96, 1385.
35. Le Doux, J. M. L., Morgan, J. R. and Yarmush, M. L. 1999, Biotechnol. Progr., 15, 397.
36. Devereux, J., Haeberli, P. and Smithies, O. 1984, Nucl. Acids Res., 12, 387.