Poxviral/Retroviral Chimeric Vectors Allow Cytoplasmic Production
of Transducing Defective Retroviral Particles

G. W. Holzer, J. A. Mayrhofer, W. Gritschenberger, F. Dorner, and F. G. Falkner[1]

Baxter/Hyland Immuno Division, Biomedical Research Center, Uferstrasse 15, A-2304 Orth/Donau, Austria

Defective vaccinia viruses were constructed that express functional Moloney murine leukemia virus-based vector genomes, giving rise to substantial titers of transduction-competent retrovirus particles after infection of a retroviral packaging cell line. For this purpose, the proviral retrovirus genome, engineered into the vaccinia virus mutant, was subjected to several modifications, including the replacement of retroviral promoter sequences by vaccinia virus sequences and the precise fusion of the transcription stop signal downstream of and the removal of such signals within the transcription unit, allowing cytoplasmic transcription of distinct full-length retroviral transcripts. Vaccinia-mediated expression of retroviral vector particles could be observed as early as 3 h postinfection and resulted in stable transduction of NIH/3T3 target cells at higher titers than the control performed by conventional plasmid transfections. Thus at least part of the vaccinia life cycle and retroviral assembly can occur concomitantly. Due to the favorable properties of vaccinia vectors, including high coding capacity, stability, and wide host range, defective vaccinia viral/retroviral chimeric vectors are promising tools for gene therapy applications.   © 1999 Academic Press

INTRODUCTION

Current viral vectors used in gene therapy include defective retroviruses (for a review, see Boris-Lawrie and Temin, 1993; Miller, 1997) and defective adenoviruses (Li *et al.,* 1993), including adenovirus minichromosomes (Hay *et al.,* 1984; Kochanek *et al.,* 1996) and adeno-associated viruses (for a review, see Berns and Giraud, 1995; Kremer and Perricaudet, 1995). Recently, chimeric virus or virus-derived systems have been described as production and potential delivery vectors for retroviruses. Semliki Forest virus-derived replication competent RNAs were electroporated into host cells and used to produce recombinant retroviral particles (Li and Garoff, 1996; Wahlfors *et al.,* 1997). Furthermore, herpesvirus amplicons expressing *gag*, *pol*, and *env* genes were shown to rescue defective lacZ retrovirus in a cell line carrying a corresponding provirus (Savard *et al.,* 1997). In addition, an adenovirus/retrovirus chimeric system has been described consisting of two adenoviruses: the first one encoding retroviral packaging functions and the second one encoding a defective retroviral genome. If both viruses coinfect one target cell, the system allows *in vivo* gene transduction (Feng *et al.,* 1997). In this report, we describe the use of vaccinia virus, one of the most widely used vectors in molecular and cell biology

(Moss, 1996; Paoletti, 1996), as a chimeric carrier for small RNA virus genomes. The development of uracil DNA glycosylase (UDG)-defective vaccinia viruses, replicating only in engineered complementing cell lines (Holzer and Falkner, 1997), has allowed us to investigate defective vaccinia as a chimeric carrier for retroviral genomes. We describe UDG-deficient vaccinia virus, carrying a modified defective provirus that, on transcription in the cytoplasm of retroviral packaging cells, induces significant titers of transducing defective retroviral particles.

RESULTS

Outline of the experimental approach

Vaccinia virus transcribes its genes, replicates its DNA, and assembles its virions in the cytoplasm. Retrovirus genomes, on the other hand, are transcribed from nuclear proviruses, further modified in the nucleus, packaged into virions at the cellular membrane, and finally released by budding (for a review, see Coffin, 1996). For the construction of a chimeric poxviral/retroviral vector, it was therefore of interest to evaluate the hypothesis that at least part of the complex life cycles of both viruses can occur concomitantly in the same cell. To test these assumptions, a defective proviral genome was modified to serve as an efficient vaccinia virus early transcription unit and inserted into a defective vaccinia virus, which was used to infect a retroviral packaging cell line. Su-

[1] To whom reprint requests should be addressed. Fax: *43-2212-2716. E-mail: falknef@baxter.com.
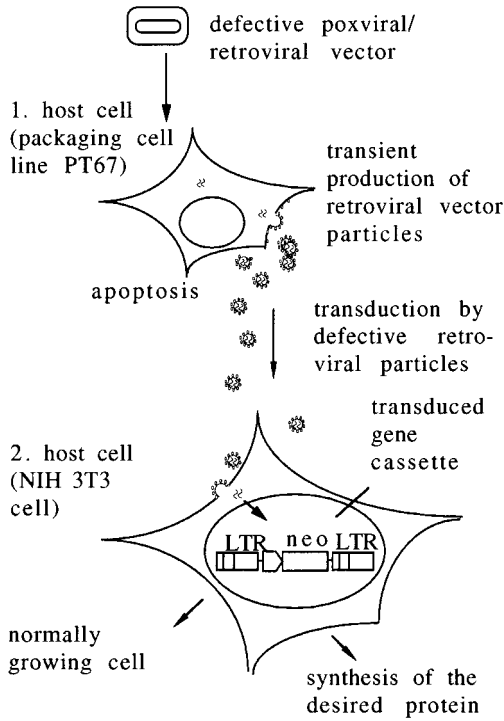
FIG. 1. Schematic representation of the poxviral/retroviral vector system. Packaging cells infected with defective chimeric vaccinia virus synthesize retroviral transcripts in the cytoplasm that are packaged into functional retrovirus vector particles. On infection of target cells by these particles, reverse transcription and stable integration of the provirus result in transduction of the expression cassette into the recipient cell.

pernatants of the infected cells were then used to transduce NIH/3T3 cells. The approach is depicted in Fig. 1.

Modification of the retroviral transcription unit

Vaccinia virus early transcripts are capped mRNAs of discrete length terminating ~50 bp downstream of a specific termination signal (Yuen and Moss, 1987). Thus early genes usually carry no termination signal (TTTTTNT) within the coding region but in the 3′ untranslated region. To convert the defective proviral DNA present in the plasmid pLXSN (Fig. 2A) (Miller and Rosman, 1989) into a functioning vaccinia early transcription unit, three internal TTTTTNT signals were removed, and the provirus was cloned into the vector pTKgpt-selP (Pfleiderer et al., 1995), linking it to a strong early–late promoter (Chakrabarti et al., 1997). The resulting plasmid was designated pTKgpt-LXSN. The structure of the defective provirus present in pLXSN is outlined in Fig. 2A; the proviral genome consists of the long terminal repeats (LTRs) with their characteristic repeat (R) and unique (U) regions, the packaging signal Ψ, and an SV40 promoter-neomycin (neo) gene cassette (Miller and Rosman, 1989). In the plasmid pTKgpt-LXSN and the corresponding virus vd-LXSN (see below), the U3 and downstream U5 re-

gions have been deleted from the provirus. The residual transcription unit has been placed under the control of a vaccinia virus promoter and is flanked by thymidine kinase (tk) gene sequences; vaccinia transcription starts upstream of the 5′-repeat (R) and stops at a TTTTTNT signal inserted downstream of the polyadenylation signal of the retroviral transcription unit (Fig. 2B).

Construction and genomic characterization of the defective virus vd-LXSN

The plasmid pTKgpt-LXSN was inserted into the tk locus of the defective vaccinia virus eVAC-1 (Holzer et al., 1998), and recombinants were plaque purified several times in the complementing cell line RK-D4R-44 (see Materials and Methods), finally resulting in the virus
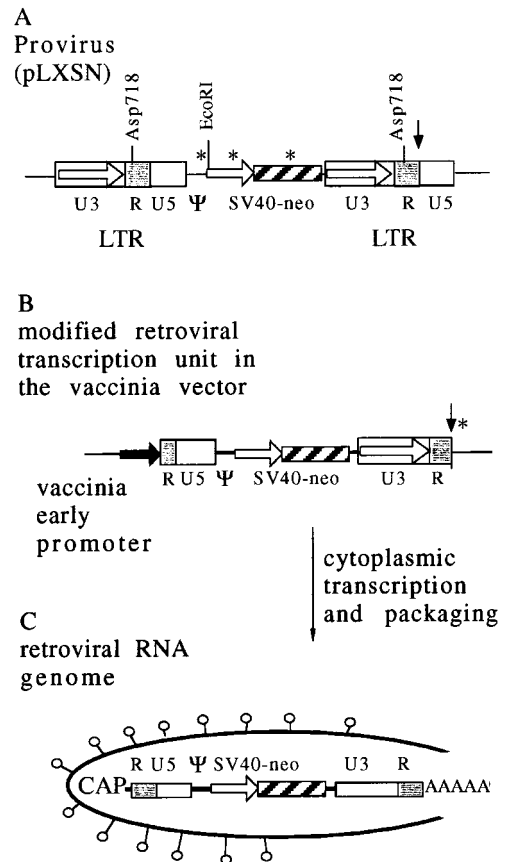

FIG. 2. Schematic representation of the retroviral transcription units in plasmid and virus constructs. (A) Transcription from the pLXSN proviral vector takes place in the nucleus and is driven by the retroviral U3 or the internal SV40 promoter (open arrows). The processing signal AATAAA (vertical arrow) defines the 3′-end of the message, whereas elements with the sequence TTTTTNT (*) are ignored by the nuclear transcription apparatus. (B) A vaccinia virus promoter (filled arrow) controls transcription, and TTTTTNT motifs, acting as early viral transcription termination signals, have been removed from the vector sequence. Transcription is terminated at such a signal downstream of the 3′-R region, resulting in a message that is recognized as genomic retroviral RNA (C) and packaged into nascent virions.
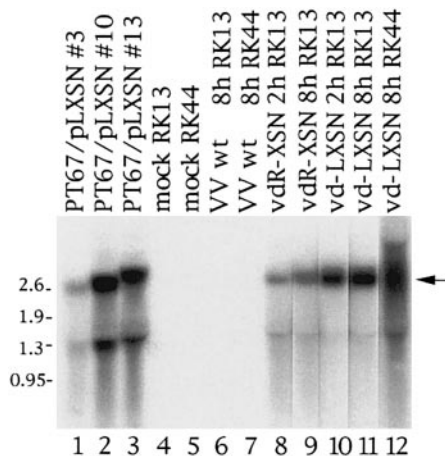
FIG. 3. Northern blot of vaccinia-transcribed retroviral genomes. RK13 cells and the complementing cell line RK-D4R-44 were infected with wild-type or recombinant vaccinia virus as indicated at an m.o.i. of 10 pfu/cell. Total RNA was isolated at 2 or 8 h p.i., whereas RNA of retroviral producer cell lines (lanes 1–3) was isolated when the monolayers reached confluency. The blot was probed with *neo* antisense transcripts. Fragment sizes of the RNA marker are given in kilobases, and the expected retroviral genomic transcript is indicated by an arrow.

vd-LXSN. In the genome of this defective virus, the proviral transcription unit contains the two retroviral "R"-regions (Fig. 2B); these repeats, although small in size (~50 bp), might destabilize the genetic construct and lead to the loss of the insert. To evaluate the stability and exclude potential contamination by parental virus, viral DNAs were prepared, cleaved with appropriate restriction enzymes, and subjected to Southern blotting using *neo*- and *tk*-specific probes. The bands predicted for vd-LXSN were found; no *tk* wild-type gene was detectable, and no evidence for instability was noted, confirming the identity and purity of the construct (data not shown). In addition, replicating virus was not detectable in the defective vaccinia stocks using a sensitive assay protocol (detection limit, one replicating in 4×10^7 defective viruses; see Holzer *et al.,* 1998).

Full-length retroviral transcripts are produced in cells infected with the chimeric virus

Due to the deletion of the UDG gene, the virus vd-LXSN abortively infects its host cells, transcribes exclusively early genes, and produces only early proteins. To check the properties of vd-LXSN in this respect, nonpermissive RK13 cells and complementing RK-D4R-44 cells were infected with the virus, and total RNAs were prepared, glyoxylated, and subjected to Northern blotting. As a positive control, retroviral producer cell lines were established by transfecting the plasmid pLXSN into the packaging cell line PT67 (Fig. 3). In both the producer cell lines (lanes 1–3) and the vd-LXSN-infected cells (lanes 10 and 11), the full-length transcript of the retroviral

genome was detectable by a neomycin-specific probe (arrow). In addition, a transcript most probably initiated by the internal SV40 promoter is visible as a 1.4-kb band in the producer lines (lanes 1–3), whereas only a faint band is detectable, with the vaccinia-induced RNA being synthesized solely by the viral transcription machinery in the cytoplasm of the host cell. This weak band migrates just below the 18S ribosomal RNA band and is probably an artifact produced by compression of RNA, resulting in a higher local hybridization background. Because the virus has a prolonged early phase, the transcripts were checked after 2 and 8 h of infection and accumulation was noted (compare lanes 10 and 11). Late viral RNA prepared from complementing cells showed the typical vaccinia late smear, consisting of heterogeneously sized transcripts (lane 12). The transcriptional analysis confirmed the expected properties of the vaccinia-incorporated proviral transcription unit.

Transduction-competent retroviral particles are produced in a retroviral packaging cell line

To demonstrate that the cytoplasmically transcribed retroviral genomes are packaged and form transducing retroviral particles, colony-forming assays were performed with cell culture supernatants from vaccinia-infected packaging cells. The supernatants were filtered through 0.45-μm filters to remove large particles and plated onto NIH/3T3 indicator cells, which were then subjected to G418 selection. As a positive control, the indicator cells were transduced by particles obtained
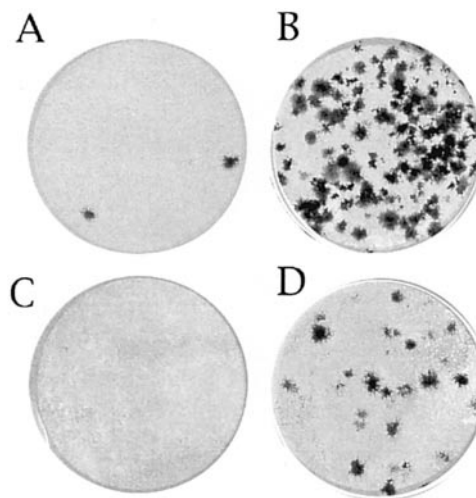

FIG. 4. Colony-forming assay in NIH/3T3 cells with vaccinia-induced retroviral particles. Dishes with subconfluent PT67 packaging cells (10^6 cells) were infected with the vaccinia viruses vd-LXSN (A) or vdR-XSN (B) at an m.o.i. of 1.0 pfu/cell or transfected with 15 μg of pTKgpt-LXSN (C) or pLXSN (D) plasmid DNA. At 8 h after vaccinia virus infection and 48 h after plasmid transfection, respectively, serial dilutions of the supernatants were used to transduce NIH/3T3 cells; 1:100 dilutions of a typical experiment are shown.

A MoMLV   U3   R

TCCGCTCTCCGAGCTCAATAAAAGAGCCCACAACCCCTCACTCGGC|GCGCCAGT|
→

B vd-LXSN   vv promoter selP   R

CTAAAAATTGAAATTTATTTTTTTTTTTTT**GGAATATAAATAAGGA**CGGC|GCGCCAGT|
<u>critical early</u>   →
                       +1

C vdR-XSN   vv promoter PR   R

GCAACTACCGGCATATGCAAAAATTGAAATACTAG|TGAATCCTATT|CGCGCCAGT|
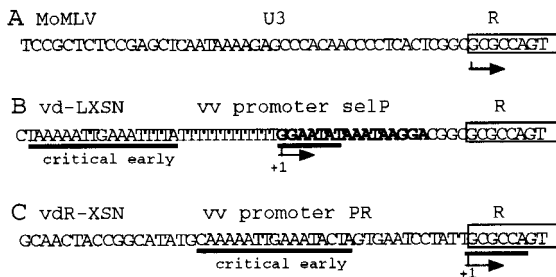   <u>critical early</u>
                       +1

FIG. 5. Sequences around the RNA start sites of the different constructs. Transcription start sites in the Moloney murine leukemia virus (MoMLV) provirus (A) and in two different vaccinia/retrovirus chimeras (B and C) are indicated by arrows. Boxes mark the first nucleotides of the regular retroviral transcript, starting with the R region. The critical early promoter motifs in the vaccinia constructs are underlined. The nonretroviral sequence of the transcript in vd-LXSN (B) is indicated in bold.

after transfection of the packaging cell line with the plasmid pLXSN (Fig. 4D). In the negative control, the plasmid pLXSN was substituted by the vaccinia insertion plasmid pTKgpt-LXSN (Fig. 4C). In the crucial experiment, the packaging cell line PT67 was infected with the virus vd-LXSN, and cell culture supernatants were subjected to the colony-forming assay. Surprisingly, the poxviral/retroviral chimeric virus induced the production of transducing retroviral particles, detectable by their ability to give rise to G418-resistant NIH/3T3 cells in the colony assay (Fig. 4A). Use of an improved chimeric virus (vdR-XSN; see below) even resulted in significantly elevated titers (Fig. 4B). Colony-forming assays, performed with cell culture supernatants obtained after transfection of the packaging cell line with the vaccinia insertion plasmid pTKgpt-LXSN, did not yield transducing colonies because in this construct, the proviral promoter region located in the U3 region of the LTR is substituted by a vaccinia promoter (see Fig. 2), which is not active in the nuclear context. In addition, transcripts originating from the SV40–neo gene cassette lack the retroviral packaging signal and are not usually packaged into particles; even if this were the case, the lack of the 5′ LTR sequences in potential SV40–neo transcripts would prevent reverse transcription and transduction. Therefore, in contrast to the use of nuclear DNA viruses as retroviral carriers, neo-positive colonies result only from retroviral transduction events.

Improvement of the system by precise promoter fusion

The titers of transducing retroviral particles obtained in the prototype vector vd-LXSN were low (see below). A closer inspection of the molecular structure of retroviral genome in vd-LXSN revealed that the fusion of the RNA initiation site of the vaccinia early promoter with the 5′ end of the retroviral genome was suboptimal (Fig. 5). A

new plasmid and an improved virus therefore were constructed, designated pR-XSN and vdR-XSN, respectively; in both, the vaccinia promoter and retrovirus insert are optimally fused (Fig. 5C). In the previous construct (vd-LXSN), transcription starts at a G residue ~20 bases upstream of the proviral R region (Fig. 5B). Because priming of reverse transcription is mediated by the R region, the extra 20 bases do not base pair and should result in inefficient reverse transcription. Therefore, in the improved construct, a synthetic early promoter motif was placed such that transcription starts precisely at the beginning of the R region (Fig. 5C), as confirmed by primer extension experiments (data not shown). The virus vdR-XSN induced the expected early genomic transcripts in infected nonpermissive cells (Fig. 3, lanes 8 and 9). Although the total amount of vector transcripts was lower than that seen in vd-LXSN (lanes 10 and 11), the improved construct vdR-XSN resulted in a retroviral titer exceeding that obtained with vd-LXSN by ~2 logs and the transfected plasmid pLXSN by 1 log (see below, and Fig. 4), showing that the vaccinia system is an efficient tool for the production of defective transduction-competent retroviral particles (see also Fig. 4B). Although the titers in repeated experiments varied with the condition of the packaging and the target cells, the ratio of the titers outlined above remained relatively constant.

The vaccinia-induced retroviral particles integrate into the host genome

Several transduced G418-resistant NIH/3T3 cell clones, obtained from the chimeric poxvirus/retrovirus system (the vdR-XSN/PT67 cell culture supernatants), were grown to larger cultures, and high-molecular-weight genomic DNA was isolated. The DNAs were cleaved with the restriction enzymes Asp718 and EcoRI, subjected to a Southern blot analysis, and hybridized with a neomycin-specific probe. The enzyme Asp718 cleaves in the R regions (see also Fig. 2) resulting in an internal fragment of 2.8 kb (Fig. 6A, arrow) common to proviral transcription units independent of the integration site in the target cell. This common 2.8-kb fragment was visible in the DNAs originating from the plasmid control pR-XSN (Fig. 6A, lane 1) and in the transduced cell lines (lanes 4 and 5). No neo-specific fragments were detectable in the negative control (lane 3).

As predicted, the fragments produced by EcoRI cleavage varied in the different transduced cell clones (Fig. 6B, lanes 4 and 5) because EcoRI is present only once in the provirus. EcoRI cleaves in the middle of the neo gene cassette, resulting in two hybridizing fragments. Each transduced cell clone displayed individually hybridizing fragments, confirming random integration. The analysis confirmed the results of the colony-forming assays; the poxviral/retroviral vectors produce transduction-compe-
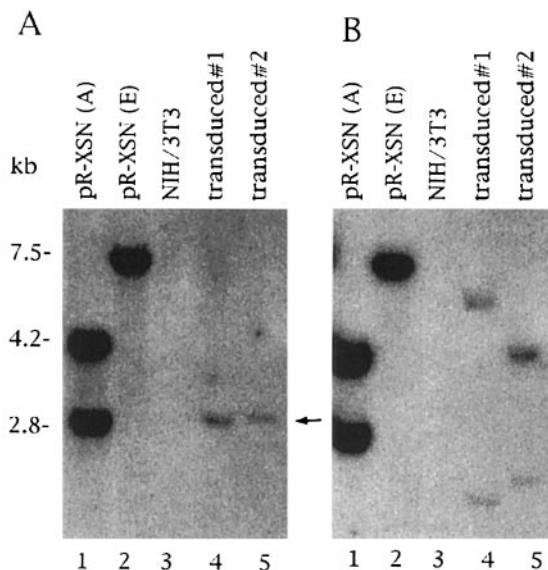
FIG. 6. Southern blots of total DNAs prepared from *neo*-positive cell clones. High-molecular-weight DNA was extracted from wild-type NIH/3T3 cells (lanes 3) and from NIH/3T3 clones transduced with vdR-XSN-induced retroviral particles (lanes 4 and 5). For the positive controls, 30 μg of NIH/3T3 DNA was spiked with 10 pg of the plasmid pR-XSN digested with *Asp*718 (A) or *Eco*RI (E) (lanes 1 and 2). As a negative control, pure NIH/3T3 cell DNA is shown in lanes 3. The cellular DNAs were digested with *Asp*718 (A) or *Eco*RI (B). The blots were probed with pSV2-neo plasmid-DNA.

$(4 \times 10^5$ cfu/ml). More surprisingly, retroviral vector particles could be harvested as early as 3 h postinfection (p.i.), reaching maximal titers at an incubation time between 6 and 9 h (5×10^4 cfu/ml), indicating that packaging, assembly, and release of the defective retroviruses are rapid processes.

DISCUSSION

Because retroviral vectors cannot be grown to high titers, tend to be unstable, and are sensitive to lysis by complement when transfused into patients, more robust retroviral delivery systems are highly desirable. In a previous report, defective adenovirus/retrovirus chimeras were constructed, which allowed *in vivo* retroviral transduction by coinfection of a host cell with two recombinant adenoviruses: one expressing the retroviral genome and the other one expressing the packaging functions (Feng *et al.,* 1997). *In situ* transduction of neighboring cells in an animal could be demonstrated after implantation of doubly infected tumor cells into nude mice. This report showed that chimeric large DNA viruses could induce the production of retroviral particles *in situ* (Feng *et al.,* 1997). A more elegant approach would be the direct use of one large carrier virus obviating the
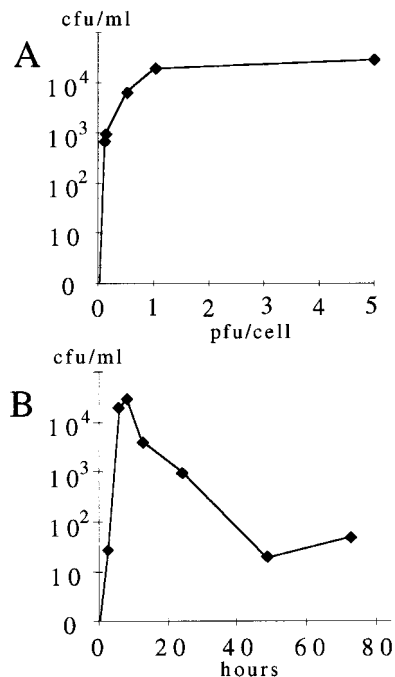
tent retroviral particles, and the provirus stably integrates into the hosts genome and is detectable in repeatedly passaged cell clones.

Optimal production of retroviral particles occurs within the first 8 h of infection

To determine the optimal conditions for the production of retroviral particles, the roles of multiplicity of infection (m.o.i.) and of incubation time in production rates were determined. The optimal infection dose with the improved poxviral/retroviral vector was determined by infecting the packaging cell line PT67 with increasing amounts of vdR-XSN and titrating the supernatants on NIH/3T3 cells in the presence of G418. As shown in Fig. 7A, transduction rates correlated with the vaccinia doses at a low m.o.i., whereas elevation of the m.o.i. over 1 plaque-forming unit per cell led to only a modest raise in retroviral titers. The optimal incubation time was determined by infecting the packaging cells with the improved vector vdR-XSN at an m.o.i. of 0.5 and titering the supernatants at the indicated time points (Fig. 7B). To compare the system with conventional protocols, pLXSN DNA was transfected into packaging cells, and supernatants were titered after 48 h. With 8×10^2 cfu/ml, the transfection-induced titer did not reach the peak values of vaccinia-induced supernatants, whereas the cloned producer line 10 (see also Fig. 3) resulted in significantly higher titers

FIG. 7. Dose dependence and kinetics of vaccinia virus-induced retrovirus production. (A) PT67 packaging cells (10^6 cells) were infected with the vaccinia recombinant vdR-XSN using increasing amounts of virus (pfu/cell). Supernatants were harvested 6 h p.i., and retroviral titers, given in cfu/ml, were determined in focus assays. (B) vdR-XSN infections were done as above at an m.o.i. of 0.5 pfu/cell, and from a total of 5 ml, 200-μl aliquots of supernatant were removed at the times indicated and titrated in focus assays.

need for double infections and implantations. This approach is currently not feasible with classic adenovirus vectors due to the limited packaging capacity (Bett *et al.,* 1993).

An ideal system for this purpose, however, may be defective vaccinia viruses expressing exclusively early genes. Due to the large coding capacity of up to 50 kb and multiple potential insertion sites, it should be feasible to construct vaccinia viruses expressing packaging functions and provirus in one vector. With the goal of obtaining subunit and/or live vaccines, human or simian immunodeficiency virus *gag*, *pol*, and *env* genes have been expressed in vaccinia, and particle formation has been demonstrated (see, for example, Haffar *et al.,* 1990; Hirsch *et al.,* 1996; Karacostas *et al.,* 1989). Our findings now show that modified retroviral genomes, transcribed by vaccinia, can be packaged in packaging cell lines into transduction-competent defective retrovirus. Potential negative effects of the vaccinia infection on the synthesis, assembly, and release of retroviral particles were not observed. A chimeric vaccinia virus expressing packaging functions and the defective provirus will therefore be feasible and may allow *in vivo* retroviral transduction of cells. Moreover, due to the cytoplasmic site of transcription, it should be feasible to construct particularly efficient retroviral transduction units. Vaccinia, in contrast to adenovirus or herpesvirus, recognizes neither splicing nor nuclear polyadenylation (pA) signals. Plasmid expression vectors used to produce proteins in vertebrate cells usually contain introns in their transcription units to enhance expression levels; in particular, the efficient expression of human clotting factor VIII requires intron-containing gene cassettes (Chuah *et al.,* 1995). Therefore, a vaccinia-produced retroviral genome containing introns and internal pA signals should allow transduction of optimal transcription units. Internal pA signals, in addition, prevent transcription of full-length retroviral genomes in the nucleus, adding to the safety of the transduced gene cassette.

Defective vaccinia vectors expressing exclusively early genes, as opposed to other (non)replicating vaccinia vectors, are particularly suited as carriers for small RNA viruses, not only for safety reasons because they do not replicate in natural hosts but also because only correctly processed and dimerized retroviral RNA, as obtained by early transcription, forms functional transducing particles. Vaccinia late transcripts are heterogeneous in size; late RNAs with the correct size and containing a packaging motif may be packaged but presumably give rise to large amounts of pseudoparticles.

With the prototype virus vd-LXSN, the titers obtained were low. On precise fusion of the RNA initiation site of the vaccinia promoter with the first nucleotide of the proviral R region, a ~100-fold increase in the transducing titer was achieved, confirming the requirement of precise

priming in the reverse transcription process. The RNA primer produced in the first step of the reverse transcription process has, with the prototype virus-produced retroviral genomes as a template, a noncomplementary overhang that, when the primer jumps to the 3′ end of the genome, interferes with but obviously does not prevent reverse transcription. A similar effect was seen with retroviral particles produced from Semliki Forest virus vectors (Li and Garoff, 1996; Wahlfors *et al.,* 1997). In conclusion, defective vaccinia vectors induce a relatively efficient production of transduction-competent defective retroviral particles in retroviral packaging cell lines. At the ideal m.o.i. of 1.0 and an incubation time of 8 h, the retroviral titer induced by vaccinia virus exceeded that obtained by the conventional plasmid transfection protocol by ~1 log. The next step in developing an *in vivo* vaccinia-based transduction system will be insertion of the retroviral packaging functions into the vaccinia vector already transcribing the retroviral genome.

MATERIALS AND METHODS

Virus and cell lines

The African Green Monkey kidney cell line CV-1 (ATCC CCL-70), the rabbit kidney cell line RK13 (ATCC CCL-37), the NIH/3T3 cell line (ATCC CRL-1658), and the Western Reserve (WR) strain of vaccinia virus (ATCC VR119) were obtained from American Type Culture Collection. The PT67 cell line was obtained from Clontech Laboratories, Inc. The cell line RK-D4R-44.20 has been described previously (Holzer and Falkner, 1997).

Construction of plasmids

pCR-LX1–pCR-LX4. To substitute the three TTTTTNT signals in the proviral genome present in pLXSN (Miller and Rosman, 1989), a PCR-dependent mutagenesis procedure was applied. Three fragments were amplified by PCR with the pLXSN template LX1-LX3 (1.6, 1.0, and 1.2 kb in size) and subcloned into the pCRII vector (InVitrogen, Inc.). To construct pCR-LX1, the primers oRV-5 (5′-TACG-TACGGC GCGCCAGTCT TCCGATAG-3′) and oRV-6 (5′-GAACCGGTCG CCCCTGCGCT GAC-3′) were used; for pCR-LX2, the primers oRV-7 (5′-AGACGTCCCA GG-GACTTTGG GGGCCGTATT TGTGGC-3′) and oRV-8 (5′-AGGCCGAGGC GGCCTCGGCC TCTGCATAAA TAAATA-AAAT TAG-3′) were used; and for pCR-LX3, the primers oRV-9 (5′-CGACCGGTTC TATTTGTCAA GACCGACCT-3′) and oRV-10 (5′-GCGGCCGCAA CTGCAAGAGG GTTTAT-TGGA-3′) were used. The plasmid pCR-LX4 was constructed by excising the fragment LX2 from pCR-LX2 with *Aat*II and *Sfi*I and inserting it into the plasmid pCR-LX1.

pTKgpt-LX3. The plasmid pTKgpt-selP (Pfleiderer *et al.,* 1995), cleaved with *Nco*I and *Not*I, was ligated with the small 0.8-kb *Nco*I–*Not*I fragment isolated from pCR-LX3,

resulting in pTKgpt-LX3. This plasmid carries the correct 3′ part of the proviral genome.

pLXSNmut. To modify a TTTTTNT signal in the *neo* ORF of pLXSN, a mutagenesis with the primers oRV35 (5′-AAATAGAACC GGTCGCCCCT GCGCTGAC-3′) and oRV-9 (5′-CGACCGGTTC TATTTGTCAA GACCGACCT-3′) using the Quickchange mutagenesis kit (Stratagene, Inc.) was performed, resulting in a new *Pin*AI site in the plasmid pLXSNmut.

pCR-LX4+. Substitution of the unique *Sfi*I–*Sac*I fragment of pCR-LX4 with the modified 1.4-kb *Sfi*I–*Sac*I *neo* cassette from pLXSNmut resulted in the plasmid pCR-LX4+; this plasmid contains the fully assembled modified provirus except for the extreme 3′ end.

pTKgpt-LXSN. Insertion of the 2.7-kb *Sna*BI–*Sac*I fragment into the *Stu*I–*Sac*I cleaved plasmid pTKgpt-LX3 resulted in pTKgpt-LXSN.

pR-XSN. The plasmid pTKgpt-LXSN was digested with *Nde*I and *Bbs*I, thereby excising the early–late vaccinia promoter upstream of the retroviral transcription unit. Ligation with the oligonucleotides oPR1 (5′-TATG-CAAAAA TTGAAATACT AGTGAATCCT ATT-3′) and oPR2 (5′-GCGCAATAGG ATTCACTAGT ATTTCAATTT TTGCA-3′) resulted in the plasmid pR-XSN, in which an optimized early vaccinia virus promoter signal is fused to the retroviral R region.

Construction of the viruses vd-LXSN and vd-RXSN

To construct the defective viruses vd-LXSN and vd-RXSN, the plasmids pTKgpt-LXSN or pTKgpt-RXSN, respectively, were transfected into RK-D4R-44 cells before infection with the defective virus eVAC-1 (Holzer *et al.,* 1998). Plaque assays were done under *gpt* selection (Falkner and Moss, 1988) in the RK-D4R-44 cell line.

Colony-forming assay

NIH/3T3 cells were infected with retroviral particles essentially as described in the user manual of the RetroXpress System (K1060–1; Clontech, Inc.). Selection was performed with 500 μg/ml G418 (Sigma Inc.). Colonies were stained with crystal violet after 10 days.

Establishment of retroviral producer cell lines

PT67 cells (Clontech, Inc.) were transfected with the plasmid pLXSN (Graham and van der Eb, 1973), and G418-resistant colonies were isolated according to standard cell culture procedures.

Northern blots

Total RNA (20 μg) was glyoxylated, resolved on a 1% agarose gel, and blotted onto a nylon membrane (Hybond N; Amersham Inc.). The blot was probed with an-

tisense RNA specific for the complete SV40 promoter and the *neo* gene.

REFERENCES

Berns, K. I., and Giraud, C. (1995). Adenovirus and adeno-associated virus as vectors for gene therapy. *Ann. N.Y. Acad. Sci.* **772,** 95–104.

Bett, A. J., Prevec, L., and Graham, F. L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* **67,** 5911–5921.

Boris-Lawrie, K. A., and Temin, H. M. (1993). Recent advances in retrovirus vector technology. *Curr. Opin. Genet. Dev.* **3,** 102–109.

Chakrabarti, S., Sisler, J. R., and Moss, B. (1997). Compact, synthetic, vaccinia virus early/late promoter for protein expression. *BioTechniques* **23,** 1094–1097.

Chuah, M. K., Vandendriessche, T., and Morgan, R. A. (1995). Development and analysis of retroviral vectors expressing human factor VIII as a potential gene therapy for hemophilia A. *Hum. Gene Ther.* **6,** 1363–1377.

Coffin, J. M. (1996). Retroviridae: The viruses and their replication. *In* "Virology," 3rd ed. (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 1767–1847. 2 vols. Lippincott-Raven, Philadelphia.

Falkner, F. G., and Moss, B. (1988). *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J. Virol.* **62,** 1849–1854.

Feng, M., Jackson, W. H., Goldman, C. K., Rancourt, C., Wang, M., Dusing, S. K., Siegal, G., and Curiel, D. T. (1997). Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector. *Nat. Biotech.* **15,** 864–861.

Graham, F. L., and van der Eb, A. J. (1973). A new technique for the assay of infectifity of human adenovirus 5 DNA. *Virology* **52,** 456–467.

Haffar, O., Garrigues, J., Travis, B., Moran, P., Zarling, J., and Hu, S. L. (1990). Human immunodeficiency virus-like, nonreplicating, gag-env particles assemble in a recombinant vaccinia virus expression system. *J. Virol.* **64,** 2653–2659.

Hay, R. T., Stow, N. D., and McDougall, I. M. (1984). Replication of adenovirus mini-chromosomes. *J. Mol. Biol.* **175,** 493–510.

Hirsch, V. M., Fuerst, T. R., Sutter, G., Carroll, M. W., Yang, L. C., Goldstein, S., Piatak, M., Jr., Elkins, W. R., Alvord, W. G., Montefiori, D. C., Moss, B., and Lifson, J. D. (1996). Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: Effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J. Virol.* **70,** 3741–3752.

Holzer, G., Gritschenberger, W., Mayrhofer, J. A., Wieser, V., Dorner, F., and Falkner, F. G. (1998). Dominant host range selection of vaccinia recombinants by rescue of an essential gene. *Virology* **249,** 160–166.

Holzer, G. W., and Falkner, F. G. (1997). Construction of a vaccinia virus deficient in the essential DNA repair enzyme uracil DNA glycosylase by a complementing cell line. *J. Virol.* **71,** 4997–5002.

Karacostas, V., Nagashima, K., Gonda, M. A., and Moss, B. (1989). Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. *Proc. Natl. Acad. Sci. USA* **86,** 8964–8967.

Kochanek, S., Clemens, P. R., Mitani, K., Chen, H. H., Chan, S., and Caskey, C. T. (1996). A new adenovirus vector: Replacement of all viral coding sequences with 28kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc. Natl. Acad. Sci. USA* **93,** 5731–5736.

Kremer, E. J., and Perricaudet, M. (1995). Adenovirus and adeno-associated virus mediated gene transfer. *Br. Med. Bull.* **51,** 31–44.

Li, K.-J., and Garoff, H. (1996). Production of infectious recombinant Moloney murine leukemia virus particles in BHK cells using Semliki

Forest virus-derived RNA expression vectors. *Proc. Natl. Acad. Sci. USA* **93,** 11658–11663.

Li, Q., Kay, M. A., Finegold, M., Stratford-Perricaudet, L. D., and Woo, S. L. (1993). Assessment of recombinant adenoviral vectors for hepatic gene therapy. *Hum. Gene Ther.* **4,** 403–409.

Miller, A. D. (1997). Development and applications of retroviral vectors. *In* "Retroviruses" (J. M. Coffin, S. H. Hughes, and H. E. Varmus, Eds.), pp. 437–473. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Miller, A. D., and Rosman, G. J. (1989). Improved retroviral vectors for gene transfer and expression. *BioTechniques* **7,** 980–990.

Moss, B. (1996). Genetically engineered poxviruses for recombinant gene expression, vacciniation and safety. *Proc. Natl. Acad. Sci. USA* **93,** 11341–11348.

Paoletti, E. (1996). Applications of poxvirus vectors to vaccination: An update. *Proc. Natl. Acad. Sci USA* **93,** 11349–11353.

Pfleiderer, M., Falkner, F. G., and Dorner, F. (1995). Requirements for optimal expression of secreted and nonsecreted recombinant proteins in vaccinia virus systems. *Prot. Expr. Purif.* **6,** 559–569.

Savard, N., Cosset, F.-L., and Epstein, A. L. (1997). Defective herpes simplex virus type 1 vectors harboring gag, pol, and env genes can be used to rescue defective retrovirus vectors. *J. Virol.* **71,** 4111–4117.

Wahlfors, J. J., Xanthopolous, K. G., and Morgan, R. A. (1997). Semliki Forest virus-mediated production of retroviral vector RNA in retroviral packaging cells. *Hum. Gene Ther.* **8,** 2031–2041.

Yuen, L., and Moss, B. (1987). Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. *Proc. Natl. Acad. Sci. USA* **84,** 6417–6421.