Efficient recovery and regeneration of integrated retroviruses

Brigitte Schott, Eugene S. Kandel and Igor B. Roninson*

Department of Genetics (M/C 669), University of Illinois at Chicago, 900 South Ashland Avenue, Chicago, IL 60607-7170, USA

Received May 21, 1997; Accepted May 27, 1997

ABSTRACT

We report a rapid and efficient PCR-based rescue procedure for integrated recombinant retroviruses. Full-length proviral DNA is amplified by long-range PCR using a pair of primers derived from the long terminal repeats (LTR), and virus is regenerated by transfecting retrovirus-packaging cells with the PCR-derived provirus. The viral yield from the PCR product is similar to that from the retroviral plasmid vector, and the representation of different inserts is accurately maintained in the recovered retroviral population. This procedure is suitable for expression cloning from retroviral libraries and should be applicable to the analysis of natural retrovirus populations.

Retroviral vectors provide one of the most efficient means for gene transfer in mammalian cells. Among other applications, such vectors are used to construct high-complexity libraries for expression cloning of genes (1–3) or genetic suppressor elements (GSEs) (4–6). An integral step in expression cloning is vector recovery from the cells selected for the phenotype of interest; the recovery should be efficient and should adequately reproduce the complexity of the insert sequences present in the selected cells. The usual procedure for the recovery of integrated retroviral vectors involves PCR amplification of inserts from integrated proviruses, which is followed by a labor-intensive step of recloning the PCR products (4). The alternative biological rescue procedures (superinfection with helper virus or fusion with retrovirus-packaging cells) are apt to change the representation of different inserts in the rescued virus population due to differences in virus production by different cells. We have now developed a protocol for rapid and efficient recovery and regeneration of integrated proviruses, which does not require cloning and maintains sequence representation in the recovered virus population. This protocol uses long-range PCR (7,8) to recover functional proviral DNA, which is then used to generate retroviral particles by transient transfection of retrovirus-packaging cells.

The recovery protocol has been developed for the most commonly used type of retroviral vectors based on Moloney murine leukemia/sarcoma viruses and typified by LNCX (9). As illustrated in Figure 1A, the pLNCX plasmid vector contains different 5' and 3' LTR sequences; after reverse transcription, both LTR of the integrated provirus acquire the U3 region from the 3' LTR and the U5 region

from the 5' LTR (10). To amplify the full-length proviral DNA, we have used a sense-oriented (LTRs) primer based on the U3 sequence of the 3' LTR (5'-AATGAAAGACCCACCTGTAGGTTT-GGCAAGCTAG-3') and an antisense-oriented (LTRas) primer from the U5 region of the 5' LTR (5'-CAAATGAAAGACCCCG-TCGTGGGTAGTCAATCAC-3'). Genomic DNA was extracted from retrovirus-transduced cells using Qiagen Blood and Cell Culture DNA kit (high molecular weight and purity of the DNA preparation are critical for the procedure).

Each PCR reaction (50 µl) contained 0.2 mM each of the four dNTPs, 0.5 µg each of LTRs and LTRas primers and 0.5 µg genomic DNA template. In earlier experiments, PCR was carried out in Taq extender buffer (Stratagene) using 10 U Taq DNA polymerase (Promega) and 10 U Taq Extender (Stratagene) per tube. In more recent experiments, we have utilized instead TaqPlus Long low-salt buffer and 5 U of TaqPlus Long polymerase mixture (Stratagene); these conditions provided higher and more reproducible PCR yield. PCR was performed in a Perkin Elmer Cetus thermocycler under the following conditions: 3 min at 94°C; 27 cycles of 1 min at 94°C, 1 min at 65°C, and 2.25 min at 72°C; followed by 5 min at 72°C. Figure 1B (lane P) shows the result of a reaction carried out on the DNA from human HT1080 cells transduced with LNCX. The reaction yields two products, a short 0.7 kb band corresponding to the LTR, and a long 4.1 kb band corresponding to the full-length provirus. These conditions have been successfully used to amplify proviral DNA for LNCX or LXSX (9) based vectors (tested with inserts of up to 1.7 kb in the cloning site) in several different types of human cells. In contrast, the same PCR conditions applied to murine cells transduced with the same vectors yielded almost no full-length proviral DNA detectable by ethidium bromide staining, due to cross-reactivity of the LTRs and LTRas primers with LTR of endogenous murine retroviruses. Amplification of proviral DNA from murine cells was made possible, however, by carrying out a second round of PCR on the provirus-size DNA which was gel-purified (without ethidium bromide staining) after the first round of PCR. This is illustrated in Figure 1C, where genomic DNA template was isolated from murine NIH 3T3 cells that were infected with retroviral vector LRSN, which carries an S65T mutant form of the green fluorescent protein (GFP) (11) in the LXSX vector. After the first round of PCR, 15 µl of the reaction were used for electrophoresis in a 1% agarose gel, and DNA was extracted from the region of the gel corresponding to 3.5–4.5 kb, using QIAquick Gel Extraction Kit (Qiagen). 1/3000 of the recovered DNA was used

*To whom correspondence should be addressed. Tel: +1 312 996 3486; Fax: +1 312 413 8358; Email: roninson@uic.edu

Figure 1. (A) Map of LNCX retroviral vector in the form of a plasmid (pLNCX) or integrated provirus. neo, neomycin phosphotransferase gene; CMV, cytomegalovirus promoter; X, insert in the cloning site of the vector. U3, R and U5 indicate the different LTR regions (10). Positions of the primers used for PCR are indicated (LTRs, LTRas, ATG). The 5' LTR of pLNCX (horizontal stripes) is derived from Moloney murine sarcoma virus, and the 3' LTR (vertical stripes) from Moloney murine leukemia virus; the origin of the LTR sequences in the provirus is indicated by the corresponding stripes. (B) Electrophoretic analysis of a long-range PCR of the DNA from HT1080 cells transduced with the LNCX vector (1% agarose gel). M, size markers (*Hind*III-digested λ DNA); P, PCR reaction; C, negative PCR control (no DNA template). (C) Electrophoretic analysis of a second round of long-range PCR (see text) of the DNA from NIH 3T3 cells that were uninfected (lane 1) or infected with the LRSN vector (lane 2). Lanes M and C are as in (B).

for the second round of PCR under the same conditions. This PCR, when carried out on the DNA from uninfected NIH 3T3 cells, yielded a single band presumably corresponding to the endogenous retrovirus LTR, traces of which remained in the gel-purified sample. In contrast, genomic DNA from LRSN-infected cells yielded bands corresponding to the endogenous and vector-derived LTR, as well as a full-length 4.1 kb LRSN provirus (lane 2).

PCR-amplified proviral DNA (combined with salmon sperm carrier DNA to a total of 15 μ g) was used to transfect BOSC 23 ecotropic retrovirus-packaging cells (12); the transfection and subsequent infection of recipient cells were carried out as previously described (13). In some experiments, PCR-amplified LNCX-based provirus, recovered from HT1080 cells by a single round of PCR, was purified using QIAquick PCR Purification Kit (Qiagen) and products of one to five PCR reactions were used for transfection. Infected cells were obtained under these conditions, but at a relatively low (<3%) rate. To maximize the viral yield from the provirus derived by a single round of PCR, we gel-purified proviral DNA from a mixture of 20 PCR reactions prior to transfection (without ethidium bromide staining). The efficiency of infection with the LRSN virus recovered under these conditions and the ability of this virus to express functional GFP and Neo proteins were evaluated either by the percentage of fluorescent cells expressing GFP (as measured 3 days after infection) or by the formation of G418-resistant colonies. Figure 2 shows the fluorescence profiles of HT1080 cells (expressing the murine ecotropic receptor, 14) that were either uninfected or infected with retrovirus produced by BOSC23 cells after transfection with

Figure 2. Flow cytometric profiles of HT1080 cells, uninfected (A) or infected with retrovirus produced by BOSC23 packaging cells that had been transfected with 1 μ g of LRSN supercoiled plasmid vector (B) or 1 μ g of the PCR-derived LRSN provirus (C). 5×10^5 cells were suspended in phosphate buffered saline containing 1 μ g/ml propidium iodide (PI). Cells were analyzed with FACSsort (Becton-Dickinson) using argon laser excitation (488 nm). PI fluorescence was detected in FL3 emission channel (650LP filter); GFP fluorescence of PI-negative (living) cells was monitored using FL1 emission channel (530/30 BP filter). The data were collected on log scale.

Figure 3. Short-range PCR analysis of representation of different-size inserts in a population of LNCX-derived proviruses. The following templates were used for PCR: genomic DNA of HT1080 cells carrying the integrated provirus population (sample 1), proviral DNA recovered from the same cells by long-range PCR (sample 2), genomic DNA from HT1080 cells transduced with the rescued provirus population (sample 3). Five distinct inserts are designated A-E. (A) Electrophoretic analysis of the PCR products in 6% polyacrylamide gel. M, size standards (123 bp ladder); C, negative PCR control (no DNA template). (B) Relative intensity of bands A-E in samples 1-3, as measured in ethidium bromide-stained polyacrylamide gel using ISO 1000 gel imaging system (Alpha Innotech). The intensity of each band is represented as the percentage of total intensity of all five bands for a given sample.

1 μ g of LRSN plasmid DNA or the same amount of gel-purified LRSN proviral DNA. The percentage of cells infected with the plasmid-derived virus was 34% by fluorescence and 30% by G418 resistance, while the corresponding values for the recovered virus were 32 and 46%, indicating that the PCR-generated linear provirus was transcribed in BOSC23 cells as efficiently as the supercoiled plasmid. In the case of LRSN recovered from murine

NIH 3T3 cells by two rounds of PCR, the product of a single PCR reaction (purified using QIAquick PCR Purification Kit) yielded the infection rate of 7.6% by fluorescence and 10% by G418 resistance (data not shown).

The maintenance of sequence representation in the recovered retrovirus population is illustrated by an experiment carried out on HT1080 cells that were infected with a normalized cDNA fragment library in the LNCX vector and selected for resistance to taxol (E.S.K., unpublished). The cDNA inserts from retroviral vectors integrated in this subpopulation were amplified by PCR using a primer (ATG) corresponding to the adaptor sequence flanking the inserts (5). Figure 3A shows electrophoretic analysis of PCR products amplified directly from genomic DNA, from full-length proviral DNA recovered by long-range PCR, or from genomic DNA of HT1080 cells infected with the recovered provirus and analyzed 3 days after infection. Each lane contains five distinct bands; their relative intensity is shown in Figure 3B. The representation of different bands is very similar in all three lanes, indicating that sequence representation in this relatively simple population has been maintained throughout the procedure.

In summary, the described provirus recovery protocol is rapid (3 days from genomic DNA extraction to the generation of infectious virus), efficient, and capable of maintaining sequence representation in the retroviral population. This protocol should be useful not only for expression cloning in retroviral vectors but also for functional analysis of sequence variability of full-length genomes in naturally occurring 'quasispecies' of different retroviruses (including HIV).

ACKNOWLEDGEMENTS

We thank Elizabeth S.Iraj for technical assistance, Donald Zuhn

and Andrei Gudkov for helpful discussions, and Randal Cox for help with the illustrations. Supported by US National Cancer Institute grants R01-CA56736 and R01-CA62099, US Army grant DAMD17-94-J-4038 (I.B.R.) and US Army predoctoral fellowship DAMD17-96-I-6050 (E.S.K.).

REFERENCES

- 1 Murphy, A.J.M. and Efstratiadis, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8277–8281.
- 2 Rayner, J.R. and Gonda, T.J. (1994) *Mol. Cell. Biol.* **14**, 880–887.
- 3 Kitamura, T., Onishi, M., Kinoshita, S., Shibuya, A., Miyajima, A. and Nolan, G.P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 9146–9150.
- 4 Gudkov, A.V., Zelnick, C.R., Kazarov, A.R., Thimmapaya, R., Suttle, D.P., Beck, W.T. and Roninson, I.B. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2131–2235.
- 5 Gudkov, A.V., Kazarov, A.R., Thimmapaya, R., Axenovich, S.A., Mazo, I.A. and Roninson, I.B. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3744–3748.
- 6 Ossovskaya, V.S., Mazo, I.A., Chernov, M.V., Chemova, O.B., Stresoska, Z., Kondratov, R., Stark, G.R., Chumakov, P.M. and Gudkov, A.V. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 10309–10314.
- 7 Barnes, W.M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 2216–2220.
- 8 Cheng, S., Fockler, C., Barnes, W.M. and Higuchi, R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 5695–5699.
- 9 Miller, D.A. and Rosman, G.J. (1989) *Biotechniques* **7**, 980–986.
- 10 Luciw, P.A. and Leung, N.J. (1992) In Levy, J.A. (ed.), *The Retroviridae 1*. Plenum press, New York and London, chap. 5, pp. 159–263.
- 11 Heim, R., Cubitt, A.B. and Tsien, R.Y. (1995) *Nature* **375**, 663–664.
- 12 Pear, W.S., Nolan, G.P., Scott, M.L. and Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8392–8396.
- 13 Schott, B., Iraj, E.S. and Roninson, I.B. (1996) *Somatic Cell. Mol. Genet.* **22**, 292–309.
- 14 Albritton, L.M., Tseng, L., Scadden, D. and Cunningham, J.M. (1989) *Cell* **57**, 659–666.