JOURNAL OF VIROLOGY, Jan. 1990, p. 424–427
0022-538X/90/010424-04$02.00/0

Vol. 64, No. 1

Human Cells Infected with Retrovirus Vectors Acquire an Endogenous Murine Provirus

DAVID T. SCADDEN,† BARBARA FULLER,‡ AND JAMES M. CUNNINGHAM*

Howard Hughes Medical Institute and Division of Hematology, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Received 24 February 1989/Accepted 18 September 1989

A 5.2-kilobase mouse RNA is expressed in human cells following infection with recombinant retroviruses propagated in mouse NIH 3T3 cells as Ψ-2 pseudotypes. This RNA is transcribed from a defective mink cell focus-forming provirus and copackaged into virions and integrated into human target cell DNA at a frequency comparable to that of the recombinant retrovirus genome.

Cell lines such as Ψ-2 (21) and Ψ-AM (4) express the retroviral genes essential for virus assembly and replication and permit the propagation of retroviruses that can serve as vectors to introduce specified genes into cells by infection (21, 32). These viruses are defective because the genes encoding the virus proteins contain deletions in sequences required for packaging into virions. However, other cellular RNAs may be packaged into retroviruses and eventually become expressed in the infected target cell (2, 3, 14, 19, 27). In this report, we identify a murine provirus transcript which is transferred into human cells by infection with defective retrovirus vectors propagated as Ψ-2 pseudotypes.

We have studied a human EJ cell line, 803, which has acquired susceptibility to infection by murine ecotropic retroviruses as a consequence of transfection with the mouse gene encoding the virus receptor (1). This cell line was identified by exposure to MSV-NEO (36), a recombinant retrovirus which is propagated in mouse Ψ-2 cells and confers survival upon infected cells maintained in culture medium containing G418. Polyadenylated [poly(A)$^+$] RNA from 803 cells was isolated and used as a template to prepare ^{32}P-labeled cDNA (specific activity, $>10^8$ cpm/μg) as part of a cDNA subtraction protocol (10, 20) initially designed to identify the ecotropic receptor gene. This cDNA was hybridized to a 50-fold excess of unlabeled poly(A)$^+$ RNA prepared from uninfected EJ cells. Unhybridized cDNA was then recovered by hydroxyapatite chromatography and applied under hybridization conditions to nitrocellulose filters which contained phage DNA from a BALB/c fibroblast cDNA library (37). Analysis of 12 phages identified by this protocol revealed that they were all derived from the same gene. Analysis of the nucleotide sequence of B5, the phage with the largest insert, demonstrated strong homology to murine leukemia viruses (MuLVs) but not the virus receptor gene (1).

To investigate the origin of the transcript homologous to B5 in the 803 cell line, we examined a Northern (RNA) blot hybridized to a ^{32}P-labeled Moloney murine provirus, clone 836 (11). Autoradiography of this blot (Fig. 1A) identified the 3.3-kilobase (kb) MSV-NEO transcript and the 7.9-kb and 2.9-kb transcripts from the Ψ-2 provirus (21) in Ψ-2 MSV-NEO, the virus producer cell line (lane 1). The MSV-NEO transcript was also identified in RNA from 803 cells (lane 3), but the Ψ-2 provirus transcripts were not present, an expected consequence of their defect in packaging into infectious virus. However, a faint 5.2-kb RNA was detected in 803 RNA (lane 3), the result of cross-hybridization with an abundant RNA homologous to the 836 provirus probe. Indeed, when B5 was applied as a hybridization probe to the same Northern blot (Fig. 1B) prepared by removal of the 836 probe, this 5.2-kb transcript and an additional 2-kb transcript were identified in RNA prepared from both 803 cells and the MSV-NEO virus producer cell line, but not uninfected human EJ cells (lane 2). This result suggested that these transcripts had been acquired by the 803 cell line as a consequence of MSV-NEO virus infection.

To confirm this, we examined RNA isolated directly from MSV-NEO virions and from two other recombinant retroviruses, ZIPgptSV(X) (23) and ZIP-DHFR^r (36), also propagated in Ψ-2 cells. We detected the 5.2-kb transcript in virion RNA (Fig. 2, lanes 3, 4, and 5), but the 2-kb transcript was not identified; it may represent a spliced version of the 5.2-kb transcript that is not efficiently packaged into virions. Examination of several human cell lines infected with amphotropic pseudotypes (Ψ-AM-derived recombinant viruses) also demonstrated the 5.2-kb and 2-kb transcripts (Fig. 2, lanes 10 to 13), proving unequivocally that they were acquired by retrovirus infection.

The steady-state level of these two transcripts varied but could exceed that of MSV-NEO RNA (Fig. 1B, lanes 3 and 4) despite selection of the infected cells for expression of the neomycin resistance gene within the recombinant virus genome. The expression of these transcripts was independent of the recombinant retrovirus structure (Fig. 2, lanes 7, 10, and 11) or the target cell type (Fig. 2, lanes 7, 12, and 13). In addition, NIH 3T3 cells expressed these transcripts (Fig. 2, lane 1), and therefore other virus-packaging cell lines derived from these cells (5, 22) may produce recombinant virus containing these transcripts.

We compared the relative frequency of transfer of the 5.2-kb and the MSV-NEO transcript by performing Southern blots with *Eco*RI-digested DNA prepared from three different clones of EJ cells (803, C-26, and C-3) infected with MSV-NEO virus. The filters were initially hybridized to a ^{32}P-labeled MSV-NEO-derived probe with the 700-base-pair (bp) *Pvu*II restriction fragment from the MSV-NEO provirus. Since this fragment does not contain an *Eco*RI site, each MSV-NEO virus integration was identified as a single hy-

* Corresponding author.
† Present address: Division of Hematology/Oncology, New England Deaconess Hospital, Boston, MA 02215.
‡ Present address: School of Veterinary Medicine, Tufts University, Boston, MA 02111.

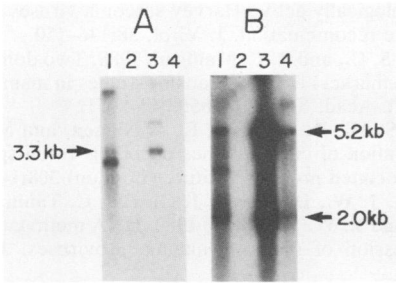

FIG. 1. Identification of a transcript expressed in a human EJ cell line 803 after infection with MSV-NEO retrovirus. An autoradiogram of the same Northern blot appears in each panel. The blot was probed with ^{32}P-labeled ecotropic provirus 836 (A) and B5 phage insert (B). Probes were generated by random primer labeling (5) to a specific activity of >108 cpm/μg. Lane 1, Ψ-2 MSV NEO virus producer cell line; lane 2, uninfected EJ cells; lane 3, 803; lane 4, 803 repeated. Lanes 1 to 3 were loaded with 5 μg and lane 4 was loaded with 500 ng of poly(A)$^+$ RNA.

bridizing band (Fig. 3A). After autoradiography, the filter was stripped of the MSV-NEO probe and rehybridized to the ^{32}P-labeled B5 (Fig. 3B) *Eco*RI insert. Comparison of the number of hybridizing bands in Fig. 3B and 3A demonstrates that integration of the 5.2-kb transcript cDNA into the target cell DNA occurred at only a slightly lower frequency than MSV-NEO integration in all three cell lines. These cell lines were identified by selection of MSV-NEO expression in G418-containing culture medium; the relative frequency of B5 integration may be higher in an unselected population of infected cells.

To obtain an exact cDNA copy of the transcripts, we used B5 as a hybridization probe on a cDNA library prepared

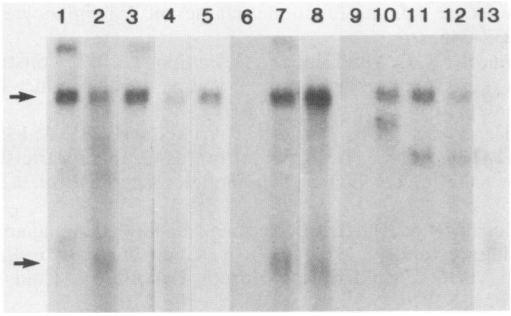

FIG. 2. B5 hybridizes to transcripts present in viral RNA and in poly(A)$^+$ RNA prepared from human cell lines infected with Ψ-2 retroviruses. A Northern blot was hybridized to the ^{32}P-labeled *Eco*RI insert of B5. Sources of RNA: lane 1, NIH 3T3 fibroblasts; lane 2, Ψ-2 MSV-NEO producer cells; lane 3, ZIPgptSV(X) virions; lane 4, MSV-NEO virions; lane 5, ZIP-DHFR^r virions; lane 6, uninfected human EJ cells; lane 7, C-26 cells (EJ cells transfected with the gene encoding the murine ecotropic retrovirus receptor, infected with MSV-NEO, and selected in G418-containing medium); lane 8, 803 cells; lane 9, HeLa cells; lane 10, EJ cells infected with amphotropic ZIP-NEOSV(X) virus (4) and selected in G418-containing medium; lane 11, EJ cells infected with amphotropic SV2his virus (9) and selected in L-histidinol-containing medium; lane 12, HeLa cells infected with amphotropic SV2his virus and selected in L-histidinol-containing medium; lane 13, HepG2 cells infected with amphotropic SV2his virus and selected in L-histidinol-containing medium. Lanes 8 and 13 contain 1 μg of RNA; all others contain 5 μg. All the data were generated from a single Northern blot. Lanes 1, 2, 3, 6, and 7 were exposed to film for 16 h, lanes 4, 5, and 9 to 12 were exposed for 24 h; lane 8 was exposed for 6 h; and lane 13 was exposed for 36 h. Arrows indicate 5.2- (top) and 2.0-kb transcripts.

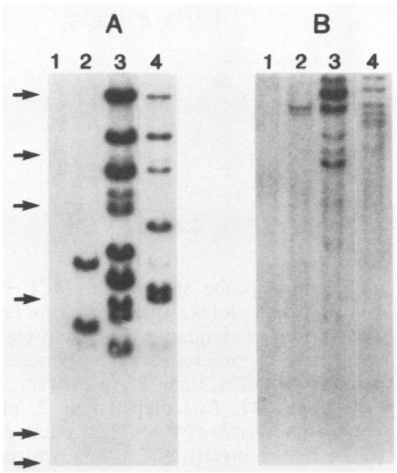

FIG. 3. Integration frequency of the provirus homologous to B5 in human EJ cells infected with MSV-NEO. Three different EJ clones that expressed the ecotropic retrovirus receptor were infected with ecotropic MSV-NEO virus and selected with G418. DNA was prepared from each cell line, digested with *Eco*RI, and analyzed by a Southern blot hybridized to a ^{32}P-labeled 0.7-kb *Pvu*II restriction fragment from the MSV-NEO provirus (A) and after removal of the probe, to the ^{32}P-labeled B5 insert (B). Lane 1, uninfected EJ cells; lane 2, C-26 cells; lane 3, 803 cells; lane 4, C3 cells. Size markers (top to bottom) are 23, 9.4, 6.5, 4.5, 2.2, and 2.0 kb.

from 803 poly(A)$^+$ RNA (1). We obtained a recombinant phage, HT4, which contained a 1.9-kb insert that reproduced the hybridization pattern of B5 (data not shown). Analysis of the nucleotide sequence of HT4 identified a 430-bp region with strong homology to the long terminal repeat (LTR) of MuLV and included a 190-bp sequence characteristic of endogenous proviruses (17, 26, 31). In addition, an oligonucleotide sequence which is unique to the envelope gene of the MCF subgroup of MuLV (25) hybridized on a Southern blot to HT4 DNA (hybridization: Gene Screen Plus; 50% formamide, 1 M NaCl, 1% sodium dodecyl sulfate [SDS], 10% dextran sulfate, 500 μg of fish DNA per ml, 42°C; wash: 2× SSC–0.1% SDS, 42°C) but not to DNA from other MuLV subgroups (Fig. 4). Therefore, analysis of both the LTR and *env* sequence of HT4 was consistent with its origin from an endogenous MCF-like provirus.

The culture medium of 803 cells did not contain reverse transcriptase activity, consistent with a lack of virus production. Furthermore, when the HT4 nucleotide sequence was compared with that of a replication-competent MCF MuLV, MCF247 (13), a large (>l kb) deletion in the HT4 envelope gene was identified. Therefore, the HT4 retrovirus is apparently defective.

Expression of defective proviruses such as HT4 may potentially have a number of diverse effects upon the host cell. For example, the HT4 provirus may encode antigenic viral proteins (16, 29), which could provoke a host immune response against the infected cells. Also, acquisition of defective proviruses similar to HT4 by retrovirus infection has been associated with host immune suppression (2, 3, 27). Furthermore, recombination (15, 28) between the HT4 provirus and the retrovirus vector genome or other sequences packaged into the virion (2, 3, 14, 19, 27, 30) could result in new combinations of functional genes (8), including replication-competent viruses (34). Finally, important cellular genes in the target cell genome may be disrupted as a

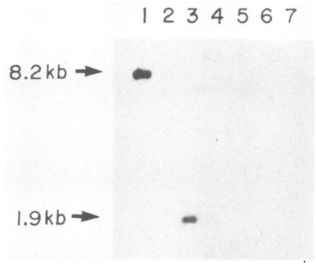

FIG. 4. Oligonucleotide probe specific for MCF subgroup hybridizes to HT4. A Southern blot was prepared from DNA obtained from plasmids that contain *env* sequences of proviruses representing different murine retrovirus subgroups and hybridized to a ^{32}P-labeled MCF-specific probe (25). Lane 1, pW247 (12), derived from cloned MCF virus MCF 247, *Pst*I digest; lane 2, pNZB-X (24), derived from a xenotropic provirus from NZB mice, *Eco*RI digest; lane 3, pHT4 1.9-kb *Eco*RI insert; lane 4, 836 ecotropic Moloney provirus (11), *Eco*RI digest; lane 5, pMX14 (J. P. Stoye, unpublished data), a 3.3-kb subclone of the ecotropic AKV provirus from the HRS/J mouse that contains *env* sequences, *Hin*dIII digest; lane 6, pMAV-(4), a recombinant provirus that contains *env* sequences derived from the amphotropic virus 4070A *Eco*RI digest.

consequence of integration of the provirus into cellular DNA (7, 18, 33). Knowledge of the presence of this "hitchiker" sequence may therefore be relevant for the use of retroviruses propagated in murine fibroblasts as insertional mutagens or in human gene therapy. Elimination of this MCF-like RNA from retroviral vectors may require their propagation in cell lines from species with minimal provirus content.

We gratefully acknowledge the support and encouragement of H. Franklin Bunn. Jonathan Stoye, Jeff Morgan, David Williams, David Wolf, and Lise Riviere provided important reagents. Michelle Hoffman and Robert Weinberg provided useful criticism of the manuscript.

D.T.S. was supported in part by a Physician-Scientist Award from the National Institutes of Health. This work was supported by the Howard Hughes Medical Institute and by a Public Health Service grant from the National Institutes of Health to J.M.C.

LITERATURE CITED

1. **Albritton, L. M., L. Tseng, D. Scadden, and J. M. Cunningham.** 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell **57**:659–666.
2. **Aziz, D. C., Z. Hanna, and P. Jolicoeur.** 1989. Severe immunodeficiency disease induced by defective murine leukaemia virus. Nature (London) **338**:505–508.
3. **Chattopadhyay, D. K., H. C. Morse, M. Makino, S. K. Ruscetti, and J. W. Hartley.** 1989. Defective virus is associated with induction of murine retrovirus-induced immunodeficiency syndrome. Proc. Natl. Acad. Sci. USA **86**:3862–3866.
4. **Cone, R. D., and R. C. Mulligan.** 1984. High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. Proc. Natl. Acad. Sci. USA **81**:6349–6353.
5. **Eglitis, M. A., P. Kantoff, E. Gilboa, and W. F. Anderson.** 1985. Gene expression in mice after high-efficiency retroviral-mediated gene transfer. Science **230**:1395–1398.
6. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–269.
7. **Gisselbrecht, S., S. Fichelson, B. Sola, D. Bordeaux, A. Haupe, C. Andre, F. Galibert, and P. Tambourin.** 1987. Frequent c-*fms* activation by provirus insertion in mouse myeloblastic leukemia. Nature (London) **329**:259–260.
8. **Goldfarb, M. P., and R. A. Weinberg.** 1981. Generation of novel, biologically active Harvey sarcoma viruses via apparent illegitimate recombination. J. Virol. **38**:136–150.
9. **Hartman, S. C., and R. C. Mulligan.** 1988. Two dominant-acting selectable markers for gene transfer studies in mammalian cells. Proc. Natl. Acad. Sci. USA **85**:8047–8051.
10. **Hedrick, S. M., D. I. Cohen, E. A. Nielsen, and M. M. Davis.** 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature (London) **308**:149–153.
11. **Hoffmann, J. W., D. Steffen, J. Gusella, C. Tabin, S. Bird, D. Cowing, and R. W. Weinberg.** 1982. DNA methylation affecting the expression of murine leukemia proviruses. J. Virol. **44**: 144–147.
12. **Holland, C. A., J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1985. At least four viral genes contribute to the leukemogenicity of murine retrovirus MCF 247 in AKR mice. J. Virol. **53**: 158–165.
13. **Holland, C. A., J. Wozney, and N. Hopkins.** 1983. Nucleotide sequence of the gp70 gene of murine retrovirus MCF 247. J. Virol. **47**:413–420.
14. **Howk, R. S., D. M. Troxler, D. Lowy, P. Duesberg, and E. M. Scolnick.** 1978. Identification of a 30S RNA with properties of a defective type C virus in murine cells. J. Virol. **25**:115–123.
15. **Itin, A., and E. Keshet.** 1983. Apparent recombinance between virus-like (VL30) and murine leukemia-related sequences in mouse DNA. J. Virol. **47**:178–184.
16. **Jongstra, J., and C. Moroni.** 1981. Lipopolysaccharide induces retroviral antigen expression in 129/J mouse lymphocytes: evidence for assembly of a defective viral particle. J. Virol. **37**:1044–1050.
17. **Khan, A. S., and M. A. Martin.** 1983. Endogenous murine leukemia proviral long terminal repeats contain a unique 190-base-pair insert. Proc. Natl. Acad. Sci. USA **80**:2699–2703.
18. **King, W., M. D. Patel, L. I. Lobel, S. Goff, and M. C. Nguyen-Huu.** 1985. Insertion mutagenesis of embryonal carcinoma cells by retroviruses. Science **228**:554–559.
19. **Linial, M.** 1987. Creation of a processed pseudogene by retroviral infection. Cell **49**:93–102.
20. **Littman, D. R., Y. Thomas, P. J. Maddon, L. Chess, and R. Axel.** 1985. The isolation and sequence of the gene encoding T8: a molecule defining functional classes of T lymphocytes. Cell **40**:237–246.
21. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
22. **Miller, A. D., M. A. Bender, E. A. S. Harris, M. Kaleko, and R. E. Gelinas.** 1988. Design of retrovirus vectors for transfer and expression of the human beta-globin gene. J. Virol. **62**:4337–4345.
23. **Mulligan, R. C.** 1982. Development of new mammalian transducing vectors, p. 133–143. *In* Y. Gluzman (ed.), Eukaryotic viral vectors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **O'Neill, R. R., C. E. Buckler, T. S. Theodore, M. A. Martin, and R. Repaske.** 1985. Envelope and long terminal repeat sequences of a cloned infectious NZB xenotropic murine leukemia virus. J. Virol. **53**:100–106.
25. **O'Neill, R. R., A. S. Khan, M. D. Hoggan, J. W. Hartley, M. A. Martin, and R. Repaske.** 1986. Specific hybridization probes demonstrate fewer xenotropic than mink cell focus-forming murine leukemia virus *env*-related sequences in DNAs from inbred laboratory mice. J. Virol. **58**:359–366.
26. **Ou, C. Y., L. R. Boone, and W. K. Yang.** 1983. A novel sequence segment and other nucleotide structural features in the long terminal repeat of a BALB/c mouse genomic leukemia virus-related DNA clone. Nucleic Acids Res. **11**:5603–5619.
27. **Overbaugh, J., P. R. Donahue, S. L. Quackenbush, E. A. Hoover, and J. I. Mullins.** 1988. Molecular cloning of a feline leukemia virus that induces fatal immunodeficiency disease in cats. Science **239**:906–910.
28. **Panganiban, A. T., and D. Fiore.** 1988. Ordered interstrand and intrastrand DNA transfer during reverse transcription. Science **241**:1064–1069.
29. **Scheele, C. M., and H. Hanafusa.** 1971. Proteins of helper-

dependent RSV. Virology **45**:401–410.
30. **Scolnick, E. M., W. C. Vass, R. S. Howk, and P. H. Duesberg.** 1979. Defective retrovirus-like 30S RNA species of rat and mouse cells are infectious if packaged by type C helper virus. J. Virol. **29**:964–972.
31. **Stoye, J. P., and J. M. Coffin.** 1987. The four classes of endogenous murine leukemia virus: structural relationship and potential for recombination. J. Virol. **61**:2659–2669.
32. **Tabin, C., J. Hoffman, S. Goff, and R. Weinberg.** 1982. Adaptation of a retrovirus as a eukaryotic vector transmitting the herpes simplex virus thymidine kinase gene. Mol. Cell Biol. **2**:426–436.
33. **Varmus, H. E., N. Quintrell, and S. Ortiz.** 1981. Retroviruses as mutagens: insertion and excision of a nontransforming provirus alter expression of a resident transforming provirus. Cell. **25**: 23–36.
34. **Varmus, H. E., and R. Swanstrom.** 1985. Replication of retroviruses, p. 369–512. *In* R. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Williams, D. A., I. R. Lemischka, D. G. Nathan, and R. C. Mulligan.** 1984. Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature (London) **310**:476–480.
36. **Williams, D. A., K. Hsieh, A. DeSilva, and R. C. Mulligan.** 1987. Protection of bone marrow transplant recipients from lethal dose of methotrexate by generation of methotrexate-resistant bone marrow. J. Exp. Med. **166**:210–218.
37. **Wolf, D., N. Harris, N. Goldfinger, and V. Rotter.** 1985. Isolation of a full-length mouse cDNA clone coding for an immunologically distinct p53 molecule. Mol. Cell. Biol. **5**:127–132.