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New Retrovirus Helper Cells with Almost No Nucleotide Sequence Homology to Retrovirus Vectors

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We prepared retrovirus packaging cell lines containing *gag-pol* genes from spleen necrosis virus (expressed from a cytomegalovirus promoter and the simian virus 40 (SV40) polyadenylation sequences) and, on a separate vector, either the *env* gene from spleen necrosis virus (expressed from the Rous sarcoma virus promoter and the SV40 polyadenylation sequences) or the *env* gene from amphotropic murine leukemia virus (expressed from a cytomegalovirus promoter and the SV40 polyadenylation sequences). The nucleotide sequences in these packaging cell lines have almost no homology to the retrovirus vectors we used. Retrovirus vectors were produced from these new helper cell lines without any genetic interactions between the vectors and sequences in the helper cells and without transfer of the packaging sequences.

Retrovirus vectors have been widely used (for reviews, see references 4 and 20). Most of these vectors are replication defective as a result of the deletion of virus coding sequences. Helper or packaging cell lines were developed for the propagation of replication-defective retrovirus vectors in the absence of helper viruses. Many studies with these helper cell lines have been reported, but problems like recombination of the vector with sequences in the helper cell to form replication-competent retroviruses, passage of packaging genomes with the vector, and unwanted changes of vector sequences during transfection have occurred (1, 10, 15, 16; this paper).

In our original reticuloendotheliosis virus (Rev) helper cells (C3 cells), we expressed the Rev *gag-pol* and *env* genes from different deleted proviruses to prevent some of these problems (22). (C3 helper cells contain spleen necrosis virus [SNV] DNAs deleted from 0.68 to 0.98 kilobase pairs [kbp] and from 0.54 to 5.9 kbp [22]. The SNV encapsidation sequences are between 0.68 and 0.86 kbp [6, 22].) However, this procedure was not sufficient to prevent the occurrence of some recombination, transfer of packaging sequences, and other unwanted genetic changes (10; this paper; unpublished data). Therefore, we constructed new helper cell lines with the *gag-pol* and the *env* genes expressed from two separate expression vectors with almost no nucleotide sequence homology to the vector sequences. Recently, others modified one of the long terminal repeats (LTRs), separated the two *gag-pol* and *env* genes for the same purpose, or both (1, 15, 26). However, gene conversion of vector sequences could still occur with such helper cells.

Unwanted genetic changes in C3 helper cells. JD220SVHy is a retrovirus vector with a U3 deletion in the right-hand LTR region except for 14 base pairs 5′ of the *Sac*I site required for integration (2) (Fig. 1). It should be noted that these 14 base pairs from the U3 region remaining in the 3′ LTR are deleted in the 5′ LTR of JD220SVHy to prevent homologous recombination between different vector molecules to regenerate a wild-type 3′ U3 region. Upon infection of sensitive cells, JD220SVHy forms a provirus with both LTRs having deleted U3 regions (2, 3). Thus, an infected cell should not contain any viral transcripts initiating from the virus LTR, and no virus can be produced.

However, some C3 helper cells infected with virus from C3 cells transfected with pJD220SVHy released infectious vector virus (R. Dornburg, personal communication). For example, in experiment 1, C3 cells were transfected with pJD220SVHy (designated step 1 cells) and produced virus of 4×10^5 CFU/ml at 3 weeks postinfection when assayed on D17 cells (8, 11, 12, 21). (Virus harvested from helper cells was clarified by centrifugation followed by immediate use. We have found that vector virus produced from helper cells is quite unstable relative to wild-type virus [unpublished data].) The virus from the step 1 pJD220SVHy-transfected C3 cells was used to infect fresh C3 helper cells. (These infected cells are designated step 2 cells.) The titer was reduced by a factor of 100 as a result of the expression of the SNV *env* gene in the C3 cells. Supernatant media were collected from the step 2 cells and were used to infect fresh D17 cells which were selected for hygromycin resistance. About 50 CFU of vector virus per ml were produced from mass cultures of the step 2 C3 cells 1 week after infection, and 10^3 CFU/ml were produced 2 weeks after infection. These results indicated that a functional U3 region in the vector LTR had been restored in the C3 cells; otherwise it would not have been possible to pass virus from the step 2 C3 cells. In addition, when the D17 cells infected with vector virus from the step 2 C3 cells were superinfected with replication-competent Rev strain A, transforming vector virus of 10^6 CFU/ml was recovered (data not shown). This result provides further evidence for the restoration of a functional U3 region of the LTR in the JD220SVHy vector in the C3 cells.

To prevent homologous genetic interactions between the viral sequences in the helper cells and the vector, we made helper cells with almost no nucleotide sequences homologous to those in our vectors.

Plasmid constructions. pJDCMV19SV (Fig. 1) was constructed in two steps. First, the *Hin*dIII fragment containing the cytomegalovirus immediate-early gene promoter isolated from pCATwt760 (19) was inserted into the *Hin*dIII site of pUC19, creating pJDCMV19. Second, the *Bam*HI-*Hin*dIII

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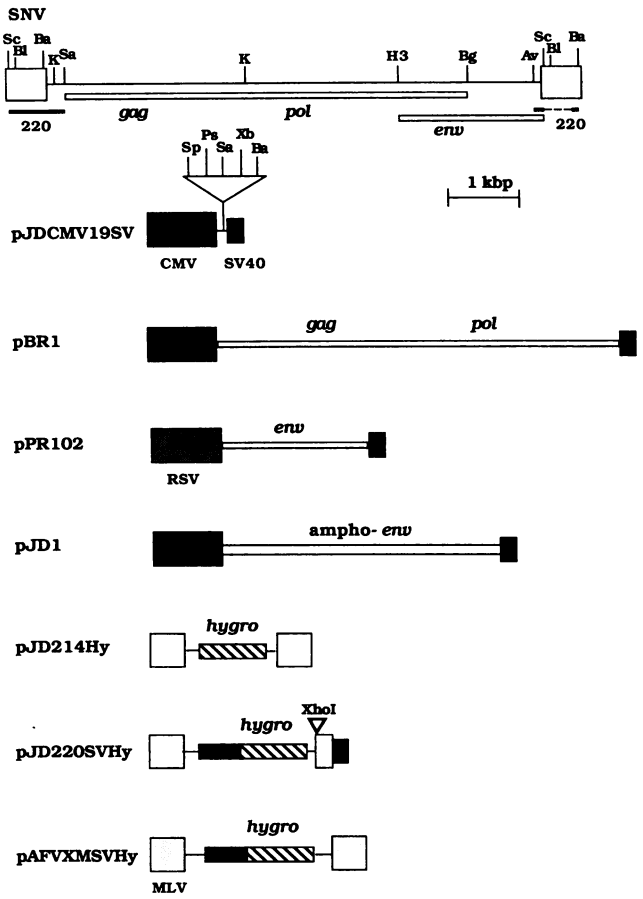

FIG. 1. Structure of constructs. A genome of SNV is presented with the restriction enzyme cleavage sites used indicated. The open bars directly underneath represent the sequences in the indicated vectors. (The LTRs of pJD220SVHy do not contain *Sac*I sites; there is a *Bal*I site only in the 5′ LTR.) The dark stippled boxes beside pJDCMV19SV, pBR1, and pJD1 represent the CMV immediate-early gene promoter; closed boxes represent the SV40 late-gene polyadenylation signal; the dark stippled box marked RSV represents the RSV LTR; open boxes represent the SNV LTR; the inverted triangle indicates a deletion of the U3 region of the SNV LTR into which a *Xho*I linker was inserted; the light stippled boxes marked MLV represent the murine leukemia-sarcoma virus LTR of AFVXM (13); the closed bars represent the SV40 late promoter; the hatched bars marked *hygro* represent the hygromycin resistance gene; *gag*, *pol*, and *env* refer to the genes of SNV; ampho-*env* refers to the envelope gene of amphotropic MLV 4070A; 220 refers to the sequences of SNV in pJD220SVHy. Sc, *Sac*I; Bl, *Bal*I; K, *Kpn*I; Ba, *Bam*HI; Sa, *Sal*I; H3, *Hin*dIII; Bg, *Bgl*II; Av, *Avr*II; Sp, *Sph*I; Ps, *Pst*I; Xb, *Xba*I.

fragment isolated from pSVL-141/+79 (24) containing the simian virus 40 (SV40) late polyadenylation signal was inserted by blunt-ended ligation into the *Sma*I site of pJDCMV19, resulting in pJDCMV19SV.

Three steps were required to make pBR1, which expresses the *gag-pol* gene of SNV by using the cytomegalovirus immediate-early promoter and the SV40 polyadenylation sequences. (i) The 3′ *Kpn*I-*Bgl*II fragment from SNV (DNA coordinates 3.5 to 6.629 kbp [25]) was substituted for the *Kpn*I-*Bgl*II fragment of pUC19, making pBRS. (ii) The 5′ *Kpn*I fragment from SNV (DNA coordinates 0.676 to 3.5 kbp) was inserted into the *Kpn*I site of pBRS, resulting in pBRSNV. (iii) The *Sal*I fragment from pBRSNV containing the SNV *gag-pol* gene was cloned into the *Sal*I site of pJDCMV19SV, creating pBR1.

TABLE 1. Virus production by DSN and DAN helper cells[a]

Helper cell	Vector	Titer (CFU/ml) on indicated cells		
		D17	Rat-1[b]	NIH 3T3
DSN	JD214Hy	7×10^5	3×10^4	10
	AFVXMSVHy	4×10^4	3×10^4	0
DAN	JD214Hy	4×10^4	5×10^3	50
	AFVXMSVHy	1×10^4	8×10^3	4×10^2

[a] The indicated helper cells were infected with helper-free vector virus from DSN cells transfected with the indicated vector DNA, and clones were picked. Vector virus production was assayed 10 weeks after infection.

[b] The titers for Rat-1 cells were from a separate experiment and are normalized to the relative titers on D17 cells.

pPR102 expresses the *env* gene of SNV by using the Rous sarcoma virus (RSV) long terminal repeat and the SV40 polyadenylation sequences. It was constructed by replacing the *Hin*dIII-*Hpa*I fragment of pRSVCAT (9) with the *Hin*dIII-*Sac*I fragment of SNV (DNA coordinates 5.653 to 7.747 kbp [22]) by blunt-ended ligation.

pJD1 expresses the *env* gene from amphotropic murine leukemia virus (MLV) strain 4070A by using the cytomegalovirus immediate-early promoter and the SV40 polyadenylation sequences. It was made by inserting the *Sal*I fragment from pTranspapp (17) containing the *env* gene from amphotropic MLV 4070A into the *Sal*I site of pJDCMV19SV.

Other vectors have been described (2, 3, 5–7, 18).

Virus production by DSN and DAN cells. D17 cells were transfected with the *gag-pol* and *env* expression vectors (Fig. 1) along with SV2neo and were selected with G418. The resulting colonies were grown and were screened for helper cell activity. Clones transfected with pBR1, pPR102, and SV2neo and containing *gag-pol* and *env* genes from SNV were obtained and were designated DSN cells. Clones transfected with pBR1, pJD1, and SV2neo and containing *gag-pol* genes from SNV and the *env* gene from amphotropic MLV were obtained and were designated DAN cells.

The DSN and DAN cells were transfected with pJD214Hy and pAFVXMSVHy (Fig. 1), and hygromycin-resistant cells were selected. Virus harvested from these cells was assayed on D17, Rat-1, and NIH 3T3 cells (Table 1). Virus production by the DSN cells was similar to that of previously described Rev helper cells (5–7). JD214Hy was produced at a higher titer than AFVXMSVHy; the titers of both vectors on rat cells was almost as high as on dog cells; there was almost no infection of mouse cells.

Vector virus was produced by the DAN cells but at a lower titer than by the DSN cells. Again, JD214Hy was produced at a higher titer than AFVXMSVHy, and the titers of both viruses on rat cells were only slightly lower than those of viruses on dog cells. Both vector viruses infected mouse cells; the titer of the MLV-based AFVXMSVHy was higher than that of the Rev-based JD214Hy. This result is expected based on those previously reported (7), which showed that transcription from LTRs of Rev-based vectors is suppressed in mouse cells.

Tests for formation of replication-competent virus and for restoration of deleted U3 sequences. DSN cells were transfected with pJD214Hy and were selected for hygromycin resistance. The resulting hygromycin-resistant cells were passaged. At 21 and 35 days after transfection, virus was harvested from confluent DSN cells and was used to infect D17 cells. On day 5 after infection of these D17 cells,

supernatant media were assayed for DNA polymerase activity; none was found (data not shown). The supernatant media were also used to inoculate fresh D17 cells and chicken embryo fibroblasts. The fresh D17 cells were selected for hygromycin resistance. No resistant colonies were found (data not shown). The media from the inoculated chicken embryo fibroblasts were, 5 days after inoculation, assayed for release of sedimentable DNA polymerase activity. No DNA polymerase activity was found (data not shown).

These results indicated that replication-competent virus was not produced by the DSN cells after transfection with pJD214Hy and passage of the transfected DSN cells and that the packaging sequences were not transferred to infected D17 cells. If replication-competent virus had been produced, the infected D17 cells would have produced virus which could infect chicken embryo fibroblasts, would have been detectable in a reverse transcriptase assay, and would have transferred the JD214Hy genome to fresh D17 cells.

In another test for the transfer of the packaging sequences or formation of replication-competent virus, supernatant medium from DSN cells transfected with pJD214Hy and passaged for 53 days was plated on G418-resistant D17 cells transfected with an SNV neomycin-resistant vector, and hygromycin-resistant colonies were selected. (The titer of virus was 10^5 CFU/ml.) On day 11 after infection, supernatant medium from a confluent plate of hygromycin- and G418-resistant cells was used to inoculate fresh D17 cells and these cells were selected for G418 resistance. No G418-resistant colonies were found (data not shown). In addition, DSN and DAN cells transfected with pJD214Hy were grown together for 30 days, and supernatant medium was used to inoculate D17 cells, which were tested for DNA polymerase activity. None was found (data not shown).

In addition, experiments with pJD220SVHy like that described above (experiment 1) were performed with the DSN and DAN helper cells. DSN and DAN cells were transfected with pJD220SVHy. Virus was harvested and assayed on fresh DSN cells, resulting in two sets of step 2 DSN hygromycin-resistant colonies. At 3 weeks postinfection, supernatant media from these colonies were assayed on fresh D17 cells, and the cells were selected for hygromycin resistance (experiment 2). No transforming virus was found. In parallel, DSN cells were transfected with pJD220SVHy, and the supernatant medium was used to infect D17 cells. A total of 10^3 CFU/ml were recovered.

In a repeat experiment, a small amount of transforming virus was found after transfection of DSN cells with pJD220SVHy followed by infection of DSN cells. However, the amount of transforming virus was much smaller than that found after transfection of C3 cells followed by infection of C3 cells (experiment 1; titers from DSN cells were 1, 10, and 150 CFU/ml at 1, 2, and 3 weeks postinfection, respectively). The transforming virus from the step 2 DSN cells could be the result of nonhomologous recombination of the plasmid DNAs during transfection. To test this hypothesis, DNAs from four D17 clones infected with virus from DSN step 2 cells were analyzed with *Xho*I, *Bal*I, and *Sac*I digestion and Southern blot hybridization. *Xho*I is present in the 3′ LTR of pJD220SVHy but not in a wild-type LTR; *Bal*I and *Sac*I are present in a wild-type LTR, but *Sac*I is not in either LTR of pJD220SVHy and *Bal*I is not present in the 3′ LTR of pJD220SVHy. In all four cases, the *Xho*I and *Bal*I sites were present in both LTRs, and the *Sac*I site was not present in the LTRs (data not shown). Thus, the transforming virus appeared as a result of some nonhomologous process.

Conclusions. We have described Rev helper cells allowing propagation of Rev and MLV vectors without any homologous genetic exchanges (recombination or gene conversion) between the vectors and sequences in the helper cells. In addition, the separation of the *gag-pol* and *env* genes on different expression vectors allowed formation of chimeric virions. Here we report the successful formation of virions with Gag and Pol proteins from SNV and Env proteins from amphotropic MLV. (Others had previously reported MLV-avian leukosis virus pseudotypes [14, 23].) In separate experiments with other helper cells constructed in an analogous manner, we have successfully formed chimeric virions with Gag and Pol proteins from SNV or MLV and Env proteins from bovine leukemia virus (J. Ban, N. First, and H. M. Temin, J. Gen. Virol., in press).

The occasional occurrence of transforming virus after transfection of DSN cells by pJD220SVHy indicates that even with these new helper cells with almost no nucleotide sequence homology to vector sequences unwanted genetic events can occur. Thus, it is necessary to clone the transfected helper cells or modify the vectors even more to prevent any unwanted genetic changes.

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LITERATURE CITED

1. **Bosselman, R. A., R. Hsu, J. Bruszewski, S. Hu, F. Martin, and M. Nicolson.** 1987. Replication-defective chimeric helper proviruses and factors affecting generation of competent virus: expression of Moloney murine leukemia virus structural genes via the metallothionein promoter. Mol. Cell. Biol. **7**:1797–1806.
2. **Dornburg, R., and H. M. Temin.** 1988. Retroviral vector system for the study of cDNA gene formation. Mol. Cell. Biol. **8**: 2328–2334.
3. **Dougherty, J. P., and H. M. Temin.** 1987. A promoterless retroviral vector indicates that sequences in U3 are required for 3′ RNA processing. Proc. Natl. Acad. Sci. USA **84**:1197–1201.
4. **Eglitis, M. A., and W. F. Anderson.** 1988. Retroviral vectors for introduction of genes into mammalian cells. BioTechniques **6**:608–614.
5. **Embretson, J. E., and H. M. Temin.** 1986. Pseudotyped retroviral vectors reveal restrictions to reticuloendotheliosis virus replication in rat cells. J. Virol. **60**:662–668.
6. **Embretson, J. E., and H. M. Temin.** 1987. Lack of competition results in efficient packaging of heterologous murine retroviral RNAs and reticuloendotheliosis virus encapsidation-minus RNAs by the reticuloendotheliosis virus helper cell line. J. Virol. **61**:2675–2683.
7. **Embretson, J. E., and H. M. Temin.** 1987. Transcription from a spleen necrosis virus 5′ long terminal repeat is suppressed in mouse cells. J. Virol. **61**:3454–3462.
8. **Emerman, M., and H. M. Temin.** 1984. High-frequency deletion in recovered retrovirus vectors containing exogenous DNA with promoters. J. Virol. **50**:42–49.
9. **Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA **79**:6777–6781.
10. **Hu, S., J. Bruszewski, M. Nicolson, J. Tseng, R.-Y. Hsu, and R. Bosselman.** 1987. Generation of competent virus in the REV helper cell line C3. Virology **159**:446–449.
11. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell.

Biol. **4**:1172–1174.
12. **Knutson, J. C., and D. Yee.** 1987. Electroporation: parameters affecting transfer of DNA into mammalian cells. Anal. Biochem. **164**:44–52.
13. **Kriegler, M., C. F. Perez, C. Hardy, and M. Botchan.** 1984. Transformation mediated by the SV40 T antigens: separation of the overlapping SV40 early genes with a retroviral vector. Cell **38**:483–491.
14. **Levy, J.** 1977. Murine xenotropic type C viruses. III. Phenotypic mixing with avian leukosis and sarcoma viruses. Virology **77**:811–825.
15. **Markowitz, D., S. Goff, and A. Bank.** 1988. A safe packaging line for gene transfer: separation of viral genes on two different plasmids. J. Virol. **62**:1120–1124.
16. **Miller, A. D., and C. Buttimore.** 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. **6**:2895–2902.
17. **Sorge, J., D. Wright, V. D. Erdman, and A. E. Cutting.** 1984. Amphotrophic retrovirus vector system for human cell gene transfer. Mol. Cell. Biol. **4**:1730–1737.
18. **Southern, P. J., and P. J. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
19. **Stinski, M. F., and T. J. Roehr.** 1985. Activation of the major immediate early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. J. Virol. **55**:431–441.
20. **Temin, H. M.** 1986. Retrovirus vectors for gene transfer: efficient integration into and expression of exogenous DNA in vertebrate cell genomes, p. 149–187. *In* R. Kucherlapati (ed.), Gene transfer. Plenum Publishing Corp., New York.
21. **Watanabe, S., and H. M. Temin.** 1982. Encapsidation sequences for spleen necrosis virus, an avian retrovirus, are between the 5′ long terminal repeat and the start of the *gag* gene. Proc. Natl. Acad. Sci. USA **79**:5986–5990.
22. **Watanabe, S., and H. M. Temin.** 1983. Construction of helper cell line for avian reticuloendotheliosis virus cloning vectors. Mol. Cell. Biol. **3**:2241–2249.
23. **Weiss, R. A., and A. L. Wong.** 1977. Phenotypic mixing between avian and mammalian RNA tumor viruses: envelope pseudotypes of Rous sarcoma virus. Virology **76**:826–834.
24. **Wickens, M., and T. Stephenson.** 1984. Role of the conserved AAUAAA sequence: four AAUAAA point mutants prevent messenger RNA 3′ end formation. Science **226**:1045–1051.
25. **Wilhelmsen, K. C., K. Eggleton, and H. M. Temin.** 1984. Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. J. Virol. **52**:172–182.
26. **Wilson, J. M., D. E. Johnston, D. M. Jefferson, and R. C. Mulligan.** 1988. Correction of the genetic defect in hepatocytes from the Watanabe heritable hyperlipidemic rabbit. Proc. Natl. Acad. Sci. USA **85**:4421–4425.