

A Conditional Self-Inactivating Retrovirus Vector That Uses a Tetracycline-Responsive Expression System

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We developed a novel conditional self-inactivating (C-SIN) vector, TL-SN, by replacement of the enhancer-promoter of the 3' long terminal repeat of Moloney murine leukemia virus with a synthetic tetracycline operator-cytomegalovirus promoter (*tetP*) from the tetracycline-responsive expression system (TRES). The other component of the TRES, a chimeric transactivator (tTA), was stably incorporated into PA317 amphotropic packaging cells, thus generating the packaging cell line PA317-tTA. C-SIN amphotropic G418-resistant virus particles were generated with a titer of 2×10^5 CFU/ml within 2 days of transinfection of PA317-tTA cells with TL-SN ecotropic virus particles. This titer was approximately 2 log units higher than that obtained by transinfection of parental PA317 cells and was due to the high level of viral transcripts originating from the *tetP* promoter at the 5' end of the transduced vector in the presence of tTA. Our C-SIN vector has the potential for use in human gene therapy since it incorporates the advantages of previous SIN vectors in having weak *tetP* promoter activity (in the absence of tTA in target cells) while at the same time achieving high viral titers with PA317-tTA packaging cells.

Replication-defective recombinant retroviruses are widely used as an efficient system for gene delivery into eukaryotic cells (for reviews see references 1, 4, 7, 16, 20, and 23 and references therein). However, standard retrovirus vectors used for therapeutic gene transfer have the potential to activate nearby cellular proto-oncogenes by their own enhancer-promoter within the long terminal repeat (LTR) (for reviews see references 2, 25, and 26 and references therein; 15, 22, 27). Furthermore, the viral LTR enhancer may affect internal promoters of vectors (5, 6, 28, 30), making regulation of transferred genes difficult. To overcome these problems, self-inactivating (SIN) retrovirus vectors (also called suicide, single-cycle, or U3-minus vectors) that contain a deletion of the 3' LTR enhancer-promoter region have been developed (3, 9, 10, 19, 30, 31). However, the utility of most SIN vectors is limited because of their low titers. For example, the highest titer obtained from amphotropic SIN retrovirus vectors was 10^2 hypoxanthine-aminopterin-thymidine (HAT)-resistant CFU/ml (30) or 10^2 to 10^3 hygromycin-resistant CFU/ml (3). When transfected ψ 2 cells were screened, the highest titer of ecotropic SIN vectors ranged from 2×10^4 to 1×10^5 G418-resistant CFU/ml (31).

We have used the tetracycline-responsive expression system (TRES) to develop a modified SIN vector which can be produced at a titer of 10^5 CFU/ml from a mixed population of transinfected producer cells. The TRES consists of two components, the transcriptional activator (tTA) and its corresponding *cis*-element, *tetP* (8). tTA is a chimeric protein composed of the tetracycline repressor fused to the activation domain of the herpes simplex virus (HSV) transcriptional activator, VP16. The target of tTA, *tetP*, is a synthetic promoter consisting of tandem repeats of the tetracycline operator and a minimal promoter sequence from the cytomegalovirus (CMV) immediate early promoter. Recently, we and others have re-

ported that retrovirus vectors containing the TRES can deliver high and regulatable gene expression from the transferred gene (11–14, 21). In the studies reported here, we have incorporated *tetP* into the 3' LTR of a retrovirus vector to create a novel conditional-SIN (C-SIN) vector, TL-SN, and developed a novel packaging cell line, PA317-tTA, which constitutively expresses tTA.

When production of G418-resistant TL-SN retrovirus particles with PA317-tTA cells was examined on murine NIH 3T3 cells, virus particles from TL-SN-transfected PA317-tTA (PA317-tTA/TL-SN) cells were produced with a titer approximately 2 log units higher (2×10^5 CFU/ml) than the titers obtained from transiently transfected cells. This higher titer appears to require tTA binding to the *tetP* promoter and is correlated with an increased level of viral transcripts in PA317-tTA/TL-SN cells. This result indicates that transcription from the *tetP* promoter in the chimeric 5' LTR is strongly activated by tTA provided *in trans*, leading to efficient synthesis of viral RNAs. Our C-SIN vector system with the modified PA317-tTA packaging cell lines can facilitate retrovirus-mediated gene transfer into target cells without adverse effects due to the presence of functional viral LTRs.

C-SIN vector TL-SN and the PA317-tTA packaging cell line. The TL-SN vector was created by replacement of the enhancer-promoter of the 3' LTR from the G1XSvNa vector (Genetic Therapy, Inc., Gaithersburg, Md.) with the *tetP* promoter from the plasmid pUHD10-3 (8) (Fig. 1). The control SIN vector, G5-SN, was constructed by subcloning the simian virus 40 (SV40) promoter-driven neomycin phosphotransferase (Neo^r) gene into the pG5 vector (Genetic Therapy, Inc.) (31), which has its enhancer deleted from the 3' LTR but maintains its promoter (Fig. 1).

The other element of TRES, tTA, was incorporated into a PA317 murine amphotropic retrovirus packaging cell line (ATCC no. CRL 9078; American Type Culture Collection, Rockville, Md.) (17) by cotransfection with two plasmids, pUHD15-1 (8), containing the transactivator tTA, and pPUR (Clontech, Palo Alto, Calif.), containing a puromycin resistance gene, and by selection with 4 μ g of puromycin (Sigma, St.

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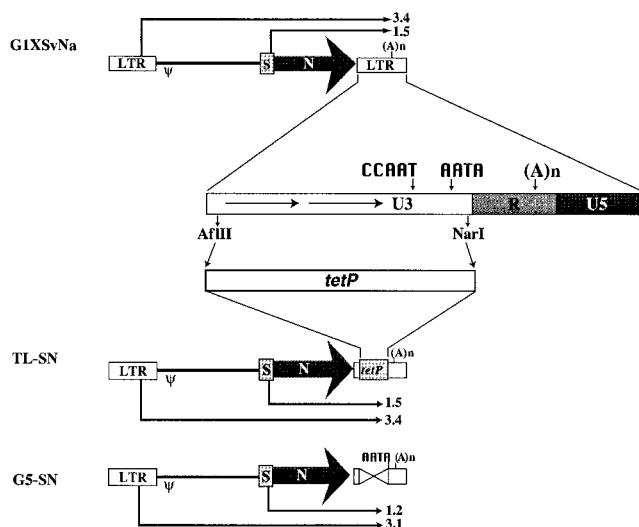


FIG. 1. Structure of the recombinant retrovirus vectors. The TL-SN vector was made by replacement of the *Afl*III-*Nar*I fragment of the 3' LTR with the *tetP* promoter from plasmid pUHD10-3. A *Nde*I-*Hind*III fragment containing the 3' LTR from the retrovirus vector pG1XSvNa was subcloned into pBS-SK (Stratagene, La Jolla, Calif.) to generate pBS-LTR. Then, the enhancer-promoter of the LTR in pBS-LTR was replaced with an enzyme linker, *Afl*III-*Xho*I-*Bam*HI-*Xba*I-*Nar*I, to create pBS- Δ LTR. A *Nde*I-*Hind*III fragment containing the modified Δ LTR fragment was exchanged with the corresponding 3' LTR fragment from pG1XSvNa. The TL-SN vector was created by insertion of an *Xho*I-*Xba*I fragment containing the *tetP* promoter from pUHD10-3 into the Δ LTR region of pG1XSvNa. The G5-SN vector was made by subcloning the SvNa fragment from G1XSvNa into pG5. A crossed line indicates the deletion of the 3' LTR enhancer. The bent arrows (\curvearrowright) indicate the approximate locations of promoters and the direction of transcription. The size of the predicted transcript is indicated in kilobases at the end of each bent arrow. TL, chimeric LTR containing *tetP*; *tetP*, synthetic tetracycline operator-CMV promoter; ψ , packaging signal sequence; S, SV40 enhancer-promoter; N, neomycin phosphotransferase (Neo^r) gene. The direct repeats of enhancer sequences (\rightarrow), CAT box (CCAAT), TATA box (AATA), and polyadenylation signal [(A)_n] in the 3' LTR region are indicated.

Louis, Mo.) per milliliter for 10 days. This modified packaging cell line, PA317-tTA, expressing tTA constitutively, was expanded, pooled, and used for transinfection experiments.

Virus production after transient transfection with retrovirus vectors. The usual approach to produce SIN vector supernatants is transient transfection, since efficient transcription from a functional 5' LTR is required for a high level of viral RNA synthesis. We therefore examined virus production by the C-SIN (TL-SN) and G5-SN vectors by using conventional transfection into PA317 packaging cells. PA317 (6×10^5) cells were applied to 100-mm-diameter tissue culture plates containing Dulbecco's modified Eagle medium with a high concentration of glucose (4.5 g/liter), supplemented with 2 mM glutamine and 10% fetal bovine serum (BioWhittaker, Walkersville, Md.). After 6 h, cells were transfected with 20 μ g of the indicated retrovirus vectors per plate (G5-SN or TL-SN) by the calcium phosphate precipitation method (5 Prime-3 Prime, Inc., Boulder, Colo.). Cells were washed, fed with fresh medium after 16 h of transfection, and incubated for an additional 48 h, after which the supernatant was collected. Vector supernatants were filtered with a 0.45- μ m-pore-size Millex-GS filter (Millipore, Bedford, Mass.) and used to measure the titers of G418-resistant virus particles. Vector supernatants obtained by transient transfection with either G5-SN or TL-SN yielded relatively low titers on NIH 3T3 cells [(5.5 \pm 1.9) $\times 10^3$ or (5.2 \pm 0.9) $\times 10^3$ CFU/ml ($n = 3$)]. The SIN vector G5-SN and the C-SIN vector TL-SN yielded an average 40-fold lower titer

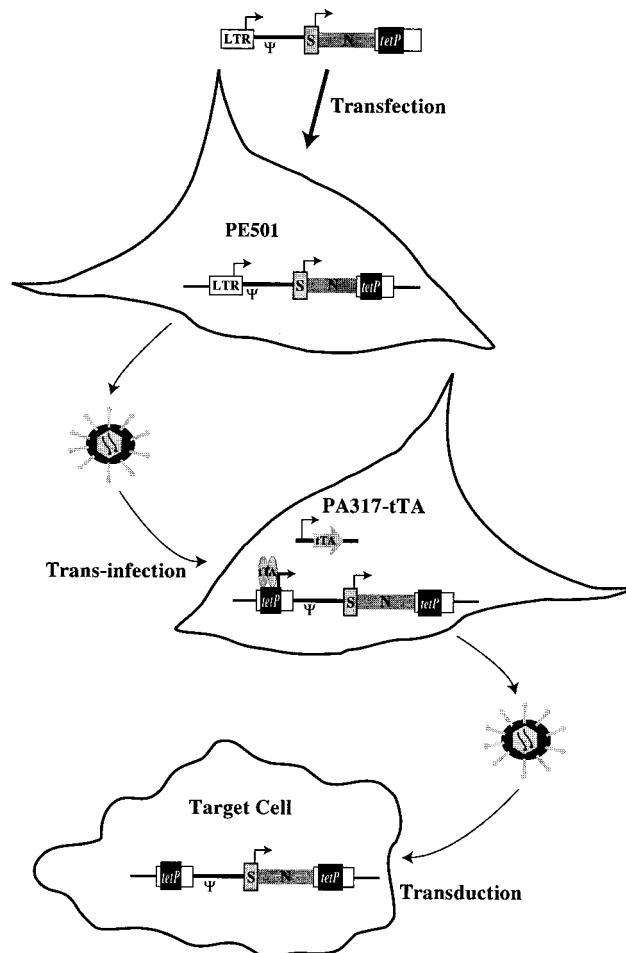


FIG. 2. Schematic illustration of retrovirus production with the PA317-tTA packaging cell line and a C-SIN vector. The C-SIN vector was transiently transfected into PE501 cells. The ecotropic retrovirus produced from the transfected PE501 cells was transduced into PA317-tTA cells. Then, amphotropic retrovirus particles carrying the C-SIN vector were transduced into target cells. The *tetP* promoter of the C-SIN provirus in PA317-tTA cells would exhibit a strong transcriptional activation in the presence of the transactivator tTA. However, the *tetP* promoter of the C-SIN provirus in the transduced target cells, which lack tTA, would only show basal promoter activity from the CMV minimal promoter. Therefore, the internal SV40 promoter of the provirus serves as a major transcription initiation site in transduced target cells. The symbols and abbreviations are defined in the legend to Fig. 1.

than other retrovirus vectors with intact LTRs at both ends of the provirus (data not shown).

Efficient virus production by transfection of PA317-tTA cells with the C-SIN vector. Our experimental rationale for producing high titers of SIN vector particles was to regulate transcription from the vector 5' LTR. Since the 3' LTR U3 serves as a template for both U3 regions during reverse transcription of the viral RNA after transfection or transduction, both LTRs of the TL-SN provirus in transinfected packaging cells will be the chimeric U3 LTR containing *tetP* (Fig. 2). Accordingly, the level of transcripts originating from the 5' LTR *tetP* promoter in transinfected PA317-tTA cells would be high due to the presence of tTA (Fig. 2). However, the same *tetP* promoter in both the 5' and 3' LTRs of TL-SN provirus transduced into target cells would only act as a weak promoter, as those cells do not express the transactivator tTA (Fig. 2).

To test the efficiency of virus production and the level of

TABLE 1. Virus production from PA317 and PA317-tTA packaging cells^a after transfection of ecotropic virus particles

Packaging cell line	Retrovirus vector	Neo ^r virus titer (CFU/ml) ^b
PA317	G5-SN	$(8.7 \pm 3.3) \times 10^3$
	TL-SN	$(5.3 \pm 1.2) \times 10^3$
PA317-tTA	G5-SN	$(9.4 \pm 4.4) \times 10^3$
	TL-SN	$(2.4 \pm 1.3) \times 10^5$

^a PA317-tTA packaging cells constitutively express tTA.

^b Neo^r virus titers are the means \pm standard errors of the means from multiple experiments ($n \geq 3$).

viral RNA transcripts originating from *tetP* of the TL-SN vector, we performed transfection of parental PA317 and modified PA317-tTA packaging cells with various vectors. Replication-defective ecotropic virus particles were produced with titers of 10^3 CFU/ml by transient transfection of PE501 cells (ecotropic packaging cells) (18) with the TL-SN and G5-SN vectors as previously described (data not shown). To produce amphotropic replication-defective viral supernatants, 6×10^5 PA317 or PA317-tTA cells were independently applied to 100-mm-diameter tissue culture plates and transfected with the ecotropic virus particles. After 16 h of incubation, the cells were washed, fed with fresh medium, and incubated for an additional 48 h, after which the supernatant was collected and used to measure titers of G418-resistant virus particles on NIH 3T3 cells. The G5-SN vector transferring only the LTR promoter generated similar titers (9×10^3 CFU/ml) from both the transfected PA317 and the PA317-tTA cells (Table 1). However, the TL-SN vector particles were produced at an average 50-fold-higher titer (2×10^5 CFU/ml) in PA317-tTA cells compared to PA317 cells (Table 1). These results indicate that efficient virus production can be rapidly achieved within 2 days following transfection of the TL-SN vector into PA317-tTA packaging cells.

Analysis of transcripts from the provirus in producer cells.

To examine whether higher titers were due to increased levels of vector-length transcripts from TL-SN in the presence of tTA, mRNA expression from the transduced proviruses was analyzed by Northern hybridization (24). Both PA317/G5-SN and PA317-tTA/G5-SN cells produced two transcripts (3.1 and 1.2 kb), generated from the LTR and the internal SV40 promoter (Fig. 1). The levels of both transcripts were similar in both cell types (Fig. 3A, lanes 2 and 3). For the cells transfected with the TL-SN vector, transcripts of 3.4 and 1.5 kb, initiated either from the 5' *tetP* promoter or from the internal SV40 promoter, were expected (Fig. 1). This was seen for both PA317/TL-SN and PA317-tTA/TL-SN cells (Fig. 3A, lanes 4 and 5; Fig. 3B, lanes 2 and 3). PA317/TL-SN cells showed a low basal level of the predicted 3.4-kb transcript originating from *tetP* in the absence of tTA. As indicated by the relative levels of the internal SV40 promoter-derived transcripts, which are common to both the G5-SN and the TL-SN vectors, the TL-SN provirus demonstrated a lower level of transcripts in transfected cells (Fig. 3A; compare the S band in lanes 2 and 3 with that in lanes 4 and 5). However, by providing tTA in *trans* the levels of TL-SN viral transcripts originating from the 5' *tetP* promoter were significantly increased and surpassed the levels of G5-SN viral transcripts in PA317-tTA/G5-SN cells (Fig. 3A, compare the V band in lane 5 to the V bands in lanes 3 and 4). When the structural integrity of proviral DNA in mixed producer cells was analyzed by Southern blot analysis, the expected fragments of 2.9 and 2.5 kb were observed in the TL-SN

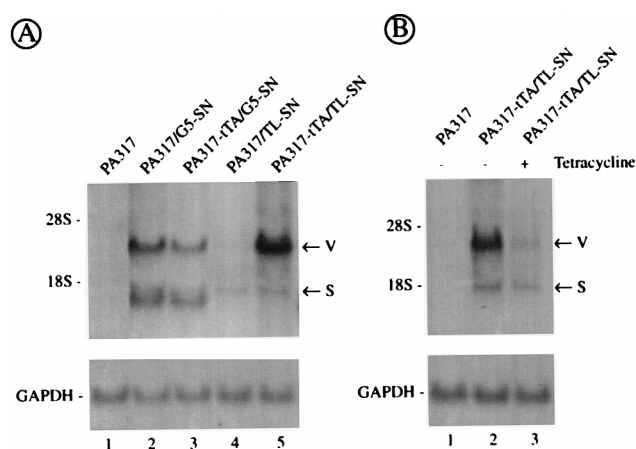


FIG. 3. Northern analysis of transinfected producer cells (A) and dependence of viral RNA synthesis on tTA binding to the *tetP* promoter (B). Total RNAs were extracted from producer cells with RNazol (Tel-Test, Inc., Friendswood, Tex.), and 20 μ g of total RNA was used for Northern analysis with labeled cDNA probes. The RNA blots were independently hybridized with a ³²P-labeled Neo^r probe (top panel) or a ³²P-labeled human GAPDH probe (bottom panel). The characteristic predicted transcripts from either the 5' LTR or the chimeric *tetP* LTR (V) and from the internal SV40 promoter (S) are indicated by arrows on the right (also see Fig. 1). The positions of 18S and 28S rRNA species are indicated on the left. (A) PA317 and PA317-tTA cells transfected with various retrovirus vectors were selected for 10 days in tetracycline-free medium supplemented with 0.6 mg of G418 (Geneticin; Gibco, Grand Island, N.Y.) per ml. (B) PA317-tTA/TL-SN cells selected with G418 were grown in either tetracycline-free medium (lane 2) or medium supplemented with 10 μ g of tetracycline per ml for 24 h (lane 3).

and G5-SN producer cells, respectively, indicating that the structure of the integrated vector is intact. Furthermore, approximately equal amounts of hybridized Neo^r signal was observed in PA317 and PA317-tTA cells transduced with either the G5-SN or the TL-SN vector (data not shown).

After quantification with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and standardization against constitutive levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, the relative level of the *tetP*-originated 3.4-kb transcripts generated in PA317-tTA/TL-SN producer cells was shown to be 14-fold higher than the level of mRNA generated from the *tetP* promoter in PA317/TL-SN producer cells. The *tetP* promoter and tTA system has been shown to function as a regulatable transcriptional system, since its *tetP*-driven transcription can be shut off in the presence of tetracycline (8, 11, 13, 29). In parallel with previous reports, the increased level of transcripts from the *tetP* promoter in PA317-tTA/TL-SN producer cells was reduced up to 85% after 24 h of incubation with 10 μ g of tetracycline (Sigma) per ml (Fig. 3B, compare lanes 2 and 3). This result indicates that the high level of viral transcripts, resulting in high titers, is due to the strong transcriptional activity of the *tetP*-tTA system.

Retrovirus-mediated gene transfer with SIN vectors is advantageous because it reduces the potential adverse effects resulting from transcription from the LTR enhancer-promoter of the integrated provirus. In addition, SIN vectors show improved function of internal promoters (30, 31) without potential promoter interference from the LTR enhancer-promoter. However, their use has been limited by the tendency to result in low titers. Our approach, using transfection of PA317-tTA packaging cells with a C-SIN retrovirus vector containing a chimeric *tetP* LTR should overcome this low-titer problem.

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REFERENCES

- Anderson, W. F. 1992. Human gene therapy. *Science* **256**:808–813.
- Bishop, J. M. 1983. Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* **52**:301–354.
- Dougherty, J. P., and H. M. Temin. 1987. A promoterless retroviral vector indicates that there are sequences in U3 required for 3' RNA processing. *Proc. Natl. Acad. Sci. USA* **84**:1197–1201.
- Eglitis, M. A., and W. F. Anderson. 1988. Retroviral vectors for introduction of genes into mammalian cells. *BioTechniques* **6**:608–614.
- Emerman, M., and H. M. Temin. 1984. Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* **39**:459–467.
- Emerman, M., and H. M. Temin. 1986. Comparison of promoter expression in avian and murine retrovirus vectors. *Nucleic Acids Res.* **14**:9381–9386.
- Gilboa, E., M. A. Eglitis, P. W. Kantoff, and W. F. Anderson. 1986. Transfer and expression of cloned genes using retroviral vectors. *BioTechniques* **4**:504–512.
- Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89**:5547–5551.
- Hawley, R. G., L. Covarrubias, T. Hawley, and B. Mintz. 1987. Handicapped retroviral vectors efficiently transduce foreign genes into hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **84**:2406–2410.
- Hawley, R. G., L. A. Sabourin, and T. S. Hawley. 1989. An improved retroviral vector for gene transfer into undifferentiated cells. *Nucleic Acids Res.* **17**:4001.
- Hofmann, A., G. P. Nolan, and H. M. Blau. 1996. Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette. *Proc. Natl. Acad. Sci. USA* **93**:5185–5190.
- Hoshimaru, M., J. Ray, D. W. Y. Sah, and F. H. Gage. 1996. Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the v-myc oncogene. *Proc. Natl. Acad. Sci. USA* **93**:1518–1523.
- Hwang, J.-J., Z. Scusic, and W. F. Anderson. 1996. Novel retroviral vector transferring a suicide gene and a selectable marker gene with enhanced gene expression by using a tetracycline-responsive expression system. *J. Virol.* **70**:8138–8141.
- Iida, A., S.-T. Chen, T. Friedmann, and J.-K. Yee. 1996. Inducible gene expression by retrovirus-mediated transfer of a modified tetracycline-regulated system. *J. Virol.* **70**:6054–6059.
- Linial, M., and M. Groudine. 1985. Transcription of three c-myc exons is enhanced in chicken bursal lymphoma cell lines. *Proc. Natl. Acad. Sci. USA* **82**:53–57.
- Miller, A. D. 1992. Human gene therapy comes of age. *Nature* **357**:455–460.
- 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.
- Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. *BioTechniques* **7**:980–990.
- Olson, P., S. Nelson, and R. Dornburg. 1994. Improved self-inactivating retroviral vectors derived from spleen necrosis virus. *J. Virol.* **68**:7060–7066.
- Osborne, W. R. A. 1991. Retrovirus-mediated gene expression in mammalian cells. *Curr. Opin. Biotechnol.* **2**:708–712.
- Paulus, W., I. Baur, F. M. Boyce, X. O. Breakefield, and S. A. Reeves. 1996. Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *J. Virol.* **70**:62–67.
- Payne, G. S., J. M. Bishop, and H. E. Varmus. 1982. Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. *Nature* **295**:209–214.
- Salmons, B., and W. H. Günzburg. 1993. Targeting of retroviral vectors for gene therapy. *Hum. Gene Ther.* **4**:129–141.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Temin, H. M. 1990. Safety considerations in somatic gene therapy of human disease with retrovirus vectors. *Hum. Gene Ther.* **1**:111–123.
- Varmus, H., and P. Brown. 1989. Retroviruses, p. 53–108. *In* D. E. Berg and M. M. Howe (ed.), *Mobile DNA*. American Society for Microbiology, Washington, D.C.
- Westaway, D., G. Payne, and H. E. Varmus. 1984. Proviral deletions and oncogene base-substitutions in insertionally mutagenized c-myc alleles may contribute to the progression of avian bursal tumors. *Proc. Natl. Acad. Sci. USA* **81**:843–847.
- Xu, L., J.-K. Yee, J. A. Wolff, and T. Friedmann. 1989. Factors affecting long-term stability of Moloney murine leukemia virus-based vectors. *Virology* **171**:331–341.
- Yarranton, G. T. 1992. Inducible vectors for expression in mammalian cells. *Curr. Opin. Biotechnol.* **3**:506–511.
- Yee, J.-K., J. C. Moores, D. J. Jolly, J. A. Wolff, J. G. Respass, and T. Friedman. 1987. Gene expression from transcriptionally disabled retroviral vectors. *Proc. Natl. Acad. Sci. USA* **84**:5197–5201.
- Yu, S.-F., T. Rueden, P. W. Kantoff, C. Garber, M. Seiberg, U. Reuther, W. F. Anderson, E. F. Wagner, and E. Gilboa. 1986. Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc. Natl. Acad. Sci. USA* **83**:3194–3198.