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IMPROVED GENE EXPRESSION BY A U3-BASED RETROVIRAL VECTOR

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SUMMARY: To improve the expression of the genes transduced by retroviral vectors, we have constructed a U3-based retroviral vector and evaluated its effect on the expression of an insert from the internal promoter. The unique feature of the vector is that the transduced gene is inserted at the U3 region of the 3' long terminal repeats (LTR). Consequently, in the infected cells the gene is duplicated and transferred to the 5'-LTR. When compared with the conventional retroviral vectors which insert the gene within the retroviral transcriptional unit, the U3-based vectors greatly enhanced the expression of the transduced gene under all three promoters tested, viz. the cytomegalovirus immediately early gene promoter (CMV), the SV40 early gene promoter (SV), and the herpes simplex virus thymidine kinase gene promoter (TK). The SV and TK promoters which were previously shown suppressed by the retroviral promoter in the conventional construction restored their potencies in the U3-based vectors. Our results therefore suggested that the U3-based vectors are more advantageous than the conventional vectors for gene expression.

Recombinant retroviral vectors are frequently used for gene transfer (1,2). Specifically, retroviral vectors have been widely applied to gene therapy (for review see 3) as well as insertional mutagenesis (4,5). In most cases, it is essential to express two genes from a single proviral genome, one the selection marker to facilitate the isolation of the infected cells and the other the gene of interest. In applications of retroviral vectors to various usages, two approaches have been used to coexpress two exogenous genes. The first involves the splicing vectors (6) which employ the primary transcript for the translation of the upstream gene and the spliced transcript for the translation of the downstream gene. The strategy mimicks that used by retrovirus to generate the *env* gene product. However, while the splicing efficiency is optimally regulated in the

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retroviral genome, i.e. approximately 1:1 of the primary transcript to the spliced transcript (7), the splicing efficiencies of the recombinant splicing vectors vary a great deal and are dependent on the construct context (8,9). Therefore, a balanced expression of these two genes from a single splicing vector has always been problematic in many cases. The second approach involves the insertion of an internal promoter in the proviral genome so that the upstream gene is expressed from the retroviral long terminal repeat (LTR) and the downstream gene is expressed from the internal promoter (10,11). The promoter-insert cassettes are usually inserted at a site downstream of the first gene and in between two LTRs. This approach has often been used in gene therapy studies (1,3). However, the problem encountered in this design is the competitive interference between these two promoters (1,11,12). Consequently, one of these two genes may not be expressed properly in individual isolates. In order to improve the expression of the transduced genes by using the retroviral vectors, we have modified the vector by inserting the promoter-insert cassettes at the 3'-U3 region at a site in front of the retroviral promoter/enhancer. The rationale of this U3-based vector has been described previously (13). In this communication, we have evaluated the expression of a gene from three different internal promoters, viz. the cytomegalovirus immediately early gene promoter (CMV) (14), the SV40 early gene promoter (SV) (15), and the herpes simplex virus thymidine kinase gene promoter (TK) (16) within the U3-based vector in two different target cells, NIH 3T3 and BW5147 (a T lymphoma cell line).

MATERIALS AND METHODS

Construction of porcine growth hormone (PGH)-containing retroviral vectors All the retroviral vectors were derived from the N2 vector (17) in which we have inserted three unique enzyme sites, *BamH*I, *Stu*I, and *Hind*III at the *Nhe*I site of the 3'-U3 for the convenience of cloning. The *Nhe*I site is located in front of the retroviral promoter/enhancer region. This vector is designated as N3 (Fig. 1). A 740-bp cDNA fragment coding for the porcine growth hormone (PGH) (a gift from Dr. Chang, W.C., National Taiwan University) was used as a test gene in this study. The PGH cDNA was first cloned at the downstream of either the CMV, the SV, or the TK promoter to generate a promoter-PGH cassette which was then inserted into either the *Xho*I site (the X-series) or the *BamH*I site (the B-series) of the N3 vector. The general schemes of the construction are depicted in Fig. 1.

Cell culture and virus infection All cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. Cell lines used included an ecotropic retrovirus packaging cell line, GP+E-86 (18), NIH 3T3, and BW5147 (a T cell lymphoma). Two micrograms of recombinant retroviral vector DNA were first transfected into GP+E-86 cells by calcium phosphate precipitation method (19). The supernatant from the G418-resistant clone that generated the highest virus titer was used to infect

NIH 3T3 and BW5147 cells, followed by 0.8 mg/ml and 1.2 mg/ml G418 selection, respectively. Total resistant clones were pooled for further analysis.

Southern blot and Northern blot analysis High molecular weight DNA was digested with proper restriction enzymes, electrophoresed on a 0.8% agarose gel and electro-transferred to a nytran membrane. The *PGH*-specific probe was used to hybridize the filter. The RNA was also extracted from cultured cells by hot phenol method (20). Poly-A RNA was selected by oligo-dT column (21) and 1 μg of poly-A RNA was subjected to formaldehyde-agarose gel electrophoresis (22), transferred to the nytran membrane and hybridized with the *PGH* probe.

Western blot analysis To analyze the PGH production in the infected clones, cells were grown in a 10-cm dish till confluent, then washed twice with phosphate saline buffer. A serum-free medium, Iscoves's modified Dulbecco's medium, was used to refresh the cells for one night, and then the culture medium was collected and concentrated by Amicon Centriprep-10. The proteins were electrophoresed on a 12.5% SDS-PAGE (23) and electro-transferred to a nitrocellulose membrane (24). The PGH protein was detected by a rabbit anti-PGH antiserum and a goat anti-rabbit IgG. The bands were visualized by the addition of substrate BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium).

RESULTS AND DISCUSSION

Duplication of the chimeric 3'-LTR Since the U3 region of the 3'-LTR is served as the template for the synthesis of both the 5'- and the 3'-U3 of the proviral DNA during retroviral replication (25), the chimeric LTR as designed in the B-series vector will be duplicated in the infected cells . Therefore an additional copy of the promoter-insert cassette will be present at the U3 of the 5'-LTR and be placed upstream of the retroviral promoter. In contrast to the conventional vectors in which the promoter-insert cassettes are within the retroviral transcriptional unit, this design might eliminate or at least reduce the negative effect of the retroviral LTR promoter, thus improving the expression from the internal promoter at the 5'-U3. To test this possibility, we have compared the expression of a transduced gene from these two types of vectors, the X-series and the B-series vectors (Fig. 1). A porcine growth hormone (PGH) cDNA was used as the test gene and transcription of it was under the control of three different promoters, viz. the CMV, the SV, or the TK promoter. The constructs were termed CX, SX and TX, respectively when the promoter-insert cassettes were inserted at the *Xho*I site of the N3 vector; or CB, SB and TB, respectively when the cassettes were inserted at the *BamH*I site of the N3 vector (Fig. 1). To understand the integrity and the status of the chimeric 3'-LTR of the proviral genome in the infected NIH 3T3 and BW5147 cells, the genomic DNA was digested with either *Kpn*I or *Hind*III. Since *Kpn*I cleaves once in the long terminal repeats, it will excise an unit length of proviral DNA (Fig. 2A). The results shown in Fig. 2B and 2C demonstrate that all the infected NIH 3T3 and BW5147cells revealed a single

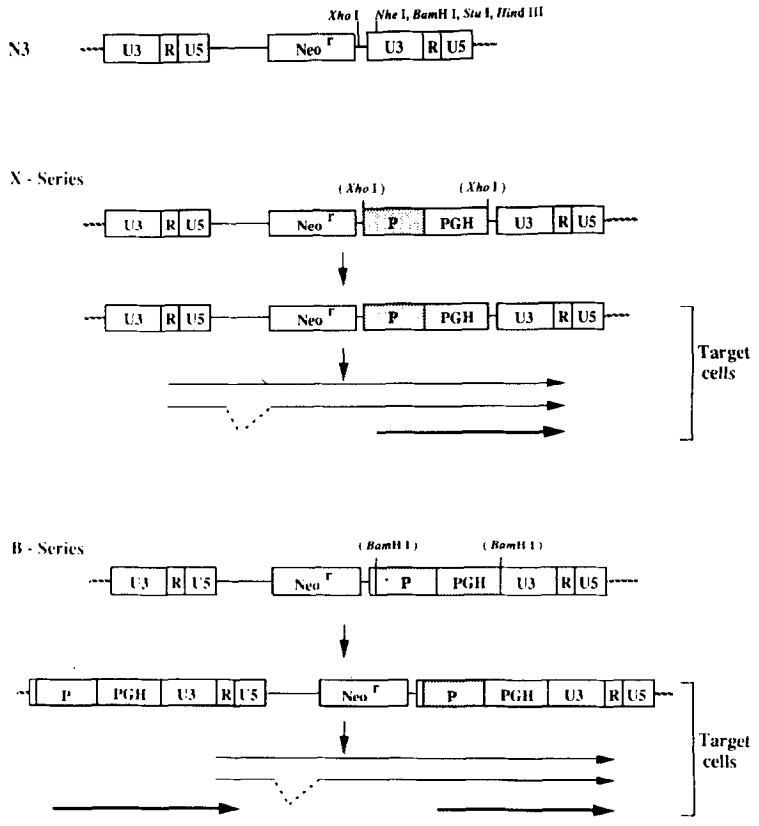

Fig. 1. Schematic diagrams of the X-series and the B-series vectors. The N3 vector was derived from the N2 vector (17) and contains three additional unique enzyme sites, *BamH*I, *Stu*I, and *Hind*III, at the 3'-U3. The X-series vectors harbor the promoter-PGH inserts at the *Xho*I site of the N3; whereas the B-series vectors insert the cassettes at the *BamH*I site. The proviral genome of the X-series or the B-series vector and the predicted transcripts derived from each in the target cells are indicated. P designated the internal promoters, viz. the CMV, the SV, or the TK promoter, respectively.

band of the expected sizes, 4.7 kb for CX and CB; 4.7 kb for TX and TB, and a smaller band for SX (3.5 kb) or SB (4.0 kb) since one additional *Kpn*I site was present at the SV40 promoter. The results therefore suggested that the proviral genomes were intact within the retroviral transcription unit. In addition, *Hind*III site was created only at the U3 of 3'-LTR in the chimeric molecules and was therefore used to detect the duplication event occurred in the B-series vector-infected cells. Again, the results shown in Fig. 2B and 2C demonstrate a distinct band for each, 4.7 kb (CB), 4.5 kb (SB) and 4.7 kb (TB) respectively, from both infected NIH 3T3 and BW5147 cells, indicating that the chimeric LTR was indeed duplicated and transferred to the 5'-LTR.

Enhanced expression of PGH from the B-series vector in both infected target cells As depicted in Fig. 1, the proviral genome derived from

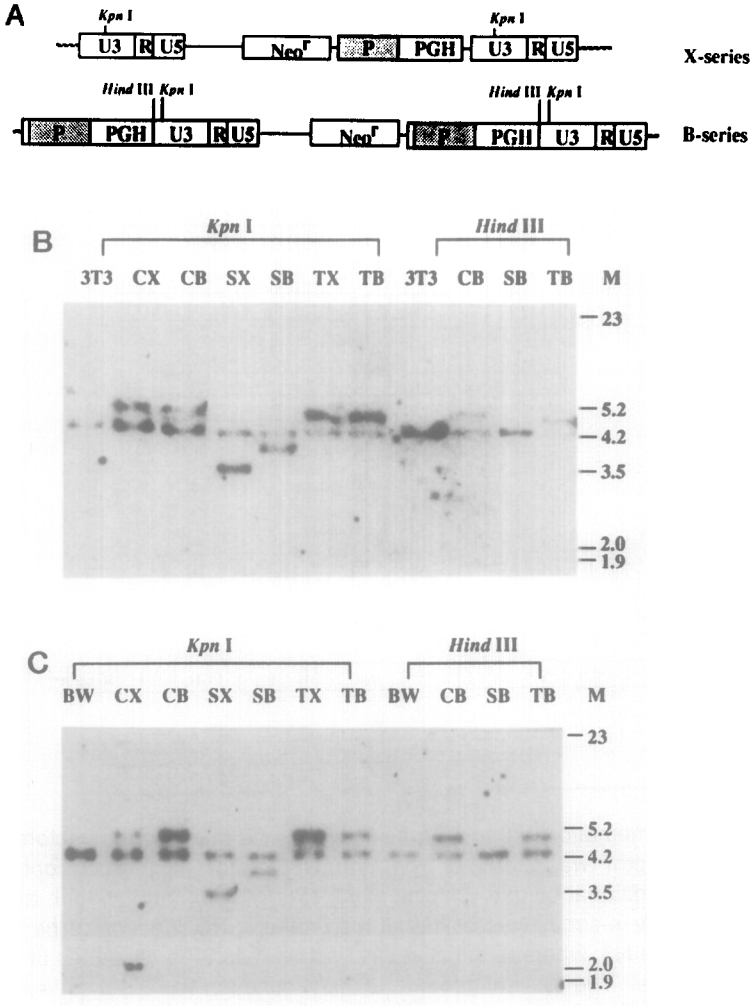

Fig. 2. Analysis of the proviral DNA in the infected target cells. Schematic diagrams indicate the interpreted proviral genome of the X-series and the B-series vector in the target cells (A). The genomic DNA extracted from the infected NIH 3T3 clones (B) or the BW5147 clones (C) were digested with *Kpn*I or *Hind* III and hybridized with the *PGH* probe. The 3T3 and the BW are the cell controls which were not infected with any virus. Symbols for each lane are described in the text. Asterisks indicate the position for the mouse endogenous growth hormone sequences which were cross hybridized with the *PGH* probe.

either the X-series or the B-series vector will transcribe three types of RNA : two are transcribed from the LTR, the full-length and the cryptically spliced RNA (17); and the third one is transcribed from the internal promoter and is served as the mRNA for PGH. But the B-series proviral genome will contain two copies of the promoter-insert cassettes, one of which is placed beyond the retroviral transcriptional unit and may increase the expression of *PGH* from the internal promoters. The results of RNA analysis shown in Fig. 3 indicate that the B-series provirus indeed expressed 5-20 folds higher of the internal promoter-initiated

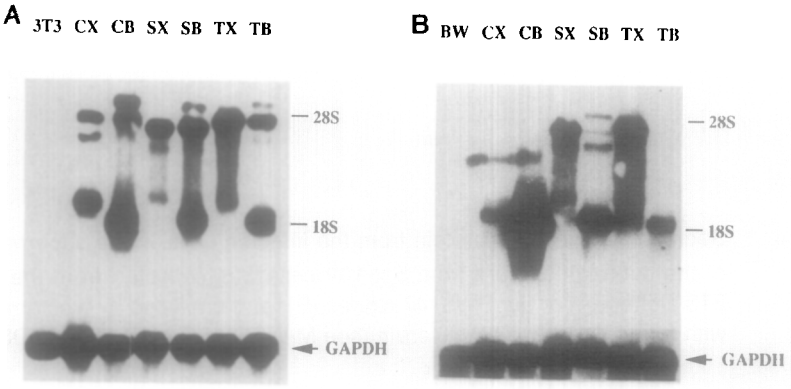

Fig. 3. Analysis of the RNA expression in the target cells. One microgram of the poly-A RNA extracted from the infected NIH 3T3 clones (A) or BW5147 clones (B) was loaded in each lane and analyzed with the *PGH* probe or the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) probe which was used as an internal control.

transcripts (the lowest band of each lane) than the X-series provirus in NIH 3T3 cells (Fig. 3A); and 2-20 folds higher in BW5147 cells (Fig. 3B). Note that the comparisons have been normalized by the amounts of the internal control, the glyceraldehyde-3-phosphate dehydrogenase (GAPGH) transcript, loaded in each lane. Furthermore, we also found interesting interactions between the LTR and the internal promoters. For example, if the CMV promoter was used, it suppressed the LTR promoter no matter it was in the X-series or in the B-series vector; whereas if the SV and the TK promoters were used, they were suppressed by the LTR promoter in the X-series vector, and *vice versa* in the B-series vector (Fig. 3A and 3B, compare the upper two bands with the lowest band) The contrast is particularly dramatic in BW5147 cells. These results were consistent with previous observations that the activity of a promoter is often reduced when placed downstream of an active promoter (12), and may explain some of the problems encountered in the expression of retrovirally transduced gene from the internal promoters (1,11,12). Among all three promoters tested the CMV promoter always revealed the highest activity in both cell lines, again suggested the universality and strength of the CMV promoter (14). An additional RNA transcript larger than the full-length RNA (the slowest migration) was detected in the B-series vectors-infected cells (Fig. 3A). Based on the sizes, we proposed that these transcripts were initiated from the 5'-internal promoter and terminated at the poly-A signal of the 3'-LTR of the provirus. The PGH protein secreted in the medium was also assayed by western blot analysis using a rabbit anti-PGH antiserum (Fig. 4). Basically, the pattern of protein production reflected that of the RNA expression, i.e. the production of PGH protein was much higher from the B-series vectors than from the X-series vectors.

In conclusion, we have demonstrated the advantages of using the U3-based retroviral vectors. The great enhancement of the expression from the

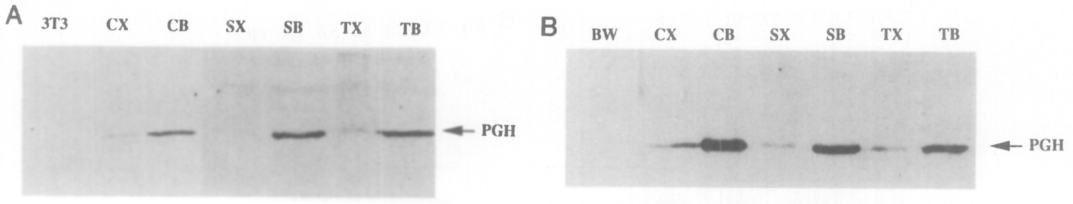

Fig. 4. Secretion of the PGH protein from the infected cells. The supernatant of the overnight culture from 5 x 10^6 cells was collected from the NIH 3T3 clones (A) or the BW5147 clones (B), and analyzed by western blot with rabbit anti-BAMHI antiserum (see MATERIALS AND METHODS).

internal promoters was clearly observed with all three promoters tested, especially for the SV promoter and the TK promoter which were previously demonstrated strongly suppressed by the LTR promoter in the conventional vectors (12 ; also see Fig. 3). The effects were even more dramatic in T lymphoid cells. The CMV promoter was not greatly suppressed in the X-series construction since its activity was stronger than the LTR promoter and possibly interfered the LTR activity no matter which position it was inserted. However, in the B-series vector the CMV promoter exhibited much higher activity, indicating that the upstream position was even superior to the downstream position. Taken together, the U3-based vectors are much more advantageous for choices of different internal promoters to express the transduced gene in a variety of cells.

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REFERENCES

1. Miller, A.D., and Rosman, G.J. (1989) BioTechnique 9, 980-990.
2. Brown, A.M.C., and Scott, M.R.D. (1987) In DNA Cloning Vol III (D.M. Glover Ed.) pp189-211, IRL Press, Oxford, England.
3. Apperley, J.F., and Williams, D.A. (1990) Br. J. Haematol. 75, 148-155.
4. Brenner, D.G., Lin-C., S., and Cohen, S.N. (1989) Proc. Natl. Acad. Sci. USA 86, 5517-5521.
5. Reddy, S., Degregori, J.V., Melchner, H. von, and Ruley, H. Earl. (1991) J. Virol. 65, 1507-1515.
6. Cepko, C.L., Roberts, B.E., and Mulligan, R.C. (1984) Cell 37, 1053-1062.

7. Coffin, J. (1983) In RNA Tumor Viruses (R. Weiss, N. Teich, H. Varmus, J. Coffin Eds) pp261-368, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
8. Arrigo, S., and Beemon, K. (1989) Mol. Cell. Biol. 8, 4858-4867.
9. Berberich, S.L., and Stoltzfus, M. (1991) J. Virol. 65, 2640-2646.
10. Gilboa, E., Eglitis, M.A., Kantoff, P.W., and Anderson, W.F. (1986) BioTechniques 4, 504-512
11. Emerman, M., and Temin, H.M. (1984) Cell 39, 459-467.
12. Emerman, M., and Temin, H.M. (1986) Mol. Cell. Biol. 6, 792-800.
13. Yu, S.F., Ruden, T.V., Kantoff, P.W., Garber, C., Seiberg, M., Ruther, U., Anderson, W.F., Wagner, E.F., and Gilboa, E. (1986) Proc. Natl. Acad. Sci. USA 83, 3194-3198.
14. Boshart, M., Webber, F., Jahn, G., Dorsch-Hasler, K., Feckenstein, B., Schaffner, W. (1985) Cell 41, 521-530.
15. Benoist, C., and Chambon, P. (1981) Nature 290, 304-310.
16. McKnight, S.L. (1980) Nucleic Acid Research 8, 5949-5964.
17. Armentano, D., Yu, S.F., Kantoff, P.W., Ruden, T.von, Anderson, W.F., and Gilboa, E. (1987) J. Virol 61, 1647-1650.
18. Markowitz, D., Goff, S., and Bank, A. (1988) J. Virol 62, 1120-1124.
19. Graham, F.L., and van der Eb., A.J. (1973) Virol. 52, 456-467.
20. Scherrer, K., and Darnell, J.E. (1962) Biochem. Biophys. Res. Comm. 7, 486-490.
21. Aviv., H., and Leder, P. (1982) Proc. Natl. Acad. Sci. USA 69, 1408-1412.
22. Lehrach, M., Diamond, D., Wozney, J.W., and Boedtker, R.H. (1977) Biochemistry 16, 4743-4751.
23. Laemmli, U.K. (1970) Nature 227, 680-685.
24. Burnette, W.H. (1981) Anal. Biochem. 112, 195-203.
25. Varmus, H., and Swanstrom, L. (1983) In RNA Tumor Viruses (R. Weiss, N. Teich, H. Varmus, J. Coffin Eds) pp369-512, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.