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An improved retroviral vector for assaying promoter activity

Analysis of promoter interference in pIP211 vector

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We recently developed a novel promoter assay system using a retroviral vector (pIP200 series). Transcription from the internal promoter, which had been inserted for the promoter assay, was shown to be interfered with by transcription from the upstream long terminal repeat (LTR). Here we report a new high-titer 'self-inactivating' vector, in which transcription interference was virtually eliminated. This new vector was constructed by introducing only a very minor mutation into the 'TATA box' in the 3′-LTR. This mutation was successfully transferred to the 5′-LTR after reverse transcription, yielding a provirus incapable of transcribing viral RNA. The viral titer was not reduced by the mutation, permitting general application of this virus.

Retroviral vector; Promoter interference; Fluorescein-di-β-D-galactopyranoside

1. INTRODUCTION

We recently developed a novel promoter assay system using a retroviral vector (pIP200 series), which enabled us to detect promoter activity in individual brain cells in primary culture [1,2]. The structure of this vector with a nuclear location signal at the amino-terminus of the β-galactosidase (β-gal) is illustrated in Fig. 1A (pIP211). Two kinds of transcripts are produced from this vector: one from the 5′-LTR and another from the inserted promoter. However, on the longer transcript, since the translational initiation codon (ATG) of the *lacZ* gene is the second ATG codon following that of the *neo* gene, translational efficiency of the β-gal from this transcript is greatly reduced in eukaryotic cells. On the other hand, on the shorter transcript, the ATG codon of *lacZ* is the first one and initiates translation efficiently. Thus, β-gal is produced reflecting the cell type-specific activation of the inserted promoter. The effect of the enhancers in LTRs were not detectable in our recent studies [1,2].

One of the problems with this vector system is the possibility that the transcription itself from the internal promoter might be affected by the readthrough transcription from the 5′-LTR (promoter interference) [3–6]. In order to determine whether there is promoter interference in this vector and to improve the system, we have constructed a new 'self-inactivating (SIN) type' retroviral vector. By using this vector with SV40 early promoter as an internal promoter, the extent of promoter interference was measured. SV40 promoter without transcription from the upstream LTR promoter gives 1.4 times more transcript than that with overriding transcription, indicating the necessity of using SIN vector for promoter assay. Previously reported SIN vectors delete the viral enhancer or both the viral enhancer and the promoter upon integration into the host genome [7–13]. Most authors have reported very low titers with these vectors, which prevented their general application. Soriano et al. reported a high-titer SIN vector [13], but since they deleted only the enhancer and self-inactivation is insufficient, that type of vector is not suitable for our purpose. Reduced titers of SIN vectors are probably due to destruction of the steric structure important for 3′ RNA processing [8]. Therefore, we have introduced a very minor mutation only in the 'TATA box' in the 3′-LTR, and succeeded in producing high-titer virus with sufficient 'self-inactivation'.

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Abbreviations: LTR, long terminal repeat; β-gal, β-galactosidase; SIN vector, self-inactivating vector; PBS, phosphate-buffered saline; FCS, fetal calf serum; FDG, fluorescein-di-β-D-galactopyranoside; PI, propidium iodide; X-Gal, 5-bromo-4-chloro-3-indolyl-β-galactoside; FACS, fluorescence-activated cell sorter.

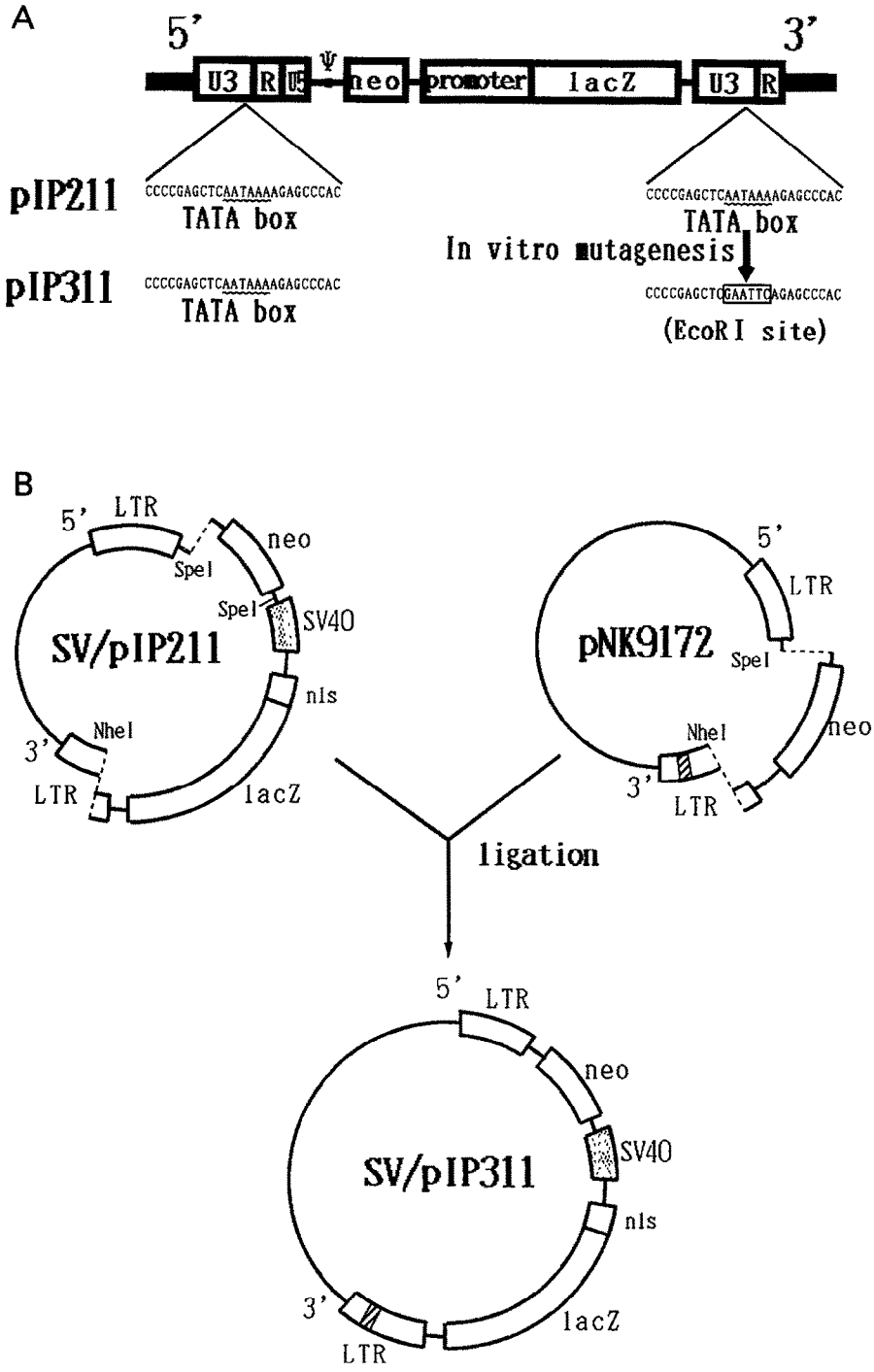

Fig. 1. Construction of SV/pIP311. (A) The sequence of 'TATA box' in the 3'-LTR of SV/pIP211 was mutated to the GAATTC sequence, which is the recognition site for *Eco*RI restriction enzyme. (B) pNK9172 plasmid is the same as pIF9171 plasmid [1] except that the 'TATA box' in the 3'-LTR was mutated (shown as a hatched region). SV/pIP311 plasmid was constructed from pNK9172 and SV/pIP211 [15] plasmids.

2. MATERIALS AND METHODS

2.1. *Construction of SV/pIP311 plasmid*

Site-directed in vitro mutagenesis using a heteroduplex intermediate was carried out in pIF9171 plasmid [1] as reported previously [14], which converted the 'TATA box' (AATAAA) in the 3'-LTR to the GAATTC sequence. The sequence of the oligonucleotide used in this process was: 5'-CCCCGAGCTCGAATTCAGAGCCCAC-3', which was synthesized by a Beckman system 1E DNA synthesizer. The 3.9-kb *Nhe*I– *Spe*I fragment of this mutated plasmid (pNK9172) was used to replace the corresponding region of SV/pIP211 plasmid [15] to construct the SV/pIP311 plasmid (Fig. 1B).

2.2. *Fluorescence-activated cell sorting*

Cells were harvested and resuspended at 3.5×10^6–4.2×10^7 cells/ml in phosphate-buffered saline (PBS) containing 10 mM HEPES and 4% fetal calf serum (FCS). Fluorescein-di-β-D-galactopyranoside (FDG) [16] was added to a final concentration of 1 mM, and the mixture was incubated at 37°C for 1 min. The reaction was stopped by adding 10 vols. of ice-cold PBS containing 10 mM HEPES, 4% FCS, and 1 μg of propidium iodide (PI) per ml. Fluorescein-positive and PI-negative cells were sorted and collected on a FACStar PLUS (Beckton Dickinson). These cells were cultured and used for the second round of cell sorting to eliminate most of the β-gal negative cells.

2.3. *Northern blot analysis*

Total RNAs were extracted by the guanidinium thiocyanate method and separated on a 1.5% formaldehyde agarose gel. The RNAs were then transferred to a Zeta-Probe membrane (Bio-Rad) with 10 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate). The 1.1-kb *Sac*I–*Eco*RI fragment of the *lacZ* gene and the 2.0-kb *Pst*I fragment of a chicken β-actin cDNA clone, pA1 [17], were ^{32}P-labeled with a Random Primer Labeling Kit (Boehringer Mannheim) and used as probes. Membranes were washed, and exposed to a Kodak X-Omat AR film at −70°C or to an imaging plate of a Bio-Image Analyzer BAS2000 (Fuji).

2.4. *Estimation of the viral titers*

Ten independent clones of G418-resistant packaging cells (φ2) were randomly selected and isolated, and the viral supernatants were collected. NIH 3T3 cells were plated at 1×10^4 cells/cm^2, cultured for 24 h, and infected with each clone of virus with 8 μg of polybrene per ml. The cells were processed for X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside: a substrate of β-gal) staining [1] 44 h after the viral infection. The numbers of X-Gal-positive cells per 1 ml of the added supernatants were calculated.

3. RESULTS AND DISCUSSION

In this study, we used SV/pIP211 vector as a model system, which has an SV40 early promoter and a nuclear location signal at the amino-terminus of β-gal [2,15]. The nuclear location signal restricts the localization of β-gal to the nucleus, which makes it very easy to identify each β-gal-positive cell. In order to construct a 'SIN' vector *without* any severe destruction of the steric structure of 3′-LTR, we changed the sequence of the 'TATA box' (AATAAA) in the 3′-LTR to a GAATTC sequence without any deletion to construct SV/pIP311 (Fig. 1A). This mutation is expected to be transferred to the 5′-LTR after reverse transcription, yielding a provirus incapable of transcribing viral RNA (Fig. 2). Recombinant retroviruses were produced as described previously [1].

We used NIH 3T3 cells as the target cells for infection, and the infected cells were collected by the FDG-FACS (fluorescence-activated cell sorter) method, because the *neo* gene is not expressed from the SV/pIP311 provirus and selection with G418 is impossible. Results of two rounds of cell sorting are shown in Fig. 3A. More than 95% of the collected cells were X-Gal-positive. Northern blot analysis of the RNA from these cells with a *lacZ* probe revealed that the longer transcript from the 5′-LTR of SV/pIP311 provirus was barely detected (Fig. 3B), which proved efficient 'self-inactivation' of this

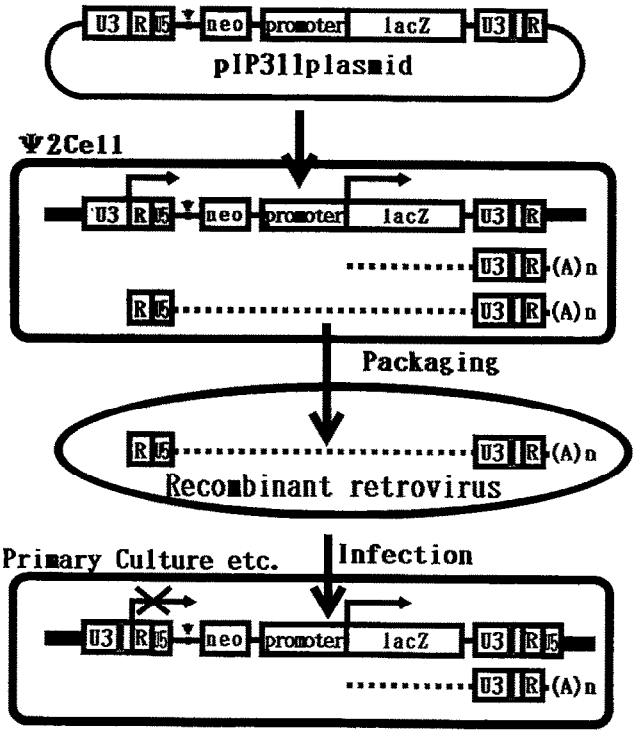

Fig. 2. Summary of production of pIP311 virus. A packaging cell line, φ2, was transfected with pIP311 plasmid containing an inserted promoter. In this cell, two kinds of transcripts are produced (dotted lines), while only the longer one is packaged to a recombinant retrovirus particle. This retrovirus was recovered as the culture supernatant and was used to infect target cells. In the infected cells, only the transcript from the internal promoter can be produced, because the mutation of the 'TATA box' in the 3′-LTR is transferred to the 5′-LTR after reverse trancsription. φ, packaging signal; shadowed region, mutation in the 'TATA box'.

virus. In addition, quantification of the bands by a Personal Densitometer PD110 (Molecular Dynamics) or a Bio-Image Analyzer BAS2000 (Fuji) showed that the transcript from the SV40 early promoter from pIP311 provirus was about 1.4 times more abundant than that from pIP211 provirus. The SV/pIP311 virus-infected cells were always more densely stained by X-Gal than those infected by SV/pIP211 virus under the same staining condition (Fig. 3A), which is in good agreement with the RNA analysis. These results clearly show that the transcription from the 5′-LTR indeed interferes with the transcription from the internal promoter in pIP211 vector.

Next we compared the titers of two recombinant retroviruses (pIP211, pIP311) by counting the X-Gal positive cells, because SV/pIP311 virus-infected cells cannot be counted as the conventional G418-resistant colony-forming units. Firstly, we estimated what proportion of the SV/pIP211-infected cells produces sufficient β-gal to be detected by X-Gal staining, because the presence of promoter suppression in SV/pIP211 pro-

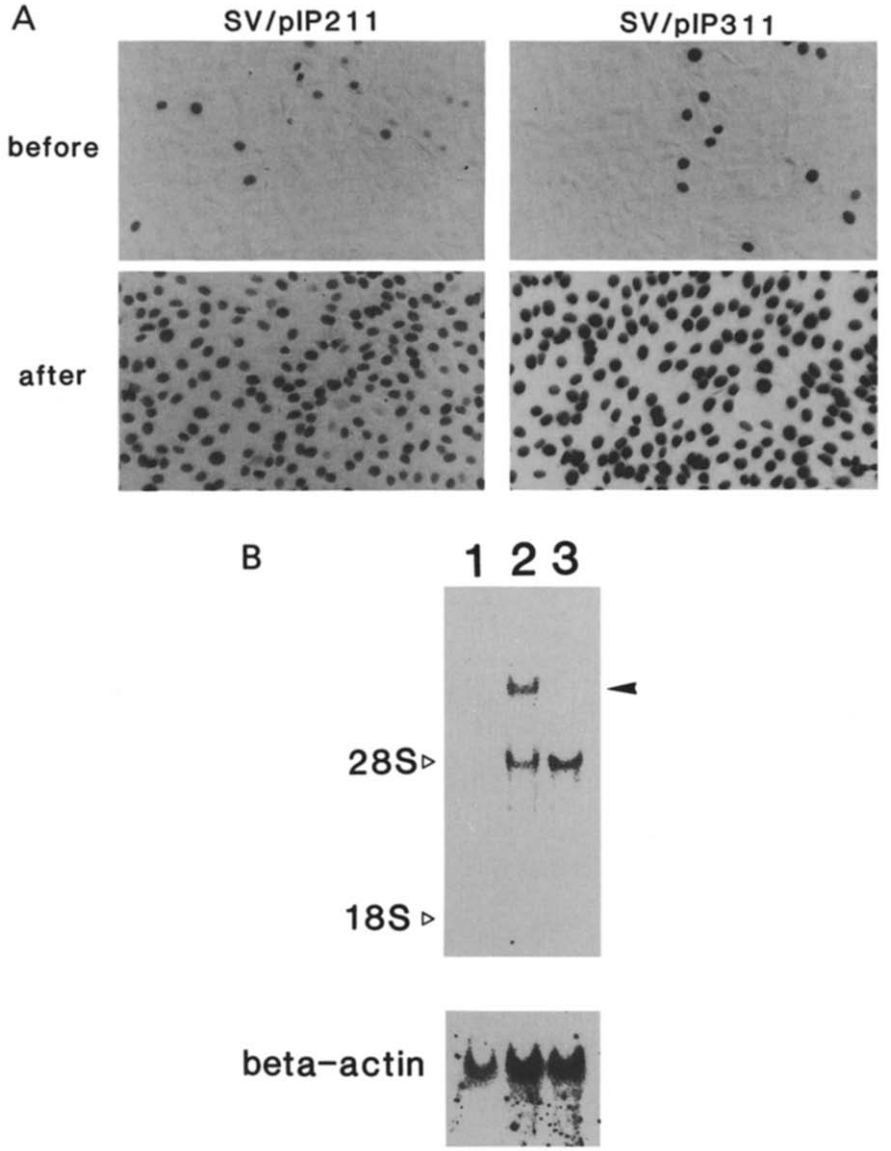

Fig. 3. RNA analysis of the cells collected by a FACS. (A) Comparison of the cells before and after two rounds of cell sorting. Portions of the collected cells were cultured overnight and stained with X-Gal. (B) Northern blot analysis with a *lacZ* probe (upper panel). Results of rehybridization of the same membrane with a β-actin probe are shown in the lower panel. The longer transcript from the 5′-LTR was barely detected in cells infected with SV/pIP311 virus (shown by the arrowhead). Lane 1, control cells without infection by any virus; lane 2, cells infected with SV/pIP211 virus; lane 3, cells infected with SV/pIP311 virus.

virus has now been proven. NIH 3T3 cells were infected with SV/pIP211 virus, cultured and selected with 1.5–2.0 mg of G418 per ml for 12 days, and processed for X-Gal staining as described previously [1]. Some of the infected cells were cultured for 9 days without G418 after selection for 9 days. Both of these cell cultures showed 95.0–95.3% X-Gal-positive cells, indicating that the titer of SV/pIP211 virus can be roughly estimated by counting the X-Gal-positive cells. Titers of 10 randomly selected clones of $\phi 2$ cells harboring SV/pIP211 and SV/pIP311 were determined as described in section 2 (Table I). Both the maximum and the average titers were higher in SV/pIP311 virus-producing cells than in SV/pIP211 virus-producing cells. At least the mutation introduced into the SV/pIP311 virus did not reduce its titer, although the titers of most of the previously reported SIN vectors were severely reduced. This is probably because only a minor mutation had been introduced and it did not affect the steric structure of the 3′-LTR severely, permitting efficient processing of the 3′-end of the RNA.

In conclusion, we have constructed a new high-titer 'self-inactivating type' pIP311 vector. RNA analysis revealed the presence of promoter interference in pIP211 vector, showing that pIP311 vector is more suitable for semi-quantitative promoter assay.

Table I

Viral titers of SV/pIP211 and SV/pIP311

SV/pIP211		SV/pIP311	
Clone no.	Number of X-Gal positive cells ($\times 10^3$ cells/ml)	Clone no.	Number of X-Gal positive cells ($\times 10^3$ cells/ml)
1	53.8	1	108.8
2	10.0	2	32.8
3	4.1	3	22.7
4	2.9	4	16.7
5	24.6	5	0.9
6	4.3	6	3.8
7	3.1	7	17.9
8	0.6	8	43.6
9	6.9	9	190.0
10	0.3	10	0.6
Ave.	11.1	Ave.	43.8

Viral titers were estimated by counting X-Gal positive cells 44 h after the viral infection. Randomly selected ten independent clones of $\phi 2$ cells harboring SV/pIP211 and SV/pIP311 were examined.

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