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Improved Mammalian Vectors for High Expression of G418 Resistance

Kazuto Katoh, Yoshiko Takahashi, Sigeo Hayashi* and Hisato Kondoh

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

ABSTRACT. Cell lines, such as those of teratocarcinoma and embryonic stem cells, fail to support high G418 resistance after transfection of *neo* vectors. To alleviate this, we modified pSV2-*neo* in two steps, first with tandem promoters of SV40 early genes and HSVtk, then by removing an improper met codon located immediately upstream of the authentic initiator codon in the mRNA sequence. In the final product, pST*neo*B, the *neo* transcription unit has *Xho*I sites at both ends. pST*neo*B yields G418-resistant transformants of teratocarcinoma cells at dramatically higher efficiencies than pSV2-*neo*. This vector extends the application of G418 selection in gene transfer to a wider range of cell types.

Eukaryotic vectors with dominant drug resistant genes are widely used to co-introduce genes of various biological interests stably into host genomes. For animal cells, especially those in culture, pSV2-*neo* (16) and its derivatives are the most versatile. The plasmid pSV2-*neo* carries the bacterial Tn5-derived coding sequence (*neo*) (1) bracketed by SV40 promoter and polyA addition signals, and animal cells that express the *neo* gene are selected with the protein synthesis-inhibiting antibiotic, G418. This selection method has less risk of chromosomal rearrangement than methods used for other drug resistant genes which render the cells starved for nucleic acid precursors.

Cell lines of certain cell types, however, survive only poorly selection for G418 resistance after gene transfer because of their too low expression of the *neo* gene. Typical examples are teratocarcinoma cells (10) and analogous embryonic stem cells of the mouse, which are extremely useful for studying the developmental regulation of gene expression (11, for review).

To overcome difficulties in the use of *neo* vectors with these cell types, we modified pSV2-*neo* in two steps to augment *neo* expression; first by using tandem promoters of SV40 and HSVtk, then by removing a nucleotide sequence of mRNA in the 5' untranslated region which interferes with normal translational initiation.

MATERIALS AND METHODS

Cell lines. Mouse teratocarcinoma cell lines OTF9 (12), PCC3/A/1 (3) and the fibroblast line, STO (9) were used. The culture medium was as described (4).

* Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309–0347, USA.

Abbreviation used: HSVtk, herpes simplex virus thymidine kinase gene

Transfection and G418 selection. The standard calcium phosphate technique (17) was used. A 0.5 ml calcium phosphate precipitate suspension containing 1 μg DNA was added to a 6 cm dish inoculated with 10^5 fibroblast or 2×10^5 teratocarcinoma cells the day before transfection. After 6 h, the culture was fed fresh medium, and after 24 h was transferred to a 9-cm dish. Forty-eight hours later G418 was added at 400 μg/ml, and after 10 days the cells were fixed then stained with Giemsa's solution.

Plasmid construction. Recombinant DNA was handled by the standard procedure (8). To construct pST*neo*, the 320 bp *Hind*III-*Bgl*II fragment of pSV2-*neo* was replaced with the 260 bp *Pvu*II-*Bgl*II fragment of HSVtk (from −197 to +56 (7)) after attaching an *Hind*III linker to the *Pvu*II end. To construct pST*neo*A, the overlapping *Pvu*II and *Eco*RI fragments of the *neo* region were excised from pST*neo*, made blunt-ended by T4 polymerase, attached with *Xho*I linkers, and cleaved at the *Bgl*II site. The resulting 580 bp *Xho*I-*Bgl*II and 2780 bp *Bgl*II-*Xho*I fragments that respectively corresponded to the promoter and *neo*-coding regions were joined at the *Bgl*II site and cloned at the *Xho*I site of plasmid vector pIBI76 (International Biotechnologies, Inc., New Haven, Connecticut). To construct pST*neo*B, the 160 bp *Bgl*II-*Nar*I fragment of the initiator met codon region was excised from pST*neo*A and cleaved with *Sau*3AI. The resultant *Sau*3AI-*Nar*I fragment was ligated to the rest of the plasmid sequence at the *Bgl*II and *Nar*I ends.

RESULTS

Insertion of the HSVtk promoter immediately downstream of the SV40 promoter. This was done by replacing the *Hind*III-*Bgl*II fragment of pSV2-*neo* (1, 16), which contained the bacterial sequence to the 5' of the *neo* gene, with the segment of the HSVtk promoter region (7) flanked by the same restriction sites (Fig. 1). The HSVtk promoter has been shown to drive the *neo* coding sequence in animal cells (2). The SV40-HSVtk tandem promoter initially was designed by Nicolas and Berg (10) in constructing pSVtk*neo*β. The resulting plasmid, pST*neo*, differs from pSVtk*neo*β in that the intron sequence is of SV40 rather than of β-globin origin. Because the removed bacterial sequence contains the prokaryotic promoter for the *neo* gene (1),

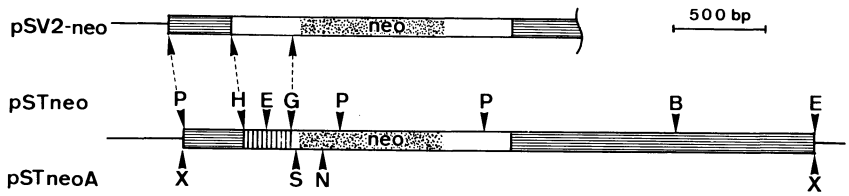

Fig. 1. Derivation of pST*neo* from pSV2-*neo*. Boxes with horizontal stripes show the SV40 promoter region (left) and SV40 intron-polyA addition site region (right). The open box shows the Tn5-derived sequence in which the *neo*-coding region is dotted. The box with vertical stripes shows the HSVtk promoter region. Horizontal lines indicate plasmid vector sequences. The orientation of the *neo* gene is the same for the plasmids, i.e. the ampicillin resistant gene is transcribed to the right. Relevant restriction sites: P, *Pvu*II; H, *Hind*III; E, *Eco*RI; G, *Bgl*II; B, *Bam*HI; X, *Xho*I; S, *Sau*3AI (only one site is indicated for this restriction enzyme) and N, *Nar*I. pST*neo* differs from pSV2-*neo* only in the replacement of the *Hind*III-*Bgl*II region. The *neo* region of pST*neo*A is the same as that of pST*neo* except for the *Xho*I sites at the termini which replace a *Pvu*II and an *Eco*RI site of pST*neo*. pST*neo*B lacks the short *Bgl*II-*Sau*3AI segment immediately in front of the *neo*-coding region.

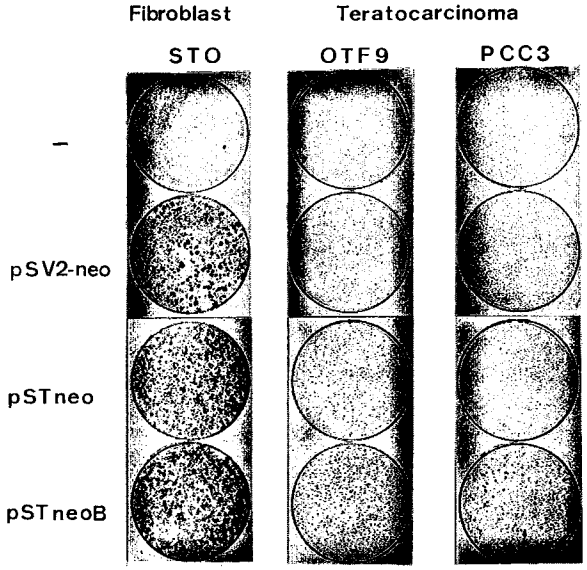

Fig. 2. Transformation of fibroblast and teratocarcinoma cell lines. G418-resistant colonies that developed from 2×10^5 (fibroblast) and 4×10^5 (teratocarcinoma) transfected cells are shown.

pST*neo* did not confer resistance for neomycin and related antibiotics (kanamycin and G418) (data not shown) as did pSV2-*neo* (16).

Transfection of the teratocarcinoma line OTF9 with pST*neo* indicated improvement in the frequency and size of G418-resistant colonies over pSV2-*neo*, but the transfection of PCC3/A/1 was still somewhat poor (Fig. 2; Table 1). Interestingly, transfection of fibroblasts, such as STO, with plasmids produced G418-resistant colonies at almost the same efficiency (Fig. 2; Table 1), evidence that in fibroblasts, the *neo* expression from pSV2-*neo* is already sufficiently high for G418 resistance in most stable transformants.

The *Pvu*II-*Eco*RI segment of pST*neo* that contained the whole *neo* transcription unit was provided with the *Xho*I linker sequence at its ends, so that the G418-resistant gene could be easily moved onto other plasmids and recloned in the pIBI76 vector. The resulting plasmid pST*neo*A was similar to pST*neo* in its G418 sensitivity in a bacterial host and in its expression of G418-resistance in teratocarcinoma and fibroblast lines (data not shown).

TABLE 1. FREQUENCY OF G418-RESISTANT TRANSFORMANTS BY TRANSFECTION WITH VARIOUS *NEO* GENES. AVERAGES OF TRIPLICATED TRANSFECTIONS SHOWN AS G418-RESISTANT COLONIES/10^6 TRANSFECTED CELLS/μg DNA

Plasmid	Cell line		
	STO	OTF9	PCC4/A/1
—	0	0	0
pSV2-*neo*	870	61	8
pST*neo*	1360	128	18
pST*neo*B	1980	411	108

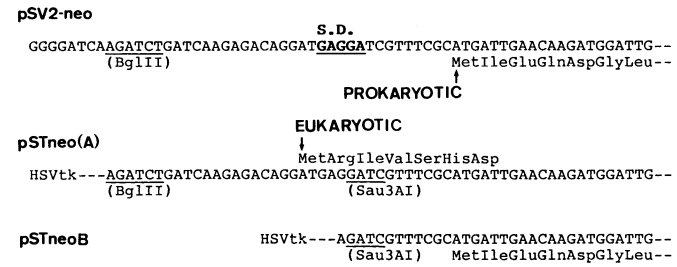

Fig. 3. DNA sequences of the translational initiator region and putative polypeptide sequences. On prokaryotic ribosomes, the met codon to the 3' of the S.D. (Shine-Dalgarno) sequence is registered as the initiator codon and directs normal *neo* expression. In contrast, on eukaryotic ribosomes, if the 5' most met codon satisfies certain conditions, such as being preceded by a purine residue at position −3 (5, 6), it primarily initiates polypeptide synthesis. Therefore, the met codon that overlaps the S.D. sequence of *neo* initiates out-of-phase polypeptide synthesis in mammalian cells and interferes with normal initiation. Removal of this non-authentic met codon by deletion of the nucleotide sequence between the *Bgl*II and *Sau*3AI sites relieves the normal translational initiation of such interference. The HSVtk sequence to the 5' of the *Bgl*II site does not contain a met codon.

Removal of the met codon immediately upstream of the normal translational initiation codon. pST*neo* was further improved for transformation of teratocarcinoma lines. Examination of the base sequences of the 5' non-coding region of the *neo* mRNA sequence suggested that an out-of-phase translation initiates immediately ahead of the normal initiation site (Fig. 3). This met codon presumably is not used in bacteria because it is not preceded by the Shine-Dalgarno sequence (15) required for the initiator codon. But, in mammalian cells the met codon is scanned from the 5' end of the mRNA for translational initiation (5). The nucleotide sequence surrounding the upstream met codon satisfies a condition for the initiator codon (6), whereas the normal met codon does not. Taking into account analogous cases studied by Kozak (6), the upstream met codon probably interferes with normal translational initiation. We therefore removed a 20 bp *Bgl*II-*Sau*3AI fragment that contained this upstream met codon from pST*neo*A and produced pST*neo*B.

Transfection of teratocarcinoma cells with pST*neo*B gave satisfactory results (Fig. 2; Table 1). The frequency of occurrence of G418-resistant colonies neared that for fibroblasts, and the resistant colonies were markedly larger than those produced with pST*neo* or pST*neo*A, evidence of high *neo* expression.

DISCUSSION

We improved the G418 vector in two steps starting from pSV2-*neo*: Tandem promoters (10) were used and an improper upstream met codon was removed. Each step contributed substantially to the higher expression of G418 resistance, and the final product gene, pST*neo*B, yielded G418-resistant transformants of teratocarcinoma cells at a frequency comparable to that obtained with fibroblasts. Although we tested only teratocarcinoma cells, pST*neo*B should prove useful for other cell lines in which pSV2-*neo* does not engender high G418 resistance.

Two additional modifications are included in our construction of pST*neo*B. First, *Xho*I linker sequences are attached at both ends of the *neo* transcription unit. This makes the *neo* gene more convenient to use because the transcription unit can

be easily moved onto other recombinant DNAs. In addition, the expression of *neo* in the bacterial host is removed. This facilitates use of the protoplast fusion technique (13, 14) for gene transfer because the *neo* gene product (neomycin phosphotransferase) is not carried over from bacterial cells and because non-fused bacterial cells can be killed by kanamycin which is inert to mammalian cells. Protoplast fusion is especially useful for gene transfer to transfection-sensitive cell lines (as with the majority of teratocarcinoma lines (e.g., ref. 4)) and results in less frequent rearrangement of the introduced genes than does transfection (our unpublished result).

Note

Plasmid pST*neo*B is obtainable from the Japanese Cancer Research Resources Bank (Gene).

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