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Expression in mammalian cells of a gene from *Streptomyces alboniger* conferring puromycin resistance

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ABSTRACT

The gene encoding a puromycin N-acetyl transferase from Streptomyces alboniger has been cloned next to the SV40 early promoter in a mammalian cells-Escherichia coli shutle vector. When this construction was introduced into VERO cells it expressed the relevant enzymic activity. Moreover, the puromycin N-acetyl transferase gene has been used as a dominant marker for the selection of transformed mammalian cells able to grow in the presence of the antibiotic.

INTRODUCTION

Streptomyces are mycelial Gram-positive bacteria possesing a highly developed secondary metabolism. They produce two thirds of the known antibiotics (1) and secret many enzymes, as cellulases, lipases, amylases, etc (2). Streptomyces DNA contains a high proportion of G:C pairs (>73%) (3). Their mRNAs show a biassed codon usage as compared to other organims and contain 3'-terminal sequences of putative stable secondary structure, which may play a role in transcription termination (4,5). The expression of Streptomyces genes in mammalian cells could be of general interest in view of the variety of enzymic activities encoded in their DNAs, but could be hampered by the structural features cited above.

The cloning and expression in Streptomyces lividans and Escherichia coli of a puromycin N-acetyl transferase (PAC) gene (pac) from Streptomyces alboniger have been recently reported (6). If expressed, this gene determines resistance to puromycin in the host (6). Taking into account that puromycin inhibits the growth of animal cells due to its blocking of protein synthesis by 80S ribosomes (7) it was considered that the

expression of pac in these cells could determine resistance to the antibiotic. This report describes the expression in mammalian cells of the Streptomyces alboniger puromycin N-acetyl transferase gene (6) cloned next to the SV40 early promoter and its use as a dominant selective marker.

MATERIALS AND METHODS

Bacterial strains, cell lines, plasmids and growth conditions

E. coli JM83, carrying plasmid pVN3.1 and E. coli AM6, a puromycin-sensitive mutant have been described elsewhere (6). E. coli strains were grown in liquid LB and LB agar medium (Miller, 1972). If required, the media were supplemented with ampicillin (100 µg/ml). Cell lines BHK21, Vero, L and HeLa were provided by The American Type Culture Collection. COS-1 cell line was provided by Y. Gluzman. The conditions for cell culture have been described (8). Plasmid pSV_2neo (9) was kindly provided by P. Berg. Plasmid pSV2ßglobin, originally prepared in the laboratory of P. Berg, was provided by F. Calvo (Laboratorios Llorente, Madrid, Spain).

Determination of enzymic activity

Cells were scrapped off the plates, and disrupted by sonication in 100 µl of 50 mM Tris-HCl pH 8.5, 2 mM EDTA, and 10% (v/v) glycerol. Enzymic activity was determined either spectrophotometrically or radiochemically as described elsewhere (6, 10).

DNA isolation and cloning procedures

Plasmid DNA from E. coli was prepared as described (11). Plasmid DNA was digested with restriction endonucleases as recommended by the suppliers (Boehringer- Mannheim, New England Biolabs and BRL). Specific DNA fragments were isolated from restricted samples of plasmid DNA by gel electrophoresis in low melting-point agarose followed by extraction of the relevant band by the cetyltrimethylammoniumbromide-assisted method (12). Ligation of DNA fragments was achieved by T4 DNA ligase (New Englad Biolabs).

Transformation of E. coli AM6 was carried out as described (6). Colonies were selected on LB plates containing ampicillin (100 µg/ml).

Transfection and transformation

Cultured cells were transfected with pSV2pac DNA using the DEAE dextran procedure (13). Transformation for puromycin or G418 resistance was done by the calcium phosphate precipitation technique (14, 15).

RESULTS AND DISCUSSION

Plasmid pVN3.1 (Fig 1, ref 6) contains the pac gene encoding the puromycin-inactivating enzyme puromycin N-acetyl transferase (PAC) from Streptomyces alboniger. The pac gene was isolated from pVN3.1 as a 1.1 kb HindIII-BamHI fragment (6) and was cloned downstream from the SV40 early promoter by substituting the ß-globin gene in pSV2ßglobin plasmid. The structure of the resulting pSV2pac plasmid and a detailed restriction map of the pac gene are shown in Figure 1. To test the expression of the PAC activity in mammalian cells, the COS-1 cell line was used. These cells constitutively express SV40 T-antigen and allow the transient amplification of SV40 derived replicons, theirby improving the detection of the expression of cloned genes (7). The cultures were transfected with pSV2pac, extracts were prepared at 48 hours post--transfection and the enzyme assayed. As shown in Table I, the cultures transfected with pSV2pac plasmid accumulated the enzyme, whereas those transfected with pSV2neo DNA showed no detectable PAC activity. Since the transfection procedure used leads to the expression of the cloned gene in only a few percent of the cells in the culture (17), it follows that the level of PAC accumulation (4-33 mU/mg) in the COS-1 cells that resulted transfected is similar to the value obtained in Streptomyces and Escherichia coli (6).

To determine whether the pac gene could be expressed permanently in mammalian cells, VERO cell cultures were transformed with a mixture of pSV2neo and pSV2pac DNAs and cell clones resistant to G-418 were selected. Out of 13 clones isolated, one (clone GP1) showed a specific PAC activity of 1.58 mU/mg of protein. This clone grew similarly in the presence of either puromycin (10 µg/ml) or G-418 (500 µg/ml). These results indicate that the pac gene can be

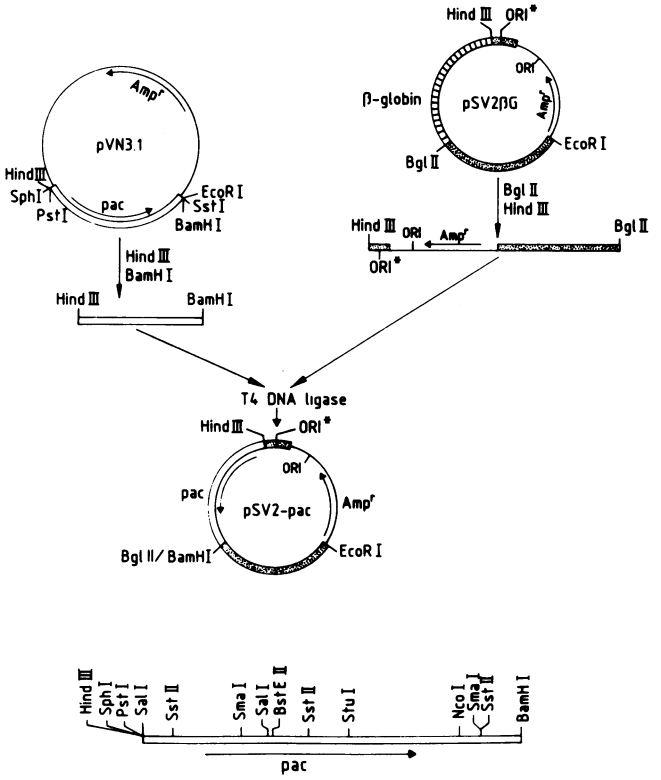

Figure 1: Strategy of the cloning of Streptomyces alboniger pac gene in pSV2 vector. Plasmids pSV2ßglobin and pVN3.1 were digested with restriction endonucleases BglII plus HindIII and BamHI plus HindIII, respectively. The resulting fragments were separated by low melting point agarose. The 4.2 kb fragment from pSV2ßglobin and the 1.1 kb fragment from pVN3.1 were isolated by the CTAB-assisted method (18) and then ligated with T4 DNA ligase. The ligation products were used to transform Escherichia coli AM6.Amplicillin-resistant (100 µg/ml) colonies were selected on agar plates and plasmid DNA was isolated as described (17). From one of the clones pSV2pac was isolated. The lower part of the figure shows a detailed restriction map of the 1.1 kb Streptomyces alboniger DNA cloned fragment. The arrows indicate the direction of transcription and the size of the pac or ß-lactamase genes. ORI indicates the origin of replication of pBR322 plasmid. ORI* refers to both origin of replication and early promoter of the SV40 virus. (——) pBR322, (▭) S. alboniger, (▭) ß-globin and (▭) SV40 DNAs.

Table I: Transient expression of Streptomyces alboniger pac gene in COS-1 cells.

Experiment	Plasmid DNA	Puromycin N-acetyl transferase[a] (mU/mg of protein)
1	pSV2neo	<0.1
	pSV2pac	5.1 ± 0.9
2	pSV2neo	<0.1
	pSV2pac	27.2 ± 5.8

a.- Replicate cultures of COS-1 cells were transfected with either pSV2neo or pSV2pac DNA. Seventy two hours post-transfection, cells were scrapped off the plates, and total cell extracts were prepared as described under Materials and Methods. Enzymic activity was determined spectrophotometrically (20).

constitutively expressed in cultured VERO cells and suggest that it may be useful for selection in gene transfer experiments with mammalian cells as a puromycin resistant marker.

Table II: Permanent expression of Streptomyces alboniger pac gene in transformed VERO cells.

Cell clone[a]	Puromycin N-acetyl transferase activity[b] (mU/mg of protein)	Growth in puromycin containing medium[c] (µg/ml)					
		0	5	10	20	50	100
a1	14.0	+++	+++	+++	+++	+++	+++
a2	3.7	+++	+++	++	+	+/-	-
a3	4.1	+++	+++	+++	++	+	-
a6	2.8	+++	+++	+++	++	++	+
E4	3.6	+++	+++	+++	++	+	+/-

a.- VERO cells cultures were transformed with pSV2pac or mock-transformed and colonies were selected for growth in medium containing puromycin (10 ug/ml). No resistant colonies were detected in mock-transformed cultures.
b.- Enzymic activity was determined by incorporation of ^{14}C-acetyl-CoA as described (17). Activity values for extracts of VERO cells were below 0.1 mU/mg of protein.
c.- Each cell clone was subcultured with a split ratio of 1:10 into media containing different concentrations of puromycin and incubated at 37°C. (+++), (++), (+), (+/-) and (-) indicate cell growth corresponding to aproximately 100, 75, 50, 25 and 0% of that observed in parallel VERO cell cultures, respectively.

This possibility was tested by transformation of VERO cells with pSV2pac DNA and selection with puromycin (10 μg/ml). The characterization of the resistant clones obtained in two independent experiments is shown in Table II. Every clone tested expressed measurable PAC activity which correlated with the *in vivo* resistance to puromycin (Table II), and contained the *pac* gene integrated into chromosomal DNA (data not shown). Therefore, the *pac* gene can confer resistance to puromycin when expressed in VERO cells. To test the generality of these results and the eventual usefulness of the *pac* gene as a dominant selectable marker, cell lines of monkey, hamster, mouse and human origin, were used in transformation experiments in which pSV2neo was included as an internal control. As shown in Table III, every cell line tested (VERO, BHK 21, L and HeLa cells) could be transformed, albeit with very different efficiencies. In the case of BHK 21 cells, the efficiency of transformation to puromycin resistance is comparable to that obtained for G-418 resistance, while it was lower for the other cell lines.

The basis for the differences in transformation efficiencies obtained for the cell lines used is not understood. It could be a reflection of either variable efficiencies of expression of the *pac* gene, or differences in the stability of the PAC enzyme or a consequence of the availability of acetyl-CoA in the cytoplasm of the different cells. It is noteworthy that this is the first example of an antibiotic acetyl transferase whose expression can be used as a selectable marker in mammalian cells. In contrast, the chloramphenicol acetyl transferase (CAT), that can be efficiently expressed in a variety of mammalian cell lines (13), has not been useful as a selective marker so far.

The results presented in this report demonstrate for the first time the expression in mammalian cells of a gene derived from *Streptomyces*, in spite of the differences in G:C content and codon usage between their corresponding DNAs. This finding opens the way for the expression of specific *Streptomyces* genes in mammalian cells and even in transgenic animals. Specially interesting are the genes responsible for

Table III: Efficiency of transformation of different cell lines with pSV2pac.

Cell line	Frequency of spontaneous pur^r mutants ($x10^{-6}$)[a]	Number of colonies resistant to[b] puromycin	G-418
BHK21	0.14	668/284	1000/1200
VERO	<0.14	1/5	66/400
L	4.4	37/19	162/800
HeLa	0.2	8/ND[c]	40/ND

a.- To several dishes containing a total of 5-17x10^6 cells, puromycin was added to a final concentration of 10 µg/ml (except for HeLa cells, that received 2.5 µg/ml). After 2-3 weeks the spontaneus resistant colonies were counted and the frequency of spontaneus puromycin resistant mutants was calculated.
b.- Two or three dishes containing 10^6 cells were transfected by the calcium phosphate precipitation technique (11-12) with 3 µg of either pSV2pac or pSV2neo DNAs. Twenty four to thirty six hours thereafter the cells were subcultured (at a 1:3 ratio for cells receiving pSV2pac or 1:10 ratio for cells receiving pSV2neo) and 48 hr later puromycin (10 µg/ml, except for HeLa cells, that received 2.5 µg/ml) or G-418 (500 µg/ml) was added, respectively. The media were changed every 3-5 days and 2-3 weeks later the colonies were stained with crystal violet and counted. The numbers indicate the average value of resistant colonies for 2x10^6 cells obtained in two independent experiments.
c.- Not determined.

antibiotic detoxification, that could serve as selective markers in the manipulation of mammalian genomes. This is in fact the case with the pac gene, which has proven useful in the selection for puromycin resistance (Table III). Moreover, the genes determining resistance to puromycin and G-418 can be expressed simultaneously in, at least, VERO cells (see above) which could have applications in insertional inactivation experiments. In addition, puromycin inhibits protozoan and insect cell growth (7, 19), and chryscandin, a puromycin-analogue antibiotic, which is inactivated by PAC (20), blocks the growth of the human pathogen Candida albicans (21). Therefore, the pac gene should be useful to develope dominant cloning vectors for these organisms.

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