Bacterial *IMPDH* Gene Used for the Selection of Mammalian Cell Transfectants

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ABSTRACT

Stable cell transfection is used for the expression of exogenous genes or cDNAs in eukaryotic cells. Selection of these transfectants requires a dominant selectable marker. A variety of such markers has been identified and is currently in use. However, many of these are not suitable for all cell types or require unique conditions. Here we describe a simple and versatile dominant selectable marker that involves bacterial IMP dehydrogenase (*IMPDH*), an enzyme essential for the replication of mammalian and bacterial cells. Although *IMPDH* is evolutionarily conserved, the bacterial enzyme is orders of magnitude more resistant to the toxic effect of the drug mycophenolic acid, which is an *IMPDH* inhibitor. We have demonstrated that transfection of human, monkey or Chinese hamster cell lines with an expression vector containing bacterial *IMPDH* and mycophenolic acid treatment resulted in the selection of colonies with a strikingly increased resistance to mycophenolic acid toxicity. Analysis of cells derived from these colonies indicated that the acquisition of this resistance was associated with bacterial *IMPDH* protein expression. As a proof of principle, we showed that mammalian cell transfection with a bicistronic *IMPDH*/GFP expression vector and mycophenolic acid treatment can be used to successfully select transfectants that express the fluorescent protein. These results indicate that bacterial *IMPDH* is a practical dominant selectable marker that can be used for the selection of transfectants that express exogenous genes or cDNAs in mammalian cells.

INTRODUCTION

The introduction of DNA sequences into mammalian cells is frequently used to express exogenous proteins. Because of a low efficiency of stable integration

of such DNA into the mammalian genome, few cells become transformed and express the exogenous gene or cDNA of interest. The selection of these cells is usually achieved by having cultures transfected with (i) a vector containing the exogenous gene or cDNA and another containing a dominant selectable marker that confers growth advantage in the presence of an appropriate cytotoxic drug or (ii) a vector that harbors both the exogenous gene or cDNA and the dominant selectable marker. Over the years, a series of dominant selectable markers has been identified and utilized (1–6; reviewed in Reference 7). However, not all of these are appropriate for all cell types or experimental systems, nor are they necessarily simple and easy to use. For example, thymidine kinase requires the use or generation of cells deficient in this enzyme (8). Others may not be easily expressed in certain cell types or may require unique media. Some cell types may exhibit an innate resistance to the selecting drug (1,2,4,8). The commonly used dominant selectable marker is the bacterial aminoglycoside phosphotransferase II gene (commonly referred to as the neomycin-resistant or *neo* gene), which confers resistance to the cytotoxic effect of the aminoglycoside antibiotic G418 (9). However, its high cost makes G418 less ideal for high-throughput protocols. In addition, G418 causes mammalian cells to release GPI-anchored proteins (10), thus making it unsuitable for the study of these proteins.

We have identified an additional dominant selectable marker, bacterial inosine monophosphate dehydrogenase (*IMPDH*), which can be used for stable transfection of mammalian cells. The advantages of this selectable marker over existing markers include the simplicity of the selection protocol, its ability to be transfected and expressed in a variety of mammalian cell types, and the cost-effectiveness of the selection drug mycophenolic acid (MPA) relative to G418, which makes it suitable for high-throughput screenings. *IMPDH* catalyzes the oxidation of IMP to XMP (11–13; reviewed in Reference 14), which is the rate-limiting step in the de novo guanine nucleotide biosynthesis pathway and, as a result, is essential for the growth and proliferation of every

cell. Although *IMPDH* is evolutionarily conserved (15,16), the bacterial enzyme is orders of magnitude more resistant than mammalian *IMPDH* to the toxic effect of MPA (17–20). Based on this property, we have demonstrated that expression of bacterial *IMPDH* in mammalian cells confers in them resistance to MPA toxicity and, moreover, that this enzyme is a useful dominant selectable marker for the expression and selection of exogenous genes or cDNAs in mammalian cell transfections.

MATERIALS AND METHODS

Cells

Chinese hamster ovary V79, human breast carcinoma MDA-MB-231, human prostate PC3, and African green monkey kidney COS7 cells were cultured in growth medium consisting of RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ L/mL streptomycin.

Construction of Vectors

Bacterial *IMPDH* was amplified from *Escherichia coli* genomic DNA by PCR with the primers 5'-CAGC-GGCCGCAATGCTACGTATCGCTA-AAGAAGC-3' and 5'-CCGGATATCGAATCAGGAGCCCCAGACGG-3', which contain flanking *NotI* and *EcoRV* restriction endonuclease sites, respectively. The PCR fragment was ligated into the pFLAG-CMV-2 vector (Sigma, St. Louis, MO, USA) to generate the plasmid pFLAG-*IMPDH* (Figure 1A). The sequence of the amplified DNA fragment was found to agree with that of the published *E. coli IMPDH* gene sequence. A bicistronic expression vector containing FLAG-tagged bacterial *IMPDH* and enhanced green fluorescent protein (*EGFP*) (pIMPDH-EGFP; Figure 1B) was constructed after excising the *IMPDH* DNA fragment from the pFLAG-*IMPDH* plasmid and ligating it into the pIRES2-EGFP vector (BD Biosciences Clontech, Palo Alto, CA, USA). A bicistronic expression vector containing the *neo* and *EGFP* genes (pNEO-EGFP; Figure 1C) was constructed after PCR-amplifying

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Table 1. Transfection Efficiency

	Cell Type	Vector	Colonies/ 1×10^6 Viable Cells
Experiment 1 ^a	V79	pFLAG-IMPDPH	150
	MDA-MB-231	pFLAG-IMPDPH	14
	PC3	pFLAG-IMPDPH	6
	COS7	pFLAG-IMPDPH	30
Experiment 2	V79	pIMPDPH-EGFP	78
	MDA-MB-231	pIMPDPH-EGFP	22
	PC3	pIMPDPH-EGFP	9
Experiment 3	V79	pIMPDPH-EGFP	400
	V79	pNEO-EGFP	620

^aTransfection with a control vector that does not contain the *IMPDPH* gene failed to yield any colonies/ 3×10^6 electroporated viable cells.

the *neo* gene from the pCDNA3 vector and ligating it into the pIRES2-EGFP vector. The constitutively expressing EGFP vector (pHygEGFP) was purchased from BD Biosciences Clontech.

Mammalian Cell Transfection

Cells (2×10^7) were washed once with PBS, resuspended in 400 μ L Cytomix buffer (21), placed in an electroporation cuvette, and transfected with 10 μ g DNA by electroporation at 200 V for 30 ms in an ElectroSquarePoratorTM T280 electroporator (BTX, San Diego, CA, USA). Immediately after electroporation, the cuvettes were placed on ice for 10–15 min. After that, the cells were washed once in PBS and 1×10^6

viable cells, as determined by trypan blue exclusion, were resuspended in 10 mL growth medium in 100-mm tissue culture plates, incubated overnight at 37°C, and treated the next day with 0.8 μ g/mL MPA (stock concentration at 14.2 mg/mL in DMSO; Sigma) or 600 μ g/mL G418. For the different assays, cells were obtained from either individual or pooled MPA-resistant colonies. For colony isolation, visible individual colonies were each picked and aspirated into a glass Pasteur pipet and transferred into a well of a 24-well tissue culture plate. The next day, after rinsing the wells with PBS and incubation for 1–2 min in a drop of a trypsin/EDTA solution, the cells within each well were dispersed by forceful pipetting. Next, the cells were incubated in growth medium to generate a monolayer culture, which was used to determine the cells' susceptibility to MPA. Cultures were also obtained from single MPA-resistant cells derived from pooled colonies by seeding 2.5 cells/mL into 96-well flat-bottom microplates in 200 μ L growth medium/

Figure 1. Schematic diagrams of bacterial IMPDPH and NEO expression plasmids. (A) FLAG-tagged *E. coli* IMPDPH gene under the control of the CMV promoter. The poly(A) sequence is from the human growth hormone gene. The backbone of the plasmid is the pFLAG-CMV-2 vector. Bicistronic expression vectors of (B) FLAG-tagged bacterial IMPDPH and EGFP or (C) neomycin resistance (NEO) and EGFP genes are under the control of the CMV promoter. The internal ribosomal entry sequence (IRES) is from the encephalomyocarditis virus. The poly(A) sequence contains the SV40 early mRNA poly(A) signal. The backbone of the plasmids is the pIRES2-EGFP vector.

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well, and only cultures deriving from wells that were initially found to contain single cells were used. MPA toxicity was determined by the methylthiazolotetrazolium (MTT) assay, while the expression of GFP was detected by fluorescence microscopy and/or flow cytometry.

MTT Assay

Cell survival and proliferation were measured using the MTT assay, which detects active mitochondria and, as a result, viable cells (22). For this assay, 2×10^4 cells were suspended in 200 μ L growth medium/well in 96-well flat-bottom microplates with various stimuli and incubated at 37°C. After 6 days, 10 μ L MTT solution (5 mg/mL in PBS) (Sigma) was added to each well, and the plates were incubated for an additional 4 h at 37°C. In the case of V79 cells, which grow twice as fast as the other cell lines, the MTT solution was added after 3 days. The plates were then centrifuged at 300 $\times$ g for 5 min, the supernatants were discarded, 100 μ L DMSO were added per well, and the plates were read at 540 nm using a microplate reader.

Western Blotting

Cells (2×10^6) were lysed in 100 μ L boiling 2 $\times$ SDS-PAGE sample dye for 15 min, after which time 10 μ L lysate samples were subjected to SDS-PAGE and electroblotted to Hybond[®]-C extra nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The FLAG epitope-tagged *E. coli* IMPDH was visualized with an anti-FLAG (M2) antibody (Sigma) and goat anti-mouse IgG-HRP (Bio-Rad Laboratories, Hercules, CA, USA) using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

RESULTS

Expression of Bacterial IMPDH in Mammalian Cells Confers in Them Resistance to MPA Toxicity

To determine the suitability of bacterial IMPDH as a dominant selectable marker for the selection of mammalian cell transfectants, we constructed a vec-

tor containing FLAG epitope-tagged *E. coli* IMPDH under the control of the cytomegalovirus (CMV) promoter (Figure 1A). Transfection of Chinese hamster V79, human MDA-MB-231 and PC3, and monkey COS7 cells with this vector and incubation for 10–20 days with 0.8 μ g/mL MPA yielded MPA-resistant colonies in the four cell types. Transfection efficiencies of the various cell types, as measured by the number of MPA-resistant colonies/ 1×10^6 viable electroporated cells, are tabulated in Table 1, experiment 1. No such colonies were obtained after transfection with the same vector but without the IMPDH gene.

To further substantiate their resistance, we initially isolated seven individual MPA-resistant V79 colonies and verified, by means of the MTT assay, that their cells were resistant to the cytotoxic effect of up to 50 μ g/mL MPA. In contrast, the parental cells failed to grow in the presence of as little as 0.8 μ g/mL of the drug. In addition, we have established single-cell-derived cultures from pooled MPA-resistant colonies from each of the four cell types and determined their susceptibility to different concentrations of MPA. The results indicated that these cells also exhibited resistance to MPA cytotoxicity (Figure 2, A–D). Growth inhibition of wild-type V79 cells, which have a doubling time of about 12 h compared to 24 h for the other cell types, was detected as early as 3 days after MPA treatment compared to 6 days for MDA-MB-231, PC3, and COS7 cells. Although on the sixth day after MPA treatment there was some MTT staining in control (not transfected parental) cultures (Figure 2, A–C), no replicating cells were observed. Moreover, after 4 weeks of this treatment, these control cultures exhibited no MTT staining above background levels (data not shown). Unlike the other cell types, the MPA-resistant COS7 cells did not proliferate as vigorously as the wild-type cells (Figure 2C).

Next, we wanted to determine whether MPA-resistant cells express the transfected bacterial IMPDH protein. To avoid potential cross-reactivity of a bacterial IMPDH antibody with the resident mammalian enzyme and to facilitate the detection of the protein, we have originally constructed the vector to

contain FLAG epitope-tagged *E. coli* IMPDH (Figure 1A). Western blot analysis with an anti-FLAG antibody indicated that cells from MPA-resistant colonies but not wild-type cells contain an antigenic protein band with a molecular mass of 53 kDa (Figure 2E), which is the predicted size of the *E. coli* IMPDH (20). The band from the MPA-resistant PC3 cells required a longer exposure time because of a lower level of bacterial IMPDH expression (Figure 2E, lower panel). Based on these results, we conclude that MPA resistance of pFLAG-IMPDH vector-transfected mammalian cells is coupled with bacterial IMPDH protein expression.

GFP Expression in Transfected V79 Cells

Because dominant selectable markers are used to isolate mammalian cell transfectants that express an exogenous gene or cDNA of interest, we decided as a proof of principle to determine whether bacterial IMPDH could be used to obtain such cells. A strategy often used to select for transfectants involves co-transfection of cells with a vector containing the dominant selectable marker along with another vector containing the exogenous gene or cDNA of interest. For this reason, we co-transfected V79 cells with pFLAG-IMPDH and pHygEGFP vectors, which harbors a GFP gene, and selected for transfectants in MPA-containing medium. Fluorescence microscopy and flow cytometry analysis of cultures derived from pooled MPA-resistant colonies indicated that only about 30% of these cells express GFP (Figure 3A). To increase the likelihood of selecting for cells expressing both bacterial IMPDH and GFP, we generated a bicistronic expression vector that contains both the bacterial IMPDH and GFP genes (Figure 1B). Transfection of V79, MDA-MB-231, or PC3 cells with this vector and treatment with the selective agent also yielded MPA-resistant colonies. The transfection efficiencies of the various cell types are shown in Table 1, experiment 2. Microscopic observation as well as flow cytometry analysis indicated that the MPA-resistant cells obtained from pooled colonies or from colonies derived from

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single cells express GFP (Figure 3B). Moreover, when cultured for 2 months in the presence of MPA, they continued to express GFP. These results indicate the usefulness of bacterial *IMPDH* in the selection of mammalian cell transfectants expressing an exogenous protein.

To further demonstrate the effectiveness of this gene as a dominant selectable marker, we compared the transfection efficiency of the *IMPDH* vector to that of the commonly used dominant selectable marker, namely the *neo* gene (Figure 1C). Because the base vector (pIRES2-EGFP) used in this study has a *neo* gene outside of the bicistronic expression vector, which contains the EGFP region, the pNEO-*IMPDH* vector was linearized with *Clal* to render the aforementioned *neo* gene nonfunctional while keeping the NEO-IRES-EGFP bicistronic genes fully intact. As a control, the p*IMPDH*-EGFP vector was also linearized with the same endonuclease. Transfection with either the linearized p*IMPDH*-EGFP or pNEO-EGFP vector resulted in 400 MPA-resistant and 620 G418-resistant colonies/ 1×10^6 viable transfected cells, respectively (Table 1, experiment 3). Although transfection efficiencies can vary from day to day (Table 1, experiments 2 and 3; V79 cells transfected with p*IMPDH*-EGFP), a direct comparison of the *IMPDH* and NEO-containing vectors indicated comparable transfection efficiencies. Expression of GFP in the MPA- and G418-resistant cells was verified by flow cytometry analysis (Figure 3, C and D).

DISCUSSION

In the present studies, we show that transfection of mammalian cells with a vector containing the bacterial *IMPDH* gene conferred in them a selective

growth advantage in the presence of the cytotoxic drug MPA. As a proof of principle, we have also demonstrated the ability of this dominant selectable marker to allow for the selection of transfectants that express an exoge-

Figure 2. Expression of bacterial *IMPDH* in mammalian cells confers resistance to MPA toxicity. MPA cytotoxicity was determined in cells of the control (not transfected parental) and of an *IMPDH* transfectant derived from the (A) MDA-MB-231, (B) PC3, (C) COS7, or (D) V79 cell line. Black bars depict the control cells, while white bars depict cells of the *IMPDH* transfectants. The viability of MDA-MB-231, PC3, and COS7 cells was examined after 6 days of incubation, while after 3 days for V79 cells. Cell viability was determined by the MTT assay and the absorbance values of the controls in the absence of MPA were arbitrarily set at 100%. Data are the $\bar{x} \pm SE$ of triplicates in an experiment, which is representative of three similar experiments. The expression of the *E. coli IMPDH* gene (E) was determined in control (not transfected parental, Wt) and *IMPDH* transfectant (Tx) cells. To determine this expression, lysates derived from 1×10^5 cells were subjected to SDS-PAGE, and the FLAG-tagged *E. coli IMPDH* was detected by Western blotting using an anti-FLAG antibody and developed by enhanced chemiluminescence. The lower panel depicts a 2-h over-exposure of the blot. Results are representative of three similar experiments.

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nous gene of interest, namely *GFP*. The ability to express this gene in four different mammalian cell types from three different species indicates the versatility of this dominant selectable marker. Because IMPDH is common in all living cells, we did not expect exogenously expressed bacterial IMPDH to have an adverse effect on mammalian cell function. Yet, ectopic expression of bacterial IMPDH caused the COS7 cells to grow at a somewhat slower rate than the parental cells (Figure 2C). This aberrant behavior is most likely due to an interaction between the simian virus 40 (SV40) origin of replication, which is a component of our vector, and the viral T-antigen, which is expressed in these cells. Such an interaction may result in high levels of vector replication before its integration into the cellular genome and, as a consequence, lead to abnormal integration

and aberrant cellular DNA replication. For this reason, the reduced replication of the COS7 transfectants may be unique to these cells and not be a problem with cell types that do not express the viral T-antigen, as exemplified by the other three cell lines tested in the present study.

MPA blocks the de novo guanine nucleotide biosynthesis pathway by inhibiting IMPDH. However, guanine nucleotides can also be synthesized via the salvage pathway by utilizing guanosine and/or guanine and the mammalian enzyme hypoxanthine-guanine phosphoribosyltransferase. Some batches of FBS may contain unusually high concentrations of guanosine and/or guanine, which could potentially reduce the effectiveness of the selection process. In this respect, the addition of high concentrations of guanine or guanosine (100–500 μ M) to the culture medium has been

reported to reverse the effects of MPA (23,24). However, it is unlikely that such concentrations are present in commercially available sera. Moreover, IMPDH inhibitors including MPA have been reported to also inhibit the activity of the guanine salvage pathway (24–29). Nevertheless, a proper dose response curve of MPA toxicity should be performed before using IMPDH for selection.

Although MPA has been utilized previously as a selectable agent in conjunction with the *E. coli* xanthine-guanine phosphoribosyltransferase (*XGPRT*) gene (1), we feel that our system is more advantageous. Selection with IMPDH requires only MPA, while selection with *XGPRT* necessitates MPA as well as xanthine, hypoxanthine, thymidine, and aminopterin. Moreover, the use of xanthine by the expressed bacterial *XGPRT* protein instead of hypoxanthine, which is used by the mammalian enzyme hypoxanthine-guanine phosphoribosyltransferase, could have unforeseen potential adverse effects.

Interestingly, MPA-resistant wild-type V79 and murine neuroblastoma cells have been reported (30–32). Resistance in these cells, which only express endogenous mammalian *IMPDH*, was obtained as a result of *IMPDH* gene amplification after a prolonged and stepwise process involving the use of increased MPA concentrations. It is unlikely that a significant fraction of naturally resistant cells would be generated by our selection protocol, which uses a higher concentration of MPA and a shorter selection time than those used in the initial selection of the cells with amplified resident *IMPDH*. In agreement with this notion, none of the cultures transfected with control vectors generated MPA-resistant colonies.

Figure 3. Expression of GFP in transfected V79 cells. GFP expression was determined by flow cytometry in (A) pooled MPA-resistant colonies from V79 cells co-transfected with pFLAG-IMPDH and pHygEGFP vectors (open histogram) or (B) an MPA-resistant culture derived from a single V79 cell originally transfected with the bicistronic pIMPDH-EGFP vector (open histogram). GFP expression was examined in (C) IMPDH-EGFP and (D) NEO-EGFP vector-transfected V79 cells (open histograms) that were transfected on the same day under identical conditions. Shaded histograms in panels A–C represent V79 cells transfected with the pFLAG-IMPDH vector only. The shaded histogram in panel D represents control (not transfected) V79 cells. Results are representative of three similar experiments.

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In addition to being new, the *IMPDH* dominant selectable marker has several advantages over existing ones. First, unlike some other markers that require specialized medium, the present dominant selectable marker can be used with a wide variety of media, and the selection procedure is simple, since selection only requires the addition of an appropriate concentration of MPA to such a medium. Second, this dominant selectable marker does not necessitate an *IMPDH*-deficient cell in contrast to certain selectable markers such as thymidine kinase, which requires a thymidine kinase-deficient cell (8). As a result, the *IMPDH* dominant selectable marker is less time-consuming to set up. Lastly, the *IMPDH* dominant selectable marker has the potential to be used in high-throughput screenings. Unlike the widely used *neo* dominant selectable marker (7), which requires a high dose range (100–1000 µg/mL) of the expensive selection drug G418, the bacterial *IMPDH* dominant selectable marker uses MPA at a dose range 2–3 orders of magnitude lower, thus making it a cost-effective alternative for use in various high-throughput screenings.

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