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Öztürk ÖZDEMİR

Transfection of Human Kidney Epithelial 293 Cells With Bacterial Cytosine-5-Methyltransferase M. *Msp1* and The SV40 Nuclear Localisation Sequence of VP1

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Department of Medical
Biology and Genetics Faculty of Medicine
Cumhuriyet University
58140, Sivas-Turkey and
Department of Medical Biology and
Biothechnology University of Sheffield
S10 2TN, UK

Abstract: The aim of this study was to construct a plasmid that would direct the expression of a bacterial DNA methyltransferase in cultured mammalian cells. It was carried out with a PCR-based strategy for introducing three components' coding sequences into the transfection vector pcDNA$_3$ (Invitrogen). The gene encoding the bacterial cytosine specific DNA methyltransferase M.*Msp*1 was first introduced into the general cloning vector pMTL23. Then, the coding sequence for glutathione-S-transferase was added into the upstream of human viral nuclear localisation sequences SV40 VPI of the prepared construct using the PCR technique. The complete synthetic fusion protein gene was finally introduced into the general purpose transfection vector pcDNA$_3$. The plasmid encoded fusion protein was demonstrated by exhibiting cytosine specific DNA methyltransferase activity by resistance to the cognate restriction enzyme R.*Msp*1 digestion following transformation into *E.coli.* Human kidney epithelial 293 cells were transfected with DNA methyltransferase vector using an antibody directed against the glutathione-S-tranferase moiety, so that the enzyme was expressed in whole cells. Furthermore, substantial phenotypic changes were observed in the 293 cells following transient transfection. Total protein and genomic DNA samples were prepared from the transfected cells and attempts were made to establish that genomic DNA was methylated at bacterial (*Moroxella spp.*) MTase M.*Msp*1 sites.

Key Words: Kidney epithelial 293 cells, C-5 MTase M.*Msp*1, NLS-VP1, cell transfection.

Introduction

DNA methylation is found in diverse organisms ranging from bacteria to mammals as well as plants. In mammals, approximately 70% of all CpG islands are methylated at position 5 of cytosine. Methylation is carried out by DNA methyltransferases (MTases). Cytosine methylation is an important mechanism of gene regulation in mammals. Changes in DNA methylation may be associated with facilitating protooncogene expression and inactivation of tumor suppressor genes. Aberrant DNA methylation may promote the genetic instability of a chromosomal locus. CpG dinucleotides, the targets of DNA methylation, are underrepresented five-fold in the genome due to the high mutability of methylated cytosine C-T transition mutations (1). The cytosine analogue 5-aza-2'-deoxycytidine has been used clinically to reactivate genes silenced by DNA hypermethylation. In particular, patients with β-Thalassemia show fetal globin expression after administration of 5-aza-2'-deoxycytidine and 5-azacytidine (2). DNA MTases with varying sequence specificities provide an excellent model system for understanding the molecular mechanism of specific DNA recognition. The cytosine-5-MTases are involved in a variety of biological processes in cells by catalysing the transfer of a methyl group from S-adenosyl-L-methionine to the C_5 position of cytosine. These enzymes access their target base by elegantly flipping it out of the DNA double helix. Wu and Santi have proposed a catalytic mechanism for C-5-cytosine DNA MTases that involves a nucleophilic attack by the enzyme on the C-6 position of the cytosine moiety (3). Bacterial MTases are known to regulate various processes such as replication, repair, recombination, transcription, restriction-modification systems and function to defend cells against phage infections (4). Most eukaryotic MTases methylate cytosine within CpG dinucleotides and contain a C-terminal segment that shares sequence with the bacterial enzymes (5). A single mammalian MTase enzyme has been

characterised and cloned (6). This enzyme displays methylation of hemimethylated CpG and *de novo* methylation of hemimethylated CpG dinucleotides (7, 8). O6-methylguanine and O4-methylthymine are potentially mutagenic DNA lesions that cause G:C→A:T or A:T→G:C transition mutations by mispairing during DNA replication. The repair of these mutations by DNA repair methyltransferases is therefore expected to prevent methylation-induced transitions (9). Specific genes can be stable or transiently introduced into cultured cells by vector-mediated gene transfer. Basically, there are two types of experiments using mammalian cell gene transfer techniques. One of these is transient transfection, which allows gene products, either RNA or protein, to be obtained within hours of DNA uptake. The other is stable transfection, which allows plasmid vector DNA integrated into the host cell chromatin to be obtained up to 24-80h following uptake of DNA. In this manner, we constructed a new PCR gene fusion product (NLS+GST+MTase M.*Msp*1 genes) and introduced it into vector $pcDNA_3$. The newly constructed plasmid was named pOZT 4 (10). In this study, the human epithelial 293 cells were transfected and co-transfected with newly constructed plasmid and pAdVantage (Invitrogen) using the $CaPO_4$ co-precipitation method (11). Using a bacterial fusion gene, the aim was to transfer it into the mammalian cell and determine its methylase activity on mammalian genomic DNA by table gene transfection.

Materials and Methods

PCR and Oligonucleotides

In order to establish an expression vector, we constructed a genetic fusion of a bacterial cytosine-5 specific DNA MTase with a vertebrate nuclear targeting sequence and marker enzyme glutathione-S-transferase genes. Amplification of the bacterial M.*Msp*1 gene was carried out using 100ng template DNA with forward and reverse primers (10). The PCR was set up according to the protocol outlined in the expand long template PCR system package insert. Primers were synthesised on a model 381 A DNA synthesiser by the biomolecular synthesis service of the Krebs Institute to create restriction sites at both ends of *Hind*III-*Eco*RI with PCR and thereby enable its insertion into the cloning vectors. The PCR product was obtained after 30 cycles of the reaction involving denaturation at 94˚C for 1.5 minutes, annealing at 50-52˚C for 2 minutes and polymerisation at 72˚C for 3 minutes. This gene was also amplified for the insertion of NLS+GST+M.*Msp*1 gene fusion into the vector (10).

Cell Transfection and Tissues

All the cell lines used in this study were kindly donated by Dr. D.P. HORNBY. The human embryonic 293 kidney cells were maintained in a MEM medium supplemented with 10% fetal calf serum. All cultures were grown at 37˚C in a humidified 5% CO_2 95% air incubator. Cells were seeded at $1x10^6$ cells per 19 cm plate, and after 24 h the cultures were co-transfected using the calcium phosphate precipitation method with 10µg of 1:10 pOZT 4 and pAdVantage. First, different concentrations of pOZT4 (5µg-20µg) and pAdVantage (1µg-5µg) were mixed and made up to 100µl with TE (10mM Tric.Cl pH:8, 1mM EDTA) buffer. Then, 100µl of x10 solution I [(18.36 g $CaCl_2$ and 0.125 M Hepes, pH 7.15 (Gibco BRL) /100µl sterile water)] and 300µl of solution II (25mM Hepes, pH 7.15) were added to be DNA. After this, 500µl o f solution III (280mM NaCl, 1.5mM Na_2HPO_4, 325mM Hepes, pH 7.15) was added dropwise to the DNA mix. Whilst solution III was being added, air was gently bubbled through the DNA solution. The final solution was left at room temperature for 5 minutes, and a very fine precipitate was formed. Finnaly, this fine DNA precipitate was added to the cell to be transfected. Approximately 24 h after transfection, cultures were seeded at $1x10^6$ cells and $20x10^6$ per 10 cm plate in a medium containing G418 (1000µg ml^{-1} neomycin). This medium with G418 was changed every 2 or 3 days until neo-resistant colonies developed (approximately 6-7 weeks). G418-resistant cell subclones were isolated and maintained as local populations in media containing 800µgml^{-1} neomycin.

Isolation of Nuclear DNA from Transfected Cells

High-molecular-weight DNA was extracted from the cells by lysis in 1% SDS/10mM NaCl/50mM Tris-HCl, pH:7.4/ 10mM EDTA, then treated with RNase A (100µg ml^{-1}) and proteinase K (1mg/ml). Following this, the reactions were extracted twice with phenol and once with chloroform and then precipitated with ice-cold ethanol and redissolved in TE (10mM Tris.Cl, pH:8, 1mM EDTA). Meanwhile, high-molecular-weight DNA was also prepared for pulsed-field gel electrophoresis. In brief, after overnight incubation of the cells in L buffer (0.1M EDTA pH:8/0.01M Tris.Cl pH:7.6/ 0.02M NaCl), the DNA was mixed with 1% melted agarose in L buffer again and the mixture stirred with a selade pipette. SDS and proteinase K were added to a final concentration of 1% and 1mg/ml respectively and the reaction was incubated at 37˚C overnight. Following incubation, the DNA was extracted, precipated and electrophoresed on 2% agarose gel.

SDS-PAGE and Immunobloting

Protein expresison and purification from the transfected, co-transfected and control cells were analysed by SDS-PAGE electrophoresis. Typically, 10-12.5% SDS polyacrilamide gels were prepared for protein separation. Protein samples were denatured by heating at 100˚C for 5-10 minutes after the addition of an equal volume of SDS gels loading buffer and run under denaturing conditions on 10-12.5% polyacrylamide gels at 100-160 volts. When the protein concentration was at an undetectable level, staining with commasie blue, western blotting and the ECL development technique were used to detect the minute amount of DNA.

Western Blot and ECL Development

After the protein separation with SDS-PAGE and retarded DNA gels, one piece of nitro-cellulose membrane (Hybond TM-ECL) and 8 pieces of Whatman paper (3mm) were soaked in transfer buffer (TTB: 3.03 g tris-methylamine +14.4 g glycine +200 ml methanol in 1 L MQ pH:8.3) for 15 minutes. The gels were placed on top of 4 sheets of Whatman paper, air bubbles were removed and placed on the nitro-cellulose membrane, and another 4 layers of Whatman paper were placed on top. This sandwich was blotted across at a constant voltage of 9 V for 30 minutes. Nitro-cellulose membranes were incubated with 1x TBS (x10 TBS: 24.228 g tris-methlamine+292 g NaCl into 1 L.MQ, pH7.5) for 30 minutes. The block membrane was blotted in 5% of blotto semi-skimmed milk powder (Marvel) in TTBS overnight at room temperature and incubated with anti-GST for 90 minutes. After washing 3 times with TTBS buffer, the nitro-cellulose membrane was incubated with a second antibody (anti-rabbit IgG- alkaline phosphate conjugate rose in goat, Sigma) at a dilution of 1:1000 also in TTBS buffer for 1 hour at room temperature. A further 3 washes were carried out with TBS. The immuno-blot was developed in an ECL reagent kit for 1 minute in 3.75 ml reagent 1 and 3.75 ml reagent 2 mixtures.

Restriction Enzyme Assay

Restriction digestion was assayed by incubating 1 µg DNA in a total volume of 20 µl with 10-20 units of restriction enzyme. Digests were carried out at the recommended temperatures (usually 37˚C) for 2-24 h, using specific buffers supplied with the enzymes. Restriction cleavages were then analysed by 1% agarose gel electrophoresis.

Assay of Methyltransferase Activity

DNA from the control, transfected and co-transfected cells was digested with R.*Msp*1 and R.*Hpa*II for detection of methylase activity of bacterial MTase M.*Msp*I on the genomic DNA of those cells. The digestion procedures were as follows: 5µl genomic DNA+2µl R.*Msp*1 or R.*Hpa*II buffer+2µl RNase+1µl R.*Msp*1 or R.*Hpa*II+9µl MQ water. The reaction mixture was incubated at 37˚C for 3 hours and DNA samples were routinely run on 1% agarose gels.

Results

In order to establish an expression vector, we constructed a genetic fusion of a bacterial cytosine-specific DNA methyltransferase (C-5 cytosine MTase M.*Msp*1) with a vertebrate nuclear targeting signal (NLS-SV40 VP1) and the marker enzyme glutathion-S-transferase (GTS). The new vector was named pOZT4 and cloned, and was expressed in a few *E.coli* strains (10,

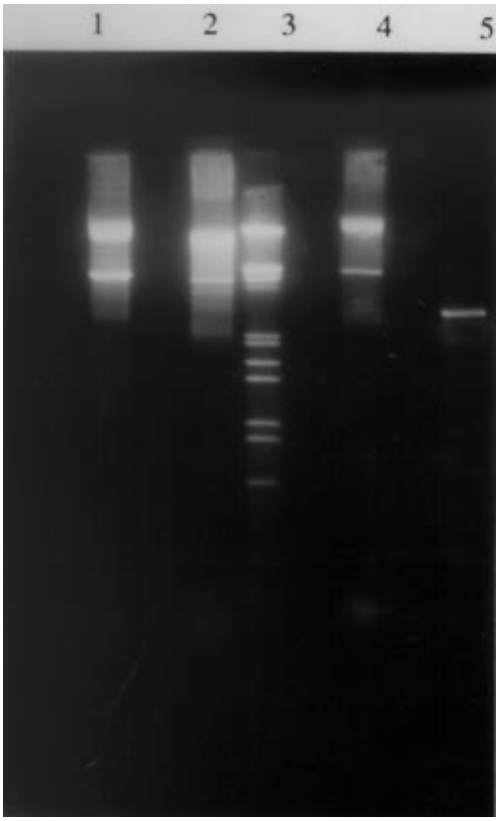

Figure 1. Agarose gel electrophoresis of plasmid DNA from transformed *E.coli* strains with pOZT4 and digested with restriction enzyme. R.*Msp*1. All plasmid DNA was fully methylated.
Lanes 1 and 2: Plasmid DNA from Top10F' strain
Lane 3: λ DNA cut with HindIII/EcoRI
Lane 4: Plasmid DNA from DHα5 strain
Lane 5: Untransformed pUC19 plasmid DNA

Figure 2. Western-immunoblot analysis and ECL development of NLS+GST+MTase M.*Msp*1 protein from transfected cell lines with pOZT4. Proteins from indicated cell lines were loaded onto a 10% SDS-PAGE gel and electrophoresed. Proteins were electroblotted onto a nitro-cellulose membrane (Hybond TM-ECL), which was probed with monoclonal antibodies specific for Ab-1 anti glutathion-S-transferase (Sigma) and Ab-2 anti-rabbit horse-radish peroxidase (Ammersham Life Science). Arrow indicates 66.45 kDs protein.

12). The prior expression and plasmid DNA was tested by restriction enzyme, R.*Msp*1 and R.*Hpa*II digestion. It was found that the methylated DNA from the transfected cell could not be cut and it exhibited higher fragment sizes in a 1% agarose gel electrophoresis in contrast to the DNA from untransfected cells (Figure 1-lanes 1, and 4). Transfection of mammalian cells with expression vector often results in suboptimal expression of the protein of interest. Co-transfection of cells with the pAdVantage vector enhances transient protein expression in a variety of cell types by increasing translation initiation. In this study, human kidney epithelial 293 cells were co-transfected with pOZT4 and pAdVantage at ratios of 1:10, 1:5 and 1:1 and grown in MEM and Dulbecco's modified Eagle's medium containing 10% fetal calf

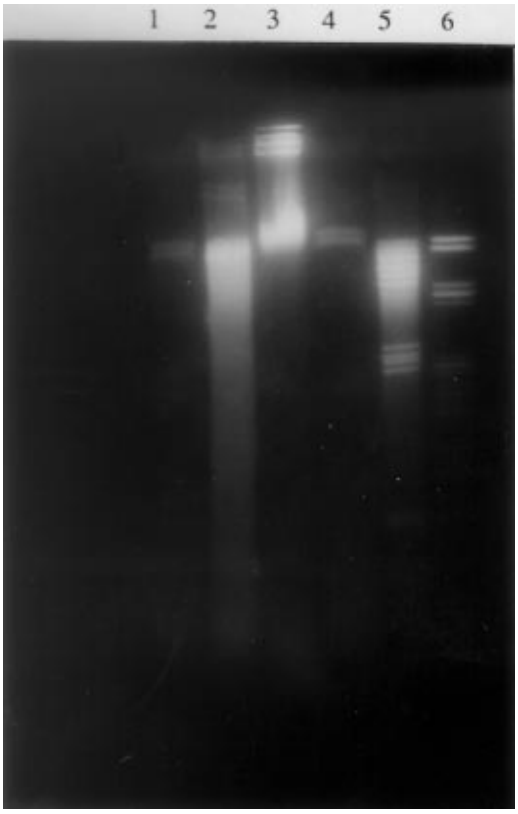

Figure 3. Genomic DNA extracted from transfected and untrasfected human kidney 293 cells and digested with R.*Msp*1.
Lanes 1 and 4: 2% gel electrophoresis of DNA from untransfected cells.
Lanes 2 and 3: DNA from transfected cells
Lane 5: λ DNA cut with HindIII
Lane 6: λ DNA cut with HindIII/EcoRI

serum. Substantial phenotypic changes were observed in the 293 cells following transient transfection. For the detection of fusion protein activity, the genomic DNA from 24 hour-45 day transfectants was analysed and pulsed-field electrophoresis, western-SDS-PAGE and DNA-protein analyses were performed. Transfected and untransfected cells were valuated by daily cell counts of erthrosin B dye exclusion. DNA and protein samples were extracted from stably and transiently transfected cells and control cells according to the standard procedure. The DNA samples were than cleaved with the isoschizomeric restriction endonucleases *Msp*1 and *Hpa*II and electrophoresed on 2% agarose gels. The results of this indicated that the inserted gene had been successfully expressed with the obvious appearance of a band on the gels at approximately 66.45 kDs in the 293 cell samples using the western-immunoblot analysis and ECL development (Figure 2). Genomic restriction digests were used once again to confirm that the recombinant plasmid

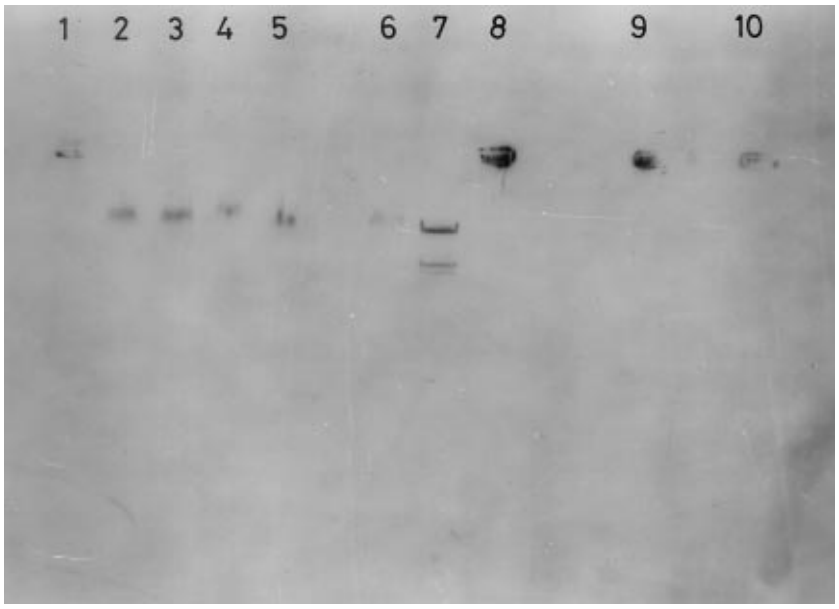

Figure 4. Pulsed-filed gel electrophoresis of R.*Msp*1 and R.*Hpa*II digested DNA from transfected cells.
Lanes 1, 8, 9 and 10: R.*Msp*1 digested DNA
Lanes 2-6: R.*Hpa*II digested DNA
Lane 7: λ DNA cut with HindIII/EcoRI

had been obtained (Figure 3). Following harvesting and separation of soluble and insoluble material, the samples were analysed by SDS-PAGE electrophoresis. It was found that the fusion gene was expressed in all transfected and co-transfected cells. The DNA from the 45-day stably transfected cells was identified as uncut high-molecular-weight DNA with low fragmentation (uncut, smear DNA) in contrast to the control (untransfected) DNA (Figure 3 - Lanes 2 and 3). In the pulsed-field gel electrophoresis, it was found that the genomic DNA from transfected cells also exhibited low fragmentation (Figure 4). R.*Msp*1 digested DNA from transfected cells exhibited low fragmentation (Figure 4, Lanes 1, 8, 9 and 10) in contrast to the R.*Hpa*II digestion (fragmented DNA), (Figure, Lanes 2-6).

Discussion

In the past decade, a number of studies have established the inverse correlation between transcriptional activity of a DNA sequence in different cell types and the presence of CpG methylation (13-15). It has been suggested that DNA methylation plays an important role in X-chromosome inactivation, imprinting, protection of the genome from invasive DNA sequences and compartmentalisation of the genome into condensed, and/or decondensed regions. Que et al. reported that the chlorella virus SC1A encodes at least five functional N6-methydeoxyadenine, 5-methyldeoxycytidine and one non-functional m5C methyltransferase (16). Schroeder and Mass proposed that the CpG methylation inactivates the transcriptional activity of the promoter of the human p53 tumor suppessor gene (17). The tumor suppressor gene p53 could therefore contribute to carcinogenesis by inactivation via methylation of a key element in cell cycle control. Using oligonucleotides encompassing the differentially methylated sites as probes in band-shift assays, Huntriss et al. identified that a nuclear protein which binds to a specific region of the mouse Xist gene was correlated with differential methylation of CpG sites of Xist expression and X-chromosome inactivation in females (18). Studies of the methylation of the transglutaminase promoter in normal and neoplastic human cells demonstrated that the promoter was methylated *in vivo* and that hypomethylation of the promoter was correlated with consitutive gene expression (19). Methylation of mammalian DNA can lead to repression of transciription and alteration of chromatin structure. Both effects are the result of an interaction between the methylated sites and methyl-CpG bindin proteins. Quantification of transcript levels by RNase protection assay has demonstrated that DNA methylation at positions other than CG or CNG sites contribute to the reduction in gene expression (20). Recent evidence suggests that the DNA sequences are methylated in CpG dinucleotides and CpCpG trinucleotides and that the methylation can be inhibited by 5-azacytidine (21). The present study examined the transfection of bacterial DNA MTase into mammalian cells using a vector construct containing human viral nuclear localisation sequences. First, the newly constructed plasmid was tested for its ability to generate bacterial cytosine-5-M.*Msp*1 MTase

activity in *E.coli* DH5α and Top10F' cell colonies, and their capacity to be maintained in an extrachromosomal form without undergoing extensive rearrangements was investigated. Then, the human kidney epithelial 293 cells were transfected with this bacterial gene fusion vector to determine the effects of the methylase activtiy of bacterial enzyme on the mammalian nuclear DNA. In the gene cloning we introduced a vector into mammalian host cells that was a DNA molecule with the capacity to replicate in parallel with the endogenous genome. The pOZT4 is typical G418 resistance vector. It contains a promoter and RNA processing signals from the SV 40 tumor virus flanking the ampicillin and neomycin drug resistance genes that allows the selection of cells as stable transfectants (10). The new vector als contains a GST marker enzyme that allows rapid screening by western or southern blot analysis for the presence of an insert (Figure 2). Phenotypical changes were observed in the 293 cells following transient transfection due to the effect of methylase activity of the fusion gene product. This phenomenon of transient expression is a useful method of studying gene regulation, because cells can be analysed very soon after exposure to the DNA. Degtyarev et al. found that a gene for Bst F 51-1 DNA MTase from *Bacillus stearohermophilus* F5 has sequence specificity of 5'-GGATG-3' recognition side (22). Xydas et al. suggested that methylation is not a source of error in pulsed-field agarose gel-based size estimation for chromosomal DNA molecules less than 1.12 Mbp in agarose gels (23). Nonglucosylated, hydroxymethylcytosine-containing T2 gt-virion DNA has a higher level of methylation than T4 gt-virion DNA, due to the methylation capabilities of T2 and T4 Dam MTases (24). Recent evidence suggests that after the transfection of 293 cells with recombine vector which contains bacterial DNA MTase gene fusion, the genomic DNA of mammalian cells is methylated at bacterial *Msp*1 recoginiton sites. Furthermore, substantial phenotypic changes were observed in 293 cells following transient transfection. As Clark et al. suggested, it has been demonstrated that in mammalian cells not only CpG sequences, but also CCGG sequences can be methylate *in vivo.* The restriction modifying enzymes R.*Msp*1 and R.*Hpa*II, which differ in their ability to cut DNA at central C methylated in CCGG recognition sequence, were used to check the fusion gene activity on transfected 293 cell DNA. The enzyme *Msp*1 will cut whether or not the C is methylated and *Hpa*II will only do so if the C is unmethylated. If DNA is digested with either *Msp*1 or *Hpa*II, both enzymes will give the same pattern of bands only if all the C residues within the recognition sites are unmethylated. In the present study, both enzymes gave different patterns of bands in transfected and untransfected cells (Figure 4). This means that bacterial enzyme was active in transfected 293 cells and transferred methyl groups to the first carbon atom of CCGG sequences in genomic DNA due to *Msp*1 methylase activity. In conlusion, it has been proved that the gene fusion in the simian virus VP1 nuclear localisation signal, glutathione -S-transferase and bacterial DNA MTase M.*Msp*1 gene combination was expressed in all the transfected cells successfully.

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