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Cell Type-Specific Activation of the Cytomegalovirus Promoter by Dimethylsulfoxide and 5-Aza-2'-deoxycytidine

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Abstract

The cytomegalovirus promoter is a very potent promoter commonly used for driving the expression of transgenes, though it gradually becomes silenced in stably transfected cells. We examined the methylation status of the cytomegalovirus promoter in two different cell lines and characterized its mechanisms of activation by dimethylsulfoxide and 5-Aza-2'-deoxycytidine. The cytomegalovirus promoter stably transfected into Chinese hamster ovary cells is suppressed by DNA methylation-independent mechanisms, which is different from the rat embryonic cardiomyoblast H9c2-*Fluc*.3 cells in which the cytomegalovirus promoter is silenced by methylation. Dimethylsulfoxide and 5-Aza-2'-deoxycytidine can activate the cytomegalovirus promoter in both cell types by overlapping mechanisms. Dimethylsulfoxide activates the cytomegalovirus promoter in Chinese hamster ovary cells by promoting histone acetylation and the activation of p38 mitogen-activated protein kinase and nuclear factor κ B signaling pathways, while 5-Aza-2'-deoxycytidine increases histone acetylation and activates the nuclear factor κ B but not the p38 mitogen-activated protein kinase pathway. In H9c2-*Fluc*.3 cells, both agents promote demethylation of the cytomegalovirus promoter, and enhance its activity exclusively through activation of the nuclear factor κ B pathway and to a lesser extent of the p38 mitogen-activated protein kinase pathway. Our findings suggest that suppression and activation of the cytomegalovirus promoter are cell type-specific. These results may be used for developing strategies to enhance the expression of transgenes and the production of recombinant proteins encoded by transgenes controlled by a cytomegalovirus promoter.

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Keywords

Cytomegalovirus promoter; Dimethylsulfoxide; 5-Aza-2'-deoxycytidine; NF- κ B; p38 MAP kinase

1. Introduction

The immediate/early promoter enhancer of cytomegalovirus (CMV promoter) is one of the most commonly used *in vitro* and *in vivo* promoter for driving the expression of transgenes in mammalian cells because of its strong promoter activity. However, many transgenes under the control of viral promoters, including the CMV promoter, have been found to be gradually silenced over a period of months in culture (Choi, Basma, Singh, & Cheng, 2005; Knust, Bruggemann, & Doerfler, 1989; Krishnan et al., 2006; Palmer, Rosman, Osborne, & Miller, 1991; Verma & Somia, 1997). The gene silencing mechanism of these promoters is not clear, although DNA methylation, histone hypoacetylation, activation of signaling molecules and altered levels of transcriptional factors have been suggested (Brooks, Harkins, Wang, Qian, Liu, & Rubanyi, 2004; Swindle & Klug, 2002). In eukaryotes, particularly in mammals, methylation is involved in the formation of transcriptionally repressive chromatin states and maintenance of genome stability. The repression is often brought about by methylation of CpG dinucleotides clustered to form CpG Islands which are usually located in the promoter and the 5' exon/intron region of a gene. Proposed mechanisms for inhibition of gene expression due to promoter methylation include direct interference of methyl group in binding of transcription factors due to steric hindrance, methyl groups mediating the binding of certain proteins to DNA by forming a protein complex that blocks the binding of transcription factors and methyl groups affecting DNA-histone interaction, resulting in chromatin compaction, thereby preventing transcription (Esteller, Corn, Baylin, & Herman, 2001; Esteller & Herman, 2002; Singal & Ginder, 1999). The acetylation status of lysine side chains in histones also can regulate gene expression. Hypoacetylation of histones leads to the condensation of DNA through interactions of the free lysine residues in histones and DNA backbones, resulting in suppression of gene expression. Measurement of the status of DNA methylation and histone acetylation in the promoter regions can serve as an indicator of the transcription activity of a specific gene (Grassi et al., 2003).

We observed a several-fold higher β -galactosidase (β Gal) activity in the pCMVLacZ stably transfected CHO cells (CHO-LacZ) freshly prepared from frozen stocks when compared with same cells maintained in culture. Upon subculture of the freshly recovered cells, the β -Gal activity returned to the same level as cells which had been maintained in culture. The only difference between these two culture conditions was the presence of 2.5% Dimethylsulfoxide (DMSO) in the culture medium containing freshly recovered cells. This amount of DMSO was carry over from the 10% DMSO used for cryopreservation of the cells. This observation led to the current study.

DMSO is a water-soluble organic solvent routinely used in cell biology. Its application includes cryoprotection of cells (Karlsson, Cravalho, Borel, Tompkins, Yarmush, & Toner, 1993), enhancement of stem cell differentiation in cardiac-, smooth-, and skeletal-muscle,

blood and neural cell lineages (Bonser, Siegel, McConnell, & Cuatrecasas, 1981; Brooks, Harkins, Wang, Qian, Liu, & Rubanyi, 2004; Dinsmore et al., 1996; Dinsmore, Ratliff, Jacoby, Wunderlich, & Lindberg, 1998; Pruitt, 1994). Some DMSO actions, such as cryopreservation of cells, may be attributed to its capability to stabilize the liquid matrix of cell membranes (Yu & Quinn, 1994). Mechanisms of other DMSO actions including our initial finding are not well understood. Our observation that the CMV promoter in CHO cells was not methylated, coupled with the finding that this promoter was activated by DMSO and 5-Aza-2'-deoxycytidine (5-Aza CdR), an agent known to inhibit DNA methylation (Krishnan et al., 2006), prompted us to study the mechanisms of the activation of unmethylated CMV promoter. To get a better understanding of the mechanisms of the activation of the CMV promoter in general, we also examined the mode of action of these two agents in rat embryonic cardiomyoblast H9c2-*Fluc*.3 cells. These cells contain a luciferase gene controlled by the CMV promoter, which was inactivated by methylation after 66 passages (Krishnan et al., 2006). The results of the studies suggest that suppression and activation of the CMV promoter are cell type-specific.

2. Materials and methods

2.1. Cell culture

CHO cells were grown in F12 Ham medium supplemented with 7.5% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin and 50 µg/mL streptomycin and 2 mM L-glutamine. The rat embryonic cardiomyoblast H9c2-*Fluc*.3 cells (passage 66) which were originally prepared by stable transfection with CMV-luciferase gene was a kind gift from Dr. Joseph C. Wu, Stanford University School of Medicine, Stanford, CA. The passage 66 H9c2-*Fluc*.3 cells, which had the CMV promoter inactivated by methylation (Krishnan et al., 2006), were grown in DMEM medium, supplemented with 5% fetal bovine serum (FBS), 10 units/ml penicillin, and 10 µg/ml streptomycin. All cell culture reagents were from GIBCOBRL (Carlsbad, CA).

2.2. Construction of the expression vector and generation of stable cell clones

Bovine C2GnT-1(L) (Li, Adler, & Cheng, 1998) was cloned into the mammalian expression vector, pcDNA3/Zeoicin (Beum, Singh, Burdick, Hollingsworth, & Cheng, 1999), which contained a CMV promoter and a bovine growth hormone polyadenylation signal (Invitrogen, Carlsbad, CA) to generate pCMV-C2GnT-L. pCMV-C2GnT-L was transfected into CHO cells to generate stable clones, CHO-C2GnT-L, selected for resistance to zeocin (300 µg/mL). CHO cells stably transfected with pCMVLacZ (CHO-LacZ) were generated by transfection with pcDNA6-LacZ/bsd (Invitrogen, USA) and selected with blasticidin (10 µg/mL).

2.3. Quantitation of LacZ expression after transient transfection

Transient transfections were performed as described previously except for the following modification (Cheng, 1996; Seki, Iwakawa, Cheng, & Cheng, 2002). Each well of the 48-well plate was exposed to a transfection solution containing 5µg *Maackia amurensis* agglutinin (EY Lab, Inc, San Mateo, CA) ligand, 3µg DMRIE-cholesterol liposome

(Invitrogen, Carlsbad, CA), and 1 μ g pCMVLacZ DNA. β -Gal activity in the transfected cells was measured after 24 h culture.

2.4. β -Gal assay

pCMV CHO-LacZ #3 cells were treated with various agents, including DMSO, 5-Aza CdR (Sigma, USA), SB203580 (SB) (EMD Calbiochem, USA) and Wedelolactone (WL) (EMD Calbiochem, USA). After 24 h treatment with DMSO or 5-Aza CdR, cells were measured for β -Gal activity using a Mammalian β -Gal Assay Reagent Kit (Pierce) as described previously (Yanagihara, Cheng, & Cheng, 2000). A UVmax microplate reader was used for measuring the optical density at 404 nm (Molecular Devices, Sunnyvale, CA), which was converted to the amounts of β -Gal using *E. coli* β -Gal enzyme as the standard. For inhibitor treatment, the cells were incubated with a MAP kinase inhibitor (SB 203580, 30 μ M) or IKK α and β kinase inhibitor (Wedelolactone, 100 μ M) for 1 h prior to DMSO treatment. The enzyme activity was expressed as ng β -Gal activity/ μ g protein.

2.5. β 1-6 N-Acetylglucosaminyltransferase (C2GnT) assay

C2GnT activity was assayed using total cell lysates prepared from T25 flasks as described previously (Ropp, Little, & Cheng, 1991). Aliquots of the homogenates were measured for enzyme activity using UDP-[3 H]GlcNAc (~3,500 dpm/nmol, American Radiolabeled Chemicals, St. Louis, MO) as the sugar donor and 2 mM Gal β 1,3GalNAc α -O-Benzyl (Toronto Research Chemicals Inc. Downsview, Ontario, Canada) as the acceptor. The products were isolated with C18 cartridges and quantitated using a Liquid Scintillation Counter (Packard, Meriden, CT). Protein concentration was measured with Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin as the standard. Enzyme activity was calculated by subtracting the endogenous activity measured without exogenous acceptor from total activity and expressed as nmol of sugar donor transferred/h/mg protein.

2.6 Luciferase assay

H9c2 *Fluc.3* cells were grown in 48-well plates. After treatment with DMSO or 5-Aza CdR for 24 h, the cells were measured for luciferase activity using a Luciferase assay system with a Reporter Lysis buffer kit as described by manufacturer protocol (Promega corporation, Madison, WI). The light produced was measured on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Protein was measured as described above. Data were expressed as Relative Light Units per μ g protein. The inhibitor study was performed as described above.

2.7. Western blot analysis

CHO-LacZ cells were grown in T-25 flasks until they were 80% confluent, and then treated with 2–4% DMSO for 24 h. Cell lysates were resolved on SDS-Polyacrylamide gel (4% stacking and 7.5% separating gels) electrophoresis. Proteins were electroblotted onto a PVDF membrane (Immobilon-P, 0.45 μ m, Millipore, Bedford, MA) overnight at 4 $^{\circ}$ C and 100 mA, then blocked with 5% nonfat milk in Tris Buffered Saline (TBS: 0.9% NaCl, 10 mM Tris, pH 7.5) at room temperature for 1 h. The membrane was then incubated for 1 h at room temperature with anti-V5 monoclonal antibody (1:2000) in 5% nonfat milk. The

membrane was washed with 5% nonfat milk in TBS for 15 min followed by two additional 5 min washes. The membrane was incubated with peroxidase-conjugated goat anti-mouse IgG secondary antibodies (1:10,000) in TBS containing 5% nonfat milk at room temperature for 1 h. The membrane was rinsed 3X with TBS as described above. ECL reagents (Pierce, Rockford, IL) were applied per the manufacturer instruction and the blots were exposed to ECL-sensitive film (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.8. Northern blot analysis

Total RNA from 0–4% DMSO-treated CHO-LacZ cells cultured in T25 flasks was isolated using Tri Reagent (MRC Inc., Cincinnati, OH). Twenty five µg of total RNA was fractionated by formaldehyde-1% agarose gel electrophoresis and transferred to a nitrocellulose membrane and UV cross-linked. The membrane was prehybridized in 50% formamide, 5X saline sodium citrate (SSC), 5X Denhardt's solution, 5 mM EDTA, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA at 42 °C for 4 h. Open reading frames (ORFs) of both glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and LacZ were prepared from the respective plasmids by EcoRI digestion and used as hybridization probes. These probes were radiolabeled with [α -³²P]dCTP (ICN, Costa Mesa, CA) using Prime-It II Random Primer DNA Labeling Kit (Stratagene, Cedar Creek, TX) and added to a prehybridization solution containing 10% dextran sulfate followed by incubation at 42 °C for 14 h. The membrane was washed with 0.1% SDS in 2X SSC at room temperature for 15 min and twice with 0.1 % SDS in 0.2X SSC at 58 °C for 15 min. and exposed to X-OMAT AR film (Kodak, Rochester, NY) with an intensifying screen at –80 °C for 48–72 h.

2.9. Bisulfite modification and methylation-specific PCR

Bisulfite treatment of 2 µg genomic DNA was carried out using the EZ DNA Methylation-gold kit (Zymo Research, Orange, CA). Bisulfite treatment deaminates unmethylated cytosines to convert them to uracils leaving methylated cytosine residues unchanged. Methylation-specific PCR (MSPCR) was performed to determine the methylation status of the CMV promoter in CHO-LacZ and H9c2 *Fluc.3* cells. Two hundred picograms of bisulfite-modified DNA was used as template. MSPCR primers specific for methylated CpG sequences were: (M) sense primer 5' CGTTTTTTATTGACGTTAATGACGG3'; antisense primer 5' TACGTCATAAAACGAAATTATTACGAC 3'. Primers specific for unmethylated CpG sequences were: (U) sense primer 5'TGTTTTTTATTGATGTTAATGATGG 3'; antisense primer TACATCAATAAAACAAAATTATTA CAAC 3'. The PCR reactions (40 cycles) were performed in a total volume of 25 µl using standard conditions, with denaturation at 95 °C, annealing at 60–64 °C, and extension at 72 °C. The CMV promoter-containing pcDNA6-LacZ/bsd vector, methylated using M. SssI (CpG) DNA methyltransferase (New England Biolabs, Ipswich, MA), was used as the positive, fully methylated control. The untreated vector served as unmethylated control and no-template DNA served as negative controls. All procedures followed the manufacturer's instructions. The PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under a UV illuminator. Detection of a discrete visible band of PCR product with methylation-specific primers was considered positive. All experiments were carried out in duplicate.

2.10. Formaldehyde cross-linking and chromatin immunoprecipitation

The formaldehyde cross-linking and chromatin immunoprecipitation assays of cultured cells were performed as described (Christenson, Stouffer, & Strauss, 2001; Kuo & Allis, 1999). Briefly, the CHO-LacZ cells were pretreated with 2–4 % DMSO, 5-Aza CdR or 2% DMSO + 5-Aza CdR (1 µg/ml). After a 12 h incubation, 37% formaldehyde (Fisher Scientific) was added directly to the cell culture medium to cross-link DNA and its associated proteins. Immunoprecipitation was carried out overnight at 4 °C using 10 µg of anti-acetyl histone (H3) antibody specific for the acetylated form of lysine-K9 and K14 of histone (H3) (Upstate cell signaling solutions, Charlottesville, VA). Non-immune rabbit serum-treated sample served as the negative control. Immunoprecipitation, washing, and elution of immune complexes were carried out as described in the ChIP assay kit (Upstate cell signaling solutions, Charlottesville, VA). Before the first wash, 10% of the supernatant from the samples without antibody for each condition was saved for determination of total input chromatin and processed with the eluted immunoprecipitates beginning at the cross-link reversal step. After final ethanol precipitation, both the samples and the inputs were eluted with 30 µl of high grade nuclease free water (Promega) (Christenson et al., 2001; Kuo & Allis, 1999).

The PCR reaction contained 2 µl of samples (IPs) or 2 µl of diluted input (1:100 diluted input-chromatin) and 2X PCR master mixes (Promega) in 25 µl of total reaction volume. After 32–34 cycles of amplification, 10–12 µl of the PCR products were subjected to electrophoresis on a 4% agarose gel, and DNA was stained with ethidium bromide and visualized under UV light. The signals (intensity) were quantitated by Alpha imager I (3.3d). Fold changes in acetylation were calculated by first dividing the sample signal intensity by the input-signal intensity (to control for varying levels of chromatin in each sample) of CHO LacZ cells. The primers used for PCR analysis were: CMV promoter sense primer 5'TCCGCGTTACATAACTTACGG3', antisense primer 5'TGCCAAGTGGGCAGTTTAC3'; CHO cell GAPDH sense primer 5'ACATCAAATGGGGTGATGCT3', antisense primer 5'CTTGGTTCACACCCATCACA3'.

2.11. Statistical analysis

The difference in means between two experimental groups was analyzed by the Student's *t*-test while statistical analysis of multiple samples was carried out using one way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. DMSO enhanced CMV promoter activity in CHO-LacZ cells

As a part of our effort to study the regulation of gene expression controlled by the CMV promoter, we generated several CHO-LacZ clones that exhibited varying degrees of LacZ expression. We discovered that the β-Gal activity in the initial culture of the cells prepared from frozen stock was much higher than that in the cells that were maintained in culture. This observation prompted us to examine whether this phenomenon could be reproduced in other frozen stocks. As shown in Fig. 1A, cells freshly prepared from stocks of 9 different clones expressed from 3 to 15-fold more β-Gal activity than cells maintained in culture.

3.2. DMSO enhanced the expression of LacZ in CHO-LacZ cells in a dose and time dependent manner

Since the initial culture of the frozen stock contained 2.5% (v/v) DMSO, we surmised that DMSO was responsible for the enhancement of the β -Gal activity. To examine this possibility, we measured β -Gal activity of a CHO-LacZ clone (#3) that expressed a moderate level of β -Gal activity as a function of DMSO concentration. As shown in Fig. 1B, β -Gal activity in this CHO-LacZ clone increased as DMSO concentrations increased. A plateau was reached at 4–5% DMSO with a reduction resulting from 6% DMSO. Because toxicity was observed in cells treated with DMSO at concentrations greater than 4%, subsequent studies were carried out using DMSO concentrations at 4% or less. A significant increase in β -Gal activity in CHO-LacZ cells was detected as early as 4 h after adding 2% DMSO to the culture (Fig. 1C). β -Gal activity in DMSO-treated cells was about 2.5-fold higher than the activity found in the control cells after 8 h, and about 10-fold higher after 24 h.

3.3. DMSO enhanced transcription and translation of LacZ gene in CHO-LacZ cells

Enhancement of β -Gal activity by DMSO may be a result of the stabilization of β -Gal by DMSO, activation of LacZ gene expression, or a combination of the two. Since exposure of cell extracts to 2 – 4% DMSO for up to 24 h did not affect β -Gal activity (data not shown), stabilization of β -Gal by DMSO was not considered to be a factor for the enhancement. To determine if increased β -Gal activity was the result of increased expression of LacZ gene, β -Gal transcript and protein levels were assessed in CHO-LacZ cells after treatment with 2, 3, or 4% DMSO for 24 h. The β -Gal transcripts as detected by northern blotting increased with increasing concentrations of DMSO while GAPDH message remained unchanged (Fig. 2A-I). In addition, the levels of β -Gal protein as detected by western blotting increased in a DMSO concentration-dependent manner as shown in Fig. 2A-II. These results suggested that the enhancement of β -Gal activity by DMSO was due to increased transcriptional activity of the CMV promoter. X-Gal staining of the DMSO-treated cells was also employed to determine whether the increased expression of the LacZ gene reflected an increase in the percent of the population of cells expressing β -Gal. X-Gal staining of this CHO-LacZ clone treated with culture medium (control), 2, 3, or 4% DMSO detected β -Gal enzyme in 9, 32, 55, and 85 % of cells, respectively (Fig. 2B).

3.4. DMSO enhanced the expression of β 1,6N-Acetylglucosaminyltransferase-L (C2GnT-L) cDNA in CHO-C2GnT-L cells

To examine if the DMSO enhancement of LacZ gene expression observed in CHO-LacZ cells could be reproduced with a different transgene, the experiment was repeated in CHO-C2GnT-L cells. Treatment of these cells with 2, 3, and 4% DMSO enhanced C2GnT activity by 4, 6, and 7-fold, respectively (Fig. 3).

3.5. DMSO enhanced the expression of β -Gal in CHO cells transiently transfected with pCMV-LacZ

DMSO also enhanced β -Gal activity in CHO cells transiently transfected with pCMV-LacZ. A 4-fold increase of β -Gal activity was observed ($p < 0.001$) in CHO cells treated with 2% DMSO for 24 h following transient transfection with CMV-LacZ cDNA for 6 h (Fig. 4).

3.6. Effects of DMSO and 5-Aza CdR on the methylation status of CMV promoter in CHO-LacZ and H9c2 Fluc.3 cells

Since methylation of the promoter region of a gene is a well recognized mechanism for its inactivation (Jaenisch & Bird, 2003), we proceeded to examine the methylation status of the CMV promoter in CHO-LacZ cells with and without treatment with 2 – 4% DMSO, 5-Aza CdR, or 2% DMSO plus 5-Aza CdR. Only unmethylated PCR products were detected in the control cells or CHO-LacZ cells treated with DMSO, 5-Aza CdR, or DMSO plus 5-Aza CdR (Fig. 5A). To validate the experimental procedures, we performed the same experiment on embryonic cardiomyoblast (H9c2 *Fluc.3*) cells (passage 66), which contained a CMV-luciferase cDNA with the CMV promoter inactivated by methylation (Krishnan et al., 2006). As shown in Fig. 5B, the CMV promoter in H9c2 *Fluc.3* cells (control) was methylated. Treatment of these cells with 4% DMSO, 5-Aza CdR, and 2% DMSO plus 5-Aza CdR resulted in demethylation of the CMV promoter (Fig. 5B). The 5-Aza CdR effect was greater than that of DMSO.

3.7. Effects of DMSO on histone acetylation in CHO-LacZ cells

To identify the DMSO-mediated methylation-independent mechanism for activation of the CMV-promoter, we evaluated the histone acetylation status of the CMV promoter by a chromatin immunoprecipitation (ChIP)-PCR assay in CHO-C2GnT-L cells. While some acetylation of the CMV promoter-associated histone H3 was detected in untreated CHO-C2GnT-L cells, treatment with 2 and 4% DMSO led to a 10 and 20% increase in CMV-associated acetylated histone H3, respectively. Treatment of these cells with 5-Aza CdR and 2% DMSO plus 5-Aza CdR increased acetylated histone H-3 by 23% and 30%, respectively (Fig. 6).

3.8. Effects of p38 MAP kinase inhibitor (SB203580), and IKK α and β kinase inhibitor (Wedelolactone) on the activation of CMV promoter by DMSO and 5-Aza CdR in CHO-LacZ cells

To further characterize the mechanism of DMSO activation of the CMV promoter, we employed SB203580, a MAP kinase inhibitor, to assess a possible involvement of p38 MAP kinase in the process. As shown in Fig. 7A, enhancement of β -Gal activity by 0.2, 2 and 2.5-fold was observed in the cells treated with 1 μ g/ml 5-Aza CdR, 2% DMSO and 4% DMSO, respectively. Treatment with a combination of 2% DMSO and 5-Aza CdR led to a greater enhancement of the β -Gal activity (3-fold above control) than the sum (2.2-fold above control) of the enzyme activities obtained by either agent alone. Exposure of CHO-LacZ cells to 30 μ M of SB203580 for 1 h prior to treatment with 2–4% DMSO, 5-Aza CdR, and 2% DMSO plus 5-Aza CdR for 24 h showed a significant decrease of β -Gal activity when

compared to the cells without treatment. No significant decrease of enzyme activity was observed in 5-Aza CdR-treated cells after treatment with SB203580 (Fig. 7A).

Since CMV promoter contains more than one NF κ B binding sites and NF κ B is a transcription factor downstream to p38 MAP kinase signaling pathway (Madrid, Mayo, Reuther, & Baldwin, 2001; Sambucetti, Cherrington, Wilkinson, & Mocarski, 1989; Wong, Zhang, Ip, & Lam, 2002), we proceeded to examine if NF κ B was involved in the activation of the CMV promoter by DMSO. Exposure of CHO-LacZ cells to 100 μ M Wedelolactone for 1 h prior to treatment with 2% DMSO, 4% DMSO, or 2% DMSO plus 5-Aza CdR for 24 h significantly reduced β -Gal activity as compared to the cells without Wedelolactone pretreatment. WL did not significantly reduce β -Gal activity in cells treated with 5-Aza CdR (Fig. 7B).

3.9. Effects of p38 MAP kinase inhibitor (SB203580), and IKK α and β kinase inhibitor (Wedelolactone) on the activation of CMV promoter by DMSO and 5-Aza CdR in H9c2 *Fluc.3* cells

H9c2 *Fluc.3* cells treated with 2% DMSO, 4% DMSO, 5-Aza CdR, and 2% DMSO plus 5-Aza CdR for 24 h showed 1.3, 9.3, 4.4 and 23-fold enhancement of luciferase activity. Pretreatment of these cells with SB203580 significantly reduced luciferase activity in the cells treated with 2% DMSO, 4% DMSO, 5-Aza CdR and 2% DMSO plus 5-Aza CdR as compared to the cells treated with each agent alone (Fig. 8A). Also, pretreatment of H9c2 *Fluc.3* cells with Wedelolactone completely prevented the activation of the CMV promoter by 2% DMSO, 4% DMSO, 5-Aza CdR, and 2% DMSO plus 5-Aza CdR (Fig. 8B).

4. Discussion

CMV promoter is a powerful promoter widely used for driving transgene expression in mammalian cells. However, it becomes inactivated over time after insertion into the host genome (Cameron, Bachman, Myohanen, Herman, & Baylin, 1999; Choi et al., 2005; Knust et al., 1989; Palmer et al., 1991; Verma & Somia, 1997). Finding ways to activate the silenced CMV promoter should help overcome this limitation and facilitate the use of this promoter for enhancing the expression of the transgenes and production of recombinant proteins (Singh et al., 2004). In this report, we describe a cell type-specific inactivation of the CMV promoter via methylation-dependent and independent mechanisms. In both cases, the CMV promoter can be activated by DMSO and 5-Aza CdR by some shared mechanisms.

Methylation of CpG dinucleotides in the promoter region is a major gene suppression mechanism for the CMV promoter-mediated transgene expression system (Swindle & Klug, 2002) and some tumor suppressor genes including the acute leukemia-associated p15^{INK4b} gene (Toyota, Kopecky, Toyota, Jair, Willman, & Issa, 2001). However, our studies indicate that this gene silencing mechanism does not directly regulate the activity of CMV promoter in CHO-LacZ cells. The reasons are: First, the magnitude of the DMSO enhancement of CMV promoter activity observed 24 h after transient transfection was similar to that of the CMV promoter observed in stably transfected CHO cells. Second, no methylation of the integrated CMV promoter was detected in CHO-LacZ cells (Fig. 5A). This unexpected finding is authenticated by the validation of the methylation analysis technique used in this

study on embryonic cardiomyoblast H9c2 *Fluc.3* cells, which display inhibition of the CMV promoter by methylation (Krishnan et al., 2006). Therefore, methylation of the CMV promoter is not involved in the inactivation of this promoter in CHO cells.

Hypoacetylation of histones is another epigenetic change associated with gene inactivation (Swindle & Klug, 2002). Our studies indicate that hypoacetylation of histones plays only a moderate role in the inactivation of CMV promoter activity in CHO cells. DMSO (2%) treatment increased the level of acetylated histone H3 associated with the CMV promoter by only 10%. In contrast, a 23% increase in the amount of acetylated histone H3 associated with the CMV promoter in 5-Aza CdR-treated cells (Fig. 6). Similar DNA methylation-independent gene inactivation, which can be activated by 5-Aza CdR, was reported in mouse centromeric heterochromatin (Takebayashi et al., 2001) and acute myeloid leukemia cells (Schmelz, Sattler, Wagner, Lubbert, Dorken, & Tamm, 2005). Treatment of CHO cells with both agents shows only an additive effect on the increase of the amounts of acetylated histone H3. However, a synergistic effect is observed on CMV promoter activity. These results indicate that histone hypoacetylation can only partially account for the inactivation of the CMV promoter activity in the CHO cells. In the H9c2-*Fluc.3* cells which show inactivation of the CMV promoter by methylation, DMSO and 5-Aza CdR alone and in combination also activate this promoter, suggesting that similar cellular pathways activated by these two agents may function in both types of cell.

Activation of gene expression can involve DNA methylation-dependent and independent mechanisms (Liang, Gonzales, Jones, Orntoft, & Thykjaer, 2002; Nguyen et al., 2002). Our results clearly show that activation of the CMV promoter activity in CHO cells is independent of methylation. However, we can not exclude the possibility that inactivation of other genes involved in the activation of CMV promoter works through methylation of the promoters of these genes. The DNA methylation-independent mechanisms involved in the activation of the CMV promoter may include activation of p38 MAP kinase (Bruening, Giasson, Mushynski, & Durham, 1998; Liang et al., 2002) and IFN γ signaling pathways (Nguyen et al., 2002), inhibition of histone H3-lysine 9 methylation, and upregulation of certain transcription factors, such as NF κ B (Lee et al., 2005). Our study shows that p38 MAP kinase (Fig. 7A) and NF κ B (Fig. 7B) are involved in the activation of the CMV promoter by DMSO. This is consistent with our observation that DMSO activation of a silenced CMV promoter in CHO cells is independent of the methylation status of the CMV promoter, and only moderately associated with histone acetylation. Activation of the CMV promoter by DMSO is correlated with activation of p38 MAP kinase-NF κ B signaling pathways. However, the activation of the methylation-independent CMV promoter by 5-Aza CdR in CHO cells does not involve p38 MAP kinase-NF κ B pathway. In the cells (H9c2 *Fluc.3*) which show inactivation of the CMV promoter by methylation (Krishnan et al., 2006), treatment with DMSO and/or 5-Aza CdR results in demethylation. MAP treatment kinase is involved to some extent while NF κ B pathway plays an essential role in the activation of this promoter by DMSO and 5-Aza CdR.

These findings raise an important data interpretation issue when DMSO is used as a solvent in biological application. Because DMSO can activate cellular genes and transgenes (Ishiguro & Sartorelli, 2004; Zhu, Smith, Ansah, & Gooderham, 2005), some of these gene

products may affect the outcome of the experiment. In this case, the effect observed by the test agent dissolved in DMSO may not represent its true action rather a result of the interactive actions of the test agent and DMSO. Therefore, the experimental results should be validated by alternative approaches when DMSO is used in an experiment.

In conclusion, we show that the mechanisms for the inactivation of the CMV promoter are cell type-specific and can operate in either methylation-dependent and independent pathways. Both types of CMV promoter inactivation can be activated by DMSO and 5-Aza Cdr. Mechanistically, histone acetylation, P38-MAP kinase, and NF κ B each plays some moderate role in the activation of the CMV promoter in CHO cells while P38-MAP kinase plays some moderate role and NF κ B plays an essential role in the activation of the CMV promoter in a methylation-dependent H9c2 *Fluc.3* cell line (Krishnan et al., 2006). For generation of a gene product controlled by a CMV promoter, activation of p38 MAP kinase-NF κ B pathway may be employed to enhance the production of the recombinant protein.

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Fig. 1.

(A) β -Gal activity in different CHO-LacZ clones freshly recovered from frozen stocks and those maintained in culture. The initial culture of cells recovered from frozen stocks contained 2.5% DMSO (cells frozen in 1 ml medium containing 10% DMSO plus 3 ml culture medium). All freshly recovered cells had significantly higher enzyme activity than the same cells maintained in culture ($p < 0.01$). Clone #3, which had a moderate β -Gal activity in the maintained culture but a 10-fold higher activity in freshly recovered stock, was chosen for subsequent study. (B) β -Gal activity in CHO-LacZ cells (clone #3) treated with DMSO at various concentrations. β -Gal activity was significantly higher in cells treated with 2–5 % DMSO ($p < 0.01$) than that in untreated cells. (C) β -Gal activity in CHO-LacZ cells as a function of 2% DMSO treatment times. The enzyme activity was not significantly increased until after 4 h ($p < 0.01$) and reached its peak after 24 h. Enzyme activity is expressed as ng β -Gal/ μ g protein (mean $\pm$ S.E.) ($n=6$).

Fig. 2.

Northern blot (A-I), western blot (A-II) and X-Gal staining (B) of CHO-LacZ cells treated with 2–4% DMSO. Analysis was performed 24 h after initial treatment. Increase of transcript and protein levels was observed in a DMSO concentration-dependent manner (A-I and A-II). The percentage of blue cells in untreated control and cells treated with 2, 3, or 4% DMSO was 9.0 ± 1.0 , 32 ± 1.5 , 55 ± 2.1 , and 85 ± 1.5 , respectively (mean $\pm$ S.E.) (n=6).

Fig. 3.

Effects of DMSO concentrations on C2GnT activity in CHO-C2GnT-L cells. Cells cultured in T-25 flasks were treated with DMSO for 24 h. The DMSO concentration (2–4%)-dependent enhancement of C2GnT activity ranged from 4.3 to 7.1-fold of the control ($p < 0.0001$) ($n = 6$).

Fig. 4. DMSO enhancement of β-Gal activity in CHO cells transiently transfected with pCMVLacZ. Enzyme activity was measured 24 h after transfection. DMSO (2%) significantly enhanced β-Gal activity in the transiently transfected CHO cells ($p < 0.001$) ($n = 6$).

Fig. 5.

Methylation-specific PCR analysis of CMV promoter containing CpG dinucleotides in CHO-LacZ (A) and H9c2 *Fluc.3* (B) cells. Primer sets used for amplification are designated as unmethylated (U) and methylated (M). No methylated PCR products were detected in untreated CHO-LacZ cells (control) and same cells treated with 2% DMSO, 4% DMSO, 5-Aza CdR and 2% DMSO + 5-Aza CdR. Methylated PCR products were detected in untreated H9c2 *Fluc.3* cells (control) and same cells treated with 2% DMSO, 4% DMSO, 5-Aza CdR and 2% DMSO + 5-Aza CdR. Unmethylated PCR products were only detected in H9c2 *Fluc.3* cells treated with 4% DMSO, 5-Aza CdR, and 2% DMSO + 5-Aza CdR. Methylated pcDNA6-LacZ/bsd was used as a positive control vector (MV) and non-methylated pcDNA6-LacZ/bsd was used as a negative control vector (NV). No-template DNA was used as a reaction control. (L, 100 bp ladder).

Fig. 6.

Chromatin Immunoprecipitation (ChIP)-PCR assay for CMV promoter sequence from CHO-LacZ. Chromatin DNA was immunoprecipitated with antibodies specific for acetylated histone H3 (acetyl-H3). DNA fragments were amplified with primers specific for CMV promoter and control GAPDH. ChIP-PCR products for CMV promoter (IP) (161 bp): Lane 1, 1 kb+ ladder; Lane 2, No DNA control; Lane 3, Negative control; Lane 4, No treatment control; Lane 5, 2% DMSO; Lane 6, 4% DMSO; Lane 7, 5-Aza CdR; Lane 8, 2% DMSO + 5-Aza CdR. ChIP-PCR products obtained for CMV promoter inputs under various conditions as described above are designated as (Input) (161 bp). ChIP-PCR products obtained for GAPDH control under various conditions as described above are designated as (GAPDH) (162 bp). The fold changes in signal intensity were measured by the ratio of IP/inputs to control GAPDH genes as described under materials and methods. Increased signal intensity observed in CHO-LacZ cells treated with various agents was: 2% DMSO, 10%; 4% DMSO, 20%; 5-Aza CdR, 23%; and 5-Aza CdR + 2% DMSO, 30% when compared with that of the untreated control cells.

Fig. 7.

Effects of MAP kinase inhibitor (SB 203580, SB) (A) and IKK α and β kinase inhibitor (Wedelolactone, WL) (B) on the activation of CMV promoter by DMSO and 5-Aza CdR in CHO-LacZ cells. CHO-LacZ cells treated 2 and 4% DMSO, 1 μ g/mL 5-Aza CdR, and 2% DMSO plus 1 μ g/mL 5-Aza CdR for 24 h showed a significant enhancement of β -Gal activity. Pretreatment of these cells with 30 μ M SB or 100 μ M WL for 1 h significantly reduced the DMSO effect but did not affect the 5-Aza CdR effect. Enzyme activity was expressed as ng β -Gal/ μ g protein (mean $\pm$ S.E.) (n=6). (2%, 2% DMSO; 4%, 4%DMSO; SB, SB203580; WL, Wedelolactone; ns, not significant; **, p<0.01; ***, p<0.001).

Fig. 8.

Effects of p38 MAP kinase inhibitor (A) and IKK α and β kinase inhibitor (B) on the activation of CMV promoter by DMSO and 5-Aza CdR in H9c2 *Fluc.3* cells. H9c2 *Fluc.3* cells treated 2 and 4% DMSO, 1 μ g/mL 5-Aza CdR, and 2% DMSO plus 1 μ g/mL 5-Aza CdR for 24 h showed significant enhancement of β -Gal activity. Pretreatment of these cells with 30 μ M SB and 100 μ M WL for 1 h significantly and completely inhibited the stimulatory effects of both agents, respectively. Enzyme activity was expressed as RLU/ μ g protein (mean $\pm$ S.E.) (n=6). (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).