

native promoter of the gene of interest is undefined, it is simpler to use a heterologous promoter. In other cases, particularly when a regulatory element has not been separated from its native promoter, or when an enhancer has a "preference" for its native promoter (6), it may be safer to use the native promoter of the gene of interest. The Pelican vectors are available with or without a minimal *Hsp70* promoter, which extends from -43 to +92 and includes a TATA box (15). Every enhancer that we have tested to date will drive transcription from the minimal *Hsp70* promoter.

The *Hsp70* minimal promoter (hs43) was isolated from the plasmid pCaSpeR-hs43- β gal3 (15,17) as an *XhoI-SmaI* fragment and cloned into the *XhoI* and *StuI* sites of pPelican.

We have included a unique *SpeI* restriction site downstream of the reporter gene in the Pelican vectors, which may be useful for two purposes: (i) placing an enhancer downstream of the reporter and (ii) replacing the reporter with a gene of interest. This site was used to replace the *lacZ* gene in pPelican with *GFP* to make the pGreen Pelican and pStinger vectors. The Pelican vectors can therefore be modified to serve as insulated gene misexpression vectors, which would be expected to show less position-effect variability than other such vectors, and may therefore require analysis of fewer transgenic lines.

We believe that these insulated GFP and β -gal transformation vectors will prove to be useful to investigators who use reporter genes to assay transcriptional *cis*-regulatory sequences in transgenic flies.

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Scott Barolo, Lucy A. Carver and James W. Posakony
*University of California
San Diego, La Jolla, CA, USA*

Treatment by Methyl Methanesulfonate Induces Up-Regulation of Cytomegalovirus Immediate/Early Promoter

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The major immediate early promoter/enhancer of human cytomegalovirus (CMV promoter) is one of the conventional promoters for expression of proteins in eukaryotic cells. The advantages of this promoter are a broad spectrum of host cells and a high level of constitutive transcription (1). However, modest levels of transgene expression from the CMV promoter have often been observed following integration of expression cassettes into chromosomal DNA (2,9). Here, we report that the CMV promoter may be dramatically up-regulated. Treatment of cells by less than 1 mM methyl methanesulfonate (Aldrich Chemical, Milwaukee, WI, USA) induces more than 100 times the stimulation of transcription from the CMV promoter.

This effect was observed during an attempt to express the fusion protein me(y)2-EGFP in mammalian cells. The me(y)2 is a small 101-amino acid protein, the mouse homologue of the *Drosophila melanogaster* e(y)2 transcription factor (12). The expression construct was prepared by subcloning the me(y)2 ORF into vector pEGFP-N1 (Clontech Laboratories, Palo Alto, CA, USA) to produce a fusion with the enhanced green fluorescent protein (EGFP). Correct in-frame insertion was verified by sequencing both strands of the resulting fusion ORF. The control pEGFP-N1 vector contains the CMV enhancer/promoter region (-582 to +7

relative to the 5' end of immediate early protein gene 1 (IE1) mRNA of HCMV) for expression of the fusion protein and the neomycin-resistance gene for selection of transfected cells. This system allows direct detection of the fusion protein expressed in transfected cells as a result of EGFP fluorescence.

To establish stable cell lines, 10(1) cells (mouse embryonic fibroblasts) (6) and human HeLa cells were transfected with pme(y)2-EGFP and pEGFP-N1 by calcium phosphate precipitation. The assessed percentage of transiently transfected cells amounted to 5%–10% by EGFP fluorescence 48 h after transfection. The cells were then plated onto 10-cm dishes and selected in a medium containing 500 µg/mL G-418 (ICN Biomedicals, Costa Mesa, CA, USA) for 14 days. However, we were unable

either during or after the selection to detect expression of the me(y)2-EGFP fusion protein by fluorescent microscopy, although EGFP fluorescence was readily detected in cell lines that stably express pEGFP-N1.

This result could be attributed to the instability of the me(y)2-EGFP fusion protein. While EGFP has been reported to be stable in eukaryotic cells with a half-life of 24 h (7), many cellular proteins undergo rapid turnover with a half-life of about 1 h, as is the case for transcription factor p53 (5). However, p53 protein can be stabilized in response to many types of DNA damage (5). There were no logical reasons to expect that the me(y)2 fusion protein could not be stabilized by the treatment of cells with a DNA-damaging agent. By using direct fluorescence detection,

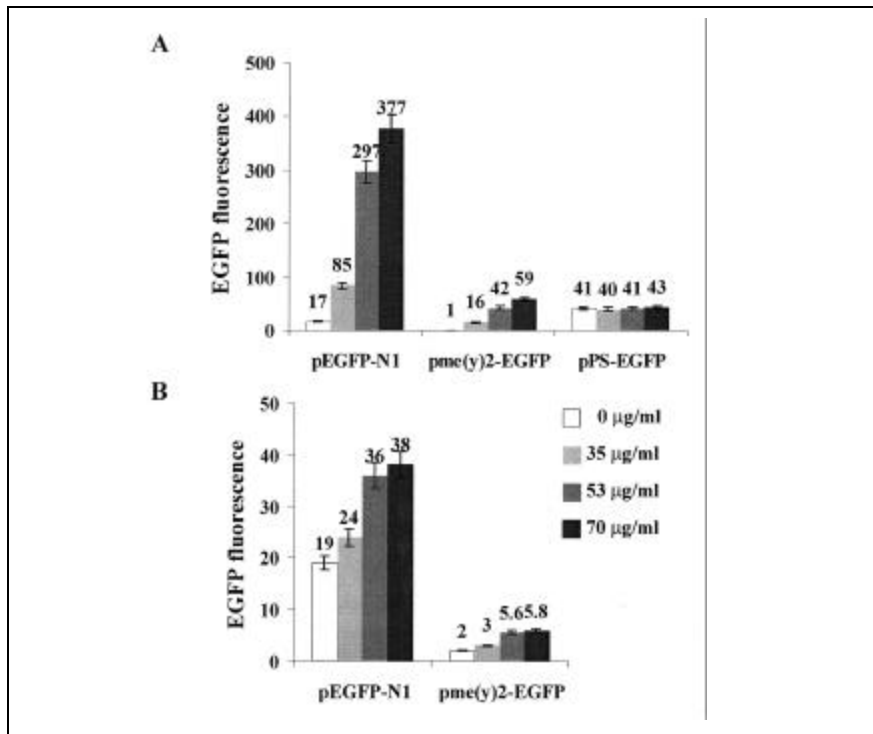


Figure 1. Increase in EGFP fluorescence after MMS treatment. Fluorometric analysis of EGFP and me(y)2-EGFP fusion protein expression. (A) 10(1)/EGFP, 10(1)/me(y)2-EGFP and 10(1)/PS-EGFP stable cell lines (50%–70% monolayer) were treated with MMS at various concentrations (0, 35, 53 and 70 µg/mL). (B) 10(1) cells were transiently transfected with pEGFP-N1 and pme(y)2-EGFP and treated with MMS at 12 h after transfection. Twelve hours after the MMS addition, cells were washed twice with ice-cold PBS and scraped into 100 µL buffer containing 50 mM NaH₂PO₄, 200 mM NaCl and 10 mM Tris-HCl, pH 8.0. After repeated freeze/thaw cycles, equal amounts of total protein were used for fluorimetric analysis with a fluorimeter (Photon Technologies International, Monmouth Junction, NJ, USA). Excitation was at 480 nm, and fluorescence was measured at 510 nm, according to the Living Colors™ User Manual (Clontech Laboratories, Palo Alto, CA, USA). The lowest level of fluorescence was registered in an extract from untreated 10(1)/me(y)2-EGFP cells. It was nearly equal to the background autofluorescence and was taken as an arbitrary unit. Each experiment was done twice with each of two independent cell lines, and the average was taken.

Benchmarks

we could easily check this possibility. We used the DNA damaging agent methyl methanesulfonate (MMS), a direct mutagen that disrupts DNA structure by methylation of bases (4). A low MMS dose of 35 $\mu\text{g}/\text{mL}$ produced no apparent detrimental effect on cell viability, yet 70 $\mu\text{g}/\text{mL}$ caused extensive cell death within 24 h.

The stable cell lines [pEGFP-N1 and pme(y)2-EGFP] were treated with MMS at various concentrations. After 12 h, the fusion protein expression was detected in the cells by fluorescence microscopy. An increase in cell fluorescence correlated with the increase in MMS concentration. At the maximal dose of MMS, an approximate 60-fold increase in EGFP fluorescence was measured and a similar increase in fluorescence was seen for the control pEGFP-N1 vector, about 20-fold (Figure 1).

Up-regulation occurs at the level of transcription. MMS strongly induces the CMV promoter in a concentration-dependent manner (Figure 2). Densitometric analysis showed that MMS treatment caused an approximate 250-fold increase in the mRNA level of me(y)2-EGFP.

MMS treatment induced the level of the me(y)2-EGFP protein (Figure 3). The me(y)2-EGFP fusion protein was nearly undetectable in nuclear extracts from untreated cells, and yet, after stimulation, a high level of an approximate 39 kDa protein (the deduced molecular weight of the me(y)2-EGFP fusion protein) was detected by polyclonal antibodies to me(y)2. The identity of this band as the me(y)2-EGFP fusion protein was verified by the probing of the same blot with antibodies to EGFP (data not shown).

The results described above were obtained using stably transfected cell lines with plasmids integrated into the cells' chromosomal DNA. To investigate the effect of MMS on non-integrated constructs, similar experiments were performed with transiently transfected cells. pEGFP-N1 and pme(y)2-EGFP plasmids were transfected into 10(1) cells as described for stable transfection. Twelve hours after transfection, the cells were treated with MMS for an additional 12 h. As shown in Figure 1B, more modest stimulation of the CMV promoter was observed. At the

maximal dose of MMS used, an approximate threefold increase in EGFP fluorescence was measured.

The MMS-mediated induction of

transcription is specific for the CMV promoter. The EGFP gene was subcloned under the control of the LTR promoter in MuMLV-based retroviral vec-

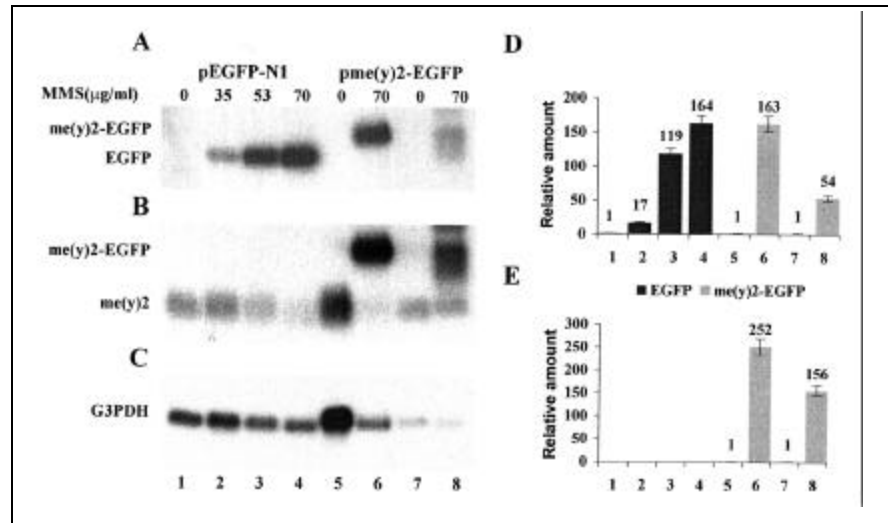


Figure 2. MMS-induced up-regulation of the CMV promoter occurs at the level of transcription. (A, B and C) Northern blot hybridization with total RNA (10 $\mu\text{g}/\text{lane}$) isolated from 10(1)/EGFP (lanes 1–4), 10(1)/me(y)2-EGFP (lanes 5 and 6) and HeLa/me(y)2-EGFP (lanes 7 and 8) stable cell lines. Cells were harvested 12 h after MMS addition: lanes 1, 5 and 7: untreated cells; lane 2, 35 $\mu\text{g}/\text{mL}$ MMS; lane 3, 53 $\mu\text{g}/\text{mL}$ MMS; lanes 4, 6 and 8, 70 $\mu\text{g}/\text{mL}$ MMS. The following fragments were used as probes: (A) EcoRI-NotI fragment from pEGFP-N1 corresponding to EGFP gene; (B) me(y)2 cDNA; (C) G3PDH cDNA; (D) and (E) Quantification of Northern blot data (A) and (B), respectively. Signals were normalized by hybridization of the same membrane with G3PDH probe. The basal level of the me(y)2-EGFP mRNA in untreated 10(1)/me(y)2-EGFP cells is used as an arbitrary unit. The autoradiographs in the linear range of exposure were scanned by a densitometer (Molecular Dynamics, Sunnyvale, CA, USA) and processed using the ImageQuant Software (version 3.3; Molecular Dynamics).

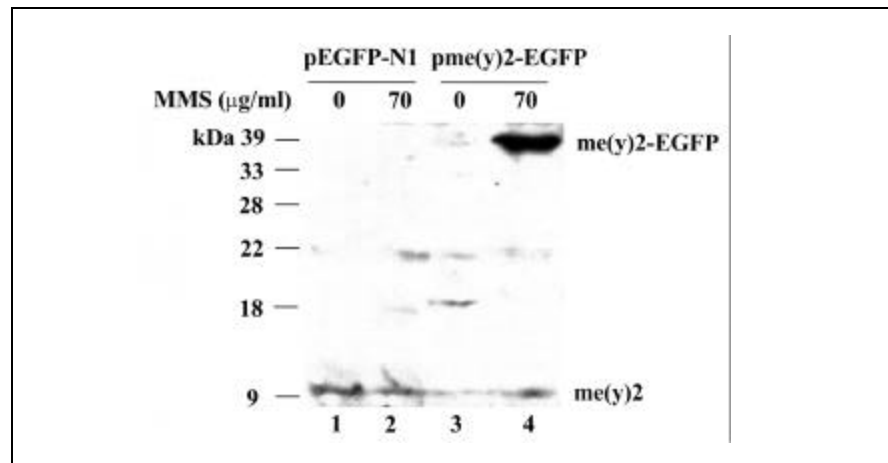


Figure 3. Western blot analysis of MMS-induced up-regulation of me(y)2-EGFP protein expression. Nuclear extracts were prepared from 10(1)/EGFP (lanes 1 and 2) and 10(1)/me(y)2-EGFP (lanes 3 and 4) stable cell lines. Cells were harvested 12 h after the MMS addition: lane 1 and 3, untreated cells; lanes 2 and 4, 70 $\mu\text{g}/\text{mL}$ MMS. Cells were lysed in 0.5 mL of a lysis buffer containing 20 mM HEPES, pH 7.6, 10 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 20% glycerol, 0.1% Triton[®] X-100, 1 mM DTT and 10 $\mu\text{g}/\text{mL}$ aprotinin. Nuclei were pelleted by low-speed centrifugation and shaken at 0°C for 45 min in 50 μL of the same buffer supplemented with 500 mM NaCl. After centrifugation at 10 000 rpm for 10 min, 50 μg nuclear extracts were separated by a 16% SDS-PAGE. Proteins were transferred to a PVDF membrane (Hybond-P[®], Amersham Pharmacia Biotech, Piscataway, NJ, USA) and were probed with rabbit polyclonal antibodies raised against the me(y)2 protein and developed using ECL[®]-Plus Western blotting reagents (Amersham Pharmacia Biotech).

Benchmarks

tor pPS-neo (10). The 10(1) cells were infected with pPS-EGFP recombinant retrovirus and selected in a G-418-supplemented medium for 14 days. The basal level of EGFP fluorescence in 10(1)/PS-EGFP cells was moderately higher than that in 10(1)/EGFP cells. However, no increase in EGFP fluorescence was observed after MMS treatment at various concentrations (Figure 1A).

The MMS effect is chemically specific. Cells were treated with various concentrations of different analogues of MMS and were found to have no effect on EGFP fluorescence (data not shown).

The results presented here are consistent with previous reports in which the specificity of CMV-driven transcription was determined. In one study, the CMV promoter was found to be up-regulated by cAMP in lymphoid cell lines via a CRE element contained in the enhancer (13). In another report, NF- κ B was implicated in activation of the CMV enhancer via cytomegalovirus gene products and T cell stimulation (11). It was shown that CMV promoter/enhancer shows some degree of tissue specificity (1). Inductions of the repressed CMV promoter by heat shock, sodium arsenite or by inhibition of protein synthesis have been previously demonstrated (2). The CMV promoter, in the context of an adenoviral vector, was susceptible to stimulation (up to 60 times) by a variety of chemical agents (15). Tumor necrosis factor alpha and phorbol 12-myristate 13-acetate stimulate the activity of the CMV promoter in immature monocytic cells (14). Note that in the previous studies (14,15) the stimulation effect depended on the cell line. Recently, Bruening et al. (3) found that stress-activated JNK1 and p38 MAP kinases are involved in regulation of transcription from the CMV promoter. In the experiments in which NIH 3T3 cells transiently transfected with the pCMV-*lacZ* plasmid were treated with a sublethal dose of sodium arsenite, a threefold increase in β -galactosidase activity was observed. As sodium arsenite has been shown to activate SAPK/JNK, the authors cotransfected pCMV-*lacZ* with pCMV-MEKK1(Act) encoding an active MEKK1, one of the kinases in the MAP kinase cascade that resulted in an approximate 15-fold increase in β -galactosidase activity.

To test whether other drugs known to activate the SAPK/JNK pathway (4) can up-regulate the CMV promoter, 10(1)/EGFP cells were treated with various concentrations of etoposide and doxorubicin. However, no increase in EGFP fluorescence was observed in 24 h (data not shown).

Our results demonstrate that MMS can up-regulate transcription from the CMV promoter by two orders of magnitude in fibroblasts and in cervical carcinoma cells. We also show that the MMuLV LTR promoter is not affected by MMS treatment.

Our results offered two conclusions. First, when designing experiments in which a transgene is expressed under the control of the CMV promoter, one should anticipate the possibility of promoter regulation. Second, we found a simple method of strong induction of expression from the CMV promoter. Such stimulation may be used in cases when the internal state of the cells does not vary. This method may be of practical value for applications of many studies that use the CMV promoter for the maximal expression of a transgene.

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Elena N. Nabirochkina and Alexey V. Ivanov
*Russian Academy of Sciences
Moscow, Russia*