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ORIGINAL ARTICLE

Effect of adenovirus infection on transgene expression under the adenoviral MLP/TPL and the CMVie promoter/enhancer in CHO cells

Mohamed A. El-Mogy [a,c,*], Moemen A.K. Abdalla [b], Vanja Misic [c], Yousef Haj-Ahmad [c]

[a] *Molecular Biology Department, National Research Centre, Dokki, Giza, Egypt*
[b] *Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt*
[c] *Department of Biological Sciences, Brock University, St. Catharines, ON, Canada*

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Abstract The adenovirus major late promoter (MLP) and its translational regulator – the tripartite leader (TPL) sequence – can actively drive efficient gene expression during adenoviral infection. However, both elements have not been widely tested in transgene expression outside of the adenovirus genome context. In this study, we tested whether the combination of MLP and TPL would enhance transgene expression beyond that of the most widely used promoter in transgene expression in mammalian cells, the cytomegalovirus immediate early (CMVie) promoter/enhancer. The activity of these two regulatory elements was compared in Chinese hamster ovary (CHO) cells. Although transient expression was significantly higher under the control of the CMVie promoter/enhance compared to the MLP/TPL, this difference was greater at the level of transcription (30 folds) than translation (11 folds). Even with adenovirus infection to provide additional elements (*in trans*), CMVie promoter/enhancer exhibited significantly higher activity relative to MLP/TPL. Interestingly, the CMVie promoter/enhancer was 1.9 folds more active in adenovirus-infected cells than in non-infected cells. Our study shows that the MLP-TPL drives lower transgene expression than the CMVie promoter/enhancer particularly at the transcription level. The data also highlight the utility of the TPL sequence at the translation level and/or possible overwhelming of the cellular translational machinery by the high transcription activity of the CMVie promoter/enhancer. In addition, here we present data that show stimulation of the CMVie promoter/enhancer by adenovirus infection, which may prove interesting in future work to test the combination of CMVie/TPL sequence, and additional adenovirus elements, for transgene expression.

* Corresponding author at: Molecular Biology Department, National Research Centre, Dokki, Giza, Egypt.
E-mail address: melmogy@hotmail.com (M.A. El-Mogy).

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1. Introduction

Active gene expression is an essential requirement in different applications including DNA vaccines, gene therapy and commercial protein production. The choice of promoters as well as other regulatory sequences that act in combination to derive active gene expression is a major optimization target to facilitate the outcomes of the transgene construct. The adenovirus major late promoter (MLP) and the cytomegalovirus immediate early (CMVie) promoter/enhancer are two of the most commonly used promoters in transgene expression [25,29]. The late phase of adenovirus infection starts when the L4-22K and L4-33K proteins are expressed at low levels to fully activate the MLP [27,46]. Once activated, the MLP will derive the expression of an abundant amount of late proteins required to form and assemble new viral capsids. Active translation in this phase is attributed to the activity of the MLP and the presence of the tripartite leader sequence (TPL). The expression of all the late viral proteins is driven by the MLP which has its full activity during the late phase of the viral infection and is transactivated by the adenoviral E1A proteins [30,51].

TPL is a 5′ untranslated sequence present in all of the late, but none of the early, viral mRNA. TPL facilitates mRNA transport and accumulation in the cytoplasm and is responsible for the selective translation of the late viral proteins in preference of the cellular proteins [49]. Translation of any TPL-attached mRNA is eIF-4F-independent [10]. The relaxed secondary structure of TPL facilitates its function in translation initiation even when eIF-4F is inhibited [11].

The CMVie promoter/enhancer is the most commonly used promoter for transgene expression in mammalian cells because it achieves high levels of transcription [1,5,6,14,19,33,34,38,40,44,45]. In the context of cytomegalovirus replication, the CMVie controls the expression of the IE1, which is a transactivator essential for viral latency and replication and belongs to the immediate early family of genes that are expressed first after infection [39,41]. In this study, we engineered two constructs that contained either the MLP/TPL sequence or the CMVie promoter/enhancer for the expression of a GFP reporter gene. We then compared gene expression levels between the two constructs in non-infected and adenovirus-infected Chinese hamster ovary (CHO) cells.

2. Materials and methods

2.1. Plasmid constructs

Two plasmids were used in this study denoted by pCG and pMTGA. pCG was constructed from pCMV-GFP plasmid that was used in a previous study in our laboratory [15]. We constructed pCG plasmid by the removal of the SV40 SD/SA fragment using SacI and AgeI and closing the plasmid by cloning of the annealing product of the following two fragments 5′cgtttagtgaaccgtcagatcgcctga3′ and 5′tcgagcaggcgatctg acggttcactaaac3′. The resulting plasmid contained CMVie promoter/enhancer, green fluorescence protein (GFP) and SV40 poly A signal. On the other hand, pMTGA was constructed by the cloning of two fragments into pUC19. The first fragment contained GFP-SV40 poly A and was obtained by PCR on pCG plasmid using the following two primers: 5′atg gtgagcaagggcgc3′ and 5′ttgttgttaacttgtttattgcagcttataatgg3′. The obtained fragment was cloned into the HincII site of pUC19. Next, a fragment that contains the major late promoter (MLP) and tripartite leader sequence (TPL) was obtained by gene construction and cloned into NheI and AgeI sites, upstream from the GFP-SV40 poly A. Both plasmids were prepared by cesium chloride gradient. The schematic diagrams of the two plasmids are shown in Fig. 1.

2.2. Cell lines and maintenance

The used Chinese hamster ovary (CHO) cells were the subclone K1 (ATCC CCL-61) derived from the parental cell line initiated by Puck and coworkers [35]. Cells were maintained as a monolayer in Petri cell culture dishes and cultured in advanced Dulbecco's Modified Eagle Medium (Advanced D-MEM: Invitrogen Corp., Gibco), containing 5% (v/v) fetal bovine serum (FBS, PAA Laboratories Inc.), 1% (v/v) penicillin/streptomycin (Invitrogen Corp., Gibco) and 1% (v/v) glutamine (Invitrogen Corp., Gibco). Growing cells were incubated in a water-jacketed incubator (Fisher Scientific,

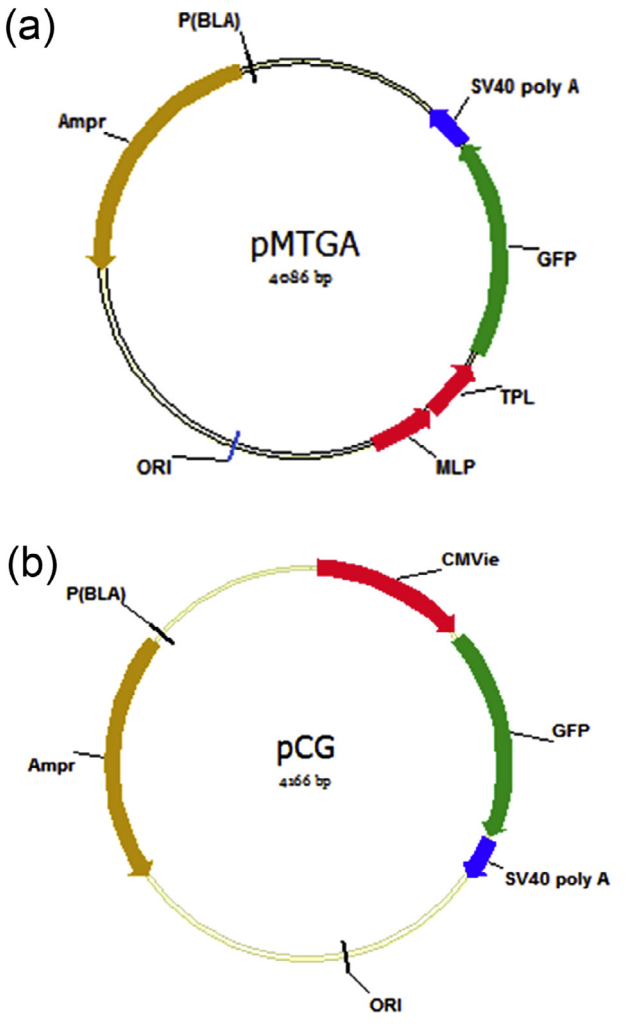

Figure 1 Schematic diagrams of pMTGA (a) and pCG (b). Both plasmids contain a common reporter gene (GFP) and poly A signal (SV40 poly A). Different regulatory elements are used to drive the expression, either the MLP-TPL (pMTGA) or the CMVie promoter/enhancer (pCG).

Pittsburgh PA) at 37 °C with 96% relative humidity and 5% CO_2.

2.3. Lipofectamine 2000 transfection

Lipofectamine 2000 (Invitrogen) was used to transfect plasmids DNA into mammalian cells. The confluency of the monolayer was ensured to be at least 70% at the transfection time. The culture medium was replaced prior to the transfection with 2 mL of antibiotic-free medium. The transfection mix for each well (of a 6-well plate) was prepared in 500 μL by mixing plasmid DNA and Lipofectamine 2000. First, 5 μg plasmid DNA was diluted in a total volume of 250 μL using Opti-MEM I Reduced Serum Medium (Invitrogen Corp., Gibco). Similarly, 5 μL Lipofectamine 2000 was diluted in 250 μL total volume using Opti-MEM. Both the diluted DNA and Lipofectamine 2000 were incubated at room temperature for 5 min, then mixed together and incubated at room temperature for additional 20 min. The 500 μL transfection mixture was added dropwise onto the well and shaken to distribute the mixture evenly. Finally, the plate was incubated at 37 °C for 6 h before changing the medium with the regular, antibiotic-containing, medium.

2.4. Adenovirus and its infection

Adenovirus dl309 was used in this study. The viral titer was determined by plaque assay according to the method described by Cromeans et al. [9]. Viral infection of mammalian cells was carried out in 6-well plates using a volume of the viral stock equivalent to multiplicity of infection (MOI) of 1 plaque forming unit (PFU)/cell since it has been proven in previous work to be more suitable for transgene expression in CHO cells [12]. This viral volume was mixed with PBS++ (0.01% $CaCl_2{\cdot}2H_2O$ and 0.01% $MgCl_2{\cdot}6H_2O$ dissolved in phosphate buffered saline) in a total volume of 500 μL/well and then added to the cell monolayer (after aspirating the medium). The 6-well plate was then incubated for 1 h at 37 °C with 96% relative humidity and 5% CO_2, with swirling the plate every 15 min. After that, 2 mL of the culture medium was added to each well and the plate was returned to the incubator.

2.5. RNA/DNA isolation

DNA and RNA were isolated, all from the same sample, using the RNA/DNA/Protein Purification Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

2.6. DNase treatment of RNA

The digestion of residual DNA in the isolated RNA samples was performed using TURBO DNase (Ambion). Each 50 μL of sample was digested in a 100 μL reaction mixture containing the provided buffer and four units of TURBO DNase, with incubation at 37 °C for 30 min.

2.7. RNA cleaning

All cleaned RNA samples were carried out using the RNA CleanUp and Concentration Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

2.8. Reverse transcription

Two to five hundred nanograms of total RNA was used in reverse transcription (RT) reactions. RNA was mixed with 0.5 μL of 100 mM oligo(dT)18 primer (Sigma), and completed to a final volume of 5 μL using RNase/DNase-free water (Ambion). This mixture was heated up for 5 min at 70 °C, and then chilled to 4 °C. During the cooling step, 15 μL of the RT reaction solution is added to the mixture. The added RT reaction solution contains 4 μL of 5X First Strand Buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl and 15 mM MgCl2), 2 μL of 0.1 M Dithiothreitol (DTT), 1 μL of 10 mM dNTPs, 0.1 μL Superscript III reverse transcriptase (Invitrogen) and 7.9 μL RNase/DNase-free water (Ambion). The reaction was continued by an incubation step at 25 °C for 5 min, followed with 90 min incubation at 42 °C and 15 min incubation at 70 °C before finally holding the reaction at 4 °C.

2.9. Quantitative PCR

Quantitative PCR (qPCR) was performed on a known concentration of template DNA or complementary DNA (cDNA), using the Bio-Rad iCycler thermal cycler. Specific primers within the GFP gene were used (GFP-F: 5′ATCCTGATCG AGCTGAATGG3′ and GFP-R: 5′TGCCATCCTCGATGT TGTG3′) with an amplicon size of 484 bp. The reaction mixture contained 10 μL of 2X SYBR GREEN master mix (Bio-Rad), and 1.2 μL of each primer (5 mM stock). The total volume of the reaction was completed with dH2O to 20 μL. A 15 min heating at 95 °C was used to activate the hotstart enzyme. Forty amplification cycles were performed as follows: 15 s at 95 °C, 30 s at 59 °C and 1 min at 72 °C. The reaction was kept at 57 °C for 1 min before starting a melting curve analysis by a 0.5 °C increment every 10 s over 80 rounds. A standard curve of known plasmid concentration (10 fg to 1 ng) was used to determine the initial concentration of plasmid in each sample.

2.10. GFP fluorescence intensity quantification

The fluorescence intensity of green fluorescence protein (GFP) was quantified directly from mammalian cells by measuring the relative fluorescence units (RFU) using the BioTek Synergy HT Multi-Mode Microplate Reader. Transfected cells were washed twice with PBS, lifted from the plate and counted. Fifty thousand cells per well were then transferred to a black rounded-bottom 96-well plate (Costar) in a total volume of 200 μL of PBS. The RFU was then measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm, using non-transfected cells as a blank.

3. Results

Gene expression levels from GFP under the MLP/TPL as well as CMVie were investigated in non-infected and adenovirus-infected CHO cells. Expression from the two plasmid constructs was investigated with infection at MOI 1 PFU/cell. Plasmids were transfected into CHO cells using Lipofectamine 2000 in two triplicate groups for each plasmid, the first group to be used in RNA and DNA isolation while the second to be

used in GFP fluorescence intensity quantification. The medium was changed 6 h post-transfection to allow the cells to recover from the transfection mix. Twelve hours after transfection, adenovirus was infected into one group of each plasmid transfected cells. In addition to the zero and 12 h (considered as the infection's 0 time) collection times, samples were collected at 36, 84 h, 7.5, 11.5 and 15.5 days post-transfection. Two negative controls were included: the first is CHO cells transfected with pUC19 and used as a control for transfected cells, while the second is pUC19 transfected and infected with the virus.

The isolated DNA was quantified spectrophotometrically and equal amounts were used in a qPCR reaction using GFP specific primers. A standard curve of known plasmid concentration was used to determine the plasmid copy number per cell for each collection time in the transfected and infected conditions (Fig. 2), since the amount of genomic DNA per CHO cell is 3.1 pg [16]. As revealed by the graphs and the statistical analysis performed on the data obtained by the two-way ANOVA using Tukey's test at a significance level of less than 0.05, all of the plasmids have an insignificant change in their stability in CHO cells with the transfection and the infection conditions. The maximum plasmid copy number was obtained at 12 h post-transfection with all of the plasmids used and the two conditions. The plasmids' copy numbers remain significantly elevated on 36 h post-transfection and reach the baseline on day 7.5 post-transfection.

The DNase treated and cleaned RNA was quantified and equal quantities were used in an RT reaction followed by qPCR. Based on the amount of total RNA per CHO cell [12], a standard curve of known plasmid concentration was used to measure the copy numbers of GFP mRNA transcripts

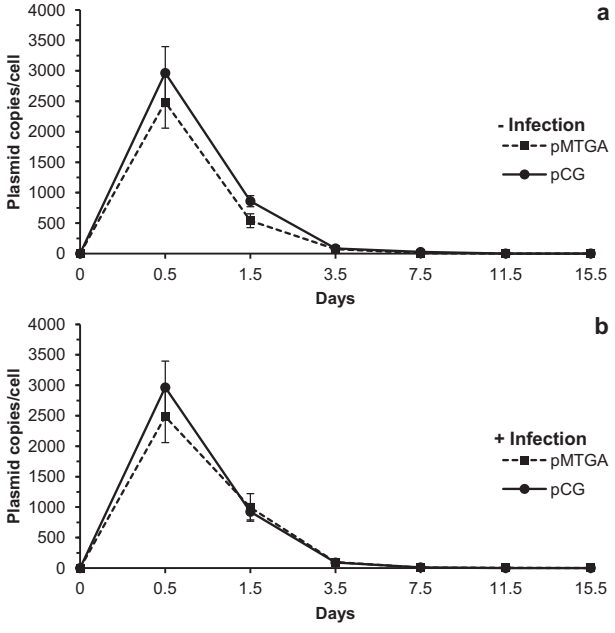

Figure 2 Copy numbers of the two plasmids over 15.5 days post-transfection into CHO cells in non-infected (a) and adenovirus-infected (b) conditions. Copy numbers were obtained by qPCR using a standard curve of known plasmid DNA concentration. qPCR was performed on equal amounts of DNA isolated from collected samples.

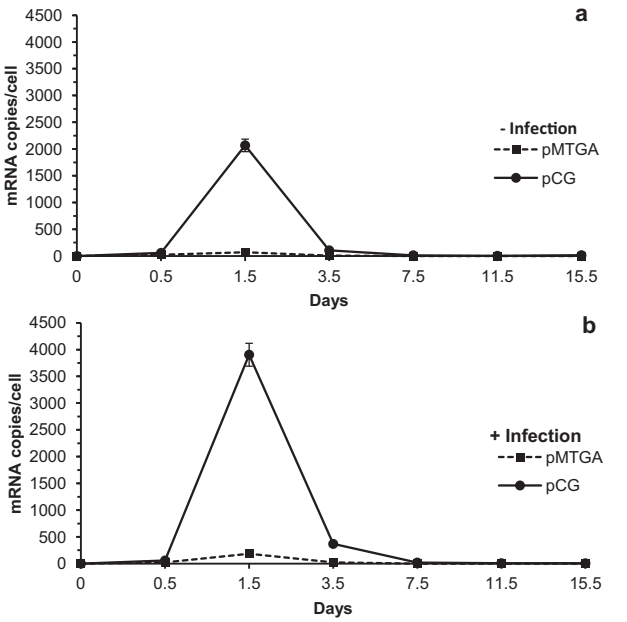

Figure 3 GFP mRNA transcripts from the two plasmids over 15.5 days post-transfection into CHO cells in non-infected (a) and adenovirus-infected (b) conditions. Copy numbers were obtained by qPCR using a standard curve of known plasmid DNA concentration. qPCR was performed on equal volumes of RT product from equal amount of RNA isolated from collected samples.

from each plasmid in the transfection and the infection conditions (Fig. 3). The trend for mRNA transcripts produced from the two plasmids over time in both the transfection and the infection conditions was similar, with a significant increase after 36–48 h. The transcripts' levels almost reached the baseline in day 7.5 and thereafter. When comparing the transcription efficiency from both plasmids on days 1.5 and 3.5 days post-transfection, pCG showed higher transcripts per cell than pMTGA. Transcripts per cell from pCG in non-infected cells were 29.6 folds and 14.3 folds on day 1.5 and day 3.5 post-transfection, respectively. Transcripts from pCG in infected cells show similar enhancement over pMTGA to reach 21.3 folds and 15.6 folds on day 1.5 and day 3.5 post-transfection, respectively (Table 1).

The second collected group was used for GFP quantification by its fluorescence intensity and measured as the RFU in the equal cell counts from the different samples. CHO cells were used as a blank and the two negative controls (pUC19-transfected and pUC19-transfected/infected CHO cell) were included. The RFUs of GFP expressed in each plasmid with both conditions are shown in Fig. 4. Three biological replicates were carried out and statistical analysis of the data obtained was performed as mentioned earlier. The two plasmids showed significant (at $P < 0.05$) increase in GFP fluorescence intensity after 12 h post-transfection, in both the transfection and the infection conditions, and last until day 7.5 post-transfection. When comparing GFP fluorescence intensity from both plasmids on days 1.5 and 3.5 days post-transfection, pCG showed higher intensity than pMTGA. The fold difference from pCG over pMTGA from non-infected cells was 11.4 folds and 3.6 folds on day 1.5 and day 3.5 post-transfection, respectively.

Table 1 Fold increase in gene expression on days 1.5 and 3.5 (1.5–3.5) post-transfection in non-infected and adenoviral-infected cells.

Condition	CMVie enhancer/promoter over MLP/TPL		MLP/TPL in infected over non-infected cells		CMVie enhancer/promoter in infected over non-infected cells	
Level	Transcription	Translation	Transcription	Translation	Transcription	Translation
Non-infected cells	29.6–14.3	11.4–3.6	1–1	1–1	1–1	1–1
Adenovirus-infected cells	21.3–15.6	14.7–2.4	2.6–3.2	1.4–1.7	1.9–3.5	1.9–1.1

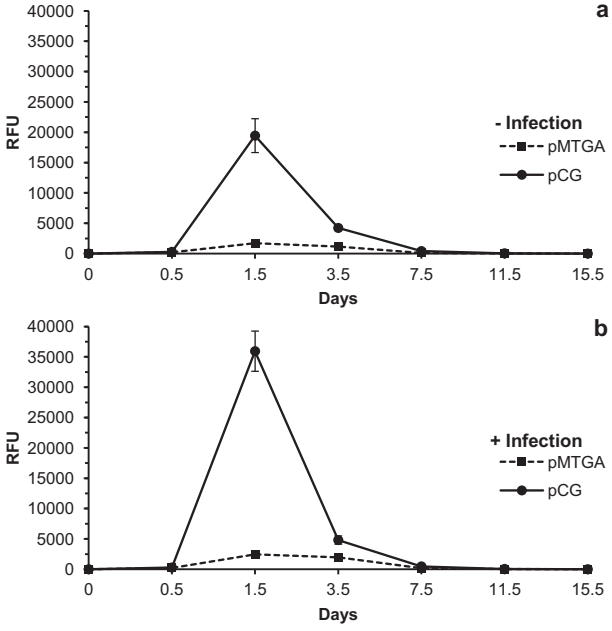

Figure 4 GFP fluorescence intensity over 15.5 days post-transfection into CHO cells in non-infected (a) and adenovirus-infected (b) conditions. Fluorescence intensity was measured as relative fluorescence units (RFU) of equal cell counts.

The fold difference from infected cells shows similar enhancement from pCG over pMTGA to reach 14.7 folds and 2.4 folds on day 1.5 and day 3.5 post-transfection, respectively (Table 1).

Gene expression efficiency in non-infected and adenovirus-infected cells transfected with pMTGA is shown in Fig. 5. There was no change in plasmid stability in both conditions; however, the effect on GFP mRNA transcripts as well as GFP expression was significantly higher in infected cells than non-infected cells on days 1.5 and 3.5 post-transfection. The enhancement in mRNA transcripts on 1.5 and 3.5 days is 2.6 folds and 3.2 folds, respectively, while the enhancement of GFP expression on the same days is 1.4 folds and 1.7 folds, respectively. On the other hand, gene expression efficiency in non-infected and infected cells transfected with pCG is shown in Fig. 6. Similarly, there is no change in plasmid stability in both conditions, with a significant enhancement of GFP mRNA transcription and GFP expression on 1.5 days post-transfection in infected cells than non-infected cells. The enhancement in mRNA transcripts as well as GFP expression on 1.5 day is 1.9 folds. On day 3.5, significant enhancement of 3.5 folds was obtained in the transcription level with non-significant enhancement of 1.1 folds in the translation levels (Table 1).

4. Discussion

Highly active gene expression driven by the adenoviral MLP and TPL sequence, observed during late stages of adenovirus infection, has led to their *in vivo* use for gene expression [18]. However, to achieve even higher levels of gene expression, use of the CMVie promoter/enhancer has been preferred for both *in vivo* and *in vitro* studies and applications [14,26,28,37].

Part of the observed difference in expression levels between these two systems may be explained by the roles they fulfill in their endogenous viral contexts. Namely, the adenovirus MLP, which facilitates expression of all late genes, is only active during the end stages of adenovirus replication and depends on transactivation by early viral products [30,51]. However, the CMVie promoter/enhancer drives expression of the very first genes required for cytomegalovirus replication and, therefore, is not dependent on the presence of other viral components for activity [39,41].

In this study, we tested whether providing additional adenovirus sequences, *in trans*, could enhance MLP-TPL activity to that observed for CMVie, using a GFP reporter gene and assaying for transcript and protein levels. For this purpose we made use of the adenovirus dl309, which has the same properties as the wild-type adenovirus serotype 5 with the exception of the E3 region [4,20,21]. CHO cells were chosen as the cellular background for this study because they are the most widely used mammalian cell line for recombinant protein production [43].

Our data confirmed that outside of their endogenous viral contexts, activity of CMVie was significantly higher than that of MLP/TPL for transgene expression. Although GFP expression driven by CMVie was higher at both transcriptional and protein levels, relative to MLP/TPL, this effect was more pronounced in the former. This was expected, since the TPL facilitates late mRNA transport and accumulation in the cytoplasm and is responsible for the selective translation of the late viral proteins instead of the cellular proteins [3,24,49]. Adenovirus E1B 55k and E4 orf6 play the main role in the active transport of viral late mRNA, containing the TPL, from the nucleus to the cytoplasm [2,8,17,23,32,31,42], while cellular mRNA transport is blocked by the same complex [13]. In addition, the TPL allows ribosome shunting, in a similar manner to the internal ribosome entry site (IRES) recognition, which avoids the scanning mechanism that is needed for cellular mRNAs and the required cap-binding [47,48].

GFP expression driven by the MLP/TPL was higher upon adenovirus infection, relative to non-infected cells. This enhancement was due to the expression of the E1A 12S and 13S protein products which stimulate cell division and growth [22]. They play an important role in activating the expression

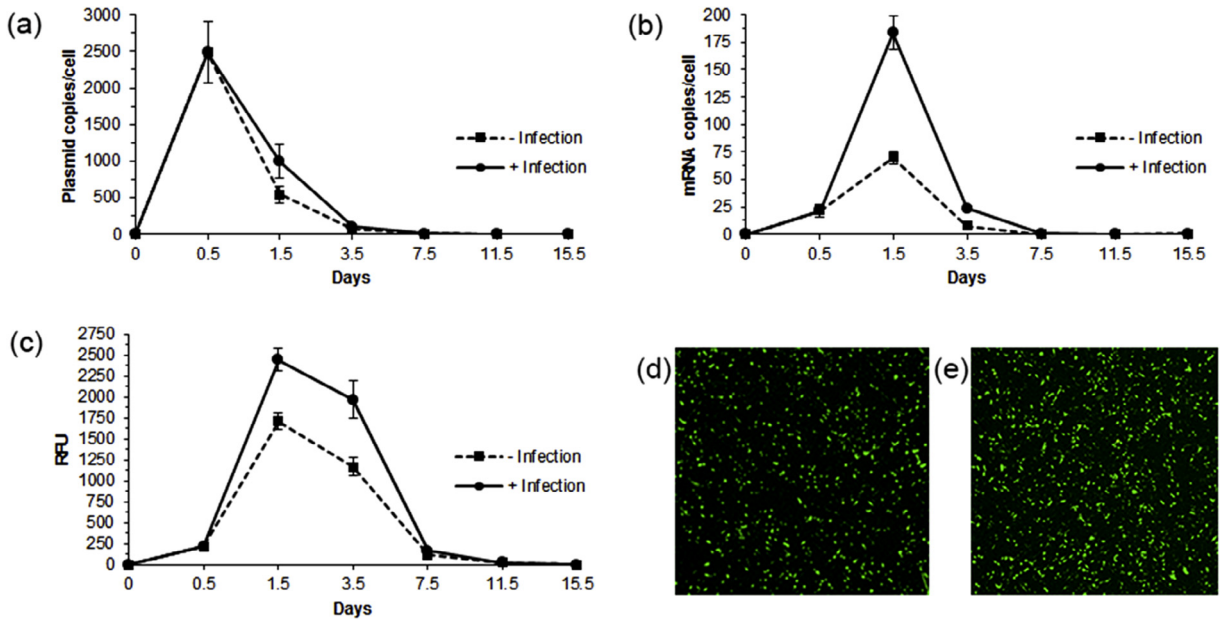

Figure 5 pMTGA in non-infected and infected conditions over 15.5 days in CHO cells. Infection with adenovirus was carried out on 12 h post-transfection. (a) Plasmid copy numbers, (b) GFP mRNA transcripts and (c) GFP fluorescence intensity. Images of GFP expression in (d) non-infected cells and (e) infected cells have been acquired with a 4× objective lens at 1.5 days post-transfection, using Nikon's confocal microscope.

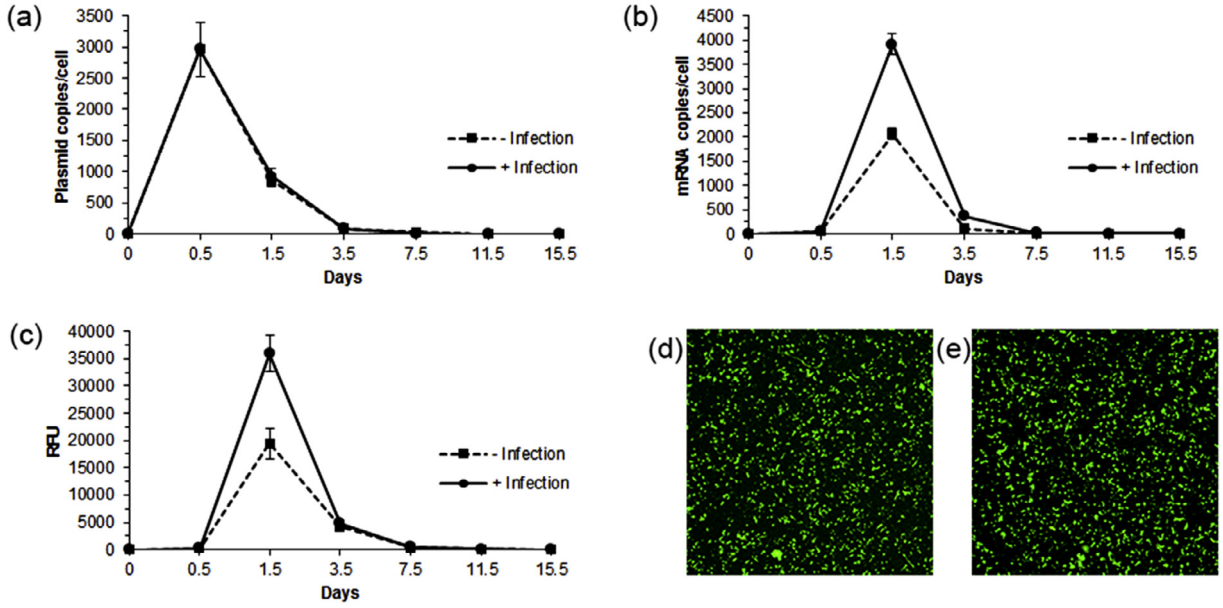

Figure 6 pCG in non-infected and infected conditions over 15.5 days in CHO cells. Infection with adenovirus was carried out on 12 h post-transfection. (a) Plasmid copy numbers, (b) GFP mRNA transcripts and (c) GFP fluorescence intensity. Images of GFP expression in (d) non-infected cells and (e) infected cells have been acquired with a 4× objective lens at 1.5 days post-transfection, using Nikon's confocal microscope.

of adenoviral E2 proteins and other cellular S-phase proteins [7]. However, even with the added activity stimulated by adenovirus infection, reporter gene expression from the MLP/TPL construct did not reach levels observed for CMVie in non-infected and Ad5dl309 infected cells. Interestingly, the CMVie promoter/enhancer also showed enhanced expression in adenovirus-infected cells, suggesting that adenoviral components, provided *in trans,* can stimulate activity of this regulatory sequence, most likely in a similar manner to that observed for MLP. In addition, the effects of adenovirus infection on reporter gene expression from both systems were greater on transcription than on translation levels, although both increased relative to non-infected control. As more information about new transcripts generated by alternative splicing

from the adenoviral genome is being uncovered [36,50]. That might reveal some specific sequences and/or proteins that can be tested to enhance transgene expression from the MLP-TPL.

5. Conclusion

The MLP-TPL derives lower transgene expression levels than the CMVie promoter/enhancer, particularly at the transcription level. The lower fold increase in translation levels than transcription levels from the CMVie promoter/enhancer shows the utility of the TPL sequence at the translation level and/or overwhelming of the cellular translational machinery by the high transcription activity of the CMVie promoter/enhancer. Moreover, expression efficiency enhancement from the CMVie promoter/enhancer cassette in adenovirus-infected cells shows the importance of studying the interaction between this promoter and other adenoviral elements, particularly, the TPL sequence. This may improve the translational efficiency from the abundant transcripts, which accumulate to high levels when the CMVie promoter/enhancer is used to drive gene expression.

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