ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Effect of Different Promoters on the Efficacy of DNA Vaccine Against Hepatitis C Virus.

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ABSTRACT

Hepatitis C is a global health problem which causes about 2.4 million deaths each year due to the absence of effective treatment or vaccine. DNA vaccine showed great results in small animals but its efficiency in human is reduced. Different strategies were used to improve the efficacy of DNA vaccine. In this study, the effect of different promoters on the efficacy of DNA vaccine was tested. We constructed DNA vaccines encoding NS3 and E1 proteins under the control different promoters; cytomegalovirus promoter, adenoviral promoters E1A and MLP. The effect of the different promoters on antigen expression and vaccine efficacy was measured. Our results showed that CMV has the highest transcription level of NS3 and E1 in HEK 293 cells. After mice immunization, we found that all constructs produced NS3 and E1 specific antibodies however plasmids with CMV promoter produced the highest antibody titer. We also examined the miRNA expression level in serum after immunization. We found a slight, not significant increase in mir-181, mir-21 and mir-296. These results showed that the promoter strength has great impact on the efficiency of the DNA vaccine. CMV promoter has been found to be stronger than adenoviral promoters.

Keywords: Hepatitis C virus; DNA vaccine; miRNA; promoter effect.

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ISSN: 0975-8585

INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of chronic liver infection. It is estimated that nearly 170 million people are infected with HCV worldwide. They are at a high risk for developing liver cirrhosis and hepatocellular carcinoma. Given the lack of an effective vaccine until now, the development of an efficient vaccine is urgently required. DNA vaccination is a promising approach that depends on the delivery and expression of the antigen in the host cell. Thus, DNA vaccines induce both humoral and cellular responses [1, 2]. DNA vaccination is an ideal approach for protection against HCV, particularly given the difficulty in growing HCV in cell culture for a live vaccine [3]. In addition, evidence has shown that cellular immunity plays a critical role in HCV clearance [4-6] and neutralizing antibodies are of vital importance in preventing HCV infection [7]. The viral genetic diversity represents a major challenge to vaccine development [8]. Therefore, choosing the correct antigen is critical in order to design an effective DNA vaccine. The NS3 gene has limited genetic variability due to its vital enzymatic activity of protease and helicase. In addition, NS3 has induces a strong immune response and correlates to viral clearance during acute infection [9, 10]. E1 is a good target for neutralizing antibodies, due to its limited variability and inter-genotype cross-reactivity compared to E2 [11]. Furthermore, the function of E1 was found to be impaired in chronic HCV infection, suggesting a role in viral clearance [12] Therefore, NS3 and E1 are ideal candidates for a prophylactic and therapeutic vaccine capable of eliciting a cellular immune response that prevents the infection and eliminates the infected cells [13, 14].DNA vaccines were evaluated against several viruses and successfully produced protective immunity in small animals but they showed lower efficacy in large animals and humans [15]. Subsequently, a number of strategies were employed to enhance the efficacy of DNA vaccines. These approaches include improving antigen expression and stability, addition of adjuvants and immune modulators, using different delivery methods and routes, codon optimization and heterogeneous boosting [16]. Antigen expression level is an important factor, affecting the immune response triggered by DNA vaccines that has yet to be optimized. Most studies of DNA vaccines have utilized standard promoters to enhance gene expression. The most widely used is the immediate early cytomegalovirus promoter (CMV) due to its strong constitutive activity in most cell types [17-19]. Therefore, the effect of different promoters on the efficiency of DNA vaccines against HCV was evaluated in this thesis study. Two different Adenoviral promoters were tested, the weak immediate early E1A promoter and the strong major late promoter (MLP), and compared to the standard CMV promoter.E1A promoter is a weak immediate early promoter responsible for transcription of the early region E1A of Adenovirus (Ad). The transcription of the E1A region is initiated immediately after infection, since it depends on cellular transcription factors [20]. The maximal and early activity of the E1A promoter is due to the interaction of cellular transcription factors E2F [21] and EF-1A [22] with the enhancer elements of the promoter.The MLP drives transcription of the major transcriptional unit of Ad which encodes the structural protein required for virion assembly. The activity of MLP is weak during the early phase of Ad infection, and greatly increases during the late infection, following viral DNA replication [23]. In this study, plasmids were constructed encoding HCV antigens NS3 or E1, under the control of the three promoters, CMV, MLP and E1A. The effects of promoters on antigen expression as well as the antibody level were evaluated. In addition, microRNAs that are involved in immune response regulation were evaluated as indicators of the immune response.

METHODS

HCV Plasmids construction:

NS3 and E1 cDNAs were obtained by RT-PCR amplification from the total RNA of the Huh 7.5 Con1/FL-Neo cell line harboring the HCV viral genome of 1b genotype (a kind gift from Dr. Charles Rice, Rockefeller University, USA). NS3 and E1 were separately cloned under 3 different promoters, CMV, MLP, and E1A. The primers used to amplify E1 and NS3 are shown in table (1), restriction sites *Nco*I and *Not*I sites were added in the forward and reverse primers respectively (underlined. Each fragment was inserted in *Nco*I and *Not*I under MLP giving the plasmids pMTE1 and pMTNS3 from which E1 and NS3 were digested for cloning under the other promoters. E1 and NS3 were cloned in *Nco*I and *Bam*HI sites under E1A giving pEE1 and pENS3 plasmids, and in *Age*I and *Not*I sites under the CMV promoter giving the plasmids pCE1 and pCNS3. All plasmids were confirmed by restriction digestion and sequencing. Plasmids were prepared using Endotoxin Free Plasmid DNA MaxiPrep Kit (NorgenBiotek Corp. ON, Canada) and resuspended in endotoxin free phosphate buffer saline (PBS) to obtain a final concentration of 1 mg/ mL.

ISSN: 0975-8585

Table 1: List of primers used for E1 and NS3 amplification

Primer	Sequence	Fragment size
E1-Fw	5' GCATGCCATGGGATATGAAGTGCGCAACGTATC3'	488 bp
E1-RV	5'CGATGATTGCGGCCGCTTAGATCCGGAGTAACTGCGATA3'	
NS3-Fw	5' CACCCTGCCATGGCGCCTATTACGGCCTACTC 3'	780 bp
NS3Rv	5' CGATGATTGCGGCCGCTACGTGACGACCTCCAGGT 3'	

***In vitro* evaluation of NS3 and E1 expression under different promoters**

The expression of NS3 and E1 was evaluated in HEK 293 cells transfected with the plasmids driven by the three promoters CMV, E1A and MLP. The expression level was quantified by qRT-PCR. Total RNA was isolated from infected cells. Reverse transcription reaction was performed using 200 ng of RNA digested with DNase I (Ambion, CA, USA). The quantity of mRNA (copy number/cell) was determined by real-time PCR using absolute quantification. A standard curve was prepared from NS3 plasmid using known concentrations. The concentration of unknown samples was then determined by simple interpolation of cycle threshold (Ct) values into the standard curve.

Animals and immunization protocol

Female BALB/c mice, 6-8 weeks old, (18-20 g) were purchased from Charles River Breeding Laboratories (ON Canada), and were housed at the animal care facilities at Brock University. All animal work was approved by the Brock University Animal Care and Use Committee (ACC) and done according to the Canadian Council on Animal Care (CCAC) guidelines required for experimentation with animals. Three animals were used in each group. Immunization with the DNA vaccines was accomplished by injecting 50 µg of plasmid DNA (in 100 µL final volume of endotoxin free PBS) into the tibialis anterior (TA) muscle, divided on the left and right muscles. Each animal received three intramuscular injections, at two-week intervals. The control animals were injected with 100 µL PBS. Mice were anaesthetized by inhalation of 5% isofluorine with oxygen prior to injection. Blood samples were collected by heart puncture 2 weeks after the last immunization. Serum was separated by centrifugation at 7000 xg for 15 min .

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to monitor for the levels of NS3-specific antibodies in the sera collected from immunized animals [24]. ELISA plate was coated with 0.1 µg/well of recombinant NS3 (Genway Biotech, CA, USA) or E1 expressed in *E.coli.* Two-fold serial dilutions of mice sera (in PBS containing 1% BSA) were incubated with the antigen for 2 h at room temperature in NS3 antigen. 100 µL of Peroxidase-conjugate goat anti-mouse IgG antibody (Sigma, ON, Canada), at a dilution of 1:5000, was added to each well. The reactions were developed by adding 200 µL/well of Ophenylenediamine substrate (SIGMA FAST™ OPD tablets). The OD was measuredat 450 nm. The titers of the antibodies are expressed as the reciprocal of the lowest sample dilution, in which the optical density of the samples is between 1.0 and 0.5 and at least twice as high as the control [25].

MicroRNA quantification

Specific miRNAs involved in the regulation of the immune response were quantified in the sera of immunized and control mice using qRT-PCR. First, Total RNA was isolated from 100 µL serum using the Total RNA Purification kit (NorgenBiotek Corp. ON, Canada) according to the manufacturer's instructions. A 20 µL RT reaction was performed using a 0.5 µL (50 mM stock) stem loop primer specific to each miRNA (mir-RT) and 200 ng of RNA. Real-time PCR was performed on the cDNA using specific forward primers for each miRNA and one universal reverse primer (miRNA-RV) (Table 2). For relative quantification of miRNA levels, the Ct values were normalized to the 5SrRNA gene. The expression levels of miRNAs were indicated as fold-difference in expression compared to the non-immunized animals. The fold change values were calculated with the ΔΔCt method .

ISSN: 0975-8585

Table 2: List of primers used for miRNAs amplification

PCR product	Primer	Sequence
	miRNA-RV	5' GTGCAGGGTCCGAGGT 3'
mir-181	mir-181-RT	5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAC 3'
	mir-181-Fw	5' AACATTCAACGCTGTCGGT 3'
mir-296	mir-296-RT	5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGGA 3'
	mir-296-Fw	5' AGGGCCCCCCCTCAAT 3'
mir-21	mir-21-RT	5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGTT 3'
	mir-21-Fw	5' TAGCTTATCAGACTGATGTTGA 3'

RESULTS

The transcription level of E1 and NS3 driven by the different promoters

The activity of three different promoters, CMV, E1A and MLP were compared in 293 cells at different time points post-transfection, including 0, 12, 24 and 48 h. The expression levels for E1 were quantified using qRT-PCR. The results showed that the CMV promoter has the highest activity starting at 12 h, which increased at 24 h and reached a maximum at 48 h (920 mRNA copies/cell). Both MLP and E1A demonstrated very low transcription levels, which reached maximum activity at 24 h (Figure 1).

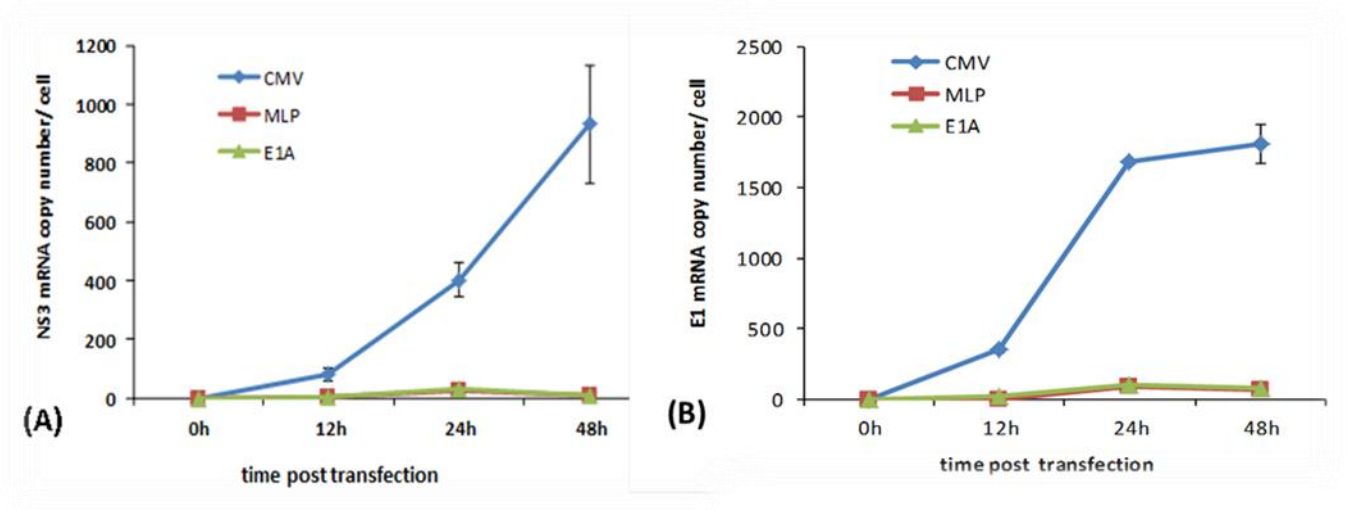

Figure 1: The level of NS3 (A) and E1 (B) transcription under different promoters (E1A, MLP, CMV), at 0, 12, 24, 48 hours post-transfection in 293 cells

Evaluation of HCV- E1 and NS3-specific antibody levels

To investigate the effect of different promoters on the immune response, sera from immunized and control mice were collected 2 weeks after the last dose. The antibody level was measured using ELISA. All immunized animals produced antibodies against the HCV E1 and NS3 proteins. The results demonstrated high titers of HCV E1 and NS3-specific antibodies in animals injected with plasmids controlled by the CMV promoter. On the other hand, plasmids that contain Ad MLP and Ad E1A promoters resulted in lower antibody titers but were still significantly higher than the control animals (Figure 2).

ISSN: 0975-8585

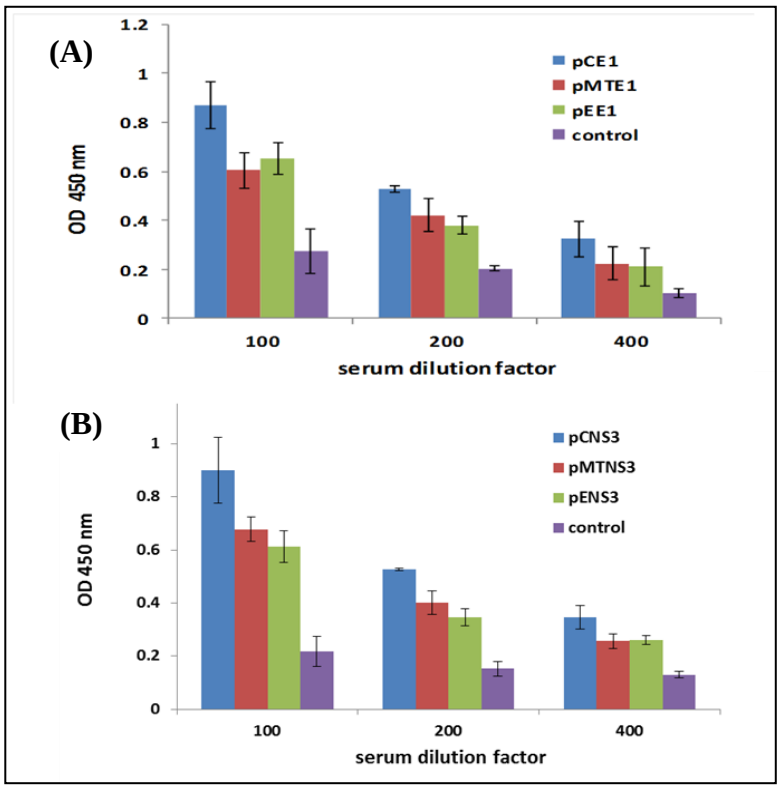

Figure 2: E1 (A) and NS3 (B) antibody levels in sera from animals immunized with plasmid DNA under different promoters: CMV, MLP, and E1A.

The effect of vaccination on serum miRNA levels

Cellular miRNAs are essential in the regulation of all cellular activities and they determine the fate of several cell types. The purpose of this experiment was to investigate the relationship between the immune response and cellular miRNA. We examined the level of mir-181, mir-21, and mir-296 in animals immunized with E1 and NS3 plasmid vaccines. The results demonstrated an upregulation of certain miRNAs. Mir-181 was upregulated in all immunized groups except the pCNS3 injected group, however the highest expression was observed in the groups immunized with pEE1. Mir-21 was also upregulated in groups immunized with pMTNS3, pMTE1 and pEE1. Mir-296 showed a slightly elevated expression in those vaccinated groups that received pMTNS3 and pMTE1. However, the increase in miRNAs was not statistically significant ($p > 0.05$) (Figure 3).

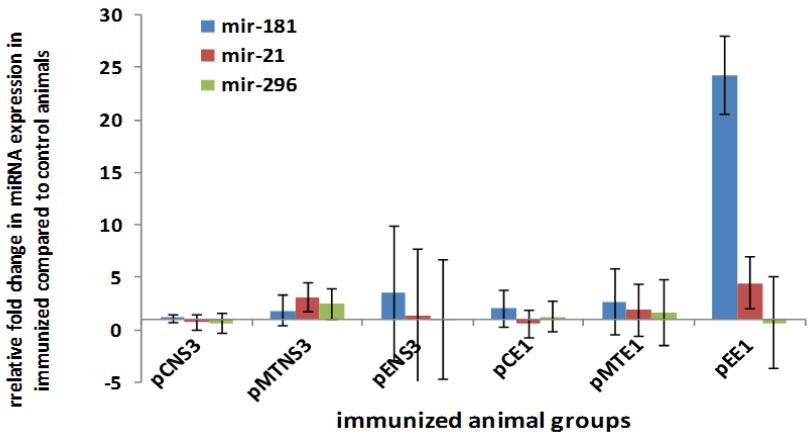

Figure 3: The expression level of miRNAs in the sera of mice immunized with E1 and NS3 plasmids.

ISSN: 0975-8585

DISCUSSION

DNA vaccination is a promising approach for challenging viruses. In addition to their ability to induce both cellular and humoral immunity, DNA vaccine technology allows plasmid modifications to enhance or broaden the immune response. However, DNA vaccine trials have shown limited immunogenicity in humans compared to small experimental animals. Therefore, optimization of the DNA vaccines is critical to enhance their efficacy. The focus of this study is to optimize antigen expression by using different promoters. Two promoters derived from adenovirus were evaluated; the weak early promoter E1A and the strong late promoter MLP. These promoters were chosen in order to compare between early and late promoters and because they were not previously evaluated in DNA vaccine although they were evaluated for in vitro expression. Antigen expression under different promoters was evaluated in vitro. The results demonstrated a higher transcription level of NS3 and E1, driven by the CMV promoter compared to MLP and E1A promoters. This can be explained by the known strength of the CMV promoter, due to the strong TATAA box, enhancers located upstream of the transcription initiation site, as well as its cyclic-AMP response elements (17, 26). This result is consistent with other studies that compared different promoters and found that CMV was more effective in transgene expression [27, 28]. On the other hand, both Ad promoters exhibited significantly lower transcriptional activity (mRNA level), which is in accordance with the results shown by Goossens et al. [29] where they found that the CMV promoter induced 6-10 fold higher activity in reporter gene expression than MLP. Another experiment using adenovirus to express IL2 under MLP showed less expression than both RSV and CMV [30]. In the case of the E1A promoter, it has been shown that its activity is 100 fold lower than the CMV promoter in neuronal cells [31]. Moreover, Ad vectors induced expression of the E1A proteins under the CMV promoter 10 fold higher than its endogenous E1A promoter [32]. The results showed that there was no significant difference between the activity of E1A and MLP, which coincides with the report that the strong activity of MLP is related to late infection, resulting from the interaction of virus-induced transcription factors with the binding sites downstream to the promoter. This is confirmed by deletion of these sites which reduced transcription activity by 25-50 folds [33]. It has been reported that Ad E1A protein supplied by 293 cells can transactivate some promoters such as MLP and CMV [34] and reduce the activity of the E1A promoter [35]. However our results showed that both E1A and MLP promoters have similar activity in 293 cells, which agrees with the finding in natural Ad infection that MLP demonstrates similar activity to the early promoters in the early phase (23, 36]. This indicates that 293 cells can be used for monitoring Ad promoters' activities, and the expression of E1 has minimal effects on their activities. In general, these results indicate that the CMV promoter is more effective in driving antigen expression. Although MLP is a strong promoter during viral infection, it is less effective when it is used separate from the virus. E1A is a weak promoter, and therefore induced less activity. These results confirmed that the strength of the promoter is more important than the temporal expression. In order to investigate the correlation between the level of protein expression and humoral immune responses elicited by the HCV vaccine constructs, the development of E1 and NS3 antibodies in immunized mice was analyzed. We compared the level of antibodies induced by each vaccine and found that all constructs were capable of inducing a humoral response, however their efficacy was variable. The CMV promoter induced the highest antibody level, while the Ad promoters E1A and MLP exhibited almost similar immune responses. These results reflected the activity of each promoter as seen in our *in vitro* expression results and confirmed the direct correlation between antigen expression and the immune response. This relationship was also proven by Galvin et al. [37]. They demonstrated that HIV-1 Gag and Env antibody responses, which were induced by the CMV promoter, were higher than that of AKV murine leukemia viral long terminal repeat promoters. The relation between the strong promoter and antibody titer is explained by the high antigen expression, which consequently enhances antigen presentation by APCs, and produces a potent immune response. Although Ad promoters were weaker than CMV, and exhibited lower antigen expression, they were still capable of inducing a significant level of antibodies which can be further enhanced by using adjuvants. This may be useful in case of antigens that if expressed in large amounts, would cause detrimental effect such as the oncogenic E7 protein of Human papilloma virus (HPV) [38]. In this study, *in vitro* and *in vivo* results showed that the early promoter did not enhance the expression or the immune response. This indicates that strong promoter like CMV is more effective in enhancing gene expressing and also vaccine immunogenicity. However, CMV was already used in DNA vaccine trials and could not induce optimal immunogenicity in humans. That underscores the need to explore more powerful approaches to enhance antigen expression. In addition, combining a strong promoter with codon optimization or a potent adjuvant would optimize the efficacy of DNA vaccines. Enhancing gene uptake is also important and could be realized by using different methods of delivery such as electroporation or gene gun technology that are more effective than intramuscular route and require a lower amount of DNA .DNA vaccines encoding E1 and NS3 produce

high levels of antibodies under the control of CMV, which indicates that they could be candidate vaccines and if given together, would maximize the protection. These plasmid vaccines would expect to protect against heterologous strains of HCV due to the relative genetic conservation of E1 and NS3 among strains. However, this study was limited in that only one time point could be evaluated. In future experiments we can monitor the immune response over longer periods to investigate the duration of the immune response and indicate the effectiveness of the vaccine. MiRNAs are small players that are involved in most cellular functions including immune cell maturation, differentiation and the ability to induce an immune response [39-41]. In this study we investigated this relationship by monitoring the change in serum miRNA levels after immunization. We have observed some changes in miRNAs expression however, these changes were not statistically significant. MiRNAs were shown to be regulated through TLR stimulation and activation of the transcription factor nuclear factor-kappa B (NF-κB) pathway. In case of plasmid DNA, the CpG motifs in the plasmid backbone stimulate the TLR-9 and induce a cascade of pro-inflammatory cytokines that is expected to up-regulate mir-21, mir-181 and mir-296 [41-43]. Mir-21 has a key role in negative regulation of TLR-dependent inflammatory cytokines to reduce their harmful effect on the host [44]. Others reported that mir-21 activates NF-κB increasing the inflammatory response [45]. Up-regulation of mir-21 in this experiment indicates the activation of the immune system after vaccination by CpG motifs in the plasmid vaccines. Mir-181 is involved in the activation of B-cells [46, 47] and T-cells as well as the increase in T cell receptor (TCR) expression. The up-regulation of mir-181 in most of the vaccinated groups suggests that it is vital to the activation of the immune response and may be one of the mechanisms by which DNA vaccine induces its effect. Mir-296 has an antiviral effect and has been shown to inhibit viral replication. Induction of mir-296 by plasmid vaccines is stimulated by IFN-B induced through CpG dependent stimulation of TLR. This confirms that DNA vaccines may induce a therapeutic effect not only by priming the immune response but also by induction of anti-HCV miRNAs such as mir-296 which is induced by IFN-B and inhibits HCV replication [58]. Since the changes in these miRNAs were not significant, optimization of the CpG motif content in the DNA vaccine may enhance miRNAs expression and hence increase immunogenicity.

CONCLUSION

In this study we found that CMV promoter is stronger than adenoviral promoters, E1A and MLP, indicating that strong early promoters are required for maximum performance of a DNA vaccine. Measuring miRNAs levels, induced after vaccination, showed that plasmid DNA stimulates miRNAs induction via innate immune system activation by CpG motifs. Although not in a significant level, these results merit further investigation to confirm the relation between the vaccine CpG content and the miRNA levels.

ACKNOWLEDGMENTS

The authors would like to thank the Egyptian Government and the Egyptian Cultural & Educational Bureau in Canada for their financial support of this project. The authors are grateful to Dr. Charles Rice (Center for the Study of Hepatitis C, the Rockefeller University, New York, NY, USA) for providing the Huh 7.5 cell line. The authors would also like to thank the staff of NorgenBiotek and the staff of animal care at Brock University for their technical assistance.

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