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A Study of Varicella Zoster Virus Glycoprotein C Regulatory Region Response to Viral Activators *in vitro*

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Abstract: A study was conducted to analyze the response of varicella zoster virus (VZV) glycoprotein C gene (ORF14) regulatory sequences downstream as well as upstream of the transcription site to VZV transactivators, IE4 and IE62 and p29, the single-stranded DNA binding protein, *in vitro* by transiently transfecting a permissive human melanoma cell line (Mewo). This glycoprotein has been shown to be an important factor in VZV pathogenesis and therefore the regulation of its expression has been of much interest. In this study, the promoter region of gC as well as another VZV glycoprotein, gI (as a positive control), was amplified and cloned into a promoter less plasmid expressing the luciferase gene as a reporter. The activities of the regulatory regions from both glycoproteins were assessed by quantifying the luciferase activity. The results show that the luciferase assay is a powerful means of measuring promoter activity; nevertheless, the promoter region and cognate downstream and upstream sequences of the true late gC gene were not responsive to these viral proteins, indicating that other viral/cellular factors and/or viral replication could be involved in gC synthesis during the VZV infection cycle.

Key words: VZV glycoprotein C, transient expression, viral transactivating proteins, luciferase reporter assay, Mewo cell line

INTRODUCTION

Varicella-Zoster Virus (VZV) is an important human herpesvirus causing a primary infection known as varicella (also called chicken pox) following by latency of the virus in the Trigeminal Ganglia (TG) and Dorsal Root Ganglia (DRG) (Quinlivan and Beuer, 2006; Arvin, 2001; Cohen and Straus, 1996). After an interval that may vary several years, the virus could reactivate, either spontaneously or following one or more triggering factors, to produce herpes zoster (also known as shingles) (Gilden, 2004).

VZV genome consists of a linear double-stranded DNA molecule composed of about 125 kb and is believed to encode a minimum of 71 ORF (Davison and Scott, 1986) and during lytic infection, VZV gene complement is expressed in three kinetic classes, Immediate Early(IE), Early (E) and Late (L) including at least eight glycoproteins (gB, gC, gE, gG, gI, gK, gL and gM) (Kemble *et al*., 2000; Ruyechan and Hay, 1999; Gershon and Gershon, 1999; Cohen and Straus, 1996).

VZV gC, the product of the true-late gene, ORF14, is believed to be required for effective viral replication when tested in human skin implants in Severe Combined Immune Deficient (SCID) mouse model and may contribute to the first evidence of an essential role for any VZV gene product in the pathogenesis of human infection (Moffat *et al*., 1998). Although like its homologue, herpes simplex virus-1 (HSV-1) gC, it has been shown to be dispensable for viral replication in cell culture (Cohen and Seidel, 1994), the fact that the live, attenuated VZV Oka vaccine is defective in VZV gC expression when compared to the wild type VZV strains, is supportive of a role for VZV gC in viral pathogenesis (Kinchington *et al*., 1990a, b).

ORF 14 is expressed during lytic but not latent infection and has recently been shown to be euchromatic (Gary *et al*., 2006). As for all VZV genes, viral transactivators play a decisive role in the regulation of ORF14 expression. VZV ORF62 product, immediate early 62 protein (IE62), transactivates the expression of all three classes of gene promoters (Inchauspe *et al*., 1989; Perera *et al*., 1992a, b). This transcription activation is a coordinate process and requires complex regulatory regions with unique promoter and binding sites. IE62 is a relatively large protein and in VZV-infected cells, IE62

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can be detected in both the cytoplasm and the nucleus. Its ability to regulate the expression of VZV genes of all three putative kinetic classes as well as its functional similarity with ICP4 of HSV certainly argues for an important role in the VZV life cycle (Perera *et al*., 1992b).

Other viral factors which are involved in VZV gene expression, include IE 4, a potent transactivator (Defechereux *et al*., 1993) that cooperates with IE62 in driving VZV gene expressions (Spengler *et al*., 2000) and 29p which is a single-stranded DNA binding protein with functions during replication of the viral genome (Kinchington *et al*., 1988; Cohrs *et al*., 2002) as well as implications in the regulation of the late gene, ORF67, the product of which is glycoprotein I (He *et al*., 2001).

The experiments in this study were designed to assess the effects of the different transactivating factors on the VZV ORF14 promoter region and several 100 bp upstream in an effort to identify cis-acting elements such as enhancers which could boost expression *in vitro*. The promoter region of VZV ORF67 expressing gI glycoprotein which has been extensively studied (Ling *et al*., 1992), was also amplified, cloned and used as a positive control to compare with gC. Several reporter constructs in which these promoters individually drive expression of the luciferase gene as a reporter, were co-transfected with expression vectors into Mewo cells. Results indicate that the luciferase reporter system is a sensitive assay for promoter activity assessment *in vitro*, as verified by the data obtained from the gI promoter region studies. However, the gC promoter appears weakly responsive to transactivators such as IE62 and IE4 as well as protein 29 and inclusion of DNA sequences more than 800 bp upstream of the transcription site in the gC promoter construct does not enhance expression.

MATERIALS AND METHODS

Cells: Mewo cells, a human melanoma cell line that supports the replication of VZV, were grown in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U of penicillin (Sigma, USA) mL^{-1} and 0.1 mg of streptomycin (Sigma, USA) mL^{-1} at 37°C in an incubator supplying 5% CO_2.

Plasmids: The cloning of pCMV62, pCMV4 and pMS29 plasmids expressing IE62, IE4 and p29, respectively, under the control of the cytomegalovirus (CMV) immediate-early (IE) promoter was described previously by Perera *et al*. (1992b). The following plasmids were constructed in this study: psgC1, psgC2 and psgI, all three vectors were derived from pGL2-Basic (Promega, Madison, USA) which carries a promoter less *luc* gene. The fragments from ORF14 promoter and upstream sequences, as well as the promoter region from gI were amplified by PCR using primers designed to carry *KpnI* at the 5'end and *HindIII* site at the 3' end. pGL2-control was propagated, purified and used as a control of transfection.

PCR: Several sets of primers (Table 1) were designed (Gene Runner Software), synthesized (Integrated DNA Technologies, Inc., USA and Bioneer, South Korea) and used in polymerase chain reactions to amplify promoter regions encompassing upstream as well as downstream sequences relative to the ORF14 transcription start. ORF67 promoter and upstream sequences was generated using VZV cosmid pVSpe21 (Kemble *et al*., 2000) as template and primers as depicted in Table 1. These cosmids are constructed by ligating 4 overlapping fragments, which represent the full-length genome of VZV, into SuperCos 1 cosmid (Stratagene, USA). The pVFsp4 cosmid containing a fragment of the VZV (Parent Oka, P-Oka) genome from nucleotide position (np) 1-33211 was used as template. For the amplification of the ORF67 promoter region, pVSpe21 cosmid carrying viral genome np 94208-124884 was used as template. To amplify the desired regions of ORF14 and ORF67 regulatory sequences, Platinum *Pfx* polymerase enzyme (Invitrogen, USA) was chosen for the polymerase chain reaction since it is known to be a highly processive enzyme with proofreading activity.

Cloning of the regulatory regions: The PCR products were purified using QIAquick PCR Purification Kit (QIAgen, USA) double-digested with *HindIII* (Takara,

Table 1: Primer sequences used for the promoter region amplification of gC and gI. Underlined sequences denote recognition sequence for *KpnI* restriction endonuclease while sequences identified in bold letters indicate *HindIII* recognition sites

Fragment	Primer sequence
gC1	Forward primer: 5'-CATG**AAGCTT**GGTGTGGGTTGAGATTC-3'
	Reverse primer: 5'- GATCGGTACCCAACGACCTCGGTGACG-3'
gC2	Forward primer: 5'-CATG**AAGCTT**GGTGTGGGTTGAGATTC-3'
	Reverse primer 5'- CATGGGTACCGTTGGGAGGTTTACTAAC-3'
gI	Forward primer: 5'- GATCGGTACCAATTGTGACAGTGAGCGT-3'
	Reverse primer: 5'- CATG**AAGCTT**TTGCAAGCTCACGTGGTC-3'

South Korea) and *KpnI* (Invitrogen, USA) and then ligated to *HindIII* and *KpnI*-digested promoter less pGL2-Basic vector in orientations such that the luciferase reporter gene represents various promoter regions of ORF 14 gene expression, to generate the reporter constructs. The ligated products were subsequently introduced into competent *E. coli* cells and plated onto selective LB plates containing ampicillin (100 μg μL^{-1}) All plasmids and cosmids were purified using QIAgen plasmid maxiprep kit (QIAgen, USA) as instructed by the manufacturers.

Transient transfections of Mewo cells: Mewo cells were transfected for transient expression with Transfectam (Promega, USA) following the manufacturer's instructions as well as dendrosomes, a lipid-based DNA porter (Sarbolouki *et al.*, 2000). Two microliters of Transfectam or dendrosome reagent was used per microgram of DNA in each transfection. In transfections performed in 12-well plates ~2×10^5 mewo cells per well were seeded in 1 mL of complete growth medium per well. The cells were 80% confluent at the time of transfection. 2.4 micrograms of a given luciferase reporter vector DNA was cotransfected with ~2 μg pCMV62 plasmid and 1 μg of other effector constructs. In each transfection, complementary amounts of pGL2-Basic plasmid was included to equalize the total amount of DNA. For every transfection, 1 μg of a β-galactosidase (β-gal) expressing plasmid, pON284, was included as an internal control to check the transfection efficiency. This plasmid carries the lacZ gene under the control of CMV promoter.

Reporter gene assays Luciferase assay: For each cotransfection, equal amount of cell extracts, transfected with luciferase reporter vectors, were prepared in 100 μL of the lysis buffer included in the Luciferase Reporter Gene Assay High Sensitivity Kit (Roche, Germany). Luciferase assays were carried out with 50 μL of cell extracts with the addition of 30 μL of the luciferin reagent in each assay after which the luciferase activity was measured in a Sirius Luminometer (Berthold Detection Systems). Duplicate transfections were performed in all experiments all results were confirmed by parallel experiments.

β-galactosidase assay: β-gal activity for each transfection was assessed using the protocol of Sambrook and Russell (2001) with minor adjustments for use in microtiter plates. Briefly, 1 μL of $100\times Mg^{+2}$ was added to 67 μL sodium phosphate buffer (pH 7.5), 20 μL 1×ONPG (Sigma, USA) and 20 μL cell lysate prepared for luciferase assay. The plates were placed in a 37°C incubator for several hours and the reactions terminated by the addition of 100 μL sodium carbonate (0.5 M). The Optical Density (OD) of all transfections were determined at 405 nm in an ELISA reader (Laboratory Systems). Final numbers following normalization with β-gal readings indicate fold induction of luciferase activity in Fig. 2 and 3.

RESULTS

PCR: Two DNA fragments from regulatory regions of ORF14 transcription start site and one fragment containing sequences from regulatory regions of ORF 67 (Fig. 1) were amplified using *Pfx* polymerase to ensure the faithful polymerization of the promoter regions under study. The identity of the putative positive clones were verified by (1) PCR of the bacterial colonies and DNA from positive clones. (2) Double digestion of individual clones using *KpnI* and *HindIII* restriction endonucleases (3) Sequencing of the putative positive clones (Rosewell Park Cancer Institute Biopolymer Facility, Buffalo, USA) and analysis by ClustalW Software (http://www.ebi.ac.uk/clustalw)

Transient transfections and reporter gene assays: The resulting reporter plasmids, psgI as well as psgC1 and psgC2 were transfected into Mewo cells in the presence or absence of pCMV62. pGL2-Control plasmid which carries SV-40 promoter and enhancer driving the high expression of luciferase (*luc*) reporter, was used as a positive control of the transfection process for every set of transfections. pGL2-Basic carrying a promoter less *luc* gene was included as the negative control. One microgram of pON284 plasmid was cotransfected while appropriate amount of pGL2-Basic plasmid was added to the pCMV62 transfections to equalize total DNA in all transfections. β-*galactosidase* assays were performed parallel to luciferase assays using the cell lysates following transient transfections to normalize the basal and IE62-mediated luciferase activities expressed from each reporter vector.

Role of IE62: The results from the luciferase assays are presented in Fig. 2 as fold induction of the luciferase activities compared to that of the promoter less pGL2-Basic control in the absence of the major VZV transactivator, pCMV62, which was normalized to 1. This promoter less plasmid was not inducible by IE62 (data not shown) therefore, any IE62-mediated induction in reporters containing the promoter elements being studied must have been due to the presence of these cis-acting elements.

Luciferase activity from plasmids containing ORF14 promoter regions did not increase beyond the promoter less plasmid. Addition of pCMV 62 in transfections carried out with ORF14 promoter plasmids caused a slight increase in fold induction of the luciferase activity. The transfection of Mewo cells with psgI plasmid containing glycoprotein I promoter region without pCMV62 did not produce luciferase activity as indicated in Fig. 2; however, the presence of IE62 expressed from pCMV62 plasmid caused a substantial increase in luciferase activity.

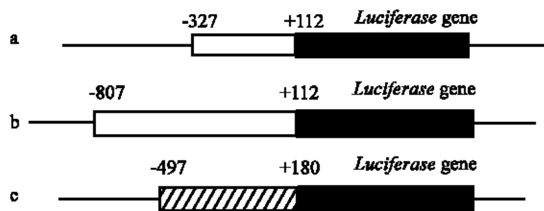

Fig. 1: Schematic representation of *luciferase* gene the expression of which is driven by regulatory sequences from gC and gI. Sequences from downstream of the transcription site were included in these regulatory regions cloned (positive numbers). Upstream regions are denoted by negative numbers. (a) construct psgC1 (b) construct psgC2 (c) construct psgI

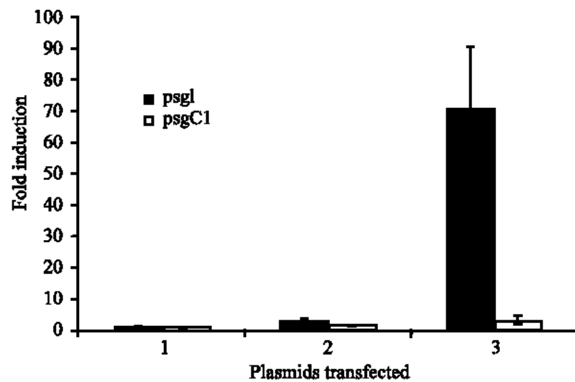

Fig. 2: gI promoter region and gC promoter region activation in the absence and presence of IE62. Numbers on the *x*-axis refer to transfections: (1) pGL2-Basic reporter plasmid alone (2) pGL2-Basic reporter containing the *luc* reporter gene under the control of the VZV gI (psgI plasmid) and gC promoter region (psgC1) in the absence of IE62 and (3) in the presence of IE62 where upmodulation of gI promoter is noted by more than 70 fold induction in *luciferase* activity

Plasmid constructions carrying promoter regions of ORF14 were tested and compared to the activity of gI promoter. As indicated in Fig. 2, gI regulatory region was positively activated in the presence of IE62 with almost 80 fold increase in luciferase activity. In case of gC, the inclusion of pCMV62-expressing IE62 plasmids in transfections with psgC1 failed to activate the gC promoter. To further investigate the role of sequences further upstream of the gC transcription start site, psgC2 (Table 1 and Fig. 1) was constructed and used for transfections into Mewo cells. Again, the increase in luciferase activity, reflecting gC promoter activity, was not significant.

Role of IE4: The product of ORF4, IE 4, directly transactivates plasmids containing homologous or heterologous promoters and has no apparent transrepressing activity (Moriuchi *et al.*, 1994). Again, as expected, IE4 expression in cotransfections with pCMV62 did increase the activity of gI promoter while the gC promoter remained minimally responsive (Fig. 3).

Role of p29: The positive effect of p29 on gI promoter activity in a CD 4+ continuous human T cell line, A3.01, has previously been shown (Boucaud *et al.*, 1998). It is apparent in this study that in Mewo cells using luciferase as a reporter, the effect on gI is not as pronounced. Inclusion of p29 in cotransfection of psgC2 with pCMV62 once again did not boost the activity of this promoter *in vitro.*

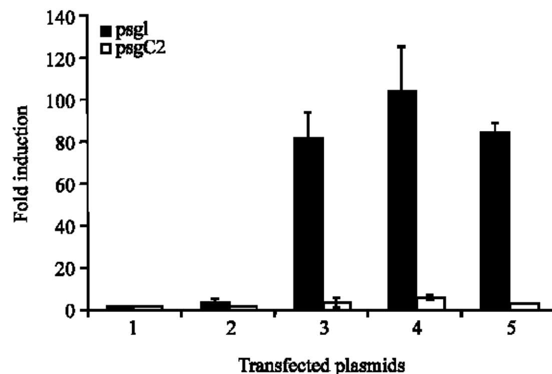

Fig. 3: Activation of gI and gC promoters in the presence of IE4, IE62 and p29. Numbers on the *x*-axis indicate:1) the promoter less pGL2-Basic plasmid 2) psgI and psgC2 in the absence of any viral proteins 3) psgI and psgC2 in the presence of IE62 expressing plasmid 4) psgI and psgC2 in the presence of IE62 and IE4 expressing plasmids 5) psgI and psgC2 in the presence of IE62 and p29 expressing plasmids

DISCUSSION

Considering the importance of gC protein in VZV pathogenesis, the experiments in this study were designed to elucidate cis-acting elements of ORF14 that could contribute to its regulation It has been shown that this ORF displays weak promoter activity *in vitro* (Perera *et al*., 1992a, b) but its expression increases in the infection cycle albeit 72 h post-infection of cells (Storlie *et al*., 2006). Research carried out on another VZV glycoprotein, gI, has indicated the involvement of at least three viral factors i.e. IE4, IE62 and protein 29 in its regulation (He *et al*., 2001). The present study proceeded to clone and test the activity of an extensive regulatory region in gC and gI in the presence of these factors *in vitro* by transfection of permissive Mewo cells and quantifying the expression of the luciferase reporter. The results indicate that luciferase reporter assay system appears more sensitive than CAT assays previously used for assessing gI activity (Perera *et al*., 1992a, b). Even so, the gC regulatory region was not responsive to these factors *in vitro* when compared to the activity of gI under the same conditions (Fig. 2 and 3).

On the other hand, studies carried out on gC promoter structure in HSV-1, show that a post-transcriptional activation of gC by the homologue of VZV IE4, ICP27 may be involved Perkins *et al*. (2003). The study proposes that ICP27 functions through positively acting elements within the transcribed body of gC and stimulates it's expression. No parallel study has been carried out in VZV but the finding by Perkins *et al*. (2003) may explain why IE4 does not induce high levels of gC at transcription and in transient transfection assays (Fig. 2).

Since He *et al*. (2001) have shown that p29 has an upmodulatory effect on gI activity, we proposed a similar role for p29 in promoting gC activity by perhaps preparing the DNA structure for replication. Subsequently, a larger region of the gC upstream sequences (Fig. 1) was used as target for transfections using IE62 and p29 as effector constructs. However, gC promoter sequences still remained refractory to these viral proteins (Fig. 2 and 3). The reason for the minimal activity *in vitro* for gC promoter is still largely unknown but since its activity is evident in viral infection, it is conceivable that the replication process and/or other viral proteins may be involved. Furthermore, as a recent study has indicated, cellular proteins seem to participate in gC regulation (Storlie *et al*., 2006) This group has shown that gC biosynthesis in Mewo cell monolayer is not evident until 72 h post-infection. Bioinformatic analysis of the ORF14 promoter area in the same study has lead to the discovery of PBX/HOX consensus binding domains in the promoter/enhancer regions of the genes for VZV, gC and IE4 protein. PBX/HOX transcription factors play essential roles in both human organ development and oncogenesis. As a result of treating VZV-infected cultures with chemicals known to induce the production of PBX/HOX transcription proteins, namely, hexamethylene bisacetamide (HMBA) and retinoic acid (RA), gC biosynthesis showed a marked increase and appeared 48 h post-infection in the infected cells.

In this study, one reason for the obvious lack of ORF14 promoter/enhancer activity when tested *in vitro* could be the negligible amounts (or absence) of these transcription factors in cultured cells 48 h post-transfection. Therefore, experiments are being designed to treat cultured Mewo cells with HMBA and RA and consequently enhance gC promoter activity. Concurrently, efforts are being directed at elucidating other VZV factors which may have a stimulatory effect on ORF14 by cotransfection of the plasmid constructs with various combinations of the 4 VZV cosmids *in vitro*. The outcome of these experiments could eventually shed light on the ever-elusive regulation of gC expression.

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