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Effects of Inducible cAMP Early Repressor (ICER) on Herpes Simplex Virus -1 (HSV-1) Infectivity

Abstract

While inducible cAMP early repressor (ICER) is known for many roles, including inhibition of anti-apoptosis transcription and regulation of hormone signaling in human / animals, little is known of the role of ICER when interacting with viral cAMP responsive elements (CREs). Prior to this study, CRE sites were found in a variety of human viruses, in locations relating to DNA binding, DNA replication, and latency. A gel shift assay confirmed that ICER can bind to viral CRE sequences, and a reporter gene assay demonstrated that the presence of ICER on a viral promoter upregulates viral expression.

In an attempt to further understand the effects of ICER on viral replication, an *in vivo* study was conducted, focused on infecting an inducible expression system and analyzing the changes of viral infectivity, using Herpes Simplex Virus-1 as a model virus. In addition, two variants of ICER (an HA tag on the carboxyl terminus and an HA tag on the amino terminus) were studied.

This study demonstrates that inducing ICER with an HA tag on the C-terminus limits the viral replication capacity in Vero cell cultures.

MONTCLAIR STATE UNIVERSITY

Effects of Inducible cAMP Early Repressor (ICER) on Herpes Simplex Virus -1 (HSV-1)

Replication

by

Rosalina Caba

A Master's Thesis Submitted to the Faculty of

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EFFECTS OF INDUCIBLE cAMP EARLY REPRESSOR (ICER) ON HERPES SIMPLEX VIRUS -1 (HSV-1)
REPLICATION

A THESIS

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Rosalina Caba

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Montclair, NJ

2021

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Chapter 1: Introduction

1.1 cAMP Responsive Elements (CREs)

1.1.1 CREs Sequence and Locations

A cAMP Responsive Element (CRE) is a short DNA sequence, which bind transcriptional activators and repressors induced by increased levels of cAMP. CREs are palindromes, with a consensus sequence of (5'-TGACGTCA-3') or half-palindrome (5'-TGACG-3' and 5'-CGTCA-3'). In a bioinformatic search of conserved CRE sites in humans and mice, more than half of the time, the CRE was located about 200 nucleotides away from the transcription start site. A total of 3,025 genes were identified; 38% genes related to transcription factors, 24% are related to metabolism, and 33% are related to genes involving cell cycle (Zhang et al, 2005). While CRE locations and target genes vary, there are three major protein families which bind to CREs: CREM, CREB, and ATF-1.

1.2 Inducible cAMP early repressor (ICER)

1.2.1 ICER Structure, Induction and Function

cAMP responsive element modulator (CREM), cAMP responsive element binding protein (CREB), and activating factor-1 (ATF-1) are proteins that bind to cAMP responsive element (CRE). Of the three protein families, only CREM produces transcriptional activators and repressors. (Molina, 1993). The CREM gene contains four promoters, where promoter 2 (P2) contains two DNA binding domains. Upon increased levels of cAMP, there is an increase in the transcription and translation of CREM, and through alternative splicing, an intron of the CREM

gene then becomes inducible cAMP early repressor (ICER), a protein that only contains a DNA binding domain, and lacks a transactivation or kinase inducible domain (Molina, 1993; Laiode, 1993).

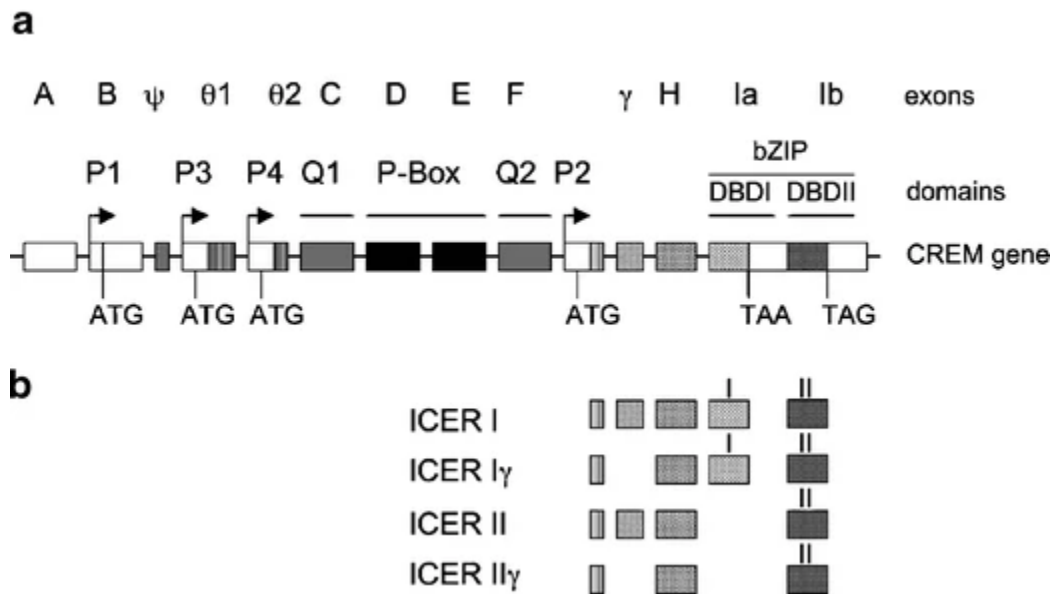


Figure 1: CREM Promoter. Figure 1a represents the intron / exons located in the promoters of cAMP Responsive Element Modulator (CREM). Figure 1b represents the different ICER isoforms. Source: Borlikova, Gilyana, and Shogo Endo. "Inducible cAMP early repressor (ICER) and brain functions." *Molecular neurobiology* 40.1 (2009): 73-86.

There are four isoforms of ICER: ICER I, ICER I γ , ICER II, and ICER II γ . ICER I γ and ICER II γ contain the gamma exon. ICER I contains DNA Binding Domain I, and ICER II contains DNA Binding Domain II (Foulkes, Borrelli, Sassone-Corsi, 1991). Due to the lack of a transactivation domain, ICER is unable to execute transcription, and only bind to CRE sequences. It can dimerize with phosphorylated CREB (pCREB) or homodimerize with itself and repress expression of pCREB / CREM through competitive binding. Therefore, after high levels of cAMP, ICER negative regulates cAMP induced gene expression (Molina, 1993).

1.2.2 ICER regulation

ICER itself is regulated on a transcriptional level. The CREM / ICER promoter (P2) contains four repeating CREs, called cAMP regulatory elements (CARE). Proteins that are able to bind to CRE also bind to CARE sequences (Molina, 1993). Therefore, after ICER competitively represses pCREB, CREM, or ATF-1, these proteins can bind to the CARE sequence in the promoter and inhibit ICER expression.

1.2.3 ICER Degradation

Cellular concentrations of ICER are maintained through degradation, rather than post-translational modifications. In an experiment performed by Folco and Koren in 1997, inhibition of the proteasome caused increased levels of ICER-ubiquitin, demonstrating that ICER is removed from the cell through ubiquitination and the proteasome (Folco, 1997).

1.2.4 ICER And Stress Signaling

ICER plays part in many critical roles in signal transduction, and its transcription is dependent on the quality and quantity of signal introduced into the cell / tissue. Stress, for example, can induce ICER expression. In a study where rats were exposed to surgical stress, an increase in ICER was found in the adrenal glands. When the pituitary gland was removed, a decrease in the production of ICER occurred. This is significant because the pituitary gland secretes adrenocorticotropin hormone (ATCH), which in turn increases levels of cAMP in the adrenal glands and leads to further production of steroids in response to stress. When the rats with removed pituitary glands are injected with ATCH, transient induction of ICER was observed

(Della Fazia, 1998). This established that ICER is involved in the regulatory cascade that activates the production of steroids in the adrenal gland.

It was hypothesized by researchers that ICER had an anti-apoptotic effect, as removal of the pituitary causes atrophy in the adrenal cortex, and an overproduction of ATCH causes hypertrophy. In addition, because this experiment already proves that CREM/ ICER is produced with the presence of ATCH in the adrenal gland, ICER perhaps can repress cyclin A and allow the cell to move forward the G2/M phase (Della Fazia, 1998).

1.2.5 ICER and Apoptosis

This hypothesis, however, contrasts *in vitro* findings of ICER and apoptosis. In neuronal cell cultures undergoing cell death, an upregulation of ICER was found. Bcl-2, an anti-apoptotic protein, is also a pCREB target. An overexpression of ICER *in vitro* therefore causes cell death by competitively binding to pCREB target of the Bcl-2 promoter; silencing anti-apoptotic expression (Konopka, et al, 1998). It is to note that when tested *in vivo*, overexpressing ICER in neuronal cells does not cause cell death; as it is probable that neuronal cell death is more tightly regulated by many signaling systems (Mouravlev et al, 2006).

1.3 CRE sequence in Viral Genomes

1.3.1 Bioinformatic Search

In an attempt to understand the pandemic the world experienced beginning in 2019, the sequence of SARS-CoV-2 virus (NCBI Reference Sequence: NC_045512.2) was previously analyzed by Michelle Oh in 2020. The canonical CRE site was not found to align, but variants of

the CRE sequence (5'-TGACATCA-3', 5'-TGATGTCA-3', 5'-TGACATAA-3', 5'-TGAGGTCA-3', and 5'-TCGATCAA-3') were found in multiple locations within the viral DNA sequence.

The search for CRE sites was then expanded to all human viruses. Many viruses were found to contain either canonical or variants of the CRE sequences. Those with canonical CREs include Human Parvovirus, Yellow Fever virus, Influenza A virus, Human adenovirus, Rotavirus, and Human herpesvirus. Those with variant CRE sequences include Human Papillomavirus, Ebolavirus, Zika virus, and Hepatitis C virus. When the location of the CRE sequences within these viruses were related to viral function, many were linked to either DNA binding, DNA replication, or latency. In order to study the *in vivo* effects of ICER on viral CREs, Herpes Simplex Virus-1 (HSV-1) was chosen as a model virus, as an attenuated safe strain for laboratory studies was available. The HSV-1 also contained green fluorescent protein (GFP), allowing for visible infectivity to cell culture.

1.4 Herpes Simplex Virus -1 (HSV-1)

1.4.1 HSV-1 Classification and Structure

Herpes Simplex Virus -1 (HSV-1) is a large enveloped alpha herpesvirus, containing double stranded DNA. There are two types of Herpes simplex virus, type I and type II. Herpes Simplex Virus type II is known as the causative agent of genital warts, and Herpes Simplex Virus type I is known as the causative agent of cold sores. The herpes virus contains 74 open reading frames, encoding 84 different proteins, all of which are transcribed by the host's RNA polymerase II. The Herpes viral genome contains two major sections, the long unique region

and the short unique region. The long unique region contains 56 of the 72 open reading frames (Boehmer et al, 2003).

1.4.2 HSV-1 Replication

HSV-1 enters host cells by binding to transmembrane receptors of the epithelial cells and fusing its viral envelope to the cell membrane, creating a pore in which the contents of the virus can enter the cell. The virion travels through the cytoplasm and upon encountering the nuclear envelope, the viral capsid binds to the nuclear envelope and injects the DNA into the nucleus. During lytic infection, HSV creates early response proteins that interrupt host protein synthesis and degrade host mRNA, in order to produce more viral double stranded DNA (Copeland et al, 2009).

Only upon latent infection does the virus travel to the terminal of sensory neurons and terminate transcription of all viral proteins, except for latency associated transcript (LAT). This transcript interferes with the host cell's apoptotic responses and allows viruses to survive silently in host cells until it is time to infect other cells. Stress signals or cell death usually cause HSV-1 to change from a latent infection to an active, lytic one (Bloom et al, 2010).

1.5 Experimental Design

To begin, Vero cells were utilized to generate an inducible system that would express ICER when exposed to doxycycline. Vero cells originate from the African Green Monkey epithelial kidney cells, a model cell line ideal for studying viral infection (Ammerman et al, 2008).

Two variants of ICER were tested. An antibody tag (HA) was placed on both the carboxyl (C)- terminus and amino (N)- terminus of the ICER protein. A pending paper by Dr. Carlos Molina suggests that blocking the N- terminus causes immediate ubiquitination and ultimate degradation of the protein, which in turn disrupts essential cell function and causes apoptosis. Therefore, efficiency of ICER was tested by utilizing the two variants. ICER was integrated to the Vero cell line using the pTETone system, which will randomly integrate ICER into the genome. As a control, luciferase was transfected in place of ICER to test the effectiveness of the Tet on system, as the protein will luminesce when the system is on.

Upon successful growth of luciferase, pTetOne-ICER-(HA-N) and pTetOne-ICER (HA-C) were tested with PCR as well as western blotting to ensure ICER had integrated into the genome and was inducible. The Vero cells were infected with Herpes simplex virus-1, that contained GFP in its genome (De Oliveira, et al, 2013). Fluorescent microscopy was then used to analyze the change of infectivity of HSV-1 in the presence and absence of ICER. The data gathered will then provide insight into the possible effect ICER has on HSV-1 capacity to replicate in mammalian cell culture.



Figure 2: pTetOne ICER Plasmids. The figures demonstrates integration of ICER into the pTetOne plasmid. On the left is ICER (N-HA) integration into pTetOne, and on the right is ICER (C-HA) integration into pTetOne.

Chapter 2: Materials and Methods

2.1 Cloning ICER into Tet-One Inducible System

Cloning of ICER followed the Clontech[®] Laboratories, Inc. Tet-One Inducible Expression System User Manual (Takara Bio, USA), using In-Fusion HD. pTet-One plasmid were linearized with EcoRI and BamHI, and primers for integration of ICER into the pTet-One plasmid are as follows:

	ICER (N-HA)	ICER (C-HA)
Forward Primer 5' – 3'	CCCTCGTAAAGAATTC CGCGTCATATGGCTAGCGT	GCAGAGATCTGGATC CGAATTCTCAGGCGTAGTCGGG
Reverse Primer 5' – 3'	GCAGAGATCTGGATCC TGCAGAATTCTCATTCGTTCTTGCA	CCCTCGTAAAGAATTC TCATATGGCTAGCGTTTAACTTAAGCTTGG

Table 1: PCR Primers for ICER integration into pTetOne. Forward and reverse primers were generated to join linearized pTetOne plasmid with ICER. Primers created for both ICER (C-HA) and ICER (N-HA).

2.2 Vero Cell Culture

Vero cells were cultured in doxycycline free Eagle's minimum essential medium (EMEM) and cultivated at 37°C with 5% CO₂.

2.3 ICER Transfection

Cell cultures was transfected with ICER following the Xfect[™] Transfection Reagent Protocol-At-A-Glance (Takara Bio, USA), using 1 ng – 100 ng of template DNA, 100 ng of the hygromycin selection marker, and transfected in 10% FBS – tetracycline free. Plasmids were selected for with 150 ug/mL of hygromycin. After 48 hours; peak transfection was read, and adherent colonies were selected.

2.3 Assay for ICER Integration in Vero Cells: PCR

Applied Biosystems AmpFLSTR™ Identifiler™ PCR Amplification Kit (Thermo Fisher Scientific, USA) , rapid DNA extraction protocol was used to amplify the region of ICER selected using PCR. The selected primers amplified the promoter and ICER; ensuring that ICER was integrated in the correct orientation. Correct integration of ICER would yield a 576 bp PCR product.

	ICER (N-HA)	ICER (C-HA)
Forward Primer 5' – 3'	ACCTCTACAAATGTGGTATGGCT GA	TGTGGGAGGTTTTTTAAAGCAAGTAAA ACC
Reverse Primer 5' – 3'	TAAAAGAGTGCTGATTTTTTGAG TAAACTTCAATTCC	TTTGAGTAACTTCAATTCCACAACACTT TTGT

Table 2: PCR Primers of ICER Integration into pTETOne. Forward and Reverse primers were created to amplify a 576 bp region, encompassing the TRE3GS promoter and the ICER gene.

2.4 Assay for ICER Inducibility: Western Blot

Colonies were tested with Western Blot to ensure ICER was inducible in the presence of 100 ng of doxycycline. Culture medium was removed, and cells were washed before fixed with 4% formaldehyde. Cells were washed again, and reheated Antigen Revival Buffer was added before rewashing with PBS. All remaining PBS was removed, and wells were placed on a plate shaker with Protein Block before the primary antibody added. After washing, the plates were imaged.

2.5 Viral Infection

Cell cultures grown in 6 well (9.6 cm²) cell culture plate were infected with 100 µL of HSV-1, and shaken every 15 minutes for an hour, and incubated for 72 hours at 37°C with 5% CO₂.

Chapter 3: Results

3.1 Transfection of ICER into Vero Cell Line

Use of negative selection marker hygromycin yielded 7 clones of ICER (N-HA) and 9 clones of ICER (C-HA). Clones were then harvested, and PCR primers were designed for amplification of the ICER sequence. Expected PCR size was 576 bp, as the PCR primers tested the orientation and location of ICER's insertion into the cell line. The PCR products showed only 4 potential clones thought to have the gene: two clones of ICER (C-HA) and ICER (N-HA). While one clone from each variant of ICER tested had expected PCR amplicon of ~576 bp (VN5 and VC4), the other clones had bands near ~1000 bp (VN4 and VC9) (Fig. 3). While this may have occurred due to double integration of ICER in the same site, it did not discount the possibility of a functional gene product.

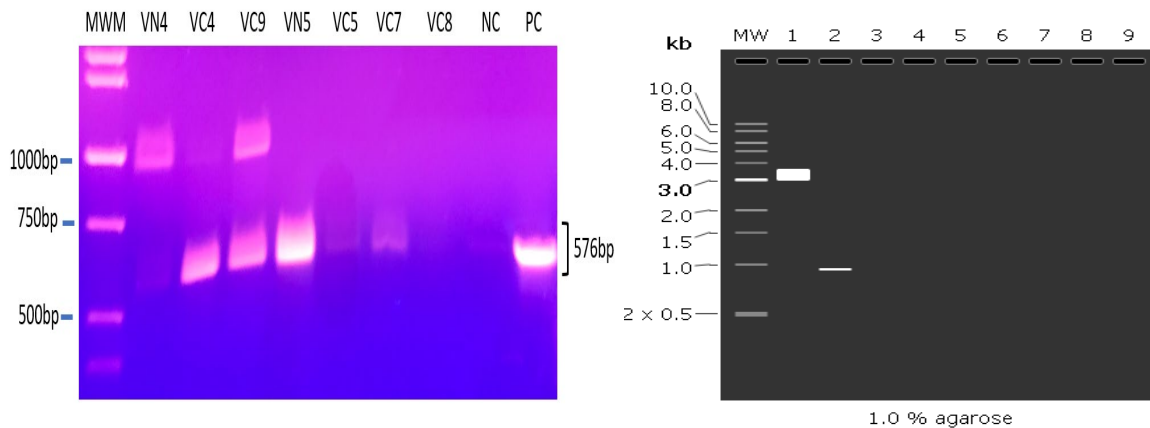


Figure 1: Amplification of ICER into pTETOne plasmids. The figure on the left shows PCR primers of ICER (C-HA) and ICER (N-HA) clones. Nomenclature: Vero cells = V, ICER (C-HA) = C, and ICER (N-HA) = N. Number denotes the clone number. VN4 and VC9 have DNA bands ~1000 bp, indicative of double integration. VC4, VC9, and VN5 have clear DNA bands near expected ICER bands (~576 bp). Negative control was PCR water, and positive control was the ICER plasmid. The figure on the right is a representation of a gel, in which the same PCR primers for amplification of ICER in the pTetOne system, has ICER double integrated in the same site (lane 2). Lane 1 is the supercoiled pTetOne-ICER plasmid.

Western blotting was used to then test the inducibility of ICER expression, when cultured with doxycycline. The expected molecular weight of ICER was 15-20 kd. Three clones were tested for inducibility, VN4, VC4, and VC5 (Fig. 4). Clone VN5 demonstrated definitive inducibility in the presence of doxycycline.

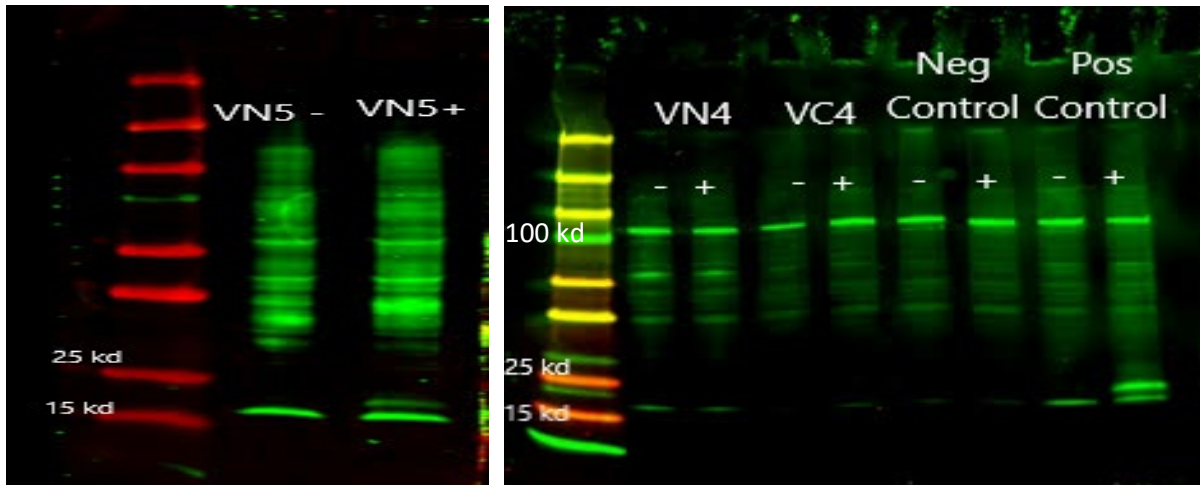


Figure 2: Western Blot of Vero-ICER clones. An anti-HA tag was used for western blotting of ICER (C-HA) and ICER (N-HA). Absence of doxycycline is represented with a (-) and presence of doxycycline is represented with a (+). Inducibility of ICER is demonstrated by a double band, present between 15-20 kd. VN5+ showed clear inducibility of ICER under doxycycline. The negative control was Vero cells without ICER present, and the positive control was Vero cells transiently infected with the ICER plasmid. Nonspecific binding occurred 100 kd.

3.2 Vero Cell Line Infection with HSV-1

ICER (C-HA), ICER (N-HA) and ICER (Luciferase) were infected with HSV-1, and fluorescence was visually assessed after 24 hours and 72 hours. At 24 hours post- infection, clones grown in the presence of doxycycline, including the luciferase control, lacked presence of viral GFP. This indicates a possible delay in viral replication in the presence of doxycycline. After 72 hours, as demonstrated in the expression of viral GFP in the presence of doxycycline, clones containing the luciferase control and the ICER (N-HA) variant demonstrated viral infectivity ..The ICER (C-HA) variant lacked production of viral GFP after 72 hours. At both 24

and 72 hours of infectivity, all clones (luciferase, ICER (C-HA), and ICER (N-HA) grown in the absence of doxycycline showed presence of viral GFP. (Fig. 5).

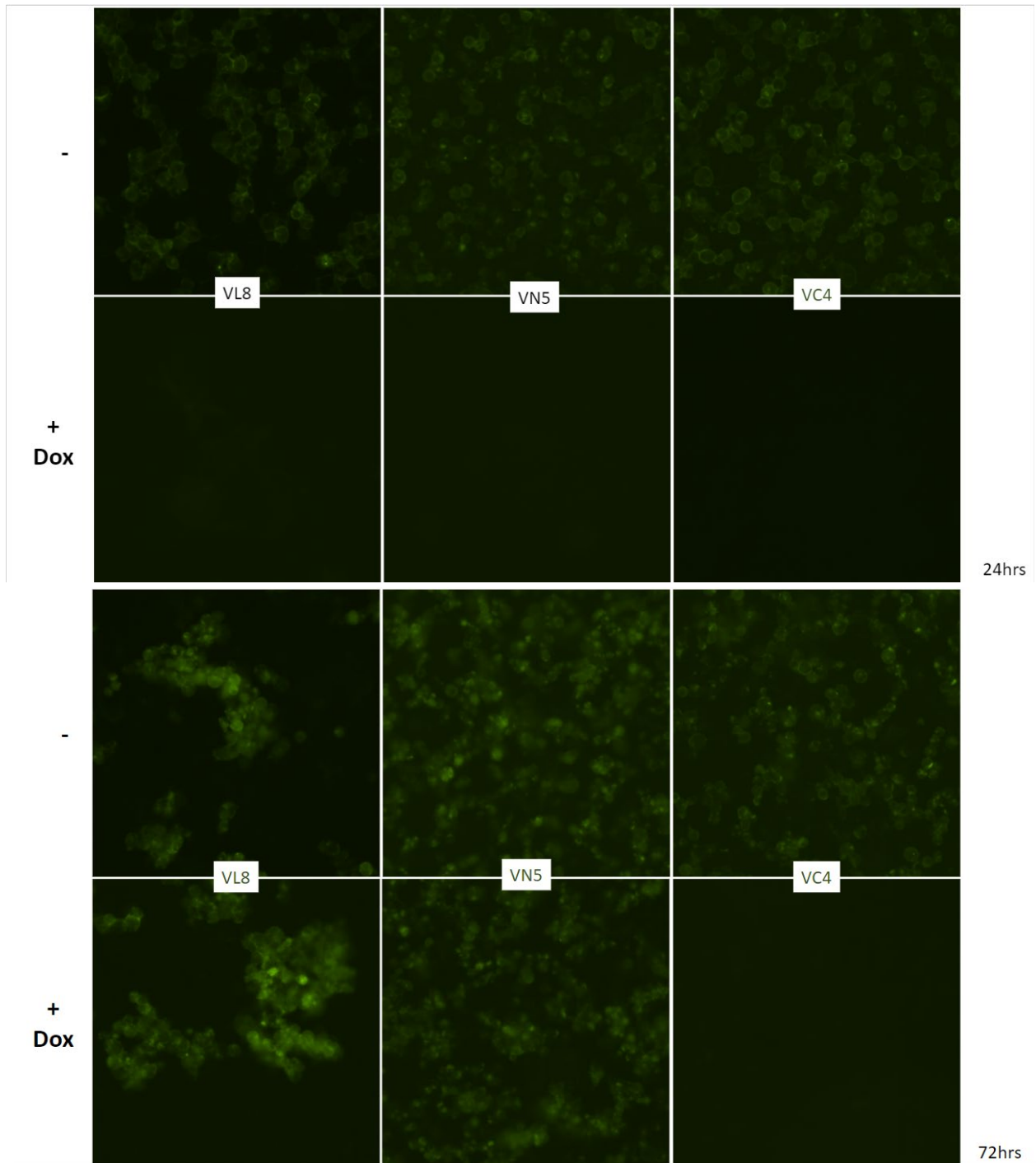


Figure 3: *Fluorescent Microscopy of viral infection.* Luciferase clone VL8, ICER (N-HA) clone VN5, and ICER (C-HA) clone VC4 were infected with HSV-1 and incubated for 24 hours and 72 hours.

Chapter 4: Discussion

Induction of ICER was not clearly observed for western blotting of ICER (C-HA) clone VC4 (Fig. 4). The amount of doxycycline used in the media is a factor, as 100 ng may not have been sufficient to cause induction.

Infectivity of Herpes simplex virus -1 (HSV-1) was not observed in the presence of ICER (C-HA) after 72 hours. Concentration of doxycycline used did not cause cell death, as cell growth and viral infectivity was observed in the luciferase and ICER (N-HA) cell cultures (Fig. 5).

There was no difference in the process of viral infection performed between the luciferase control, ICER (N-HA), and ICER (C-HA) cell cultures, and therefore rules out procedure / process of infection as cause of the repression of infectivity in the ICER (C-HA) cell line. While it is possible there was not enough ICER present during western blotting to be induced in 100 ng of doxycycline, it is possible that when cultured for 72 hours, there may have been enough induction of ICER to limit viral infectivity. If ICER were not present in the VC4 clone in the presence of doxycycline, results would appear as they do for the VC4 clone without doxycycline, where ICER is not expressed.

Follow up studies that may be performed to confirm ICER (C-HA) inhibits viral infectivity is performing western blotting / infection with higher concentrations of doxycycline, to ensure ICER is expressed in sufficient quantities in the cell line.

Other factors to challenge the results are the cell line chosen and the mechanism of ICER integration into the cell line. The Vero cell line was used as it is a model cell line to study

viral infection. However, a human cell line may yield different results, as it aligns closer to *in vitro* effects of HSV-1 in the presence of ICER. Transfection of the pTetOne into the cell line caused random integration of the plasmid into the genome. A different technique to use would be a site-specific integration of the plasmid into the cell line, ensuring ICER is not integrated in a part of the genome related to cell function / cell proliferation.

However, if further studies still determine that ICER (C-HA) can limit viral infectivity, this evidence supports the hypothesis that inhibiting the amino terminus of ICER causes a non-function protein product. In addition, further studies can be done to study the mechanism of action between HSV-1 and ICER. While results generated during this study contrasts results previously generated during a reporter gene assay of a viral promoter and ICER, perhaps the infection stage of HSV-1 reacts differently in the presence of ICER. HSV-1 CRE sequences are found on LAT genes, which are expressed during lytic and latent infection, and are found to repress expression of viral proteins during latency. While HSV-1 is in latency, ICER has been found to upregulate the expression of viral proteins, and reestablish a lytic phase (Colgin et al, 2001). There is little research about the lytic phase of HSV-1 and effects in the presence of ICER. If indeed ICER can repress lytic viral infection, then this may be used as a source of treatment for these infections.

Chapter 5: Conclusion

Infecting cell cultures that express an ICER with a blocked carboxyl terminus is shown to repress HSV-1 replication abilities. Further experiments should be performed to challenge the results generated, and if results prove to be consistent, two avenues of investigation can be investigated. The first would be understanding the difference in viral infectivity capabilities between ICER (C-HA) and ICER (N-HA). The second avenue of research would be determining why and how ICER is able to repress lytic viral replication. Data generated from these avenues of research will allow deeper insight for the use of ICER as a potential biologic anti-viral treatment for not only HSV-1, but any viruses that contain CRE sites.

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