Selective protein synthesis during vaccinia virus-induced host shutoff

by

Pragyesh Dhungel

B.Tech., Kathmandu University, 2013

AN ABSTRACT OF A DISSERTATION

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Abstract

During infection, poxvirus makes host cells conducive for viral replication by causing host shutoff that is marked by global inhibition of host protein synthesis. Host shutoff facilitates the reallocation of cellular resources for viral replication and evasion of host antiviral immune responses. However, it poses a challenge for continuous synthesis of crucial cellular proteins and viral proteins that are important for viral replication. It is unclear whether and how viral and specific cellular proteins are selectively synthesized during poxvirus-induced host shutoff.

In this dissertation, we elucidated that vaccinia virus boosts viral post-replicative protein synthesis by using the 5'-poly(A) leader at the 5'-UTR. Vaccinia virus has evolutionarily optimized the length of the poly(A) leader, and uninterrupted poly(A) leader is required for promoting poxvirus protein production. During vaccinia virus-induced shutoff, poly(A) leader stipulates viral post-replicative mRNAs an adaptive mechanism to translate efficiently. The poly(A) leader translation was not mediated by an internal ribosome entry site (IRES) mode, albeit poly(A) leader mediates cap-independent mode of translation. Through further investigation, we uncovered a cellular RNA-binding protein La-Related Protein 4 (LARP4) that was repurposed to augment vaccinia virus post-replicative mRNA translation. During VACV infection, LARP4 is enriched in the virus factory where VACV post-replicative mRNAs are translated. A decrease of LARP4 protein level reduces VACV replication, blocks post-replicative protein synthesis, and decreases 5'-poly(A) leader mediated translational advantage. Further studies showed that LARP4 is vital for the cap-independent mode of translation from poly(A) leader.

We also showed that infection of vaccinia virus, the prototypic poxvirus, induced selective synthesis of cellular proteins involved in oxidative phosphorylation. Using

simultaneous RNA-seq and ribosome profiling, we determined the mRNAs encoding proteins for oxidative phosphorylation complexes had increased relative translation efficiency. Indeed, vaccinia virus infection increased the activity of oxidative phosphorylation. Inhibition of oxidative phosphorylation function suppressed vaccinia virus replication significantly. Moreover, the mRNAs of oxidative phosphorylation have short 5'-UTRs with a less complex secondary structure that could confer oxidative phosphorylation mRNAs a translational advantage in vaccinia virus-infected cells during host shutoff.

Together, these studies advanced our understanding of how vaccinia virus selectively synthesizes viral and cellular proteins for efficient viral replication during host shutoff. The findings may facilitate the development of novel anti-poxvirus strategies and the improvement of poxviruses as vaccine vectors and anti-cancer agents.

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Approved by:

Major Professor
Zhilong Yang, Ph.D.

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Dedication

I would like to dedicate this dissertation to my family!

Chapter 1 - Introduction

Poxvirus

Viruses and the importance of virology

Viruses (from Sanskrit *visah* and Latin *vīrus*, both meaning poison) are ubiquitously present microscopic infectious agents that require host cells in order to replicate (1). Viruses infect both eukaryotes (Animals, Plants, and Fungi) and prokaryotes (Archaea and Bacteria), utilizing host machineries to conduct different phases of the replication cycle, thus making them an obligatory intracellular parasite. Viruses have genomic material (DNA or RNA) inside a protein coat called a capsid and—in some cases—an extra layer of lipid envelope.

The study of viruses (virology) is important not merely due to them being the most abundant lifeforms—with an estimated 10^{31} viruses on earth (2,3)—but also because viruses are imperative for maintaining the biogeochemical cycle by killing 20% of the microbial biomass daily in the ocean. Viruses through causing diseases in plants, animals, and humans drive the evolution of both host and virus (4). Virology contributes to knowledge regarding viruses and the cells they infect. In fact, the study of viruses provides an instrumental understanding of various basic mechanisms such as DNA replication, transcription, translation, RNA processing, and immunology, among others (5–8). Throughout history, viral diseases have crippled the world and the greatest threat is currently posed by diseases from viruses such as HIV, Ebola, Nipah, measles, monkeypox, influenza, MERS-CoV, and Zika. Basic knowledge regarding these pathogens provides scientists with the tools necessary to develop vaccines against these deadly viruses and therapeutics for the diseases they cause. Insight into important fundamental aspects of viruses has also led to the development of beneficial applications of viruses as tools against non-infectious diseases including cancer, and anti-microbial resistance (AMR). Notable

examples include therapies such as oncolytic virotherapy against various cancers and phage therapy against AMR (9,10). The scientific capital gained from studying viruses will have broader applications in science and medicine, subsequently attributing to the discovery of new basic cellular mechanisms to broaden our horizon of knowledge.

Poxviridae virion structure and classification

The virions of poxviruses are membrane-bound structures with diverse morphology, though they are mostly brick- or oval-shaped. The internal structures of virions consist of a walled dumbbell-shaped core flanked by lateral bodies. The size of the prototypic poxvirus-vaccinia virus (VACV) virion is 360 x 270 x 250 nm (11). Furthermore, different forms of virion are produced that vary mainly in the outer membrane layers, including immature virion (IV), mature virion (MV), wrapped virion (WV), and extracellular enveloped virion (EV). IVs have a genome and core proteins encapsulated by a single membrane of crescent envelope. Further maturation of membrane proteins in IV form MV. When MVs acquire two extra membranes from the *trans*-golgi network, a WV with three membrane is formed. During egress, the outer membrane of the WV is fused with the outer plasma membrane to release two membraned EV (12).

Poxviridae members have a broad host range that includes butterflies, moths, birds, sheep, cattle, crocodile, rabbit, swine, and humans. Based on this host range, they are classified into two subfamilies: *Chordopoxvirinae* and *Entomopoxvirinae*. Subfamily *Chordopoxvirinae* contains members infecting vertebrate hosts and are divided into 11 genera (*Avipoxvirus*, *Capripoxvirus*, *Centapoxvirus*, *Cervidpoxvirus*, *Crocodylidpoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, *Suipoxvirus*, and *Yatapoxvirus*). Members of the subfamily *Entomopoxvirinae* infect insect hosts and are classified into three genera based on

virion morphology, genome size, and host range (*Alphaentomopoxvirus*, *Betaentomopoxvirus*, and *Gammaentomopoxvirus*). Among the different genera of poxviruses, only *Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, and Yatapoxvirus* genera can infect humans (12,13).

Among the *Poxviridae* members that can infect humans, *Orthopoxvirus* is the most widely studied. The infamous variola virus (VARV)—the causative agent of smallpox—and monkeypox virus (MPXV)—the causative agent of monkeypox—belong to the *Orthopoxvirus* genus. Molluscum contagiosum virus (MOCV), belonging to the *Molluscipoxvirus* genus, causes a human skin disease called molluscum contagiosum (12).

Poxviridae genome and gene expression

Poxviridae is a family of viruses that have a linear, non-segmented, double-stranded DNA genome with a unique feature of replicating in the host cell cytoplasm (12). The genome ranges from 130 to 375 kilobase pairs (kbp) in length with a hairpin loop at each terminus (14). To sustain cytosolic replication, transmission between hosts, and the evasion of the host immune system, approximately 150-300 proteins are encoded by the genomes of different poxviruses. They encode, among many others, enzymes responsible for viral DNA replication, transcription, mRNA modification-like capping, 2'O methylation, decapping, polyadenylation-, at least 15 proteins for entry (4 for binding and 11 for fusion), and a plethora of immunomodulatory enzymes alongside protein kinases and proteases (15). Viral DNA replication, post-replicative gene transcription, translation, and virion assembly occur in distinct perinuclear compartments referred to as viral factories (VFs). VFs are specialized cytoplasmic foci formed by the accumulation of viral DNA from actively replicating virus that arise from a single poxvirus virion (16).

During infection, poxvirus virions attach and fuse with the cell membrane to release their core into the host cell cytoplasm (17,18). After the release of the core, poxvirus gene expression occurs in a temporal cascade manner (19,20). Immediately following the release of the core into the cytoplasm, early genes are transcribed, processed in the core, and diffused outside in the cytoplasm for subsequent translation by host ribosomes (21). Early genes encode for DNA polymerase, RNA polymerase, intermediate transcription factors, growth factors, and many immunomodulatory proteins (12). After early gene expression, core uncoating occurs followed by DNA replication in perinuclear spaces called VFs (22). The daughter strand of DNA is then used for the transcription of post-replicative genes (intermediate and late genes) (23). The intermediate gene encodes for late transcription factors, whereas late genes primarily encode for structural proteins—along with other core enzymes and early transcription factors—to be packaged in the virion (12). Virion assembly begins by the formation of a crescent around a dense mass of core protein known as the viroplasm (24). Maturation of this crescent is accompanied by the encapsidation of the IV genome form. Further proteolysis, the addition of membrane proteins to IV, and movement of IV outside VFs form MVs (25,26). MVs are the most abundant particle produced during infection. Some MVs are wrapped by a *trans*-Golgi-derived membrane to form WVs (27), which travel to the cell surface via microtubules. The outer membrane of the WV fuses with the plasma membrane to release EV (12).

Diseases caused by poxvirus infection

Poxviruses cause diseases in humans and other animals. VARV and MOCV exclusively infect humans, as the only known natural reservoir for these viruses are humans. Many other poxviruses can also infect humans though infections occurring via the zoonotic route from infected animals—monkeypox being a notable one. Tanapox virus and yabapoxvirus also cause

infections in humans and are transmitted via insect vector. Orf virus, pseudocowpox virus, bovine popular stomatitis virus, and seal parapoxvirus also infect humans via direct contact with occupational hazards. Lumpy skin disease virus (LSDV), myxoma virus (MYXV), and goatpox virus (GTPV) are some prominent poxviruses causing fatal diseases in their respective animal hosts, subsequently causing economic losses to global agribusiness. Prominent poxvirus-derived human diseases are discussed in the following section in greater detail (12).

Smallpox

Smallpox, which is caused by VARV, is the deadliest infectious disease in human history, killing 300-500 million people in the 20th century alone. Smallpox was transmitted by contact with infected individuals or contaminated objects, as well as inhalation of an airborne aerosol containing virus. Smallpox was endemic worldwide and was described as early as the 4th century in China. The fatality rate of smallpox ranged from 10-40%. From ancient times, continuous efforts were made to cure or prevent smallpox. One common prophylactic measure, known as “variolation”, was practiced in China by subcutaneously or intranasally inoculating smallpox scab material to unexposed individuals (28), which prevented future lethal VARV infection.

In the late 17th century, English physician Edward Jenner observed that milkmaids who were exposed to cowpox virus (CPXV) during the milking process were protected from future VARV infection. This observation led him to conduct an experiment in 1796, where he inoculated material from the CPXV lesions of milkmaid to a boy susceptible to smallpox. After recovery from cowpox, the boy was challenged with VARV (29) and did not develop smallpox. This led to the discovery of a safe alternative process to variolation known as “vaccination”, with the cowpox material used for injections being the "vaccine”. The term comes from the Latin word *vaccinae* meaning "of or relating to cows”.

After Jenner's findings, vaccine material for smallpox vaccination was obtained from poxvirus lesions from milkmaids as well as animals such as sheep, horses, and cows. This practice provided different vaccine materials to be mixed and potentially increased mutations in virus during transfer in different hosts. It seems that during this time, VACV emerged as the vaccine material. The original source of VACV remains debated, though recent findings indicate that horsepox virus (HPXV) is the most likely candidate (30). While Jenner used CPXV as a vaccine, the VACV that emerged from the practice of maintaining vaccine material provides an equal—if not higher—degree of effective protection from smallpox.

In 1966, the World Health Organization (WHO) initiated a global vaccination campaign using VACV to eradicate smallpox. In 1980, the WHO announced the eradication of smallpox. Although smallpox is eradicated from nature, it still poses threats. In fact, in the early 21st century, a United States intelligence agency claimed that smallpox can potentially be used as a biological weapon (31). Presently, there remain two stocks of VARV in government laboratories in the USA and Russia (32). Unclaimed stocks of VARV have also been discovered in the past (33). Advancements in synthetic biology makes it possible for the *de novo* synthesis of VARV, as synthetic HPXV was recently generated from commercially synthesized DNA fragments (34). Hence, everyone must be vigilant about the possibility of smallpox re-emergence.

Molluscum contagiosum

Molluscum contagiosum is a cutaneous infection caused by MOCV, which causes painless round bumps on the skin and is mostly observed in children. In the US, there are 200,000 cases per year, with this infection being implicated in 1 in 500 outpatient visits per year. MOCV is distributed throughout the world. The transmission of MOCV occurs by sexual contact as well as direct or indirect contact via shared towels. MOCV reinfection in other areas of the skin occurs frequently due to autoinoculation by scratching the original lesion. Unlike other

poxviruses, MOCV have a stringent constraint on replication. The reason researchers are unable to determine the molecular basis of such blockage is the inability to grow MOCV in tissue culture and the lack of an animal model (12).

Monkeypox

Monkeypox is a zoonotic disease caused by MPXV. It is endemic in Central and Western Africa. MPXV infects rodents and sometimes non-human primates; however, there has been zoonotic transmission to humans through cuts during the handling of bush meat, the inhalation of contaminated aerosol, or bites from infected animals. The natural reservoirs of MPXV are rodents; however, they were first discovered in 1958 when colonies of monkey were infected and thus, named monkeypox virus. The appearance of monkeypox disease is similar to smallpox, as records of human-to-human transmission exist. Although monkeypox in less fatal (approximately ~ 10%) than smallpox, there is neither proven treatment nor approved vaccine for monkeypox. However, smallpox vaccination can reduce the risk of monkeypox infection in humans and other animals. Since 1970, there have been numerous outbreaks in 10 countries in Africa, the USA, Israel, and the UK. The Democratic Republic of Congo has reported >1000 cases of monkeypox annually since 2005, and, as per Nigeria, the outbreak remains uncontained. With outbreaks not being contained, the clinical similarity to smallpox, human-to-human transmission, and no proven treatment or vaccine, monkeypox represents a disease of grave concern since it can become an efficient human pathogen under favorable conditions (35,36).

Poxviruses as gene expression tools, vaccines vectors, and oncolytic agents

The majority of research on orthopoxviruses has been performed on VACV, which is a member of the orthopoxviruses and shares genetic and antigenic similarities with other orthopoxvirus members, and is the reason why VACV was effectively used as a live vaccine to eradicate smallpox. This feat of eradicating smallpox is considered one of the greatest

achievements of vaccinology. Long after smallpox eradication, VACV remains intensively studied due to its role as a vector in vaccine development and cancer treatment—making its study imperative for biomedical research. VACV continues to be used in the laboratory as a tool to understand the basic virological properties of the orthopoxviruses and cells. VACV is a convenient and safe alternative to the more pathogenic viruses (e.g. VARV and MPXV); therefore, it is the most commonly studied orthopoxvirus (12).

VACV may also be used in gene therapy, in the treatment of some cancers, and as a vector for rabies wildlife vaccines. Much of the work on oncolytic VACV development has focused on strains deficient for the viral thymidine kinase (TK) gene, many of which are further attenuated by the inactivation of additional virus genes, including viral growth factor (VGF) (37).

VACV has many advantages over other expression systems, including the ease of isolating recombinant viruses, the broad host range, the ability to take large amounts of DNA, a high rate of protein expression, and its relative safety (38,39). All of these features make VACV a great tool for understanding the basic biology of cells, vaccine vectors, cancer therapeutics, and gene therapy agents (38–40).

Poxvirus-induced Host shutoff

Viruses are obligatory intracellular parasites that require a host cell to replicate. In many virus-infected cells, the synthesis of host macromolecules is adversely affected, whereas viral macromolecules are synthesized unsurpassed. This leads to the accumulation of viral proteins concurrently with a decline in global host protein synthesis, a phenomenon termed “host shutoff”. To induce host shutoff, the virus targets the various steps of gene expression: DNA synthesis, transcription, mRNA processing, and translation (41).

Viruses have a limited number of genes to perform replication. However, they do not possess genes for energy production and protein synthesis. Hence, all viruses are obliged to rely on host cells to co-share produced energy and translation machinery. In other words, competition exists between viruses and hosts to use the limited amount of energy molecules and cellular translation machinery available. This situation instigates the virus to induce rapid and profound decline in global host protein synthesis. Viruses encode proteins to induce host shutoff by affecting various steps of gene expression. Studies on virus-induced host shutoff to date have indicated that viruses allocate relatively fewer proteins to inhibit DNA synthesis and transcription; conversely, the viruses encode an arsenal of proteins to target mRNA processing and translation. One of the most energy-consuming processes in the cell is mRNA translation. By usurping host mRNA translation, viruses can gain competitive advantage over available energy and translational machinery, thereby enhancing the expression of viral mRNA to protein (42).

During infection with a virus, host cells induce innate immune response as first line of defense, which then primes the adaptive immune response to clear any viral infection. Despite the host cell having an efficient, innate, and adaptive immunity-mediated antiviral response, viruses have evolved diverse strategies to impede, modulate, and evade such antiviral response. In addition to encoding multiple classes of immunomodulatory proteins, viruses also take advantage of host shutoff. The depletion of host proteins, including those involved in antiviral response, provides viruses with an additional strategy to counteract immune response. In addition to host shutoff and production of immunomodulatory proteins, poxviruses such as VACV have evolved strategies to diminish formation of pathogen associated molecular patterns (PAMPs) such as dsRNA and recognition of such PAMPs by host cell pattern recognition receptors (PRRs)

(43). The multiple strategies available to curb antiviral response allows for unchecked viral replication (41,44).

Host shutoff must occur within the time frame of the virus replication cycle—for many viruses, this could be merely a few hours. Within such short period of time, a virus uses a myriad of tricks to induce host shutoff and divert cellular resources towards the synthesis of viral macromolecules. During infection, poxviruses such as VACV modulate host cells in a multifaceted manner. Immediately following infection, VACVs modify cytoplasmic cytoskeleton by making cells circular. Furthermore, VACVs make host cells conducive for viral replication by inhibiting host cell cytopathic effect, altering cellular metabolism, and inducing global host protein synthesis shutoff. Furthermore, to induce host shutoff, VACV infections cease the synthesis of all three major macromolecules in the flow of genetic information from host DNA through RNA to proteins. Some of the strategies used by various viruses are discussed in the following section, with a focus on poxvirus.

Cellular DNA synthesis inhibition and the induction of host DNA degradation

DNA is the central genetic material that encodes information that proceeds to transcripts and proteins. Viruses have evolved means to modulate host DNA molecules. Some plant and animal viruses increase host DNA synthesis to induce tumorigenic growth that favors virus replication and spread. Members of the Geminivirus family, beet curly top virus (BCTV) and tomato golden mosaic virus (TGMV) induce host DNA synthesis to cause tumorigenic growth through the C4 and AL1 genes, respectively (45,46). Similarly, animal viruses have pleiotropic transforming proteins—E1A in adenovirus, E6/7 in papillomavirus, and large T antigen in simian virus—to prompt normal cells to transform into cancer cells (46–48). In contrast, many other classes of viruses decrease host DNA synthesis or degrade them to induce host shutoff and

reallocate cellular resources for viral DNA synthesis. Notable examples include the virion-associated protein of frog virus 3 (FV3), S1 gene of reovirus, ICP10 of herpes simplex virus type 2 (HSV-2), and a small segment at 3' end of vesicular stomatitis virus (VSV) genome inhibits host DNA synthesis via an unknown mechanism (49–52). Bacteriophage T4D-induced exo- and endodeoxynucleases degrade bacterial host DNA within 5 minutes post infection (54).

Poxvirus infection induces a rapid decrease in host cell DNA synthesis and an increase in DNA degradation. To decrease host cell DNA synthesis, VACV infection declines host nuclear DNA polymerase activity as early as 2 hours post infection. Studies measuring ^{14}C- or ^{3}H-labeled thymidine incorporation into host and viral DNA during VACV infection observed an immediate decrease in labeled thymidine incorporation in host DNA, whereas labeled thymidine incorporation in viral DNA increased until 3 hours post infection, corresponding to the time when viral DNA replication occurs (55,56). Heat-inactivated and UV-irradiated non-infectious VACV inhibited host DNA synthesis, indicating that protein(s) in the virion were responsible for the inhibition (56,57). Additionally, VACV encodes two DNases that hydrolyze single-stranded DNA. Exonuclease is active at pH 5 and endonuclease is active at pH 7.8, similar to the pH found in the nucleus, which might be a mechanism to selectively degrade host DNA present in nucleus (57–59). By negatively affecting the host DNA, which is a source of genetic information to synthesize host proteins, vaccinia virus induces host shutoff by operating at multiple levels.

Prevention of cellular RNA synthesis

RNA is transcribed by three different DNA-dependent RNA polymerase enzymes in eukaryotic cells. Transcription by RNA polymerase I (RNAPI) synthesizes ribosomal RNAs (rRNAs), while RNA polymerase II (RNAPII) yields messenger RNAs (mRNAs), and RNA polymerase III (RNAPIII) produces transfer RNAs (tRNAs), small non-coding RNAs (snRNAs),

micro RNAs (miRNAs), 5S RNA (5S), and small nucleolar RNAs (snoRNAs) (61,62). Prevention of cellular mRNA synthesis provides viruses with the advantage of accessing cellular translation machinery unchallenged. Hence, viruses use different proteins to target enzymes, RNAPII, that synthesize mRNAs. Foot-and-mouth disease virus (FMDV) encodes 3C proteases that cleave histone H3, thus affecting the regulatory domain necessary for transcription (63). Influenza A virus (IAV) uses multiple strategies to diminish host transcription. Early during infection, IAV inhibits RNAPII elongation followed by RNAPII degradation later during infection. IAV infection also induces stress that leads to the failure of RNAPII termination at the polyadenylation signal (PAS), which negatively affects host mRNA (61–63). Like IAV, herpes simplex virus type 1 (HSV-1) also induces the failure of RNAPII to terminate at PAS by an unknown mechanism (67). Additionally, HSV-1 infection decreases RNAPII occupancy in two-thirds of cellular genes (68). In fact, HSV-1 proteins ICP4 and ICP22 are known to negatively regulate RNAPII-mediated transcription initiation and elongation (66–68).

VACV infection inhibits host cell RNA synthesis by selectively inhibiting RNAPII, which functions to catalyze the transcription of DNA to synthesize pre-mRNA (72,73). In previous studies, the rate of RNA synthesis was determined using ^{3}H and ^{14}C-uridine uptake experiments. These experiments demonstrated that, compared to the uninfected condition, a 60% and 90% reduction in uridine uptake during VACV infection occurred at 3 and 9 hours post infection, respectively (57,73). Unlike the inhibition of DNA polymerase activity, infectious VACV and the expression of early viral genes are required to inhibit RNAPII activity. Additionally, VACV infection induces the global degradation of host and viral mRNA, subsequently decreasing the half-life of host/viral mRNA during infection. However, the

inhibition of RNA synthesis was shown to be independent of RNA degradation (73), though the mechanism remains elusive.

Targeting various mRNA processing mechanisms

mRNA that codes for protein are synthesized as pre-mRNA. Pre-mRNA synthesized during transcription undergo processing to form mature mRNA. Viruses encode proteins that block each of the steps involved in mRNA maturation, including mRNA processing-capping, polyadenylation or splicing, and the export of mRNA from the nucleus to cytoplasm. Viruses most frequently induces host mRNA degradation.

Capping: During transcription, when the length of mRNA reaches 25-30 nucleotides, 7-methylguanosine cap (m^7G) is added to the 5′ end of the transcript, which protects the mRNA from 5'-3' exoribonucleases such as Xrn1. Many viruses target the removal of the 5' cap to induce mRNA degradation. Influenza virus cleaves 5' caps and uses the cleaved oligonucleotide as a primer for its own transcription through a mechanism known as "cap snatching" (74). To perform cap snatching, polymerase basic protein 2 (PB-2) binds to host mRNA cap through an extensive conformation change, followed by the cleavage of cap from the host mRNA by the endonuclease in polymerase acidic protein (PA). PB-1 uses the cleaved cap as a primer for viral mRNA transcription (75). Hantavirus also performs cap snatching using its N protein, which binds to cellular mRNA cap and rescues capped mRNA fragments from P bodies post-mRNA degradation. The capped mRNA fragment is further processed by viral RNA-dependent RNA polymerase to be used as a primer for viral mRNA synthesis (76). Recently, African swine fever virus (ASFV) was shown to encode a decapping protein, ASFV-DP, which removes the m^7G cap from both host and viral mRNAs (77).

To take advantage of cellular cap-dependent translation initiation mode, VACV mRNAs have a 7-methylguanosine (m^7G) cap at the 5' end. VACV mRNAs capping is carried out in three reactions performed by viral enzymes. In this process, the newly synthesized transcript with a 5' triphosphate is cleaved to a diphosphate by RNA triphosphatase. Guanosine monophosphate (GMP) is then added by RNA guanylyl-transferase, and subsequently methylated at N7 position by RNA (guanine-N7) methyltransferase. VACV encodes the heterodimeric capping enzyme complex of D1/D12 protein. D1 functions as RNA triphosphatase and RNA guanylyltransferase, whereas D12 performs RNA (guanine-N7) methyltransferase activities (12). Capped viral transcripts promptly compete for the limited translational machinery and energy in the host cell. Additionally, VACV encodes VP39 protein (J3R), which adds a methyl group at the 2'-O position of the first transcribed nucleotide (cap 1) adjacent to the 5' cap (cap 0). Such modification is present in eukaryotic mRNA, but is usually absent in viruses (78). The host's innate immune system recognizes hypomethylated viral RNA by interferon-induced proteins with tetratricopeptide repeats 1 (IFIT-1) to distinguish between self and non-self mRNA, thereby preventing non-self mRNA translation (79). By methylation at the 2'-O position of cap 1, VACV evades host immune response and boosts its mRNA translation (80). Another strategy that VACV uses is to completely remove the 5' cap or decap mRNA by two proteins (D9 and D10) expressed at the early and post-replicative periods of the viral replication cycle, respectively (81,82). The decapped mRNAs are then degraded by 5'-3' exonucleases such as Xrn1 (83,84). The decapping process leading to mRNA degradation is discussed further in mRNA degradation section.

Splicing: The pre-mRNA contains coding exons and non-coding introns. During the maturation of pre-mRNA, the introns are removed and exons are joined through a process called

splicing. Viruses inhibit host gene expression by targeting splicing. HSV-2 encodes ICP27 protein, which binds directly to pre-mRNA and prevents splicing (85). Unlike HSV-2, human immunodeficiency virus 1 (HIV-1) Vpr protein binds to spliceosome protein SAP145 to inhibit host pre-mRNA splicing (86). Blocking splicing will lead to mRNA with a premature stop codon or the disruption of the open reading frame, resulting in the production of aberrant proteins that are promptly degraded.

VACV has been evolutionarily optimized to lack gene-containing introns; hence, splicing is dispensable for VACV. However, VACV targets host-splicing mechanism to induce host shutoff. The serine (S)/arginine (R)-rich protein (SR protein) family are an important protein family required for spliceosome assembly (87). SR proteins are hyper-phosphorylated at a serine residue that is required for its function (88). Like adenoviruses, VACVs can inactivate SR protein, thereby controlling the host cell's RNA splicing machinery. VACV hypo-phosphorylates SR protein, mediated by VACV phosphatase H1, to inhibit its function in splicing (89). A recent study demonstrated that CPXV infection significantly upregulates the proteins of RNA splicing at 2 hpi; however, during the course of infection (6 hpi and 10 hpi) many splicing factors are downregulated (90). Since splicing is not required for VACV, it can inhibit cellular splicing machinery to induce host shutoff without negatively affecting viral growth.

Polyadenylation: Polyadenylation is the process of adding a stretch of adenine bases at the 3’ end of mRNA. It is an essential process for mRNA maturation that is tightly coupled with transcription termination. Polyadenylation protects mRNA from 3’-5’ exoribonucleases and assists in the optimal translation of mRNAs (91). Viruses have evolved methods to target the polyadenylation process to affect mRNA stability. Nonstructural protein 1 (NS1a) of IAV restricts the polyadenylation of nascent host mRNA by interacting with cleavage-

polyadenylation specificity factor 30 (CPSF30) (92). CPSF are responsible for recognizing polyadenylation signal in pre-mRNA and cleave the pre-mRNA, followed by the polyadenylation of upstream cleaved product (93). In contrast to IAV's strategy to block polyadenylation, Kaposi's sarcoma-associated herpesvirus (KSHV) hyper-adenylates host transcripts through SOX protein, which also leads to decreased transcript stability (94,95).

To add a poly(A) tail at the 3' end of viral mRNA, VACV encodes the heterodimeric poly(A) polymerase complex of VP55/VP39 proteins early during infection. VP55 (E1L) protein functions as a catalytic component, whereas VP39 (J3R) protein acts as a processivity factor. During VACV infection, apart from catalyzing polyadenylation in viral mRNA VACV poly(A), polymerase polyadenylates cellular RNAs such as tRNA, small nuclear RNA (snRNA), and mRNA to form non-translating polyadenylated short sequences (POLADS) (96). In this cell-free protein synthesizing system, the addition of POLADS inhibits cellular mRNA synthesis by up to 70%, while viral mRNA had minimal inhibition (94–96). The selective host protein synthesis inhibitory property of the POLADS is due to the poly(A) tail, as the increased length of the poly(A) tail in POLADS corresponds to increased inhibitory activity (96,98). Moreover, the addition of PABP reversed host cell mRNA translation inhibition by POLADS, suggesting that PABP becomes the limiting factor for host cell mRNA translation during VACV infection (96,98,99). Another interesting strategy that VACV uses to thwart the negative regulator for viral replication caused by host microRNA (miRNA) involves viral poly(A) polymerase (100). During VACV infection, VP55 protein becomes essential and adequate to polyadenylate host miRNA, thereby leading to its degradation and relieving its negative regulation on viral replication (100).

mRNA export: mRNA translation occurs in the cytoplasm, thus the transcribed mRNA must be exported from the nucleus. Viruses target the export of host mRNAs by disrupting the

nuclear-cytoplasmic transport machinery. Rhinovirus 2A and 3C protease cleaves vital constituents of nucleocytoplasmic mRNA export machinery (101). IAV protein NS1 and VSV matrix protein downregulate and competitively bind Nup98 protein, respectively, which is required for mRNA export (102,103). Additionally, IAV's NS1 protein interacts with and sequesters key mRNA export machinery proteins (p15, E1B-AP5, Rae1, and NXF1) to form an inhibitory complex, thereby inducing mRNA export blockage (102). During poxvirus infection, no known inhibition of nuclear-cytoplasmic transport machinery has been reported to date.

mRNA degradation: A common feature of many viruses is that they encode exoribonucleases and endoribonucleases to cleave host mRNA. Severe acute respiratory syndrome-Coronavirus (SARS-CoV) NSP1 protein, IAV PA-X protein, HSV virion host shutoff (VHS) protein, Epstein-Barr virus (EBV) BGLF5 protein, and KSHV SOX protein have exo- or endoribonuclease activities and decrease global host protein synthesis by decimating host mRNAs (101–105).

VACV infection promptly induces host cellular RNA degradation. Cellular housekeeping genes for mRNA, such as ß-actin and α-tubulin mRNA, are progressively degraded over time during VACV infection, and they are almost entirely degraded by 10 hours post infection (109). Earlier studies postulated that RNA degradation during VACV infection could occur due to the following reasons: 1) Synthesis of viral RNases; 2) Rapid turnover of mRNA due to re-compartmentalization of mRNA in morphologically altered infected cells; 3) Interferon-induced 2′5′-oligoadenylate synthetase (OAS) through the activation of endonuclease RNase L, which does not discriminate between host and viral mRNA. Although the aforementioned postulates could be true, subsequent findings have demonstrated that VACV encodes two enzymes (D9R and D10R) with the Nudix hydrolases motif (81,82). Enzymes with the Nudix motif hydrolyze a

nucleoside diphosphate linked to any moiety X. Initial reports demonstrated that D9R and D10R negatively regulated gene expression independent of the promoter used. However, the expression of a gene under an encephalomyocarditis (EMC) virus leader sequence that underwent 5'-cap-independent translation using IRES was not negatively affected by D9R and D10R overexpression (110). These significant findings demonstrated that D9R and D10R degraded the transcripts that are capped (m^7GpppN) at the 5' end—a feature of both VACV and host mRNAs. In fact, by cleaving the 5'-cap, the Nudix motif of D9R and D10R presumably renders the mRNA for degradation by 5'-3' exonuclease Xrn1 (84). The importance of D9R and D10R is further accentuated by the fact that a homolog of D9R is found in all chordopoxviruses, whereas the D10R homolog is conserved in all known poxviruses (15). A recent study demonstrated that the depletion of cellular mRNA is a major contributor to VACV-induced host protein synthesis shutoff (111). Degradation of host mRNA leading to host shutoff is vital for VACV replication, as it not only usurps host innate immune response, but also reallocates the translation machinery to viral mRNAs.

Usurping host mRNA translation

mRNA translation is the most energy-consuming process in the cell. Cellular mRNA translation has three major steps: initiation, elongation, and termination. During initiation, eukaryotic initiation factor 2 (eIF2) forms a ternary complex (TC) with initiator-methionine tRNA (Met-tRNAi) and GTP. Consequently, TC binds to 40S ribosome in complex with eIF1, eIF3, and eIF5 to form the 43S pre-initiation complex. A rate-limiting translation initiation step is then followed by the recruitment of a hetero-trimeric complex called eIF4F on mRNA. eIF4F consists of m^7G cap-binding protein eIF4E, RNA helicase eIF4A, and scaffold protein eIF4G. eIF4E binding protein 1 (4EBP1) is a translation repressor that limits the availability of eIF4E to

form the eIF4F complex (112). Hyperphosphorylated (4 sites) 4EBP1 releases eIF4E for eIF4F complex formation. Once formed, the eIF4F complex binds m^7G cap, and the scaffold protein eIF4G interacts with the poly(A) binding protein (PABP) bound to 3' poly(A) tail to promote transient 5'-3' communication known as the closed loop model. Subsequently, by binding to the eIF3 complex, eIF4G helps recruit 43S pre-initiation complex on the mRNA to form 48S pre-initiation complex. The 48S pre-initiation complex then scans the mRNA in 5'→ 3' direction until it reaches the start codon, usually AUG. Once the start codon is recognized, eIF5B mediates the hydrolysis of eIF2-bound GTP. This change prompts the joining of the 60S ribosome to 48S pre-initiation complex to form an elongation-competent 80S complex (113). This sophisticated process is followed by translation elongation, termination, and ribosome recycling.

Since translation initiation is the rate-limiting step of translation, many viruses target different proteins involved in this process. The various proteins involved in translation initiation that are targeted by viruses are discussed in greater detail in the following paragraphs.

eIF4F: The multi-subunit complex eIF4F is one of the primary targets for viruses to seize host translation. Viruses belonging to different families target all three proteins in the eIF4F complex. FMDV protease 3C cleaves both eIF4As to block host translation (114). HSV-1 VHS protein binds to eIF4A—a RNA helicase—to gain close proximity to cleave and degrade host mRNAs (115), while various viral proteases target eIF4G to inhibit host translation. Proteases such as enterovirus (EV) 2A protease, HIV-1 protease, FMDV leader protease, and feline calicivirus 3C protease cleave eIF4G (113–116). Viral proteins such as rhinovirus 2A and rotavirus NSP3 bind eIF4G and displace PABP to prevent the closed loop conformation required for translation in many host mRNAs (120,121). Cap-binding protein eIF4E is a common target for many viruses to decline host translation. Adenovirus shutoff protein 100 K, tobacco mosaic

virus (TMV) VPg protein, *Cripavirus*, and influenza virus by unidentified proteins bind and recruit eIF4E to viral mRNA (119–122). Enteroviruses (EV) use a distinctive approach to target eIF4E, where virus-induced miR-141 suppresses eIF4E mRNA translation (126). Many viruses also target the phosphorylation state of translation repressor protein 4EBP1. Hyperphosphorylated 4EBP1 then releases eIF4E to mediate translation initiation. SV40 Small T Ag carries out the PP2A-dependent dephosphorylation of 4EBP1, whereas VSV M protein and reovirus p17 dephosphorylate 4EBP1 by inactivating Akt-mTOR (124–126). In doing so, viruses induce host shutoff since the majority of host mRNAs depend on eIF4F complex-reliant cap-dependent translation.

eIF1, eIF2, eIF3, and eIF5 multifactor complex: The multifactor initiation complex is formed when the N-terminus of eIF3 interacts concurrently with eIF5 and eIF1, and is further augmented by the interaction of eIF5 with the N-terminus of eIF2β (130,131). Viruses are known to alter the multifactor initiation complex function to induce host shutoff. Alphaviruses (Sindbis and Semliki Forest virus) induce the phosphorylation of eIF2α to block global host translation (132). eIF3 is multiprotein complex composed of 13 different proteins, and viruses bind to different proteins of eIF3 to decimate host mRNA translation. Measles N protein binds eIF3g, whereas rabies M protein binds eIF3h to negatively affect host translation (133,134). Similarly, SARS-CoV and infectious bronchitis virus (IBV) spike protein binds eIF3f to impair host mRNA translation (135). Enteroviruses encode 3C proteases cleave eIF5B, thus impairing translation by preventing eIF5B from interacting with eIF1A and the ribosome to accurately position met-tRNA on the start codon of an mRNA (136). Some viruses, such as FMDV, use multiple modes to induce host shutoff. In addition to cleaving eIF4A and eIF4G, FMDV infection induces the cleavage of eIF3a, eIF3b, and PABP (114).

***PABP*:** PABP binds to the 3' poly(A) tail of an mRNA to enhance RNA stability, and this interaction is vital for translation initiation complex formation in the cytoplasm (137). Many viruses target PABP to induce host shutoff. Norovirus and enterovirus protease 3C cleave PABP to inhibit its function (138,139). HIV-1 protease, Rubella capsid protein, and influenza NS1 bind PABP and suppress translation (136–138). Rotavirus NSP3 displaces PABP from eIF4G and interacts with RoXaN to cause nuclear accumulation of PABP (121,143). Similarly, the SOX and K10 proteins of KSHV and HSV-1 UL47 protein bind PABP to cause nuclear accumulation and prevent its function in the cytoplasm (144,145).

VACV uses distinct tactics to modulate the extremely synchronized and high energy consuming protein synthesis mechanism from cellular mRNAs. During VACV infection, prompt and selective inhibition of cellular protein synthesis occurs. Earlier findings have demonstrated that VACV infection results in the inhibition of protein synthesis via a surface tubular element (STE) displayed on the VACV membrane. However, VACV STE did not affect either cellular RNA or DNA synthesis. When the authors exposed cells to purified STE, a decrease in the polyribosome occurred, accompanied by an increase in the free ribosome pool. Although these observations suggest that STE negatively affects translation initiation, further studies are necessary to determine the exact mechanism (146).

Another very remarkable factor present in virions that is implicated in inducing host shutoff is phosphorylated 11 kDa protein (147,148), which is encoded by F17R in Copenhagen strain (F18R in WR strain) (145–148). Purified 11 kDa/F17 protein from VACV virion and cell-free extract from VACV-infected cells prevents methionyl-tRNA_fMet-40S initiation complex formation to induce host shutoff (147,148). The importance of this small protein is further augmented by the fact that preventing expression of F17 protein interrupts VACV

morphogenesis (149). It was recently demonstrated that F17 sequestering Rictor and Raptor dysregulates mTOR to counter antiviral response while retaining mTOR-mediated enhancement of viral protein synthesis (152). These findings indicate that F17 has multiple roles, from inducing host shutoff and countering antiviral response to enhancing viral protein synthesis during infection.

Among the many approaches used to usurp host mRNA translation, VACV expresses protein 169 early during infection, and is confined in the host cell cytoplasm. In the cytoplasm, protein 169 impairs host protein synthesis, thereby facilitating the inhibition of host antiviral responses. Unlike other factors that induce host shutoff, protein 169 targets translation initiation by affecting both cap-dependent and cap-independent mechanisms. Although protein 169 is not vital to VACV replication and spread, it is required to regulate virulence, as VACV lacking protein 169 causes severe infections that induce stronger immune responses and are thus promptly cleared. By inducing host protein synthesis shutoff, VACV protein 169 suppresses host antiviral response and hence regulates virulence (153).

Apart from these general mechanisms to induce host shutoff, VACV also targets some specific translation initiation factors. Translation initiation is thought to be the rate-limiting step during mRNA translation, as it is highly regulated via various translation initiation factors. VACV modulates translation initiation factors such as eIF4E, eIF4G that are part of the cap-binding complex eIF4F. VACV infection phosphorylates eIF4E, though the broader implications of such modification are not well studied to date (154). However, it is well established in eukaryotes that higher eIF4E phosphorylation is associated with increased translation (151–154). Conversely, other studies have suggested that non-phosphorylated eIF4E have a higher affinity for a 5' cap in an mRNA than the phosphorylated eIF4E counterpart (159). Furthermore,

phosphorylated eIF4E is not mandatory for translation initiation (160), and cap-dependent translation is inhibited by phosphorylated eIF4E (161). If any of the latter situation is true during VACV infection, phosphorylation of eIF4E may be one of the mechanisms used to subvert the cap-dependent translation of host mRNA.

VACV also sequesters eIF4E and eIF4G into foci near the virus factory, suggesting that the initiation factors are re-localized to enhance VACV mRNA translation (16,162). Recently, antiviral granules (AVGs) and RNA granules were found adjacent to and within viral factories (163,164). In fact, AVGs contain translation initiation proteins (eIF3h, eIF4E, and PABP), leading to speculation that such redistribution of translation initiation factors could be a mechanism to limit the availability of these factors for host mRNA translation (163).

As discussed earlier, VACV poly(A) polymerase induced POLADS sequester PABP in order to make them inaccessible for host mRNA translation to induce host shutoff. The different strategies used by VACV to induce host shutoff is summarized in **Fig 1-1**.

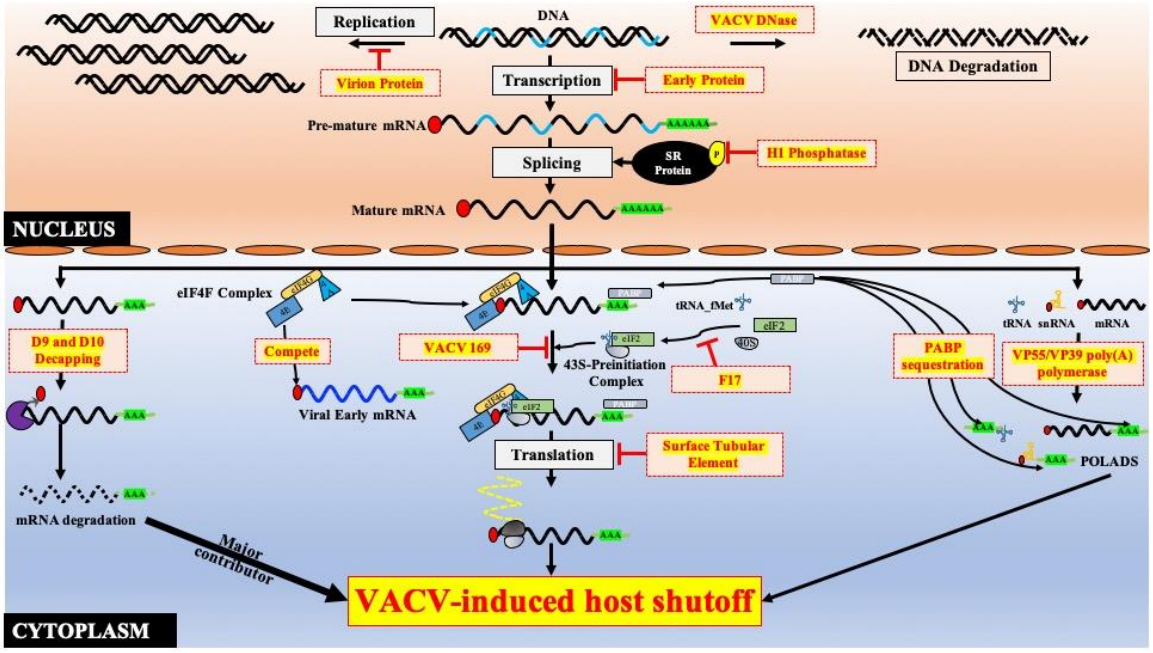

Figure 1-1: Summary of VACV-induced host protein synthesis shutoff

VACV targets and ceases synthesis of all three major macromolecules in central dogma of genetics, i.e. DNA, RNA and Proteins. To induce host shutoff, VACV inhibits DNA replication (Virion proteins), degrades host DNA (VACV DNases), inhibits transcription (Early protein/s) and splicing (H1 Phosphatase). Further, VACV hinders 43S preinitiation complex formation (F17), decreases polysome bound mRNAs (Surface Tubular Element) and inhibits both cap-dependent and cap-independent mode of translation (VACV 169). Additionally, VP55/VP39 heterodimeric poly(A) polymerase adds poly(A) tail to tRNA, snRNA and mRNAs to form POLADS. These POLADS sequesters PABP to negatively affect translation. Remarkably, the major contributor to host shutoff is host mRNA degradation that is carried out by sequential decapping by two viral decapping enzymes D9 and D10 followed by degradation by cellular Xrn1 5'-3' exonuclease. Using all these multiple strategies, VACV induces host shutoff.

Selective Protein Synthesis during Virus-induced Host Shutoff

Virus-induced global host shutoff is beneficial for viruses, as it helps evade host antiviral immune response. Additionally, shutoff also leads to the reapportion of cellular machinery and critical host processes to confer a replication advantage for the virus. Although shutoff provides advantages to the virus in regard to immune evasion and the reallocation of cellular resources, the selective synthesis of crucial cellular proteins and viral proteins is necessary to sustain virus replication.

Strategies employed by poxvirus to synthesize proteins during host shutoff

Many viruses have evolved various strategies to boost viral protein synthesis during host shutoff. Most viruses have acquired critical elements in mRNA to induce selective viral protein synthesis. A common element in viral mRNA is the internal ribosome entry site (IRES). Notable viruses that use IRES to mediate translation initiation via initiation factor independent mode include SARS, HCV, CSFV, HIV, and CrPV (165). Influenza B virus induces combined translation of both M1 and BM2 protein by the base pairing of mRNA with 18S ribosomal RNA to promote the re-initiation of translation (166). Adenovirus uses ribosome shunting to enhance viral mRNA translation, where the tripartite leader in the non-coding region of viral late mRNAs exhibits high complementarity with 18S rRNA, which promotes the ribosome shunting mechanism (167). Viruses are notorious for recruiting translation initiation factors to their mRNA to promote viral mRNA translation. Calicivirus VPg protein binds and recruits eiF3 and eIF4E to viral mRNA (168,169) and HSV-11 ICP27, while UL47 protein binds PABP (145). As such, this recruitment of initiation factor to viral mRNA stimulates its translation. During the replication cycle, viruses produce dsRNA that is sensed by innate immune molecule PKR, leading to self and downstream eIF2α phosphorylation (170). When eIF2α is phosphorylated,

global inhibition of translation initiation occurs (171). To avert this situation, viruses use various strategies to hinder PKR-mediated eIF2α-phosphorylation. Influenza NS1 HCMV's two related proteins (TRS1 and IRS1) sequester dsRNA and prevent PKR phosphorylation (172,173). HSV-1 US11, EBV SM, and KSHV ORF57 directly sequester PKR to thwart PKR activation (174,175). HPV E6 and HSV ICP34.5 protein regulate eif2α phosphatase to dephosphorylate eiF2α (176,177).

To mediate selective translation during poxvirus infection, VACV modulates many proteins that regulate translation initiation. VACV possesses cis-acting sequences and trans-acting factors that may regulate selective viral mRNA translation. Selectively synthesized proteins and the evolutionarily optimized strategy used during VACV-induced host shutoff are discussed below.

VACV's strategies to modulate translation initiation factor

eIF4F Complex:

The eIF4F complex is composed of a cap-binding subunit (eIF4E), an RNA helicase (eIF4A), and a scaffold protein (eIF4G). Among these proteins, the activity of eIF4E is highly regulated by many factors that converge into two major signaling pathways: PI3K/AKT/mTOR and MAPK/ERK (178,179).

eIF4E is repressed by hypo-phosphorylated eIF4E-binding proteins (4E-BPs) (180). Activation of PI3K/AKT/mTOR pathway leads to hyperphosphorylation of 4E-BP by mTORC1 triggering the release of eIF4E and thereby, increasing the pool of eIF4E available for translation initiation (181,182). At the early time during infection, VACV induces surface β1-mediated PI3K activation leading to hyperphosphorylation of 4E-BP1 and subsequent release of cap-binding protein eIF4E (183,184). Hyperphosphorylated 4E-BP1 is consequently degraded by

proteasome during VACV infection (162). Activation of PI3K augments the formation of eIF4F complex enhancing VACV protein synthesis (184). Another poxvirus, Myxoma virus activates AKT by host range protein MT-5 (185,186). However, the role of AKT activation on a translation during MYXV infection is not studied yet.

A second signaling pathway that regulates eIF4E is MAPK/ERK pathway. Activation of the MAPK/ERK pathway phosphorylates and activates MNK1/2. Activated MNK1/2, in turn, phosphorylates eIF4E at the Serine 209 residue (178,187), leading to an increase in translation initiation (151–154). VACV infection activates MAPK/ERK pathway stimulating phosphorylation of eIF4E by MNK1 (162). VACV protein synthesis was severely affected when cells were treated with MNK inhibitor CGP57380 and in MNK1 knocked out MEF cell line suggesting the importance of eIF4E phosphorylation by MNK1 (162,188). The previous report showed that 40S-bound eIF3e (an indicator of 43S pre-initiation complex formation) is prerequisite for recruitment of eIF4E kinase MNK1 to eIF4G during VACV infection (154). Interestingly, phosphorylated eIF4E has lower affinity for 5' cap (159), and it is predicted that eIF4E phosphorylation occurs after 43S pre-initiation complex formation to promote the release of the eIF4F complex to initiate ribosome scanning (189). As discussed earlier, phosphorylation of eIF4E could have negative regulation in host translation initiation leading to dual purpose during VACV infection.

eIF1, eIF2, eIF3 and eIF5 multifactor complex:

VACV infection generates a huge amount of double-stranded RNA (dsRNA) due to the overlapping nature of viral transcripts. dsRNA is sensed by the host anti-viral defense mechanism through the N-terminal domain of protein kinase R (PKR). Upon dsRNA binding, PKR is activated by autophosphorylation reaction followed by phosphorylation of eIF2α leading

to global inhibition of translation in infected cells (190). VACV has developed multiple strategies to evade PKR activation. One of the strategies used by poxvirus to prevent PKR mediated eIF2α phosphorylation is to encode pseudosubstrates of PKR such as K3L in VACV, M156R in MYXV, C8L in swine poxvirus (187–189). These pseudosubstrates isolate PKR to prevent activation of downstream effector-phosphorylated eIF2α. Another approach poxvirus uses to deter, or delay PKR activation is by producing dsRNA binding protein, E3L in VACV and M029 in MYXV, that sequester dsRNA to make it inaccessible to PKR (194,195). Despite the presence of PKR antagonists, poxviruses also use different strategies to minimize the production of dsRNA. VACV D9 and D10 are decapping enzymes that keep the amount of virus-induced dsRNA at a minimum during infection, preventing the activation of PKR and 2'5'-oligoadenylate synthetase (OAS)/RNase L-associated RNA decay that inhibits viral protein synthesis (83,84). VACV also takes advantage of the cellular 5'-3' mRNA exonuclease Xrn1 to reduce the number of potential PKR and RNase L substrates (83,84).

Selectively translated cellular proteins

During VACV induced host shutoff, mRNA degradation is the major contributor; however, a small fraction of host transcripts are not affected. One study showed that interleukin 6 signal transducer (IL-6ST) and Apurinic/apyrimidinic endonuclease 2 (APEX2) mRNA are constant, whereas, solute carrier family 4-member 3 (SLC4A3) mRNA abundance is marginally decreased (196). In contrast, pericentrin (PCNT2) and Wiskott-Aldrich syndrome protein (WASP) mRNA are upregulated (196). Later it was discovered that WASP is important for VACV infection in the host cell (197). However, the mechanism by which host factors such as WASP are selectively upregulated remains to be investigated.

Recent global, simultaneous RNA-seq and Ribosome profiling, analysis showed that, during VACV infection, mRNA encoding proteins for oxidative phosphorylation (OXPHOS) are translationally upregulated (111). OXPHOS are responsible for ATP production, and elevated ATP level is crucial for VACV infection (111,198,199). Previously, it was shown that VACV increases explicitly nicotinamide adenine dinucleotide dehydrogenase 4 (ND4) and cyclooxygenase-2 (COX2) protein levels, two mitochondrial proteins that function in the electron transport chain to generate ATP (199). However, the mechanism on how the synthesis of these mitochondrial proteins is regulated also remains to be elucidated. We have shown that synthesis of protein of members of oxidative phosphorylation is selectively upregulated during VACV infection. The short 5′ untranslated region with the less complex secondary structure of the oxidative phosphorylation mRNAs contributed to the translational upregulation (111). Additionally, mRNA encoding proteins for ribosomal subunits were also significantly enriched at 2 hpi, but higher TE was not significantly different at later times. These findings are discussed in detail in Chapter 5 of this dissertation.

VACV mRNA translation

All the viruses inducing the shutoff of translation can continue to translate at least part of their mRNAs using non-canonical translation such as IRES, Ribosome shunting, CITE or VPG initiation. In many instances, the expression of viral proteins seems to occur through cap-independent mechanisms. The 5’-poly(A) leader at the 5' end of the 5' UTR is the feature of all VACV post-replicative mRNAs. 5’-poly(A) leader conferred a selective translational advantage to post-replicative mRNA in poxvirus-infected cells (200,201). Post-translational modification of small ribosomal protein RACK1 by VACV kinase B1 is necessary for poly(A) leader mediated translational advantage (200). These poly(A)-headed mRNAs can be efficiently translated in

cells with impaired cap-dependent translation. Moreover, the 5’-poly(A) leader is not an IRES (201). Similar 5' leader sequence, Omega prime, found in tobacco mosaic virus (TMV) also enhances translation (202,203). Unlike VACV 5'-poly(A) leader, TMV omega prime enhances translation by promoting recruitment of eIF4F complex (201,204). The result of 5'-poly(A) leader mediated translational advantage during poxvirus infection is discussed in detail in Chapter 3 of this dissertation. We very recently, determined a cellular protein LARP4 is essential for poly(A) leader translation, and these results are discussed in detail in Chapter 4 of this dissertation.

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Chapter 2 - *In-Vitro* Transcribed RNA-based Luciferase Reporter Assay to Study Translation Regulation in Poxvirus-Infected Cells

Pragyesh Dhungel[1], Fernando Cantu[1], Candy Hernandez[1], Zhilong Yang[1]

1. Division of Biology, Kansas State University, Manhattan, KS, USA

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Summary

We present a protocol to study mRNA translation regulation in poxvirus-infected cells using In-Vitro Transcribed RNA-based luciferase reporter assay. The assay can be used to study translation regulated by cis-elements of an mRNA, including 5'-untranslated region (UTR) and 3'-UTR. Different translation initiation modes can also be examined using this method.

Abstract

Every poxvirus mRNA transcribed after viral DNA replication has an evolutionarily conserved, non-templated 5'-poly(A) leader in the 5'-UTR. To dissect the role of 5'-poly(A) leader in mRNA translation during poxvirus infection we developed an in-vitro transcribed RNA-based luciferase reporter assay. This reporter assay comprises four core steps: (1) PCR to amplify the DNA template for in-vitro transcription; (2) In-vitro transcription to generate mRNA using T7 RNA polymerase; (3) Transfection to introduce in-vitro transcribed mRNA into cells; (4) Detection of luciferase activity as the indicator of translation. The RNA-based luciferase reporter assay described here circumvents issues of plasmid replication in poxvirus-infected cells and cryptic transcription from the plasmid. This protocol can be used to determine translation regulation by cis-elements in an mRNA including 5'-UTR and 3'-UTR in systems other than poxvirus-infected cells. Moreover, different modes of translation initiation like cap-dependent, cap-independent, re-initiation, and internal initiation can be investigated using this method.

Introduction

According to the central dogma, genetic information flows from DNA to RNA and then finally to protein (1,2). This flow of genetic information is highly regulated at many levels including mRNA translation (3,4). Development of reporter assays to measure regulation of gene expression will facilitate understanding of regulatory mechanisms involved in this process. Here we describe a protocol to study mRNA translation using an in-vitro transcribed RNA-based luciferase reporter assay in poxvirus-infected cells.

Poxviruses comprise many highly dangerous human and animal pathogens (5). Like all other viruses, poxviruses exclusively rely on host cell machinery for protein synthesis (6–8). To efficiently synthesize viral proteins, viruses evolved many strategies to hijack cellular translational machinery to redirect it for translation of viral mRNAs (7,8). One commonly employed mechanism by viruses is to use cis-acting elements in their transcripts. Notable examples include Internal Ribosome Entry Site (IRES) and cap-independent translation enhancer (CITE) (9–11). These cis-elements render the viral transcripts a translational advantage by attracting translational machinery via diverse mechanisms (12–14). Over 100 poxvirus mRNAs have an evolutionarily conserved cis-acting element in the 5'-untranslated region (5'-UTR): a 5'-poly(A) leader at the very 5' ends of these mRNAs (15,16). The lengths of these 5'-poly(A) leaders are heterogeneous and are generated by slippage of the poxvirus-encoded RNA polymerase during transcription (17,18). We, and others, recently discovered that the 5'-poly(A) leader confers a translation advantage to an mRNA in cells infected with vaccinia virus (VACV), the prototypic member of poxviruses (19,20).

The in-vitro transcribed RNA-based luciferase reporter assay was initially developed to understand the role of 5'-poly(A) leader in mRNA translation during poxvirus infection (19,21).

Although plasmid DNA-based luciferase reporter assays have been widely used, there are several drawbacks that will complicate the result interpretation in poxvirus-infected cells. First, plasmids are able to replicate in VACV-infected cells (22). Second, cryptic transcription often occurs from plasmid DNA (18,23,24). Third, VACV promoter-driven transcription generates poly(A)-leader of heterogeneous lengths consequently making it difficult to control the poly(A)-leader length in some experiments (18). An in-vitro transcribed RNA-based luciferase reporter assay circumvents these issues and the data interpretation is straightforward.

There are four key steps in this method: (1) polymerase chain reaction (PCR) to generate the DNA template for in-vitro transcription; (2) in-vitro transcription to generate mRNA; (3) transfection to deliver mRNA into cells; and (4) detection of luciferase activity as indicator of translation (**Figure 2-1**). The resulting PCR amplicon contains the following elements in 5' to 3' direction: T7-Promoter, poly(A) leader or desired 5'-UTR sequence, firefly luciferase open reading frame (ORF) followed by a poly(A) tail. PCR amplicon is used as the template to synthesize mRNA by in-vitro transcription using T7 polymerase. During in-vitro transcription, m7G cap or other cap analog is incorporated in newly synthesized mRNA. The capped transcripts are transfected into uninfected or VACV-infected cells. Cell lysate is collected at desired time after transfection to measure luciferase activities that indicate protein production from transfected mRNA. This reporter assay can be used to study translation regulation by cis-element present in 5'-UTR, 3'-UTR or other regions of an mRNA. Furthermore, the in-vitro transcribed RNA-based assay can be used to study different mechanisms of translation initiation including cap-dependent initiation, cap-independent initiation, re-initiation and internal initiation like IRES.

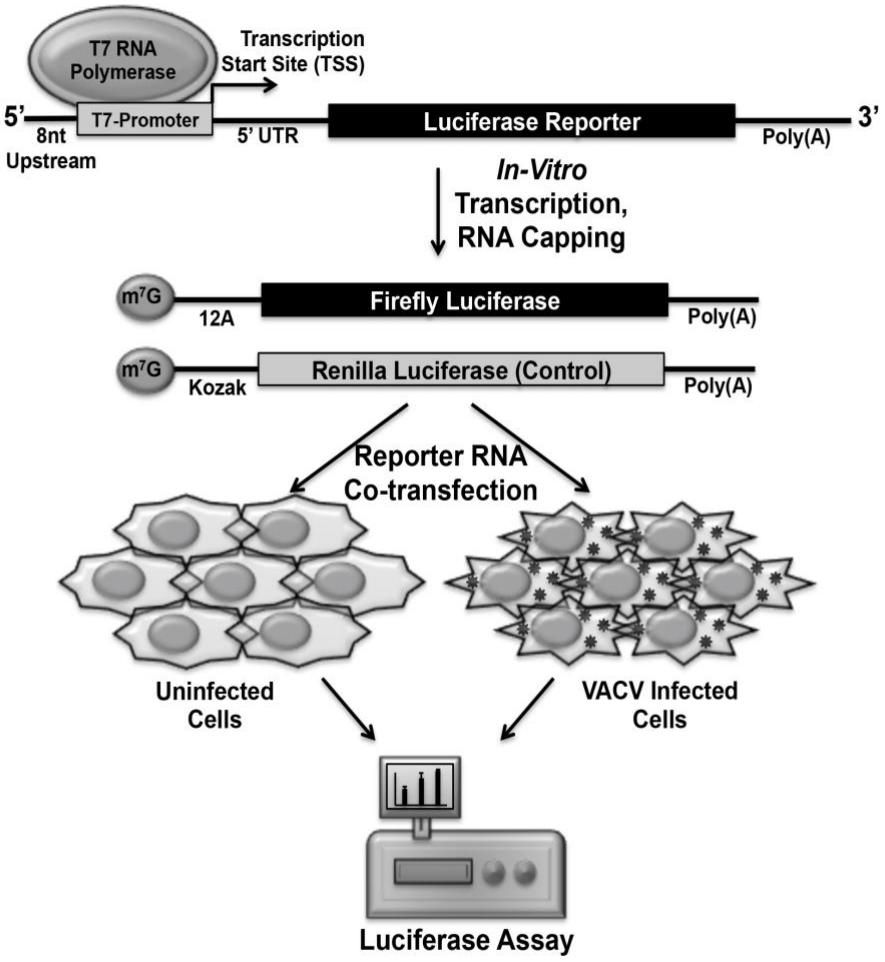

Figure 2-1: Schematic of the experimental procedure.

PCR is used to generate a DNA template with desired elements. mRNA encoding a luciferase reporter gene is synthesized in-vitro using a T7 RNA polymerase-based system. A Firefly luciferase (Fluc) mRNA is co-transfected with a Renilla luciferase (Rluc) mRNA into uninfected or VACV-infected cells. Luciferase activities are measured using a luminometer with dual luciferase capability.

Protocol

Note: Information about the Material/Equipment used in this protocol can be found in the Table of Materials.

1. Prepare DNA template by PCR for in-vitro transcription

1.1. To prepare DNA template by PCR, design primers. When designing primers consider crucial characteristics like primer length, annealing temperature (Tm), GC content, 3' end with G or C etc.

Note: Discussed in detail in these literature (25–27).

1.2. Design primers to generate PCR amplicon containing the following elements in 5' to 3' direction: T7-Promoter, poly(A) leader, firefly luciferase ORF and a poly(A) tail referred hereafter to as T7_12A-Fluc. Design primers (Forward and Reverse) to encompass all the additional elements not present in the template DNA (**Figure 2-2A**).

Note: The sequence of all elements can be found in **Table 2-1**.

Table 2-1: Sequences used in the method

The table contains the sequences of T7 promoter, poly(A) leader, Kozak sequence, poly(A) tail.

Elements	**Sequence**
T7 Promoter	TAATACGACTCACTATAGGG
Poly(A) leader	AAAAAAAAAAAA, ranging from 3 to 51 As
Kozak sequence	GCCACC
Poly(A) tail	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA……

1.3. Include several extra nucleotides in forward primer (5'-3') (28), followed by T7-promoter, poly(A) leader or desired 5'-UTR sequence and approximately 20 nucleotides, adjust based on Tm, corresponding to the 5' end of the reporter gene's ORF. Ensure the corresponding region in the primer is identical to the sense strand (+ strand) of the gene.

Note: For long 5'-UTR, synthesize two DNA fragments: one with T7 promoter followed by long 5'-UTR and second with reporter gene's ORF. Join these two fragments using overlap extension PCR (29).

1.4. Design reverse primer (5'-3') to include a poly(A) tail and approximately 20 nucleotides, adjust based on Tm, corresponding to the 3' end of the reporter gene's ORF. Ensure the corresponding region in the primer is identical to the anti-sense strand (- strand) of the gene and an in-frame stop codon is present before the poly(A) tail.

Note: The desired length of A's in a poly(A) leader or poly(A) tail can be customized in the primers. For example, to add 50 A's in the poly(A) tail, the reverse primer should entail 50 T's. Similarly, to add 20 A's in the poly(A) leader, the forward primer should entail 20 A's.

1.5. For internal control, design another set of primers containing the following elements in 5' to 3' direction: T7 Promoter, a random 5'-UTR coding sequence containing Kozak sequence, renilla luciferase ORF and poly(A) tail referred hereafter to as T7_Kozak-Rluc.

1.6. In a PCR tube, add the reagents in the following order: DNase free water, 2X high-fidelity DNA polymerase, primers and sequence confirmed luciferase template DNA (**Table 2-2**).

Table 2-2: PCR reaction

The order and the volume of components added in PCR reaction.

Components	**Volume**
DNase free water:	38 µl
2X High-fidelity DNA polymerase Master mix:	50 µl
Forward Primer (10 µM):	4 µl
Reverse Primer (10 µM):	4 µl
Luciferase DNA Template* (1-10 ng/µl):	4 µl
Total:	**100 µl**

Note: Amounts of individual components in the mixture should be adjusted according to the reaction volume.

*Source for Fluc DNA template is PTK-Fluc plasmid.

*Source for Rluc DNA template is PTK-Rluc plasmid

1.7. Use a standard 3-step (Denaturation, Annealing, Extension) PCR cycle to generate DNA template as shown in **Table 2-3**.

Table 2-3: PCR Program

The steps for PCR program along with temperature, time and cycle.

Step	**Temperature**	**Time**	**Cycle**
Initial denaturation	95 ℃	2 minutes	(1X Cycle)
Denaturation	95 ℃:	15 seconds	(25X Cycle)
Annealing	**X ℃**:	30 seconds	
Extension	72 ℃:	**T minutes**	
Final Extension	72 ℃:	7 minutes	(1X Cycle)
Hold	4 ℃:	∞	

Note: Annealing temperature X ℃ depends on the primer set being used and extension time T minutes depend on the PCR amplicon size and DNA polymerase used.

1.8. Detect the PCR product by running 5-10% of PCR reaction in 1% agarose Tris-acetate-EDTA (TAE) gel electrophoresis (containing 0.1µg/ml ethidium bromide) along with commercially available molecular weight standard. Visualize the gel under a UV illuminator to determine the size of the PCR product.

1.9. After determining the correct size of the PCR product as shown in **Figure 2-2B**, ~1.7 kb for T7_12A-Fluc and ~1.0 kb for T7_Kozak-Rluc, purify it using a commercially available PCR purification kit. Elute the DNA using 100 µl nuclease free water.

1.10. Once purified, determine the concentration of the DNA and the A260/A280 ratio (~1.8-2.0 is acceptable).

1.11. Store purified DNA at -20 °C or use for in-vitro transcription immediately.

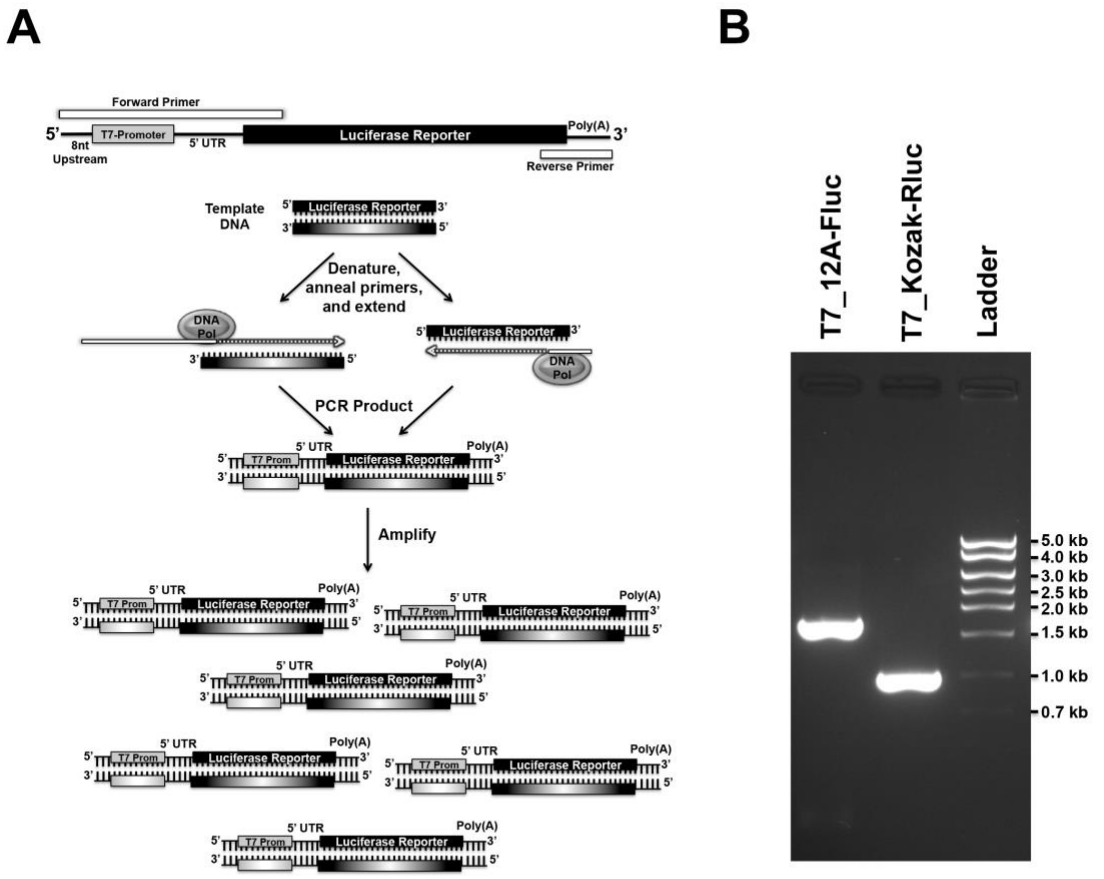

Figure 2-2: Primer design and PCR-based DNA amplification.

(A) Forward primer is synthesized to include an 8nt random sequence, T7 promoter followed by a desired 5'-UTR and part of the 5' end of the luciferase reporter gene, while the reverse primer includes a T-tract to generate a poly(A) tail and the 3' end of the luciferase reporter gene. By overhang extension PCR using a plasmid template containing luciferase gene, a DNA template is generated. (B) DNA band of the desired size from PCR reaction was detected using 1% agarose TAE gel electrophoresis.

2. Generate mRNA by in-vitro transcription

2.1. Synthesize RNA from the PCR product in vitro, using an in-vitro transcription kit (**Figure 2-3A**).

Note: T7_12A-Fluc and T7_Kozak-Rluc DNA templates are used to synthesize 12A-Fluc and Kozak-Rluc mRNAs, respectively.

2.2. In a microcentrifuge tube, add the reagents in the following order: DNase-RNase free water, NTP Buffer Mix, Cap Analog, Template PCR Product, T7-RNA polymerase Mix (**Table 2-4**).

Table 2-4: *In-vitro* transcription reaction

The order and the volume of components added for in-vitro transcription reaction.

Components	**Volume**	
DNase-RNase free water:	up to 20 µl	
NTP Buffer Mix (20 mM of each rNTP):	2 µl	
Cap Analog (40 mM):	4 µl	
Template PCR Product (400 ng)*:	**X** µl	(Concentration dependent)
T7-RNA polymerase Mix:	2 µl	
Total:	**20 µl**	

Note: Other capping system can also be used to cap RNA sequentially after in-vitro transcription following manufacturer's instruction.

2.3. Mix thoroughly and incubate at 37 °C for 2 hours.

2.4. Proceed to purification of the synthesized RNA using an RNA purification kit.

2.5. Run purified RNA in 1.5% agarose Tris-borate-EDTA (TBE) gel (containing 0.5 µg/ml ethidium bromide) to check the RNA. Visualize the gel under a UV illuminator (**Figure 2-3B**).

2.6. Determine the concentration of the RNA and the A260/A280 ratio (~1.8-2.0 is acceptable).

2.7. Aliquot the purified RNA and store at -80 °C.

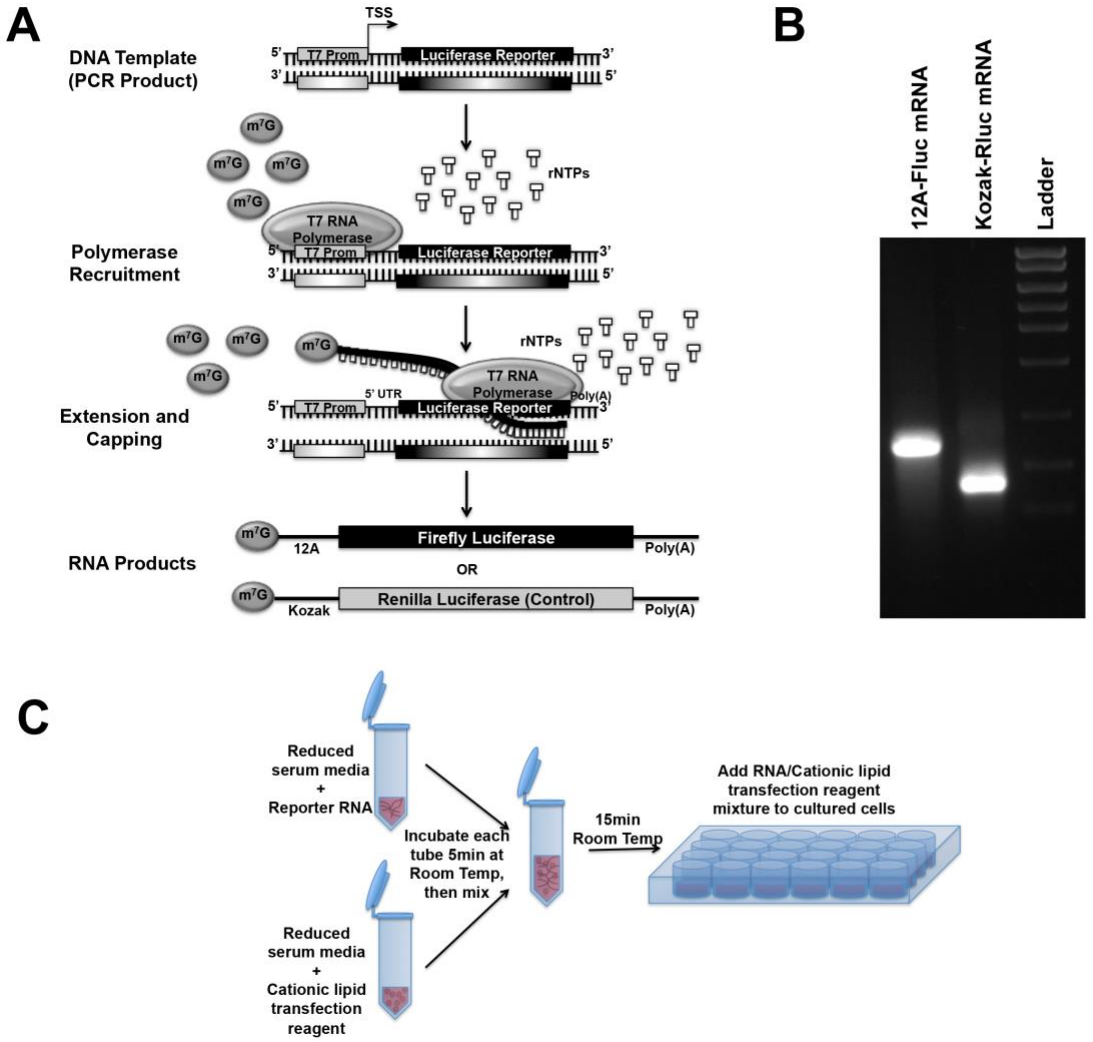

Figure 2-3: mRNA synthesis and transfection.

(A) Schematic of in-vitro transcription. DNA amplified by PCR containing the luciferase gene downstream from the 5'-UTR of interest and the T7 promoter is used as the template. The T7 RNA polymerase is recruited to the promoter and adds ribonucleotides, shown in white, from 5' to 3' direction. Once mRNA is 25-30nt long, m7G cap is added using an anti-reverse cap analog, ARCA. (B) RNA bands from in-vitro transcription was detected using 1.5% agarose TBE gel electrophoresis. (C) Schematic demonstrating the transfection of reporter mRNA into cells.

Medium containing either the reporter mRNA or cationic lipid transfection reagent in separate tubes is allowed to equilibrate at room temperature for 5 minutes. The solutions are then mixed followed by incubation at room temperature for 15 minutes after which the RNA/transfection reagent mixture is added into cells in culture plates.

3. Transfect mRNA to cells

3.1. Seed HeLa cells in a 24-well plate (to be approx. ~80-90% confluent next day) and incubate overnight in an incubator at 37 °C with 5% CO2.

3.2. Infect HeLa cells with vaccinia virus (VACV) at a Multiplicity of Infection (MOI) of 5 or keep uninfected HeLa cells for comparison.

Note: MOI is number of infectious viral particles per cell.
MOI of X= {[(Number of cells * X) / Virus Titer] * 1000} µl of virus per 1 ml medium.

3.3. After desired hours post infection (hpi) (we often transfect at 10-12 hpi), transfect mRNA (500 ng of total mRNA per well of 24-well plates) using a cationic lipid transfection reagent as shown in **Figure 2-3C**.

3.3.1. For one well of a 24-well plate, mix 480 ng of 12A sequence bearing firefly luciferase (12A-Fluc) mRNA and 20 ng of Kozak sequence bearing renilla luciferase (Kozak-Rluc) mRNA in one microcentrifuge tube. In another microcentrifuge tube add 1.1 µl of cationic lipid transfection reagent.

3.3.2. Add 55 µl of reduced serum medium in both tubes. Mix and incubate at room temperature for 5 minutes.

3.3.3. After 5 minutes of incubation, add 55 µl cationic lipid transfection reagent containing reduced serum medium in mRNA containing tube.

3.3.4. Mix gently but thoroughly, and incubate at room temperature for 15 minutes.

3.3.5. During the incubation, remove the cell culture medium and add 400 µl of reduced serum medium per well of 24-well plates.

3.3.6. After incubation, add 100 µl of the mixture dropwise and evenly to one well of 24-well plates.

4. Measure luciferase activities

4.1. Five-hours post-co-transfection of 12A-Fluc and Kozak-Rluc mRNA, measure luciferase activity using a Dual-Luciferase Assay Kit (DLAK).

4.2 Remove reduced serum medium and lyse the cells by adding 150 µl 1X Passive lysis buffer, a component of DLAK.

4.3. After 10 minutes incubation at room temperature, collect the lysate by scrapping the cells and transfer to a microcentrifuge tube.

4.4. Centrifuge the lysate at 12,000 X g for 10 minutes at 4 ℃ to pellet cell debris.

4.5. Add 30 µl of supernatant in opaque-walled 96 well white assay plate with a solid bottom.

4.6. Measure the dual luminescence using DLAK and a multimode plate reader luminometer.

4.7. The measurement is taken using kinetics function (all steps on a per-well basis) using the settings described in **Table 2-5**.

Table 2-5: Luciferase Measurement Settings

The steps for luciferase measurement with recommended volume or time.

Steps	**Volume/Time**
Inject Luciferase Assay Substrate (Fluc):	30 µl
Wait / Incubation time:	2 sec
Luminescence Measurement (Fluc):	10 seconds
Stop & Glo Substrate (Rluc):	30 µl
Wait / Incubation time:	2 sec
Luminescence Measurement (Rluc):	10 seconds

Note: The reading can also be taken using manual luminometer. Add equal volume of lysate and substrate for Fluc in a cuvette. Wait for 2 seconds and measure for 10 seconds using luminometer. Following Fluc measurement, quickly take out cuvette from luminometer and add equal volume of substrate for Rluc manually. Again, wait for 2 seconds and measure for 10 seconds using luminometer.

4.8. Export the luminescence reading data into desirable file format.

4.9. Determine relative translation rate from 12A-Fluc mRNA in uninfected and VACV infected HeLa cells by dividing Fluc value by internal control Rluc value.

Results

The four steps of in-vitro transcribed RNA-based luciferase reporter assay: PCR to generate DNA template for in-vitro transcription, in-vitro transcription to generate mRNA, mRNA transfection, and luciferase measurement, can be seen in the schematic diagram (**Figure 2-1**). Designing of primers for both DNA templates (Fluc and Rluc) and the general scheme of overhang extension PCR is illustrated in the schematic (**Figure 2-2A**). After PCR, the correct sized PCR product was detected by TAE agarose gel electrophoresis (**Figure 2-2B**). Subsequently, the PCR product is used as the template to synthesize RNA in-vitro (**Figure 2-3A**), which is purified and run in TBE gel electrophoresis to verify the size (**Figure 2-3B**). The purified and verified mRNA is transfected into cells using cationic lipid transfection reagent (**Figure 2-3C**).

The in-vitro transcribed RNA-based luciferase reporter assay was developed to understand the role of 5'-poly(A) leader in mRNA translation during poxvirus infection. Using this assay, we tested the translation efficiency of a Fluc mRNA that contains a 5'-poly(A) leader (12nt) in uninfected and VACV-infected cells. The Fluc value was normalized using Rluc value in both uninfected and VACV-infected cells to determine the relative Fluc activity (i.e. Fluc activity/Rluc activity) (**Figure 2-4A**). The division of Fluc by Rluc normalized the transfection efficiency and RNA stability in a particular well. Using this analysis approach, we determined that a 5'-poly(A) leader containing mRNA has a translational advantage during VACV infection (**Figure 2-4B**). The advantage in infected cells was not due to differential transfection efficiency or mRNA stability as the RNA level was similar in uninfected and VACV infected cells 5 hours post mRNA transfection (19).

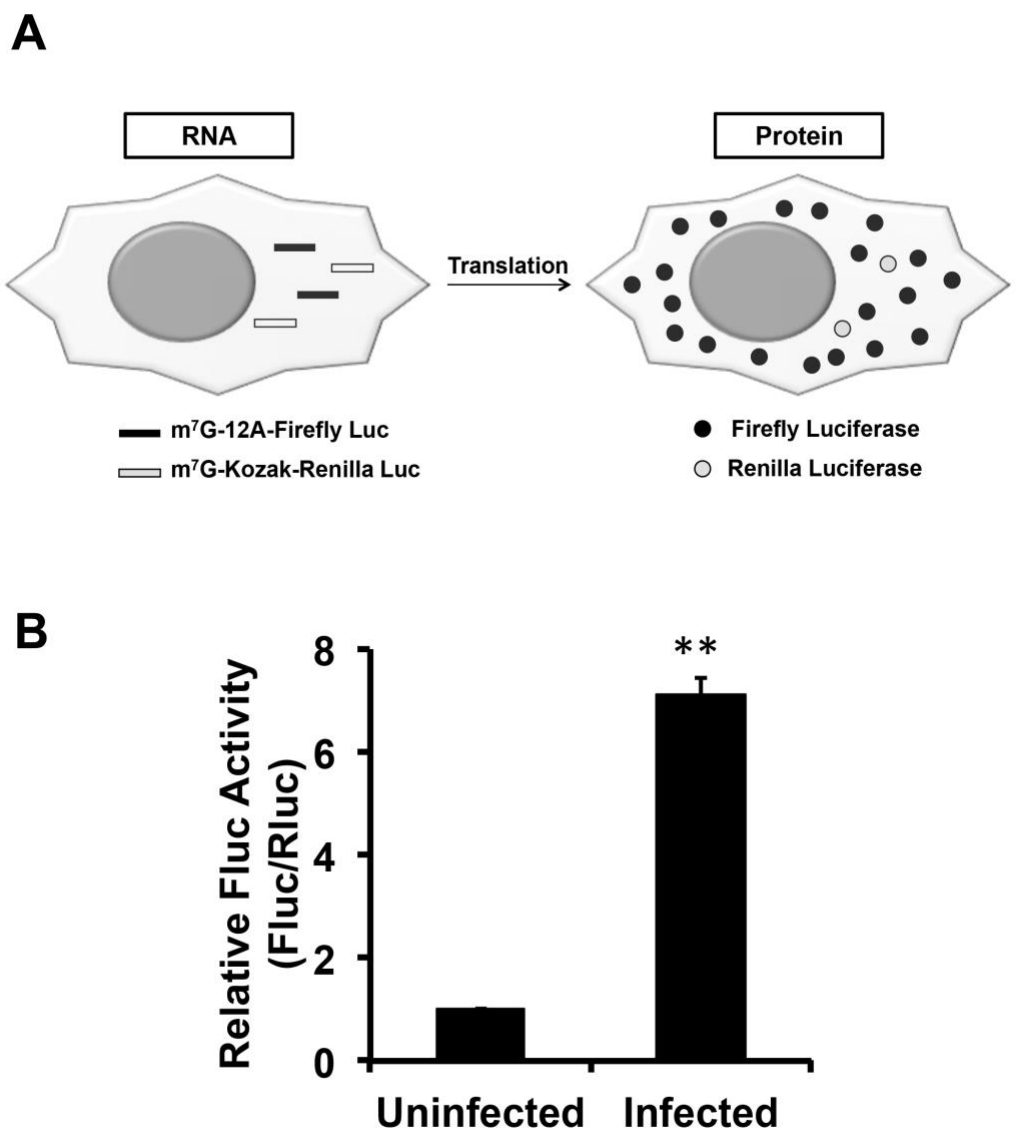

Figure 2-4: Increased translational efficiency of mRNA containing a 5'-poly(A) leader. (A) Fluc mRNA containing a poly(A) leader in the 5'-UTR and Rluc mRNA with the Kozak consensus sequence in the 5'-UTR are co-transfected into cells. (B) Fluc mRNA with 5'-poly(A) leader was transfected in uninfected and VACV-infected cells along with Rluc mRNA. Five-hours post-transfection, luciferase activity was measured using a luminometer. Rluc normalized Fluc activity is represented in uninfected and VACV-infected cells. Error bars indicate the standard deviations (SD) of at least three repeats. Student's t-test was used to determine P-values; ***P value < 0.001.

Discussion

All four-core steps are critical to the success of the in-vitro transcribed RNA-based luciferase reporter assay. Special attention should be given to primer design, especially for the T7 promoter sequence. T7 RNA polymerase starts transcription from the underlined first G (GGG-5'-UTR-AUG-) in T7 promoter added before the 5'-UTR sequence. Although the transcription start site (TSS) starts from the first G at the 5' end, decreasing the number of G's less than three in T7 promoter region decreased the RNA yield/output from in-vitro transcription. During the experiment, we observed that gel purified DNA product was not the best for in-vitro transcription as both yield and quality of RNA were lower. We only ran 5-10% of the PCR reaction in 1% agarose gel electrophoresis to determine the size and purified the rest 90-95%, using a PCR purification kit, to be used for in-vitro transcription. In case of non-specific amplification from PCR, cutting the desired sized band from gel and using gel purified DNA fragment is recommended. As the yield might be low, we suggest increasing the reaction volume for in-vitro transcription reaction. Similar to other transfection-based methods, DNA/RNA may stimulate DNA/RNA sensing pathways that may globally or selectively suppress translation. Therefore, data should be interpreted with cautions, although we did not experience problems potentially caused by this issue in our experiments.

The proposed method is suitable for use in different model systems with some modifications like the method of mRNA delivery, internal control to be used, a suitable time for translation, sample preparation and analysis of data. The main limitation of this method is that it is a reporter assay to quickly test translation regulation by cis elements that does not completely reflect physiological conditions. Therefore, this method should be corroborated by other complementary experiments, if possible.

Compared to DNA modification, the roles of RNA modifications are less well understood. However, with the discovery of enzymes that write, read and erase RNA modifications (30–35), it is now possible to study the influence of RNA modification in gene expression (31–35). The in-vitro transcribed RNA-based luciferase reporter assay may be modified to incorporate different RNA modifications and used to test their effects on RNA translation. For example, this method can incorporate different cap analogs that have various modifications (30,31). Additionally, supplementing an internal RNA modifying enzyme during or after in-vitro transcription can possibly incorporate internal RNA modification. Addition of a modification to cap 0, cap 1, and an internal RNA modification will provide a tool to study the roles of these RNA modifications in translation.

The in-vitro transcribed RNA-based luciferase reporter assay has a great potential and broad application in understanding basic biology about RNA translation. Different mechanisms for the initiation of translation, including cap-dependent initiation, cap-independent initiation, re-initiation and internal initiation such as IRES can be studied using this method. On top of these advantages, this assay can be employed to test translation regulation by cis-elements at 5'-UTR and 3'-UTR in an mRNA. The described protocol uses PCR product, which provides the advantage to avoid lengthy cloning and quickly examine the effects of RNA elements on translation. To minimize potential errors during PCR, high fidelity polymerase and low PCR cycle number should be used. Alternatively, if a template is used frequently, the desired 5-'UTR and luciferase ORF can be cloned into a plasmid as the template of in-vitro transcription. Together, the protocol consolidates transcription and mRNA capping in a single reaction and utilizes conventional transfection and analysis that make in-vitro transcribed RNA-based

luciferase reporter assay a user-friendly, quick, and straightforward method to study mechanisms of mRNA translation.

Acknowledgments

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Author contribution

The experiments in this study were conceived and designed by P.D. and Z.Y. Experiments were performed by P.D., F.C. and C.H. The schematics were made by F.C. and P.D. The paper was written by P.D. and Z.Y., and critically evaluated by all the authors.

Disclosures

The authors would like to declare no competing financial interest.

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Chapter 3 - The 5’-poly(A) leader of poxvirus mRNA confers a translational advantage that can be achieved in cells with impaired cap-dependent translation

Pragyesh Dhungel, Shuai Cao, Zhilong Yang*

Division of Biology, Kansas State University, Manhattan, Kansas, United States of America

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Abstract

The poly(A) leader at the 5'-untranslated region (5'-UTR) is an unusually striking feature of all poxvirus mRNAs transcribed after viral DNA replication (post-replicative mRNAs). These poly(A) leaders are non-templated and of heterogeneous lengths; and their function during poxvirus infection remains a long-standing question. Here, we discovered that a 5'-poly(A) leader conferred a selective translational advantage to mRNA in poxvirus-infected cells. A constitutive and uninterrupted 5'-poly(A) leader with 12 residues was optimal. Because the most frequent lengths of the 5'-poly(A) leaders are 8–12 residues, the result suggests that the poly(A) leader has been evolutionarily optimized to boost poxvirus protein production. A 5'-poly(A) leader also could increase protein production in the bacteriophage T7 promoter- based expression system of vaccinia virus, the prototypic member of poxviruses. Interest- ingly, although vaccinia virus post-replicative mRNAs do have 5'- methylated guanosine caps and can use cap-dependent translation, in vaccinia virus-infected cells, mRNA with a 5'-poly(A) leader could also be efficiently translated in cells with impaired cap-dependent translation. However, the translation was not mediated through an internal ribosome entry site (IRES). These results point to a fundamental mechanism poxvirus uses to efficiently translate its post-replicative mRNAs.

Author Summary

Poxviruses continue to impact public health significantly, despite the eradication of smallpox, the deadliest disease in human history. As a tool, poxviruses are being engineered to treat various infectious diseases and multiple cancers. All poxvirus mRNAs transcribed after viral DNA replication have a poly(A) leader in their 5'-untranslated regions, the function of which remains elusive and represents a major gap in our understanding of the mechanisms fundamental to controlling poxvirus gene expression. In poxvirus-infected cells, a 5'-poly(A) leader was found to confer on poxvirus mRNAs a translational advantage that could be achieved in cells with impaired cap-dependent translation, which is used for translation of most eukaryotic mRNAs. Furthermore, since viruses typically exploit existing cellular functions, it is highly likely that these results point to an unknown cellular mRNA translation mechanism. Thus, the findings should facilitate targeting of poxvirus post-replicative mRNA translation for the development of novel antiviral strategies. The poly(A) leader can also be used to increase foreign gene expression when using the bacteriophage T7 promoter-based poxvirus expression systems.

Introduction

All viruses rely entirely on their infected host cells for protein synthesis. Not surprisingly, to boost production of viral proteins, many viral evolutionary strategies usurp the host translation machinery. In doing so they target every step of protein synthesis, from mRNA production and stability, to translation initiation, elongation, and termination (1–4). Such mechanisms include mRNA sequence elements that enhance translation. A striking and unusual feature is found in all vaccinia virus (VACV) mRNAs transcribed after viral DNA replication; all of these mRNAs have a 5'-poly(A) leader in their 5'-untranslated regions (5'-UTRs) (5–8). It is well established that the 5'-UTR of an mRNA plays an important role in regulating eukaryotic mRNA translation (9); for the VACV mRNAs, however, it is unclear whether the 5'-poly(A) leader contributes to efficient translation of these VACV mRNAs. This lack of knowledge represents a major gap in understanding the fundamental gene expression mechanism of poxviruses.

Poxviruses comprise a highly dangerous class of emerging and re-emerging pathogens of humans and other vertebrates (10). Their large double-stranded DNA genomes encode hundreds of genes that are expressed in cascade at early, intermediate, or late stages of infection (10). The early genes initiate expression soon after viral entry, without the need for viral DNA replication; in contrast, intermediate and late genes can only be expressed after viral DNA replication. The intermediate and late genes are collectively referred to as post-replicative genes, and mainly function to form virions. In VACV, the prototypic poxvirus, 53 genes initiate transcription in the intermediate stage and 38 genes initiate transcription in the late stage (11, 12). All the VACV post-replicative mRNAs contain a non-templated 5'-poly(A) leader that is likely formed during transcription initiation, when the viral RNA polymerase slips at the conserved promoter sequence containing three A residues (6–8, 13). The 5'-poly(A) leaders of these mRNAs are of

heterogeneous length ranging from 3 to 51 A residues, with most between 8 and 12 A residues (5). For most VACV post-replicative mRNAs, the 5'-poly(A) leaders comprise the entire 5'-UTR because the 5'-poly(A) leader and the first A residue of the start codon AUG overlap (5). Similar to eukaryotic cellular mRNAs, VACV early mRNAs and post-replicative VACV mRNAs are capped by methylated guanosine (14, 15). The 5' cap is required for launching cap-dependent translation that is the dominant translation mode in eukaryotic cells (16). Therefore, like eukaryotic cellular mRNAs, these VACV mRNAs can be translated in a cap-dependent manner.

To examine the function of the VACV poly(A) leader, we first developed an in vitro transcribed RNA-based reporter assay. We used this assay to demonstrate that the 5'-poly(A) leader of an mRNA can confer a translational advantage in VACV-infected cells. Remarkably, the translational advantage can be achieved in cells with impaired cap-dependent translation, suggesting an adaptation mechanism poxvirus uses to replicate in unfriendly cellular environments.

Results

The 5'-poly(A) leader of an mRNA confers a translational advantage during VACV infection

To understand how the 5'-poly(A) leader may facilitate mRNA translation during VACV infection, we intended to develop a convenient reporter system. Though DNA plasmid-based luciferase expression is usually used to develop reporter system, technical issues posed barriers of the utility of it in VACV-infected cells as plasmids are able to replicate in VACV-infected cells (17); such plasmid replication makes it difficult to compare uninfected and VACV-infected cells. In addition, VACV promoter-driven transcription would generate mRNAs that are heterogeneous with respect to poly(A)-leader length (5); and, with plasmid templates, cryptic transcription would likely further complicate interpretation of the data (5, 11, 18).

To work around these complications, we developed an RNA-based luciferase reporter assay (**Fig 3-1A**). Using PCR, we first generated a DNA fragment containing the following elements in 5' to 3' order: a T7 promoter, the desired 5'-UTR sequence, the firefly luciferase (Fluc) ORF, and sequence encoding a poly (A) tail. The mRNA was transcribed from a bacterial T7-phage-promoter-based, in vitro transcription system, during which an m7G cap or its analog was incorporated. The resulting Fluc reporter mRNA was transfected into cells, together with a renilla luciferase (Rluc) reporter mRNA as the control of transfection efficiency. The Rluc mRNA contained a 5'-UTR bearing the Kozak consensus sequence that is important in translation initiation of eukaryotic mRNA (19). The normalized firefly luciferase activity was used as the indicator of translational potential of mRNAs with different 5'-UTRs.

Using this approach, we tested the translational efficiency of an Fluc mRNA whose 5'-UTR bears a 20-residue 5'-poly(A) leader. Translational efficiency was compared in uninfected

and VACV-infected HeLa cells. The transfection was carried out at approximately 12 hpi, during the VACV post-replicative stage of infection (11). We tested the luciferase activities at 1, 2.5, 5 and 8 h post transfection. As expected, both firefly and renilla luciferase activities increased over time. At 5 h, the luciferase activities did not reach or just reached the highest value in both uninfected and infected cells. In the following experiments, the luciferase activities were measured at approximately 5 h post transfection. Notably, compared to uninfected cells, VACV-infected cells showed a significant increase of the normalized Fluc activity (**Fig 3-1B**). The control 5'-UTR from the cellular RNF165 mRNA, which contains no poly(A) leader, did not confer a translational advantage in VACV-infected cells **(Fig 3-2C).** The Fluc mRNA with a poly(A) leader amounts in VACV-infected and uninfected cells at 5 h post transfection were similar, shown here by quantitative RT-PCR (**Fig 3-1C**), ruling out the possibility of difference in luciferase activities due to different amounts of transfected RNA in VACV-infected cells. Interestingly, the translational advantage was not observed during the early replication stage as evidenced by using an A23 gene-deleted VACV (A23Δ) that arrested viral replication at the DNA replication stage (**Fig 3-1D**) (20). Together, these results showed that, in VACV-infected HeLa cells, mRNA with a 5'-poly(A) leader was more efficiently translated during the post-replicative stage of VACV replication.

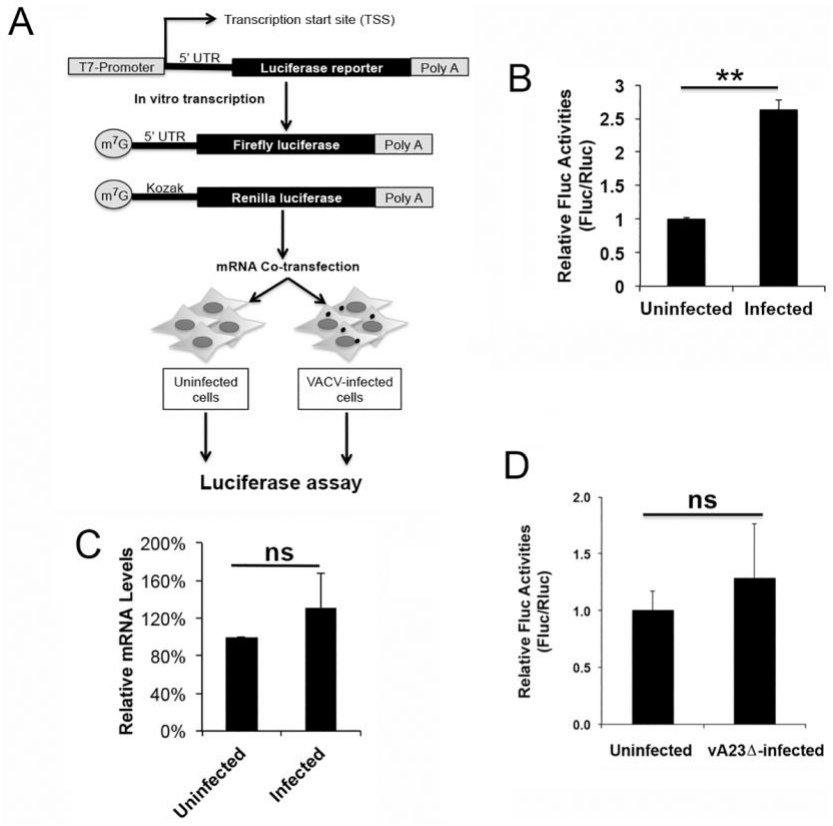

Figure 3-1: The 5' poly(A) leader confers an mRNA translational advantage during the post-replicative stage of VACV replication.

(A) Schematic of experimental approach. Messenger RNA was synthesized by a T7 promoter-based in vitro transcription system. The firefly luciferase (Fluc) reporter mRNA under a 5'-UTR to be tested was transfected into cells together with a renilla luciferase (Rluc) reporter mRNA under a 5'-UTR-containing Kozak sequence. Luciferase activities were measured at 5 h post transfection using a luminometer with a dual luciferase assay system. (B) Fluc mRNA with a 5'-poly(A) leader of 20 residues was transfected into uninfected or wild-type VACV-infected HeLa cells (12 hpi) together with an Rluc mRNA. Luciferase activities were measured at 5 h post transfection. The Rluc-normalized Fluc activity was normalized as 1 in uninfected HeLa cells. (C) The relative levels of Fluc mRNA from uninfected and VACV-infected HeLa cells were quantitated at 5 h post transfection by quantitative RT-PCR (qRT-PCR). The amount of mRNA in uninfected cells was normalized as 1. (D) Fluc mRNA with a 5'-poly(A) leader of 20 residues was transfected into uninfected, or A23-deleted recombinant VACV-infected HeLa cells (12 hpi) together with an Rluc mRNA. Luciferase activities were measured at 5 h post transfection. The Rluc-normalized Fluc activity was normalized as 1 in uninfected HeLa cells. Error bars represent

standard deviation (SD) of at least three experiments. P-values were obtained using the Student's t-test; **P value < 0.01, ns = Not Significant (i.e. P value > 0.05).

12 residues is the optimal length of the 5'-poly(A) leader for conferring a translational advantage during VACV infection

Among VACV post-replicative mRNAs, the length of the 5'-poly(A) leader varies from 3 to 51 residues, with most leaders between 8 and 12 residues (5). To determine the optimal length for conferring the translational advantage, we generated Fluc mRNAs with different poly(A) leader lengths, ranging from 4 to 20 residues. In VACV-infected cells, our reporter assays showed the translational advantage of the Fluc mRNAs increased as the length of the 5'-poly(A) leader increased to 12 residues (approximately 8-fold), and then decreased when leaders became longer (**Fig 3-2A**). The absolute luciferase activity was also the highest for the Fluc mRNA with a 5'-poly(A) leader of 12 residues in VACV-infected cells, further indicating the optimal length for conferring a translational advantage during VACV infection was 12 residues. We also examined the translational advantage during the VACV post-replicative stage by transfecting the 12A-headed RNA at 2.5, 5, 7.5, 10 and 15 hpi. The results showed translational advantage during all the times examined.

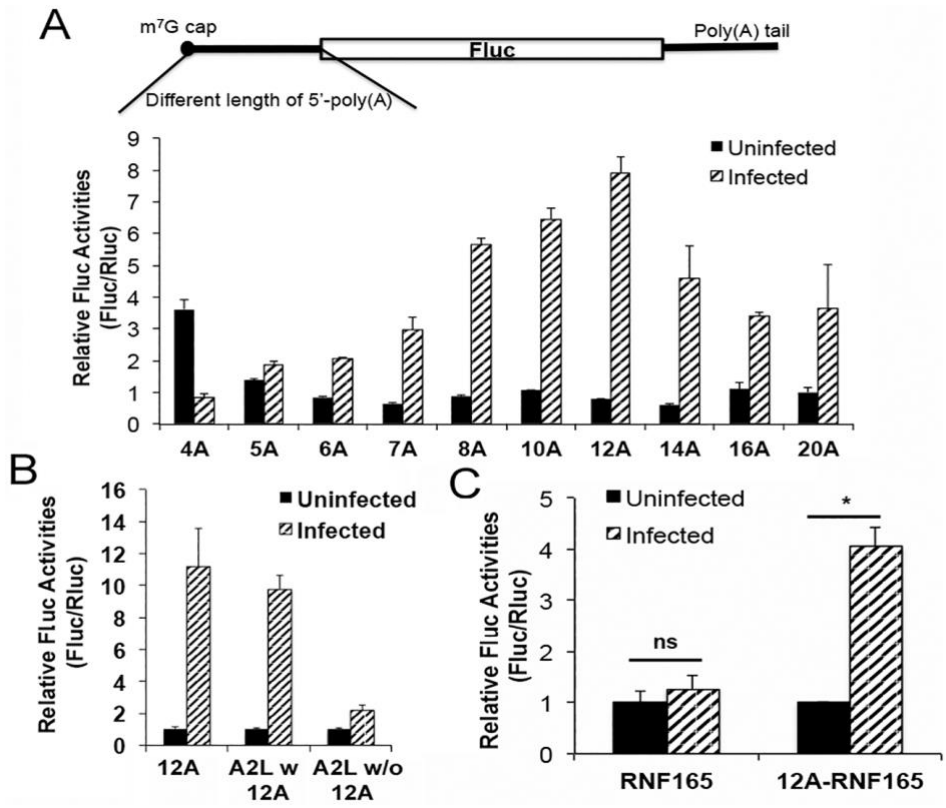

Figure 3-2: The optimal length of 5'-poly(A) leader in conferring translational advantage is 12 residues.

(A) The Fluc reporter mRNAs with different 5'-poly(A)-leaders lengths were transfected into uninfected and VACV-infected HeLa cells, together with an Rluc mRNA. Luciferase activities were measured at 5 h post transfection. (B) Fluc reporter mRNA containing the A2L mRNA 5'-UTR with (w) or without (w/o) a poly(A) leader, was transfected into uninfected and VACV-infected HeLa cells, together with an Rluc mRNA. Luciferase activities were measured at 5 h post transfection. The Rluc-normalized Fluc activity was normalized as 1 in uninfected cells. (C) Fluc reporter mRNA containing RNF165 5'-UTR with (w) or without (w/o) an A-tract addition at the 5'-end was transfected into uninfected and VACV-infected HeLa cells together with an Rluc mRNA. Luciferase activities were measured at 5 h post transfection. Error bars represent standard deviation (SD) of at least three experiments. P- values were determined using the Student's t-test; *P value < 0.05.

Addition of a 5'-poly(A) tract at the 5' end of a non-poly(A) UTR confers mRNA a translational advantage in VACV-infected cells

Although most VACV post-replicative mRNAs have a 5'-poly(A) leader immediately upstream of the start codon, some also contain a non-poly(A) intervening sequence (5), e.g., the viral A2L mRNA has a 32-nucleotide, non-poly(A) sequence between the poly(A) leader and the start codon (5). In VACV-infected cells, we tested the A2L mRNA 5'-UTR lacking a 5'-poly(A) tract, and found that it offered only slight translational advantage (Fig 2B). Adding a 12-residue A-tract to this mRNA at the 5' end of the 5'-UTR, however, significantly increased its expression (**Fig 3-2B**).

To test whether a poly(A) leader can enhance translation potential of non-viral 5'-UTR during VACV infection, we added 12 A residues to the 5' end of the RNF165 5'-UTR, which on its own only slightly enhance translation in VACV-infected cells (**Fig 3-2C**). In VACV-infected cells, addition of a 5'-end, 12-nucleotide A-tract to the RNF165 5'-UTR greatly enhanced translation of a downstream Fluc reporter (**Fig 3-2C**). These results further demonstrated that a 5'-poly(A) leader conferred a translational advantage in VACV-infected cells.

An uninterrupted 5'-poly(A) leader is essential for optimal translation in VACV-infected cells

We next investigated which A, amid the 12 residues, was vital for the poly(A) leader-mediated translational advantage. We generated Fluc mRNAs, with A to U point mutations at each of the 12 positions. The mutation was represented as 12A_mut_AN, where mutation A to U was at the position 'N' upstream of start codon AUG (**Fig 3-3A**). Any A to U change in the poly(A) leader affected the translational advantage (**Fig 3-3B**). We also mutated the A to G in

several positions and these mutations all impaired the translational advantage. This result indicated that an uninterrupted 5'-poly(A) leader is critical for the 5'-poly(A) leader-mediated translational advantage.

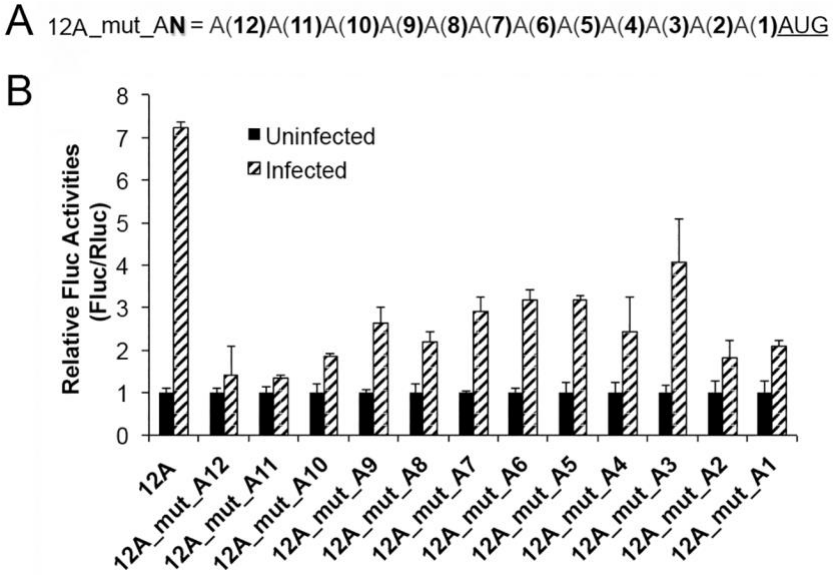

Figure 3-3: An uninterrupted 5'-poly(A) leader is essential for optimal translation in VACV-infected cells.

(A) Schematic of representation showing individual 5'-poly(A) residues mutated to U. (B) Fluc reporter mRNA containing each of the mutated 5'-poly(A) leaders were transfected into uninfected and VACV-infected HeLa cells, together with an Rluc mRNA. Luciferase activities were measured at 5 h post transfection. The Rluc normalized Fluc activity was normalized as 1 in uninfected HeLa cells. Error bars represent standard deviation (SD) of at least three experiments.

A 5'-poly(A) leader confers a translational advantage in multiple cell types and in myxoma virus infection

We examined the effect of the 5'-poly(A) leader on translation in different cell lines infected with VACV. During VACV infection, the translational upregulation of mRNA with a poly(A) leader was also observed in human foreskin fibroblasts (HFFs), rabbit RK13, and

monkey BS-C-1, though the enhancement levels varied (**Fig 3-4A**). We also tested the effect of the 5'-poly (A) leader on translation in RK-13 cells infected with Myxoma virus, a leporipoxvirus, and it similarly enhanced translational efficiency (**Fig 3-4B**).

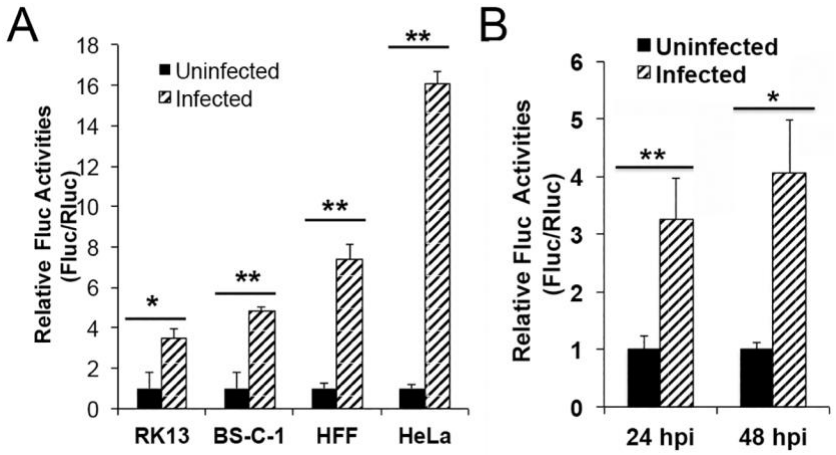

Figure 3-4: A 5'-poly(A) leader confers a translational advantage in multiple cell types and in myxoma virus infection.

(A) An Fluc reporter mRNA with a 12-residue 5'-poly(A) leader was transfected into uninfected or VACV-infected HeLa, HFF, BS-C-1 or RK13 cells, together with an Rluc mRNA, at 24 and 48 hpi. Luciferase activities were measured at 5 h post transfection. The Rluc-normalized Fluc activity was normalized as 1 in uninfected cells. (B) An Fluc reporter mRNA with a 12-residue 5'-poly(A) leader was transfected into uninfected or myxoma virus-infected RK13 cells, together with an Rluc mRNA. Luciferase activities were measured at 5 h post transfection. Error bars represent standard deviation (SD) of at least three experiments. P-values were determined using the Student's t-test; **P value < 0.01, *P value < 0.05.

Messenger RNA with a 5'-poly(A) leader is more efficiently translated than mRNA without a 5'-poly(A) leader in VACV-infected cells

We investigated whether, in VACV-infected cells, mRNAs containing a 5'-poly(A) leader have a translational advantage over those without a 5'-poly(A) leader. In previous study, we monitored the levels of active mRNA translation in VACV-infected HeLa cells by

simultaneous mRNA sequencing (RNA-Seq) and ribosome profiling (21, 22). Here, we calculated the genome-wide, relative translation efficiency (TE) of all VACV post-replicative mRNAs by the ratio of ribosome-protected mRNA to total mRNA, mapped to each transcript during the post-replicative stage at 4 and 8 hpi. VACV replication had proceeded to the post-replicative stage at 4 hpi under the experimental condition, when most viral mRNAs have 5'-poly(A) leaders (21). Although this calculation likely underestimated VACV mRNA translation efficiency, due to extensive read-through of VACV post-replicative mRNAs (5, 12), the VACV mRNAs were translated significantly more efficiently than cellular mRNAs (both in mock and VACV-infected cells), in a genome-wide manner (**Fig 3-5**). In fact, although there was an overall decrease of mRNA translation efficiency at 8 hpi compared to 4 hpi in VACV-infected cells, the translation efficiency of viral post-replicative mRNAs was still significantly higher than that of cellular mRNAs in mock-infected cells. These results indicated that, during a VACV infection, the post-replicative mRNAs with 5'-poly(A) leaders were more efficiently loaded with ribosomes, indicating higher translation efficiency of viral post-replicative mRNAs than that of host mRNAs (without 5'-poly(A) leaders).

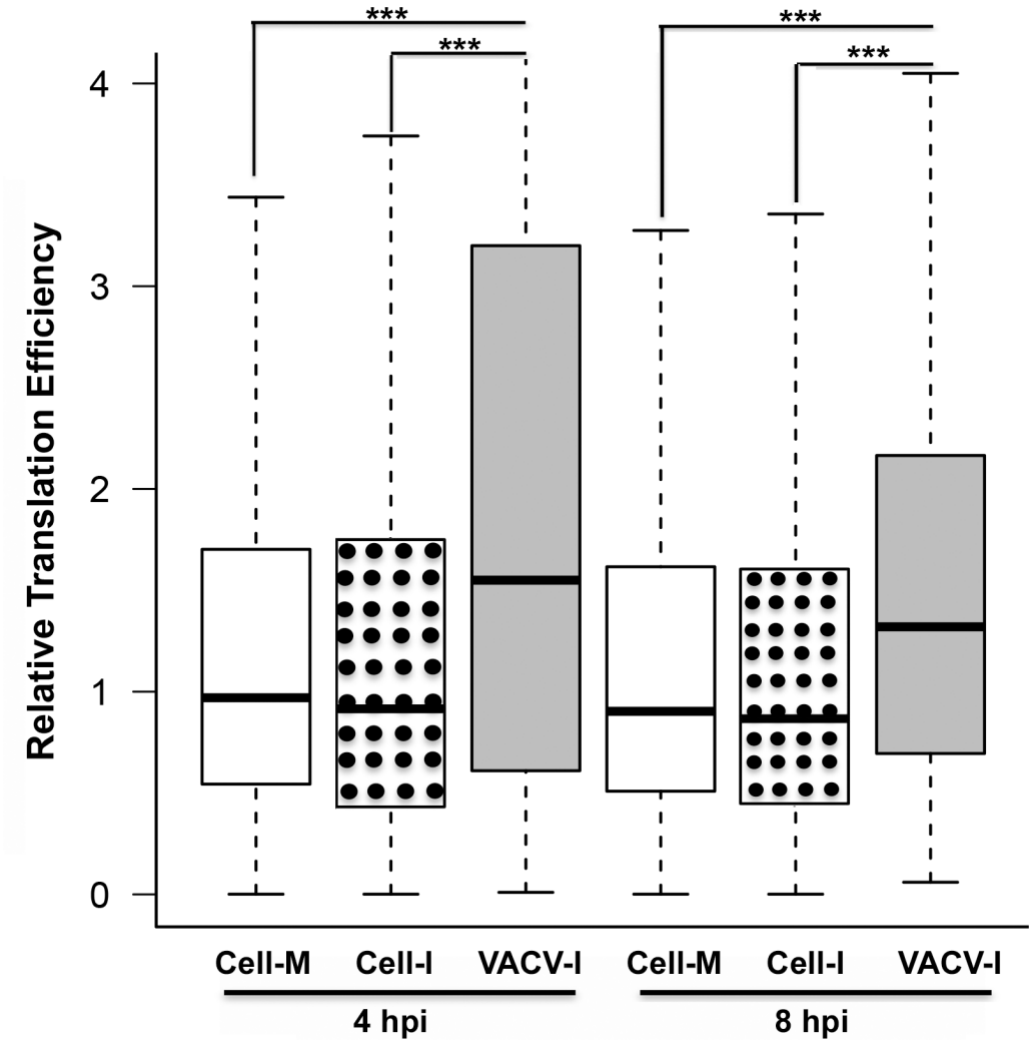

Figure 3-5: VACV post-replicative mRNAs are more efficiently translated than cellular mRNAs.

Boxwhisker plots of relative translation efficiency of VACV post-replicative mRNAs and host mRNAs at 4 and 8 hpi. Relative translation efficiency of mRNAs from uninfected HeLa cells is also shown. The bottom and top of the box indicate the first and third quartiles, respectively, with the line in the middle representing the median. Cell-M, cellular mRNAs from mock-infected cells. Cell-I, cellular mRNAs from VACV-infected cells. VACV-I, VACV mRNAs. The highest relative translation efficiency of VACV-I was off scale. ***indicates a P value < 0.001.

To examine further the role of the 5'-poly(A) leader, we carried out two experiments. In the first we constructed two plasmids in which an eGFP gene was under the control of the T7 promoter. An A-tract or a sequence containing a Kozak element was inserted between the ATG and the T7 promoter transcription start site, respectively. We transfected the plasmid into HeLa cells and then infected them with a recombinant VACV, vT7LacOi, which encodes a T7 polymerase gene whose expression is induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) (23). Because both plasmids were transfected into VACV-infected cells, the effect of VACV infection on plasmid replication was similar. Nevertheless, when cells were treated with IPTG, significantly more GFP was expressed from the plasmid containing the A-tract (**Fig 3-6A**). Small amount of eGFP protein expression was observed without IPTG induction, suggesting highly efficient translation of residual amount of mRNA from leaky expression in this inducible system. We quantified the eGFP mRNA levels by qRT-PCR and found similar mRNA amounts from A-tract-containing plasmid at 5 μM of IPTG induction to that from Kozak sequence-containing plasmid at 25 μM of IPTG (**Fig 3-6B**), while the protein level from A-tract-containing plasmid was much higher (**Fig 3-6A**).

To establish further the role of the 5'-poly(A) leader in enhancing the translational efficiency of VACV post-replicative mRNA, we made two recombinant viruses expressing IPTG inducible eGFP based on the parental virus vT7LacOi. In the recombinant viruses, the T7 promoter was followed by a Kozak sequence (vT7LacOi_Kozak-GFP) or an A-tract containing 12 residues (vT7LacOi_A12-GFP) driving the eGFP ORF. With increasing concentrations of IPTG, both recombinant viruses responded with increasing GFP expres- sion (**Fig 3-6C**). Notably, the levels of eGFP expression were strikingly higher in vT7La- cOi_A12-GFP-infected cells (**Fig 3-6C**). Again, small amount of eGFP protein expression was observed without IPTG

induction, suggesting efficient translation of mRNA from leaky transcription. qRT-PCR showed vT7LacOi_Kozak-GFP infected cells expressed more GFP mRNA, from at 100 and 1000 μM, compared to that from vT7LacOi_A12-GFP-infected cells at 5 μM IPTG induction; however, the protein level was much higher from the latter vT7LacOi_A12-GFP-infected cells at 5 μM (**Fig 3-6D**). We further compared the ratio of ribosome-bound GFP mRNA to total GFP mRNA with or without a poly(A) leader in VACV- infected cells by taking advantage of the two recombinant viruses. The result indicated that the relative ratio of the GFP mRNA transcribed from the vT7LacOi_A12-GFP genome (with a poly(A) leader) was significant higher (~3-fold) than that from the vT7LacOi_Kozak-GFP genome (without a poly(A) leader) (**Fig 3-6E**). In contrast, the ratios were similar for F17 mRNA, a late viral transcript with 5'-poly(A) leader from both viruses (**Fig 3-6E**). Taken together, these data supported that a poly(A) leader rendered VACV post-replicative mRNAs a translational advantage.

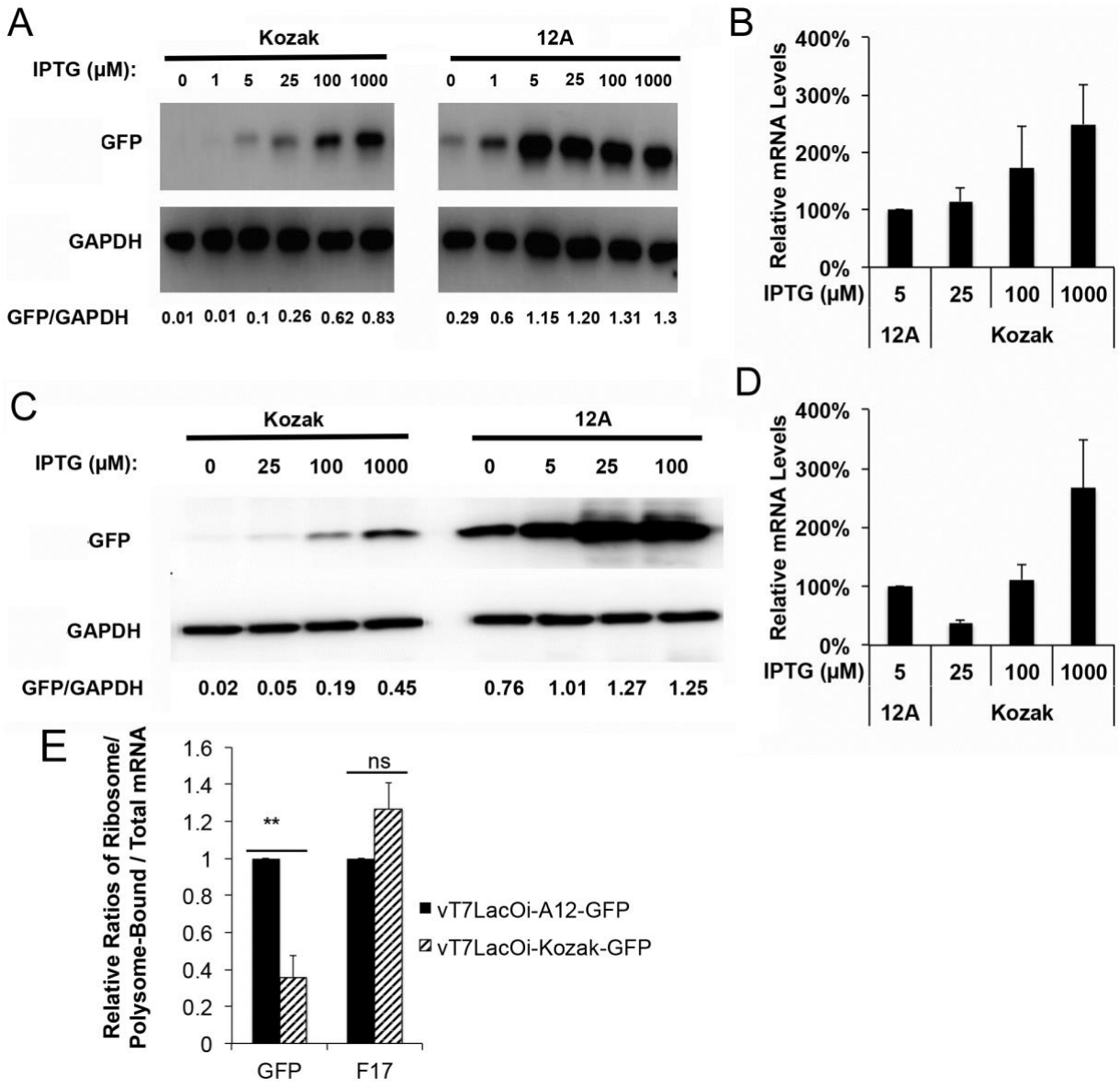

Figure 3-6: Messenger RNA with a 5'-poly(A) leader is more efficiently translated than mRNA without a 5'-poly(A) leader in VACV- infected cells.

(A) HeLa cells were transfected with plasmids expressing eGFP from a T7 promoter and containing an A-tract or a Kozak sequence between the ATG and the T7 promoter. Cells were infected with vT7LacOi 24 h post transfection and different concentrations of IPTG were added to the media as indicated. At 24 hpi, Western blotting tracked eGFP and GAPDH (loading control) expression levels; and eGFP intensity was normalized to GAPDH, shown below. (B) Levels of eGFP mRNA levels for (A) were measured by qRT-PCR, and normalized to 18S rRNA. (C) HeLa cells were infected with recombinant VACV vT7LacOi_Kozak-GFP or vT7LacOi_A12-GFP. Indicated concentrations of IPTG were added to the culture media. At 24

hpi Western blotting tracked eGFP and GAPDH (normalization and loading control) expression, shown below. (D) Levels of eGFP mRNA expression of (C) were measured by qRT-PCR, and normalized to 18S rRNA. (E) HeLa cells were infected with vT7LacOi_A12-GFP or vT7LacOi_Kozak-GFP. Total and ribosome/polysome-bound RNAs were isolated at 15 hpi. The GFP and F17 mRNAs were measured by qRT-PCR and normalized to 18S rRNA. The ratios of ribosome/polysome-bound mRNA to total mRNA were calculated for GFP and F17, respectively. The ratios from vT7LacOi_A12-GFP-infected cells were normalized to one. **indicates a P value < 0.01. ns = Not Significant (i.e. P value > 0.05).

Messenger RNA with a 5'-poly(A) leader capped by an ApppG cap analog can confer a translational advantage in VACV-infected cells

Only approximately 5 to 10% of the mRNA expressed from the bacteriophage T7 promoter in the VACV expression system contains the m7G cap structure (24), suggesting a possibility that the VACV post-replicative mRNAs with 5'-poly(A) leaders can be translated in a cap-independent manner in the experiment of **Fig 3-6C**. An ApppG cap cannot initiate cap-dependent translation but does protect mRNA from degradation and so is often used to test cap-independent mRNA translation (25, 26). We compared Fluc mRNA synthesized with an ApppG-cap to those with an m7G-cap, both with a 5'-poly(A) leader. Equal amounts of the mRNAs were transfected into VACV-infected HeLa cells, together with an m7G-capped Rluc mRNA, and luciferase activities were measured at 5 h post transfection. As expected, in uninfected cells, a 100-fold higher luciferase activity was produced from the m7G-capped mRNA compared to the ApppG-cap mRNA. Strikingly however, in VACV-infected cells, mRNAs capped with an ApppG were also efficiently translated (**Fig 3-7A**). The levels of ApppG-capped, as well as the m7G-capped mRNA were similar at 5 h post transfection in uninfected and VACV-infected cells (**Fig 3-7B**), ruling out the possibility of difference in mRNA levels as a source of different luciferase activities. The translational advantage of ApppG-capped mRNA in VACV-infected

cells was also observed in different cell types. We tested ApppG-capped mRNAs with different lengths of 5'-poly(A) leaders and observed much higher luciferase activity in VACV-infected cells compared to uninfected cells (**Fig 3-7C**). Interestingly, the length dependence in ApppG-capped mRNA was different from m7G-capped mRNA, which is likely due to different translation factors used in ApppG-capped and m7G-capped mRNA translation. In addition, large variations of ApppG-capped RNA translation in uninfected cells also contributed to the difference. Nevertheless, the conclusion of length dependence should be based on the mRNAs with m7G- capped mRNAs as the viral mRNAs are capped in VACV infected cells. These results suggested that mRNA with a 5'-poly(A) leader capped by an ApppG cap analog could confer a translational advantage in VACV-infected cells.

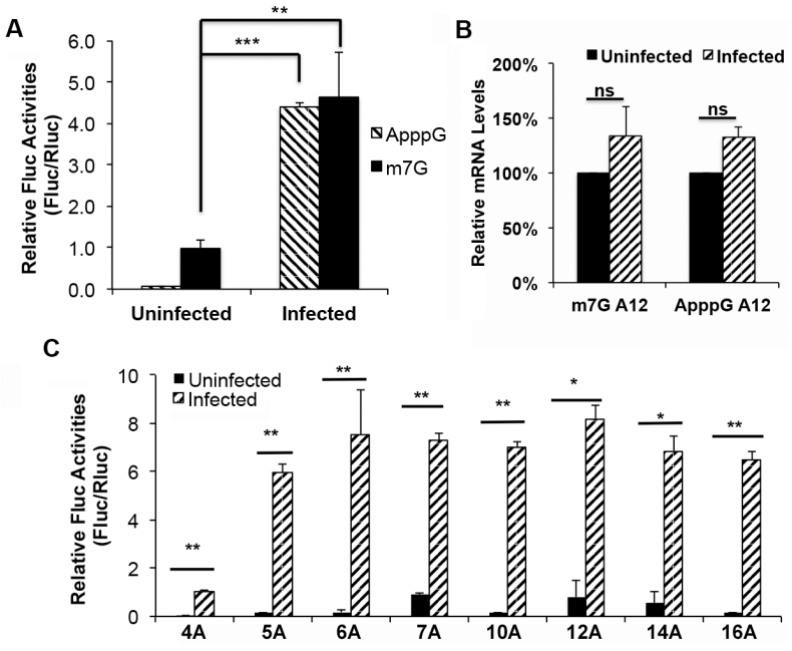

Figure 3-7: Messenger RNA with a 5'-poly(A) leader capped by ApppG cap analog can confer the translational advantage in VACV-infected cells.

(A) Relative luciferase activity from Fluc mRNA with a 5'- poly(A) leader capped with ApppG or m7G was measured at 5 h after transfection into uninfected or VACV- infected HeLa cells. Fluc activity was normalized by a co-transfected Rluc mRNA. Fluc activity from m7G- capped

mRNA in uninfected cells was normalized as 1. (B) Quantitative RT-PCR (qRT-PCR) compared the relative levels of Fluc mRNA capped by ApppG or m7G from uninfected and VACV-infected HeLa cells at 5 h post transfection. The amount of mRNA in uninfected cells was normalized as 1. (C) ApppG-capped Fluc reporter mRNAs, with different lengths of 5'-poly(A) leaders, were transfected into uninfected and VACV- infected HeLa cells together with an m7G Rluc mRNA. Luciferase activities were measured at 5 h post transfection. Error bars represent standard deviation (SD) of at least three experiments. P-values were determined using the Student's t-test; ***P value < 0.001, **P value < 0.01, *P value < 0.05, ns = Not Significant (i.e. P value > 0.05).

Messenger RNA with a 5'-poly(A) leader can confer a translational advantage in cells with impaired cap-dependent translation

Cap-dependent translation depends on the eukaryotic translation initiation factor 4E (eIF4E), which binds the 5' cap (m7G) and recruits other translation-initiation components. The eIF4E binding protein 1 (4E-BP1) can bind to eIF4E and inhibit translation initiation; and 4E-BP1 hyperphosphorylation disassociates it from eIF4E, allowing translation to initiate (27). To inhibit 4E-BP1 hyperphosphorylation, we suppressed PI3-kinase with the small molecule, LY294002, at 1 hpi and 8 hpi (28) (**Fig 3-8A**). The treatment reduced luciferase activities from transfected Fluc mRNA (with a poly(A) leader) and Rluc mRNA (with a 5'-UTR containing a Kozak sequence) in uninfected cells. In VACV-infected cells, mRNA with a 5'-poly (A) leader was still more efficiently translated, although somewhat less than in untreated cells, indicated by the ratios (3–5 fold) of firefly luciferase activities from VACV-infected cells to that from mock-infected cells (**Fig 3-8B**). In contrast, the corresponding ratios of renilla luciferase activities from co-transfected mRNA containing a Kozak element in its 5'-UTR were lower than one (**Fig 3-8B**). It is worth noting that we did not use renilla luciferase activities to normalize the firefly luciferase activities as the impairment of cap-dependent translation presumably affected renilla

mRNA translation. Moreover, in VACV-infected HeLa cells treated with LY294002 from 8 hpi, nascent viral protein synthesis was only slightly inhibited by LY294002, whereas translation of cellular proteins in uninfected cells was noticeably lower (**Fig 3-8C**). The treatment at 8 hpi rather than at earlier time of infection was to reduce the impact of unphosphorylated 4E-BP1 on VACV replication as early treatment of cells with LY294002 could inhibit VACV replication (28).

We employed another approach to impair cap-dependent translation by using three specific siRNAs that knocked down eIF4E at various levels in HeLa cells (**Fig 3-8D**). The knockdown of eIF4E reduced luciferase activities from transfected Fluc mRNA (with a poly(A) leader) and Rluc mRNA (with a 5'-UTR containing a Kozak sequence) in uninfected cells, corresponding to the levels of eIF4E knockdown. In contrast, in VACV-infected cells with eIF4E knockdown, the mRNA containing a poly(A) leader was efficiently translated, indicated by the high ratios of firefly luciferase activities from VACV-infected cells to that from uninfected cells (**Fig 3-8E**). The corresponding ratios of renilla luciferase activities from co-transfected mRNA containing a Kozak element in its 5'-UTR were much lower (**Fig 3-8E**), suggesting selective translation of poly(A)-headed mRNA in eIF4E knockdown cells infected with VACV. Consequently, the knockdown of eIF4E only reduced VACV gene expression at late time by approximately 2-fold using a reporter VACV expression firefly luciferase gene under the control of an early/late promoter (29) (**Fig 3-8F**). Similarly, there was only less than 2-fold reduction of virion production by a plaque assay (**Fig 3-8G**). Together, these findings demonstrated that even if cap- dependent translation was inhibited, mRNA with a 5'-poly(A) leader was still efficiently translated during the post-replicative stage of VACV replication.

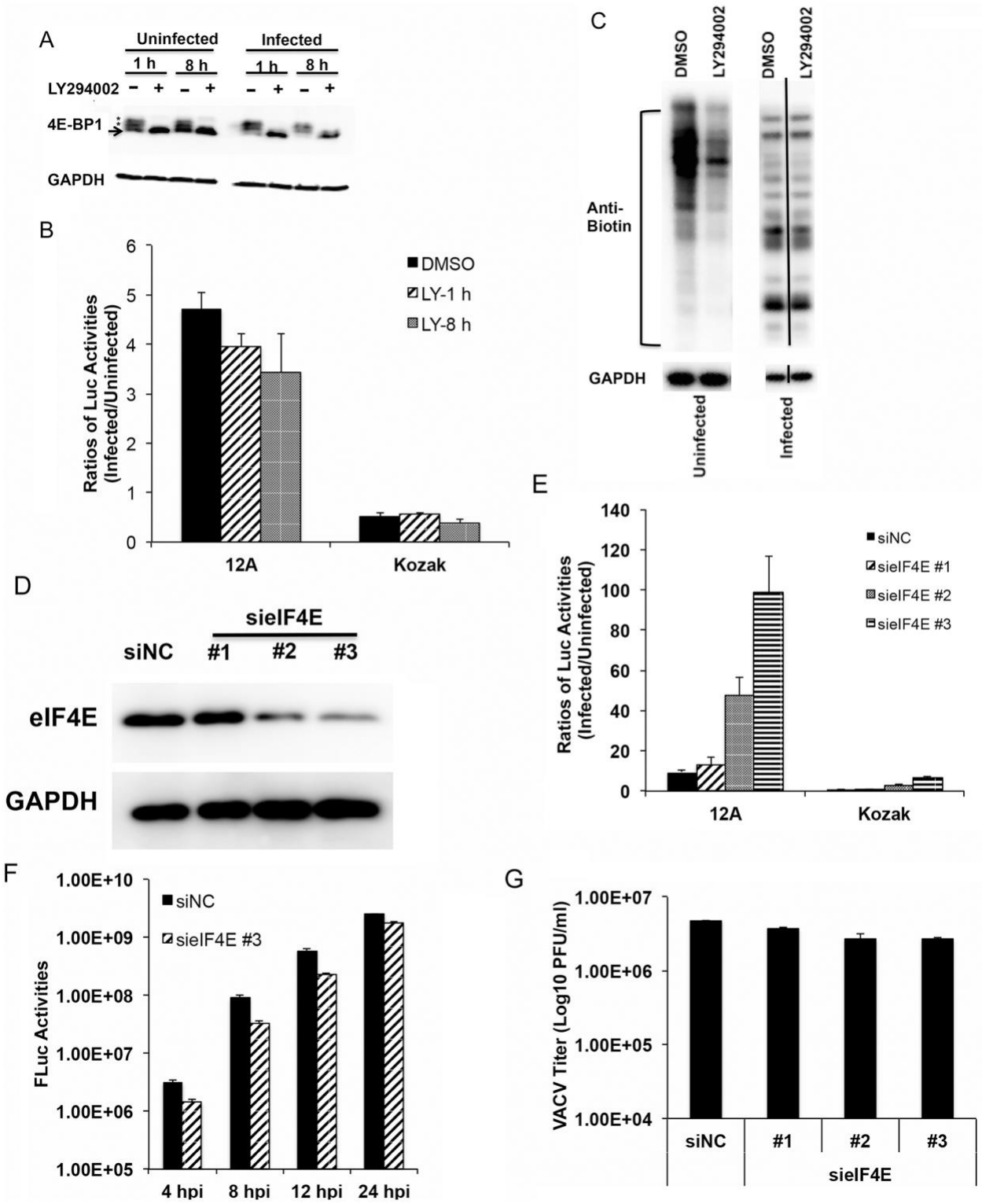

Figure 3-8: VACV post-replicative mRNAs are efficiently translated when eIF4E is inhibited.

(A) Hypophosphorylation of 4E-BP1 was induced by LY294002 in uninfected and VACV-infected HeLa cells at 1 hpi or 8 hpi and the cells were harvested for Western blotting analysis probed using anti-4E-BP1 antibody. An asterisk indicates hyperphosphorylated 4E-BP1. The arrow indicates hypophosphorylated 4E-BP1. (B) HeLa cells were treated with LY294002 at 1 or 8 hpi. An Fluc reporter mRNA with a 12A leader was transfected into uninfected and VACV-infected cells together with an Rluc mRNA at 12 hpi. Luciferase activities were measured at 5 h post transfection. The ratios of Fluc and Rluc activities from VACV-infected to uninfected HeLa cells were calculated, respectively. (C) HeLa cells infected with VACV and treated with LY294002 at 8 hpi. Newly synthesized proteins were labeled by AHA at 20 hpi and detected using antibodies against biotin conjugated to AHA using the Click-IT chemistry-based technique. The blots of infected-cell lysates were from different lanes on the same gel. (D) HeLa cells were transfected with control (siNC) or siRNAs targeting eIF4E. The protein levels of eIF4E and GAPDH were detected using specific antibodies 48 h post transfection. (E) HeLa cells were transfected with control or eIF4E siRNAs as in (D). An Fluc reporter mRNA with a 12A leader was transfected into uninfected and VACV-infected cells together with an Rluc mRNA at 12 hpi. Luciferase activities were measured at 5 h post transfection. The ratios of Fluc and Rluc activities from VACV-infected to uninfected HeLa cells were calculated, respectively. (F) HeLa cells were transfected with control or eIF4E siRNAs as in (D). 48 h post-transfection, the cells were infected with a recombinant VACV expressing firefly luciferase gene under a viral early/late promoter. Luciferase activities were detected at 8 hpi. (G) HeLa cells were transfected with control or eIF4E siRNAs as in (D). 48 h post-transfection, the cells were infected with VACV. The viral titers were determined at 24 hpi using a plaque assay. Error bars represent standard deviation (SD) of at least three experiments. P-values were determined using the Student's t-test; **P value < 0.01.

The 5'-poly(A) leader is not an internal ribosome entry site (IRES)

The IRES is the best-studied cap-independent mechanism and is used by many RNA viruses, e.g., poliovirus uses an IRES to initiate cap-independent translation (30, 31). To test whether an A-tract can function as an IRES, we synthesized a bicistronic mRNA in which renilla luciferase (Rluc) and firefly luciferase (Fluc) coding sequences are connected by a poliovirus IRES (polio IRES), an A-tract with 20 A residues, or a Kozak-containing 20-nt sequence (**Fig 3-9A**). The synthetic mRNA had an m7G cap so Rluc translation was cap-dependent. The polio IRES should drive Fluc translation whereas the Kozak-containing sequence should not. Since our reporter system was based on mRNA rather than a plasmid we could rule out interference from unexpected Fluc transcripts driven by cap-dependent translation. Synthetic RNA was transfected into uninfected or VACV-infected HeLa cells and the Fluc and Rluc activities were measured. As expected, Fluc driven by a poliovirus IRES was translated considerably in both uninfected and VACV-infected cells (**Fig 3-9B**); however, Fluc expression driven by the A-tract was negligible in both uninfected and VACV-infected cells, and at a level similar to that from the Kozak-containing sequence (**Fig 3-9B**). Interestingly, a comparison of Fluc and Rluc activities in uninfected and VACV infected cells indicated that the poliovirus IRES-driven Fluc mRNA translation was enhanced in VACV-infected cells, as evidenced by the significantly higher Fluc/Rluc ratio of the poliovirus IRES bicistronic mRNA (**Fig 3-9B**). These data indicated that the poly(A) leader did not function as an IRES.

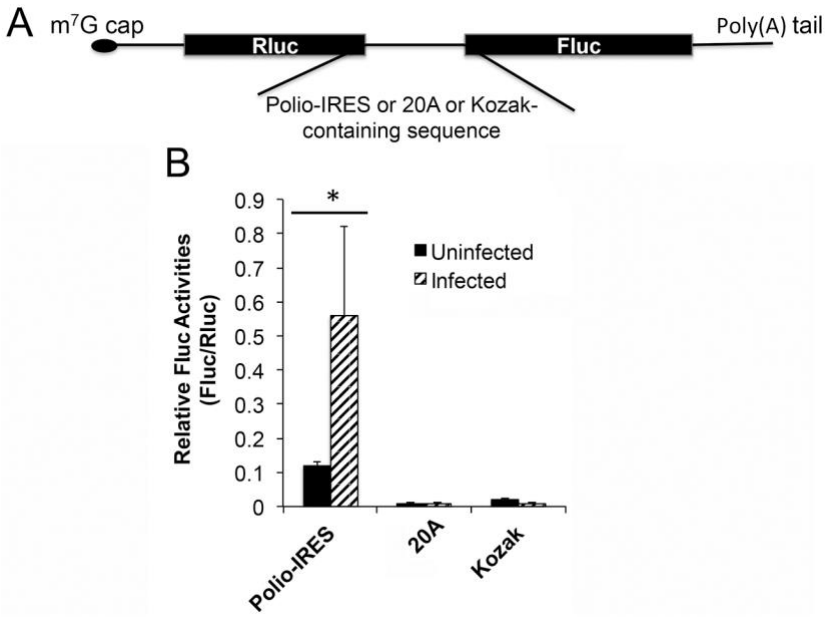

Figure 3-9: The 5'-poly(A) leader is not an internal ribosome entry site (IRES).

(A) Schematic of the bicistronic reporter mRNAs flanked by a poliovirus IRES, 20 A residues, or a Kozak-containing sequence. (B) The in vitro transcribed bicistronic mRNA was transfected into uninfected and VACV-infected HeLa cells and luciferase activities were measured 5 h post transfection. The ratios of Fluc to Rluc activities were calculated and displayed. Error bars represent standard deviation (SD) of at least three experiments. P-values were determined using the Student's t-test; *P value < 0.05, ns = Not Significant (i.e. P value > 0.05).

Discussion

Conferring a translational advantage by the 5'-poly(A) leader during poxvirus infection

After infection, VACV takes over host cell machineries to synthesize viral proteins rapidly and shut off cellular protein synthesis globally (32, 33). This can be largely attributed to cellular mRNA depletion and production of a large amount of viral mRNAs (11, 22). Cellular mRNA decay is accelerated due to VACV-encoded decapping enzymes and cellular transcription is also inhibited (34–39). In the meantime, virus-encoded, DNA-dependent RNA polymerase efficiently transcribes VACV mRNAs. Several hours after infection of HeLa cells, 70% of total mRNA is viral (11, 12). In VACV-infected cells, although the role of mRNA manipulation in host protein synthesis shutoff seems clear, it remains largely elusive whether and how the translational control contributes. As the takeover of protein synthesis in infected cells occurs after VACV DNA replication, it is conceivable that the VACV post-replicative mRNAs exert a translational advantage.

The 5'-poly(A) leader in several post-replicative VACV mRNAs was initially discovered almost three decades ago (6–8, 13). Our genome-wide survey demonstrated that all viral post-replicative mRNAs contain 5'-poly(A) leaders (5). The 5'-UTR can regulate translation efficiency of an mRNA (9). Nevertheless, it was unclear whether the 5'-poly(A) leader has a specific function or is simply because the VACV RNA polymerase "accidentally" slips during transcription at three T residues on the template strand. This study demonstrated that poxviruses use the 5'-poly(A) leader to efficiently synthesize viral post-replicative proteins, most of which are the viral building blocks. The finding here demonstrated the role of the 5'-poly(A) leader in efficient translation of VACV post-replicative mRNAs during VACV replication. However, our findings do not rule out other mechanisms that confer a translational advantage of VACV post-replicative mRNAs. Interestingly, although the enhancement of poly(A)-headed mRNA in

VACV-infected cells in all experiments, the enhancement levels varied in different experiments. The variations were likely due to quick VACV replication that resulted in variations of cellular environments at the time of RNA transfection. Most of the 5'-poly(A) leaders of VACV post-replicative mRNAs are between 8 and 12 A residues (5); this agrees with data showing 5'-poly(A) leaders within that range confer the optimal translational advantage. Thus, the 5'-poly(A) leader is convincingly an evolutionarily optimized, transcriptionally and translationally coordinated element that VACV utilizes to maximize its protein production.

A-rich tracts with various lengths are present in other eukaryotic mRNA 5'-UTRs. In yeast, A-rich tracts can be found in over 3,000 5'-UTRs. Interestingly in yeast, protein abundance correlates with the size of the A-rich tracts and mRNAs containing 5'-UTRs with 12 consecutive A residues generate large amounts of protein. Although the number of A residues is similar to those in the 5'-poly(A) leaders of VACV mRNAs, the yeast A-tracts are usually embedded internally in the 5'-UTR (40). In contrast, poly(A) leaders of VACV mRNAs are located at the very 5' end and usually are the only 5'-UTR sequence. An A-rich tract was also found in the 5'-UTRs of other viruses, such as crucifer-infecting tobamovirus and avian herpesvirus (41, 42); however, as in yeast, they are usually present inside of the 5'-UTRs rather than at the very 5' end. Additionally, unlike many of the other A-rich tracts, the 5'-poly(A) leader of VACV mRNA is not an IRES, although it can be efficiently translated in cells with impaired cap-dependent translation. These differences suggest poxvirus mRNAs employ a distinct mechanism.

Although our findings strongly indicate higher translation efficiency of poly(A)-headed mRNA in VACV-infected cells, especially since there was no significant difference with transfected RNAs after 5 h, the possibility that poly(A)-headed mRNA transcribed from viral

genome is more stable in VACV-infected cells has not been ruled out. In fact, we observed higher levels of 12A-headed mRNA (IPTG at 5 μM) than Kozak sequence-headed mRNA (IPTG at 25 μM) transcribed from recombinant VACV genome (**Fig 3-6D**), which could be attributed to higher mRNA stability and/or more active transcription of the 12A-headed mRNA.

Could the 5'-poly(A) leader mediate cap-independent translation during poxvirus infection?

Many RNA viruses, such as piconaviruses, crucifer-infecting tobamovirus, hepatitis C virus, and Foot-and-mouth disease virus can synthesize their proteins through a cap-independent translation mode (41, 43–45). The most studied mechanism is through viral IRESs, which usually bear highly complex structures to recruit 40S ribosome (46). Some RNA viruses use other cap-independent mechanism such as 3' cap-independent translational enhancer (3'CITE) in their mRNAs to recruit ribosome subunits (47, 48). Cap-independent translation is less appreciated in DNA viruses. In this study, we show that a poly(A)-headed mRNA is efficiently translated in cells with impaired cap-dependent translation (**Fig 3-8**) as well as without an m7G cap (**Fig 3-7**), which strongly suggests that a short, unstructured 5'-poly(A) leader may mediate cap-independent translation in VACV-infected cells. In literature, Mulder et al. showed that VACV protein synthesis only requires a low level of intact translation initiation factor eIF4F (49). In another in vitro study, Shirokikh et al. showed that the translation initiation complex could be formed on a 5'-poly(A) leader mRNA without the need of eIF4E, a rate-limiting and cap binding translation factor (50). Additionally, in yeast, crucifer-infecting tobamovirus, and avian herpesvirus, an A-rich tract in some 5'-UTRs is suggested to function as an IRES (26, 41, 42), an RNA element allowing for a form of 5' cap-independent translation initiation.

Recruitment of poly(A) binding protein (PABP) through the A-rich tracts plays an

important role in IRES-mediated cap-independent translation initiation. During the review process of this manuscript, Jha et al. reported that a small ribosomal subunit protein, receptor for activated C kinase (RACK1), is important for efficient translation of VACV post-replicative mRNAs (51). RACK1 was previously found to be important for IRES-mediated cap-independent translation of several RNA viruses (52). These findings support the possibility that the poly(A) can mediate cap-independent translation although it does not serve as an IRES (**Fig 3-9**).

VACV encodes its own capping enzymes, which cap VACV mRNAs with methylated guanosine, including the post-replicative mRNAs (15). The mRNA translation occurs in the "viral factory", the site of viral replication (53). Cap-dependent translation initiation factors are recruited to and concentrated within discrete subcellular compartments of the viral factory (54). VACV mRNAs can be translated in a cap-dependent manner. The possibility that a cap-independent translation mode can be employed by the VACV post-replicative mRNAs to confer a translational advantage does not exclude cap-dependent translation used by these mRNAs. It is likely that VACV uses both cap-independent and cap-dependent translation modes to maximize the translation potential of viral post-replicative mRNAs in different cellular environments. VACV infection globally shuts off host protein synthesis after DNA replication, coinciding with viral post-replicative mRNA synthesis (32, 33). During this stage, it is conceivable that the ability to utilize cap-independent translation is important because the global protein synthesis shutoff downregulates expression of most host proteins, including cap-dependent translation initiation factors. Meanwhile, a large amount of viral mRNAs need to be translated. Cap-independent translation could also ensure efficient viral mRNA translation in other physiological conditions, such as during cell mitosis and various stress conditions (including poxvirus infection itself), in which cap-dependent translation is suppressed (55). In fact, under

different conditions, some eukaryotic cellular mRNAs can employ both translational modes to ensure synthesis of necessary corresponding proteins (46). Similar to these cellular mRNAs, the VACV post-replicative mRNAs may switch between cap-dependent and cap-independent translation according to the availability of eukaryotic translation initiation factors and cellular environments.

While the experiments using an ApppG-capped RNA strongly support a cap-independent translation mode during the post-replicative stage of VACV replication (**Fig 3-7**), a caveat of the experiments is that VACV encodes both decapping and recapping enzymes that may remove and recap the transfected ApppG-capped mRNA in VACV-infected cells (14, 34, 35, 56). We do not rule out the possibility that the poly(A)-headed mRNA employs an alternative cap-dependent translation mode that requires a minimal amount of cap-binding translation initiation factor eIF4E. In fact, a minimal requirement of the eIF4F translation initiation complex has also been observed during the late stage of cytomegalovirus infection (57). Future experiments will further investigate these aforementioned different possibilities. Recombinant VACVs with defective decapping or capping enzyme expression will be useful in such study.

As all viruses rely on host translation machinery to synthesize viral proteins, VACV likely employs and modulates an existing cellular mechanism for its mRNA translation. The data presented here is an important step in uncovering this novel cellular translation mechanism. The 5'-poly(A) leader can also be used to increase foreign gene expression when using poxvirus-based vectors, as demonstrated in this study.

Materials and methods

Cells and viruses

HeLa cells (ATCC-CCL2) and human foreskin fibroblasts (HFFs, kindly provided by Dr. Nicholas Wallace) were cultured in Dulbecco's modified eagle's medium (DMEM, Quality Biological) with 10% fetal bovine serum (FBS, Peak Serum). BHK21 (C13) (ATCC CCL10), RK13 (ATCC CCL37) and BS-C-1 (ATCC CCL-26) cells were cultured in Eagle's Minimum Essential Medium (EMEM, Quality Biological) with 10% FBS (Peak Serum). All cells were incubated in a 5% CO2 atmosphere at 37°C. The recombinant VACV with a firefly luciferase gene under the control of a viral early/late promoter was a gift from Dr. Bernard Moss and described previously (29). The recombinant VACV with intermediate transcription factor gene A23 deletion (vA23Δ) was a gift from Dr. Bernard Moss and was described elsewhere (20). Preparation, infection and titration (by plaque assay) of the VACV Western Reserve (WR) strain (ATCC VR-1354) and recombinant VACVs derived from it were carried out as described elsewhere (58). Myxoma virus was kindly provided by Dr. Stefan Rothenburg.

In vitro RNA synthesis

Primers were designed to produce DNA fragment containing the T7 promoter followed by a 5'-UTR sequence of interest, reporter gene (firefly or renilla Luciferase) and poly(A) tail coding sequence. These primers were used to synthesize DNA fragments by PCR using a Q5 High-Fidelity 2X Master Mix (New England Biolabs). The synthesized DNA was used as template to generate RNA using a HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). The synthesized RNA was capped using m7G (Anti-Reverse Cap Analog (ARCA)) or ApppG cap analog (New England Biolabs) according to the manufacturer's instructions. The resulting RNA was purified using a PureLink RNA Mini Kit (Thermo Fisher Scientific) and

quantified using a NanoDrop ND-2000 instrument (Thermo Fisher Scientific). The IRES reporter plasmid pcDNA3 RLUC POLIRES FLUC used for biscistronic mRNA production was a gift from Nahum Sonenberg (Addgene plasmid # 45642) (31).

Transfection and luciferase assay

Capped and polyadenylated firefly luciferase RNA (480 ng in one well of a 24-well plate) was transfected into cells using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. When necessary, renilla luciferase reporter RNA (20ng in one well of a 24-well plate) was co-transfected as an internal control. The luciferase activities were measured at 5 h post transfection using a dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions, on a microplate luminometer (Promega).

Knockdown of eIF4E using small interfering RNA (siRNA)

The siRNAs were purchased from Integrated DNA technologies (IDTDNA). HeLa cells were transfected with sieIF4E or siNC (negative control) at the final concentration of 5 nM using Lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were harvested to examine the effects of siRNA knockdown by Western blotting analysis or infected with VACV for various assays 48 h post transfection.

Western blotting analysis

Cells were lysed and heated in sample buffer. The cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a polyvinylidene difluoride membrane, which was blocked with 5% bovine serum albumin (BSA) in TBST solution (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.05% Tween 20) at room temperature for 1 h. The membrane was then incubated with primary antibody in TBST-BSA

buffer for 1 h at room temperature or overnight at 4°C, washed with TBST, incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, washed with TBST, and developed using a chemiluminescent substrate. The intensities of the bands were quantified using Image J.

Antibodies and chemicals

Anti-GFP, anti-eIF4E and anti-4E-BP1 antibodies were purchased from Cell Signaling Technology. Anti-GAPDH was purchased from Abcam. LY294002 was purchased from Cayman Chemical.

Pulse-chase labeling and detection of newly synthesized proteins with AHA (L-azidohomoalanine)

The experiment was carried out using Click-iT AHA nascent protein kit (Thermo Fisher Scientific). Briefly, HeLa cells were cultured in a T-75 flask to a confluence of 95%. The cells were infected or mock infected with VACV at a MOI of 10. The cells were treated with DMSO or LY294002 (25 μM) at the desired times of infection. Cell culture medium was replaced with methionine-free medium at the indicated times. After incubation in methionine-free medium, AHA was added to the medium at 100 μM for 2 h. The cells were scraped off the flask and collected by centrifuging. Cell pellets were then suspended with 500 μl of lysis buffer containing 500U of benzonase for 30 min. After centrifuging at 12,000 g at 4°C for 10 min. The proteins were precipitated with methanol and chloroform and resolubilized in 50 mM Tris-HCl containing 1% SDS, pH 8.0. AHA-containing peptides were labeled with alkyne-biotin, by subjecting 200 μg of proteins to the click reaction for 30 min using the Click-iT protein labeling kit according to the manufacturer's instruction (Thermo Fisher Scientific). Proteins were re-precipitated with

methanol and chloroform, solubilized with 50mM Tris-HCl containing 1% SDS, pH 8.0, for Western blotting analysis.

Recombinant VACV generation

The recombinant viruses used in this study include vT7LacOi-Kozak-GFP and vT7LacOi-A12-GFP. The vT7LacOi-Kozak-GFP and vT7LacOi-A12-GFP were derived from the parental virus vT7LacOi that is capable of isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible T7 promoter-controlled expression of foreign genes (59). The eGFP-encoding sequence downstream of either a Kozak encoding sequence (A ATT GTG AGC GCT CAC AAT TCC CGC CGC CAC C; vT7LacOi-Kozak-GFP) or 12 A residues (vT7LacOi-A12-GFP), under the control of a T7 Promoter, were inserted between the VACWR146 and 147 ORFs.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Ambion) followed by purification using a PureLink RNA Mini Kit (Thermo Fisher Scientific). The RNA was used to synthesize cDNA using SuperScript III First-strand synthesis (Invitrogen) according to the manufacturer's instructions using random hexamers. Quantitative RT-PCR was carried out using iTaq Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer's directions and specific primers of desired genes.

Isolation of total RNA and ribosome- and polysome-bound RNA

HeLa cells were treated with 100 μg/ml cycloheximide for 15 minutes at 37°C before harvesting. The harvested cells were processed to isolate total mRNAs or ribosome- and polysome-bound mRNAs as described with modifications (60). The total RNA was isolated using TRIzol reagent followed by purification using a PureLink RNA Mini Kit. For ribosome/polysome-bound RNA, the harvested cells were resuspended in ribosome homogenization buffer (50 mM Tris-HCl (pH

7.5), 5 mM MgCl2, 25 mM KCl, 1% Triton X-100, 100 μg/ml cycloheximide, 10 mM Vanadyl ribonucleoside complex and 0.2 M sucrose) and incubated for 20 min on ice. After centrifuging at 20,000 g for 10 min at 4°C, the supernatant was collected and gently layered over sucrose cushion buffer (50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 25 mM KCl, 100 μg/ml cycloheximide, 10 mM Vanadyl ribonucleoside complex and 2 M sucrose) and ribosome homogenization buffer (1:1 ratio). The sucrose cushion was centrifuged at 35,000 rpm using SW41 Ti rotor for 20 hrs. The ribosome and polysome-bound mRNA was isolated from the pellet using TRIzol reagent followed by purification using a PureLink RNA Mini Kit. DNA was removed using DNase I from RNA samples.

Relative translation efficiency analysis

Relative translation efficiency (TE) was defined as the ratio of ribosome-protected RNA reads to mRNA reads as described elsewhere (61, 62). The mRNA and ribosome-protected RNA reads were obtained from the studies described elsewhere (21, 22).

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Author contribution

The conceptualization of the study was done by ZY and PD. Funding acquisition, project administration, supervision was done by ZY. PD is the lead author in this study and contributed to all figures except Fig 3-5 and 3-8 C. The original draft was written by PD and ZY, reviewed and edited by PD, ZY and SC.

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Chapter 4 - La-Related Protein 4 (LARP4) is required for 5'-poly(A) leader-mediated translational advantage of Vaccinia Virus mRNA

Pragyesh Dhungel, Zhilong Yang*

Division of Biology, Kansas State University, Manhattan, KS, 66506

* Address correspondence to Zhilong Yang, zyang@ksu.edu

Abstract

The 5'-poly(A) leader, a unique feature among all poxvirus post-replicative mRNAs, confers a translational advantage to viral post-replicative mRNAs. The poly(A) leader present at the very 5' end of the 5'UTR bestows a translation advantage in cells with impaired cap-dependent translation. In the present study, we identified a cellular RNA-binding protein, La-related protein 4 (LARP4) that is required for 5'-poly(A) leader-mediated translational advantage. We carried out an immunofluorescence assay to determine the factors enriched in the virus factory, where poxvirus post-replicative mRNAs translate. During VACV infection, LARP4—unlike poly(A) binding protein (PABP)—is enriched in the virus factory. Depletion of LARP4 significantly reduced the VACV titer and blocked post-replicative protein expression. Despite the moderate decrease in VACV DNA replication and post-replicative gene transcription observed during LARP4 depletion, the major effect is observed on the translation of post-replicative mRNAs. In fact, a decrease in LARP4 protein level diminishes 5'-poly(A) leader-mediated translational advantage. Importantly, LARP4 is crucial for the cap-independent mode of translation from 5'-poly(A) leader. Further studies showed that LARP4 is dispensable for mRNA stability during VACV infection. These results indicate that LARP4 is required for efficient VACV post-replicative mRNA translation.

Introduction

Viruses induce the global shutdown of host protein synthesis—termed host shutoff—that facilitates host immune evasion and the reapportion of cellular resources (1–4). To selectively synthesize viral proteins during host shutoff, viruses use numerous approaches to regulate translation machinery and/or possess a sophisticated evolutionary element in their mRNA (5–9). Intriguingly, poxvirus devised a dual and coordinated approach to selectively synthesize viral proteins: (I) by modulating factors of host translation machinery (10–12) and (II) by acquiring an evolutionarily optimized cis element in its post-replicative mRNA (13–16). The cis element present in all poxvirus post-replicative mRNAs is known as the 5'-poly(A) leader (13–16). Previous studies have shown that the 5'-poly(A) leader provides a translational advantage to viral post-replicative mRNAs during poxvirus-induced host shutoff (10,17). Remarkably, poxviral poly(A) leader-mediated translation advantage can be achieved using both cap-dependent and cap-independent modes to maximize their protein synthesis during an adverse condition of host shutoff (17,18). However, the mechanism and specific factors required for the poly(A) leader-mediated translational advantage remains elusive.

RNA-binding proteins play a crucial role in regulating the translation of mRNAs. One such family of RNA-binding proteins is the La protein family that comprises the La RNA-binding motifs and the RNA recognition motif (19,20). As a member of the La protein family, La-related protein 4 (LARP4) is known to bind to poly(A) RNA sequences, the receptor for activated C kinase 1 (RACK1), and poly(A) binding protein (PABP) (19). LARP4 promotes mRNA stability by binding to the 3' poly(A) tail and preventing deadenylation (21). The RNA-binding La motif, RNA recognition motif, and poly(A) binding protein-interacting domain in LARP4 are necessary for its mRNA stability function (21). Moreover, the predominantly

cytoplasmic LARP4 is also postulated to play a role in translation as it is associated with actively translating ribosomes (19).

Although LARP4 is suggested to be important in translation and known to bind to RACK1, which is required for poly(A) leader-mediated translational advantage, whether LARP4 is imperative for poxvirus 5'-poly(A) leader bearing mRNA translation remains unknown. In the present study, using an immunofluorescence assay, we showed that LARP4 is enriched in the virus factory during infection with VACV, the prototypic poxvirus. Further experiments demonstrated that LARP4 depletion is unfavorable for VACV replication and negatively affects post-replicative gene expression. Moreover, LARP4 is essential for 5'-poly(A) leader-mediated translation, especially for the cap-independent translation mode. During VACV infection, LARP4 binding to 5'-poly(A) leader is dispensable for mRNA stability. Our results suggest that LARP4 promotes poly(A) leader translational advantage probably via direct binding to the 5'-poly(A) leader cis element, thereby enhancing translation initiation.

Results

The RNA-binding protein La-related protein 4 (LARP4) is enriched in the virus factory during VACV infection

The unique 5'-poly(A) leader of poxvirus post-replicative mRNA provides a translational advantage to these viral mRNAs in cells with impaired cap-dependent translation (10,17). Since the 5'-poly(A) leader is at the 5'-UTR region that regulates translation, 5'-poly(A) leader-mediated translational advantage can be achieved through both cap-dependent and cap-independent translation, which supports the notion that certain trans-factors may bind directly to the 5'-poly(A) leader of an mRNA to initiate translation, thereby critically influencing VACV post-replicative gene expression (17,22). VACV replicates in the cytoplasm forming a cytoplasmic DNA foci known as a virus factory (23). Since VACV post-replicative mRNA translation occurs within the virus factory, trans-factor binding to the poly(A) leader must be enriched in the virus factory (11,24). To gain insight, we examined the sub-cellular localization of proteins that have been known to bind to the poly(A) sequence in RNA (19,25,26). Our first idea was to test for well recognized poly(A) RNA-binding protein, PABP (25). Determination of the sub-cellular localization of PABP in uninfected and VACV-infected primary human foreskin fibroblasts (HFFs) was determined by immunofluorescence assay. For this assay, uninfected and VACV-infected cells (MOI=2, 12 hpi) were fixed with formaldehyde and stained with antibodies for PABP and nuclear stain DAPI. On top of staining cellular nuclei, DAPI also stains the virus factory (indicated by **V**). As expected, PABP was localized in the cytoplasm in uninfected HFF (**Fig 4-1A, top row**). Notably, we observed that PABP was excluded from the virus factory during VACV infection (**Fig 4-1A, bottom row**). Although initially surprising, we discovered literature indicating that—unlike other translation initiation factors—PABP is not enriched in the

virus factory during VACV infection (11,27). These observations suggest that PABP might not be required for poly(A) leader-mediated function.

In pursuit of a potential poly(A) RNA sequence binding protein, we came across LARP4, a member of the La protein family that is known to bind to poly(A) sequence at the 3' end of an mRNA (19,21,28). Our immunofluorescence assay showed that endogenous LARP4 primarily localizes in the cytoplasm of uninfected HFFs (**Fig 4-1B, top row**). During VACV infection, LARP4 enrichment occurs in multiple regions of the cytoplasm. Notably, the LARP4 enrichment region co-localizes to the virus factory where VACV post-replicative mRNA translation occurs (**Fig 4-1B, middle row**). The LARP4 enrichment in virus factory was detected as early as 4 hpi and continued to a tested time of 24 hpi. Next, we sought to understand whether LARP4 enhancement in the virus factory requires VACV DNA replication and/or post-replicative gene expression. To answer this question, we utilized the intermediate transcription factor gene A23 deleted VACV (VACV-ΔA23), which undergoes DNA replication (forming virus factory); however, the post-replicative gene expression is blocked. HFFs were infected with VACV-ΔA23, and sub-cellular localization of LARP4 was determined. At 12 hpi, we observed many virus factories. However, LARP4 was remarkably not enriched but excluded from these factories (**Fig 4-1B, bottom row**). These data demonstrate that LARP4 is enriched in the virus factory during VACV infection and that post-replicative gene expression is necessary for this enrichment.

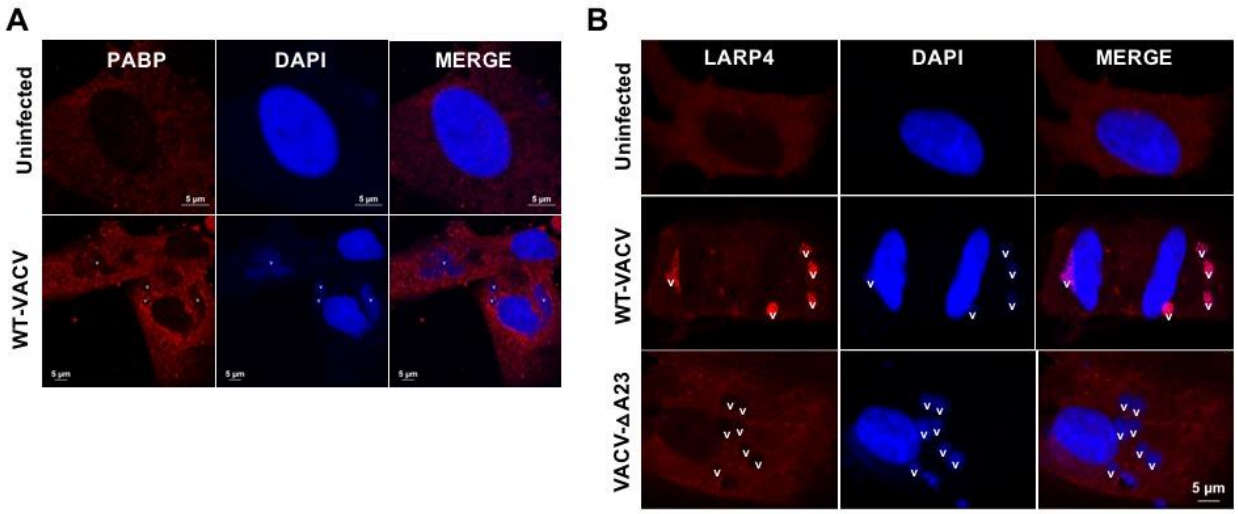

Figure 4-1: LARP4 is enriched in Virus factory during VACV infection.
(A) Immunofluorescence assay of uninfected and WT-VACV infected HFF (MOI=2, 12 hpi). Anti-PABP was used to determine localization of PABP (red) and DAPI (blue) was used to stain cellular nuclei and virus factory (indicated by V). Panel shows representative image. Scale bars: 5 µm. (B) Immunofluorescence assay of uninfected and WT-VACV or VACV-△A23 infected HFF (MOI=2, 12 hpi). At 12 hpi, HFFs were fixed and stained with respective antibodies. Anti-LARP4 was used to determine localization of LARP4 (red) and DAPI (blue) was used to stain cellular nuclei and virus factory (indicated by V). Panel shows representative image. Scale bar: 5 µm.

Reduction of LARP4 protein level reduces VACV replication

Most of the VACV post-replicative genes encode for structural proteins required for virion formation and is thus vital for viral replication (23). If LARP4 is necessary for the 5'-poly(A) leader, a feature of VACV post-replicative genes—depletion of LARP4—would affect VACV replication. To test this, HFFs depleted with LARP4 were infected with VACV at the multiplicity of infection of three. At 1 hpi, culture media was changed with or without the DNA replication inhibitor cytosine arabinoside (AraC, 40 µg/mL). At 12 hpi, we tested the cytopathic effect (CPE) of VACV infection on HFFs. Basic light microscopy showed that

LARP4 depletion with two siRNAs (#1 and #2) protected the cells from VACV-induced CPE compared to negative control siRNAs (**Fig 4-2A**). AraC treatment prevents VACV DNA replication; however, early gene expression continues up to 12 hpi (29). In fact, certain VACV early gene can induce CPE regardless of the replication status of the virus (30). This indicates that the minor CPE observed in the AraC treatment was instigated by early gene expression (**Fig 4-2A**). To determine VACV replication during LARP4 depletion in HFFs, we performed a plaque assay at 24 hpi. Compared to the control siRNAs, both siRNAs (#1 and #2) targeting LARP4 reduced VACV titer by 10-fold and 14-fold, respectively (**Fig 4-2B**). These results showed that a decrease in LARP4 leads to a concomitant reduction in VACV replication. Moreover, a previous study indicated that LARP4 depletion reduces cellular protein synthesis by 10-15% (19). During siRNA-mediated depletion of LARP4 in HFFs, we did not observe any decrease in the puromycin-labeled nascent cellular protein synthesis (**Fig 4-2C**). This result rules out the possibility of decreased VACV titer due to the decline of nascent cellular protein synthesis during LARP4 knockdown.

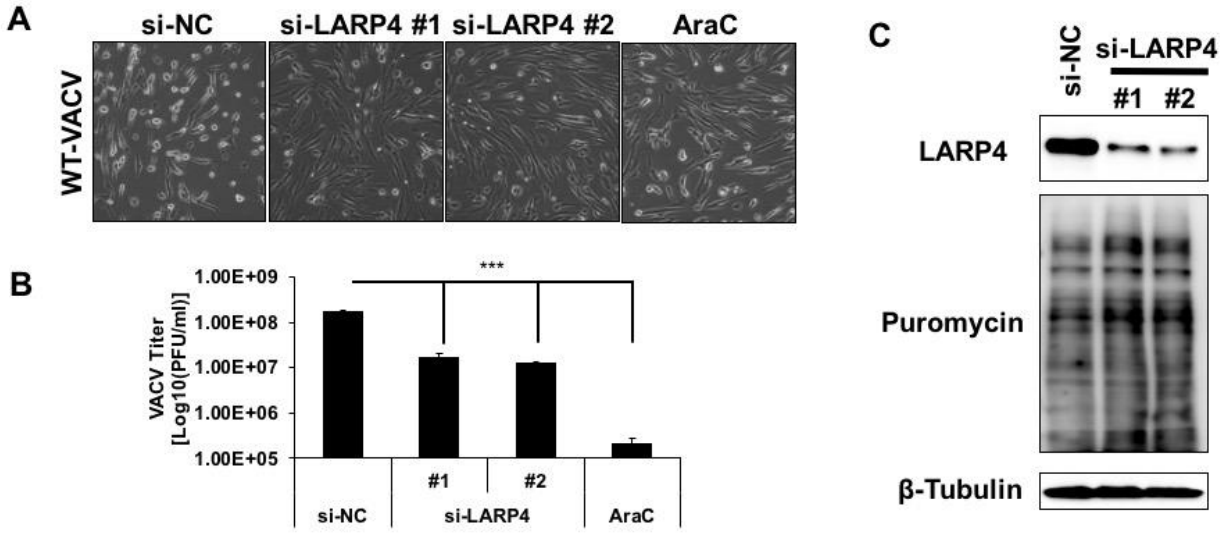

Figure 4-2: Decrease of LARP4 protein level reduces VACV Replication.

At 72 hours post siRNA transfection, HFFs were infected with VACV at a MOI of 3 (**A** and **B**). AraC (40 μg/mL) treatment was used as a positive control. (**A**) VACV infected cells were observed 12 hours post infection by microscopy. (**B**) At 24 hpi, HFFs were collected for plaque assay. Titer of the VACV during indicated treatment was determined by plaque assay using BSC-1 cells. (**C**) HFFs were transfected with negative control siRNA (si-NC) or siRNAs targeting LARP4. Nascent protein synthesis was determined by treating cells and labelling nascent protein with puromycin (10 μg/ml) for 20 minutes at 37°C. Level of LARP4, puromycin-labelled nascent protein, and β-Tubulin proteins were detected using specific antibodies 72 hours post siRNA transfection. Error bars represent standard deviation (SD) of at least three experiments. P-values were obtained using the Student's t-test; ***P value <0.001.

LARP4 knockdown suppresses post-replicative gene expression

To determine whether the reduction of VACV replication during LARP4 depletion was due to impaired VACV protein synthesis, Western blotting analysis was performed under similar condition. VACV protein synthesis occurs in a temporal manner, where early genes are expressed before DNA replication, whereas intermediate and late genes are expressed after DNA replication (28–30). As a result, intermediate and late genes are collectively termed post-replicative genes (34). Before infection, the control or LARP4 siRNAs were transfected into HFFs for 72 hours. The HFFs were then infected with VACV at MOI of 5 in presence or absence of AraC. Since VACV early, intermediate, and late proteins are synthesized at different times post infection, we collected lysate from VACV-infected HFFs at 4, 8, and 12 hpi. The samples were evaluated for LARP4, GAPDH, VACV early (E3L), intermediate (D13L), and late (A10L/P4a) proteins. During LARP4 depletion, synthesis of VACV early protein (E3L) was similar to control siRNA treated cells. Remarkably, the synthesis of VACV intermediate (D13L), and late (A10L/P4a) proteins was nearly undetectable until 12 hpi during LARP4 depletion (**Fig 4-3A**). As expected, post-replicative gene expression was completely blocked in the AraC

treatment. Immunoblotting of GAPDH demonstrates equal loading (**Fig 4-3A**). During LARP4 reduction, a block in viral post-replicative protein synthesis could be attributed to a change in post-replicative mRNA level. We performed qRT-PCR to determine VACV early (E3L), intermediate (Gaussian), and late (A10L/P4a) mRNAs level at 12 hpi. Compared to control siRNAs, LARP4 knockdown using siRNA #1 did not reduce VACV early and intermediate mRNA levels; however, a 30% reduction in late mRNA was observed (**Fig 4-3B**). LARP4 knockdown using siRNA #2 showed marginal non-significant reduction of early genes, leading to a 34% and 80% reduction in intermediate and late mRNA level, respectively. Notably, AraC treatment that prevents post-replicative gene expression led to a 98% and 99.9% reduction in intermediate and late mRNA level, respectively (**Fig 4-3B**). These findings indicate that LARP4 knockdown completely blocks post-replicative protein synthesis while instigating only a moderate decrease in intermediate mRNA level and a significant decrease in late mRNA level, which is expected in cascade gene regulation.

We subsequently investigated whether the drastic block in VACV post-replicative protein synthesis could be due to the inhibition of viral DNA replication, which is a pre-requisite for post-replicative gene transcription. To test this question, we collected total DNA from control and LARP4 si-RNA transfected VACV-infected HFFs at 1 hpi and 24 hpi. AraC treatment was used as a positive control. The amount of VACV DNA was determined using qRT-PCR using specific primers for the C11 gene. Since VACV DNA synthesis occurs around 2 hpi, we determined the fold change in VACV DNA at 24 hpi compared to the time where the DNA replication hasn't occurred at 1 hpi (34,35). Compared to control siRNAs, LARP4 siRNAs #1, #2, and AraC induced 3-fold, 4.5-fold, and 450-fold decreases in DNA level, respectively (**Fig 4-3C**). During LARP4 knockdown, a complete blockage of VACV post-replicative protein

synthesis occurs (**Fig 4-3A**), while minor decrease in VACV DNA replication (**Fig 4-3C**) and a significant yet modest post-replicative gene transcription (**Fig 4-3B**) was observed. Altogether, these data suggest that the major contributor to the impairment of VACV post-replicative gene expression during LARP4 depletion is the inhibition of post-replicative mRNA translation.

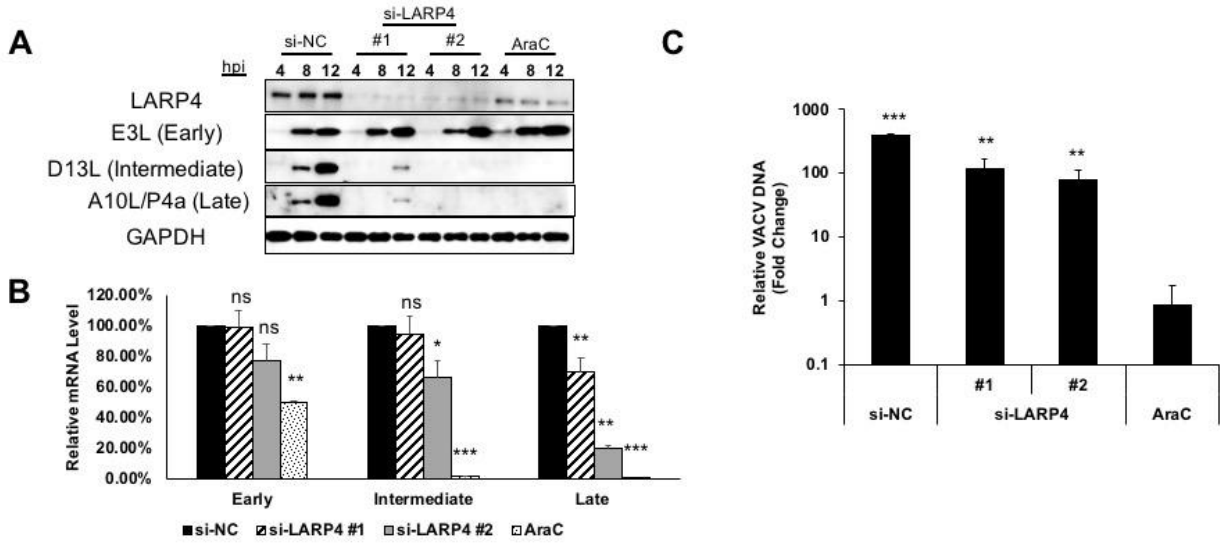

Figure 4-3: Depletion of LARP4 negatively affects post-replicative gene expression. HFFs cells were transfected with control or LARP4 siRNAs. 72 hours post transfection, HFFs were infected with VACV (A) or gaussian luciferase-expressing recombinant VACV (B and C) at a MOI of 5. AraC (40 μg/mL) treatment was used as a positive control. (A) At indicated times the HFFs were lysed, and the lysates were analyzed by western blot to determine the levels of LARP4, VACV early (E3L), intermediate (D13L), late (A10L/P4a) and GAPDH proteins. (B) At 12 hpi, levels of VACV early (E3L), intermediate (G8R driven Gaussian), late (A10L/P4a) mRNAs were measured by qRT-PCR, and 18S rRNA was used for normalization. (C) At 1 and 24 hpi, total DNA was extracted from the infected HFFs with indicated treatment. Level of VACV DNA at 1 and 24 hpi was determined and represented as fold change of VACV DNA at 24 hpi to 1 hpi. Error bars represent standard deviation (SD) of at least three experiments. P-values were obtained using the Student's t-test; *P value <0.05, **P value < 0.01, ***P value <0.001, ns = Not Significant (i.e. P value > 0.05).

LARP4 depletion decreases 5'-poly(A) leader-mediated translational advantage

Following VACV DNA replication, post-replicative mRNAs are transcribed. Although LARP4 depletion moderately altered DNA replication and intermediate mRNA transcription, the major block in post-replicative gene expression may occur at the translation level of post-replicative mRNAs. All VACV post-replicative mRNAs have a 5'-poly(A) leader. We then examined whether LARP4 is required for poly(A) leader-mediated translational advantage (17). Following 72 hours of LARP4 depletion, HFFs were mock-infected or infected with VACV (MOI=5). The 5'-poly(A) leader-containing the Fluc reporter mRNA and Kozak-headed Rluc mRNA were co-transfected into uninfected and VACV-infected HFFs at 12 hpi. Both Fluc and Rluc reporter mRNAs were either m^7G capped (**Fig 4-4A**) or ApppG capped (**Fig 4-4B**). Relative luciferase activities were measured 5 hours after reporter mRNA transfection by normalizing 5'-poly(A) leader-driven Fluc activity against co-transfected Kozak sequence-driven Rluc activity. LARP4 knockdown decreased translation from the m^7G capped 5'-poly(A) leader in uninfected HFFs (**Fig 4-4A, top**) and VACV-infected HFFs (**Fig 4-4A, bottom)**. Similarly, LARP4 depletion also significantly decreased translation from ApppG-capped 5'-poly(A) leader mRNA in VACV-infected cells (**Fig 4-4B, bottom**). A non-significant but small decrease in ApppG-capped 5'-poly(A) leader mRNA translation was observed in uninfected cells (**Fig 4-4B, top**). Unlike VACV-infected cells, AraC treatment did not alter poly(A) leader-mediated translation in uninfected HFFs (**Fig 4-4A and B, top**). Moreover, in uninfected cells, LARP4 depletion decreased translation from m^7G capped (significantly) and ApppG-capped (non-significantly) 5'-poly(A) leaders, thereby supporting the notion that LARP4 is required for poly(A) leader-mediated translation in both uninfected and VACV-infected cells (**Fig 4-4A** and **4-4B**).

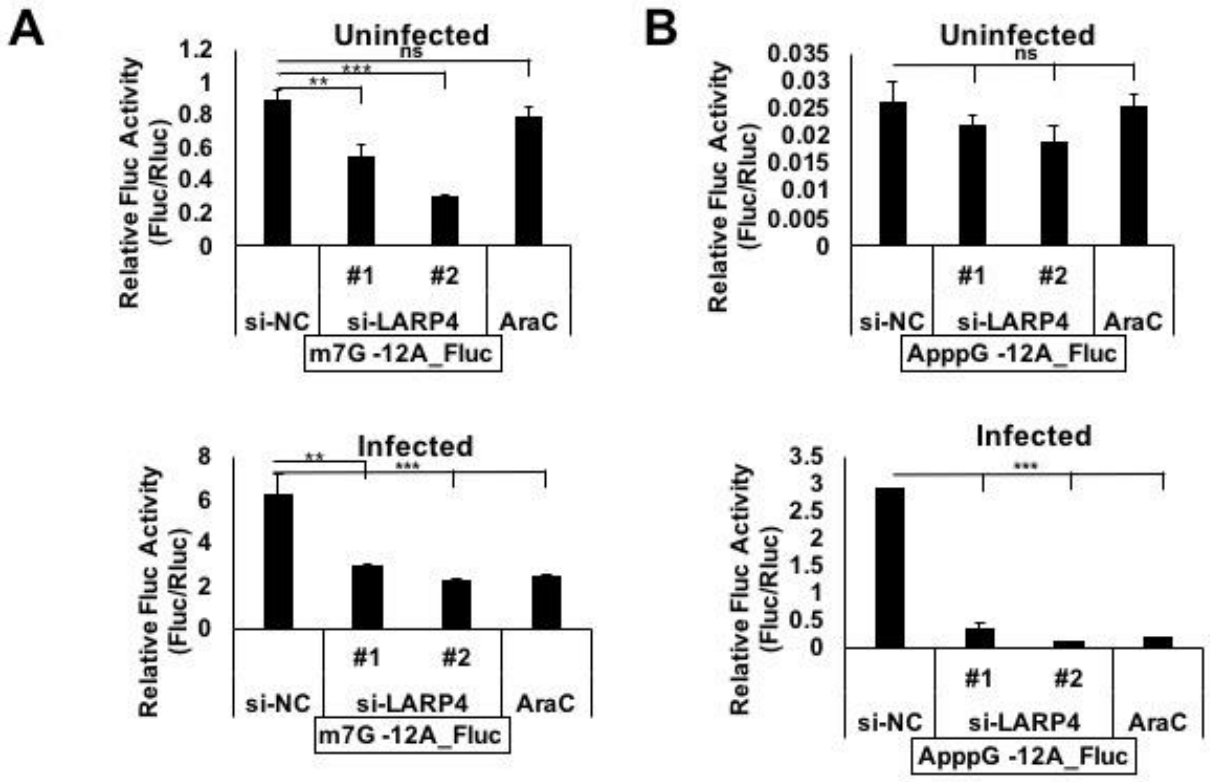

Figure 4-4: LARP4 is required for 5'poly(A) leader mediated translation.

HFFs, after 72 hours post transfection of control or LARP4 siRNAs, were either infected with VACV (MOI=5) or kept uninfected (A and B). AraC (40 μg/mL) treatment was initiated after 1 hour of infection or uninfected condition. 5'Poly(A) leader containing Fluc reporter mRNA and Kozak headed Rluc mRNA were transfected into uninfected and VACV infected HFFs at 12 hpi. Both Fluc and Rluc mRNAs were capped by either m^7G (A) or ApppG (B). Relative luciferase activity was measured 5 hours after reporter mRNA transfection by normalizing 5'Poly(A) leader driven Fluc activity by co-transfected Kozak sequence driven Rluc activity. Error bars represent standard deviation (SD) of at least three experiments. P-values were obtained using the Student's t-test; **P value < 0.01, ***P value <0.001, ns = Not Significant (i.e. P value > 0.05).

LARP4 is crucial for cap-independent translation from a 5'-poly(A) leader

During our study, we noticed that LARP4 depletion decreased the poly(A) leader bearing mRNA translation from ApppG-capped mRNAs more than m^7G capped mRNAs in the VACV-infected condition. In fact, compared to control siRNAs, the decrease in ApppG-capped poly(A)

translation by LARP4 siRNA #1 and #2 were 8.75-fold and 26.27-fold, respectively (**Fig 4-4B, bottom**). Whereas, compared to control siRNAs, the decrease in m^7G capped poly(A) translation by LARP4 siRNA #1 and #2 were 2.15-fold and 2.75-fold, respectively (**Fig 4-4A, bottom**). To investigate further, we carried out the experiment as per **Fig 4-4** in the presence or absence of cap-dependent translation initiation inhibitor LY294002. The chemical LY294002 inhibits hyper-phosphorylation of eIF4E-binding protein (4EBP-1). Notably, hypo-phosphorylated 4EBP-1 does not release the cap-binding protein eIF4E, thus inhibiting the cap-dependent initiation of translation. In the absence of LY294002, the poly(A) leader translation from ApppG-capped mRNA decreased more (2.5-fold and 8.4-fold in LARP4 depletion using siRNA #1 and #2, respectively, compared to control siRNA) than m^7G capped mRNA (1.6-fold and 2.48-fold in LARP4 reduction using siRNA #1 and #2, respectively, compared to control siRNA) (**black bars, Fig 4-5A, top and bottom**). In the presence of LY294002, translation from the m^7G capped poly(A) leader showed a minimal decrease (1.47-fold) in the negative control siRNA transfected cells, the level of which was still higher than that of LARP4-depleted cells. Intriguingly, during LY294002 treatment, translation from the m^7G capped poly(A) leader mRNAs in LARP4-depleted cells decreased to a basal level of approximately 30% compared to the cells transfected with control siRNAs (**white bars, Fig 4-5A, top**). In LY294002-treated cells, poly(A) leader translation from ApppG-capped mRNA decreased 3-fold in the control siRNAs transfected condition. The poly(A) leader containing mRNAs capped with ApppG during LY294002 treatment exhibited a severe defect in translation in the LARP4-depleted condition, leading to 10-fold decrease compared to control siRNA transfected cells (**White bars, Fig 4-5A, bottom**). In these experiments, AraC treatment, which blocks the post-replicative gene expression and necessary for poly(A) leader-mediated translation, was thus used as a positive

control (17). These results suggest that LARP4 is required for the cap-independent mode of translation from the poly(A) leader.

To further establish the role of LARP4 in the poly(A) leader's cap-independent mode of translation, we carried out an immunofluorescence assay to determine the localization of LARP4 in WT-VACV- and vD9muD10mu-infected cells. VACV decapping enzymes (D9 and D10) remove caps from both host and viral mRNAs. To test whether the cap status of mRNAs is necessary for LARP4 enrichment in the virus factory, we infected A549 cells with WT-VACV and DKO-A549 cells with vD9muD10mu that cannot decap the mRNAs (36). At 12 hpi, we determined the enrichment status of LARP4 in virus factory. We observed that LARP4 was highly enriched in virus factories during WT-VACV infection compared to vD9muD10mu infection (**Fig 4-5B**). The quantification of LARP4 and DAPI in the viral factory showed significantly higher (2.05-fold) LARP4 and similar (1.04-fold) DAPI intensity during WT-VACV infection when compared to vD9muD10mu infection (**Fig 4-5C**). This result indicates that WT-VACV with a functional decapping enzyme enhances LARP4 level in the virus factory, whereas decapping incompetent vD9muD10mu virus does not enrich LARP4 in the virus factory. Collectively, these results (**Fig 4-5 A, B and C**) indicate that LARP4 is required for both the poly(A) leader's cap-dependent and cap-independent mode of translation, and is especially critical for the cap-independent translation mode from a poly(A) leader containing mRNAs.

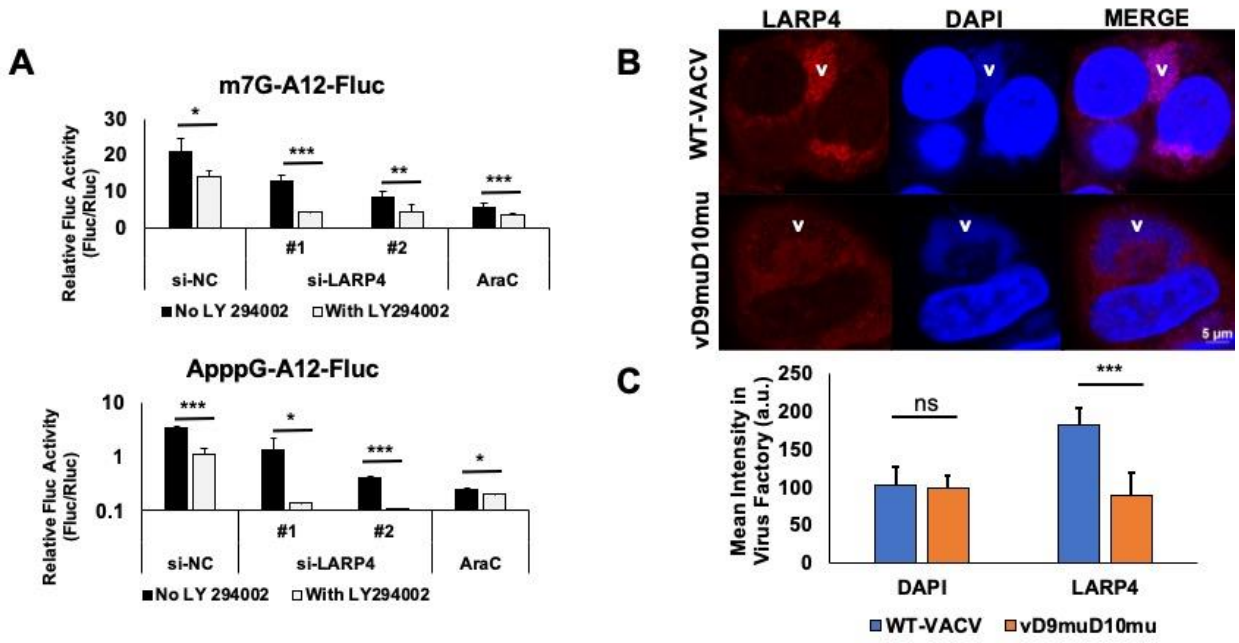

Figure 4-5: LARP4 is crucial for cap-independent mode of translation from 5'Poly(A) leader.

(A) Control or LARP4 siRNAs were transfected into HFFs. After 72 hours, HFFs with indicated siRNAs treatment were infected with VACV (MOI=5). HFFs were either mock-treated or treated with LY294002 (25 µM) and/or AraC (40 µg/mL) at 1 hpi. The 5'Poly(A) leader containing Fluc reporter mRNA and Kozak headed Rluc mRNA were transfected into HFFs with indicated treatment. Relative Luciferase activity was measured 5-hour post reporter mRNA transfection by dividing Fluc activity by Rluc activity. (B) Immunofluorescence assay of WT-VACV or VACV-D9muD10mu infected A549 cells and DKO cells, respectively (MOI=2). At 12 hpi, cells were fixed and stained with respective antibodies. Anti-LARP4 was used to determine localization of LARP4 (red) and DAPI (blue) was used to stain cellular nuclei and virus factory. Panel shows representative image. Scale bar: 5 µm. (C) Quantification of the intensity of LARP4 and DAPI in the virus factory during of WT-VACV or VACV-D9muD10mu infection.

LARP4 is dispensable for mRNA stability during VACV infection

Previous studies have shown that LARP4 binds to the 3' poly(A) tail in mRNAs and enhance stability (19,21). To determine the possibility of LARP4 binding to the 3' poly(A) tail and/or 5' poly(A) leader during VACV infection, aids in mRNA stability we synthesized the

5'-poly(A) leader containing reporter mRNAs without poly(A) tail. These reporter mRNAs were used to determine translation and stability during LARP4 depletion (**Fig 4-6A**). Following LARP4 depletion, HFFs were infected with VACV (MOI=5, 12 hpi). At 12 hpi, m^7G and ApppG-capped 5'-poly(A) leaders bearing mRNA without the 3' poly(A) tail were transfected in VACV-infected HFFs. After 5 hours following reporter mRNA transfection, the samples were prepared for analysis of translation (**Fig 4-6B**) and mRNA levels (**Fig 4-6C**). Despite lacking a 3' poly(A) tail, both m^7G and ApppG-capped mRNAs bearing the 5' poly(A) leader exhibited a decrease in translation during LARP4 knockdown (**Fig 4-6B**). This observation indicates that LARP4's role in poly(A) leader-mediated translational advantage is through direct interaction with the 5' poly(A) leader, which is—to a major extent—independent of binding to the 3' poly(A) tail. However, LARP4 binding to the 5' poly(A) leader may enhance either translation initiation, mRNA stability, or both. To test this, we determined the mRNA level at 5 hours post-transfection of reporter mRNAs without poly(A) tail. Interestingly, we observed no apparent difference in the mRNA level of transfected reporter mRNA and endogenous GAPDH mRNA during LARP4 depletion (**Fig 4-6C**). Altogether, our results suggest that LARP4 depletion decreases poly(A) leader-bearing mRNA translation without altering mRNA stability, which suggests that LARP4 probably by binding to the 5' poly(A) leader enhances translation initiation in VACV-infected cells.

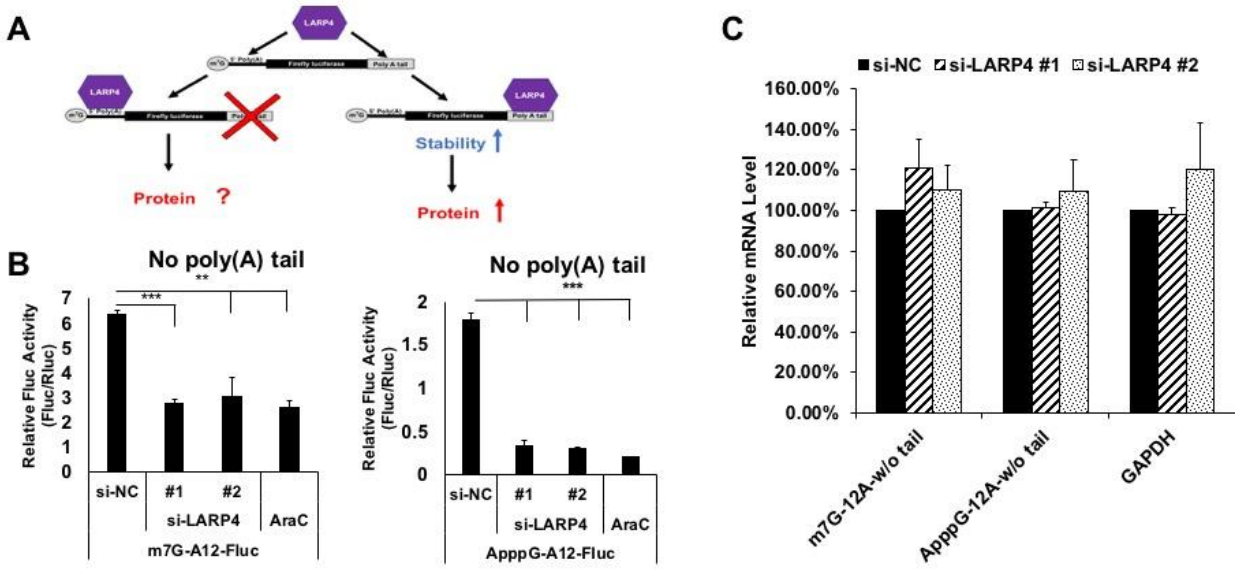

Figure 4-6: LARP4 binding to 5'poly(A) leader is dispensable for mRNA stability during poxvirus infection.

(A) Schematic showing LARP4 can bind either to poly(A) leader or poly(A) tail or both. To rule out the effect of LARP4 binding to poly(A) tail, reporter RNAs (12A-Fluc and Kozak-Rluc) were synthesized without poly(A) tail. (B and C) After transfecting HFFs with control or LARP4 siRNAs for 72 hours, cells were infected with VACV (MOI=5). At 12 hpi, reporter RNA (12A-Fluc and Kozak-Rluc) capped by either m^7G or ApppG but without poly(A) tail were transfected into HFFs with indicated treatment. (B) Relative Luciferase activity was measured 5-hour post reporter mRNA transfection by dividing Fluc activity by Rluc activity. (C) Levels of transfected m^7G or ApppG capped reporter RNAs (without poly(A) tail) and GAPDH mRNAs were measured by qRT-PCR and normalized using 18S rRNA. Error bars represent standard deviation (SD) of at least three experiments. P-values were obtained using the Student's t-test; **P value < 0.01, ***P value <0.001.

Discussion

Poxviruses induce global host protein synthesis shutoff to cause devastating diseases in humans and animals. During host shutoff, poxviruses such as VACV have evolved strategies to increase their protein synthesis. One such approach is to acquire an evolutionarily optimized 5'-poly(A) leader in the translation regulatory 5'UTR region (13,15,16,22). The poly(A) leader provides a translational advantage to VACV post-replicative mRNAs that encode for highly critical VACV structural proteins (10,17,23). Since poly(A) leader-mediated translation is independent of mRNA cap status, we hypothesized the existence of a poly(A) RNA-binding protein linked directly to the 5'-poly(A) leader to promote VACV post-replicative mRNA translation.

For identification of the 5'-poly(A) leader binding protein, we evaluated for proteins known to bind to a poly(A) RNA sequence (19,25). Using immunofluorescence assay, we identified LARP4 as a cellular protein enriched in the virus factory, which is the site for VACV post-replicative mRNA translation. Since LARP4 is known to bind to poly(A) RNA sequences and RACK1, and is essential for poly(A) leader-mediated translational advantage, we further explored the role of LARP4 (10,19). Additional experiments indicated that a decrease in LARP4 protein level reduced VACV titer and blocked post-replicative gene expression completely, with a modest decrease in VACV DNA replication and a significant yet small decrease in post-replicative gene transcription. During VACV infection, depletion of LARP4 decreased translation from poly(A)-headed mRNAs, especially from ApppG-capped reporter mRNAs and during the inhibition of cap-dependent translation. Furthermore, LARP4 depletion decreased translation from 5'-poly(A)-bearing mRNA in uninfected cells and reporter mRNAs lacking the 3' poly(A) tail, signifying its importance for poly(A)-headed mRNA translational advantage.

Moreover, we found that silencing LARP4 expression did not affect mRNA stability during VACV infection. These findings suggest that LARP4 is vital for both the cap-dependent and cap-independent mode of translation initiation in mRNAs bearing a 5'-poly(A) leader.

VACV B1 kinase phosphorylates S^{278} of RACK1 STSS motif, and this post-translational modification is required for poly(A) leader-mediated translation advantage (10). However, RACK1 did not bind to the poly(A) leader and was not enriched in the viral factory in our study (data not shown), thus suggesting that RACK1 phosphorylation may have another function during poly(A)-headed mRNA translation. Since the STSS motif is present at the extended loop of RACK1 and is known to interact with ribosomal protein at the exit site (10), we suspect that RACK1 phosphorylation changes the conformation of the exit site in the ribosome, thus making a conducive environment for poly(A)-headed mRNA translation. In Jha et al. 2017, the reporter system contained 35 A's; so we do not rule out the possibility that heterogenous poly(A) leader length may recruit different proteins to enhance translation.

A recent study showed that LARP4-NTD (N-terminal domain) is essential for poly(A) RNA binding (28). LARP4-NTD also contains the PAM2w motif, which is necessary for the interaction between the MLLE domains of PABP and LARP4. Cruz-Gallardo et al. 2019, subsequently demonstrated that LARP4-NTD binding is mutually exclusive with a PABP or poly(A) RNA sequence (28). This supports our and others' observations that PABP is excluded from the virus factory (11,27) and is nonessential for LARP4-facilitated poly(A) leader translation.

One interesting observation we observed in our study was that LARP4 enrichment in virus factory diminished during vD9muD10mu virus infection compared to WT-VACV infection (**Fig 4-5B and C**). This observation prompts two possibilities. First, in vD9muD10mu infection,

LARP4 is requisitioned by cellular mRNAs more than by VACV mRNAs, leading to a uniform distribution of LARP4 throughout the cytoplasm. Second, LARP4 recruitment to viral mRNAs and consequent enrichment in the virus factory depends on the decapping status of the VACV post-replicative mRNAs. Although both situations are possible, we view the possibility that LARP4 is recruited to decapped VACV post-replicative mRNAs as more probable since our data suggest that LARP4 is vital for a poly(A) leader bearing mRNA's cap-independent mode of translation (**Fig 4-4B, 4-5A bottom and 4-6B**). Future studies will attempt to shed light on exploring these possibilities.

The data presented in this study demonstrated that LARP4 is required for poly(A)-headed mRNAs translation in uninfected and VACV-infected HFFs. In uninfected cells, LARP4 may play a role in mRNA stability and translation initiation. In VACV-infected cells, LARP4 may have a minimal, if any, function in mRNA stability; however, LARP4 increases translation initiation by presumably binding to poly(A) leader. In uninfected cells, we propose that LARP4 will recruit other translation initiation factors and this recruitment of unknown translation initiation factors is enhanced by one or more unidentified VACV/host proteins, thus allowing the 5'-poly(A) leader to confer a translational advantage during VACV infection (**Fig 7**). Future studies will focus on identifying the VACV/host protein(s), and other eukaryotic translation initiation factors required for poly(A) leader-mediated translational advantage and on determining the unknown pre-existing eukaryotic translation mechanism.

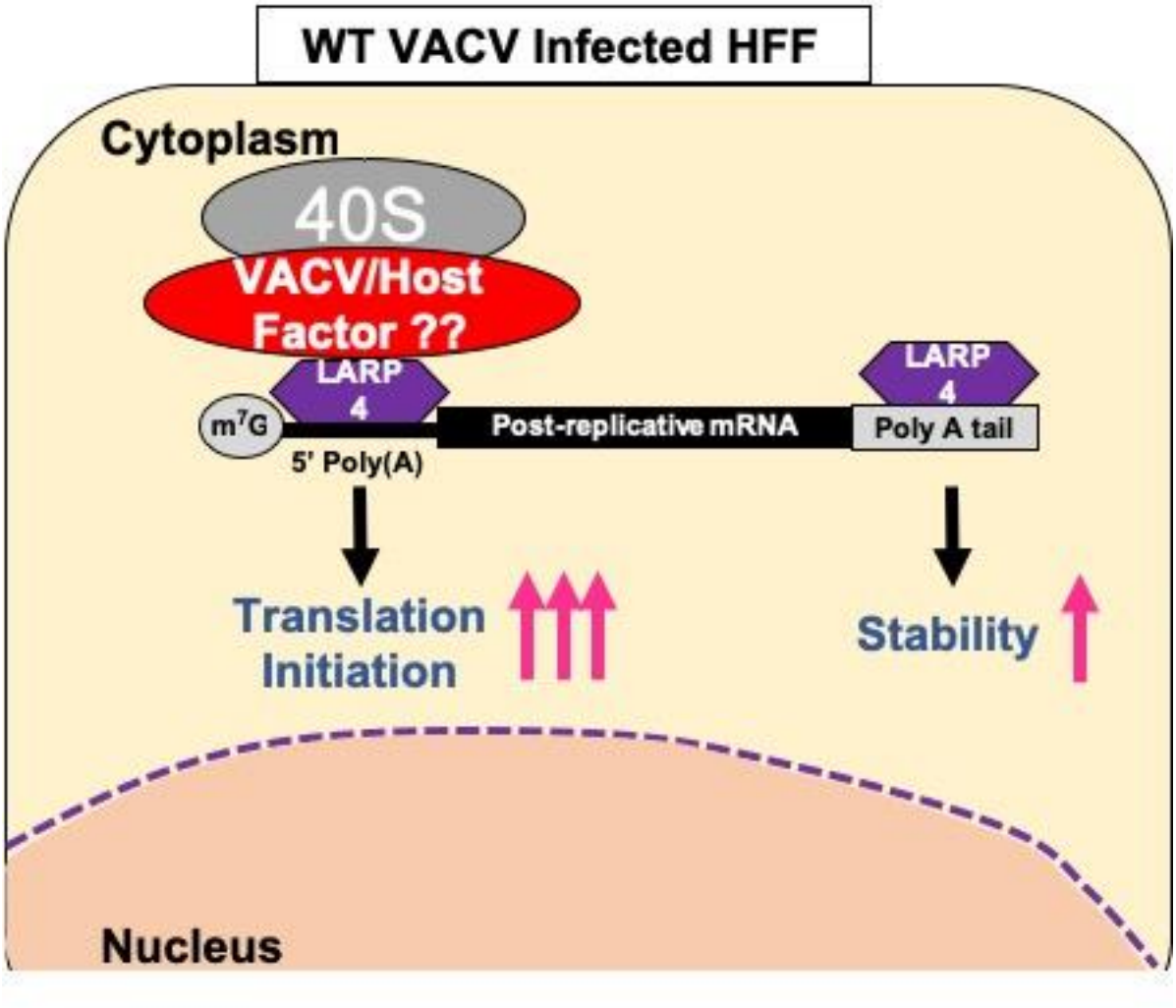

Figure 4-7: Schematic summary of the findings and working model.

LARP4 binds to both poly(A) leader (this report) and poly(A) tail (19). During VACV infection, we hypothesize that LARP4 binding to poly(A) leader enhances recruitment of 40S ribosome to initiate translation and provide poly(A) bearing mRNA a translational advantage. The recruitment of 40S via LARP4 can be mediated by unknown eukaryotic initiation factor in uninfected condition, however, during VACV infection enhancement in LARP4 mediated 40S recruitment to poly(A) leader is mediated by unidentified viral protein/s and/or repurposed unknown host factor/s.

Materials and methods

Cell culture and virus infection

Human foreskin fibroblast (HFF, gift from Dr. Nicholas Wallace) were cultured in Dulbecco's modified eagle's medium (DMEM, Quality Biological) containing 10% fetal bovine serum (FBS, Peak Serum) and 2 mM L-Glutamine (Quality Biological). Cells were incubated in a 5% CO_2 atmosphere at 37°C. VACV Western Reserve strain (ATCC VR-1354), vD9muD10mu double mutant and intermediate transcription factor gene A23 deleted VACV (VACV-ΔA23) were kindly provided by Dr. Bernard Moss and was reported earlier (37). Virus titer was determined by plaque assay using BSC-1 cells as described elsewhere (38).

Antibodies and chemical inhibitors

Anti-LARP4 (A5108), anti-PABPC1 (A14872) and anti-RPS6 (A6058) antibodies were purchased from ABclonal Inc. Anti-RPL5 (51345) antibody was purchased from Cell Signaling Technology. β-Tubulin and GAPDH (sc-365062 HRP) antibodies were purchased from Santa Cruz Biotechnology. Anti-Puromycin (MABE343) was purchased from Sigma Aldrich. Anti-D13L and anti-A10L/P4a antibody was a gift from Dr. Bernard Moss. The anti-E3L antibody was a present from Dr. Yan Xiang. LY294002 was purchased from Cayman Chemical. DAPI (D1306) was purchased from Thermo Fisher Scientific. Cytosine arabinoside (AraC) was purchased from Sigma-Aldrich.

Western blotting analysis and nascent protein synthesis analysis

Protein level was evaluated by preparing sample as described before (39). The sample was resolved in the SDS-PAGE gel followed by transferring to a polyvinylidene difluoride (PVDF) membrane. For detection of protein, membranes were blocked in either 5% BSA or 3% Milk for 1 hour at room temperature followed by incubation with primary antibody for 1 hour at room

temperature or overnight at 4°C and finally, incubation with secondary antibody added in 1X TBST (with either 3% Milk or 5% BSA). The image was developed as described elsewhere (40). Nascent protein synthesis was determined by treating the cells with puromycin (10 µg/ml, P8833, Sigma Aldrich) for 20 minutes at 37°C. Treatment was aborted by removing the cell culture media containing puromycin and washed once with 1X-PBS. NP-40 lysis buffer was added directly on the cells and the cells were subsequently scraped. Lysis was carried out by rotating the sample at 4°C for 30 minutes and centrifuged at 12000 X g at 4°C for 10 minutes. The supernatant was used for sample preparation and evaluated by western blot analysis as described previously (39).

RNA extraction and quantitative RT-PCR

At desired time cell culture media was removed, cells were washed with 1X-PBS, TRIzol reagent (Ambion) was added, and cells were collected for RNA purification. Total RNA was extracted using the PureLink RNA Mini Kit (Thermo Fisher Scientific). The purified RNA was next used to synthesize cDNA using SuperScript III First-strand synthesis (Invitrogen) with the addition of either random hexamer or oligo-dT primers. The cDNA was used to perform quantitative RT-PCR with iTaq Universal SYBR Green Supermix (Bio-Rad) using specific primers of desired genes.

***In vitro* transcribed RNA-based Luciferase Assay**

The RNA-based luciferase assay was used to determine the translation of 5'-Poly(A) leader containing mRNA during LARP4 depletion using the protocol described previously (17). The RNA used in this study were capped with either m^7G (Anti-Reverse Cap Analog [ARCA], S1411L) or ApppG cap analog (S1406S, New England Biolabs). To test LARP4 specificity for

5'-Poly(A) leader, we synthesized reporter mRNA with or without poly(A) tail by modulating poly(A) tail in DNA template.

Knockdown using small interfering RNA (siRNA)

The small interfering RNAs (siRNAs) used for this study were purchased from Integrated DNA technologies (IDT-DNA). Lipofectamine RNAiMax (Thermo Fisher Scientific) was used to transfect si-LARP4 or si-NC (Negative Control) to HFF. The si-RNAs were used at the final concentration of 12.5 nM. At 72-hours post-transfection, the cells were either collected for western blot analysis to determine siRNA mediated depletion of the target protein or infected for desired hours for further analysis (titration, cytopathic effect, western blot, qRT-PCT, luciferase assay, and mmunofluorescence assay).

Immunofluorescence assay

After desired treatment cells were fixed with 4% formaldehyde (28908, Thermo Fisher Scientific) in 1X-PBS for 20 minutes. The cells were then permeabilized with 0.1% Triton X-100 (9002-93-1, Thermo Fisher Scientific) in 1X-PBS for 15 minutes. Cells were washed 3X with 1X-PBS and blocked for 1 hour at room temperature with 2% BSA containing 1X-PBS. Then, cells were incubated with the desired primary antibody (1:100 dilution in 2% BSA containing 1X-PBS) for 1 hour at room temperature. Again, after 3X washes, cells were incubated with secondary antibody conjugated with a fluorophore (1:500 dilution in 2% BSA containing 1X-PBS) for 1 hour at room temperature. Following 2X washes with 0.1% Triton X-100 in 1X-PBS, DAPI stain (5µM) in 1X-PBS was added to the cells for 20 minutes at room temperature. Finally, 2X washes with 1X-PBS were done to remove any unbound DAPI stain. The image was taken using Carl Zeiss LSM 880 confocal microscope. ZEISS Efficient Navigation (ZEN) software was used for analysis of the images.

Acknowledgements

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Chapter 5 - Ribosome Profiling Reveals Translational Upregulation of Cellular Oxidative Phosphorylation mRNAs during Vaccinia Virus-Induced Host Shutoff

Aimei Dai[a#], Shuai Cao[b#], Pragyesh Dhungel[b#], Yizhao Luan[a], Yizhi Liu[a], Zhi Xie[a*], Zhilong Yang[b*]

[a]State Key Lab of Ophthalmology, Guangdong Provincial Key Lab of Ophthalmology and Visual Science, Zhongshan Ophthalmic Center, Sun Yatsen University, Guangzhou, China;

[b]Division of Biology, Kansas State University, Manhattan, Kansas, USA

[#] co-first authors; *corresponding authors

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Abstract

Vaccinia virus infection causes a host shutoff that is marked by global inhibition of host protein synthesis. Though the host shutoff may facilitate reallocation of cellular resources for viral replication and evasion of host antiviral immune responses, it poses a challenge for continuous synthesis of cellular proteins that are important for viral replication. It is, however, unclear whether and how certain cellular proteins may be selectively synthesized during the vaccinia virus-induced host shutoff. Using simultaneous RNA sequencing and ribosome profiling, two techniques quantifying genome-wide levels of mRNA and active protein translation, respectively, we analyzed the responses of host cells to vaccinia virus infection at both the transcriptional and translational levels. The analyses showed that cellular mRNA depletion played a dominant role in the shutoff of host protein synthesis. Though the cellular mRNAs were significantly reduced, the relative translation efficiency of a subset of cellular mRNAs increased, particularly those involved in oxidative phosphorylation that are responsible for cellular energy production. Further experiments demonstrated that the protein levels and activities of oxidative phosphorylation increased during vaccinia virus infection, while inhibition of the cellular oxidative phosphorylation function significantly suppressed vaccinia virus replication. Moreover, the short 5' untranslated region of the oxidative phosphorylation mRNAs contributed to the translational upregulation. These results provide evidence of a mechanism that couples translational control and energy metabolism, two processes that all viruses depend on host cells to provide, to support vaccinia virus replication during a host shutoff.

Importance

Many viral infections cause global host protein synthesis shutoff. While host protein synthesis shutoff benefits the virus by relocating cellular resources to viral replication, it also poses a challenge to the maintenance of cellular functions necessary for viral replication if continuous protein synthesis is required. Here we measured the host mRNA translation rate during a vaccinia virus-induced host shutoff by analyzing total and actively translating mRNAs in a genome-wide manner. This study revealed that oxidative phosphorylation mRNAs were translationally upregulated during vaccinia virus-induced host protein synthesis shutoff. Oxidative phosphorylation is the major cellular energy-producing pathway, and we further showed that maintenance of its function is important for vaccinia virus replication. This study highlights the fact that vaccinia virus infection can enhance cellular energy production through translational upregulation in the context of an overall host protein synthesis shutoff to meet energy expenditure.

Introduction

All viruses depend on infected host cells for protein synthesis. Many viruses, for example, poxviruses, influenza virus, picornavirus, and herpesviruses, cause a host shutoff after infection that is marked by global cellular protein synthesis inhibition (1–8). While this host shutoff conserves cellular resources and blunts host antiviral responses that benefit viral replication, it also presents a challenge to maintenance of the integrity of cellular functions necessary for viral replication, as continuous synthesis of certain host proteins may be needed. However, knowledge is scarce about selectively synthesized cellular proteins and their biological relevance during a virus-induced host shutoff. Determination of selectively synthesized proteins in this process will facilitate the identification of cellular functions that are important for viral replication.

Ribosome profiling is a technique based on deep sequencing of ribosome-protected mRNA fragments, providing genome-wide information of mRNAs that are actively translated (9–12). We have previously identified many unexpected translation products of vaccinia virus (VACV) by using ribosome profiling (13). Several other studies also identified large numbers of unexpected translation products from human cytomegalovirus (HCMV), coronavirus, and Kaposi's sarcoma-associated herpesvirus by using ribosome profiling (14–16). Importantly, ribosome profiling can also simultaneously reveal the translation of host mRNAs during a viral infection. In a recent study, translational changes in host genes upon HCMV infection were studied by simultaneous transcriptome sequencing (RNA-Seq) and ribosome profiling (17). That study revealed many host genes translationally activated or repressed by HCMV, which does not induce a host shutoff. Given its great sensitivity and resolution, we reasoned that ribosome

profiling is a powerful tool for the study of selectively translated cellular mRNAs by analyzing actively translating and total mRNAs of individual genes during a virus-induced host shutoff.

Poxviruses are a family of large DNA viruses that include highly pathogenic members that infect humans and economically important animals and are exemplified by the variola virus that causes the deadly human disease smallpox (18). Poxviruses are also developed as agents for cancer therapy and as vaccine vectors (19, 20). VACV, the prototypic poxvirus, was the vaccine strain used to eradicate smallpox. However, vaccination with VACV may also cause complications such as keratitis, conjunctival disease, and iritis in immunocompromised individuals (21). It has long been known that VACV rapidly takes over the host translational machinery for viral protein synthesis and causes a global host protein synthesis shutoff (4, 5, 22). This shutoff is at least partially attributed to VACV-encoded decapping enzymes D9 and D10. These decapping enzymes efficiently remove 7-methylguanosine caps on the 5' termini of mRNAs (23–28). This decapping renders the mRNAs sensitive to degradation by exonucleases and results in a rapid depletion of mRNAs (24, 25, 29, 30). In addition to mRNA degradation, transcription inhibition may also play a role in this process (31). The global depletion of cellular mRNAs is assumed to contribute to the host shutoff. However, it is largely unknown whether cellular proteins are selectively translated and, if so, whether those selectively synthesized proteins are important for VACV replication.

In this study, we analyzed dynamic transcriptional and translational landscapes of host cells over the course of VACV infection by simultaneous RNA-Seq and ribosome profiling of VACV- and mock-infected cells. Our study indicates that cellular mRNA depletion plays a dominant role in the shutoff of host protein synthesis. Though the cellular mRNA amounts were significantly reduced, the relative translation efficiency (TE) of a subset of cellular mRNAs

increased, particularly for those involved in oxidative phosphorylation that are responsible for cellular energy production. We further demonstrated that the protein levels and activities of oxidative phosphorylation increased during VACV infection, while inhibition of oxidative phosphorylation suppressed VACV replication. A short 5' untranslated region (UTR) of the oxidative phosphorylation mRNAs contributed to the translational upregulation. This study revealed that a viral infection enhanced oxidative phosphorylation through translational upregulation in the context of an overall host shutoff.

Results

Simultaneous RNA-Seq and ribosome profiling of VACV-infected cells.

To understand translational regulation and identify selectively translated cellular mRNAs during a VACV-induced host shutoff, we carried out simultaneous RNA-Seq and ribosome profiling of VACV- and mock-infected HeLa cells at 2, 4, and 8 h postinfection (hpi) (**Fig. 5-1A**). Under these conditions, VACV replication was in the early stage when viral DNA replication did not occur yet at 2 hpi, whereas at 4 hpi and later times, VACV replication had proceeded to the post-DNA replication stage (28). RNA-Seq and ribosome profiling quantitatively measured the levels of total and actively translating mRNAs, respectively (9, 10). We obtained 114.6 to 135.8 million reads from individual RNA-Seq samples and 38.0 to 98.7 million ribosome-protected fragments (RPFs) from individual ribosome profiling samples that were mapped to human and VACV genomes, providing sufficient depth of sequencing reads for further analyses. The analysis of viral mRNA translation from part of the sequencing data was reported previously (13).

Analysis of the cellular RPFs indicated that the ribosome profiling experiments were highly reproducible, as evidenced by the very strong correlation coefficient (r, >0.98) of two sets of completely independent ribosome profiling experiments (biological replicates) under the same infection conditions at each time point (**Fig. 5-1B**). The high reproducibility of the RNA-Seq and ribosome profiling experiments was also evidenced by the strong correlation of the reads of mock-infected cells at 2, 4, and 8 h, where the cells were under the same culture conditions without VACV infection. The correlation coefficients were >0.98 in all of the pairwise comparisons of experiments from the three time points (data not shown). The high quality of cellular ribosome-protected RNA fragment (RPF) reads was evidenced by the expected high

mapping rates in coding DNA sequences (CDSs) and 5′ UTRs and low mapping rates of 3′ UTRs and introns of cellular genes for both mock infection (**Fig. 5-1C**) and VACV infection (**Fig. 5-1D**) experiments. We concluded that the data sets were of high quality and suitable for systematic analyses.

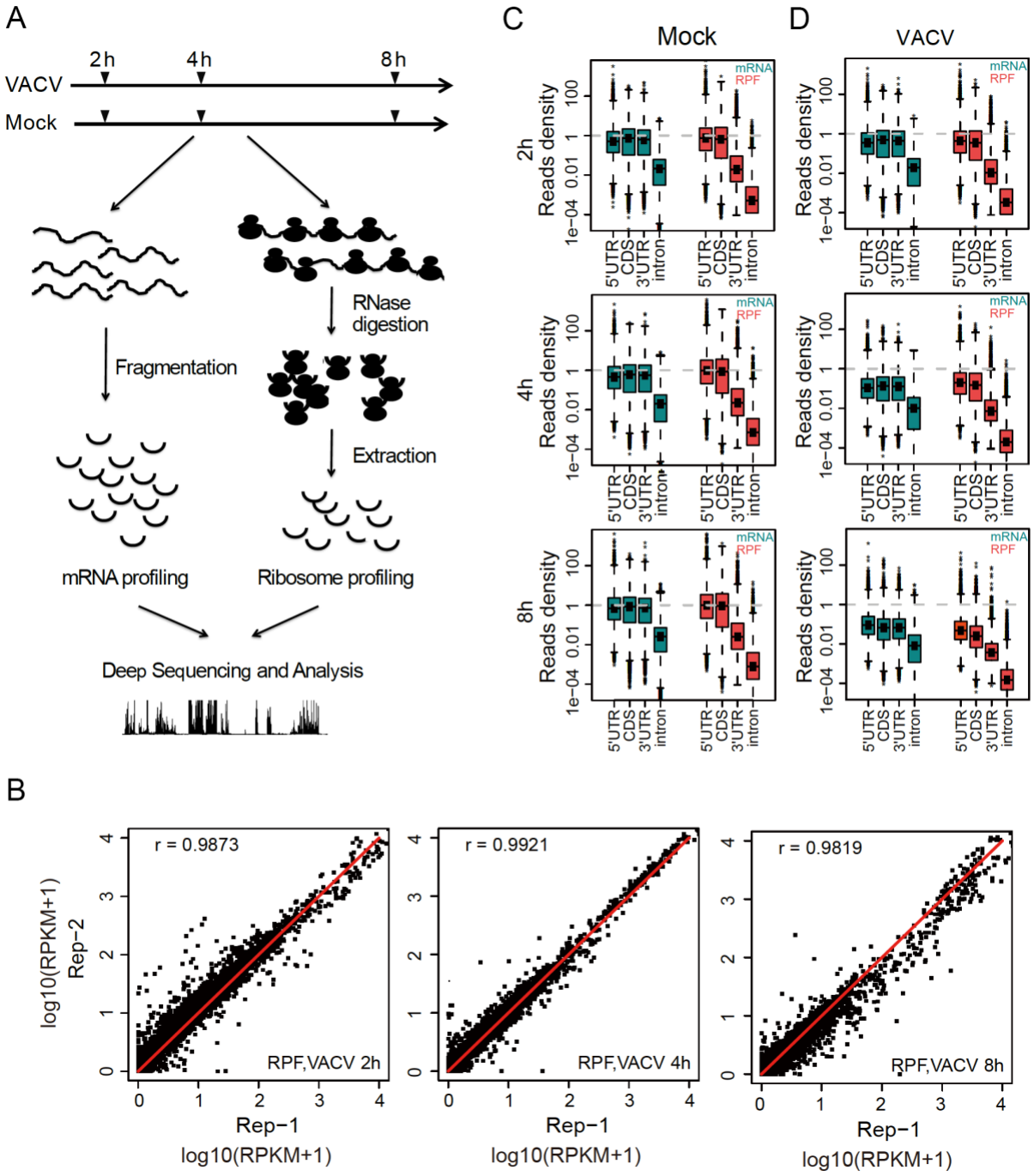


Figure 5-1: Experimental approach for simultaneous RNA-Seq and ribosome profiling during VACV infection.

(A) Overall experimental design. HeLa S3 cells were mock or VACV infected; harvested at 2, 4, and 8 hpi; and subjected to RNA-Seq and ribosome profiling. (B) High reproducibility of ribosome profiling experiments. Correlation analysis of cellular reads between ribosome profiling experiments with two biological replicates under VACV-infected condition at 2, 4, and

8 hpi is shown along with Pearson's correlation coefficient. (C, D) Read densities (number of mapped reads divided by length) of genomic regions of 5′ UTRs, CDSs, 3′ UTRs, and introns of cellular mRNAs are shown. mRNAs are green and RPFs are red under mock-infected (C) and VACV-infected (D) conditions.

mRNA depletion plays a dominant role in VACV-induced host shutoff.

We first evaluated the contribution of mRNA depletion to VACV-induced host protein synthesis shutoff by analyzing the proportions of cellular RNA reads and RPFs in the total reads. The multidimensional scaling (MDS) plot showed a dramatic overall change in cellular mRNA and RPFs at 4 and 8 hpi (**Fig. 5-2A**), whereas the change at 2 hpi was subtle compared to that in mock-infected cells. This finding is consistent with the fact that VACV replication had proceeded to the postreplicative stage at 4 hpi, when the host shutoff occurred. During the host shutoff, the proportion of cellular mRNA reads decreased significantly at 4 and 8 hpi. The cellular RPFs also decreased significantly at 4 and 8 hpi (**Fig. 5-2B**). We next characterized the global changes in cellular mRNAs and RPFs in VACV-infected cells versus mock-infected cells at 2, 4, and 8 hpi. **Figure 5-2C** shows that the distribution of RPKMs (reads per kilobase of transcript per million reads) of mRNA and RPF reads virtually overlapped in the mock infection experiments at 2, 4, and 8 hpi. In contrast, the overall RPKMs in VACV-infected cells decreased dramatically after infection, indicating a host shutoff at both the transcriptional and translational levels. Because the total cellular and viral mRNA amounts extracted from mock- and VACV-infected cells were similar, these results suggested that cellular mRNA depletion was the major contributor to VACV-induced host protein synthesis shutoff.

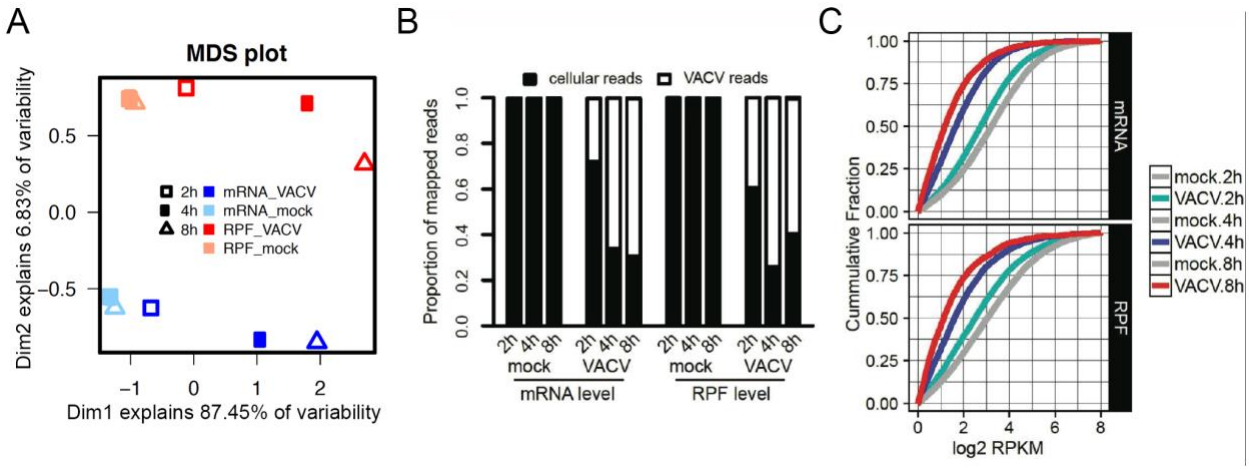


Figure 5-2: Global analysis of mRNA and RPF reads reveals the characteristics of VACV-induced host shutoff.

(A) MDS plot of ribosome profiling and RNA-seq data sets used to plot the sample relationship. The log2-fold changes in the 500 most variable genes between samples of ribosome profiling and RNA-seq data sets were approximated. Dimensions 1 and 2 of the MDS plot are presented, where dimension 1 explains 87.45% of the variability and dimension 2 explains 6.83% of the variability. (B) Mapping efficiency of mRNA/RPF reads of the human (black) and VACV (white) genomes at 2, 4, and 8 hpi. (C) Cumulative distribution of gene expression at mRNA (top) and RPF (bottom) levels, with time points shown in different colors.

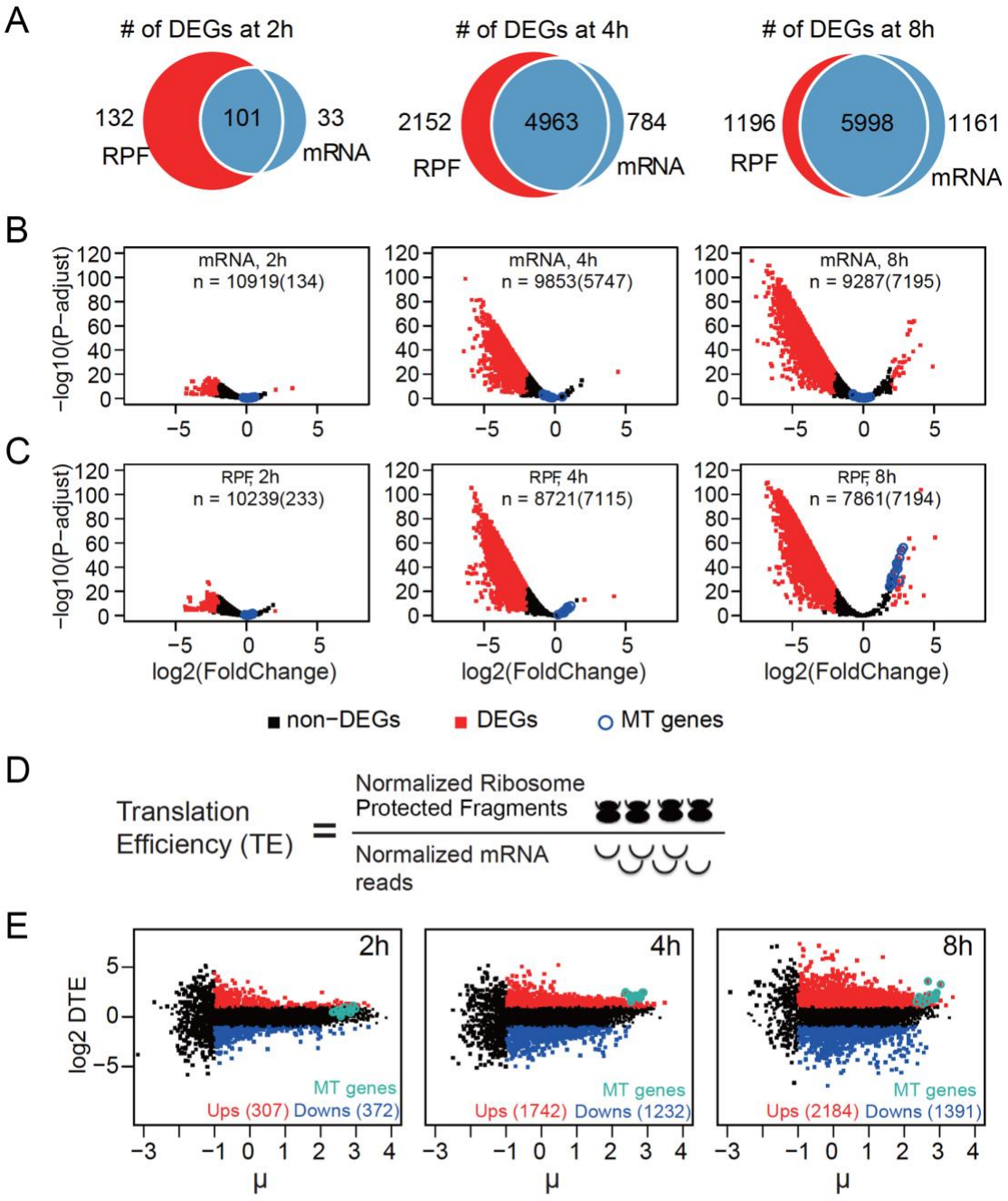
Relative TE of host mRNAs during VACV-induced host shutoff.

While only a limited number of cellular genes are differentially expressed at 2 hpi, a large number of cellular genes are differentially expressed at 4 and 8 hpi at both the mRNA and translation (RPF) levels (**Fig. 5-3A**). The differentially expressed genes also overlapped considerably at the mRNA and RPF levels, particularly at 4 and 8 hpi (**Fig. 5-3A**). Though large numbers of cellular genes are differentially expressed at 4 and 8 hpi, the majority of them were downregulated, with only a small number of genes upregulated (**Fig. 5-3B and C**). Since the host cells underwent a global shutoff at both mRNA and RPF levels, almost all of the cellular functions were downregulated. Here we focused on the genes with increased levels of mRNAs

and RPFs because they are the candidates for selectively synthesized genes that are resistant to the general trend of host shutoff. However, gene set enrichment analysis (GSEA) showed no significantly enriched pathways at both the mRNA and RPF levels when using a cutoff with an adjusted P value of <0.05 by Benjamini and Hochberg's false discovery rate (FDR), which was not a surprise because of the global shutoff and only a very limited number of genes being upregulated.

We then employed a relative TE analysis, which is defined as the ratio of normalized RPFs to mRNA reads (**Fig. 5-3D**). It has been shown to be an effective way to identify differentially translated mRNAs in HCMV infection (32). Though the mRNA levels decreased significantly, we observed extensive TE regulation of cellular mRNAs responding to VACV infection revealed by a large number of mRNAs with differential TE (DTE) in VACV- versus mock-infected cells, particularly at 4 and 8 hpi (**Fig. 5-3E**). We hypothesized that the upregulation of the relative TE of mRNAs can serve as a compensating mechanism to maintain or increase protein levels in the context of decreased mRNA levels. The functions of the genes involved may be important for VACV replication or cell survival. The genes with upregulated relative TE (>2-fold) are listed in Data Set S1 in the supplemental material.

To examine this hypothesis, we carried out a GSEA of the host genes that responded to VACV infection with differential relative TE. Using the same cutoff, an adjusted P value of <0.05 by FDR, the GSEA of the genes yielded several enriched pathways with elevated relative TE, where oxidative phosphorylation was the only enriched function at all three time points (**Fig. 5-3F**), suggesting that it was selectively targeted by translational upregulation in VACV-infected cells.



Pathway	P (adjusted)		
	2h	4h	8h
Ribosome	0.002	NS	NS
Oxidative phosphorylation	0.010	6.99E-09	0.042
Phenylalanine metabolism	NS	NS	0.042
Drug metabolism-cytochrome p450	NS	NS	0.042
Type I diabetes mellitus	NS	NS	0.042
Graft-versus-host disease	NS	NS	0.046

Figure 5-3: Differential mRNA, RPF, and relative TE analyses of cellular genes during VACV infection.

(A) Venn diagram of numbers of differentially expressed genes (DEGs; adjusted P value, ≤0.05; absolute value of log2-fold change, ≥2) present at the mRNA and RPF levels at 2, 4, and 8 hpi. (B, C) Volcano plots of mRNA (B) and RPF (C) levels with DEGs in red and mitochondrial (MT) genes in blue. (D) Relative TE is defined as the ratio of normalized RPFs to the normalized mRNA read density in the CDS region. (E) Scatterplot of the mean log2 RPKM (μ) under mock- and VACV-infected conditions of mRNA/RPF reads versus the logarithmic TE difference value under the VACV-infected condition to that under the mock-infected condition (log2 DTE). Translationally upregulated cellular genes (log2 DTE, ≥1; μ, −1 or greater) are red, and downregulated genes (log2 DTE, −1 or greater; μ, −1 or greater) are blue. Mitochondrial genes are green. Numbers of up/down-regulated genes are also shown. (F) GSEA of genes with upregulated TE based on the KEGG pathway data set. P values were adjusted for multiple testing with FDR. NS, not significant, where the adjusted P value is >0.05.

Oxidative phosphorylation capacity is enhanced during VACV infection, while inhibition of oxidative phosphorylation suppresses VACV replication.

Oxidative phosphorylation is the metabolic pathway in which ATP is produced through a series of biochemical reactions in mitochondria. The oxidative phosphorylation genes include >100 genes that are encoded in both the nuclear and mitochondrial genomes of mammalian cells. In human cells, the mitochondrial genome encodes 13 proteins that are subunits of oxidative phosphorylation (33, 34). The relative TE of mRNAs involved in oxidative phosphorylation was increased significantly, while the mRNA levels were decreased at 4 and 8 hpi of VACV infection (**Fig. 5-4A and B**). A heat map of the DTE of oxidative phosphorylation mRNAs clearly illustrates the translational upregulation of almost all of the oxidative phosphorylation genes (**Fig. 5-4C**). The RPF levels of a limited number of genes were even increased >2-fold after

VACV infection, which includes 9 of the 13 protein-encoding genes in the human mitochondrial genome (**Fig. 5-4D and 5-2C**).

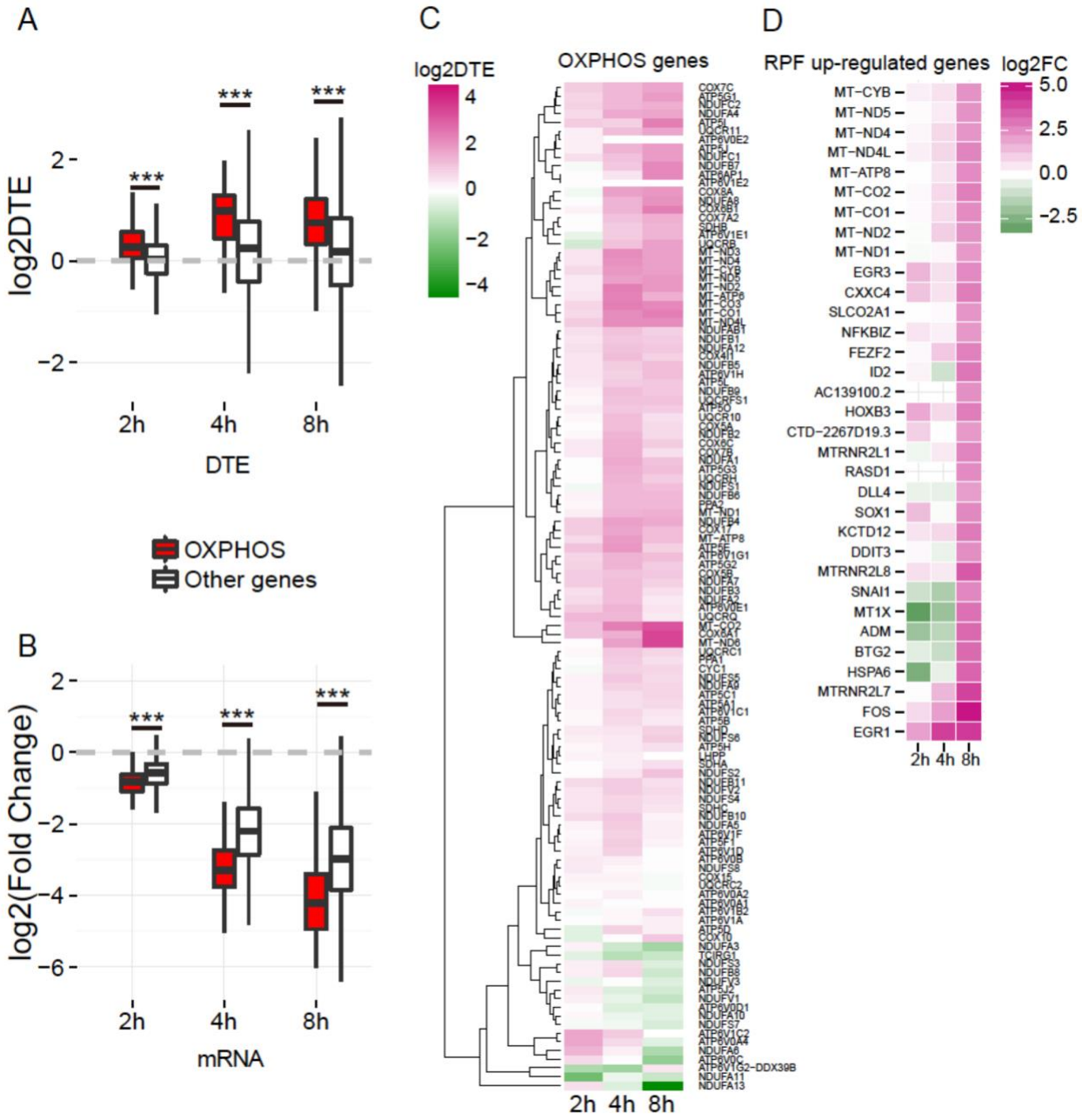


Figure 5-4: Oxidative phosphorylation genes are enriched in mRNAs with enhanced relative TE.

(A, B) Box plots of oxidative phosphorylation genes (red) and other cellular genes (white) at the DTE (A) and mRNA (B) levels. ***, P < 0.001 (Mann-Whitney U test). (C) Heat map of DTE of

oxidative phosphorylation genes at 2, 4, and 8 hpi. (D) Heat map of cellular genes with increased RPFs (>4-fold) at 2, 4 or 8 h after VACV infection. FC, fold change.

The downregulation of mRNA levels and upregulation of protein levels involved in oxidative phosphorylation were verified by quantitative reverse transcription (RT)-PCR and Western blotting of nuclear genome-encoded SDHB and mitochondrial genome-encoded MT-CO1 and MT-CO2, which are subunits of the oxidative phosphorylation complexes (**Fig. 5-5A and B**). To further examine whether oxidative phosphorylation proteins continued to be synthesized during the shutoff after VACV infection, we examined newly synthesized proteins in VACV-infected cells by labeling with AHA (l-azidohomoalanine) by a Click-iT chemistry technique. The newly synthesized proteins were then precipitated and detected with a total oxidative phosphorylation human antibody cocktail that recognizes five proteins of the oxidative phosphorylation complexes. The results indicated higher levels of UQCRC2, SDHB, and MT-CO2 in VACV-infected cells than in mock-infected cells, while the ATP5A level remained stable (or slightly increased) and the NDUFB8 level decreased. The newly synthesized ATP5A, SDHB, and MT-CO2 could be clearly detected in VACV-infected cells at higher levels than in mock-infected cells (**Fig. 5-5C**). Interestingly, NDUFB8 was one of the few oxidative phosphorylation mRNAs with decreased relative TE at 4 or 8 hpi (**Fig. 5-4C**). These findings suggested that some of the mRNAs involved in oxidative phosphorylation were translationally upregulated, which resulted in continuous protein synthesis under the condition of VACV-induced host shutoff.

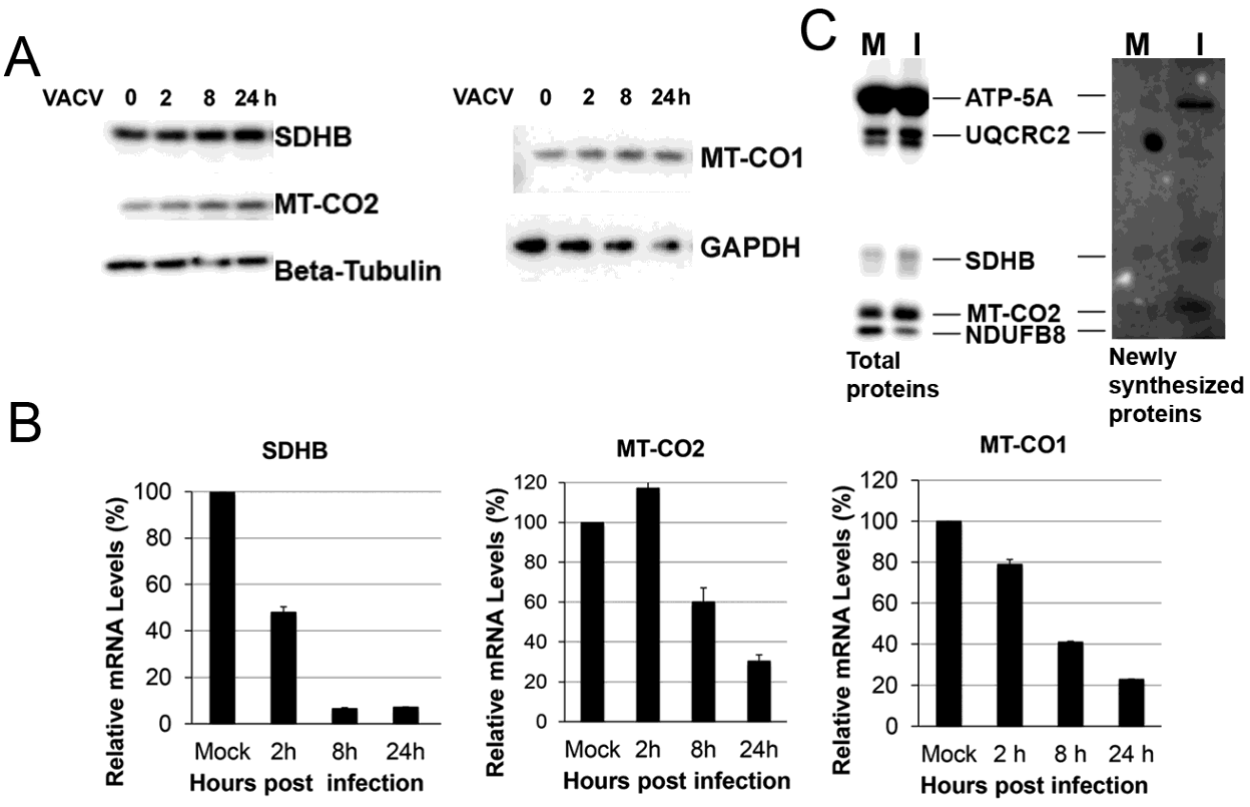


Figure 5-5: Increased synthesis of oxidative phosphorylation proteins during VACV-induced host shutoff.

(A) Western blotting analyses of SDHB, MT-CO1, and MT-CO2 levels during the course of VACV infection (MOI of 5). Beta-tubulin and GAPDH were used as loading controls. Results representative of at least three independent experiments are shown. (B) Quantitative RT-PCR analyses of SDHB, MT-CO1, and MT-CO2 gene mRNA levels. The mRNA levels were normalized to 18S rRNA levels at different time points. Each result is an average of at least three independent experiments. The error bars indicate the standard deviation of three experiments. (C) HeLa cells infected with VACV (MOI of 5) or mock infected were starved in methionine-free medium and then incubated in medium containing AHA between 3 and 7 hpi. Total proteins (left) and newly synthesized proteins labeled with AHA-containing peptides with alkyne-biotin (right) that were precipitated with streptavidin beads were subjected to Western blotting analyses with a total oxidative phosphorylation human antibody cocktail. M, mock infection; I, infection.

VACV infection has been reported to enhance the oxygen consumption rate (35), an indicator of cellular respiration, suggesting that VACV infection may increase oxidative phosphorylation-based ATP production. In fact, we observed that the ATP synthase activity and ATP level increased significantly in VACV-infected HeLa cells (**Fig. 5-6A and E**). Moreover, we tested the effects on VACV replication of carbonyl cyanide m-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation, and antimycin A, an inhibitor of oxidative phosphorylation complex III, at concentrations that did not affect cell viability (**Fig. 5-6B**). The addition of both drugs to the culture medium significantly suppressed VACV replication by >10-fold (**Fig. 5-6C**). Interestingly, CCCP inhibited VACV replication in a dose-dependent manner (**Fig. 5-6D**), which was correlated with a dose-dependent decrease in ATP levels by the CCCP treatment of VACV-infected cells (**Fig. 5-6E**). The block of VACV replication by CCCP and antimycin A was at or before late viral protein expression, as evidenced by a much lower VACV late protein expression level in cells treated with CCCP or antimycin A (**Fig. 5-6F**). Further experimentation with a reporter VACV containing a firefly luciferase gene under the control of a VACV early/late promoter indicated that the expression of both early and late VACV genes was affected by CCCP or antimycin A treatment (**Fig. 5-6G and H**). Because the chemicals were added at 1 hpi, this suggested that the impairment of oxidative phosphorylation started to affect VACV replication at the stage of early gene expression after entry. These results demonstrated that the oxidative phosphorylation function is important for VACV replication and its ATP generation capacity is enhanced during VACV infection.

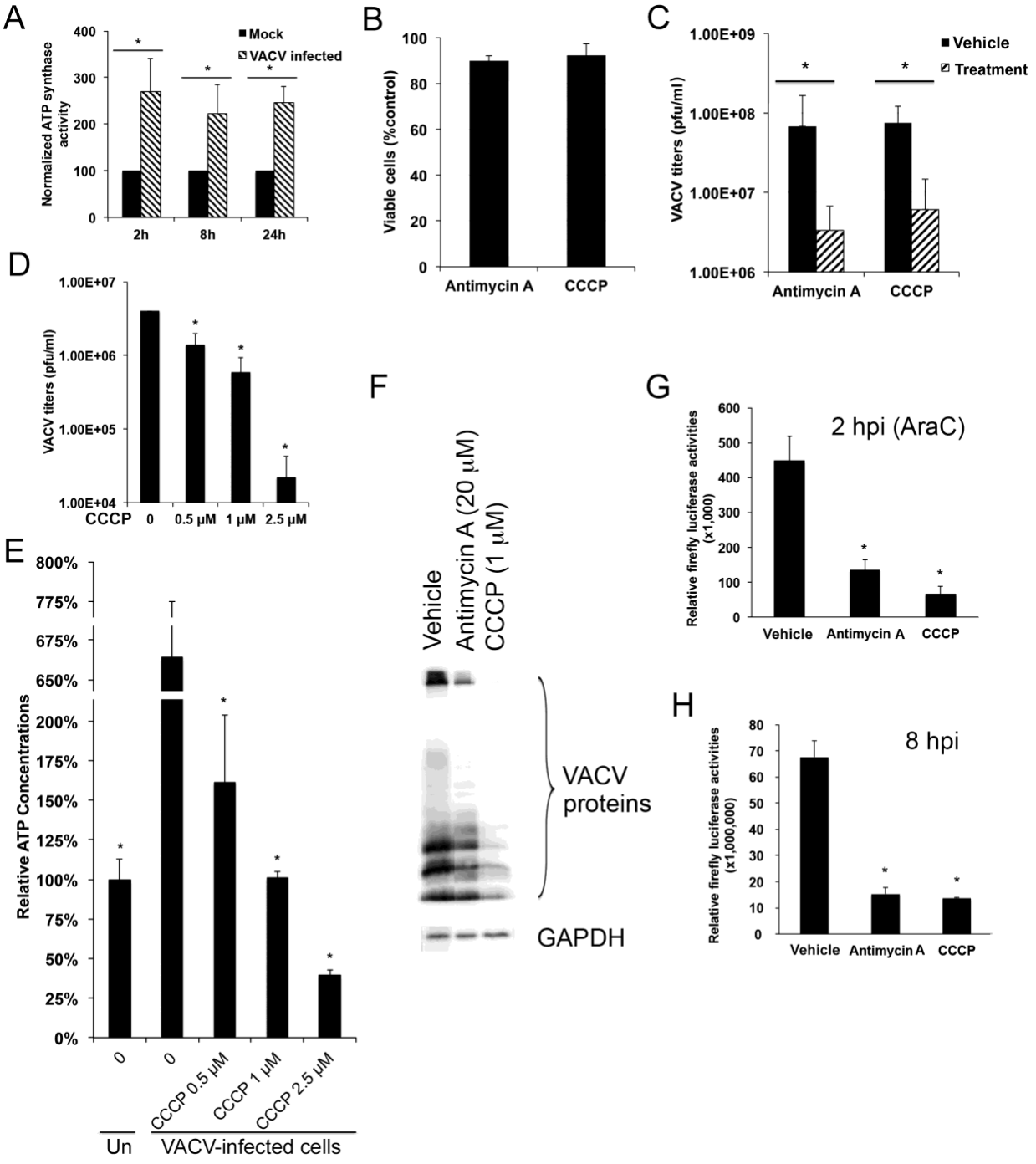


Figure 5-6: Oxidative phosphorylation activity plays an important role in VACV infection. (A) ATP synthase activity is enhanced in VACV-infected HeLa cells. HeLa cells were infected with VACV at an MOI of 3, and ATP synthase activity was determined at 2, 8, and 24 hpi. ATP synthase activity was measured with an ATP synthase enzyme activity microplate assay kit. Significant differences, defined by a P value of <0.05, are indicated by asterisks. (B) HeLa cells

were treated with the chemicals indicated (CCCP (1 μM) and antimycin A (20 μM)), and cell viability was determined at 24 h posttreatment. (C) Inhibition of VACV replication by the mitochondrial function inhibitors CCCP (1 μM) and antimycin A (20 μM). HeLa cells were infected with VACV at an MOI of 3. The chemicals indicated were added to the medium at 1 hpi. VACV titers were determined at 24 hpi by a plaque assay. Significant differences, defined by a P value of <0.05, are indicated by asterisks. (D) HeLa cells infected with VACV were treated with CCCP at the concentrations indicated, which did not significantly affect cell viability (not shown). VACV titers were determined by a plaque assay at 24 hpi. Significant differences from the titer with no CCCP treatment, defined by a P value of <0.05, are indicated by asterisks. (E) ATP levels of uninfected HeLa cells (Un) or HeLa cells infected with VACV were treated with CCCP at the concentrations indicated. ATP levels were determined 16 hpi with an ATP determination kit. Significant differences from the ATP level in VACV-infected cells without CCCP treatment, defined by a P value of <0.05, are indicated by asterisks. (F) Western blotting of VACV protein expression with the anti-VACV serum treatment indicated at 16 hpi. The vertical lines in the VACV protein expression blot were generated because of a limitation of the Western blot imaging system when processing strong signals. (G and H) HeLa cells were infected with WRvFire VACV that contained a firefly luciferase gene under the control of a viral early/late promoter with the treatment indicated. Luciferase activity was measured at 2 hpi in the presence of AraC (G) or at 8 hpi without AraC treatment (H). AraC is a DNA replication inhibitor that arrests VACV replication at the early gene expression stage. Significant differences from vehicle treatment, defined by a P value of <0.05, are indicated by asterisks.

Short 5′ UTRs of oxidative phosphorylation mRNAs can confer a translational advantage on VACV-infected cells.

The 5′ UTR plays an important role in the regulation of mRNA translation (36). We therefore examined whether 5′ UTRs may contribute to the upregulation of oxidative phosphorylation mRNA TE. We did not observe apparent consensus sequences in the 5′ UTRs of oxidative phosphorylation mRNAs. Interestingly, we observed instead that the 5′ UTRs of human oxidative phosphorylation mRNAs are shorter than the overall mRNAs. The median

length of the longest forms of individual 5′ UTRs is 102 nucleotides (nt) for oxidative phosphorylation mRNAs and 161 nt for the overall mRNAs (**Fig. 5-7A**). The minimum free energy (MFE) for RNA folding of the oxidative phosphorylation mRNA 5′ UTRs is also significantly higher than that of the overall cellular mRNA 5′ UTRs (**Fig. 5-7B**), indicating a less complex secondary structure. A shorter 5′ UTR with a less complex secondary structure may contribute to the higher TE of the corresponding mRNAs in VACV-infected cells (37). To test this possibility, we selected four short 5′ UTRs from nucleus-derived oxidative phosphorylation mRNAs and generated mRNAs containing individual 5′ UTRs upstream of a firefly luciferase reporter gene by in vitro transcription. Each of the mRNAs was transfected into uninfected or VACV-infected HeLa cells at 2 hpi together with an RNA containing a Renilla luciferase reporter gene as a transfection efficiency control. Luciferase activity was measured as an indicator of mRNA translation. The use of RNA rather than DNA reporters in this assay ruled out the possibility of a difference in luciferase activities attributable to transcription. All of the 5′ UTRs tested exhibited higher luciferase activities in VACV-infected cells, though the enhancement does not correspond linearly to the length of the 5′ UTRs (**Fig. 5-7C**). As a control, a longer 5′ UTR (119 nt) of TRIM73 mRNA, which is not an oxidative phosphorylation gene product, did not confer higher luciferase activity on VACV-infected cells (**Fig. 5-7C**). These results suggested that short oxidative phosphorylation mRNA 5′ UTRs can be one mechanism that confers a translational advantage on VACV-infected cells.

To further test whether a shorter 5′ UTR may have a translational advantage in VACV-infected cells, we generated another firefly luciferase reporter mRNA containing three tandem COX6A1 5′ UTR copies. The use of multiple 5′ UTR copies rather than a longer 5′ UTR from a different mRNA was to rule out the contribution of different sequence elements other than length

as much as possible. The mRNA with three 5′ UTR copies did not give VACV-infected cells a translational advantage over uninfected cells (**Fig. 5-7D**). Interestingly, the luciferase activity of the reporter with the tandem 5′ UTR copies was higher than that with one copy of the COX6A1 5′- UTR in uninfected cells (data not shown), suggesting that the tandem 5′-UTR copies did not downregulate the translation of the reporter mRNA in uninfected cells. These experimental results, together with the global analysis, suggested that a short 5′ UTR could confer a translational advantage of oxidative phosphorylation mRNAs on VACV-infected cells during host shutoff. However, it is worth noting that various elements in a 5′ UTR can regulate mRNA translation in addition to a short 5′ UTR and these results do not exclude other mechanisms.

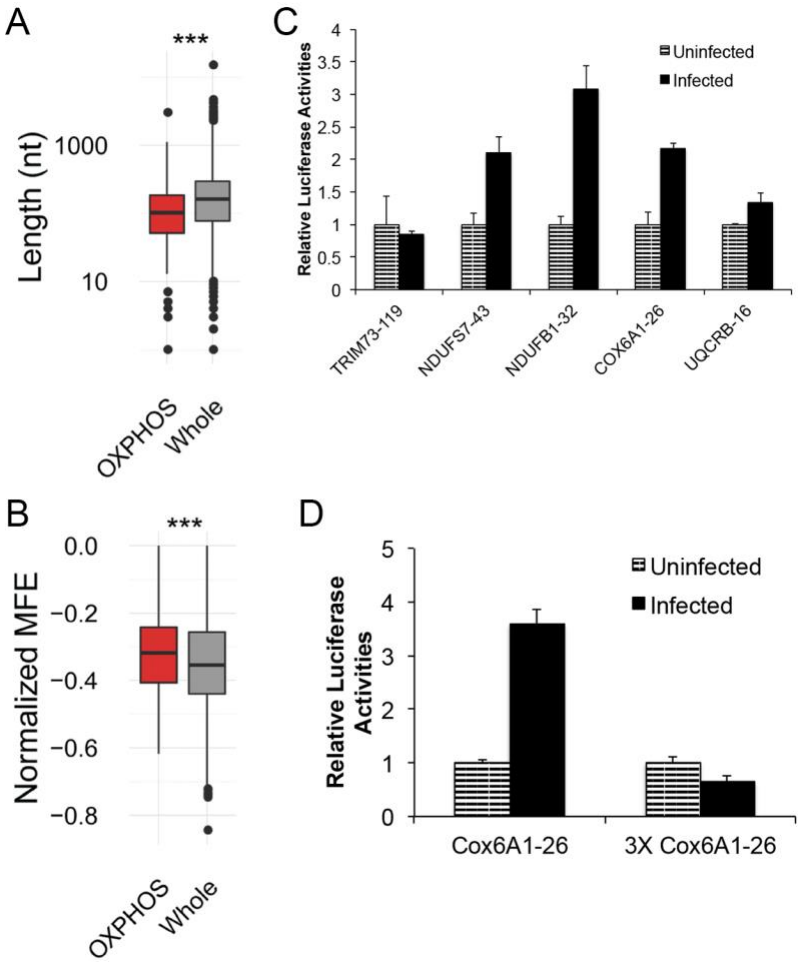


Figure 5-7: Short 5′ UTRs of oxidative phosphorylation mRNAs can confer a translational advantage on VACV-infected cells.

(A, B) Box plots of the lengths (A) and normalized MFE (B) of oxidative phosphorylation and whole cellular mRNAs. ***, $P < 0.001$ (Mann-Whitney U test). (C) Relative firefly luciferase activities of reporter mRNAs under the control of various oxidative phosphorylation mRNA 5′ UTRs in mock- and VACV-infected cells. Transfection was carried out at 2 hpi, and luciferase activity was measured at 7 hpi. The number following the gene name indicates the length of the 5′ UTR. The firefly luciferase activity was normalized to a cotransfected Renilla luciferase mRNA (transfection control). For each reporter mRNA, the luciferase activity in mock-infected cells was normalized as 1. Each result is an average of at least three independent experiments. The error bars indicate the standard deviation. (D) Relative luciferase activities of firefly reporter mRNAs under the control of one copy or three tandem copies of the Cox6A1 5′ UTR in mock- and VACV-infected cells. The experiment was carried out as described for panel C.

Discussion

VACV infection causes cellular mRNA degradation. This degradation occurs through a combined action of VACV-encoded decapping enzymes D9 and D10 and cellular nuclease XRN1 (23–25, 30). VACV infection also inhibits host cell transcription (31). Our analyses showed that mRNA depletion plays a major role during the VACV-induced host shutoff that results in similar reductions of ribosome-associated cellular mRNAs. The result is consistent with several previous studies that showed the cellular mRNAs are globally downregulated after poxvirus infections (22, 28, 38–40). However, this does not rule out the possibility that cellular mRNA translation is also suppressed during VACV infection. In fact, a recent study showed that the gene product of VACV open reading frame 169 (ORF169) suppresses general cellular protein translation (41). An important question is how some of the cellular mRNAs can escape from mRNA depletion and translational repression to maintain the integrity of infected cells at a level sufficient for viral replication. Since the cellular mRNAs and nascent cellular proteins are dramatically downregulated during VACV infection (4, 5, 22, 28), measurement of steady-state levels of mRNA and protein during VACV-induced host shutoff may not be able to sensitively identify those selectively expressed genes. Global TE analysis by simultaneous RNA-Seq and ribosome profiling provides a highly sensitive approach to the identification of selectively translated mRNAs during virus-induced host shutoff (11, 32).

Oxidative phosphorylation in the mitochondrion is the major source of cellular energy production in the form of ATP (42). Even in cancer cells or rapidly dividing cells, in which a larger portion of ATP is produced by substrate phosphorylation during glycolysis in the cytoplasm, oxidative phosphorylation is still a major source of ATP production (43). Like the replication of all other viruses, that of VACV depends entirely on host cells to provide energy.

Interestingly, in the context of a host shutoff, the oxygen consumption rate, an indicator of energy metabolism, increases in VACV-infected cells (35), which may require increased expression of proteins involved in oxidative phosphorylation through selective protein synthesis. In fact, our analysis of mRNAs with elevated TE identified oxidative phosphorylation as the primary and consistent target of translation upregulation during VACV infection. Experimental evidence further demonstrated that oxidative phosphorylation activity is important for VACV replication. Therefore, these data support a model in which oxidative phosphorylation mRNA translation is selectively upregulated to meet the energy expenditure when mRNA levels are reduced during a VACV-induced host shutoff. Our finding is in concert with the notion from several recent publications that VACV infection can reprogram the cellular metabolism to favor viral replication. A study by Fontaine et al. showed that VACV depends more on glutamine than on glucose for efficient replication (44). While it is possible to produce ATP energy through glycolysis by using glucose, a pathway that does not need oxidative phosphorylation, oxidative phosphorylation is required when using glutamine as cellular fuel. Studies by Mazzon et al. showed that VACV infection increases the synthesis of some precursors of cellular energy metabolism utilized in viral replication, which may also require an elevated oxidative phosphorylation capacity (45, 46). Interestingly, a study of protein abundance in VACV-infected cells by mass spectrometry showed that several oxidative phosphorylation proteins and proton-transporting ATP synthase were among the overabundant proteins, supporting the conclusion of this study (47).

Mitochondrial functions are damaged in many viral infections. For example, herpes simplex virus 1 infection causes the degradation of host mitochondrial DNA (48), while enterovirus 71 and dengue virus infections cause an oxidative phosphorylation dysfunction in

infected cells (49, 50). On the one hand, the disruption of mitochondrial functions may help viruses to avoid eliciting innate immune responses. On the other hand, this impairment can hurt the energy production function of mitochondria, on which both viral replication and cellular survival depend. Impaired oxidative phosphorylation may be able to provide sufficient energy for some viruses. However, for viruses that need a large amount of energy, the impaired oxidative phosphorylation function could be a factor that restricts viral replication. VACV is a large DNA virus that has been annotated to encode >200 ORFs, with the potential to have an additional >500 nonclassical ORFs from our recent analysis of VACV mRNA translation (13, 51). Protein translation is one of the most energy-consuming processes; it uses 30 to 40% of all cellular energy (52–54). Compared to many small viruses that encode only one or a few ORFs, translation of a large number of proteins at high levels may need an increased rate of energy production. In fact, it has been shown that the oxygen consumption rate increases during VACV infection (35). The increased ATP synthase activity observed in the present study suggests that at least part of the increased oxygen consumption contributed to the production of ATP, which is the usable cellular energy source.

We have, in fact, analyzed upstream ORFs (uORFs), internal ribosome entry site elements, and potential conserved sequences in the oxidative phosphorylation mRNA 5′ UTRs. However, we did not observe significant differences from overall cellular mRNAs. Rather, our analyses suggest that a short, less complex 5′ UTR is at least partially responsible for the translational upregulation of some oxidative phosphorylation mRNAs in VACV-infected cells. A short, less complex 5′ UTR in Drosophila is responsible for the higher TE of some oxidative phosphorylation mRNAs under the condition of dietary restriction (55). Interestingly, the lengths of the VACV 5′ UTRs are also short. The 5′ UTRs of early mRNAs vary from 3 to 601 nt, with a

median length of 21 nt, while the 5′ UTRs of intermediate and late mRNAs all have a poly(A) leader with a length of up to 51 nt (56, 57). As the poly(A) leaders are also short, less complex 5′ UTRs, it is possible that both the viral mRNAs and some of the host cellular mRNAs utilize this feature and some common factors for efficient protein synthesis during VACV-induced host shutoff. However, it is unlikely that a short 5′ UTR is the only mechanism employed to elevate the translation of oxidative phosphorylation mRNAs during VACV-induced host shutoff. Other sequence characteristics of 5′ UTRs, CDSs, or 3′ UTRs may contribute to the difference. In fact, the CDSs, 3′ UTRs, and full-length transcripts of oxidative phosphorylation mRNAs are also significantly shorter than total human cellular mRNAs (data not shown). These features may also affect other steps of mRNA translation, such as elongation. Again, VACV ORFs encode shorter CDSs than cellular ORFs (data not shown). The 3′ UTRs of VACV early mRNAs are generally short, while the 3′ UTRs of intermediate and late VACV mRNAs are heterogeneous (56–59). Again, the resemblance of short 5′ UTRs, CDSs, and full-length transcripts of VACV and oxidative phosphorylation mRNAs suggests that they may employ some common strategies for efficient translation during a VACV-induced host shutoff, though the advantages of a shorter 5′ UTR, CDS, or 3′ UTR may provide during this process remain elusive.

While this study showed translation upregulation as a mechanism to enhance oxidative phosphorylation capability during VACV infection, future studies will be devoted to further understanding the viral and cellular mechanisms involved in this process. Oxidative phosphorylation in mammals involves >100 genes encoded in both the nuclear and mitochondrial genomes. Translation of oxidative phosphorylation mRNAs occurs in both the cytoplasm and mitochondria by using two distinct translation systems. A recent study has suggested that translation in the two organelles is synchronized and that coordination is controlled and initiated

by cytosolic translation (60). The elevation of the relative TE of both mitochondrion- and nucleus-encoded oxidative phosphorylation mRNAs that occurs during VACV infection supports this notion. However, the translational control of oxidative phosphorylation mRNAs is complex and the mechanism that coordinates their translation in mammalian cells is largely unknown. It has been suggested that mTORC1 can selectively promote the translation of mitochondrion-related mRNAs via inhibition of the eukaryotic translation initiation factor 4E-binding proteins (4E-BPs) (61). VACV infection can inhibit 4E-BP1 by stimulating the hyperphosphorylation of 4E-BP1 (62). It is possible that inhibition of 4E-BP1 is partially responsible for the enhancement of oxidative phosphorylation mRNA translation during VACV infection. In addition, while this study addressed the role of protein synthesis in the host cell response to VACV infection, we do not exclude other posttranslational mechanisms, for example, protein stability regulation, in this process.

As host protein synthesis shutoff is caused by infections with many different viruses, selective protein synthesis through translational upregulation during virus-induced host shutoff may be a common mechanism to continuously translate proteins that are important for cells to survive for a period of time sufficient to support viral replication. In fact, while this report was under review, another study revealed that the mRNAs important in cell maintenance processes such as oxidative phosphorylation are less affected during influenza virus-induced host shutoff, and that is important for viral replication (63). Identification of these selectively translated proteins is important to elucidate the functional relevance of and mechanism involved during a virus-induced host shutoff.

Materials and Methods

Cell culture and virus infection.

Suspension HeLa S3 (ATCC CCL2.2) cells were cultured in minimum essential medium (MEM) with spinner modification and 5% equine serum in a 5% CO2 atmosphere at 37°C. Infection of HeLa S3 cells was carried out as described elsewhere (28). Adhesion HeLa (ATCC CCL2) cells were cultured in Eagle's MEM supplemented with 10% fetal bovine serum. The VACV Western Reserve strain (ATCC VR-1354) and WRvFire expressing luciferase under the control of a VACV synthetic early/late promoter were gifts from Bernard Moss (64). Preparation, titration, and infection of VACV were performed as described previously (65).

Antibodies and chemical inhibitors.

Anti-MT-CO2, anti-MT-CO1, anti-SDHB, total oxidative phosphorylation human antibody cocktail, and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibodies were purchased from Abcam (Cambridge, MA). Anti-tubulin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-VACV serum was a gift from Bernard Moss. The oxidative phosphorylation inhibitors CCCP and antimycin A were purchased from Sigma-Aldrich (St. Louis, MO). The VACV DNA replication inhibitor cytosine arabinoside (AraC) was also purchased from Sigma-Aldrich.

Ribosome profiling and RNA-Seq.

The experimental procedures for ribosome profiling and RNA-Seq were described previously (13). Briefly, ribosome profiling was carried out as described elsewhere, with minor modifications (10). VACV-infected (at a multiplicity of infection (MOI) of 10) and mock-infected HeLa S3 cells pretreated with the translational inhibitor cycloheximide were lysed and treated with DNase (Thermo Fisher Scientific, MA), and the lysate was clarified. mRNA was

isolated from a portion of the lysate with oligo(dT) and fragmented with RNase III (New England BioLabs, MA). The mRNA fragments between 50 and 80 nt were extracted. The RPFs were separated by electrophoresis after the lysate was digested with RNase I (Thermo Fisher Scientific), the ribosomes were then isolated by sucrose cushion centrifugation, and the RPFs between 28 and 34 nt were isolated. The purified mRNA and RPFs were used to generate libraries for deep sequencing as described previously (10). The purified libraries were sequenced with a HiSeq 2000 system.

Mapping, quantification, and differential gene expression analysis.

The adaptors of RNA-Seq and ribosome profiling reads were trimmed with FASTX Toolkit (v0.0.13.2, fastx_clipper: -l 25 –n –v –Q33; fastx_trimmer: -f 1 –Q33). The tRNA and rRNA were removed by Bowtie (v1.0.1, -l 20) (66). The DNA reference sequences encoding tRNAs were obtained from the Genomic tRNA Database (http://gtrnadb2009.ucsc.edu/download.html) (67). The DNA reference sequences encoding rRNAs were obtained from iGenomes (Illumina, Inc., San Diego, CA). The reads were mapped to both the human genome (Ensembl, GRCh37) and the VACV genome (NC_006998.1) with Tophat (v2.0.11, –library-type fr-firststrand) (68). Raw counts of protein-encoding genes were quantified by HTSeq (v0.6.1p2) (69). Raw counts of the host genes and the VACV genes were combined, and RPKMs were calculated by edgeR (70). The raw read numbers were normalized to the effective library sizes of total reads of the host and VACV. Genes with mean RPKMs of <1 were not included for further analysis. The RPKMs of the host genes were used to calculate differentially expressed genes at both the mRNA and RPF levels by comparing expression levels in VACV-infected cells to those in mock-infected cells. P values from multiple testing were adjusted by the Benjamini-Hochberg procedure. Genes with an

adjusted P value of ≤0.05 and a ≥2-fold absolute logarithmic change value were identified as differentially expressed.

Relative TE and GSEA.Relative TE was defined as the ratio of normalized RPF density to normalized mRNA density (17, 32). DTE was calculated as the ratio of TE under VACV-infected conditions to TE under mock-infected conditions. Among genes with a mean log2 RPKM of four related samples (mRNA and RPF from mock- and VACV-infected cells at each time point) larger than −1, those with a logarithmic DTE value of >1 were defined as translationally upregulated genes, and those with a logarithmic DTE value of less than −1 were defined as translationally downregulated genes. GSEA was carried out with the Bioconductor package piano (71).

5′ UTR, 3′ UTR, CDS, and transcript length analysis.

Sequences of protein-encoding transcripts were obtained from Gencode (v19, http://www.gencodegenes.org/releases/19.html). 5′ UTR, CDS, 3′ UTR, and transcript sequences were extracted from the aforementioned sequences, and only the longest isoforms were used for length and MFE analyses. MFE was calculated by the ViennaRNA package (v2.1.8), followed by normalization to sequence length.

Western blotting.

Cell lysates were denatured by heating, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane for Western blotting as described previously (72).

RNA extraction and quantitative RT-PCR.

Total cellular RNA was prepared with TRIzol (Thermo Fisher Scientific, MA) according to the manufacturer's procedure. Total RNA (1 μg) was used for RT with a random hexamer. The reverse-transcribed products were used for quantitative PCR of specific genes.

ATP synthase activity assay and ATP level determination. ATP synthase activity was measured with an ATP synthase enzyme activity microplate assay kit (Abcam). Briefly, samples of mock- or VACV-infected HeLa cells (MOI of 5) were collected at different times postinfection. ATP synthase from these samples was immunocaptured within the wells, and its enzyme activity was measured by determining the production of ADP, which is coupled with oxidation of NADH to NAD+ and monitored as a decrease in absorbance at 340 nm. ATP levels were measured with the ATP determination kit (Molecular Probes) by a bioluminescence assay with a recombinant firefly luciferase and its substrate luciferin in accordance with the manufacturer's protocol.

Cell viability assay.

HeLa cells were treated with chemicals or solvent vesicle at the concentrations indicated. The cells were then trypsinized and resuspended with DMEM after 24 h of treatment, the cell suspension was mixed with trypan blue at a ratio of 1:1, and cell viability was measured with a LUNA II automated cell counter. The data from at least three independent experiments were collected and averaged.

In vitro transcription.

RNAs were generated with the HiScribe T7 High Yield RNA synthesis kit (New England BioLabs). Briefly, template DNA containing the T7 promoter sequence, 5′ UTR, firefly or Renilla luciferase, and poly(A) tail coding sequences was generated by PCR. In vitro transcription and 5′ capping of the RNAs with methylated guanine were carried out in

accordance with the manufacturer's instructions. The resulting RNAs were purified with the PureLink RNA minikit (Thermo Fisher Scientific) and quantified for transfection.

RNA transfection and luciferase activity measurement.

Equal amounts of firefly luciferase reporter RNA were transfected into mock- or VACV-infected HeLa cells at 2 h after VACV infection with Lipofectamine 2000 (Thermo Fisher Scientific). Renilla luciferase reporter RNA was cotransfected as an internal control for experimental variation. Luciferase activity was measured at 7 h after VACV infection. A luminometer was used to measure luciferase activities with appropriate reporter assay reagents (Promega, WI) according to the manufacturer's instructions. Each result was an average of at least three independent experiments.

Detection of newly synthesized proteins.

Newly synthesized proteins were detected with the Click-iT AHA nascent protein kit (Thermo Fisher Scientific) combined with immunoprecipitation of the AHA-labeled proteins. Briefly, culture medium was replaced with methionine-free medium and incubated for 2 h. AHA was then added to the medium at 100 μM and incubated for 4 h to label nascent proteins. The cells were collected by centrifugation at 1,000 × g for 5 min and lysed with radioimmunoprecipitation assay lysis buffer at 4°C for 30 min. Cell lysates were collected by centrifugation at 12,000 × g for 10 min at 4°C, and the proteins in the supernatant were precipitated with methanol and chloroform and resolubilized in 50 mM Tris-Cl containing 1% SDS. A total of 200 μg of protein was subjected to click reaction for 30 min to label the AHA-containing peptides with alkyne-biotin with the Click-iT Protein Reaction Buffer kit according to the manufacture's instructions. The proteins were then precipitated with methanol and chloroform and resolubilized in 50 mM Tris-Cl containing 1% SDS, and then an equal volume of 50 mM Tris-Cl containing 6% NP-40

was added. The AHA-labeled nascent proteins were precipitated with streptavidin beads, eluted, and detected with specific antibodies.

Data availability.

The sequencing data obtained in this study have been deposited at the National Center for Biotechnology Information Sequence Read Archive (accession no. SRP056975 and SRP093314).

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Author contribution

This study was initiated, and the experiments were conceived and designed by Z.Y. and Z.X. The sample preparation and data analysis from RNA-seq and Ribosome profiling were performed by A.D., S.C., Z.Y. and Z.X. Experiments like western blot analysis, qRT-PCR,

titration, biochemical assays, reporter RNA assays were performed by S.C. and P.D. A.D., S.C. and P.D are co-first authors. P.D. contributed figures 5-6 and 5-7 (2 out of 7 figures). All authors critically examined the manuscript.

Supplemental material

Supplemental material for this article may be found at https://doi.org/10.1128/ JVI.01858-16.

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Chapter 6 - Conclusion

The findings of this dissertation improved our understanding of selective cellular and viral protein synthesis during poxvirus-induced global inhibition of host protein synthesis. The results presented herein have aided in advancing our understanding of how certain cellular proteins are selectively synthesized, and the strategies that poxviruses have evolved to boost viral protein synthesis during poxvirus-induced host shutoff.

During infection, poxvirus makes host cells conducive for viral replication by inhibiting host cell cytopathic effect, altering cellular metabolism, and causing a host shutoff that is marked by global inhibition of host protein synthesis. Although this host shutoff may facilitate the reallocation of cellular resources for viral replication and the evasion of host antiviral immune responses, it poses a challenge to maintain viral replication if continuous synthesis of crucial cellular proteins and viral proteins is vital for the viral replication. However, it remains unclear whether and how certain cellular and viral proteins may be selectively synthesized during poxvirus-induced host shutoff.

Chapters 3, and 4 of this this dissertation present the strategy that VACV uses to boost viral protein synthesis during host shutoff. Through a literature review, we identified an unusually striking feature of all poxvirus mRNAs transcribed after viral DNA replication (post-replicative mRNAs) at the 5'-untranslated region (5'-UTR), which is known as the poly(A) leader. These 5'-poly(A) leaders are non-templated and of heterogeneous length, and their function during poxvirus infection remained a long-standing question. To dissect the role of the 5'-poly(A) leader in mRNA translation during poxvirus infection, we developed an in-vitro transcribed RNA-based luciferase reporter assay (**Chapter 2**). The RNA-based luciferase reporter assay described here circumvents issues of plasmid replication in poxvirus-infected cells

and cryptic transcription from the plasmid. This protocol can be used to determine translation regulation by cis-elements in an mRNA including 5'-UTR and 3'-UTR in systems other than poxvirus-infected cells. Moreover, different modes of translation initiation such as cap-dependent, cap-independent, re-initiation, and internal initiation can be investigated using this method.

Using in-vitro transcribed RNA-based luciferase reporter assay and other experiments, we determined that a 5'-poly(A) leader conferred a selective translational advantage to mRNA in poxvirus-infected cells (**Chapter 3**). A constitutive and uninterrupted 5'-poly(A) leader with 12 residues was optimal. Since 5'-poly(A) leaders were frequently 8–12 residues in length, the result suggests that the poly(A) leader has been evolutionarily optimized to enhance poxvirus protein production. A 5'-poly(A) leader also could increase protein production in the bacteriophage T7 promoter-based expression system of VACV. Interestingly, although VACV post-replicative mRNAs do have 5'- methylated guanosine caps and can use cap-dependent translation, in vaccinia virus-infected cells, mRNA with a 5'-poly(A) leader could also be efficiently translated in cells with impaired cap-dependent translation. However, translation was not mediated through an IRES. These results point to a fundamental mechanism that poxvirus uses to efficiently translate its post-replicative mRNAs.

The 5'-poly(A) leader is present at the 5'UTR region associated with translation regulation, and poly(A) leader-mediated translational advantage does not depend on the status of the mRNA cap. This supports the notion that translation initiation factors may bind directly to 5'-poly(A) leader of an mRNA to initiate translation, thereby critically influencing VACV post-replicative gene expression. We performed immunofluorescence assays to determine the trans-factors enriched in the virus factory, where viral post-replicative mRNAs translate and are

thus required for translational advantage. During VACV infection, poly(A) RNA sequence binding protein La-related protein 4 (LARP4)—unlike poly(A) binding protein (PABP)—is enriched in the virus factory (**Chapter 4**). A decrease in LARP4 protein level reduces VACV replication, blocks post-replicative protein expression, and decreases 5'poly(A) leader-mediated translational advantage. Further studies have suggested that LARP4 is crucial for the cap-dependent and cap-independent mode of translation from the 5'-Poly(A) leader. LARP4 binding to the 5'-poly(A) leader is dispensable for mRNA stability during poxvirus infection. These results suggest that LARP4 by presumably binding to the 5'-poly(A) leader augments the post-replicative mRNA translation of VACV.

In **Chapter 5** of this dissertation, we performed simultaneous RNA sequencing and ribosome profiling (quantifying genome-wide levels of mRNA and actively translating mRNA, respectively) to measure the host mRNA translation rate during a VACV-prototypic poxvirus-induced host shutoff. This allowed us to analyze the responses of host cells to VACV infection at both the transcriptional and translational levels. The analyses showed that cellular mRNA depletion played a dominant role in the shutoff of host protein synthesis. Though the cellular mRNAs were significantly reduced, the relative translation efficiency of a subset of cellular mRNAs increased, particularly those involved in oxidative phosphorylation that are responsible for cellular energy production. Further experiments demonstrated that the protein levels and activities of oxidative phosphorylation increased during VACV infection, while the inhibition of cellular oxidative phosphorylation function significantly suppressed VACV replication. Moreover, the short 5' untranslated region and less complex secondary structure of the oxidative phosphorylation mRNAs contributed to translational upregulation. These results provide evidence of a mechanism that couples translational control and energy metabolism, which are

two processes that all viruses depend on host cells to offer in order to support VACV replication during host shutoff.

In this dissertation, *we have addressed* the major gap in understanding the fundamental gene expression mechanism of poxviruses during host shutoff. An improved understanding of this mechanism will help us design better poxvirus vectors to treat various infectious and non-infectious disease such as cancers. Another, more significant impact will be in the poxvirus-based vaccine field. Scientists have been using poxvirus-based vaccines since these can induce both innate and adaptive immunity, which has shown to have higher protection. The findings of this dissertation will aid in achieving higher and more efficient production of antigens that are difficult to express to elicit effective immune response and will help overcome this limiting factor for vaccine development. To address this limitation, our research has shown that having a 5'-poly(A) leader in the 5'UTR of any foreign gene can significantly enhance protein production (antigen) using poxvirus as a vector. Furthermore, identification of the 5'-poly(A) leader binding protein required for translational advantage will facilitate in the development of new target for anti-poxviral therapy. Like other viruses, VACV exploits a pre-existing cellular translation mechanism, and identification of the poly(A) leader binding protein could lead to the discovery of unknown cellular translation mechanisms. Additionally, in poxvirus-based expression systems, the use of poly(A) leader upstream of any foreign gene followed by incorporation the poly(A) leader binding protein will make the expression system more self-sufficient.

Another *important contribution* in pursuit of determining the selectively synthesized cellular proteins during poxvirus-induced host shutoff, was revealing that proteins involved in oxidative phosphorylation were synthesized at the same or higher-level during shutoff. Since mRNAs for oxidative phosphorylation decreased over time during infection, translational

efficiency was higher from the low quantity of enduring mRNA-maintained protein level. Interestingly, our results suggest a mechanism hijacked by the virus to facilitate poxvirus replication during shutoff by enhancing energy metabolism via the coupling of translational control and energy metabolism—two processes that all viruses depend on host cells to provide.

Future studies will focus on determining the specific translation initiation factors required for 5'-poly(A) leader-mediated translational advantage and for translation from short 5'UTR-bearing oxidative phosphorylation mRNAs during poxvirus infection. Since the poly(A) leader has a translation advantage during poxvirus infection, future studies will emphasize on identifying the viral or cellular protein/s required for poly(A) leader-mediated enhanced translation. We will also determine the roles that LARP4, RACK1, and decapping enzymes (D9 and D10) play in the overall advantage from the poly(A) leader. All these avenues may lead to an unidentified translational mechanism that would contribute greatly to the existing knowledge on this important step of gene regulation at the mRNA translation level.

The summary of this dissertation that increased our understanding of the selectively synthesized cellular proteins and VACV post-replicative proteins during VACV-induced host protein synthesis shutoff is shown in **Fig 6-1**.

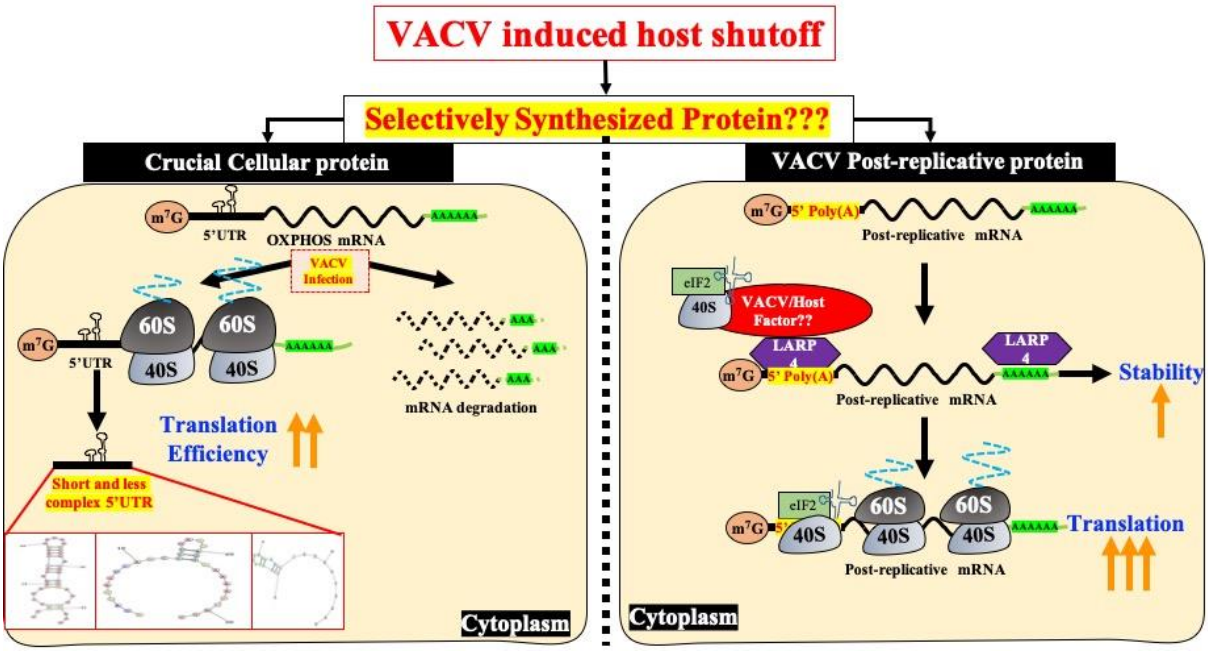

Figure 6-1: Selective protein synthesis during VACV-induced host shutoff

During VACV-induced host shutoff, viral and specific cellular proteins are selectively synthesized to drive efficient viral replication. The viral post-replicative mRNAs use evolutionary optimized 5'-Poly(A) leader at the 5'-UTR region to mediate translation advantage to VACV post-replicative mRNAs. The 5'-poly(A) leader recruits RNA binding protein LARP4 and potentially other initiation factors to mediate translation in both uninfected and VACV infected cells. During VACV infection, however, viral protein/s augments the translational initiation to provide translational advantage to 5'-poly(A) leader bearing VACV post-replicative mRNAs. The crucial cellular protein necessary for VACV replication are proteins encoding for different complexes of oxidative phosphorylation (OXPHOS). The mRNAs encoding for proteins of OXPHOS have shorter and less complex secondary structure bearing 5'-UTR that provides higher translation efficiency during VACV infection. These findings advanced our understanding on selectively synthesized proteins during poxvirus-induced host shutoff.