AN ABSTRACT OF THE DISSERTATION OF

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Title: Translation of Dengue Virus RNA: Influence of the Untranslated Regions on 5´-cap Dependent Translation and Ribosome Scanning

Abstract approved:

Theo W. Dreher

In this thesis, I studied the translation of dengue virus RNA using a luciferase reporter gene system in Vero cells. The dengue reporter mRNA construct, which harbors 5´-terminal viral nts and 3´-terminal nts, could be translated efficiently compared to an alpha globin reporter construct. The 5´-cap structure and 3´-untranslated region (UTR) of dengue virus RNA were found to contribute to strong enhancement of translation. The 5´-cap and 3´-UTR act synergistically to enhance translation like the 5´-cap and 3´-polyA tail of a cellular mRNA. Deletion analysis of each individual structural element in the dengue 3´-UTR indicated a cumulative effect in enhancing dengue translation. The cyclization sequences, which are present in the 5´ and 3´ portion of the genome to facilitate the end-to-end interactio, were tested for their importance in translation. The finding that a mutation of the sequence in the 3´-UTR, but not in the 5´, exhibited a deteriorating effect, indicates that cyclization of the genome via the sequences is not required for efficient dengue translation. The importance of a 5´-cap in dengue RNA translation was supported by using an inhibitor of cap-dependent translation, LY294002,

whose presence significantly inhibited dengue reporter RNA translation in vivo. Dengue viral replication was also found to be sensitive to the presence of inhibitor, indicating that dengue viral translation does not take advantage of inhibition of cap-dependent cellular translation, unlike mRNA with an internal ribosomal entry site. Consistent with our finding of 5´-cap dependency of dengue RNA translation, introduction of a stable stem loop structure in front of the dengue reporter construct significantly inhibited translation, strongly suggesting that dengue RNA does not harbor an internal ribosome entry site.

To examine whether ribosomes scan through the 5´-UTR of dengue RNA, various upstream AUGs (uAUG) were introduced in the 5´-UTR of a dengue construct with a green fluorescent reporter protein. uAUG containing reporter constructs showed reduced translation from the authentic AUG in vivo and in rabbit reticulocyte lysate, suggesting that dengue translation is utilizing the conventional scanning mode of ribosome for AUG recognition. All together, dengue virus utilizes the cap-dependent scanning mode for translation initiation.

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Translation of Dengue Virus RNA: Influence of the Untranslated Regions on 5´-cap Dependent Translation and Ribosome Scanning

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Wei-Wei Chiu

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APPROVED:

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Wei-Wei Chiu, Author

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CONTRIBUTION OF AUTHORS

Dr. Geroge Rohrmann assisted with discussion and helped designing experiments of Chapter 4.

Dr. Daiki Matsuda assisted with data collection in all the electroporation experiments and the critical reading.

Dr. Richard M. Kinney provided all the crucial materials and protocols to begin the project.

Andrew Regan assisted 4 of the plasmid constructs used in Chapter 2.

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Dr. Connie Bozarth provided construction of 1 plasmid construct used in Chapter 2 and 5 plasmid constructs used in Chapter 4.

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To my mother, my father, my sisters
my dear husband and my two sweet kitties, Cola and QQ

Translation of Dengue Virus RNA: Influence of the Untranslated Regions on 5´-cap Dependent Translation and Ribosome Scanning

Chapter 1 INTRODUCTION

Dengue virus causes a major public health problem in tropical and subtropical areas of the world (Halstead, 1980b). Yearly, over 100 million cases of dengue infection worldwide are estimated to occur and have become not only a huge health problem but also a serious economical burden to those areas that are dengue endemic (World Health Organization and Center for Disease Control and Prevention). Dengue virus is a mosquito-borne, positive strand RNA virus. There are four serotypes of dengue virus (DEN1, DEN2, DEN3 and DEN4), which are known to cause a broad spectrum of nonspecific viral syndromes, ranging from fever and headache to severe and fatal hemorrhagic disease, known as dengue hemorrhagic fever and dengue shock syndrome. Dengue hemorrhagic fever is a severe febrile disease that is characterized by abnormalities of homeostasis and increased membrane permeability, also characteristic of dengue shock syndrome (Halstead, 1980a).

1.1 Dengue epidemics

The first cases of dengue fever were described in 1779 in Cairo, Egypt and in Jakarta, Indonesia (Siler, 1926), and a year later dengue fever was reported in Philadelphia, United States. Subsequent outbreaks were reported between 1823 and 1905 in India, the Caribbean and Hong Kong. In the twentieth century, several major outbreaks were reported in United States, Australia, Greece, Taiwan, and Japan. In the late twentieth century, dengue virus was responsible for epidemics in Southeast Asia and central America. New epidemic patterns of dengue disease emerged, with increased incidence of dengue hemorrhagic fever and dengue shock syndrome in tropical and subtropical areas in which the four serotypes of dengue viruses are endemic (Halstead, 1966). It has been suggested that the rapid dissemination of dengue in Central and South America is

attributed to increasing airplane travel, the failure to control mosquito populations, mainly *Aedes aegypti*, and ecological conditions in Central and South American cities (Gubler, 1989a; Gubler, 1989b). In the United States, dengue fever was eliminated by mosquito vector control in the 1920s but the reintroduction of dengue infection has happened occasionally in southern States (Hafkin, 1982). Suspected cases of dengue reintroduction were reported by the Centers for Disease Control in Texas in 1980 and Florida in 1982 with the identification of dengue fever and/or *Aedes aegypti* populations infected with dengue viruses. Since the principal vector, *Aedes aegypti,* is found in the southern United States, it is possible for dengue to reoccur in this area.

1.2 Dengue virus transmission

Transmission of dengue virus is potentially carried out by *Aedes aegypti* and *Aedes albopictus*. *Aedes aegypti* originated in Africa and has spread throughout most of the world and can be found between 30 degrees north latitude and 20 degrees south latitude (Rico-Hesse, 1990; Service, 1992). The female mosquito, which feeds during daytime (Halstead, 1984), has the ability to spread dengue virus to another host after feeding on a viremic host or can transmit the virus 8-10 days after it has amplified in the salivary gland (Gubler and Rosen, 1976). Once mosquitoes are infected with dengue virus, they have the ability to transmit the virus throughout their entire life (Halstead, 1984). Transovarian transmission is possible for dengue virus-infected *Aedes aegypti*; its eggs, which are highly resistant to drying, allow for the extended preservation of the viruses in the environment (Cheong, 1986; Khin and Than, 1983; Rosen et al., 1954). *Aedes albopictus,* which has a higher biting frequency than *Aedes aegypti,* originated in

Southeast Asia, and recently has been introduced into the United States, Europe, and Nigeria (Lim, 1979). The strain of *Aedes albopictus* found in Europe is a cold-resistant strain, and it has been suggested that it may result in future dengue outbreaks (Kautner et al., 1997).

1.3 Dengue virus classification

Dengue virus belongs to the genus *Flavivirus* in the family *Flaviviridae* (Westaway et al., 1985). The *Flavivirus* genus includes yellow fever virus, dengue virus, West Nile virus, Japanese encephalitis virus, Kunjin virus, St. Louis encephalitis virus and tick-borne encephalitis virus. Dengue virus was initially identified as yellow fever virus due to their similarity in epidemiological characteristics and serological reactions (Craig, 1911; Craig, 1920). It has been found that human anti-dengue sera could cross-react with yellow fever virus, West Nile virus, and Japanese encephalitis virus (Sabin, 1950). The separation of dengue virus from yellow fever virus did not occur until the development of haemagglutination inhibition assay by Casals and Brown (1954) and the discovery of a specific epitope on dengue envelope protein that could distinguish dengue virus from the other flaviviruses (Henchal et al., 1982). Two other closely related genera in the family are *Pestivirus*, which includes viruses such as bovine viral diarrhea virus and swine fever virus, and *Hepacivirus*, which includes hepatitis C virus. These two genera of the *Flaviviridae* have similar gene arrangement as the genus *Flavivirus*, but they do not carry a 5′-^{7m}GpppN cap structure in the genomic RNAs, which are translated using internal ribosomal entry sites (Kolupaeva et al., 2000; Lukavsky et al., 2000).

1.4 Dengue viral genome organization

1.4.1 Dengue untranslated regions and conserved elements

1.4.1.1 Dengue 5´- and 3´-untranslated regions

Dengue virus contains a single-stranded, positive-sense RNA genome with a type I cap structure ($^{m7}GpppA_m$) at the 5´-end of the genome, but lacks a polyA tail at the 3´-end (Lindenbach, 2001). The RNA genomes of dengue viruses are about 11 kilobases in length and encode a single open reading frame with about 100 nucleotides of 5´ untranslated region (UTR) followed by the first AUG codon, from which a long open reading frame (ORF) is initiated. The secondary structures of the 5´- and 3´-UTRs of dengue viruses and other flaviviruses have been predicted (Brinton and Dispoto, 1988; Olsthoorn and Bol, 2001; Proutski et al., 1997), and several predicted structures are conserved among the flaviviruses. Although the detailed functions of each structural element in viral translation, replication and encapsidation are still not clear, deletions of various 3´-structure elements using replicon constructs of dengue and West Nile viruses have shown that structural elements in the 3´-end of the viral genome are important for viral replication (Alvarez et al., 2005a; Lo et al., 2003). The stem loop (SL) structure at the very 3´ end (3´-SL) of the flavivirus genome is absolutely required for viral replication (Yu and Markoff, 2005). It has also been suggested that dengue NS3 protease can interact with the dengue 3´-SL structure (Cui et al., 1998). In addition, the cellular protein translation elongation factor 1A and auto-antigen La protein have the ability to interact with 3´-SL structure of several Flaviviruses and the 3´-SL structure itself has the ability to regulate viral replication (Blackwell and Brinton, 1997; De Nova-Ocampo et al., 2002). Although some studies have addressed the conserved elements predicted in

flavivirus 3´-UTRs, their detailed functions for viral translation, replication, and encapsidation are still unclear.

1.4.1.2 Dengue virus cyclization sequences

The conserved sequences (CS and cCS) located in the 3´-UTR and 5´ capsid coding region of flaviviruses, respectively, have been shown to be involved in direct 5´-3´ interaction that is important for viral replication (Alvarez et al., 2005b; Khromykh et al., 2001a; Westaway et al., 2003). The requirement of 5´-3´ interaction for viral RNA synthesis has been demonstrated by using dengue RNA-dependent RNA polymerase in an *in vitro* transcription system (You et al., 2001). Direct visualization of the 5´-3´ interaction of dengue RNA has been demonstrated using atomic force microscopy along with discovery of a 16-nucleotide element at the 5´-end of viral genome that is required for an additional pairing with segments within the 3´-SL and that is important for viral replication (Alvarez et al., 2005b). In Kunjin virus, the 5´-cCS and 3´-CS complementarity was predicted by computer modeling using the full-length viral genome (Khromykh et al., 2001a). Furthermore, mutation of 5´-cCS or 3´-CS in a Kunjin virus replicon abolished replication, while restored ability for mutated 5´-cCS and 3´-CS elements to base pair allowed the rescue of replication. Similar findings were also found by using dengue and West Nile virus replicons, suggesting that the 5´-3´ genome cyclization is important for flavivirus replication (Alvarez et al., 2005a; Lo et al., 2003).

1.4.2 Dengue virus coding region

The dengue genome encodes a single open reading frame that could be processed in to 3 structural proteins (capsid, pre-membrane and envelope proteins), and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Fig. 1.1).

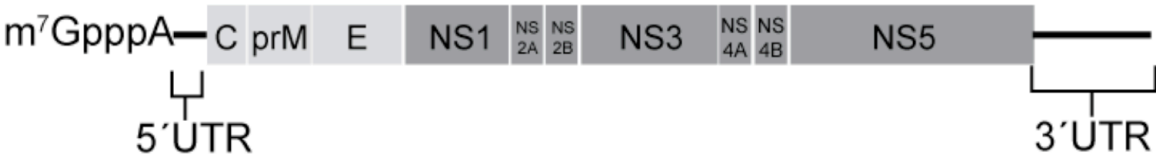

Fig 1.1 DEN2 viral genome
DEN2 genome with 5´-^{m7}G cap and 3´ non polyA tail. Single ORF encoded by DEN2 could be processed into 3 structural proteins, such as capsid (C), premembrane protein (prM), envelope protein (E), and 7 non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.

1.4.2.1 Dengue viral structural proteins

Dengue capsid protein has a molecular weight of about 13.5 kDa and is highly basic (rich in lysine and arginine residues) (Deubel et al., 1988; Hahn et al., 1988; Mason et al., 1987). It has been suggested that the capsid protein interacts with dengue genomic RNA non-specifically through its highly basic character. Dengue capsid protein contains four α-helices and forms a dimer in solution (Jones et al., 2003; Ma et al., 2004). The three-dimensional structure of dengue capsid protein as solved by NMR showed that dimeric capsid proteins expose hydrophobic domains at one side of the dimer, which may interact with the lipid bilayer of the envelope. Positively charged domains are placed at the other side of the capsid dimer, which may allow for interaction with viral RNA (Jones et al., 2003; Ma et al., 2004). Flaviviral capsid proteins are important in the selection of viral RNA for encapsidation, as cells infected with tick-borne encephalitis virus that lacks the capsid gene produce subviral particles, that contain no RNA genome (Ferlenghi et al., 2001).

Cleavage by host-encoded signalase is responsible for separation of capsid protein and pre-membrane protein, while cleavage adjacent to the C-terminal hydrophobic domain of the capsid protein by the viral encoded protease, NS3, is responsible for release of mature capsid protein (Mason et al., 1987; Wengler et al., 1987).

Mature membrane protein of 8-kDa is produced in host Golgi vesicles by cleavage by the host encoded enzyme, furin, during viral particle maturation. This is crucial for viral release, virus morphology and viral infectivity (Guirakhoo et al., 1992; Kuhn et al., 2002; Mackenzie and Westaway, 2001). By using cryo-electron microscopy, premature viral particles containing pre-membrane protein were observed as larger virions (~600 Å in diameter) containing 60 spikes on the surface compared to the mature viral particle (of ~500 Å in diameter) with a smooth surface (Zhang et al., 2003a; Zhang et al., 2003b). It is believed that the immature particles play a role in preventing premature activation of the fusogenic particle after exposure to acid pH and in facilitating virus release (Zhang et al., 2003b).

Dengue envelope proteins (54.5 kDa), folded largely into β-sheets, contain three distinctive domains: domain I, the N-terminus; domain II, the fusion (or dimerization) domain; and domain III, the IgG-like domain (Crill and Roehrig, 2001). Envelope protein appears to be a homodimer in solution and is located on the virion surface. There are two glycosylated asparagines on each dengue envelope subunit: Asparagine-153 on domain I and Asparagine-67 on domain II (Modis et al., 2003). The mannose found at the glycosylated site appears to be important for viral entry through receptor binding (Hung et al., 1999). Exposure of envelope protein dimers to low pH in early endosomes after viral uptake results in irreversible conformational change of envelope protein to a trimer conformation (Heinz et al., 2004; Kimura and Ohyama, 1988; Modis et al., 2003; Stuart and Gouet, 1995). The process of pH-triggered dimer-to-trimer transition is still not clear, but the crystallographic structure of liposome-associated envelope protein trimer has been resolved recently at high resolution (2.0 Å) (Modis et al., 2004). The images suggest that

envelope proteins in the dimer form undergo a dramatic conformational change through the interdomain linkers that results in irreversible envelope protein homotrimer formation. This pH-induced trimer formation is capable of destroying the tight packing on the outer virion surface.

1.4.2.2 Dengue viral nonstructural proteins

NS1 (38.7 kDa) contains two N-glycosylation sites and appears to associate with membrane upon dimerization (Winkler et al., 1988). NS1 has been shown to be involved in the early steps of flaviviral replication. Mutation in NS1 protein of dengue virus has led to the appearance of attenuation or a delay in the appearance of cytopathic effects (Butrapet et al., 2000; Crabtree et al., 2005). Glycosylation of NS1 protein is important for its dimerization (Pryor and Wright, 1994). A portion of the NS1 proteins expressed during viral infection in mammalian cells (but not in insect cells) (Flamand et al., 1999), is secreted to the outside of the cells. Extracellular NS1 is secreted as a dimer (Pryor and Wright, 1993) or a higher ordered oligomer (Crooks et al., 1994). Recent study of Kunjin virus has shown that an NS1 mutant with disrupted dimerization could still be secreted to the outside of the cell (Hall et al., 1999). Circulating NS1 protein could also be found in sera of patients infected with dengue virus type 1 (Libraty et al., 2002). NS1 secretion is found to be glycosylation-dependent, as treatment with glycosylation inhibitors resulted in no extracellular NS1 secretion during viral infection (Flamand et al., 1999). NS1 has also been used for vaccine development due to its ability of induce a cytolytic immune response (Costa et al., 2005a; Costa et al., 2006; Costa et al., 2005b; Wu et al., 2003).

NS2a (20 kDa), identified as a hydrophobic protein with several transmembrane domains, is required for the correct proteolysis of NS1 at the C terminus (Falgout et al.,

1989). NS2b hydrophobic protein (14.5 KDa) is a cofactor for NS3 protease activity. Although NS3 has a protease activity even in the absence of NS2b, NS2b has been shown to be required for *trans* cleavage of a cloned polyprotein substrate (Yusof et al., 2000).

NS3 is a 70 kDa, hydrophobic, multifunctional protein. It functions as a serine protease (Biedrzycka et al., 1987; Falgout et al., 1991; Li et al., 1999; Preugschat et al., 1990), RNA helicase (Wu et al., 2005), nucleotide triphosphatase and RNA 5´-triphosphatase (Bartelma and Padmanabhan, 2002; Bartholomeusz and Wright, 1993; Cui et al., 1998). The N-terminal sequence of NS3 shares homology with other trypsin-like serine proteases. Its activity has been demonstrated in vitro using various types of substrates (Arakaki et al., 2002; Leung et al., 2001; Yusof et al., 2000). The RNA helicase, nucleotide triphosphatase and RNA 5´-triphosphatase activities of full-length NS3 or its C-terminal region (residues 169-619), have also been demonstrated in vitro (Bartelma and Padmanabhan, 2002; Benarroch et al., 2004b) and are considered to be important for RNA replication and 5´-^{m7}GpppN-capping activity. The 5´-cap structure of cellular and viral RNAs are formed utilizing similar mechanisms that involve three enzymatic reactions: cleavage of 5´-triphosphate terminus of an RNA transcript to diphosphate by triphosphatase, then addition of GMP to the 5´ end of the transcript by RNA guanylyltransferase, and finally methylation of the N7 position of guanine by methyltransferase (Shuman, 1995). It is likely that flaviviruses encode their own capping enzyme since they replicate in the cytoplasm. The methyltransferase activity of dengue for capping may be provided by NS5 (Koonin, 1993), the RNA dependent RNA polymerase, which contains a methyltranserase domain, and its activity has been demonstrated in vitro using purified expressed protein (Egloff et al., 2002). Previous

studies have shown that the helicase and nucleoside triphosphatase activity of NS3 could be enhanced by the NS5 protein, the protease domain of NS3 and the presence of RNA (Li et al., 1999; Yon et al., 2005).

NS4a and NS4b are both hydrophobic proteins whose functions are still not clear. It is suggested that NS4a and NS4b may be involved in viral replication (Salonen et al., 2005). A previous study in yellow fever virus has shown that the interaction of NS4a and NS1 is important for yellow fever virus replication (Lindenbach and Rice, 1999). Kunjin virus NS4a and NS4b cannot be complemented and this result suggests that NS4a and NS4b are both cis-acting elements for Kunjin viral replication (Khromykh et al., 2000).

NS5, a 150 kDa RNA-dependent RNA polymerase containing a Gly-Asp-Asp (GDD) domain, is the largest protein encoded by dengue virus. Unphosphorylated NS5 was found to interact with NS3 in vivo and in vitro through its C-terminal region (Kapoor et al., 1995). It is thought that the phosphorylation of NS5 at serine residues would allow for the disruption of the NS3-NS5 interaction, resulting in nuclear localization of phosphorylated NS5 (Uchil et al., 2006). It is also known that the interaction site of NS5 and NS3 contains a nuclear localization domain, and the binding of NS5 to NS3 prevents the nuclear localization of NS5 protein (Yon et al., 2005). It is still unclear, however, about the role of phosphorylated NS5 in the nucleus. RNA-dependent RNA polymerase activity of NS5 protein has been demonstrated in vitro using dengue infected insect cells or Vero cells expressing NS5 protein (Ackermann and Padmanabhan, 2001; Bartholomeusz and Wright, 1993) and *Escherichia coli* expressed NS5 (Tan et al., 1996). These RNA-dependent RNA polymerases show template specificity and have the ability

for de novo negative strand synthesis as well as primed synthesis using “fold-back” of the 3´-end, depending on the conformation of NS5 (Ackermann and Padmanabhan, 2001).

1.5 Viral attachment and entry

Dengue virus enters a cell through receptor-mediated endocytosis through binding of envelope proteins and receptors (Chen et al., 1997; Martinez-Barragan and del Angel, 2001; Mukherjee et al., 1993; Munoz et al., 1998; Ramos-Castaneda et al., 1997; Salas-Benito and del Angel, 1997). After the interaction of envelope protein with the receptors, envelope protein undergoes conformational changes to allow the fusion of plasma membrane with dengue viral envelope in a pH-dependent manner. The virus entry can be blocked by using antibody against envelope protein (Feighny et al., 1994; Men et al., 2000; Putnak et al., 1991; Raviprakash et al., 2000), or using protease to remove the cell surface receptors (Daughaday et al., 1981). Dengue virus can also attach to macrophage or monocyte cells mediated by reactive immunoglobulin G (IgG) binding to Fc-receptors (Daughaday et al., 1981; Fanger and Erbe, 1992; Fanger et al., 1989). The cells bearing Fc-receptor normally produce higher titer of virus when infected with dengue virus in the presence of serologically cross-reactive but not neutralizing antisera. This immunological phenomenon is known as “antibody-dependent enhancement” and is thought to contribute to the pathogenesis of dengue hemorrhagic fever and dengue shock syndrome (Halstead, 1989; Halstead et al., 1980).

1.6 Translation modes of dengue virus RNA

1.6.1 Cellular translation and translation mechanisms of other viruses

1.6.1.1 Cellular mRNA translation

Initial translation of the dengue genome is required for producing proteins for replication. Translation of the dengue genome utilizes the cellular translation machinery since dengue virus does not encode any translational components. There are at least two major mechanisms that facilitate ribosome recruitment to the mRNA and translation initiation at the AUG site: cap-dependent and cap-independent translation initiation. Cap-dependent translation initiation is achieved by interaction of the RNA with cap-binding protein complexes, known as eukaryotic initiation factor 4F (eIF4F), containing the proteins of eIF4E, eIF4G, and eIF4A (Michel et al., 2000; Pestova and Kolupaeva, 2002; Pestova et al., 2001; Prevot et al., 2003). The cap-binding complexes interact with the 5´-^{m7}GpppN cap structure and recruit the 40S ribosome subunits to the 5´ terminus of the mRNA via eIF3 (Jivotovskaya et al., 2006). The 40S ribosome with the ternary complex, containing an initiator tRNA^{Met}, eIF2 and GTP, has the ability to scan in the 5´-3´ direction toward an initiation site, typically the first AUG (Pestova and Kolupaeva, 2002). The 60S ribosome then later joins the initiation complex to form the 80S initiation complex with AUG at the ribosomal P site.

PolyA-binding protein (PABP) bound to the 3´-polyA tail can interact with eIF4G to circularize the mRNA (Borman et al., 2000; De Gregorio et al., 1999; Kahvejian et al., 2005; Michel et al., 2000). Fully active translation of regular mRNA relies on the 5´-cap structure and 3´polyA tail, which both attract initiation factors and promote the 5´-3´ circularization for efficient ribosome recycling and translation (Dever, 1999; Gallie, 1998; Wakiyama et al., 2000).

1.6.1.2 Special viral translation modes

1.6.1.2.1 IRES-containing, cap-independent viral translation

Several viruses have RNAs that contain no 5´-cap structure. Some of these RNAs are able to interact with the initiation complex directly through cis-elements that can promote translation initiation. Another strategy used by viruses with uncapped RNAs is to regulate the cellular translation machinery by modulating the functions of eIF4E, eIF4G and PABP (Bushell and Sarnow, 2002; Sarnow et al., 2005). For example, poliovirus infection results in inhibition of cellular translation of host mRNAs, but not the viral RNAs. The translation inhibition is caused by the cleavage of eIF4G and PABP by viral proteases (Barco et al., 2000; Kuyumcu-Martinez et al., 2004; Ventoso et al., 1998). Poliovirus infection also leads to the modification (dephosphorylation) of eIF4E-binding protein (4E-BP) to allow it to sequester eIF4E from the cellular translation machinery (Gingras et al., 1996). Poliovirus genomic RNA has an ability to interact with the cleaved eIF4G and PABP to facilitate its own translation via a cap-independent mechanism (Bergamini et al., 2000). On the other hand, encephalomyocarditis virus (EMCV) infection does not result in the cleavage of eIF4G (Gingras et al., 1999) but EMCV RNA has the ability to interact with the central region of eIF4G directly without the requirement for a 5´-cap structure (Pestova et al., 1996). In the course of EMCV infection, 4E-BP is dephosphorylated to prevent eIF4E from participating in the cellular cap-dependent translation initiation (Gingras et al., 1996; Rousseau et al., 1996).

As an extreme case of a virus using cap-independent translation initiation, cricket paralysis virus RNA recruits a 40S ribosome for translation initiation without any canonical translation initiation factors (Wilson et al., 2000a; Wilson et al., 2000b). Cricket paralysis virus is a positive sense single-stranded RNA insect virus whose

genomic RNA encodes two non-overlapping open reading frames that are both initiated via an internal ribosome entry site (IRES). Cricket paralysis virus RNA can also form 80S-IRES complexes without the requirement of GTP hydrolysis and initiator ternary complexes (Wilson et al., 2000a). Hepatitis C virus has the ability to recruit 40S ribosomes without any initiation factors like cricket paralysis virus, but unlike cricket paralysis virus, formation of HCV 80S-IRES complex requires the recruitment of a ternary complex (Jan and Sarnow, 2002; Otto and Puglisi, 2004; Wilson et al., 2000a).

1.6.2 Dengue translation

1.6.2.1 Cap-dependency of dengue viral translation

Genomic RNAs of the genera *Pestivirus* and *Hepacivirus* in the family *Flaviviridae* lack a 5´-cap structure and have IRES structures for internal initiation (Frolov et al., 1998; Rijnbrand et al., 1997). However, there is no evidence for genomic RNAs of the *Flavivirus* genus, which contain a 5´-cap structure, to translate using an internal initiation mechanism (Lindenbach, 2001). Attempts to study dengue translation have been carried out using various systems, such as reporter construct, dengue replicon construct and infectious viral RNA in cells and in cell-free extracts.

Dengue virus RNA is not known to contain an IRES and it does possess a 5´-^{7m}GpppA cap. Further, efficient translation of dengue in reporter constructs requires a 5´-^{7m}GpppN cap, which has a synergistic effect in combination with the dengue 3´ UTR (Chiu et al., 2005). However, it may be possible for dengue RNA to be translated under certain conditions when cap-dependent translation is inhibited. A study by Edgil *et al.* (2006) using inhibitors of cap-dependent translation, such as LY294002 and wartmannin, has shown that the translational expression from a 5´-^{7m}GpppN-capped dengue reporter

construct is insensitive to inhibitor treatment and that 5´ApppN-capped dengue reporter mRNA could be translated in the presence of inhibitors to a level comparable to ^{7m}GpppN-capped dengue reporter constructs. In the absence of inhibitors, expression from 5´ApppN-capped RNAs was very low. Similar results were also obtained using 5´-ApppN-capped full-length dengue viral RNA. In the presence of inhibitor, but not in its absence, this form of viral RNA could replicate to the level of wild type viral RNA. In the same study, no IRES activity was detected using a standard bicistronic reporter assay. Edgil *et al*. suggested that dengue RNA does not contain any IRES, but could be translated cap-independently (Edgil et al., 2006). In our laboratory, using the same strategies as Edgil *et al*., we obtained similar observations that a dengue reporter construct has no IRES activity by placing a stable stem loop structure in front of a dengue reporter construct. However, we were unable to observe cap-independent translation in either the reporter construct or the infectious viral RNA system using inhibitor LY294002 (see chapter 2). It is clear that dengue virus RNA is translated in a cap-dependent manner and dengue has no ability to shutoff cellular translation like poliovirus; therefore, it remains unknown which point of the dengue virus replication cycle may require cap-independent translation initiation. Therefore, the cap-independent translation of dengue RNA may still need to be further investigated.

1.6.2.2 Dengue viral translation is enhanced by dengue 3´UTR in reporter construct but not in a dengue replicon construct

Several predicted structural elements in the dengue 3´-UTR also contribute to dengue translation in a reporter construct system. Deletion study of 3´-UTR elements using reporter constructs have shown that removal of the stem loop structure located at the very 3´ end results in the reduction of translatability to about 40-50% from two

independent studies (Holden and Harris, 2004; Chiu et al., 2005). This stem loop structure has been shown to be conserved among flaviviruses and to be important for viral replication. Deleting other predicted secondary structural elements within dengue 3´-UTRs reduces translation to about 25 to 75%, and translational enhancement provided by the 3´-UTR seems to be derived from contributions by each of the conserved features within the 3´-UTR (Chiu et al., 2005). Another interesting finding is that deletion of the upstream variable region of the dengue 3´UTR reduced translation to 25%, suggesting involvement of this region in translation (Chiu et al., 2005).

Interestingly, a study of dengue 3´UTR in translation using a replicon system has shown no 3´-enhancement effect for initial translation; translation of the replicon RNA was not affected by deletion of the aforementioned elements in the 3´-UTR (Alvarez et al., 2005a). This observation is consistent with the finding in a 3´deletion study in West Nile virus replicon RNA (Tilgner et al., 2005). It is unclear would result in the .

Translational enhancement by 3´-UTR from many viruses has been reported. The translation of turnip yellow mosaic virus, alfalfa mosaic virus and rotavirus RNAs is described below.

1.6.2.2.1 Translation of turnip yellow mosaic virus RNA is enhanced by 3´ tRNA-like structure

Turnip yellow mosaic virus RNA contains a 5´-cap and a 3´-tRNA-like structure. Using a reporter system, the 3´-UTR of turnip yellow mosaic virus was shown to enhance activity from mRNAs containing 5´cap with a 25-fold of increase in translation (Matsuda and Dreher, 2004). The 5´-cap and 3´-UTR of turnip yellow mosaic virus have been shown to have a synergistic effect. A previous study by Matsuda and Dreher (2004) also showed that the maximum translational enhancement requires the turnip yellow mosaic

virus 3´-tRNA-like structure to be capable of aminoacylation. In the same study, mutations negatively affecting eEF1A binding to the tRNA-like structure led to diminished translation enhancement activity, suggesting that actual binding of eEF1A to the tRNA-like structure is involved in translational enhancement for turnip yellow mosaic virus.

1.6.2.2.2 Coat protein of alfalfa mosaic virus enhances viral RNA translation

The coat protein of alfalfa mosaic virus is required for efficient translation of RNA1 to 3 of the viral genome. It is known that RNA4, a subgenomic RNA encoding coat protein, or the viral coat protein is required for successful infection of alfalfa mosaic virus, a phenomenon called genome activation (Jaspars, 1999). The coat protein of alfalfa mosaic virus has been known to interact with seven hairpin structures in the 3´-UTR of genomic RNA, and this interaction provides strong translation enhancement activity like enhancement by a polyA tail. Therefore, it is thought that the action of the alfalfa mosaic virus coat protein binding to alfalfa mosaic virus 3´-UTR is functionally similar to the interaction of polyA binding protein to a polyA tail (Neeleman et al., 2001).

The alfalfa mosaic virus 5´-UTR also contains a translation enhancer and the enhancement activity has been demonstrated in tobacco protoplast cells (Gallie et al., 1987) and in vitro translation lysate (Jobling et al., 1988; Jobling and Gehrke, 1987). The enhancement activity is eIF4G-dependent since HeLa cells infected with poliovirus virus would not support the translation of alfalfa mosaic virus translation (Hann and Gehrke, 1995).

1.6.2.2.3 Rotavirus translation

The N-terminus of NSP3, a 36 kDa protein, of rotavirus has been shown to interact with the 3´-terminal conserved sequence, GACC, of its mRNA (Poncet et al., 1994). In rotavirus-infected cells, the C-terminal domain of NSP3 has shown ability to interact with eIF4G to enhance translation through the binding of the 3´-terminal GACC sequence of rotaviral RNA. By co-immunoprecipitation assay, NSP3 was found to be able to interact with eIF4G-I and this interaction could disrupt the interaction of eIF4G with polyA binding protein (Piron et al., 1998). Competitive binding of eIF4G and NSP3 appear to inhibit the cellular mRNA translation, as the availability of eIF4G is limited.

1.6.2.3 Dengue 5´ UTR in translation

The contribution of dengue 5´-UTR in translation has been studied in both viral RNA systems and reporter construct systems. Deleting various regions of the 5´-UTR in dengue virus type 4 viral RNA has led to translation efficiency in vitro ranging from 40 to 160% compared to the wild type viral RNA (Cahour et al., 1995). These data correlate with the findings in the reporter construct system (see chapter 2): deletion analysis of dengue 5´-stem loop structures demonstrated translation changes ranging from 30 to 150%.

The dengue 5´conserved stem loop structure is located three nucleotides after the 5´cap structure (Brinton and Dispoto, 1988) with predicted free energy of -20.9 kcal/mol. Previous studies have shown that introducing a stable stem loop structure with free energy of -30 kcal/mol at the very 5´ end of an mRNA could result in the blocking of ribosome loading and reduction of translation of the mRNA (Kozak, 1988). It is unclear how ribosomes could load at the very 5´ end of the dengue genome with only a three nucleotide single-stranded region for ribosome attachment and how efficient helicase (i.e.

eIF4A) would be in opening the dengue 5´-stem loop structure before ribosome loading or during ribosome scanning. My early studies on dengue reporter construct translation in Vero cells showed translation similarly as efficient as a globin reporter construct, suggesting that the dengue 5´stem loop is highly functional for dengue translation (Chiu et al., 2005). Interestingly, when placed at the 5´-terminus of an α-globin reporter mRNA construct, the dengue 5´-stem loop structure was slightly inhibitory for translation (See Chapter 3). Thus, the effect of dengue 5´-stem loop structure may depend on the sequence context in the mRNA and may act on translation in cis with the dengue RNA sequence.

1.6.2.4 Dengue virus AUG selection and the effect of the capsid coding region.

The AUG start codon of dengue virus RNA has a poor translation initiation context: UCUCUGAUGA surrounding the AUG codon, compared to the optimized context: (A/G)CC(A/G)CCAUGG [the most influential nucleotide locations being –3 and +4 relative to A of AUG] (Kozak, 1987; Kozak, 1989; Kozak, 1997). The poor context is likely to result in "leaky" recognition by scanning ribosomes, which then move downstream toward the next available AUG (Kozak, 1995; Kozak, 1999; Kozak, 2001). It is believed that dengue virus requires translation initiation from the first AUG start codon for a functional viral life cycle. A recent study suggests that a conserved stem loop structure located in the dengue capsid coding region could assist in the recognition of the AUG start codon despite its poor context by reducing the chance of ribosome leaky scanning (Clyde and Harris, 2006). Abolishing the base pairing in this secondary structure resulted in a higher frequency of leaky scanning and increased translation from

an AUG 14 amino acids downstream of the first AUG in the reporter construct system. This finding is consistent with previous findings in vitro using rabbit reticulocyte lysates (Kozak, 1990). Translation initiation from the second AUG of dengue viral RNA would produce a shorter form of the capsid protein and its function is unknown. In the same study, Clyde *et al*. also found that virus viability in mammalian cells and insect cells requires the existence of both the first and second AUGs, although it remained to be determined whether these two AUG triplets are involved in translation, in viral replication or in a cellular regulatory function.

Translation is certainly required for initial viral protein synthesis upon dengue virus infection but how important the quantity of initial translation is for dengue viral life cycle is still unknown. A previous study has shown that the differences between the 3´-UTR of a Nicaraguan strain and that of prototype dengue virus type 2 can be directly linked to the difference in the translation level of the viruses, which further contributes to the viral infectivity of the two viruses (Edgil et al., 2003). This study has shown that the expression level of the viral protein and chimeric reporter constructs is correlated with the infectivity of the two viruses. On the other hand, another study has shown that an improvement of the first AUG initiation context at the -4 position resulted in attenuation of viral replication (Alvarez et al., 2005b). This finding suggests that improvement of translation does not necessarily enhance the viral replication or increase the viral titer because the mutation that disrupts translation might affect other stages of the virus lifecycle as well. In the study by Edgil and Harris (2003) the effects of differences in the 3´-UTR in these two strains on replication were not determined, nor were the 3´-UTR translation enhancement activity in dengue replicon construct and dengue viral RNA

determined. Thus, it remains unclear what level of the initial translation is required for virus to progress to successful replication. An experimental system allowing to modulate the amount of expressed protein or input of viral replication without affecting cis-acting signals for replication (or other stages of virus lifecycle) can possibly answer this question.

1.7 Dengue viral replication and assembly

Dengue virus replication can be detected as early as 3-4 hours post infection and the replication site is located in the perinuclear region of the infected cell that is associated with smooth membrane (Brinton, 1986; Takeda et al., 2004). Three replication forms of RNA can be isolated from dengue virus infected cells by using sucrose gradient centrifugation. 20S-22S RNA consists of the replicative forms that are RNase-resistant; 20-28S RNA replicative intermediate form that is partially RNase-resistant; and the 42S RNAs consists of the RNase sensitive form (Chu and Westaway, 1985; Stollar, 1967; Wengler et al., 1978). Conversion of replicative form to 42S RNA can be achieved by heat and other denaturing agents (Cleaves et al., 1981). The replicative form contains full-length, double stranded RNA with positive strand annealed to negative stranded RNA. The replicative intermediate form contains partial double stranded RNA that arises during the strand displacement of RNA synthesis.

Regulation of dengue viral RNA replication is not fully understood; it is likely that the RNA polymerase complexes contain different components during early and late replication, which are possibly responsible for the different synthesis rates of negative and positive strand viral RNAs. It has been known that late replication prefers synthesis

of the positive strand RNA and the increasing capsid concentration is likely to interact with positive strand RNA and sequester it from being a substrate for replication (Stollar, 1967; Westaway and Ng, 1980). A recent study in Kunjin virus has shown that encapsidation occurs only when the virus is undergoing replication; therefore, the replication and encapsidation appears to be a coupled event (Khromykh et al., 2001b).

Assembly of viral particles begins with the formation of nucleocapsid, followed by budding through the membrane containing the envelope and pre-membrane protein to form the immature particle. The immature particles are transported via the Golgi trafficking system for further maturation by host protease furin, which is located inside the Golgi network and cleaves the pre-membrane protein before exiting the cell. The maturation of the viral particle and the conformation changes of dengue virus envelope proteins are required for viral infectivity or later entry to new cells.

1.8 Prevention and control-dengue vaccine development

Although dengue vaccine development began in the 1940s, there is still no effective dengue vaccine available. Dengue vaccine development focuses on development of a tetravalent dengue vaccine due to concerns about antibody-dependent enhancement, which is described as a form of enhanced virus uptake into macrophage cells through the binding of non-neutralizing antibody against another serotype of DEN. The vaccine needs to be capable of inducing equal seroconversion against all four types of dengue. Dengue tetravalent vaccine development involves different attenuated viral vectors, such as Dengue virus type 2 PDK-53 (Huang et al., 2000; Huang et al., 2003; Huang et al., 2005) yellow fever virus (Guirakhoo et al., 2001; Guirakhoo et al., 2002;

Guirakhoo et al., 2004), (Monath et al., 2002), or rDEN4Δ30 (Blaney et al., 2005; Durbin et al., 2001; Whitehead et al., 2003).

PDK-53 strain was derived from passaging the wild-type DEN2 in primary dog kidney (PDK) cells. This attenuated vaccine candidate has a decreased infectious rate (50%) in a cell culture system and produces no clinical symptoms with ability to incite prolonged antibody production for at least two years in humans. PDK-53 tetravalent vaccine candidates were generated from dengue virus type 2 PDK-53 by replacement of the pre-membrane and envelope genes with those from other types of dengue viruses. These chimeric PDK-53 tetravalent vaccine candidates are capable of infecting cell culture and mice (Huang et al., 2003).

Tetravalent chimeric yellow fever/dengue (ChimeriVax-DEN) vaccine candidates, were constructed in which the yellow fever virus pre-membrane-envelope gene was replaced by each of the four dengue virus pre-membrane-envelope genes. Monkeys immunized with the formulation of yellow fever/dengue tetravalent vaccine were able to produce specific antibodies against the four types of dengue and survive further challenge.

Another attenuating strategy involves deleting 30 nucleotides in the 3´-UTR of dengue using infectious clone technology. This strategy was derived from the dengue virus type 4 vaccine candidate, rDEN4Δ30, which is safe, attenuated, and immunogenic in rhesus monkeys and human (Durbin et al., 2001). Deleting the same set of nucleotides of dengue virus type 1 using reverse genetic technology results in attenuated vaccine candidate: rDEN1Δ30 (Whitehead et al., 2003). Chimeric viruses using rDEN4Δ30 as a vector were also made by replacing pre-membrane-envelope gene of rDEN4Δ30 with

those of dengue virus type 2 and 3 to make rDEN2/4Δ30 and rDEN3/4Δ30. Tetravalent vaccine was formulated with equal concentrations of rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30 and rDEN4Δ30 and tested in monkeys. This vaccine candidate was able to produce antibody against four types of dengue virus. However, the four types of dengue vaccine candidate in monkey exhibited preferential replication resulting in unequal seroconversion against the four types of dengue virus at day 28, although second inmmunization resulted in nearly equal seroconversion (Robert Putnak et al., 2005). This particular vaccine candidate protects monkey from dengue virus type 1, 3, and 4 challenge with one dose, yet requires a second dose to protect from dengue virus type 2 challenge.

Although every tetravalent dengue vaccine candidate claims a promising preclinical report, unequal seroconversion against four types of dengue and possible preferential replication are still potential factors for developing disease-like symptom in vaccinees. Tetravalent dengue vaccines that are currently under human phase I and II trials in the US and Thailand are attenuated vaccines made by passaging wild type dengue in PDK cells or primary African green monkey kidney cells. Primary results of the trials indicate that these attenuated vaccine candidates are safe and immunogenic in humans, although some tetravalent formulations failed to induce neutralizing antibodies and/or equivalent T-cell responses against all four types of dengue viruses (Kanesa-thasan et al., 2001) possibly due to preferential replication among the four serotypes of dengue vaccine candidates. Therefore, it is likely that we are not going to have live attenuated dengue vaccine available in the near future.

Inactivated dengue virus vaccines have been developed and were found to be able to produce protective amounts of antibody against dengue virus and to induce T-cell response against dengue virus in mice and rhesus monkey (Eckels and Putnak, 2003). The status of the inactivated dengue virus is at preclinical trial stage (Putnak et al., 1996a; Putnak et al., 1996b). This approach may be used to develop effective dengue vaccine in the future.

Subunits vaccines using expressed dengue viral proteins, such as pre-membrane-envelope proteins, using *Escherichia coli*, yeast and insect cell expression systems have been tested in laboratory animals. Several studies using expressed dengue virus envelope protein and fusion version of pre-membrane-envelope protein from various systems have shown an ability to produce neutralizing antibodies in mice and are able to protect mice from further challenge (Eckels et al., 1994; Feighny et al., 1992; Lai, 1989; Men et al., 1991; Putnak et al., 1996b; Putnak et al., 1991). Recombinant NS1 protein has limited efficacy in antibody production and induces protection against virus infection in mice (Feighny et al., 1992). The purified NS1 protein from dengue virus type 2 infected cells, however, can efficiently induce protective antibody production and could protect mice from lethal challenge (Schlesinger et al., 1987).

1.9 Treatments

Anti-viral drugs under development against dengue mainly focus on antagonizing the activity of capping, helicase, protease, and RNA-dependent RNA polymerase (Benarroch et al., 2004a; Egloff et al., 2002; Murthy et al., 2000; Wu et al., 2005). Antisense oligos, such as morpholinos, have shown ability to inhibit dengue viral translation in BHK cell and viral replication in Vero cells (Holden et al., 2006; Kinney et

al., 2005). Most of the non-specific antiviral drugs, such as the use of mycophenolic acid as an immunosuppressant agent, has shown certain efficacy for inhibiting dengue virus replication in vitro and in cell culture system (Diamond et al., 2002; Takhampunya et al., 2006). Although mycophenolic acid does not inhibit initial translation, it affects the viral RNA synthesis (Diamond et al., 2002).

Currently, dengue vaccine and antiviral treatments for dengue infection do not exist. Supportive care and hydration are the only treatments available for dengue virus infected patients especially for those who developed hypertension with dengue hemorrhagic fever. We hope that by understanding the dengue viral life cycle and viral host infection will help to develop an anti-viral drugs and vaccines in the future.

Chapter 2 Control of translation by the 5´ and 3´ terminal regions of the flaviviral genome

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2.1 ABSTRACT

The genomic RNAs of flaviviruses such as dengue virus (DEN) have a 5´-^{m7}GpppN cap like those of cellular mRNAs, but lack a 3´-poly(A) tail. We have studied the contributions to translational expression of 5´ and 3´ terminal regions of the DEN serotype 2 genome using luciferase reporter mRNAs transfected into Vero cells. DCLD RNA contained the entire DEN 5´ and 3´-untranslated regions (UTRs), as well as the first 36 codons of the capsid coding region fused to the luciferase (LUC) reporter gene. Capped DCLD RNA was as efficiently translated in Vero cells as capped GLGpA RNA, a reporter with UTRs from the highly expressed α-globin mRNA and a 72-residue poly(A) tail. Analogous reporter RNAs with regulatory sequences from West Nile or Sindbis viruses were also strongly expressed. Although capped DCLD RNA was expressed much more efficiently than its uncapped form, uncapped DCLD RNA was translated 6 to 12 times more efficiently than uncapped RNAs with UTRs from globin mRNA. The 5´-cap and DEN 3´-UTR were the main sources of translational efficiency of DCLD RNA, and they acted synergistically in enhancing translation. The DEN 3´-UTR increased mRNA stability, although this effect was considerably weaker than the enhancement of translational efficiency. The DEN 3´-UTR thus has translational regulatory properties similar to those of a poly(A) tail. Its translation-enhancing effect was observed for RNAs with globin or DEN 5´ sequences, indicating no codependency between viral 5´ and 3´ sequences. Deletion studies showed that translational enhancement provided by the DEN 3´-UTR is attributable to the cumulative contributions of several conserved elements as well as a non-conserved domain adjacent to the stop

codon. One of the conserved elements was the conserved sequence CS1 that is complementary to cCS1 present in the 5´-end of the DEN polyprotein open reading frame. Complementarity between CS1 and cCS1 was not required for efficient translation.

2.2 INTRODUCTION

Dengue virus (DEN) is a member of the mosquito-borne group of flaviviruses that includes yellow fever, Japanese encephalitis and West Nile (WNV) viruses. It is the causative agent of millions of human infections annually in the tropical regions of the world (Messer et al., 2002). The virus has a vertebrate host range limited to humans and monkeys, but replication and amplification also occur in mosquito vectors, principally of the *Aedes* genus (Burke, 2001).

The DEN genome is an 11 kb positive strand RNA with a ^{m7}GpppA cap at the 5´ end but lacking a poly(A) tail at the 3´ end (Lindenbach, 2001b). It encodes a single long polyprotein (Fig. 2.1A) that is processed through the action of viral and host proteinases to generate ten mature structural and non-structural proteins. The maturation pathway, the cleavage sites and the responsible proteinases have been well described (Lindenbach, 2001b). However, systematic studies of the roles of the untranslated regions (UTRs) of the viral RNA in the gene expression of DEN or other flaviviruses are lacking. The UTRs are expected to play an important early role during viral infection in coordinating viral gene expression and the onset of RNA replication.

Translation of the typical cellular mRNA relies on the recruitment of ribosomes by features in the UTRs at both ends of the RNA (Gallie, 1998; Jackson and Kaminski,

1995; Mangus et al., 2003; Sachs et al., 1997; Schneider and Mohr, 2003). The 5´ cap is recognized by the cytoplasmic cap binding protein eIF4E, which contacts initiation factor eIF4G. This large protein acts as a scaffold for further protein-protein interactions that support initiation. One of those interactions contacts eIF3, a complex factor that is itself bound to the small ribosomal subunit. This chain of interactions thus recruits the ribosomal subunit to the 5´-terminus of the RNA. The 3´ regions of an mRNA also enhance translation. One mechanism by which this can occur is through an interaction between eIF4G and PABP that is bound to the poly(A) tail (Tarun et al., 1997). The translation enhancing contributions of the cap and poly(A) tail are synergistic (Gallie, 1991; Tarun and Sachs, 1995), and the most actively translated mRNAs are thought to exist in a cyclized or closed-loop state that is considered to enhance initiation and allow for improved ribosome recycling while ensuring that truncated, damaged mRNAs are poorly translated (Gallie, 1998) (Jacobson, 1996; Sachs et al., 1997). After initial loading at the 5´-end of the RNA, the small ribosomal subunit scans in the 3´ direction along the RNA in search of an initiation site (Kozak, 1999a; Pestova and Kolupaeva, 2002; Pestova et al., 2001). This is usually the 5´-most AUG triplet, at which initiation frequency is in part determined by the immediate sequence context, GCC(A/G)CCAUGG being the optimal context in mammalian cells (Kozak, 1999a).

Genomes of positive strand RNA viruses often lack a 5´-cap and/or a poly(A) tail, yet must compete with cellular mRNAs for ribosomes to support robust viral gene expression. Viral RNAs that lack a 5´-cap often possess an internal ribosome entry site (IRES) in the 5´-UTR that directs interaction with the small ribosomal subunit directly or through the binding of an initiation factor such as eIF4G (Gale, 2000; Hahn et al., 1987;

Pestova and Kolupaeva, 2002; Schneider and Mohr, 2003). This can lead to initiation at an AUG triplet that is not 5´-proximal, as occurs with the picornaviruses (Jackson and Kaminski, 1995) and the members of two genera (*Hepacivirus* and *Pestivirus*) of the family *Flaviviridae* (Lindenbach, 2001b). Since the genomes of DEN and other members of the genus *Flavivirus* have a 5´-cap and initiate translation at the 5´-most AUG triplet, standard cap-dependent initiation rather than internal ribosome entry has been considered to be the gene expression strategy used by these viruses (Lindenbach, 2001b).

The 3´-UTRs of some viral RNAs that possess a 5´-cap but lack a poly(A) tail promote translation to a similar extent as a poly(A) tail, and through a similar synergistic interaction with the cap. This is true of rotavirus mRNA (Vende et al., 2000b), whose 3´-terminal GACC sequence acts as a translational enhancer (Chizhikov and Patton, 2000) by binding the viral protein NSP3, which competes with PABP for interaction with eIF4G (Vende et al., 2000a). It is also true of some plant viral RNAs whose 3´-UTRs terminate in a tRNA-like structure — *Tobacco mosaic virus, Brome mosaic virus* (Gallie and Kobayashi, 1994; Gallie and Walbot, 1990) and *Turnip yellow mosaic virus* (Matsuda et al., 2004b) — although in these cases the molecular interactions have not been identified.

We report here a systematic analysis of the properties of the 5´ and 3´-terminal regions of the DEN serotype 2 genome in directing translation in Vero cells, using a luciferase (LUC) reporter system. Both 5´ and 3´ DEN sequences were shown to participate in the regulation of expression, principally by modulating translational efficiency rather than RNA stability.

2.3 MATERIAL AND METHODS

Clones and cell culture. Dengue virus serotype 2 (DEN2) sequences were derived from the pD2/IC-30P-A infectious cDNA clone of virus strain 16681 (GenBank Accession No. U87411) (Kinney et al., 1997). West Nile virus sequences were derived from genomic clones of a NY Flamingo isolate (GenBank Accession No. AF196835). Sindbis virus sequences were obtained from pToto54, a full-length genomic clone (GenBank Accession No. NC_001547) provided by Dr. James Strauss (Caltech). Vero (African green monkey kidney) cells were grown in DMEM (Gibco) containing antibiotics and 10% fetal bovine serum (FBS) at 37ºC in an atmosphere containing 5% CO_2.

Plasmid constructs. The basic plasmid for in vitro transcription of reporter mRNA with DEN2 5´ and 3´-UTRs and encoding firefly luciferase (LUC) was constructed in the following steps to produce pDLD, shown in Fig. 2.1B. PCR amplification with the appropriate sequence-modifying primers was used to produce a DNA fragment comprising the DEN2 5´-UTR fused to the T7 promoter and flanked by a *Not*I site at the 5´-end and a *Pst*I site at the 3´-end. The *Pst*I site was positioned to correspond to the third and fourth codons of the reporter ORF (encoding Leu-Gln). The PCR product digested with *Pst*I was cloned into the *Pst*I and blunted *Hin*dIII sites of pUC18. The LUC ORF was modified during PCR amplification by addition of an in-frame *Pst*I site in front of the second codon of the LUC ORF (Glu) and by silent mutation of the last two codons to incorporate a *Hin*dIII site immediately before the termination codon. At the same time, a *Bam*HI site was added on the 3´-side of the LUC ORF, and the PCR product digested with *Pst*I and *Bam*HI was cloned into the same sites of the

above plasmid. Finally, the DEN2 3´-UTR was added into the *Hin*dIII and *Sma*I sites after PCR amplification to yield the 3´-UTR with a *Hin*dIII site placed immediately in front of the native DEN2 TAG stop codon and a *Sna*BI site appended to the 3´-end.

Derivatives of pDLD (Fig. 2.1B) were made by analogous PCR amplification of the desired 5´ and 3´-UTR fragments as described above, and subcloning into the *Not*I-*Pst*I sites and *Hin*dIII-*Kpn*I sites, respectively (a *Kpn*I site is present in the polylinker downstream of *Sna*BI). The UTRs of rabbit α-globin mRNA (GenBank J00658) were made by annealing synthetic DNA fragments. Substitution mutation and deletion variants were made by PCR-mediated mutagenesis and subcloning. Deleted 3´-UTR (D) was made by removal of sequences between the *Bam*HI and *Xho*I sites of an intermediate plasmid, resulting in a 15 nucleotide non-viral 3´-UTR (sequence AAAUGGAUCUCGA). The sequences of all subcloned segments were verified by DNA sequencing. Table I lists the relevant sequences of clones reported here.

RNA transcription and transfection of cells. Plasmids of the pDLD family were linearized by cleavage with a restriction enzyme as indicated in Fig. 2.1B. RNAs were produced in vitro by transcription with T7 RNA polymerase and uniformly labeled with low levels of [a-^{32}P]CTP (0.2 μCi per 20 μl reaction) to facilitate quantification by liquid scintillation counting of TCA-precipitable material; 5´-capped RNAs were produced in the presence of a 6-fold excess of ^{m7}GpppG (Epicentre) over GTP (33). Template DNA was removed by digestion with ribonuclease-free deoxyribonuclease, and RNA quality was analyzed via native 1% agarose gel electrophoresis.

In vitro transcribed reporter RNA (1 pmol) was electroporated into 6 x10^5 trypsinized Vero cells in electroporation buffer (25% of DMEM and 75% of "cytosalts", which contain 120 mM KCl; 0.15 mM $CaCl_2$; 10 mM potassium phosphate, pH 7.6; 5 mM $MgCl_2$) using 0.2 cm cuvettes and a Gene Pulser Xcell device (BioRad) as recommended (BTX protocol at http://www.btxonline.com/applications/protocols/mammalian/). Electroporated cells were diluted with 4 volumes of DMEM containing 10% FBS, transferred to tissue culture plates and held at 37ºC in an atmosphere containing 5% CO_2. Cells were harvested at various time points by lysis with 50 μl of 1x Passive Lysis Buffer (Promega). A number of the experiments reported in this paper were also conducted using lipofection to deliver RNAs into cells (as used in ref. 18). Although broadly similar results were obtained, we have found electroporation to be more reliable, as well as permitting direct estimation of translational efficiency and functional RNA half-life.

Analysis of luciferase activity. Portions of each extract (10 ml) were loaded into the wells of clear-bottom black 96-well plates, and mixed with 50 μl of Luciferase Assay Reagent (Promega) for luminometry in a 1450 MicroBeta TriLux counter (Wallac). Luciferase activities were normalized to total protein concentration determined with protein assay reagent (BioRad), and expressed as relative light units (RLU) per mg protein.

Assessment of physical stabilities of RNAs after transfection. RNA transcripts were uniformly radiolabeled in the presence of [a-^{32}P]CTP and delivered by

electroporation as usual into Vero cells. At various time points after electroporation, RNAs were extracted using Trizol (Invitrogen), denatured by glyoxalation and separated by electrophoresis in 1% agarose gels run in phosphate buffer (Matsuda et al., 2004a). After drying, gels were exposed to phosphorimager plates and full-length RNA bands were quantitated after phosphorimagery.

In vitro translation and assessment of the relative specific activities of LUC variants with altered N-termini. LUC reporter RNAs were translated in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Amersham). Proteins were separated by SDS-PAGE and dried gels were exposed to phosphorimager plates. Luciferase bands were quantitated after phosphorimagery. Dilutions of translation reactions were used for estimation of LUC activity in the standard assay. Taking into account the number of methionine residues in each form of LUC, LUC specific activities were expressed as relative light units per pmole of LUC fusion protein.

2.4 RESULTS

2.4.1 Use of luciferase as a reporter for studying the effects of DEN RNA terminal regions on translational expression in primate cells

Firefly luciferase (LUC) has been widely used as a reporter in translational expression studies because of its sensitivity and ease of assay. We modified a cloned LUC coding region to place it under the control of a T7 promoter and to permit convenient cassette replacement of upstream and downstream control regions (Fig. 2.1B). In vitro transcription with T7 RNA polymerase of linearized reporter constructs produced defined mRNA transcripts that were electroporated into Vero (monkey kidney) cells for subsequent assay of LUC activity in cell lysates.

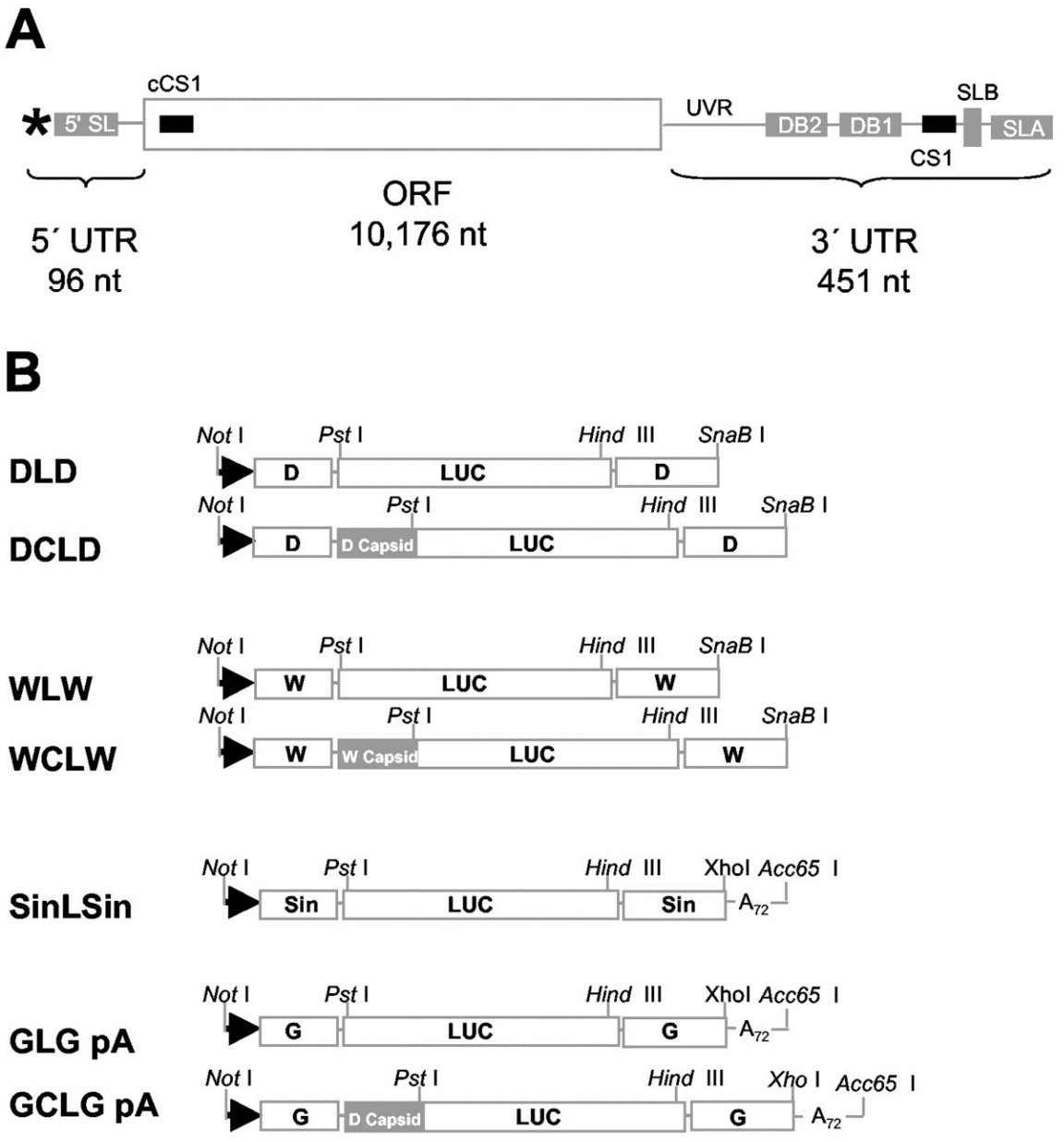


Fig. 2.1 Luciferase reporter constructs used for assaying the roles of flaviviral UTRs in regulating translational expression

(A) The 10.7 kb DEN2 genome is diagrammed, highlighting the 5´-cap (star), 5´-UTR, the single long open reading frame (ORF), and the 3´-UTR that ends in a conserved stem-loop (SLA) and has no poly(A) tail. Distinctive features of the UTRs are indicated. Gray boxes indicate structural features that are conserved among mosquito-borne flaviviruses: 5´SL in the 5´-UTR (Brinton and Dispoto, 1988), SLA and SLB at the 3´-end (Brinton et al., 1986; Matsuda and Dreher, 2004), and the pseudo-repeated elements DB1 and DB2 further upstream in the 3´-UTR. DB1 and DB2, which resemble stalked dumbbells (Olsthoorn and Bol, 2001), include a conserved sequence (CS2, not shown). Black boxes

indicate the conserved sequence elements, CS1 and cCS1 (Hahn et al., 1987), that are complementary over 11 nucleotides. UVR represents the upstream variable region of the 3′-UTR that is not well conserved among flaviviruses. (B) LUC reporter mRNA constructs with DEN2, WNV, Sindbis virus (SIN) or rabbit α-globin UTRs. All pUC-based LUC reporter constructs have the upstream *Not*I site and T7 promoter (black arrowhead), and downstream *Kpn*I, *Acc*65I/*Sac*I and *Eco*RI sites (not always shown). They also include a LUC coding region modified with an in-frame *Pst*I site at the 5′-end (placed in front of the second natural codon, GAA, of the LUC ORF) and a *Hin*dIII site just upstream of the stop codon (created with silent mutations introduced into the LUC ORF). DLD RNA, which has DEN 5′ and 3′-UTRs, is made by transcription from the indicated template after linearization with *Sna*BI. The terminal sequences of DLD RNA differ from authentic DEN RNA by substitution of a G for A residue at the 5′-end and addition of UAC at the 3′-end. Translation starts with the first two authentic DEN codons and terminates with the authentic DEN UAG stop codon. DCLD RNA differs from DLD RNA by containing the first 36 codons of the DEN capsid coding region, which includes cCS1 and corresponds roughly to the capsid coding region present in flavivirus subgenomic replicons (Khromykh and Westaway, 1997; Lo et al., 2003; Molenkamp et al., 2003; Pang et al., 2001). WLW and WCLW RNAs are analogous to DLD and DCLD RNAs, with all viral sequences from WNV. In these RNAs, a non-viral G residue is present at the 5′-end and an additional UAC at the 3′-end. Thirty-one codons of the capsid ORF are present in WCLW RNA. SinLSin RNA has the 59-nucleotide SIN 5′-UTR (with an additional G residue at the 5′-end to facilitate transcription), the first two codons of the SIN nonstructural polyprotein ORF, and the 318-nucleotide SIN 3′-UTR (GenBank J02362) with an *Xho*I site added to the 3′-end. A 72-nucleotide poly(A) tract that ends with an *Acc*65I site is present downstream of the *Xho*I site. GLG RNA contains 5′ and 3′-UTRs (36 nt and 87 nt, respectively) derived from rabbit α-globin mRNA (GenBank J00658). The first two codons of the α-globin coding region precede the two codons encoded by the *Pst*I site of the modified LUC ORF. GCLG RNA is a derivative of GLG RNA that encodes the same capsid-LUC fusion protein as DCLD RNA. Variants with and without an A_{72} tail can be made by plasmid linearization with *Acc*65I or *Xho*I, respectively.

The basic LUC reporter construct with 5′ and 3′ terminal regions from DEN2 RNA, from which DLD RNA (named for DEN 5′-UTR/LUC/DEN 3′-UTR) can be transcribed, is shown in Fig. 2.1B. A unique *Not*I site was placed upstream of the T7 promoter, which is immediately adjacent to the 96 nt-long DEN2 5′-UTR. The 5′-UTR was fused to the first 2 codons of the polyprotein ORF in order to preserve the natural

AUG context, and a unique *Pst*I site was engineered in-frame between these codons and the LUC ORF. To facilitate T7 transcription, the 5´-A residue of the genomic sequence was replaced with a G. At the other end of the LUC ORF, the last two codons were modified with silent mutations to incorporate a *Hin*dIII site just upstream of the stop codon. The native DEN2 stop codon and entire 451 nt-long 3´-UTR was placed between this *Hin*dIII site and a *Sna*BI site placed at the 3´-end (Fig. 2.1B). Linearization of pDLD plasmid DNA with *Sna*BI permits the production of transcripts with only three non-native nucleotides at the 3´-termini. To produce RNAs with other UTRs, pDLD was modified by replacement of *Not*I-*Pst*I or *Hin*dIII-*Sna*BI fragments with various PCR-generated inserts.

We constructed pDCLD (named for DEN/Capsid/LUC/DEN; Fig. 2.1B) to investigate the influence of the 5´-proximal part of the DEN ORF, which includes the cCS1 element that permits RNA cyclization by hybridization with the CS1 element in the 3´-UTR (Fig. 2.1A). pDCLD includes the first 36 codons of the DEN capsid coding region fused in front of the *Pst*I site and placed in-frame upstream of the LUC coding sequence (Fig. 2.1B). This results in the production of a capsid-LUC fusion protein. To compare translation driven by viral sequences to that driven by UTRs from a highly expressed cellular mRNA, we have made pGLGpA and pGCLGpA (Fig. 2.1B). These constructs include the 5´ and 3´-UTRs derived from rabbit α-globin mRNA and a poly(A) tail 72 residues long. In pGLGpA, the first two codons of the α-globin ORF are fused to the *Pst*I site and in frame with the LUC coding sequence, while in pGCLGpA, the entire coding region is identical to that in pDCLD. RNAs with and without a poly(A)

tail can be made from pGLGpA and pGCLGpA after linearization with *Acc*65I and *Xho*I, respectively.

The specific activities of all the forms of N-terminally modified LUC made by these and other RNAs studied in this paper have been determined and verified to be similar (Fig. 2.2). This was assessed by determination of the light yields from known amounts of ^{35}S-methionine-labeled LUC proteins synthesized by in vitro translation. We have previously observed consistent specific activities for LUC forms with different N-terminal extensions (Matsuda et al., 2004a).

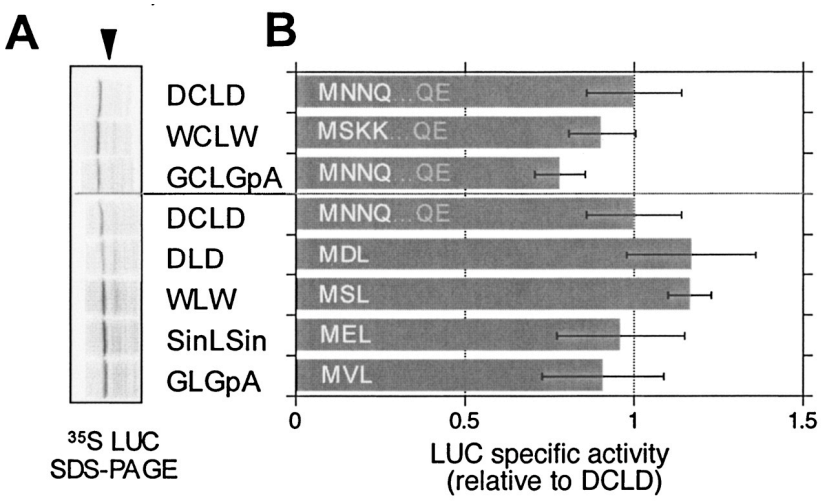


Fig. 2.2 LUC variants with N-terminal fusions have similar specific activity.
The indicated RNAs were translated in a rabbit reticulocyte lysate in the presence of ^{35}S[methionine]. (A) Translation products were separated by SDS-PAGE (10%); the major product, LUC, is indicated by an arrowhead. These samples are shown to indicate the quality of the LUC product, not the translational efficiencies of the various RNAs. (B) Relative specific activities (relative light units per pmole) of LUC proteins made in reticulocyte lysates (averages from 3 replicates with error bar representing one standard deviation). The N-terminal sequences of LUC proteins made by these RNAs are indicated (single letter amino acid code); intervening capsid sequences are indicated as "..."

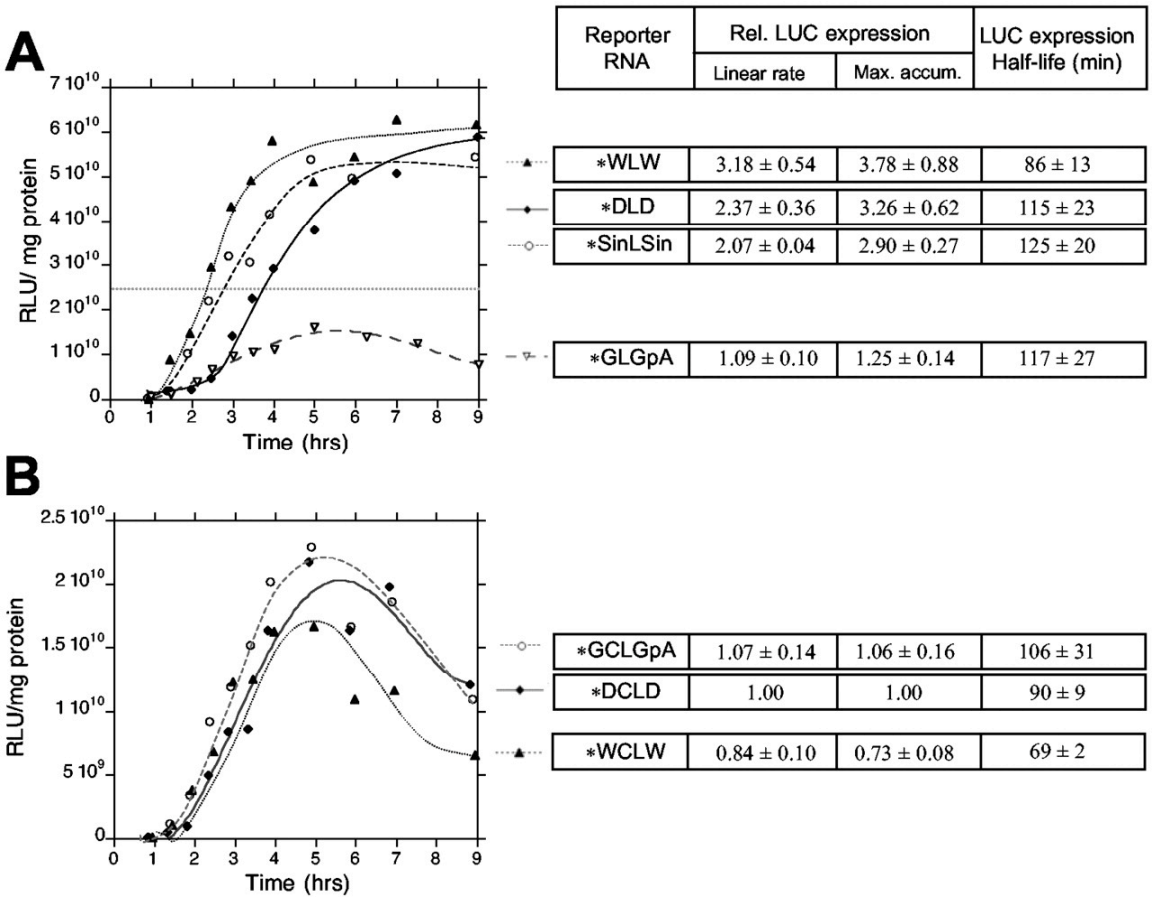
2.4.2 Flavivirus UTRs support highly efficient translation in Vero cells

To assess the overall efficiency with which flavivirus UTRs are able to support translation in Vero cells, we studied LUC expression from reporter RNAs with DEN or WNV UTRs: DLD, DCLD, WLW and WCLW RNAs (Fig. 2.1B). Expression was compared to that from GLGpA RNA and from SinLSin RNA, which has UTRs from Sindbis virus (Fig. 2.1B). SinLSin RNA was included as a representative of a virus capable of rapid amplification and highly efficient gene expression (Strauss and Strauss, 1994). All RNAs contained 5´ ^{m7}GpppG caps (indicated by * in Fig. 2.3). In time courses following LUC expression up to 9 h after RNA delivery by electroporation, DLD, WLW and SinLSin RNAs supported the synthesis of considerably more LUC activity than did GLGpA RNA (Fig. 2.3A).

We derive three parameters relevant to UTR function from LUC expression time courses. Estimates of the maximum rate of increase in LUC activity ("linear rate" in Fig. 2.3 and subsequent Fig.s), which is observed during an early phase of linear increase, are taken to reflect translational efficiency (Gallie, 2002; Matsuda et al., 2004a). The maximum accumulation of LUC activity reflects the expression capacity of each RNA. This capacity is influenced by the translational efficiency of the mRNA and by the stabilities of the mRNA and translated protein. The combined RNA and protein stabilities can be monitored by determining the longevity of continued LUC expression. From the time courses, we estimate the half-life of LUC expression as the time taken for the maximum rate of expression (discussed above) to fall to half that rate. Rates are determined as slopes of the lines of best fit for each expression time course. Among reporter RNAs encoding the same protein, differences in LUC half-life reflect differences in RNA functional stability (the RNA's capacity to direct protein synthesis; refs. (Gallie,

2002; Matsuda et al., 2004a)). When the encoded LUC proteins vary at their N-termini, differences in protein stability may also be reflected in the LUC protein half-life. The expression half-lives of LUC expressed from DLD, WLW, SinLSin and GLGpA RNAs were all similar (Fig. 2.3A). LUC synthesis from the RNAs with viral UTRs were expressed with 2- to 3-fold higher translational efficiency than LUC expressed from GLGpA RNA (Linear rate, Fig. 2.3A). The flaviviral reporter RNAs expressed LUC at least as efficiently as did SinLSin RNA. LUC expression from WLW and SinLSin RNAs consistently appeared earlier than from DLD RNA (Fig. 2.3A), suggesting differences in the ability to recruit ribosomes for the initial round of translation.

Variants of DLD and WLW that included the first 36 and 31 codons, respectively, of each viral capsid coding region (DCLD, WCLW RNAs; Fig. 2.1B) were constructed to more closely represent the translation of natural viral RNAs. The presence of additional 5´ viral sequences allows local folding into a predicted stem-loop that includes the initiation AUG (mfold, ref. (Zuker, 2003)) and long-distance pairing between CS1 and cCS1. Further, under some circumstances, the proximal part of an ORF can affect the efficiency of its own translation (Frolov and Schlesinger, 1996; Kozak, 1990). The same fragment of the DEN capsid ORF was present in GCLGpA RNA as a control. The specific activities of the capsid-LUC fusion proteins varied little from those of the corresponding LUC proteins lacking the capsid ORF (Fig. 2.2B), while expression half-lives were marginally shorter (Fig. 2.3). The decrease in LUC activities after 6 hr suggests a shorter half-life for the capsid-LUC fusion proteins.



Reporter RNA	Rel. LUC expression		LUC expression Half-life (min)
	Linear rate	Max. accum.	
*WLW	3.18 ± 0.54	3.78 ± 0.88	86 ± 13
*DLD	2.37 ± 0.36	3.26 ± 0.62	115 ± 23
*SinLSin	2.07 ± 0.04	2.90 ± 0.27	125 ± 20
*GLGpA	1.09 ± 0.10	1.25 ± 0.14	117 ± 27
*GCLGpA	1.07 ± 0.14	1.06 ± 0.16	106 ± 31
*DCLD	1.00	1.00	90 ± 9
*WCLW	0.84 ± 0.10	0.73 ± 0.08	69 ± 2

Fig. 2.3 Efficient LUC expression from 5´-capped flavivirus reporter mRNAs in Vero cells

Vero cells were electroporated with the indicated RNAs, and LUC extracts were prepared after various incubation times. LUC activities and protein levels were assayed in each extract to determine corrected LUC yields (relative light units per mg protein). Representative time courses from single experiments (average of duplicates) are shown in the time courses of A and B. The tabulated results report the LUC expression relative to that from DCLD RNA, with the (maximal) linear rate representing translational efficiency. The maximum LUC accumulation for each RNA is also reported, as is the LUC expression half-life, which reflects the combined RNA and LUC protein stability. The tabulated results reflect averages from at least 2 separate transfection experiments performed on different days, in each case involving duplicate sets of independently made RNA transcripts. Standard errors are indicated. The * in front of each RNA name represents a 5´-cap.

The efficiency and maximal expression of LUC from DCLD and WCLW RNAs were 2- to 5-fold lower than from DLD and WLW RNAs, respectively (Fig. 2.3B vs.

2.3A). Nevertheless, DCLD and WCLW RNAs supported strong LUC expression at levels similar to that from GLGpA RNA. The presence of DEN capsid coding sequences in GCLGpA RNA had little effect on LUC expression (compare GCLGpA and GLGpA, Fig. 2.3), in contrast to their effect on DLD RNA. Decreased expression is thus not a property of the encoded protein, but likely a property of the RNA (such as different folding). The capsid-coding regions include cCS1, thereby permitting long-distance hybridization to the complementary sequence, CS1, that is present in the viral, but not globin, 3´-UTRs. Together with additional long-distance 5´-3´ pairing involving sequences just upstream of the initiation AUG as suggested recently (Thurner et al., 2004), these RNA-RNA interactions may dampen the efficiency of ribosome access in the case of RNAs with viral 5´ and 3´ terminal regions.

2.4.3 Translational regulation by DEN 5´ and 3´ UTRs

Efficient translation of cellular mRNAs depends heavily on the 5´-cap and 3´-poly(A) tail, which enhance expression synergistically (Gallie, 1991; Tarun and Sachs, 1995). This response was confirmed in Vero cells using transcripts from pGCLGpA. Detecting synergy requires study of the expression of capped and uncapped versions of an RNA, as well as variants with and without a poly(A) tail. In the presence of a 5´-cap, the addition of polyadenylated globin 3´-UTR (3´-GpA) enhanced translational efficiency (linear rate) by 108-fold with reference to an RNA with a minimal vector-derived 3´-UTR (*GCLGpA vs. *GCLD; Figs. 2.4A, 2.4C). Far less enhancement (5.5-fold) was observed in response to adding 3´-GpA when RNAs were uncapped (GCLGpA vs. GCLD; Figs. 2.4B, 2.4C). The ratio between these responses (108/5.52 = 19.6) represents synergistic enhancing effects between the 5´-cap and polyadenylated 3´-UTR,

implying collaboration between these features. Note that the presence of 3´-GpA also increased the LUC expression half-life by 3-fold (82/27; Fig. 2.4A). Since GCLGpA and GCLD RNAs encode the same form of LUC, we can conclude that there is a 3-fold difference in the functional half lives of these RNAs.

Similar analyses were conducted to study the contribution of the DEN 3´-UTR to translational expression. Like the 3´-GpA, the DEN 3´-UTR is clearly important for high-level expression of LUC (Fig. 2.4A; compare *DCLD with *DCLD RNA). Addition of the DEN 3´-UTR to DCLD RNA (to produce DCLD RNA) enhanced translational efficiency 32-fold in the presence of a 5´-cap (*DCLD vs. *DCLD; Figs. 2.4A, 2.4C) and 10.5-fold in the absence of a cap (DCLD vs. DCLD; Figs. 2.4B, C). This indicates the existence of a synergistic interaction between the cap and DEN 3´-UTR (synergy value of 32.3/10.5 = 3.1), although considerably less so than that between a cap and 3´-GpA.

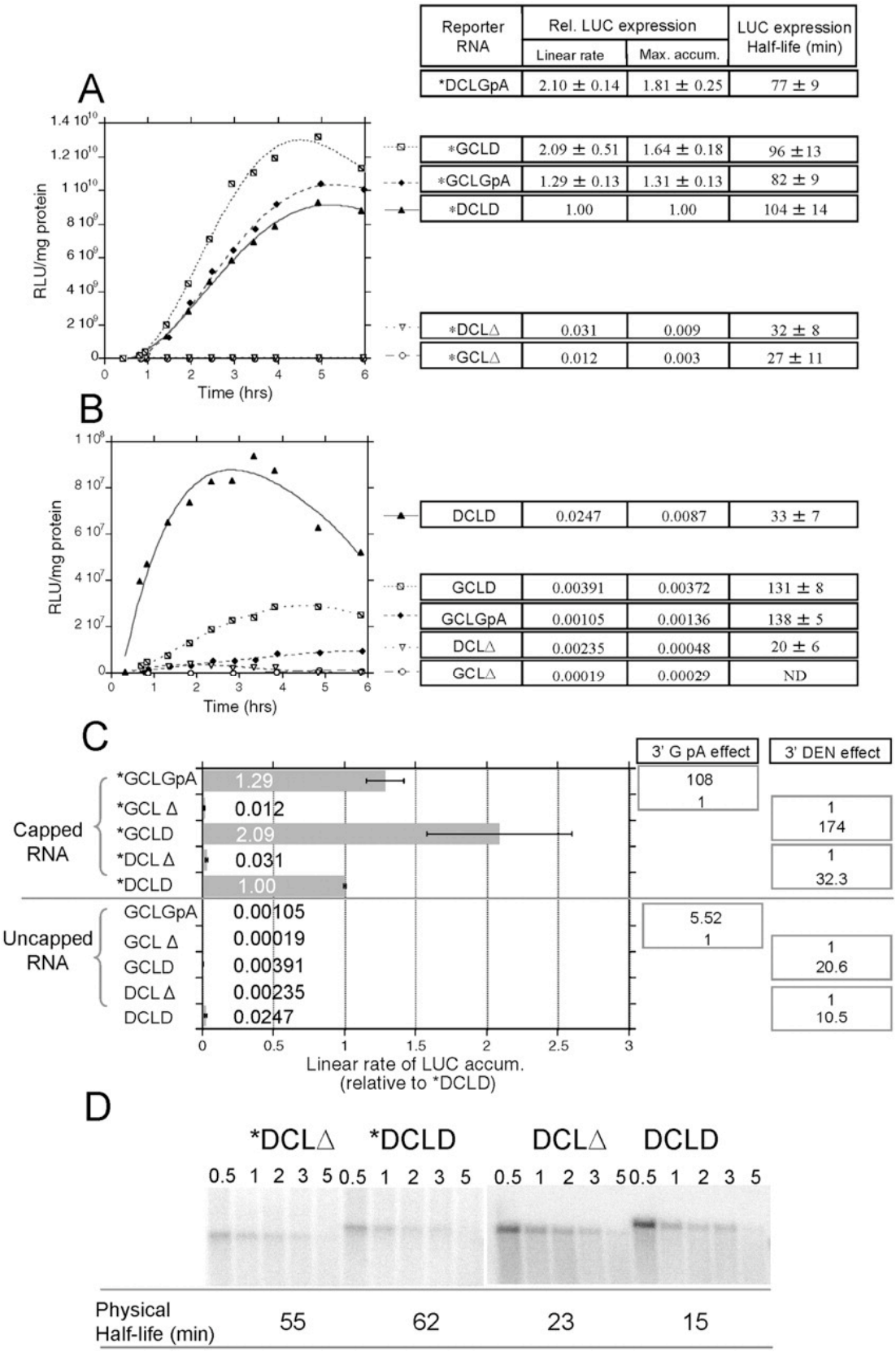
Studies with the uncapped forms of RNAs, which support relatively low LUC expression, emphasized the importance of a 5´-cap. Among uncapped RNAs, however, elevated levels of LUC expression were observed from RNAs with DEN UTRs. This is obvious for DCLD RNA in the time course of Fig. 2.4B. An enhancing effect of 5´ DEN sequences can be seen for DCLD RNA in comparison with GCLD RNA, while an effect of the DEN 3´-UTR is evident when comparing expression from GCLD and GCLGpA RNAs (Fig. 2.4B and C). The enhancing effect was stronger for the DEN 5´-UTR, which supported the synthesis of LUC 6 to 12 times more efficiently (linear rate; Figs. 2.4B, 2.4C) than 5´-G in RNA counterparts with the same 3´-UTR. No such 5´-DEN-

dependent enhancement was seen in the capped state, since capped DCLD, GCLD and GCLGpA RNAs were similarly expressed (Fig. 2.4A).

LUC expression half-life estimates indicated that the GpA and DEN 3´-UTRs and the 5´-cap all contribute to RNA stability. Capped DCLD, GCLD and GCLGpA RNAs (which encode the same form of LUC) had similar functional half-lives, and removal of the 3´-UTR decreased the half-life by about 3-fold (*DCLD and *GCLD RNAs; Fig. 2.4A). Uncapped DCLD RNA had a similar half-life as capped DCLD or GCLD RNAs, evident from the early peak in LUC activity (DCLD time course in Fig. 2.4B). While RNA stability differences associated with the 5´-cap and 3´-UTR are important, they were much smaller in magnitude than the enhancements in translational efficiency provided by these features. Analyses of the physical stabilities of RNAs during expression time courses confirmed that RNA stability differences were relatively small among the RNAs studied in Fig. 2.4 (Fig. 2.4D). In these assays, ^{32}P-labeled RNAs were electroporated and incubated in cells before being extracted and examined by electrophoresis (see Materials and Methods).

Expression from DCLGpA and GCLD RNAs was studied to assess whether there are any cooperative effects between DEN 5´ and 3´-UTRs. LUC expression from both of these capped RNAs was comparable to expression from capped DCLD and GCLGpA RNAs (Fig 2.4A), clearly indicating that the DEN UTRs do not need to be coupled to support strong translation. Addition of the DEN 3´-UTR to GCLD RNA enhanced translational efficiency 174-fold in the presence of a 5´-cap and 20.6-fold with no cap (Fig. 2.4C). There is thus stronger synergy between the 5´-cap and DEN 3´-UTR (synergy value of 174/20.6 = 8.4) for these RNAs with a globin 5´-UTR than observed

with the DEN 5´-UTR (synergy value of 3.1; see above). As evident when comparing DCLD and DCLD RNAs, the DEN 3´-UTR in GCLD RNA enhanced the RNA functional half-life about 3-fold (Fig. 2.4A). We conclude that the DEN 3´-UTR is a general enhancer of translation and RNA stability whose action does not require a matched DEN 5´-UTR.



Reporter RNA	Rel. LUC expression		LUC expression Half-life (min)
	Linear rate	Max. accum.	
*DCLGpA	2.10 ± 0.14	1.81 ± 0.25	77 ± 9
*GCLD	2.09 ± 0.51	1.64 ± 0.18	96 ±13
*GCLGpA	1.29 ± 0.13	1.31 ± 0.13	82 ± 9
*DCLD	1.00	1.00	104 ± 14
*DCLΔ	0.031	0.009	32 ± 8
*GCLΔ	0.012	0.003	27 ± 11
DCLD	0.0247	0.0087	33 ± 7
GCLD	0.00391	0.00372	131 ± 8
GCLGpA	0.00105	0.00136	138 ± 5
DCLΔ	0.00235	0.00048	20 ± 6
GCLΔ	0.00019	0.00029	ND

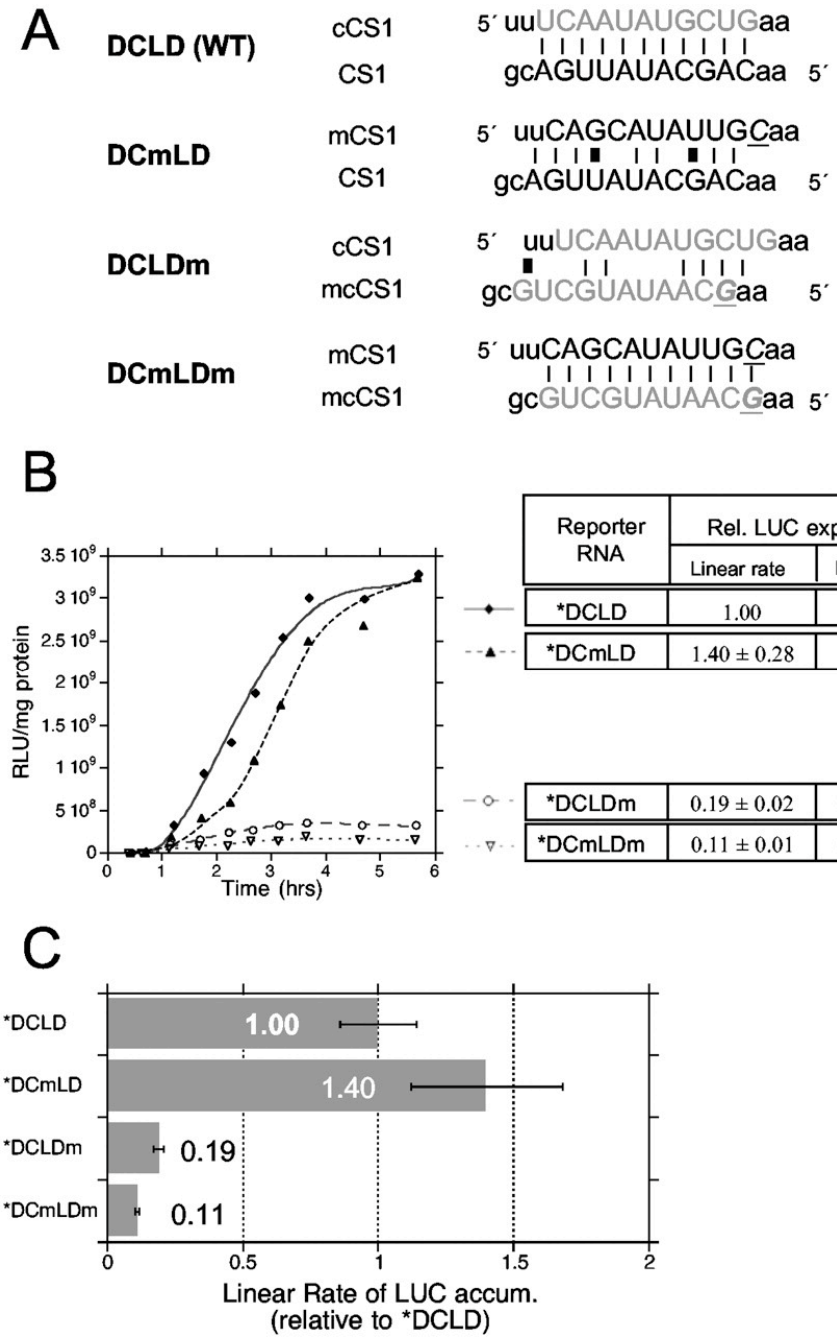
Fig. 2.4 Contributions of DEN 5´ and 3´ UTRs to translational expression in Vero cells
LUC expression from the indicated capped (A) or uncapped (B) RNAs is described as in Fig. 3. DCLD and GCLD RNAs have 3´-UTRs comprised of only 15 vector-derived nucleotides (UAAAAUGGAUCUCGA). Note the different scales on the Y axes in A and B. Expression data for *DCLGpA RNA are tabulated at the top of A, but the graphed time course is not shown be avoid cluttering the graph. For clarity, standard errors of LUC expression have been omitted for the less efficiently expressed RNAs in A and B; standard errors for these determinations averaged about 20% of the means. In C, the relative linear rates of LUC expression from the various RNAs are compared graphically. ND, not determined. (D). Assay of the physical stabilities of selected ^{32}P-labeled transcripts electroporated and incubated in Vero cells for the indicated time before extraction and analysis in the denatured state in a 1% agarose gel. The estimated half-lives are written at the bottom.

2.4.4 Complementarity between cyclization sequences CS1 and cCS1 is not needed for translation

The results already discussed failed to detect any codependency between 5´ and 3´-DEN sequences in regard to the control of translation. To explore this issue further, we wished to test directly for a translational role of the conserved cyclization sequences and their potential long-distance hybridization. The RNAs of Fig. 2.5A represent a set in which CS1 or cCS1 have replaced one another singly and together, resulting in DCmLD and DCLDm RNAs that have diminished potential for long-distance cCS1/CS1 interaction, and the double mutant DCmLDm RNA in which CS1 and cCS1 have been switched and cyclization is possible. Note that one nucleotide of each conserved sequence was changed to avoid introducing a stop codon with CS1 placed in the 5´ location. Expression from capped forms of these RNAs was studied in Vero cells.

Replacement of cCS1 with the modified CS1 sequence had little impact on LUC expression or expression half-life (*DCLD vs. DCmLD, Fig. 2.5). By contrast, replacement of CS1 with the modified cCS1 decreased LUC expression efficiency to

19%, with only a small effect on expression half-life (*DCLD vs. *DCLDm, Fig. 2.5). CS1 and cCS1 thus appear to have differential roles in translational regulation. Significantly, the inability of the double mutation (DCmLDm) to rescue the depressed LUC expression level of DCLDm RNA (Figs. 2.5B, 2.5C) suggests that RNA cyclization via cCS1/CS1 interaction is not required for translation of the reporter RNAs used here. The minimal differences in the half-lives of LUC expression from the RNAs in Fig. 2.5 indicate that these RNAs have similar stabilities.



Reporter RNA	Rel. LUC expression		LUC expression Half-life (min)
	Linear rate	Max. accum.	
*DCLD	1.00	1.00	84 ± 13
*DCmLD	1.40 ± 0.28	1.11 ± 0.12	79 ± 5
*DCLDm	0.19 ± 0.02	0.14 ± 0.02	60 ± 4
*DCmLDm	0.11 ± 0.01	0.09 ± 0.01	75 ± 7

Fig. 2.5 Complementarity between cCS1 and CS1 is not required for LUC expression from DCLD RNA

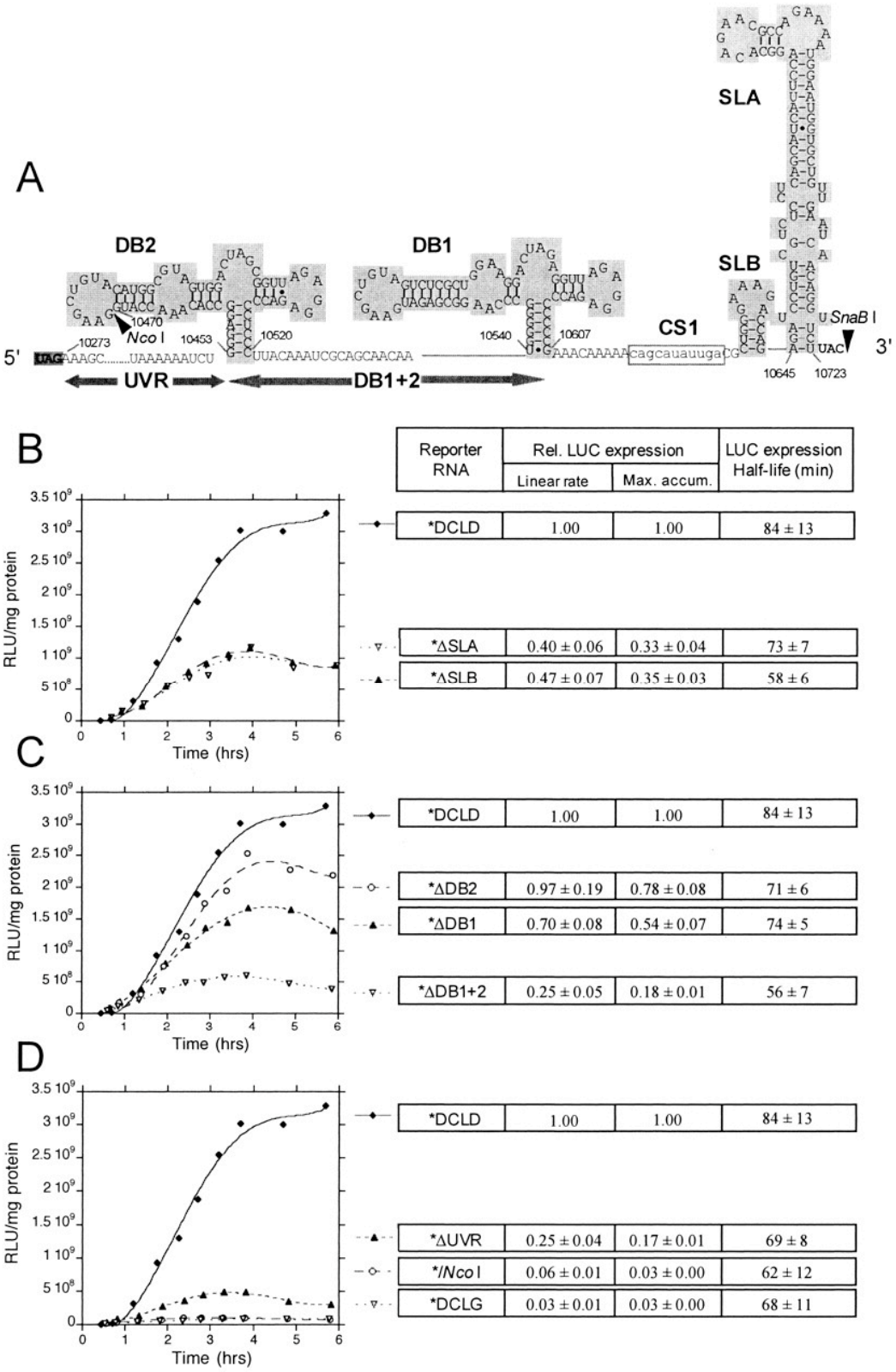
(A). The sequences of wild type and variant cyclization sequences cCS1 (5′, indicated in gray lettering) and CS1 (3′, black lettering) are shown. The wild type cCS1 and CS1 sequences, which form a perfect 11 bp duplex, are shown in upper case. The three mutant RNAs were constructed by transposing these sequences to the other end of the genome. Single base changes in mCS1 and mcCS1, indicated with underlined italics, were necessitated to avoid insertion of a UGA stop codon within the capsid ORF when CS1 was moved to the 5′ location. The most stable long-distance base-pairing schemes

are shown, with the sequence in the 5´-region of the mRNA in the upper line. (B). LUC expression from the indicated capped (*) RNAs is described as in Fig. 3. In C, the relative linear rates of LUC expression from the various RNAs are compared graphically.

2.4.5 Contribution of conserved elements in the 3´-UTR to translational expression

The above results indicated an involvement of CS1 in translation. In order to identify contributions from other elements in the 3´-UTR, the conserved features SLA, SLB, DB1 and DB2, as well the upstream variable region (UVR) (see Figs. 2.1A, 2.6A) were deleted in turn from the 3´-UTR of capped DCLD RNA. The endpoints of deletions were chosen to cleanly delete the predicted structures as shown in Fig. 2.6A, and this was confirmed by mfold (Zuker, 2003) predictions of the folding of the 3´UTRs. A large 3´-truncation was made by transcription from a pDCLD template linearized with *Nco*I, which cleaves at DEN nucleotide 10470 (Fig. 2.6A). This produced a variant of DCLD RNA (*/NcoI; Fig. 2.6) truncated near the upstream end of DB2 and thus lacking SLA, SLB, CS1, DB1 and most of DB2. LUC expression from capped RNAs was studied in Vero cells (Fig. 2.6).

Deletion of either of the two 3´-terminal stem-loops SLA and SLB decreased the efficiency of LUC translation to about half (Fig. 2.6B; linear rate). Deletion of DB1 resulted in a smaller, though significant, decrease in the efficiency of LUC translation, while deletion of DB2 had little effect (Fig. 2.6C). Simultaneous deletion of DB1 and DB2 reduced the efficiency of translation to 25%, suggesting redundancy in the contributions of these quite similar features. Deletion of the UVR, just downstream of the stop codon, also resulted in a relative translational efficiency of 25% (Fig. 2.6D).



Reporter RNA	Rel. LUC expression		LUC expression Half-life (min)
	Linear rate	Max. accum.	
*DCLD	1.00	1.00	84 ± 13
*ΔSLA	0.40 ± 0.06	0.33 ± 0.04	73 ± 7
*ΔSLB	0.47 ± 0.07	0.35 ± 0.03	58 ± 6
*DCLD	1.00	1.00	84 ± 13
*ΔDB2	0.97 ± 0.19	0.78 ± 0.08	71 ± 6
*ΔDB1	0.70 ± 0.08	0.54 ± 0.07	74 ± 5
*ΔDB1+2	0.25 ± 0.05	0.18 ± 0.01	56 ± 7
*DCLD	1.00	1.00	84 ± 13
*ΔUVR	0.25 ± 0.04	0.17 ± 0.01	69 ± 8
*/NcoI	0.06 ± 0.01	0.03 ± 0.00	62 ± 12
*DCLG	0.03 ± 0.01	0.03 ± 0.00	68 ± 11

Fig. 2.6 Several features from the DEN 3´-UTR contribute to efficient translation.

Fig. 2.6 Several features from the DEN 3´-UTR contribute to efficient translation. (A). The sequences of conserved features of the DEN2 3´-UTR are shown in their proposed foldings. The native DEN stop codon UAG is shown at left and the additional non-viral nucleotides at the 3´-end of DCLD RNA are shown at right. The *Nco*I restriction site used for producing the /NcoI variant of DCLD RNA is marked. The folding of DB1 and DB2 are according to ref. 37, while the folding of SLA and SLB are according to refs. 3 and 48. The numbered nucleotides (numbering from the 5´-end of the DEN genome) represent the deletion boundaries in the D RNA derivatives of DCLD studied in B-D. (B-D). LUC expression from the indicated capped (*) RNAs is described as in Fig. 3. Y axes are the same in each case.

Translational enhancement provided by the 3´-UTR thus seems to derive from several features, including contributions by each of the conserved features. Individual deletion of these features led to partial loss of activity. Expression from each of the RNAs with a single element deleted was higher than from RNA entirely lacking a DEN 3´-UTR (*DCLD; Fig. 2.7A). Presence of the UVR feature alone (*/NcoI RNA) increased expression only slightly from this basal level (Fig. 2.7A), emphasizing the importance of combined contributions to translational enhancement by the 3´-UTR.

Interestingly, DCLG RNA, which has the 87 nucleotide-long globin 3´-UTR but no poly(A) tail, supported the same basal level of LUC expression as DCLD RNA (Fig. 2.7). High levels of expression were dependent on the poly(A) tail (*DCLGpA RNA, Fig. 2.7A). This emphasizes the similar role of the DEN 3´-UTR and poly(A) tail in enhancing translation.

It is important to note that no noteworthy differences in functional RNA stability were observed for the RNAs with various partial deletions of the DEN 3´-UTR, although most of these capped RNAs had somewhat shorter half-lives than capped DCLD RNA (Fig. 2.7B); this conclusion was supported by analysis of the physical stabilities of these

RNAs (not shown). The RNAs containing partial 3´-deletions were all more stable than DCLD RNA, (Fig. 2.7B). It appears that, as for translational enhancement, RNA stabilization (roughly 3-fold) provided by the DEN 3´-UTR (noted earlier) derives from multiple features in the UTR. The globin 3´-UTR also appears to enhance RNA stability (2-fold stabilization observed in DCLG RNA relative to DCLD; Fig. 2.7B), with little if any further stabilization provided by a poly(A) tail (DCLGpA RNA; Fig. 2.7B). The contributions to translational enhancement were just the opposite, being primarily derived from the poly(A) tail and not measurably from the 3´G sequence (Fig. 2.7A).

Although it has been observed that a 3´-UTR can stabilize RNA and enhance expression by virtue of its length, in a sequence non-specific effect (Tanguay and Gallie, 1996), this is unlikely to be a consideration among the 3´-UTR domain deletions. Those deletions remove no more than 154 nt (DDB1+2), leaving at least a 297 nt-long UTR, and, conversely, the addition of the 87-nt 3´G did not enhance translation (Fig. 7A). The non-specific length effects were observed for 3´-UTRs shorter than 27 nt (Tanguay and Gallie, 1996).

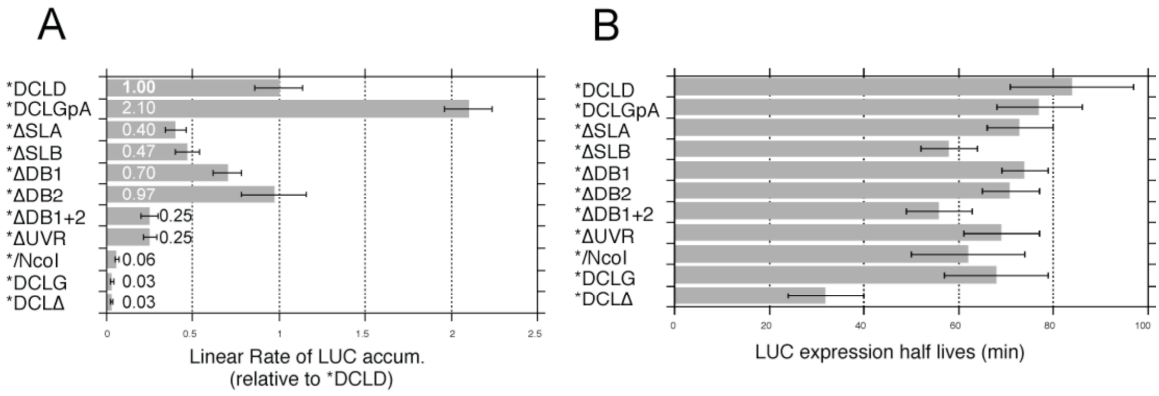


Fig. 2.7 Translational efficiencies and RNA functional half-lives of 5´-capped DCLD variants with deletions in the 3´-UTR

Fig. 2.7 Translational efficiencies and RNA functional half-lives of 5´-capped DCLD variants with deletions in the 3´-UTR.

RNA functional Graphical representations of (A) relative translation efficiencies (linear rates of LUC accumulation) and (B) LUC expression half-lives are shown for the indicated RNAs. Because all of the RNAs encode the same form of LUC, LUC expression half lives are proportional to RNA functional half-lives. Data are taken from Figs. 4 and 6. Error bars represent standard errors.

2.5 DISCUSSION

2.5.1 Flaviviral 5´ and 3´ terminal genome sequences support highly efficient translational expression

Using a luciferase reporter system and comparison with reporter RNAs that support highly efficient translation, we have shown that the 5´ and 3´ terminal regions from the DEN2 and WNV genomes are able to support strong translation in Vero cells. Translational expression from 5´-capped DCLD and WCLW RNAs with 5´ and 3´ sequences from DEN and WNV, respectively, was similar to expression from capped GLGpA RNA and only 2- to 3-times lower than expression from SinLSin RNA (Fig. 2.3). GLGpA RNA has UTRs from the α-globin mRNA, while SinLSin RNA has UTRs derived from Sindbis virus, an alphavirus that has been developed as a gene expression vector because of its ability to support potent translational expression (Agapov et al., 1998).

The viral sequences present in DCLD and WCLW RNAs included part of the capsid coding region in addition to the 5´ and 3´ UTRs. The capsid coding regions were included because they contain the conserved cCS1 sequence element that permits potential cyclization of the RNA by annealing to its complement, CS1, present in the 3´-UTR. A surprising finding was that omission of the capsid coding regions (DLD and WLW RNAs) increased translational expression by 2- to 4-fold (Fig. 2.3). This is not

due to an elongational block of translation associated with the capsid ORF, since LUC was similarly expressed from GLGpA and GCLGpA RNAs (Fig. 2.3). We have also verified that the LUC and capsid-LUC fusion proteins have similar luciferase specific activities (Fig. 2). It seems most likely that the presence of the proximal part of the capsid ORF lessens translational efficiency by affecting ribosome initiation, perhaps by altering the folding of the 5´-UTR through local interactions or as a consequence of long-distance hybridization with CS1.

In any case, it is clear that the capped flaviviral 5´-UTRs are capable of directing very high translational expression in Vero cells. This was rather unexpected in view of the conserved secondary structure positioned immediately adjacent to the 5´-end (Brinton and Dispoto, 1988; Pelletier and Sonenberg, 1985) and suboptimal consensus around the initiation AUG (UCU*C*UGAUG*A* for DEN, AUC*U*CGAUG*U* for WNV). Secondary structure at the 5´-terminus is detrimental to cap-dependent translation initiation (Kozak, 1989) and weak consensus nucleotides at the –3 and +4 positions (bold italic above) can diminish translation by up to 10-fold (Kozak, 1999b). Schrader and Westaway (Schrader and Westaway, 1990) have also commented on the efficient translation of flaviviral (Kunjin virus) RNA in Vero cells despite suboptimal AUG context.

2.5.2 The DEN 3´-UTR enhances translation and RNA stability similarly to a poly(A) tail

The contribution of the DEN 3´-UTR to translational expression was studied by determining the effect of replacing a short non-viral 3´-UTR (DCLD or GCLD RNAs) with the DEN 3´-UTR (DCLD and GCLD RNAs). For capped RNAs with either the DEN or globin 5´-UTRs, the DEN 3´-UTR significantly increased RNA stability (about 3-fold increase in functional half-life; Fig. 2.4A). Far greater increases were observed in

translational efficiency, represented by maximum LUC expression rate in our assays ("linear rate" value in Figs. 2.3-2.6). In the presence of a DEN 5´-UTR, the rate was increased 32.3-fold (*DCLD vs. *DCLD; Fig. 2.4C), while in the presence of a globin 5´-UTR, the increase was 174-fold (*GCLD vs. *GCLD; Fig. 2.4C). These responses are very comparable to those provided by a polyadenylated 3´-UTR. Capped GCLGpA RNA was 3 times as stable and was translated with 108 times the efficiency of capped GCLD RNA (Fig. 2.4C), and capped DCLGpA RNA was translated with 70 times the efficiency of capped DCLD RNA (Fig. 2.7A). The ability of the DEN 3´-UTR to enhance translational efficiency and RNA stability in the presence of either DEN or globin 5´-sequences demonstrates this to be a free-standing regulator that is not codependent on viral 5´-sequences. Stimulation of the translation of LUC reporter mRNAs by the DEN2 3´-UTR has also recently been reported by Holden and Harris (Holden and Harris, 2004).

The poly(A) tail is known to enhance translation synergistically with a 5´-cap (Gallie, 1991; Tarun and Sachs, 1995). Such synergy is established by determining the extent to which the addition of a poly(A) tail has a greater effect in the presence than in the absence of a cap. The polyadenylated globin 3´-UTR (3´-GpA) enhanced translational efficiency 108/5.52 = 19.6 times more when a cap was present (GCLGpA and GCLD RNAs; Fig. 2.4C), confirming the strong synergy that others have reported. Similar analyses of the role of the DEN 3´-UTR also indicated the existence of synergy with a 5´-cap, although this was weaker than the synergy observed with a poly(A) tail and it differed for different 5´-UTRs. Thus, the cap/3´-DEN synergy value was 8.4 for RNAs with a globin 5´-UTR (GCLD and GCLD RNAs, 174/20.6; Fig. 2.4C) and 3.1 for RNAs with a DEN 5´-UTR (DCLD and DCLD RNAs, 32.3/10.5; Fig. 2.4C).

If cap/poly(A) synergy reflects the existence of bridging interactions that cyclize the mRNA as described in the Introduction, we may think of the DEN 3´-UTR recruiting proteins capable of interacting with translation initiation factors assembled around the 5´-cap. Such a scenario has been demonstrated for rotaviral mRNAs, with the bridging interaction mediated by the viral protein NSP3 (Vende et al., 2000b). For the DEN 3´-UTR, however, the synergy we have observed does not involve a viral protein, since the only viral protein present in our assays is a fragment of the capsid protein.

The quite significant difference in synergy observed with globin or DEN 5´-UTRs is an interesting observation. Both the GCLD and DCLD RNAs used in our synergy studies possess the cCS1 and CS1 elements and therefore have the potential to cyclize through long-distance hybridization. Although this annealing would be disrupted by elongating ribosomes passing through cCS1, it may influence the initiation of translation. By forming a "closed loop" through direct RNA-RNA interaction rather than though protein mediation, translation may potentially be enhanced. This type of effect has been observed with *Barley yellow dwarf virus* RNA through direct RNA interaction between 5´ and 3´-UTRs (Guo et al., 2001). Alternatively, cCS1/CS1 interaction may be inhibitory towards translational initiation. This is suggested by the lower LUC expression from DCLD RNA compared with DLD (Fig. 2.3) or DCLGpA RNAs (Fig. 2.4), which contain only CS1 or cCS1. Recent modeling of the RNA folding of flaviviral RNAs has suggested the existence of RNA pairing between part of the 5´-UTR just upstream of the AUG initiation codon and the 3´-UTR (Thurner et al., 2004). For DCLD but not GCLD RNA, this pairing would occur in addition to the cCS1/CS1 interaction. Further experiments are needed to investigate whether these combined interactions are

responsible for the lower cap/3´-UTR synergy observed for DCLD compared with GCLD RNA, and for the lower translational expression from DCLD compared with DLD RNA (Fig. 2.3).

2.5.3 Complementarity between cCS1 and CS1 is not necessary for efficient translation driven by DEN UTRs

The above speculation explored a possible inhibitory interaction between 5´ and 3´ genomic sequences that has yet to be tested experimentally. We did, however, test the opposite effect, that is, whether cCS1/CS1 complementarity — and these sequences themselves — are required for efficient translation. Replacement of cCS1 with a modified version of CS1 had little influence on translational expression (*DCmLD; Fig. 2.5). By contrast, replacement of CS1 decreased translational efficiency to about one-fifth (*DCLDm), and there was a further reduction when these mutations were combined to permit potential hybridization and RNA cyclization (*DCmLDm; Fig. 2.5C). These results indicate that cCS1/CS1 complementarity is not an attribute that supports translation. Indeed, the marginally increased translational efficiency of DCmLD over DCLD RNA and the lower efficiency of DCmLDm relative to DCLDm RNA support the idea that the long distance RNA/RNA interactions interfere with translation. Such long-distance interactions have been implicated in flaviviral RNA replication (Lo et al., 2003; You et al., 2001; You and Padmanabhan, 1999), and it is intriguing to consider that this interaction may play a role in regulating the transition from the translation to the replication phase of the infection. If the cCS1/CS1 interaction can be stabilized, for instance by the influence of bound proteins, this might simultaneously inhibit translation and enhance replication. We have recently described an analogous switch involving

translation factor eEF1A binding to the 3´-terminal region of *Turnip yellow mosaic virus* RNA (Matsuda and Dreher, 2004; Matsuda et al., 2004b).

The low translational expression from DCLDm RNA suggests an independent role for CS1 in supporting translation. Such a role was not revealed by the only previous study of CS involvement in translational expression, performed using a WNV reporting replicon (Lo et al., 2003). In that study, mutation of neither cCS1 nor CS1 detectably influenced the yield of the LUC reporter protein during the initial phase of viral gene expression that was not dependent on RNA replication. Further studies involving a variety of CS1 sequence variants and a comparison of the DEN and WNV systems are needed to clarify the situation. There may also be sensitivity differences between the replicon assay and the mRNA reporter assays we have used.

2.5.4 Multiple features of the DEN 3´-UTR enhance translational expression

CS1 is only one of a number of conserved elements present in the 3´-UTRs of flaviviral genomes (Fig. 2.1). By deleting each of the other elements (SLA, SLB, DB1 and DB2) from DCLD RNA, we tested their contribution to the stimulatory effect of the DEN 3´-UTR. Our results suggest that both RNA stability and translational enhancement are conferred by multiple features (Fig. 2.7). At this point, we cannot be certain that lost function is directly due to the absence of the deleted element. Indirect effects due to structural rearrangements in the deleted 3´-UTRs could have occurred even though folding predictions suggested this was not the case. Using a reporting replicon, Lo et al. (Lo et al., 2003) recently reported that each of the conserved sequence elements in the 3´-UTR of WNV RNA could be mutated or deleted without noticeably affecting

translational expression in BHK cells. As noted above, these differences between the DEN reporter RNA and WNV replicon systems will need to be explored.

Among the features that contributed to fully efficient translation were the conserved 3´-terminal stems SLA and SLB. Deletion of either of these decreased translational efficiency to 40-50% that of DCLD RNA (Fig. 2.7). Holden and Harris (Holden and Harris, 2004) have also recently observed that simultaneous deletion of the DEN2 SLA and SLB (3´SL in their nomenclature) resulted in a similar decrease in translational expression in BHK cells from a reporter RNA like ours. In contrast, Brinton and colleagues (Shi et al., 1996) have viewed SLA and SLB as negative regulators of translation in the WNV system. That conclusion arose from the observation that addition of WNV SLA + SLB to the 3´-UTRs of reporter mRNAs suppressed translation considerably in vitro, though only marginally in BHK cells (Li and Brinton, 2001). Further investigation will be needed to explain these differences.

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Chapter 3 5´-cap-dependent translation of Dengue virus type 2 RNA and the influence of the 5´stem loop structure

3.1 INTRODUCTION

Dengue virus (DEN), a member of *Flavivirus*, contains a single-stranded RNA genome with 5´-^{m7}GpppA cap and nonpolyadenylated 3´-UTR (Chambers et al., 1990; Lindenbach, 2001a). The genome encodes a single polyprotein that is expressed by the host translation machinery and further processed into 10 proteins by host- and viral-encoded proteases (Arias et al., 1993; Bazan and Fletterick, 1989; Stadler et al., 1997). Successful polyprotein expression is indispensable for later steps in the viral replication cycle, such as negative and positive-strand RNA synthesis. Indeed, using a luciferase reporter system in Vero cells, we previously demonstrated that DEN RNA can be translated as efficiency as α-globin mRNA (Chiu et al., 2005), strongly suggesting that DEN can successfully compete with a pool of cellular mRNAs for cellular translational machineries.

The 5´-m7GpppN cap and polyA tail are required for efficient translation initiation of cellular mRNAs (Michel et al., 2000). During cap-dependent translation, eukaryotic initiation factor 4E (eIF4E) binds to the ^{m7}GpppN cap at the 5´-end of the mRNA and further interacts with eIF4G (adaptor protein) and eIF4A/B (helicase complex) to form a cap binding complex, eIF4F. eIF4F recruits the small ribosome subunits through the interaction of eIF3 (Dever, 1999; Pestova et al., 2001). The 3´-polyA tail is also known to contribute to translation initiation through interaction of polyA binding protein (PABP) with eIF4F bound to the 5´-end (Kahvejian et al., 2005). Although cap-dependent translation is commonly used by cellular mRNA translation, many positive-sense viruses including members of the *Picornaviridae* and the *Pestivirus*

(Kolupaeva et al., 2000; Pestova and Hellen, 1999) and *Hepacivirus* genera (Wang et al., 1993) of family *Flaviviridae,* have evolved to circumvent the requirement of cap-dependency on translation initiation (Bushell and Sarnow, 2002). Internal ribosomal binding site (IRES), which is often present in those viral genomes lacking a 5´-cap structure, allows recruitment of small subunit ribosome directly to mRNA or indirectly via interaction with eIF4G. Although DEN genomic RNA certainly contains a 5´-cap (Lindenbach, 2001a), it is still unclear whether DEN RNA concomitantly possesses an IRES element or whether DEN RNA can be translated competitively under a conditions which cap-dependent translation is inhibited.

Cap-independent translation can be verified by demonstrating an ability to efficiently express encoded protein when cap-dependent translation is inhibited. Such experimental conditions include 1) the presence of excess cap-analogue for in vitro lysate study, 2) the use of uncapped RNA or cap analog other than ^{m7}GpppN at the 5´-terminus, 3) cells infected with poliovirus whose proteases cleave eIF4G (Johannes and Sarnow, 1998), and 4) application of inhibitors such as LY294002 and wartmannin that reduce the cellular cap-dependent translation in cell culture. LY294002 and wartmannin are inhibitors of phosphatidylinositol 3 kinase (PI3 kinase). PI3 kinase is involved in a pathway that leads to promotion of cap-dependent translation by inactivating eIF4E binding protein (eIF4E-BP) through phosphorylation. Thus, administration of LY294002 or wartmannin in cells results in downregulation of cap-dependent translation of cellular mRNAs by sequestration of eIF4E (Brunn et al., 1997; Feigenblum and Schneider, 1996; Gingras et al., 1999).

In this study, we assessed the ability of cap-independent translation initiation on DEN RNA in Vero cells using LY294002 and wartmannin. Unlike a control mRNA containing a known IRES, the translation of 5´-capped DEN reporter RNA and infectious viral RNA were strongly debilitated by the drugs as in a reporter mRNA harboring 5´- and 3´- UTR of α-globin mRNA. Consistent with this result, no internal ribosomal loading activity was found in 5´- UTR of DEN RNA. We further investigated the highly conserved 5´ DEN stem loop (DENSL) structure by analyzing multiple deletion mutations. Although the structure was found to contribute to translation to a certain degree, it is dispensable for translation.

Our result of inhibitor assay contrasts with data published recently by Edgil *et al.* (2006), which suggested that DEN genomic RNA could be translated cap-independently in the presence of inhibitors. The incoherent results may be derived from the cell type differences, cell harvesting time, timing for the drug application, and/or other unknown reasons. In this study, we have evaluated these factors mentioned above that could potentially result in discrepancy of the two systems.

3.2 MATERIAL AND METHODS

Plasmid constructions. The bicistronic reporter construct that contains the EMCV sequence (Carter and Sarnow, 2000) was kindly provided by Dr. Peter Sarnow. T7-EMCV IRES sequence was amplified by PCR from the bicistronic reporter plasmid construct and cloned into the *Not* I and *Bgl* II sites of pDCLD reporter plasmid (Chiu et al., 2005), which contains DEN 5´-UTR, DEN Capsid coding region, Luciferase and DEN 3´-UTR. The resulting plasmid was named pEMCV-CLD. pEMCV-CLGpA was made by replacing the portion between *Not* I and *Pst* I with that from pEMCV-CLD.

Dengue infectious cDNA (pD2/IC-30P-A) (Kinney et al., 1997) was a kind gift from Dr. Richard Kinney. For the construction of 5´-deletion variants of pDCLD, PCR with mutagenic primers was performed to synthesize T7-5´-deletion fragments (Δ51, Δ52, Δ53, Δ33, Δ33-69, Δ18-54, Δ55-69), which were then digested with *Not*I and *Pst*I to clone into pDCLD. DEN 5´ SL (nucleotide 69 of DEN2) was placed in front of the α-globin 5´-UTR to make pDENSL/GCLD by PCR mutagenesis. Thirteen base-pair artificial stem loop (SL) structure (GAAGCTTGGGCCCAGAAATGGGCCCAAGCTTGGATCC) derived from Dr. Daniel Gallie (Gallie et al., 2000) was introduced in front of DEN 5´-UTR and globin 5´-UTR to make pSL-DCLD, pSL-GCLD, and pSL-GCLGpA.

Cell culture. Vero (African green monkey kidney) cells were grown in DMEM (Invitrogen) containing antibiotics and 10% fetal bovine serum (FBS) at 37ºC in an atmosphere containing 5% CO_2, or 5% FBS if inoculated with dengue virus. BHK21 (Baby hamster kidney) cells were kindly provided by Dr. Richard Kinney. BHK cells were grown in MEM-α (Invitrogen) containing antibiotics and 10% FBS with supplement of glutamine, and 5% FBS if incubated with dengue virus.

RNA transcription and transfection of cells. Plasmids containg DEN 3´-UTR were linearized with *SnaB*I. Plasmids with the globin 3´-UTR were linearized by cleavage with *Xba*I or *Acc65*I to generate non-polyA or polyA RNA, respectively. EMCV containing bicistronic reporter construct was linearized at the *BamH*I site. DEN infectious construct was linearized with *Xba*I, which results in generating the exact end in transcripts as in the virus. RNAs were produced by in vitro transcription with T7 RNA polymerase in the presence of trace amounts of [α-^{32}P] CTP, as described previously

(Chiu et al., 2005). RNA quality was analyzed by native 1% agarose gel electrophoresis. A-capped reporter mRNA was made in the same way except for replacing ^{m7}GpppG to ApppG (Ambion) in transcription reaction. Methylated G- and A-capped DEN infectious RNA transcripts were made by replacing ^{m7}GpppG cap with ^{m7}GpppA cap and ApppA cap, respectively (New England Bioab).

In vitro transcribed reporter RNA (1 pmol) was electroporated into Vero cells as described previously (Chiu et al., 2005). Electroporated cells were diluted with DMEM containing 10% FBS, transferred to tissue culture plates and held at 37ºC in an atmosphere containing 5% CO_2. Lipofection experiments were conducted using Lipofectamine 2000 (Invitrogen) in 96-well plate format based on the manufacturer's instructions. Lipofectamine was replaced with the complete media after 60 min. Subsequently, the cells were further for incubated at 37ºC in an atmosphere containing 5% CO_2 until they were harvested 5-hr post lipofection with 50 μl of 1x Passive Lysis Buffer (Promega) per well. Luciferase activity from each lysate was analysed, and normalized by total protein amount as described before (Chiu et al., 2005).

Infectious DEN2 viral RNAs generated from pD2/IC-30P-A were electroporated into cells as described above and then seeded on to 6-well plates. Supernatant was harvested 5-day post electroporation for plaque assay to determine the viral titer. Plaque assay was performed as described previously by Dr. Richard Kinney (Butrapet et al., 2002). In brief, Vero cells in 6-well plates were adsorbed with 200 μL inoculum for 1.5 h at 37°C, followed by the addition of 4 ml of agarose overlay medium containing 1% LE agarose in nutrient medium (0.165% lactalbumin hydrolysate (Difco Laboratories), 0.033% yeast extract (Difco Laboratories), Earle's balanced salt solution, 25 mg of

gentamycin sulfate (Sigma) and 1.0 mg of Fungizone (Sigma) per liter, with 2% of FBS. Vero cells adsorbed with virus with overlay were incubated in 37ºC in an atmosphere containing 5% CO_2 for 6 days. At day 6, second agarose overlays containing 80 mg/mL neutral red (Sigma) were applied. Plaque numbers were counted 24 hr after addition of the second overlay.

LY294002 and wartmannin treatments. LY294002 (Cell Signaling Technology) or Wartmannin (Cell Signaling Technology) was used at indicated final concentrations 60 min prior or post electroporation and remained present until harvest of the cells. Toxicity of LY294002 and Wartmannin to Vero cells during experiments was also monitored by microscopy.

3.3 RESULTS

3.3.1 Effect of LY294002 and Wartmannin in cells electroporated with cap-GLGpA RNA

Initially, the efficacy of LY294002 and Wartmannin in Vero cells was confirmed using a α-globin mRNA reporter construct, cap-GLGpA, which contains Globin 5´-UTR; Luciferase; Globin 3´-UTR; and PolyA tail. (Fig. 3.1a). This reporter mRNA is translated in a cap-dependent manner (Chiu et al., 2005), and used as a positive control for the effect of these inhibitors. Vero cells were treated with the inhibitor 1 hr before electroporation, 1 hr after electroporation, or without any inhibitor. Four μM wartmannin and 40 μM of LY 294002 were used in this experiment. Linear luciferase accumulation rate (as a measurement of translation efficiency) from cap-GLGpA RNA was decreased to 35% or 30% when the treatment of wartmannin began 1 hr after or before electroporation, respectively (Fig. 3.1b). Likewise, cells treated with LY294002 showed a decreased linear accumulation rate from cap-GLGpA to 18% (drug treatment 1 hr

before electroporation) or 17% (1 hr after electroportation) relative to those of cells without inhibitor treatment (Fig. 3.1c). Cells treated either with wartmannin or LY294002 did not show obvious sign of stress during 8 hr time courses. These data showed that at these administered concentrations, LY294002 imposes a stronger effect on cap-dependent translation than wartmannin does in Vero cells; therefore, all experiments shown below were conducted using 40μM LY294002. These results also indicate that the effect of the drug remains similar whether drug is added before or after eletroporation.

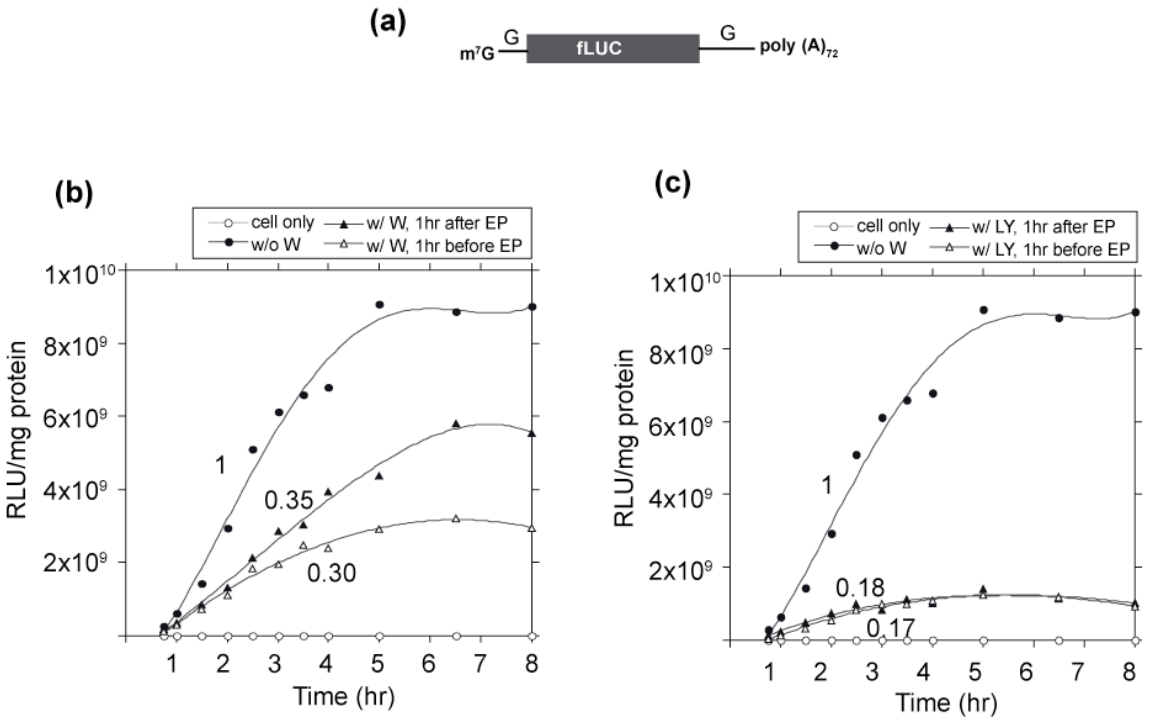

Fig. 3.1 Negative effect of wartmannin or LY294002 on translation of cap-GLGpA RNA (a) Capped GLGpA reporter construct, which contains globin 5´ UTR followed by firefly luciferase (fLUC) reporter gene and globin 3´-UTR with 72-mer polyA tail. (b) Time course of capped GLGpA RNA with the treatment of 4 μM wartmannin (W) 1 hr after electroporation, 1 hr before electroporation, or without any inhibitor in Vero cells. The linear accumulation rate relative to that for untreated capped GLGpA are labeled on graph. (C) Time course of ^{m7}G GLGpA RNA with the treatment of 40 μM LY294002 (LY) 1 hr after electroporation, 1 hr before electroporation, or without any inhibitor in Vero cells. Vero cells elelctroporated with no transcript were also reported as "cell only" for background reference.

3.3.2 Dosage effect of LY294002 using EMCV IRES-containing bicistronic reporter construct

In order to verify that LY294002 only affects cap-dependent translation in Vero cells, IRES-driven translation was examined under drug administration in a bicistronic reporter mRNA construct. The bicistronic mRNA contains a highly structured portion derived from a non-functional EMCV IRES (indicated as ΔE in Fig. 3.2a) in front of the functional EMCV IRES to prevent ribosome reinitiation from occuring (Carter and Sarnow, 2000; Wilson et al., 2000). Expression of the first gene, renilla luciferase reporter (rLUC) represents the activity of cap-dependent translation, while expression of the second gene, firefly luciferase (fLUC) represents the activity of cap-independent translation. Fig. 3.2b shows the expression of rLUC and fLUC from the bicistronic reporter mRNA when cells were treated with 40μM of LY294002 or mock treatment. As expected from the results in Fig. 3.1, the linear accumulation rate of rLUC was reduced (to 50%) upon LY294002 treatment, while that of fLUC remained unaffected. While the effect on the cap-dependent translation by the drug was relatively smaller than on GLGpA (Fig. 3.1c) for unknown reasons, the clear differential effect of the drug on cap-dependent and IRES-dependent translation initiation successfully shows that LY294002 specifically inhibits cap-dependent translation.

Detailed dosage effect of LY294002 (10 to 100 μM) on ratio of rLUC to fLUC synthesis was monitored through out the experiments up to 8 hrs post transfection (Fig. 3.2c). As shown above with 40 μM, LY294002 imposes no obvious effect on IRES-driven fLUC expression even up to the highest concentration tested (i.e. 100 μM, not shown). LY294002 concentrations above 20 μM exhibit similar effects on relative

luciferase syntheses throughoutly (Fig. 3.2c); upon the drug treatment, the ratio of rLUC to fLUC expression was reduced to about 3 from 8, which means rLUC expression was reduced to about 50%. Cells treated with LY294002, again, showed no significant stress under the microscope even up to 100 μM during the 8 hr observation period. The experiments in this section indicate that LY294002 can be used effectively to assess cap-independency on translation of an mRNA.

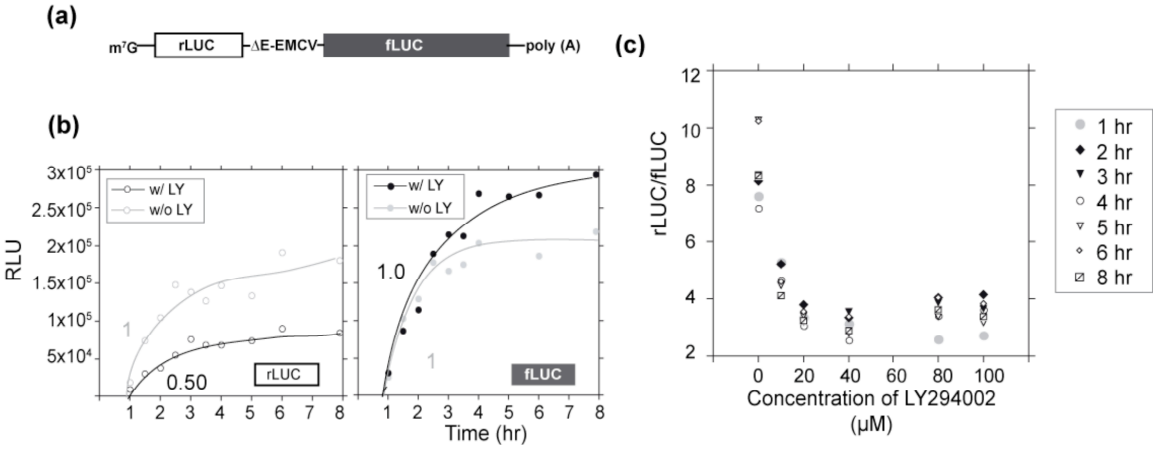

Fig. 3.2 Effect of LY294002 on translation of EMCV IRES containing bicistronic reporter construct

(a) Diagram of EMCV IRES-containing bicistronic reporter construct. The first cistron encodes renilla luciferase (rLUC) followed by a nonfunctional EMCV IRES (ΔE) to prevent ribosome read through. EMCV IRES is placed in front of a second cistron, encoding firefly luciferase (fLUC) that reports the IRES activity. The expression of rLUC and fLUC represents the cap-dependent and cap-independent translation activity, respectively. (b) rLUC (left panel) and fLUC (right panel) expression profile repeating translation of EMCV IRES bicistronic reporter construct with or without the 40 μM LY294002 treatment in Vero cells. Cells treated with 40 μM LY294002 are indicating by in black circles (for rLUC expression) and black dots (for fLUC expression). Experiments without LY294002 treatment are labeled in gray circles and gray dot. Relative light unit (RLU) was reported without protein concentration correction and the two graphs are in the same scale. Relative linear accumulation rate of each expression is also labeled on the graph. (c) Dosage effect of LY294002 in Vero cells using bicistronic reporter constructs. Ratio of rLUC to fLUC was reported at each time point (hr after electroporation) of indicated concentration of LY294002 applied in Vero cell.

3.3.3 Translation of dengue reporter construct, globin reporter construct and EMCV IRES-containing monocistronic reporter constructs in the presence or absence of inhibitor

The effect of LY294002 on EMCV IRES-driven translation was further examined in monocistronic format of the reporter mRNA as in DCLD or GCLG to allow direct comparison. EMCV-CLD, EMCV-CLGpA, and EMCV-CLG nopA transcripts were synthesized with or without 5´-^{m7}G cap and translated in Vero cells in the presence or absence of LY294002. Uncapped reporter mRNAs containing EMCV IRES showed 1.6- to 4-fold higher translatability than the ^{m7}G-capped counterparts (Fig. 3.3a and b), suggesting that two modes of translation initiation are naturally competitive when present in cis. Consistent with these results, capped EMCV IRES-mRNAs were expressed somewhat higher when cap-dependent translation was inhibited by the application of LY294002 (Fig. 3.3a).

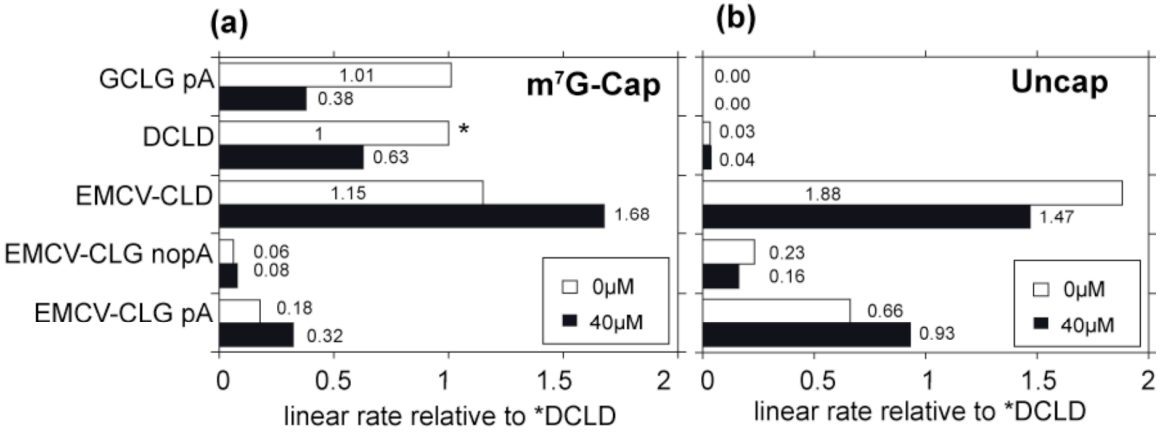

Fig. 3.3 Translation of DEN and globin reporter mRNA are sensitive to LY294002 but the EMCV IRES-containing monocistronic reporter construct is not sensitive

Various constructs with ^{m7}G cap or uncap were tested in Vero cells in the presence or absence of 40μM LY294002. Each construct contains fLUC gene fused with 36 amino acids of capsid sequence. Time course was conducted and linear accumulation rate was measured. Relative linear LUC accumulation rate was plotted using ^{m7}G-capped DCLD as 1.0. (a) ^{m7}G-capped RNA with 40μM LY294002 or without LY294002 treatment in

Vero cells. (b) Uncapped mRNA with 40 μM LY294002 or without treatment in Vero cells.

The contribution of a polyA tail to translation that is directed by EMCV IRES was found to be about 3-fold (Fig. 3.3a, b; ratio of EMCV-CLGpA to EMCV-CLG nopA in both capped and uncapped format). These results recapitulate the effect of polyA on EMCV IRES-dependent translation in cell-free HeLa-cell translation system (Bergamini et al., 2000). Interestingly, DEN 3´-UTR could stimulate EMCV translation to the level even higher than polyA tail whether it is ^{m7}G capped or uncapped. The superiority of DEN 3´-UTR over polyA tail in translation enhancement was observed in cap-dependent translation of a reporter mRNA harboring α-globin 5´-UTR (i.e. GCLD vs GCLGpA) (Chiu et al., 2005).

Unlike EMCV IRES-containing capped reporter constructs whose expression is elevated in the presence of LY294002, capped DEN and globin reporter mRNA were translated to 63% and 38%, respectively, when the inhibitor was applied. Consistently, GCLGpA, a control for cap-dependent translation, was translated at much lower rates when it was uncapped. The DCLD translation effect followed this control mRNA. These results indicate that DEN RNA is translated in a cap-dependent manner.

This conclusion disagrees with the observation of Edgil *et. al* (2006), who reported that DEN RNA could be translated in a novel cap-independent way. In their results, expression of ^{m7}G-capped DEN reporter mRNA was not affected by LY294002 treatment, and A-capped counterparts could take advantage of LY294002 treatment by expressing about 10 times more protein than in cells untreated with LY294002. We suspect that experimental factors, such as 1) we used DEN2 capsid-fLUC fusion reporter construct but they used non-fusion fLUC reporter construct; 2) we used uncap mRNA but

they used ApppA cap to assure the RNA stability; 3) electroporation was used in our system but lipofection was used by them for RNA delivery, may result in differences of the LY294002 treatment observed. Therefore, the differences in the experimental system mentioned above were also tested in our lab to see if we could reproduce their results.

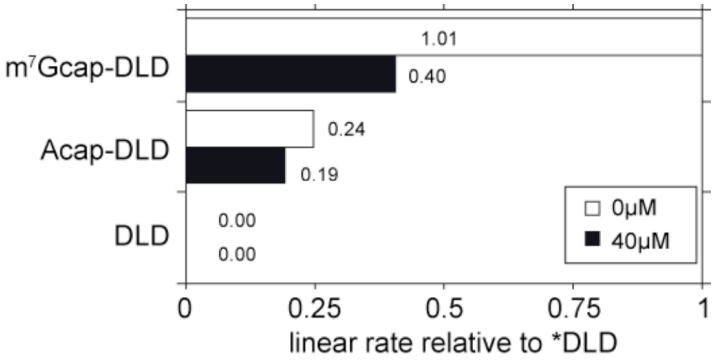

Fig. 3.4 Translation of ^{m7}G capped DEN reporter without capsid sequence are debilitated in the presence of LY294002

^{m7}G-capped, A capped and uncapped DLDs were electroporated and translated in Vero cells with 40 μM LY294002 or without LY294002 treatment and relative linear LUC accumulation rate was reported as bar graph using G cap methylated DLD as reference.

3.3.4 Cap-dependent translation of reporter mRNA translation containing no DEN2 capsid sequences and ApppA capped DEN reporter constructs via lipofection

^{m7}G-capped DLD, A-capped DLD and uncapped DLD, which are devoid of DEN capsid coding region (*c.f.* DCLD), were electroporated into Vero cells in the presence or absence of 40 μM LY294002 (Fig. 3.4) to examine whether cap-independent translation can be observed. Using linear LUC accumulation rates directed by of ^{m7}G-capped DLD as reference, we found that A-capped DLD and uncapped DLD showed translatability of 0.24 and 0.001, respectively, indicating the cap-dependency of these reporter mRNAs. Furthermore, upon the application of LY294002, translatability of ^{m7}G-capped DLD and A-capped DLD reduced to 40% (from 1 to 0.4) and to 80% (from 0.24 to 0.19), again

confirming the cap-dependency of DEN RNA and the specificity of the inhibitor to capped RNA. Further, the same conclusion was drawn when the same set of experiments was also conducted using lipofection as a means of mRNA delivery to Vero cells (Fig. 3.5a, b). In this experiment, ^{m7}G capped, A-capped and uncapped GLGpA were also used as control to assure LY294002 efficacy when using lipofection. We found that ^{m7}G-capped DLD reduced translatability to 17% (from 1 to 0.17), while expression from A capped DLD and uncapped DLD were not inhibited, but rather increased about 1.18 fold (from 0.19 to 0.22) and 1.5 fold (from 0.02 to 0.03), respectively, when 40 μM of LY294002 was applied (Fig. 3.5a). As a control, ^{m7}G-capped GLGpA was reduced to 30 % (from 1.68 to 0.51) in the presence of LY294002 while the translatability of A-capped GLGpA and uncapped GLGpA translation remained at very low levels even when LY294002 was applied (Fig. 3.5b). Again, these data failed to confirm the observation that dengue reporter mRNA is capable of cap-independent translation in Vero cells as reported (Edgil et al., 2006).

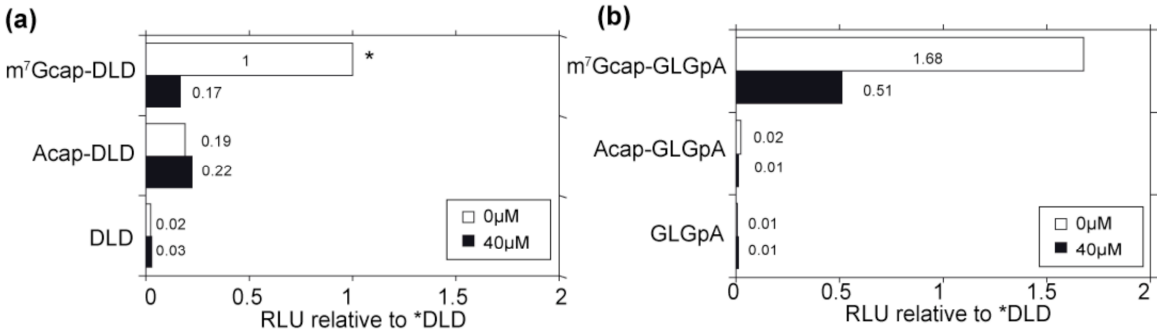

Fig. 3.5 Lipofection as a means of delivering reporter mRNA confirms the cap-dependency of DEN RNA translation

^{m7}G-capped, A capped and uncapped (a) DLD and (b) GLGpA were lipofected in Vero cells with 40 μM LY294002 or without LY294002 treatment. Samples were harvested at 5-hr post lipofection and luciferase activity was measured. Relative RLU is reported using ^{m7}G-capped DLD as reference (*).

3.3.5 Debilitated replication of uncapped and A-capped DEN infectious viral RNAs under LY294002 treatment in BHK21 cells

Unable to reproduce the cap-independent translation of reporter DEN RNA, we next examined the observation that the A-capped form of genome DEN RNA could produce comparable amount of viral progeny to ^{m7}G-capped infectious viral RNA when LY294002 and wartmannin were applied to BHK21 cells. The infectious RNA construct and the BHK cell line used in this experiment were the same as those in Edgil *et al.* (2006).

^{m7}G-capped, A capped and uncapped dengue genomic RNA made from the same DNA clone were electroporated into BHK21 cells and progeny were harvested for plaque assay. Clearly, titer of virus derived from ^{m7}G-capped DEN infectious RNA reduced 6 logs-fold when cell was treated with LY294002 (from $2.5x10^{10}$ to $2x10^{4}$ PFU/mL), and similar trend was also observed for A capped genomic RNA, which was reduced about 5 logs (from $9.5x10^{8}$ to $1x10^{4}$ PFU/mL) and uncapped genomic RNA, which was reduced about 2.5 and 4 logs (from $2.5x10^{5}$ to 10 PFU/mL) in BHK21 cells (Fig. 3.6b). These data clearly showed that progeny production of A capped and uncapped genomic RNA were unable to recover to a comparable level of ^{m7}G-capped infectious RNA. Again, these data failed to confirm the possibility of cap-independent translation using full length dengue genomic RNA.

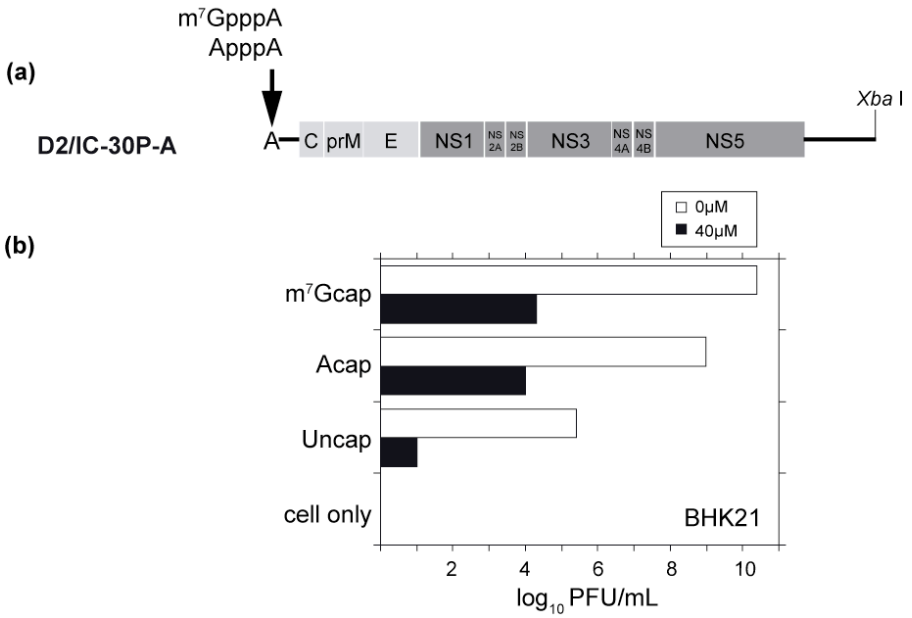

Fig. 3.6 DEN replication is abrogated by LY294002 treatment
(a) Diagram of dengue infectious viral RNA construct, D2/IC-30P-A, containing structural gene, which includes capsid (C), pre-membrane (prM) and envelope (E) genes, and nonstructural (NS) genes, which includes NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 genes. ^{m7}G-capped, A capped and uncapped genomic RNA was prepared by using T7 polymerase and electroporated into BHK21 cells treated with or without 40 μM LY294002. Progeny of infectious viral RNAs were harvest at 5-day post electroporation and plaque assay was performed. (b)Virus titers measured from those infectious viral RNAs electroporated BHK21 cells in unit of $\log_{10}$ PFU/mL.

3.3.6 Internal ribosomal loading in dengue reporter construct and Dengue 5´ stem loop structure may be slightly inhibitory

In order to further confirm the cap-dependency of DEN RNA, a stable 13 base-pair stem loop (SL; predicted free energy of –26.9 kcal/mol) was placed in front of DCLD reporter construct to see whether ribosome has ability to bypass the stem loop structure and direct loading internally. The 13 base-pair stem loop structure designed by Dr. Daniel Gallie has been found to be able to inhibit ribosome loading to the very 5´ end of a mRNA (Gallie et al., 2000). We found that placing 13 base-pair SL in front of DCLD resulted in reducing expression to 11.7% (SL-DCLD), and similar result was observed with SL-GCLGpA (reduced to 10.6%). Similarly, SL-GCLD also showed reduced translatability to 17% comparing to GCLD (from 1.54 to 0.26). All these data suggested to us that the 13 bp SL structure could block the ribosomal entry to DEN reported constructs and globin reporter construct and there is no internal ribosomal loading site in the DEN 5´ UTR.

Interestingly, a stem loop structure with predicted free energy of -20.9 kcal/mol is present at 5´-terminus of DEN RNA. We examined if this 5´-DEN stem loop structure (DENSL) is inhibitory for 5´-terminal ribosome loading like the 13 bp SL is. The 5´-DENSL was placed in front of the globin reporter construct. Diagrams of each construct used in this study are shown in Fig. 3.7a and all the mRNAs used in this experiment were ^{m7}G-capped. All the constructs were subjected to mfold RNA folding prediction (Zuker, 2003) to assure the 5´-structure elements remain unaltered with the addition of the stem loop structure. Addition of DENSL in front of GCLD (DENSL-GCLD) resulted in 47%

reduction of translatability (0.72 of DENSL-GCLD to 1.54 of GCLD), suggesting a relatively minor negative effect of DENSL structure in translation.

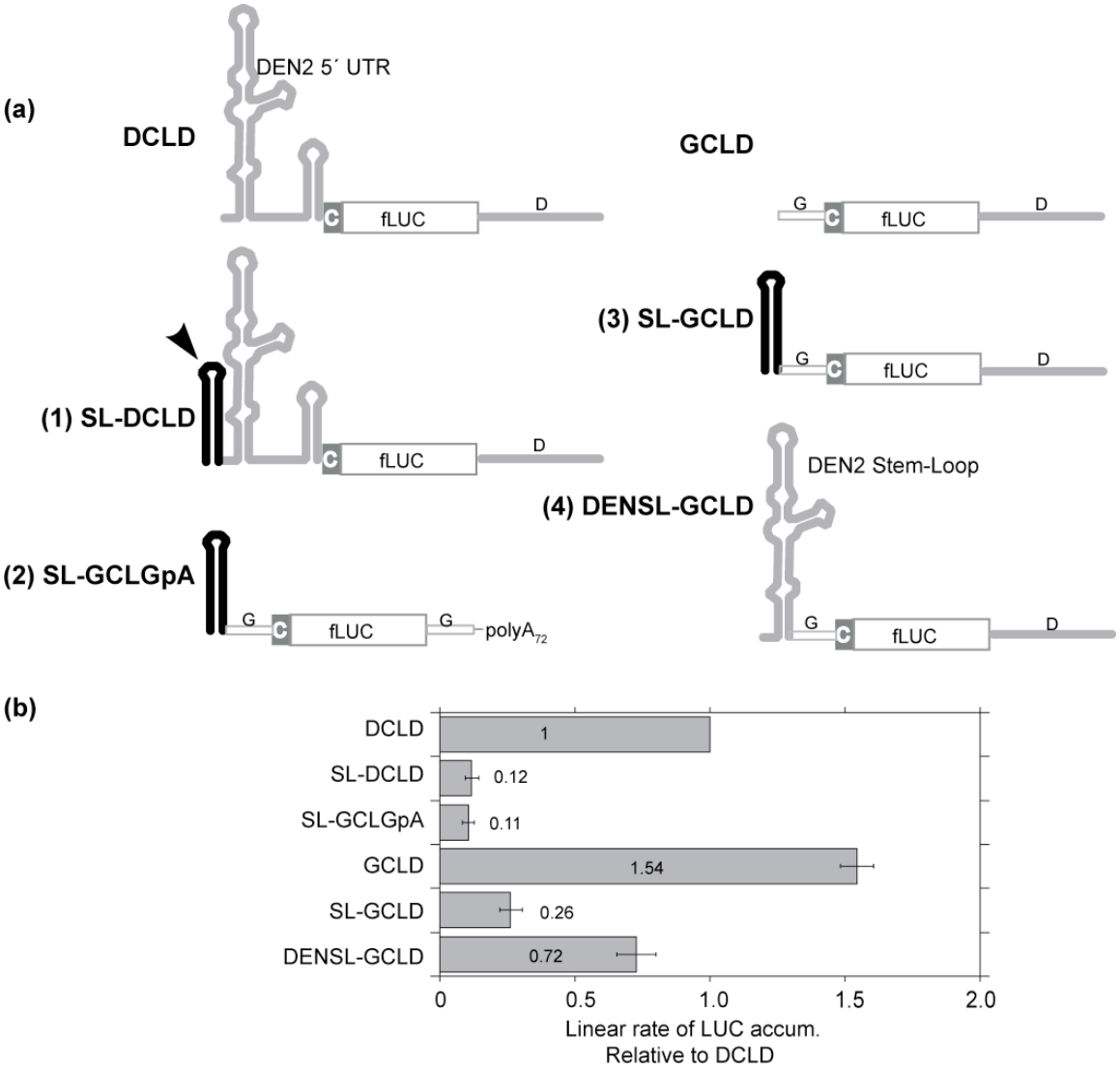

Fig. 3.7 Translation of DEN reporter is sensitive to the addition of 13bp artificial stem loop structure at the 5´-terminus

(a) Diagram of constructs with addition of 13 base-pair stem loop (SL) structure at very 5´-end of the mRNA, such as (1) SL-DCLD (2) SL-GCLGpA and (3) SL-GCLD and constructs with additional DENSL at its 5´-end, such as (4) DENSL-GCLD. DCLD and GCLD are also shown at the top of the panel. (b) Capped mRNAs were electroporated to Vero cells and samples were harvested at specified time points. Linear luciferase accumulation rate was measured and bar graph was plotted using ^{m7}G-capped DCLD as reference.

3.3.7 Dispensability of Dengue 5´ SL in DEN RNA

To determine whether DENSL has important role in DEN RNA translation, 5´-DENSL in the DEN 5´-UTR was removed and replaced with the globin 5´-UTR sequence (construct DENSL/G-DCLD). For the same purpose, serial deletions of DENSL structure in DCLD were made to map the putative elements in DENSL that may be crucial for DEN RNA translation. Diagram of deletion series is shown in Fig. 3.8a. Structure predictions of these 5´ deletion constructs was done by mfold and most of the deletion constructs have very little structure or no structure except Δ 53, which has the side stem loop removed. All the mRNAs used in this study were capped. We found that DENSL/G-DCLD had no significant change in translation comparing to DCLD suggesting that DENSL in the DEN 5´-UTR may be replaceable with other sequences (Fig. 3.8b). Deleting of base stem of DENSL (Δ51), top stem loop of DENSL (Δ52) and side stem loop of DENSL (Δ53) resulted in 50% translatability of DCLD. Deleting first half of DENSL (Δ2-33) has no impact in translation, but deleting the second half of DENSL (Δ33-69) reduced translation to 36% compared to DCLD. Deletion of nucleotides 18 to 54, including the top stem loop, upper two internal bulges and side stem loop, of DENSL resulted in 60% of translatability comparing to DCLD. Deleting nucleotides 55 to 69 of DENSL resulted in 33% translatability of DCLD. In this experiment, most of the deletion constructs retained 30-60% of translation ability of DCLD except Δ2-33, which did not affect translation at all. These data indicated that DENSL is dispensable for dengue reporter translation, yet each element in DENSL structure may still play a role in regulating

translation. These results are consistent with our idea of the conventional mode of translation initiation in DEN RNA.

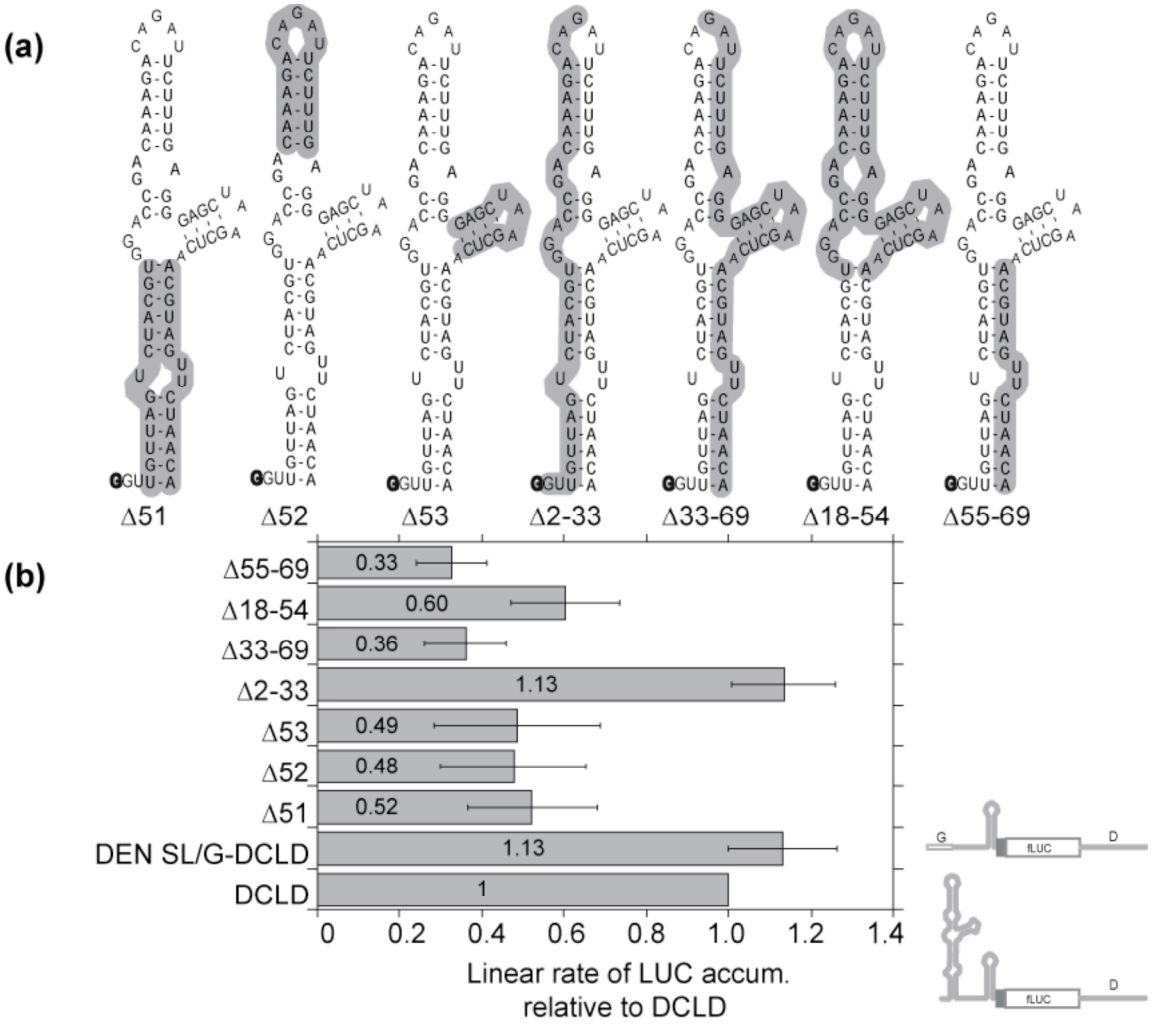

Fig. 3.8 Deletion study of DEN 5´-stem loop structure reveals its minor contribution to DEN RNA translation

(a) Diagram of 5´-DENSL deletion constructs in DCLD. Deleted sequences were highlighted with gray color. Nucleotide A at very 5´ end of viral genome was replaced with G in reporter constructs and this 5´-most G is outlined in the diagram. Another construct used in this study was DENSL/G-DCLD, which has globin 5´ UTR sequence in the place of DENSL structure to test the dispensability of DENSL structure in translation (diagram shown in b panel next to the bar graph). (b) Translation of 5´-DENSL deletion series using DCLD (wild type) as reference was monitored in Vero cells. Samples were taken at indicated time points and relative linear rate of LUC accumulation was measured. RNAs used in this study were all ^{m7}G capped.

3.4 DISCUSSION

In this study, the previously reported cap-independent mode of DEN translation (Edgil et al., 2006) was explored in both Vero and BHK cells with inclusion of two key control RNAs: EMCV and α-globin luciferase reporter mRNA, which serves as positive and negative control for cap-independent translation, respectively. Application of LY294002 to Vero cells exclusively inhibits cap-dependent translation of α-globin reporter mRNA (Fig. 3.1b, c and 3.3a) without down regulating EMCV IRES-directed translation from bicistronic mRNA (Fig. 3.2b). DEN reporter mRNA harboring the complete 5´- and 3´-UTR, was found to mirror the LY294002 effect on α-globin reporter mRNA (Fig. 3.3a, 3.4 and 3.5a), suggesting an absence of cap-independent translation mode in DEN RNA. In further supporting this conclusion, the host cellular translation activity was not affected by DEN replication (Edgil et al., 2006; Westaway, 1973), unlike Poliovirus, which shuts down host translation by cleaving eIF4G (Barco et al., 2000; Bushell et al., 2000).

Interestingly, translation of monocistronic fLUC mRNA under the control of EMCV IRES was found to be slightly less active when ^{m7}G-cap is present than when it is absent from the RNA (Fig. 3.3a vs 3.b). This can be explained by the potential competition between 5´-cap and IRES-driven translation initiation (Attal et al., 2000; Hambidge and Sarnow, 1991). Consistent with this finding, capped EMCV IRES reporter translation upon LY294002 application was better than untreated, suggesting that the inhibition of cap-dependent translation by the inhibitor could reduce the competition of

cap and IRES driven translation. We did not confirm this inhibitor-dependent increase in DEN RNA translation (Fig. 3.3a). The cap-dependent translation of DEN RNA is further supported by significantly diminished translation of uncapped DEN RNA (Fig. 3.3b and (Chiu et al., 2005)). Furthermore, by placing artificial introduced stem loop structure in front of DEN 5´ UTR, we found no potential internal ribosome loading activity of DEN translation and this finding is confirmed by the bicistronic reporter data of Edgil *et. al.*(2006).

These data clearly contrast to the finding of Edgil *et al.*, who reported that are equivalent DEN reporter construct is insensitive to inhibitor treatment, and ApppN capped DEN reporter treated with inhibitor could be translated as well as the ^{m7}G-capped DEN reporter construct. It is unclear to us what may cause the difference between the two systems because our attempt to reproduce the data of Edgil *el al.*(2006) using the same condition to the best of our knowledge failed. The experimental systems employed by Edgil *el al.*(2006) that are different from ours include: use of DLD vs DCLD and lipofection vs electroporation for RNA delivery. These differences were tested and found to be negative for the source of the difference (Fig. 3.4 and 3.5).

We further found that DEN progeny accumulation in BHK cells is sensitive to LY294002 regardless of the cap structure at the very 5´end of the viral genome (Fig. 6), and again this finding does not support results of Edgil *et. al.* (2006) that A-capped DEN RNA replicates about ten-fold higher upon LY294002 application. In our data, it is still unknown why replication of A-capped or uncapped DEN RNA were affected by the drug when the translation of respective counterparts of reporter mRNA were not (Fig. 3.3b). Successful virus replication is a result of complex interaction of host and viral proteins,

such as proteases that process DEN polyprotein into functional mature peptides. We suspect that host proteins that are necessary for DEN replication were affected by the inhibitor, by so doing, replication of DEN was debilitated significantly. In addition, the treatment period of the inhibitor was longer (5 days) in virus replication experiment than in reporter translation study (usually up to 8 hrs). Despite the usage of the same concentration of the drug as Edgil *el al*. (2006), 40 μM may be too much for longer incubation. The use of other viruses containing IRES as a positive control for the experiment should be considered in the future. However, the limitation of these positive control viruses certainly exists as the host factor requirement of these viruses is likely different from that of DEN.

DEN RNA certainly contains a ^{7m}GpppN cap at the 5´-terminus. In further support, the RNA triphosphatase activity of NS3 protein has been described in DEN and WNV (Benarroch et al., 2004; Wengler and Wengler, 1993) and a typical methyltransferase core, GTP binding site and methyltransferase activity could also be found in NS5 of DEN (Egloff et al., 2002). There is no doubt that the capping functions are important for its life cycle.

Despite the clear contrast in behavior of DEN RNA translation from cap-independent translation, such as supported by the EMCV IRES, it is interesting to find that translation of DEN is slightly more resistant to LY294002 than globin reporter mRNA (Fig. 3.3a). In addition, we found that 5´-DENSL structure with free energy of -20.9 kcal/mole is less inhibitory than 13 bp stem loop structure if placed in front of globin 5´-UTR. Based on Kozak's finding, hairpin structure with free energy of -30 kcal/more or more would stall 40S ribosome and prevent it from loading and it is most

inhibitory if the structure is located at very 5´ end of the mRNA (Kozak, 1986; Kozak, 1989; Kozak, 1991). Furthermore, our 5´-DENSL deletion assay revealed that the individual elements inside of DENSL structure contribute to translation to some extent (Fig. 3.8). All these results suggest that DEN RNA translation may be different from a truly standard 5´-cap dependent translation as in α-globin mRNA.

DENSL structure is a highly conserved element among *flaviviruses*, and it may also be important for viral replication and other viral function (Brinton and Dispoto, 1988). Previous deletion study of 5´stem loop structure of DEN type 4 resulted in 40% to 160% of total viral translation and low viral titer production (Cahour et al., 1995) and this supports our finding and also suggests that 5´-UTR may be important for viral replication. A very recent study by Dr. Andrea Gamarnik has revealed that DEN 5´-UTR could interact with NS5 RNA-dependent-RNA polymerase and act as promoter for negative strand synthesis (unpublished data).

Tobacco mosaic virus has 5´cap structure but could be translated well in vitro in a condition when cap analogue or inhibitor was applied (Altmann et al., 1990; Altmann et al., 1997; Hickey et al., 1976). The leader sequence of tobacco mosaic virus called omega has ability to enhance translation (Gallie et al., 1987; Gallie et al., 1988; Sleat et al., 1987; Sleat et al., 1988) and could interact with eIF4F through the binding of heat shock protein 101(Gallie, 2002; Wells et al., 1998). By contrast, DEN translation is certainly sensitive to the application of cap analogue in vitro (Edgil et al., 2006) and application of LY294002 (from this study). However, whether DEN 5´-UTR or other DEN elements has ability to recruit initiation factors directly, or indirectly like those of tobacco mosaic

virus omega enhancer sequence under a certain special condition, needs further investigation.

3.5 ACKNOWLEDGMENTS

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Chapter 4 Using upstream AUG initiation codons as a tool to understand mechanism of translation of dengue RNA

4.1 INTRODUCTION

Dengue virus (DEN), a mosquito-born *Flavivirus* (Wengler and Strauss, 1995), contains a single-stranded positive-sense RNA of 11 kb in length that encodes a single open reading frame. The protein product is further processed into 10 proteins (from N- to C-terminus: C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, where C is capsid protein, prM is pre-membrane protein, E is envelope protein, NS is nonstructural protein). This maturation occurs is done co- and post-translationaly by viral and cellular proteases (Chambers et al., 1990; Wengler and Strauss, 1995). DEN RNA contains a 5´-^{m7}GpppA cap, thus and presumably is translated cap-dependently. Indeed, a strong translation enhancement by the 5´-cap was observed in Vero cell using a firefly luciferase reporter mRNA construct, which contains DEN 5´- and 3´-untranslated region (UTR) ((Chiu et al., 2005), Chapter 2).

During cap-dependent translation, eukaryotic initiation factor 4F (eIF4F) binds to the cap structure and allows the binding of the 40S subunit ribosome through eIF3 to form preinitiation complex 48S (Pestova and Kolupaeva, 2002). The 48S complex scans through the 5´-UTR until it reaches an AUG start site (Dever, 1999; Pestova and Kolupaeva, 2002; Pestova et al., 2001).

The AUG selection by ribosome is strongly affected by 1) the initiation context sequence surrounding the AUG (Kozak, 1987; Kozak, 1989), 2) the length of the leader sequence (Kozak, 1991a; Kozak, 1991b), and 3) relative location of secondary structure to AUG (Kozak, 1986; Kozak, 1990). Kozak's consensus context sequence revealed that GCC (A/G)CCAUGG is the optimal context for initiation of translation in mammalian cells (the A of the AUG codon is designated as +1 position and A or G is preferred in -3

position and a G in position +4). The strong contributions of A at -3 position and G at +4 position have been confirmed in various translation systems (Kozak, 1989). When AUG is located closer to the 5´-end (typically shorter than 20 nucleotides), a ribosome has less chance to recognize the AUG (Kozak, 1991a). A stable secondary structure (-30 kcal/mol or more) at the 5´-end of an mRNA can potentially inhibit AUG recognition by perturbing the initial loading of the ribosome small subunit (Kozak, 1991c). By contrast, a positive effect of a secondary structure can be observed when located at approximately 14 nts downstream of an AUG start codon with a sub-optimized initiation context. It is thought that the structure causes ribosome pausing and create the chance for AUG recognition (Kozak, 1990). Based on Kozak's scanning model, the ribosome typically initiates at the first 5´ AUG codon with a good initiation context (called first AUG rule) (Kozak, 2002). The second (or later) AUG is also accessible by ribosome via leaky scanning or reinitation from the open reading frame generated by first AUG but normally less efficiently (Kozak, 2001; Kozak, 2002).

We have previously found that DEN translation requires ^{7m}GpppN cap under regular translation mode (Chiu et al., 2005) and DEN contains no internal ribosomal entry site (IRES) (Edgil et al., 2003) (Chiu and Dreher., Chapter 3). DEN translation can be enhanced by various elements in the DEN 3´-UTR, which has a synergistic relationship with the 5´-cap, similar to the relationship between the 5´-cap and 3´-polyA tail of cellular mRNAs.

To test wheather scanning mode is used for translation initiation, we introduced an upstream AUG (uAUG) in the DEN 5´-UTR. If ribosomes scan through the 5´-UTR, the uAUG should result in perturbation of translation initiation at the authentic AUG

(aAUG). uAUG and upstream open reading frame (uORF) are commonly seen in regulation of gene expression, such as with GCN4 regulation (Hinnebusch, 1994; Hinnebusch, 1997).

We found that translation from aAUG in Vero cells is reduced upon the introduction of uAUGs but the changes are relatively mild, suggesting that the ribosomal recognition of the introduced uAUGs is potentially leaky. In the course of reaching this conclusion, we found that the use of a luciferase mRNA construct containing DEN capsid coding region complicates the interpretation of uAUG effect on aAUG, because this region harbors another AUG (dAUG; downstream AUG), which also produces a functional luciferase, indistinguishable in the luminometer from the one initiated from aAUG. Therefore, we switched the reporter gene to enhanced green fluorescent protein (EGFP), which is small enough to distinguish the protein products originating from the different AUGs (i.e. uAUG, aAUG and dAUG) in SDS-PAGE. Our results suggest that the scanning mode is used by DEN translation and the translation of uAUG in DEN reporter construct could be leaky.

4.2 MATERIAL AND METHODS

Plasmid constructions. Four uAUGs were independently introduced by single nucleotide substitution or insertion, and named uAUGa to uAUGd from 5´-most location to 3´-location within the DEN 5´-UTR. The variants with improved initiation context of the uAUGs are correspondingly named as uAUG1 to uAUG4. The sequence changes for the uORF variants from the wild type DEN 5´-UTR are shown in Fig. 4.1 and Fig. 4.2. pDCLD containing uAUG, uAUG knockout (uAUGKO) or uAUGs with authentic AUG knockout (aAUGKO, which is the AUG used for the viral polyprotein translation

initiation) were amplified using PCR mutagenesis. PCR fragments were digested with *Not*I and *Pst*I and cloned into pDCLD (Chiu et al., 2005). Likewise, uAUG, uAUGKO and aAUGKO in pDLD, which lacks DEN capsid coding region (Chiu et al., 2005), were constructed using *Not*I and *Pst*I. DEN reporter constructs with EGFP as a reporter gene were constructed by replacing fLUC of pDCLD with EGFP using *Pst* I and *Hind* III sites. A EGFP fragment containing *Pst* I and *Hind* III sites were obtained by PCR using pEGFP (a kind gift from Dr. Gorge Rohrmann) as a template and cloned into pDCLD to make pDCEGFPD, which contains a fusion of DEN2 capsid-EGFP coding region. pDCEGFPD containing uAUG1, 2, 3 or 4 were also constructed by cloning a DCLD *Not*I-*Pst*I fragment containing uAUG into pDCEGFPD, and construction of uAUGs in pDCEGFPD with aAUGKO was done similarly. Two nucleotides "CA" were inserted after +6 location relative to $uA^{+1}UG$ position of uAUG4/DCEGFP and uAUGd/DCEGFP to fuse the uORF with DEN capsid-EGFP reading frame (the resulting constructs are named uAUG4F/DCEGFP and uAUGdF/DCEGFPD, respectively). uAUG4FKO in DCEGFPD is constructed and used as a control for the effect of context mutations and nucleotide insertion in uAUGF constructs. The uAUG was knocked out by the uAUG to AUU (wild type sequence) (Fig. 4.7).

Cell culture. Vero (African green monkey kidney) cells were grown in DMEM (Invitrogen) containing antibiotics and 10% fetal bovine serum (FBS) at 37ºC in an atmosphere containing 5% CO_2.

RNA transcription and transfection of cells. Plasmids used in this study were all linearized with *SnaB*I before in vitro transcription with T7 RNA polymerase using Megascript T7 kit (Ambion). In vitro trancription reaction was performed in the presence

of trace amounts of [α-^{32}P] CTP to quantify RNA concentration by liquid scintillation counter (Chiu et al., 2005). RNA quality was analyzed by native 1% agarose gel electrophoresis.

In vitro transcribed luciferase reporter RNA (1 pmol) was electroporated into Vero cells as described previously (Chiu et al., 2005). Electroporated cells were diluted with 4 volumes of DMEM containing 10% FBS, transferred to tissue culture plates and held at 37ºC in an atmosphere containing 5% CO_2. Samples were harvested at designated times. Luciferase activity from each lysate was analysed using a luminometer, and normalized by total protein amount as described before (Chiu et al., 2005).

In vitro transcribed EGFP RNAs (1 pmol) were lipofected using Lipofectamine 2000 (Invitrogen) in 6-well plate format based on the manufacturer's instructions. RNA and lipofectamine mix were replaced with the complete mediun after 60 min. Lipofected cells were harvested at designated times same as for electroporated cells (Chiu et al., 2005). Samples were later subjected to the western blot assay.

Western blot assay. Translation products of DEN capsid-EGFP constructs in cell lysates were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose paper (Hybond-ECL) (Amersham) and blotted with monoclonal anti-EGFP antibody (Cell Signaling Technologies) (kindly provided by Dr. Gorge Rohrmann) for visualization of DEN capsid-EGFP fusion protein, and monoclonal anti-actin antibody (kindly provided by Dr. Jeffery Greenwood) for normalization of the EGFP signals. Quantification was conducted by using IQMAC v1.2 (Molecular Dynamics).

Translation using rabbit reticulocye lysate (RRL). Ten μL of RRL (Promega) translation reaction was programmed with the in vitro transcripts based on manufacture instructions. In brief, the reaction contained 7 μL of RRL, 0.2 μL of 1M amino-acid mix without methionine (Promega), [^{35}S]-Methionine (0.86 μM, 43.5 TBq/mmol; Perkin Elmer), and 0.01 pmole RNA. Reaction was than incubated in 30ºC for 90 min. Portion of the reaction was then fractionated on 12% SDS-PAGE and exposed to phosphorimaging plate (Molecular Dymamics, Amersham) overnight. The image was then quantified by using IQMAC v1.2 (Molecular Dynamics).

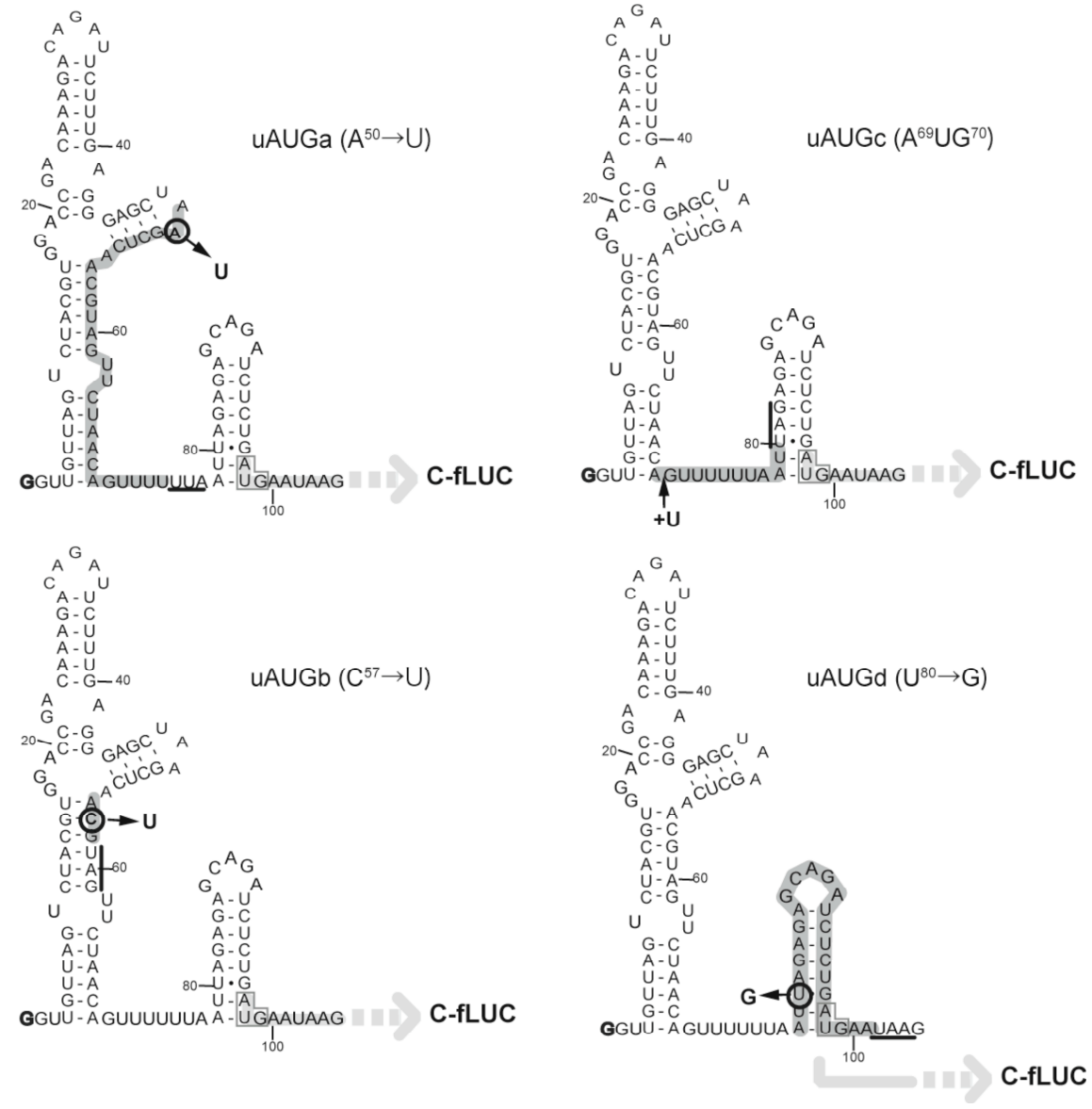
4.3 RESULTS

4.3.1 Complication of interpretation for uAUG effect in translation initiation at aAUG: lack of obvious effect of uAUG introduction in the 5´-UTR of DCLD

Translation of DEN RNA needs ribosome loading at the very 5´ ends (Chapter 3). The importance of a 5´-cap has been shown in reporter mRNA harboring DEN 5´- and 3´-UTR (DCLD, (Chiu et al., 2005)). After loading, ribosomes are likely to scan through the 5´-UTR and locate the first AUG (at 97 nts from the 5´-terminus) (Kozak, 2002). If this hypothesis is true, introduction of an AUG in the DEN 5´ UTR should result in reduction of translation initiation at the authentic AUG at nt 97 (aAUG) in the DEN reporter construct. To test this hypothesis, AUG introduction at four individual locations within the 5´-UTR of DCLD was achieved by a single nucleotide change or insertion in each case. The location of these four upstream AUG (uAUG a through d) codon and the detail of the resulting uORF sequences are shown in Fig. 4.1a. The effect of uAUG introduction can be measured by changes in expression level of the reporter luciferase gene that would be initiated at aAUG. Introduction of uAUGs at position a and b in DCLD had a mild effect on luciferase production: uAUGa/DCLD had 77% translatability

relative to wild type DCLD, and uAUGb/DCLD had 74% translatability of DCLD. Introduction of uAUGd resulted in literally no effect on translation (110% compared to DCLD). Interestingly, uAUGc/DCLD had 33% the translatability of DCLD. As control for mutation of the original sequence, testing that correlates with the creation of an AUG triplet, U of the uAUG was mutated to G to abrogate the translation initiation at uAUG. All uAUG knockout (uAUGKO) constructs that were tested here (position a to c) showed a rebound in luciferase production (80-87% of DCLD), suggesting with the observed effects of uAUG are a result of being recognized by ribosome and limiting the access of translation-initiation competent ribosomes to the downstream AUG.

(a)


uAUGa (A50→U)
U
GGUU-AGUUUUUUA A-UGAAUAAG
100
C-fLUC
uAUGc (A69UG70)
+U
GGUU-AGUUUUUUA A-UGAAUAAG
100
C-fLUC
uAUGb (C57→U)
U
GGUU-AGUUUUUUA A-UGAAUAAG
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100
C-fLUC

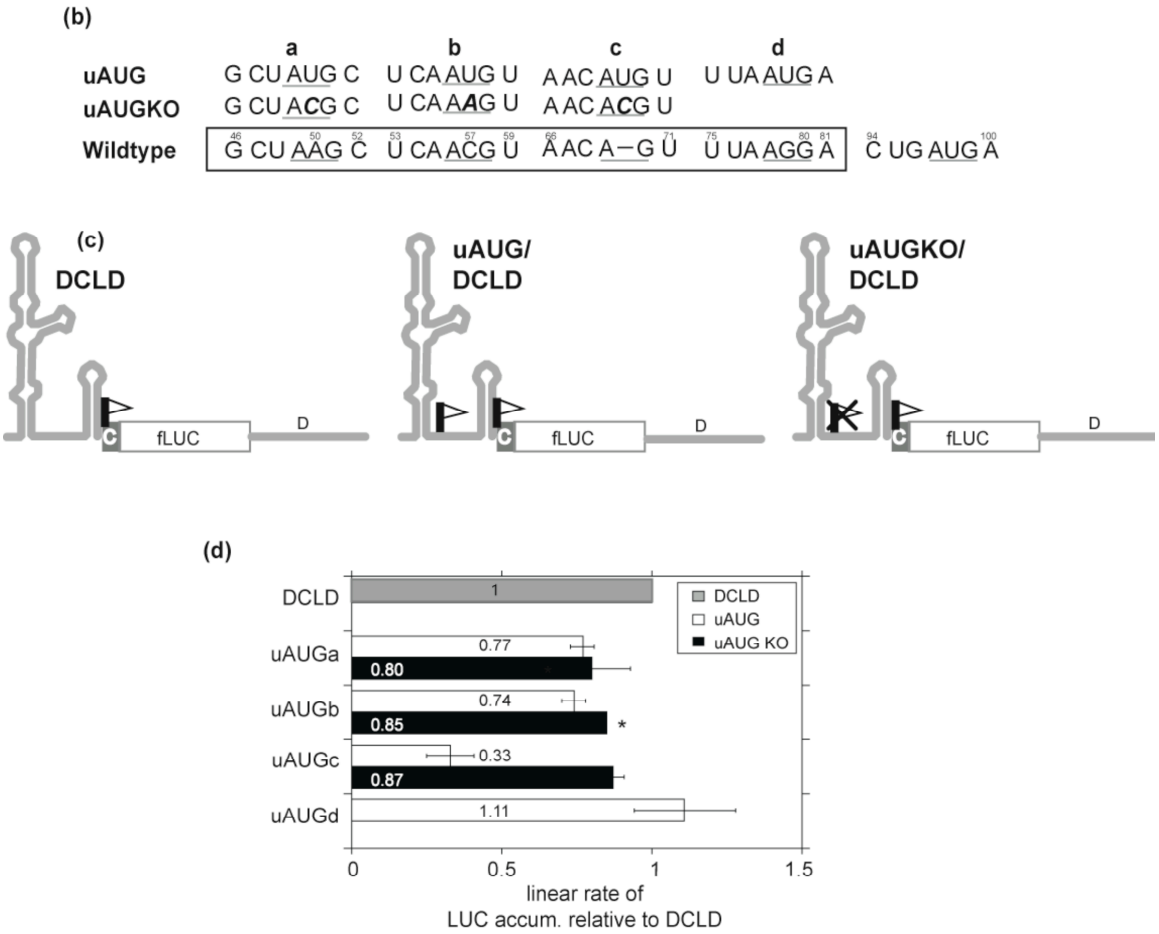

Fig. 4.1 Translation of uAUG-containing DEN reporter constructs

(a) Detailed diagrams of uAUGa to uAUGd in DEN5´-UTR. Circled nucleotides indicate the mutation sites; darker shadows represent the uORFs created by uAUGs; lighter shadows represent the DEN capsid-fLUC fusion translated from authentic AUG site (aAUG) that is boxed with gray line. uAUGb does not create an upstream open reading frame (uORF) due to a stop codon following right after the uAUGb. uAUGd creates an uORF overlapping C-fLUC ORF and ending soon after the aAUG start site. (1) uAUGa : mutation at A50 to U, (2) uAUGb : mutation at C57 to U, (3) uAUGc: insertion an U between A69 and G70, (4) uAUGd : mutation at U80 to G. (b) Wild type, uAUGs and uAUGKO sequences used in this experiment. uAUGKOs were also performed to assure the potential uAUG effect is due to the interference of uAUG introduction but not due to the sequence specificity. Wild type sequences at four different locations are boxed with numbers labeled on them; aAUG sequence is listed at right side of the box. Locations of the AUG triplets are underlined and the knockout nucleotide sequences are in bold and italic. uAUGd KO was not done (*). (c) Cartoon for translation start sites of uORFs constructs. Flags represent the AUG stat sites and the cross over the flag represents the AUG knockout. Wild type DCLD construct would translate from the aAUG; uAUG-containing DCLDs have two potential start sites, one from uAUG and one from aAUG; uAUGKO containing DCLDs would have one potential start sites at uAUG. (d)

Translation efficiency of uAUG-containing DCLD and uAUGKOs containing DCLD compared to DCLD. G methylated capped RNAs were electroportrated into Vero cells and time course over 7 hrs was done. Relative linear rate of LUC accumulation was measured using DCLD as reference and plotted as bar graph. Four individual data were collected for uAUG (a to d) containing DCLDs and SE is plotted on the graph. Experiment for uAUGbKO-containing DCLDs was conducted only once.

Having a rather minor effect of uAUG introduction, we hypothesized that non-optimized context of those uAUG would allows leaky scanning that results in translation initiation at aAUG. Indeed, uAUGc/DCLD, which exhibited the largest effect (Fig. 4.1c,d) has slightly better initiation context of uAUGc than those of other uAUGs. Therefore, we have changed the nucleotide at -3 and/or +4 initiation context positions of each uAUG initiation context in hope of improving the recognition of uAUGs (Fig 4.2a). Theoretically, these minimal changes should lead to better initiation contexts, but not to the optimal context ($GCCR^{-3}CCAUGG^{+4}$, R=A/G) (Kozak, 1987; Kozak, 1997), which would require more sites to be mutated within DEN 5´-UTR. The variants with improved initiation contexts are numbered 1 through 4, corresponding to the a through d positions of the uAUG. The initiation context improvement appeared to be effective in the ribosomal recognition of uAUGa, b and d, as the measured luciferase activity from the constructs harboring these uAUGs were decreased by 20-24% from the non-improved derivatives (compare Fig 4.1d vs 4.2c). The control experiments with uAUG knockout (KO) to AGG, again, showed rebounding expression of luciferase gene, suggesting that the uAUG introduction is serving to divert ribosome and decrease the protein expression from aAUG. Therefore, these results suggest that ribosomes, which are loaded at the 5´-terminus, scan through the 5´-UTR to reach aAUG. Interestingly, the improvement of

uAUGc by U^{+4} to G appeared to abrogate the uAUG effect for unknown reason (uAUG3, Fig. 4.2c) (compare to figure 4.1c).

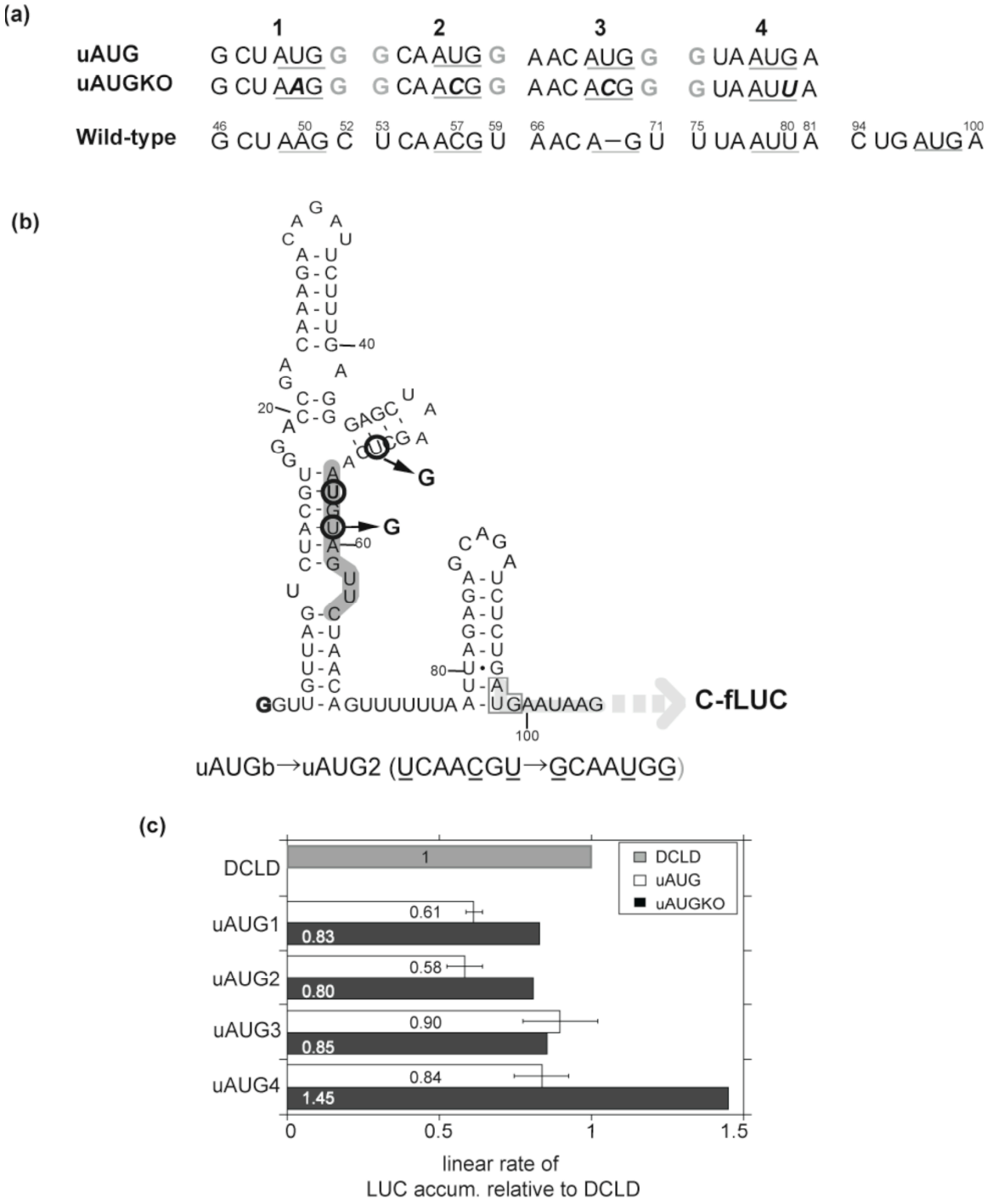

Fig. 4.2 In vivo translation of uAUG-containing DEN reporter constructs with improved initiation contexts

(a) Nucleotide sequences surrounding uAUG, uAUG knockout (uAUGKO) and the corresponding region in the wild type DEN are shown. uAUG1 to 4 contain initiation contexts improved from uAUGa to d. Each position of uAUG1 to 4 corresponds to that of uAUGa to d (see Fig. 4.1a). The improved initiation contexts were designed based on Kozak's rules at position -3 and +4: $R^{-3}CC\underline{A^{+1}UG}G^{+4}$ (R= A or G). As for uAUG4, the initiation context of uAUGd was changed at the -3 position from U to G, and the native A^{+4} was retained. Nucleotides changed for the initiation context improvement are highlighted in lighter shade, while that for AUG knockout are in bold-italic. AUG triplets are underlined; wild type sequences are shown in the box with numbers labeled on them. (b) Diagram shows the uORF created by uAUG2. uAUG1, uAUG3 and uAUG4 in DCLD would create uORFs in identical length as uAUGa, uAUGc and uAUGd in DCLD but uAUG2 in DCLD could create an actual uORF which did not occur in uAUGb in DCLD (see Fig.4.1(a)). Nucleotides changed are circled; the new uORF is highlight with a gray line; aAUG start site is boxed in the graph; lighter shaded box represents the initiation of the C-fLUC ORF. (c) Translation efficiency of uAUG-containing DCLD and uAUGKO containing DCLD comparing to DCLD (labeled in gray). G methylated capped RNAs were electroportated into Vero cells and the time course of LUC expression over 7 hrs was followed. Relative linear rate of LUC accumulation was measured using DCLD as reference and plotted as bar graph. Four individual data points were collected for uAUG-containing DCLDs and SE is reported. Experiment for uAUGKO containing DCLDs shown in this graph was conducted only once (2 data points for single RNA transcript).

4.3.2 Complication of interpretation for uAUG effect in translation initiation at aAUG: potential usage of AUG downstream of the aAUG leading to production of functional luciferase

Despite the observation of uAUG effect, the reduction of luciferase expression was not drastic. Thus, we suspected that AUG at nt 139 (downstream AUG, dAUG) could act as an active translation initiation site to produce a functional luciferase serving to complicate the analysis to underestimate the uAUG effect. dAUG may be recognized by ribosomes that fail to initiate translation at uAUGs and aAUG (leaky scanning ribosomes) or that re-initiate after the termination of uORFs. In order to test that dAUG is recognized by ribosome, we first mutated aAUG to AGG in DCLD. A comparable luciferase expression was detected upon the aAUG knockout (aAUGKO) (Fig. 4.3b). Likewise, uAUG-containing DCLD derivatives with aAUGKO were also tested in this experiment. Again, we observed similar expression levels of luciferase to the

corresponding counterparts that contain aAUG. Fig. 4.4 shows the possible translation initiation of DCLD reporter constructs. It is most likely that translation of DCLD is started from these two AUGs perhaps at varying ratios and luciferase measurement by luminometer is unable to differentiate the protein forms (Fig. 4.4a). Having shown that functional luciferase can be produced in the absence of aAUG, these results suggest a possible drawback of the experimental system using DCLD to measure uAUG effect specifically on aAUG.

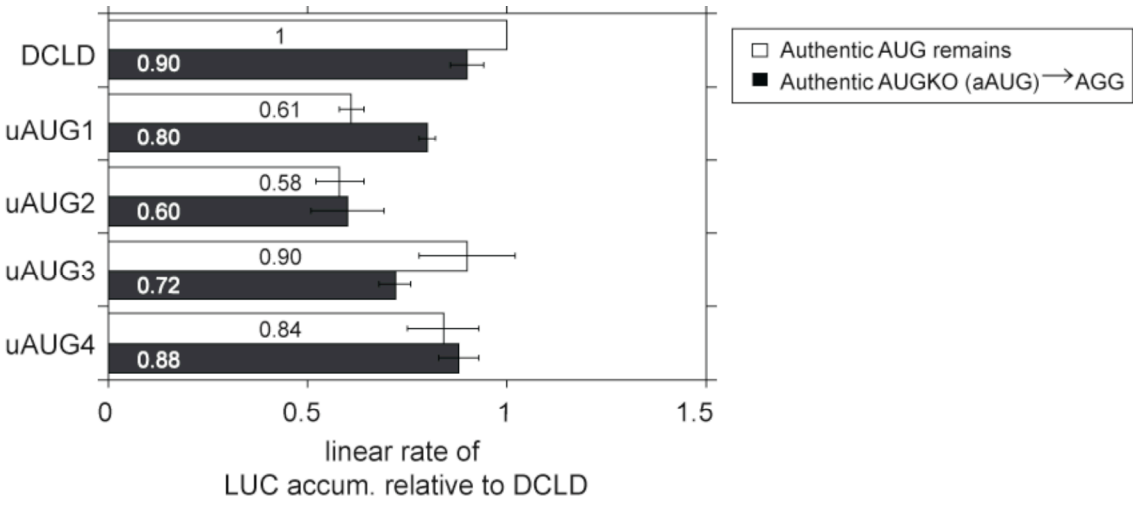

Fig. 4.3 Translation of uAUG-containing DEN reporter constructs with aAUGs mutated (knockout mutants).
Relative linear LUC accumulation rate of reporter constructs containing uAUGs with or without mutation of the AUG codon are reported as bar graph using DCLD wild type as reference.

We could test whether the downstream AUG is used for translation initiation by (1) using DLD mRNA (Chiu et al., 2005), which contains no capsid–coding sequence and dose not contain the second downstream AUG, for evaluating uAUG effects, and (2) fractionating the in vivo translation products from aAUG and dAUG on SDS-PAGE for subsequent western blot detection. Since it is technically difficult to separate the two

firefly luciferase-fusion proteins that only differ by 14 amino acids (the translation products from aAUG vs dAUG), enhanced green fluorescent protein (EGFP), a 26.3 KDa protein, was used as a reporter gene, following the second strategy.

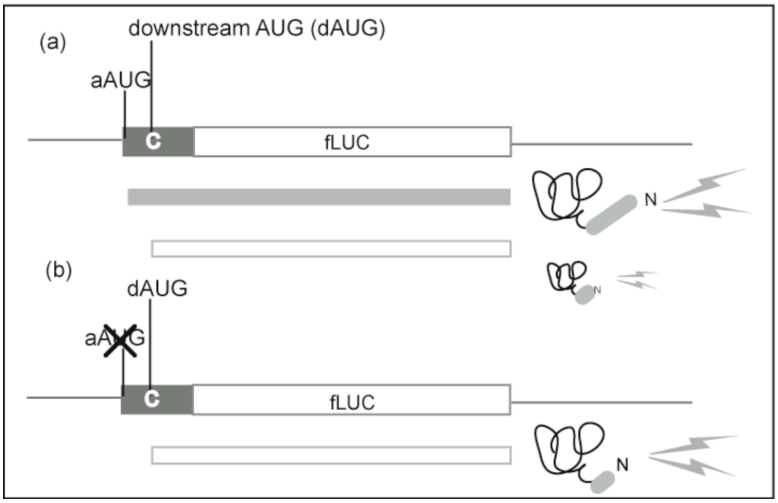

Fig. 4.4 Possible scenarios for LUC expression from DEN capsid-fLUC reporter constructs

4.3.3 uAUG resulted in drastic reduction of translation initiation at aAUG in DLD lacking dAUG

We consistently observed a greater effect of uAUGs in the DLD version of DEN reporter mRNA constructs: uAUG1/DLD showed 31% expression level of DLD, uAUG2/DLD had 15% translatability, uAUG3/DLD had 28% translatability, and uAUG4 had 60% translatability (Fig 4.5b). We also confirmed that aAUG is the main translation start site for luciferase expression in DLD by mutating aAUG to AGG, which resulted in 95% reduction in luciferase production (Fig 4.5b). These data obtained in an experimental format that avoids differential initiation sites provides stronger evidence that ribosomes scan the DEN 5´-UTR. The differences compared to uAUG-containing DCLD constructs are attributable to a certain proportion of ribosomes starting from the downstream AUG (dAUG) in the capsid-coding region and contributing to the luciferase activity. The inhibitory effect of uAUGs clearly could be detected in DLD reporter

constructs. Although greater uAUG effect was observed in DLD than in DCLD variants, DCLD may be a better representative for studying the translation of DEN than DLD, which lacks the capsid coding regions. Indeed, these two reporter mRNAs behaves differently: DLD is about two-fold more translation competent than DCLD (Chiu et al., 2005). From the results reported so far, it is unclear how much LUC expression initiated from uAUG codons and translation from dAUG could be affected by uAUG. Using EGFP constructs, we could address these questions.

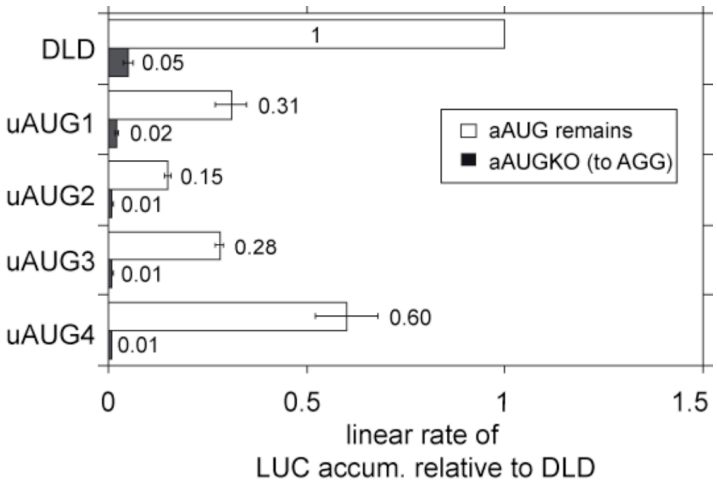

Fig. 4.5 uAUG-containing DEN reporter constructs without DEN-capsid coding region (DLD family of reporters).
Relative linear LUC accumulation rate of reporter constructs containing uAUGs with (aAUG remains) or without (aAUGKO) the authentic initiation site are reported as bar graph using DLD wild type as reference.

4.3.4 Translation termination of uORF before dAUG leading to increased recognition of dAUG in Vero cells

The advantage of using an EGFP reporter construct over a luciferase reporter is that the former allows us to monitor the protein expression from aAUG and dAUG at the same time by Western blot analysis using antibody against EGFP. Because of the better transfection efficiency, lipofection instead of electroporation was used to deliver

DCEGFPD mRNA, an EGFP version of DCLD, to Vero cells. Individual reporter protein expression analyzed by Western blot was averaged from independent triplicate experiments, and actin signal was used for normalization of sample loading, which allows us to compare not just internally (i.e. translation from aAUG/dAUG), but also externally to other lanes.

The pilot experiment to monitor reporter gene expression from DCEGFPD up to 7 hrs post transfection (Fig. 4.6a) showed that the accumulation of protein both aAUG and dAUG reached plateau at around 5 hr (Fig. 4.6b). Thus we used sample harvested at 6 hr post transfection for the rest of the Western analysis. We also verified the migration position in Western blots of EGFP that is produced from aAUG by loss of the respective band for aAUGKO. EGFP initiations at the downstream AUG codon (dAUG) migrated faster and could be resolved in Western blots (Fig. 4.6a). There are no other downstream AUGs capable of initiating the synthesis of similar sized EGFP.

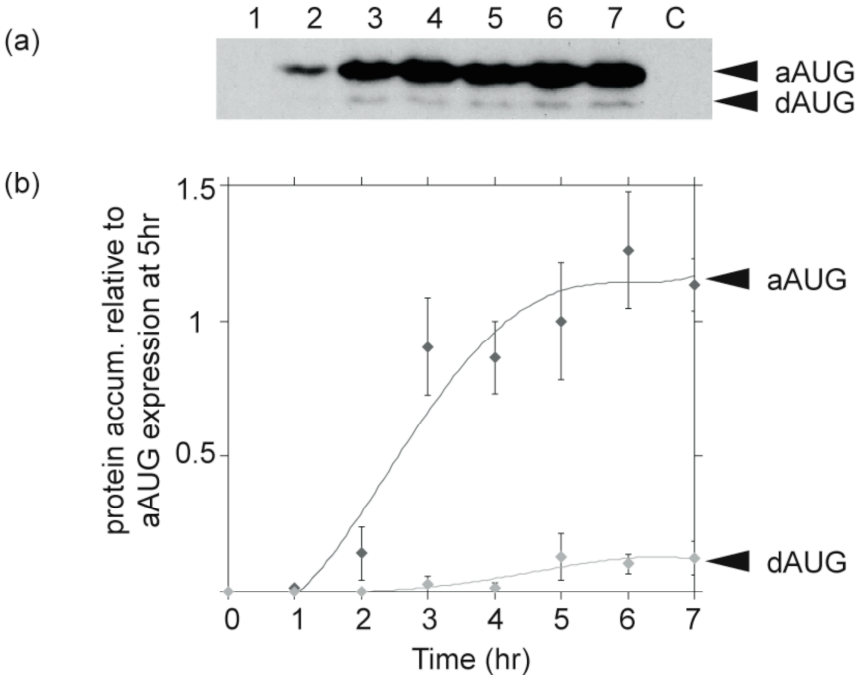

Fig. 4.6 Expression of DEN capsid-EGFP fusion proteins over time from DCEGFPD RNA in Vero cells.
(a) Western blot image of capsid-EGFP fusion expression from 1-hr to 7-hr post lipofection. Lane C is a control experiment with water only lipofected cells harvested at 6-hr post lipofection and with detection using anti-GFP antibodies. Migrations of capsid-EGFP initiation at aAUG and dAUG are indicated. Lanes 1 to 7 represent the samples harvested from 1-hr to 7-hr post lipofection. (b) Quantified data of relative protein accumulation from aAUG and dAUG from DCEGFPD RNA using aAUG expression at hr 5 as reference.

Consistent with the results from the luciferase reporter constructs, the presence of uAUG resulted in a reduction of translation from aAUG (Fig 4.7b), suggesting ribosome scanning in the 5´-UTR. Interestingly, translation from dAUG become more evident after uAUG introduction (i.e. about 4-fold increase; Fig. 4.7b and c). Next, we monitored expression from uAUG4 by inserting the dinucleotide CA to make a construct which uORF4 is in-frame with the EGFP open reading frame. The resulting mRNA construct, uAUG4F showed that uAUG is indeed recognized by ribosomes to produce a higher molecular weight band than that from aAUG (Fig 4.7b, band marked “Fusion”-lane 7, 8). As suspected from previous experiments using luciferase (Fig 4.2, 4.3, and 4.4), the recognition of uAUG is not efficient (i.e., 55% of aAUG in wild type).

The fusion of uORF to EGFP, which eliminates the translation termination before the dAUG, led to a similar level of protein expression from dAUG as in wild type, about 10% of aAUG. This result contrasts to about 4-fold increase in protein production from dAUG in uAUG-containing derivatives (uORF 1-4), strongly suggesting that translation reinitiation scanning occurs at dAUG in those cases.

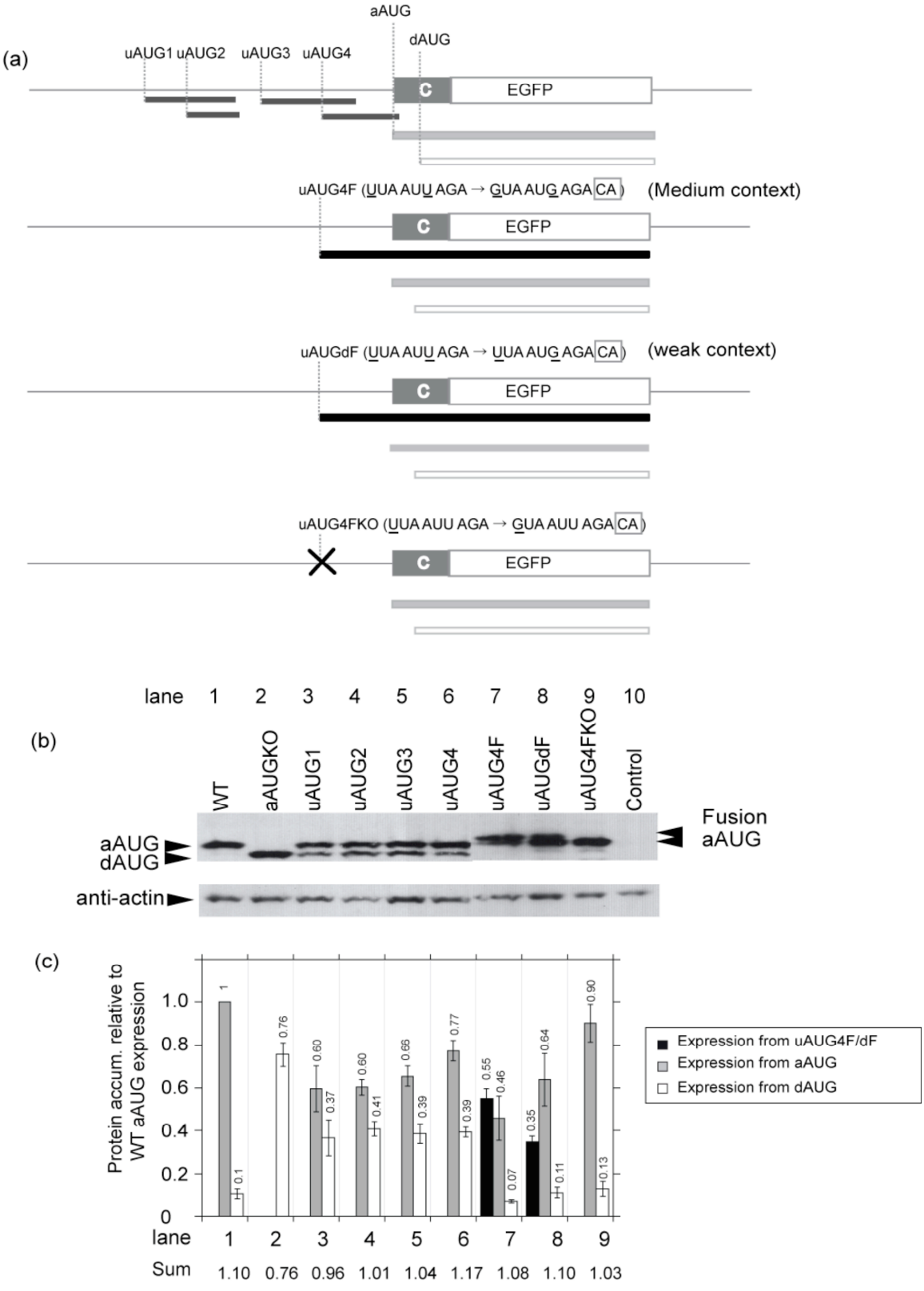
(a)
aAUG
dAUG
uAUG1 uAUG2 uAUG3 uAUG4
C
EGFP
uAUG4F (UUA AUU AGA → GUA AUG AGA CA) (Medium context)
uAUGdF (UUA AUU AGA → UUA AUG AGA CA) (weak context)
uAUG4FKO (UUA AUU AGA → GUA AUU AGA CA)
(b)
lane 1 2 3 4 5 6 7 8 9 10
WT
aAUGKO
uAUG1
uAUG2
uAUG3
uAUG4
uAUG4F
uAUGdF
uAUG4FKO
Control
aAUG
dAUG
Fusion aAUG
anti-actin
(c)
Protein accum. relative to WT aAUG expression
1.0
0.8
0.6
0.4
0.2
0
1 0.1 0.76 0.60 0.37 0.60 0.41 0.66 0.39 0.77 0.39 0.55 0.46 0.07 0.35 0.64 0.11 0.90 0.13
lane 1 2 3 4 5 6 7 8 9
Sum 1.10 0.76 0.96 1.01 1.04 1.17 1.08 1.10 1.03
Expression from uAUG4F/dF
Expression from aAUG
Expression from dAUG

Fig. 4.7 Expression of uAUGs-, uAUG4F-, uAUGdF- and uAUG4FKO-containing DCEGFPD constructs in Vero cells

(a) Diagram of uAUGs, uAUG4F, uAUGdF and uAUG4FKO in DCEGFPD construct with ORFs displayed. (1) Short uORFs expressed from uAUGs are shown as black boxes; DEN capsid-EGFP fusion proteins expressed from aAUG and dAUG are in gray and white strips, respectively. Short uORFs are not detectable by western blot using anti-GFP antibodies. (2) uAUG4F construct expresses a fusion version of uORF/ DEN capsid-EGFP protein that can be monitored by western blot. uAUG4F was constructed by inserting two nucleotides "CA" right after the +6 location relative to the $A^{+1}UG$ codon in the uAUG4-containing DCEGFPD construct. The three forms of protein translated from uAUG4F in DCEGFPD that could be detected by western blot are shown in the diagram. (3) uAUGdF containing DCEGFPD construct expresses the same three proteins as uAUG4F containing DCEGFPD except uAUG4F has better context than uAUGdF containing DCEGFPD. (4) uORF4FKO-containing DCEGFPD has uAUG4 revert back to wild-type sequence for understanding the possible effects of CA insertion and context mutation (U to G). We should only be able to detect two proteins expressed from this construct, as shown in the diagrams. (b) Western blot detection with anti-GFP antibodies of protein expression from uAUGs-, uAUG4F-, uAUGdF- and uAUG4FKO-containing DCEGFPD constructs in Vero cells. Actin signals are also shown at the same time to serve as internal control for sample loading in each lane. (c) Quantified data were summarized in bar graph using the amount of protein accumulated from aAUG of DCEGFPD as reference.

We found that EGFP expression from uAUGdF (original initiation context) is less than uAUG4F (improved initiation context) (Fig. 4.7b; lane 4.7, 4.8). Correspondingly, the expression from aAUG was lower in uAUG4F construct than uAUGdF. When uAUG4F is knocked out at AUG site in DCEGFPD (Fig. 4.7b, c; lane 9) we could clearly see the translation of aAUG revert to about 90% of wild-type expression, consistent with the observation that expression reduction for uORF4d or uORF4 constructs is genuinely due to the presence of an uAUG. DCEGFPD with aAUGKO was also shown in Fig. 4.7(b) lane 2 with about 76% translatability of DCEGFPD showing that dAUG could be used for translation initiation when aAUG is knocked out. The total expression of all forms of EGFP from each RNA construct is summarized at the bottom of the graph in Fig. 4.7c. It seems that total protein expression from each of the variant RNAs tested is

rather constant (Fig. 4.7c, row labeled “Sum”). Effects of uAUGs in aAUGKO DCEGFPD (authentic AUG knock-out) were also tested (Fig. 4.8b). By comparing to DCEGFPD-aAUGKO, uAUG1-containing DCEGFPD reduced translation to 55%, uAUG2- and uAUG3-containing DCEGFPD reduced translation to 68%, and uAUG4-containing DCEGFPD reduced translation to 82%. These results mirror the effect of uAUG in aAUG recognition seen in Fig 7b, lane 3-6. Importantly, protein expression from dAUG was increased by 19-fold upon aAUG knockout (Fig 4.8; lane 1, 2; 0.76 vs 0.04). These results further support the ribosome-scanning model of searching for a translation initiation site in DEN RNA.

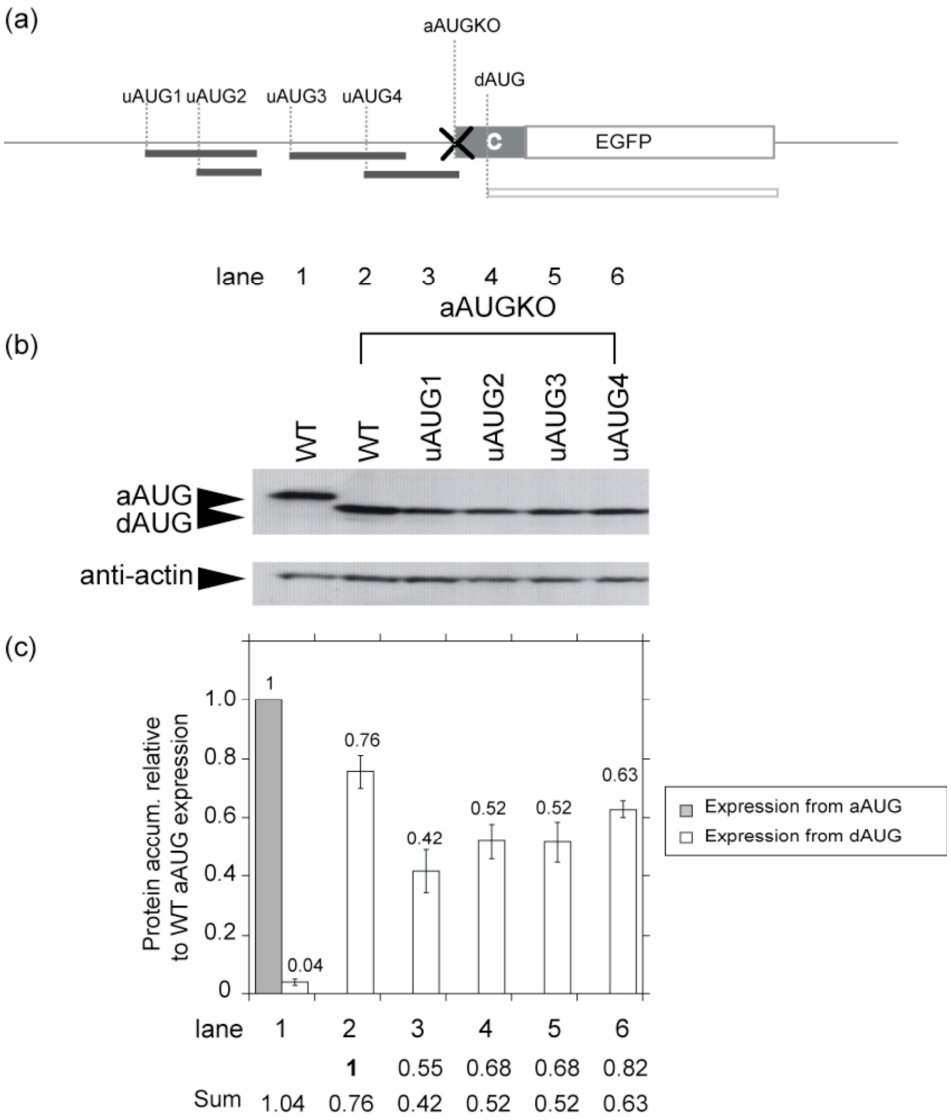

Fig. 4.8 Expression of uAUG-containing DCEGFPD in the absense of a functional aAUG (aAUGKO) in Vero cells.

(a) Diagram of ORFs expressed from uAUG-containing DCEGFPD-aAUGKO constructs. Short uORFs produced from uAUGs cannot be detected by EGFP antibody and only EGFP initiation from dAUG can be detected. (b) Western blot detection of protein expression with anti-EGFP antibody from uAUG-containing DCEGFPD constructs in Vero cells. Detection of actin was used to assure the relative equal amount of protein loaded in each lane. (c) Quantified data were summarized in bar graph using expression from aAUG of DCEGFPD as reference.

4.3.5 uAUG effect recapitulated in rabbit reticulocyte lysate

The inhibitory effects of the introduced uAUG codon on translation from aAUG were recapitulated in Rabbit reticulocyte lysate (RRL): the EGFP expression from aAUG was reduced to 37 to 60% upon the introduction of uAUG in the 5´-UTR (Fig. 4.9). The recognition of uAUG in the DEN 5´-UTR was confirmed in RRL with mRNA constructs that have uORF4 in-frame with EGFP (i.e. uAUG4F and uAUGdF) (Fig. 4.9b; lanes 7, 8). Moreover, uAUG4FKO allowed translation from aAUG to recover to the wild type level, suggesting that no side effect in RRL translation were due to either initiation codon context mutation or CA nucleotide insertion in the uAUG4 fusion construct.

By contrast to in vitro translation in Vero cells, translation from dAUG was found to be much more active (Fig. 4.9a, b; lane 1; 70% of aAUG) than in Vero cells (10%; see Fig 4.7b). This result suggests that ribosomes bypass aAUG more frequently in RRL than in Vero cells and continue scanning to dAUG. We also found that when aAUG is knocked out in DCEGFPD, translation from dAUG increased 3.8-fold (from 0.7 to 2.65), supporting the ribosomal scanning to locate AUG in DEN RNA, as seen in Vero cells (see Fig 4.7c and 4.8c). Consistent with these results indicative of scanning ribosomes, all four uAUGs reduced translation of dAUG in DCEGPFD-aAUGKO to 36-47% (Fig. 4.9c), which are very similar to the uAUG effects to aAUG translation in uAUG-containing DCEGFPD (Fig. 4.9b, lanes 3-6, gray bars).

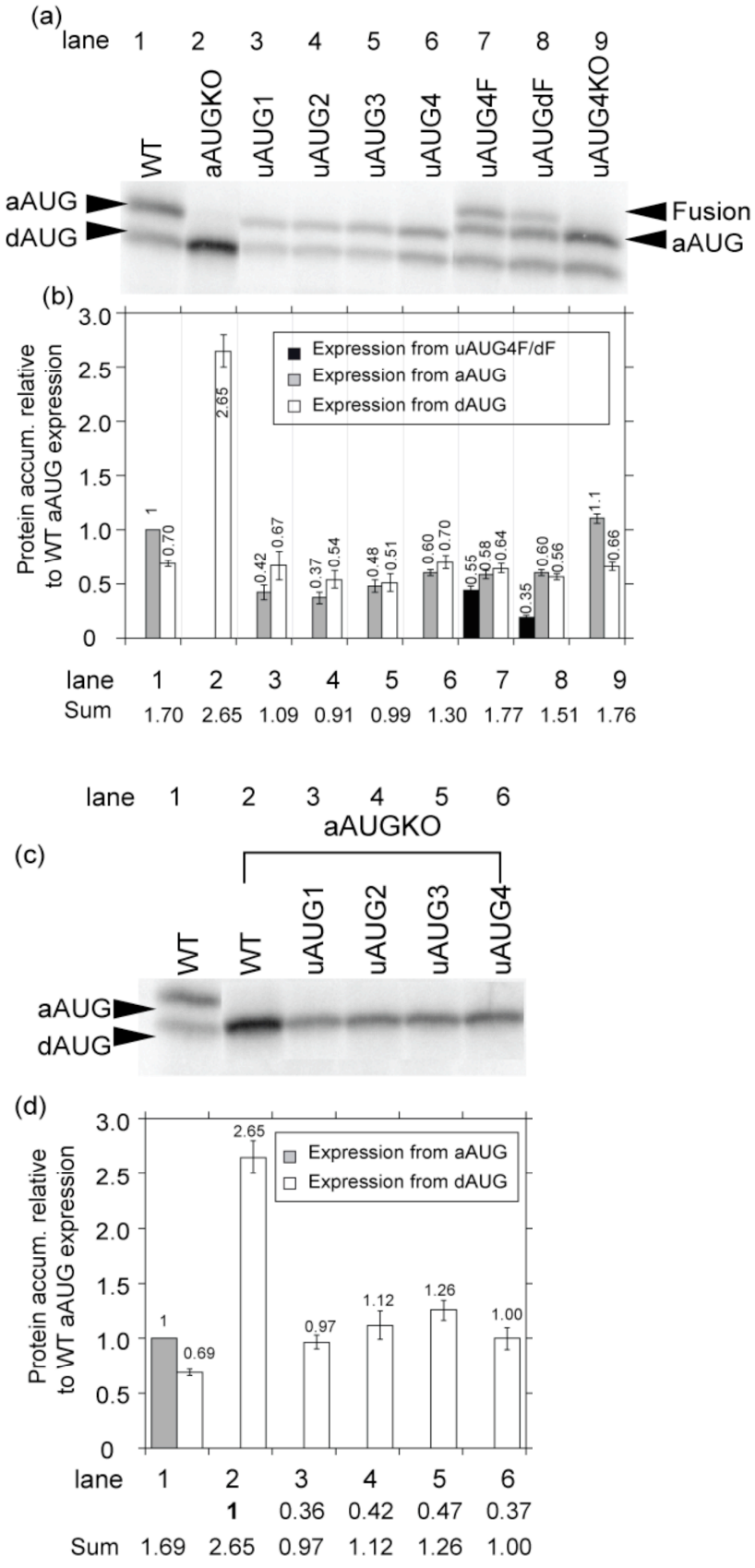
(a)
lane 1 2 3 4 5 6 7 8 9
WT
aAUGKO
uAUG1
uAUG2
uAUG3
uAUG4
uAUG4F
uAUGdF
uAUG4KO
aAUG
dAUG
Fusion
aAUG
(b)
Protein accum. relative to WT aAUG expression
Expression from uAUG4F/dF
Expression from aAUG
Expression from dAUG
lane 1 2 3 4 5 6 7 8 9
Sum 1.70 2.65 1.09 0.91 0.99 1.30 1.77 1.51 1.76
lane 1 2 3 4 5 6
aAUGKO
(c)
WT
WT
uAUG1
uAUG2
uAUG3
uAUG4
aAUG
dAUG
(d)
Protein accum. relative to WT aAUG expression
Expression from aAUG
Expression from dAUG
lane 1 2 3 4 5 6
1 0.36 0.42 0.47 0.37
Sum 1.69 2.65 0.97 1.12 1.26 1.00

Fig. 4.9 Translation of EGFP constructs in rabbit reticulocyte lysate.
(a) and (c) Gel images of RRL *in vitro* translated protein traced with [^{35}S]- methionine. Two gels were shown in (a) and (c), and which are the same set of constructs in Fig. 4.7 and 4.8. (b) and (d) Quantified data were normalized for varying numbers of methionine residues in each protein and relative protein expressions are shown in the bar graph using expression from aAUG of DCEGFPD as reference.

Another aspect of DEN RNA translation in RRL that differs from in vivo translation in Vero cells was the relatively constant translation from dAUG in all uAUG-containing mRNA constructs including uAUG4F and uAUGdF, which do not have translation termination before dAUG (Fig. 4.9). This result suggests that recognition of aAUG by ribosomes in RRL is extremely "leaky" and overrides translation re-initiation at dAUG, which was suggested in uAUG1-4 constructs in Vero cells (Fig. 4.7c).

4.4 DISCUSSION

We previously reported that the 5´-^{m7}GpppG structure is important for the translation (Chiu et al., 2005) and replication competence of DEN RNA (see Chapter 3). While finding the significant role of the 5´-cap in DEN RNA translation, Edgil et al. (2006) reported that translation of DEN reporter mRNA with a 5´-ApppG was up-regulated by 10-fold in BHK cells when an inhibitor for cap-dependent translation was applied. Similarly, DEN genomic RNA with a 5´-ApppG was also replicated upon inhibitor application (Edgil et al., 2006). Although these results could not be reproduced in our careful attempts, even with the inclusion of a positive control mRNA that employs a cap-independent translation (Chapter 3 of this thesis), it is possible that DEN RNA use an unorthodox mode of translation that dose not employ ribosomal scanning through the

5´-UTR after ribosomes are loaded at the 5´-terminus by a cap-dependent mechanism. This possibility of a non-scanning ribosome access to the initiation codon was suggested by the following results from Chapter 3: 1) a 5´-terminal stem-loop structure of DEN RNA is not as inhibitory to translation as a 13 nt base-paired stem-loop structure and this observation may indicate that robosomes could bypass the structure during scanning; 2) deletion analysis on the DEN stem-loop structure suggested that the various parts in the structure contribute to full competency of DEN translation.

In this study, we provide further evidence for the conventional scanning mode for ribosome locating the authentic DEN AUG. The introduction of uAUG at four different locations in the 5´-UTR led to a reduction of translation from aAUG where translation initiation normally occurs in DEN RNA. This observation was made in Vero cells with firefly luciferase reporter gene (Fig. 4.1d, 4.2c, 4.5b,), and with the EGFP reporter gene in vero cells (Fig. 4.7c) and in the RRL in vitro translation system (Fig. 4.9b). We further showed that uAUG is certainly a target for translation initiation by ribosomes, which led to the decreased protein expression from aAUG (Fig. 4.7c and 4.9b; lanes 7, 8). The nucleotide mutation of uAUG to AGG resulted in near wild type level of protein expression from aAUG (Fig 4.7c and 4.9b; lane 9), indicating that the presence of uAUG, but not nucleotide change in the same region, is responsible for the reduction of translation from aAUG. Therefore, we reason that ribosomes scanning in the 5´ to 3´ direction through the 5´-UTR encounter uAUG first and consequently limit the number of ribosomes available to initiate translation at aAUG. The scanning model of DEN RNA is further supported by the elevated translation from dAUG when aAUG is changed to AGG to force ribosomal scanning toward dAUG (Fig. 4.7c; lane 2, Fig. 8c; lanes 2-6,Fig.

4.9b; lane 2, Fig. 4.9d; lanes 2-6). Consistent with the scanning mode, uAUGs also reduced expression from dAUG when aAUG was mutated and the extent of effect is comparable to those of uAUGs on aAUG expression (Fig. 4.8c; lanes 3-6, Fig. 4.9d; lanes 3-6)

Despite the clear demonstration of uAUG recognition by ribosomes (Fig. 4.7c and 4.9b), the effects of uAUGs in reducing translation initiation from aAUG was rather moderate. This is not due to uAUG being too close to the 5´-cap. Kozak previously found that the AUG recognition requires leader length beyond 20 nucleotides from cap structure in order for AUG to be recognized efficiently and to provide high fidelity translation initiation (Kozak, 1991a; Kozak, 1991c). All four positions of uAUGs in this study were located beyond the 20 nts limit (the 5´-most uAUG1 is at ~50 nucleotides away from the 5´-cap structure) and theoretically should be recognized efficiently by the scanning ribosome. Improvement of initiation context of uAUGs did not show a radical reduction of protein expression from aAUG, although the trend was obvious (Fig 4.1d vs 4.2c; Fig. 4.7c; lanes 7, 8, and Fig. 4.9b; lanes 7, 8) except uAUGc for unknown reason. A previous study in HeLa and HaCaT cells using a synthetic reporter leader sequence has shown that ribosomes have ability to translate main ORF with 40% to 80% of translatability in the presence of a single uORF, depending on the uORF context (Wang and Rothnagel, 2004). Translation initiation at the main ORF with constructs containing four uORFs with favorable initiation context was still observed at nearly 10% of translatability comparing to construct containing no uORF (Wang and Rothnagel, 2004). Whether the case is extreme or not, it is very possible for DEN to have such leaky scanning at uAUG in spite of improved initiation contexts at the uAUG's.

Another complexity in introduction of uAUG became evident when we found translation initiation able to occur at dAUG and give rise to reporter protein (Fig. 4.3b). Translation initiation from dAUG was increased by about 4-fold upon uAUG introduction (Fig. 4.7c; lanes 3-6) whereas uAUG4F and 4d mRNA used dAUG at the same low rate as wild type. This difference is most likely due to the lack of translation termination before dAUG in the latter two mRNA constructs. Thus, these results strongly suggest that translation reinitiation at dAUG is occurring by ribosomes that engaged in translation of uORF. Previous study have shown that a shorter uORF could potentially promote reinitiation at downstream AUGs (Kozak, 2001; Luukkonen et al., 1995). Indeed, the uORFs generated by uAUGs in this study are no more than 10 codons in length, and thus seem compatible with reinitiation. On the other hand, reinitiation from an AUG codon that is positioned too closely behind uORF may be skipped because ribosomes have not yet reacquired the Met-tRNAi needed for initiation completely (Abastado et al., 1991; Dever, 1997; Dever et al., 1995; Pain, 1994; Valasek et al., 2002). This could be the case with aAUG, which is closer to the termination codon of uORFs, and thus the inhibitory effect of uAUG could be observed.

Time courses of DCEGFPD expression in Vero cells clearly showed a preference of expression from aAUG rather than the dAUG in spite of the suboptimal context of aAUG (TCTCTG<u>AUG</u>A). This was possibly due to the secondary structure located 14 bases downstream of aAUG start codon, which allows slowing down the scanning and therefore allows more time for recognition of the preceding AUG codon (Clyde and Harris, 2006). Recent study has shown that DEN infectious viral RNA with aAUG site knockout or dAUG site knockout would not yield viable viral progeny (Clyde and Harris,

2006). dAUG is located within the complementary cyclization sequence (cCS) which has been shown to be important for viral replication (Alvarez et al., 2005a), and the sequence surrounding the aAUG site was also suggested to be involved in cyclization of DEN viral genome in addition of CS and cCS (Alvarez et al., 2005b). Therefore, changing nucleotides at either the aAUG or dAUG codons may not only effect the translation initiation but also the cyclization (long distance hybridization) known to be necessary for viral replication (Alvarez et al., 2005b; Khromykh et al., 2001; Lo et al., 2003). We are unclear about the expression requirement of the N-terminal 14 amino acid of capsid protein for viral function absent when translation initiation at dAUG. The N-terminus of the capsid protein is positively charged and may have ability to interact with viral RNA for encapsidation (Ma et al., 2004) but a role in viral replication and viral function in general is unclear. Therefore, separating the effect of translation and replication of the aAUG and dAUG knockout mutant is necessary.

Upstream AUGs have been commonly found to regulate gene expression (Iacono et al., 2005; Kozak, 1984; Kozak, 2002; Meijer and Thomas, 2002). An uAUG occurs naturally in attenuated viral strain DEN2 PDK53, a vaccine candidate. DEN2 PDK53 was obtained from DEN2 11681 strain after 53 passages in primary dog kidney cells (PDK) (Kinney et al., 1997) and has been used to develop vaccine against West Nile virus and tetravalent DEN vaccine (Huang et al., 2003; Huang et al., 2005). Mutation sites in PDK 53 were further analyzed and one of the PDK53 mutations shown to be responsible for attenuation is located in the 5´-UTR of the genome and would generate a uAUG uAUGb in this study). Previous study has shown that single mutation of this PDK53 uAUG has ability to attenuate the virus with smaller plaque form and less viral titer production in

C6/36 cells (Huang et al., 2003). In this present study, we observed reduction of DEN reporter expression in the presence of uAUG2, a context improved uAUGb, to 60% but we are uncertain whether 60% reduction in viral protein expression is going to attenuate viral function. It is possible that the mutation may have strong impact directly on viral replication or a different function other than translation. Effect of PDK53 uAUG in DEN replicaion needs to be determined to further understanding its attenuation mechanism.

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Chapter 5 Conclusions

In this thesis, I report the study of translational expression of dengue virus RNA, mainly using reporter gene mRNA system in Vero cells. We found that the 5´-cap and the 3´-UTR of dengue virus RNA synergistically contribute to efficient translation. Luciferase reporter RNAs with 5´- and 3´-UTRs from dengue virus RNA were translated at levels comparable to reporter RNAs with α globib UTRs (Chapter 2). The 3´-UTR of dengue virus RNA contributed to RNA stability and translational enhancement in similar to a 3´-polyA tail. Translational enhancement activity was decreased by removal of several predicted secondary structures located in the dengue 3´-UTR. Although the detailed mechanism of how these structures enhance translation is not clear, the involvement of the 3´stem-loop in translation enhancement has been confirmed by Holden *et al*. (2004) and Alvarez *et al*. (2005). RNA cyclization via hybridization of CS1 and cCS1, which are located in the 5´-and 3´-regions of dengue RNA, respectively, was found to be dispensable for translation of the dengue reporter constructs. In accord with this finding, a study by Alvarez *et al*. (2005) using dengue reporter replicon showed that complementarity of the CS1 and cCS1 is not required for efficient initial translation, but required for viral replication.

The importance of a 5´-cap in the translation of dengue virus RNA in Vero cells was indicated by sensitivity to inhibitor of cap-dependent translation (Chapter 3). This result contrasts to efficient translation of the EMCV-IRES containing reporter mRNA constructs in the presence of the inhibitor. The same result was obtained using dengue infectious RNA in BHK cells in the presence of inhibitor, consistent with dengue viral replication requiring the functional cap-dependent translation for viral protein synthesis. Although our data suggest that dengue virus gene expression does not employ cap-

independent translation, Edgil *et al*. (2006) have reported the opposite result, ie., that cap-independent translation is detectable in reporter constructs and full-length viral RNA. It is still unclear what may cause the disagreement of the results from the two laboratories, and certainly the possibility of dengue RNA translating cap-independently will need further evaluation. Dengue reporter RNA translation assay showed no sign of the presence of an internal ribosome entry site in the 5´-UTR, a result that has been confirmed by the study of Edgil *et al*. (2006) using bicistronic reporter constructs.

The conserved structure located in the 5´-UTR of the dengue genome showed modest inhibitory effects when placed in front of the α-globin 5´-UTR and could be replaced by α-globin 5´-UTR in dengue reporter construct. But a deletion study showed that individual elements of the 5´-stem loop structure also contribute to translation of the dengue virus RNA to a certain level suggesting that dengue 5´-UTR is functional for translation.

By adding of upstream AUGs (uAUG) in the dengue 5´-UTR and monitoring the reduction of authentic AUG using luciferase reporter constructs, I was able to elucidate that ribosomes scan through the 5´-UTR and recognize the introduced uAUG. The addition of uAUGs resulted in the reduction of luciferase expression from dengue reporter RNA constructs lacking the capsid coding sequence, suggesting that uAUGs inhibit ribosome access to the dengue authentic AUG codon. This result was less clear with luciferase reporter constructs containing the capsid coding sequence due to expression from an in frame downstream AUG within the capsid coding region. The use of capsid-EGFP reporter constructs encoding a shorter reporter protein, allowed separation if the proteins expressed from the authentic AUG and downstream AUG on

Western blots. Translation initiation from one uAUG was confirmed using mRNA constructs in which the uAUG was in frame with the capsid and EGFP coding regions. The results clearly demonstrated that the recognition of uAUG reduced translation from the authentic AUG, suggesting that dengue translation initiation utilizes scanning ribosomes. Data reported in this thesis strongly support the idea that dengue virus RNA is translated in a conventional cap-dependent scanning mode.

Translation of the genome is important for positive strand RNA viruses and it is vital for the viral life cycle. By manipulating the dengue viral translation and other events during viral life cycle, it may be possible to control the viral disease or developing the antiviral drug for treatment. Further, experimental system that can monitor the translation initiation in viral RNA in addition to reporter RNAs would allow obtaining direct evidence of how dengue virus regulates translation initiation in vivo.

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