MOTIF II OF JAPANESE ENCEPHALITIS VIRUS NS3 PROTEIN IS NOT ESSENTIAL FOR RNA BINDING ACTIVITY

Andi Utama[1*] and Hiroyuki Shimizu[2]

[1]Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia
[2]Department of Virology II, National Institute of Infectious Diseases, Tokyo Japan
*andiutama2002@yahoo.com

ABSTRACT

The role of motif II (DEAH; Asp_{285}-Glu_{286}-Ala_{287}-His_{288}) of Japanese encephalitis virus (JEV) NS3 protein on RNA binding activity was studied. A point mutation was introduced to the motif and the RNA binding activity of each mutant protein was analyzed. Truncated form of each protein with a His-tag was expressed in *Escherichia coli* BL21(DE3)pLysS and purified by metal affinity resin. Asp-285 and Glu-286 was respectively substituted with Ala, Ala-287 was replaced by Cys, Gly, or Ser. His-288 was mutated to other 19 amino acids. In total, 24 mutant proteins were produced and analyzed. As results, all mutants showed quite similar RNA binding activity, indicating that motif II of JEV NS3 is not related to RNA binding activity. The same finding was reported for hepatitis C viruse (HCV) NS3 protein, suggesting the similar structure of NS3 protein in flavivirus.

Keywords: Japanese encephalitis, RNA, binding, motif

INTRODUCTION

Japanese encephalitis virus (JEV), a causative agent of central nervous diseases such as meningitis and severe encephalitis, belongs to the genus of flavivirus of the family Flaviviridae (Kuno *et al.*, 1998). JEV has a positive, single-strand RNA genome of approximately 11 kb in length. The genome RNA is translated into a single polyprotein precursor, which is subsequently processed into three structural (C, M, and E), and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins.

Base on sequence alignment, the flavivirus NS3 protein belongs to helicase superfamily 2 (SF2) that can be subdivided into three families (DEAD, DEAH and DExH) (Gorbalenya and Koonin, 1993). The NTPase/RNA helicase domain of flavivirus NS3 protein is a member of DExH family that has seven conserved motifs (I, Ia, II, III, IV, V and VI) (Fig. 1), along with NTPase/RNA helicase domain of hepatitis C virus (HCV) NS3 and vaccinia virus (VV) NPH-II proteins. These conserved motifs are thought to be involved in RNA binding, NTP binding, NTP hydrolysis, and RNA helicase activities (Caruthers and McKay, 2002). In particular, Motif II of DExH proteins is one of the most important sequence motifs presumably involved in NTP hydrolysis. In particular, the histidine moiety is well conserved among DExH proteins and is thought to participate in the linkage between NTP hydrolysis and RNA binding. In this study, we targeted amino acid substitutions in motif II (Asp-Glu-Ala-His, DEAH) of the recombinant JEV NS3 protein and compared the RNA binding activity of wild and mutant proteins.

Recently, important role of the conserved motifs, including motif II, in the enzymatic activities have been demonstrated for some DExH proteins by amino acid substitution and X-ray crystallographic analysis (Mancini *et al.*, 2007; Mastrangelo *et al.*, 2007; Sampath *et al.*, 2006; Xu *et al.*, 2005; Utama *et al.*, 2000b; Gross and Shuman, 1995). Particularly, the conserved motifs of HCV NS3 protein have been extensively studied by both mutational and crystallographic analysis (Wardel *et al.*, 1999; Cho *et al.*, 1998; Heilek and Peterson, 1997, Kim *et al.*, 1997; Yao *et al.*, 1997). From the analysis of HCV NS3 protein, it is shown that motif I (GxGKS/T) involved in NTP binding by forming

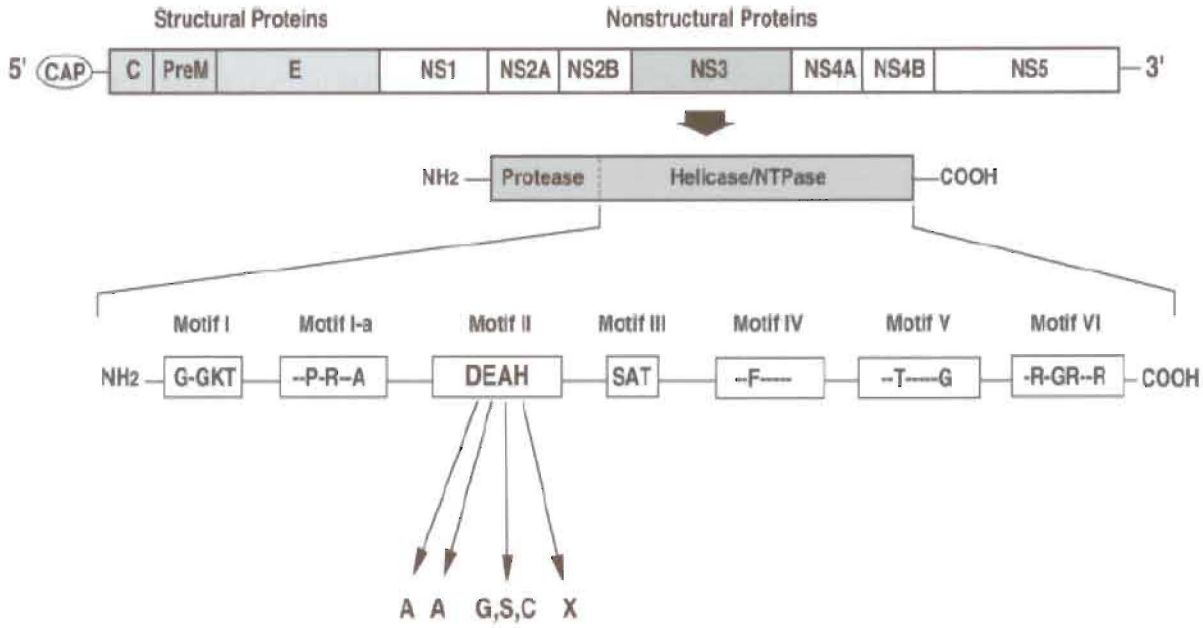

Figure 1. The structure of the JEV genome and conserved motifs in the NTPase/helicase region. There are seven conserved motifs presumably responsible for NTPase RNA binding and RNA helicase activities of the JEV NS3 protein. The amino acid residues, substituted in this study, are also shown.

P loop with β phosphate of NTP. Motif II (DECH), in close proximity to motif I, has been shown to bind to Mg^{2+} and assist in orienting the Mg^{2+}-ATP substrate for ATP hydrolysis. Motif III (TATPP) is though to function as a switch or hinge region to couple conformational changes involved in NTP hydrolysis and unwinding.

The role of motifs VI (QRxGRxGR) is controversial among DExH proteins. Motif VI in the HCV NS3 protein is thought to be critical for ATP hydrolysis but not RNA binding, while in some DExH protein is thought to be critical for RNA binding. Although the function of each motif is predicted to be similar among the same family of helicase, in fact the function of amino acid in the motifs was slightly different among the proteins. Therefore, the function of amino acid in the motifs of the DExH proteins may be different from one to another. Yet, no mutation or X-ray crystallographic analysis of JEV NS3 protein and its relation to RNA binding activity has been performed. In this study, we analyzed the function of each amino acid in motif II (DEAH) of JEV NS3 against RNA binding activity.

Materials and Methods

Constructions of NS3 expression plasmids

The expression plasmids were constructed as previously described (Utama *et al.*, 2000a; Utama *et al.*, 2000b). Briefly, a cDNA fragment derived from Japanese JEV strain JaO0566 was amplified using the primers 5'TCG ATT TCC AGC GCC ATC GTG CAG-3' and 5'-ACG TCG ACT CTC TTT CCT GCT GC-3' (generated restriction sites are underline). The 1.4 kb PCR-amplified fragment was digested with *Eco* RI and *Sal* I, and subcloned into the corresponding restriction site of pET-21b (Novagen, Madison, WI). The expression plasmid encodes amino acids 163-619 of the JEV NS3 protein with His-tag at the C-terminus.

Expression and purification of NS3 proteins

Expression and purification of NS3 proteins were performed as reported previously (Utama *et al.*, 2000a; Utama *et al.*, 2000b). In brief, the expression plasmid containing JEV NS3 genes was transformed into *E. coli* BL21(DE3)pLysS (Stratagene, La Jolla, CA). After the IPTG induction at 37°C for 3 h, the cells were collected by centrifugation (1200x*g*, 20 min). The cells

were resuspended in buffer B (10 mM Tri-HCL buffer (pH 8.5), 100 mM NaCl, 0.25% Tween 20) and disrupted by sonication on ice. The soluble fraction of the clarified cell lysate was mixed with TALON resin (Clontech, Palo Alto, CA). After gentle mixing for more than 1 h at 4°C, the resin was collected by a brief centrifugation and washed by buffer B containing 10 mM imidazole. Resin-bound protein was eluted with buffer B containing 400 mM imidazole. Purified protein was dialyzed against dialysis buffer (10 mM Tris-HCl buffer (pH 8.5), 100 mM NaCl, 10% glycerol) at 4°C overnight, and stored at -30°C before used.

Site-directed mutagenesis

Site-directed mutagenesis was introduced to DEAH motif of JEV NS3 protein by QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacture's instruction. Two partially complement oligonucleotide primers were synthesized to carry the desired mutations. The nucleotide sequence of the entire coding region was determined and the desired mutation was confirmed. The mutant plasmids were transformed into *E. coli* BL21(DE3)pLysS, then expressed and purified similar to the wild type protein.

Asp-285 or Glu-286 in motif II ($D_{285}E_{286}AH$) was substituted with alanine, and the resultant mutant proteins were designated AEAH and DAAH, respectively (Fig. 1). The Ala-287 in $DEA_{287}H$ motif was substituted to Cys, Gly, or Ser to produce, respectively, DECH, DEGH, or DESH mutant. The His-288 in $DEAH_{288}$ was mutated to other 19 amino acids to produce DEAx mutants.

Preparation of substrate and RNA binding assay

The ^{32}P-labeled ssRNA fragment was synthesized by using plasmid pSP72 (Promega, Madison, WI) as template. In brief, the plasmid pSP72 was linearized with *Bam* HI, and used as a template for *in vitro* transcription using the Riboprobe System-T7 (Promega). The RNA transcript was extracted following manufacture's instruction, and purified by spin column Micro SELECT-D (5Prime → 3Prime Inc., Boulder, CO). The RNA was diluted in RNase-free water, and used for RNA binding assay. RNA binding assay was carried out in 20 µl of binding buffer containing 25 mM MES (pH 6.0), 2 mM DTT, 2 mM $MgCl_2$, 1.25 units of RNasin (Promega), 0.32 fmol of the labeled ssRNA and the purified NS3 protein. The reaction mixture was incubated at 37°C for 10 min, and 5 µl of loading buffer (100 mM Tris–HCl (pH 7.4), 5 mM EDTA, 50% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue) was added to terminate the reaction. A 10 µl of mixture was analyzed by 10% native-PAGE, and the labeled RNA was quantified by BAS 2000 Image Analyzing System (Fuji Photo Film, Tokyo, Japan).

Results

Expression and purification of the truncated JEV NS3 protein

A cDNA corresponding to C-terminal 457 residues of JEV NS3 protein was subcloned to pET-21b vector and expressed in *E. coli*. The recombinant truncated protein was purified by metal affinity resin as described in Materials and Methods. After introduction a point mutation, resultant mutated protein was expressed and purified in a similar with the wild type protein. SDS-PAGE analysis of the 1 µg of each purified NS3 proteins showed mostly single band of protein which has 54 kDa molecular weight for all wild-type and mutant proteins(Fig. 2A, 3A, and 4A), coincides with the estimation by sequences analysis.

Mutagenesis of Asp and Glu in DExH motif of JEV NS3 protein

To analyze the functional significance of the Asp-285 and Glu-286 in motif II ($D_{285}E_{286}AH$) of the JEV NS3 protein, an alanine substitution was introduced to each residue. Both mutant proteins (**A**EAH and D**A**AH, boldface represents mutation position) showed RNA binding activity mostly identical to that of the wild type protein (Fig. 2B, Table 1). It is suggested that Asp-285 and Glu-286 are not significant for RNA binding activity.

Mutagenesis in the x residue of the DExH motif of JEV NS3 protein

We then investigated the effect to mutagenesis of alanine residue in motif II ($DEA_{287}H$) of JEV NS3 protein on RNA binding

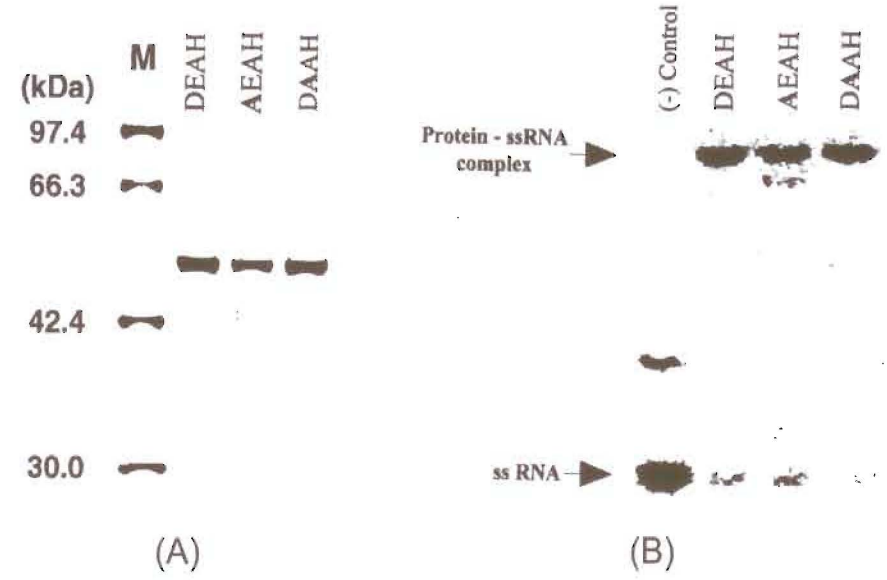

Figure 2. SDS-PAGE analysis (A) and RNA binding activity (B) of wild-type and mutant JEV NS3 (AEAH and DAAH) proteins. (A) Purified JEV NS3 proteins (1 μg/lane) were subjected to 10%-PAGE and stained with Coomassie Blue. (B) After RNA binding reaction, 10 μl of mixture was analyzed by 10% native-PAGE.

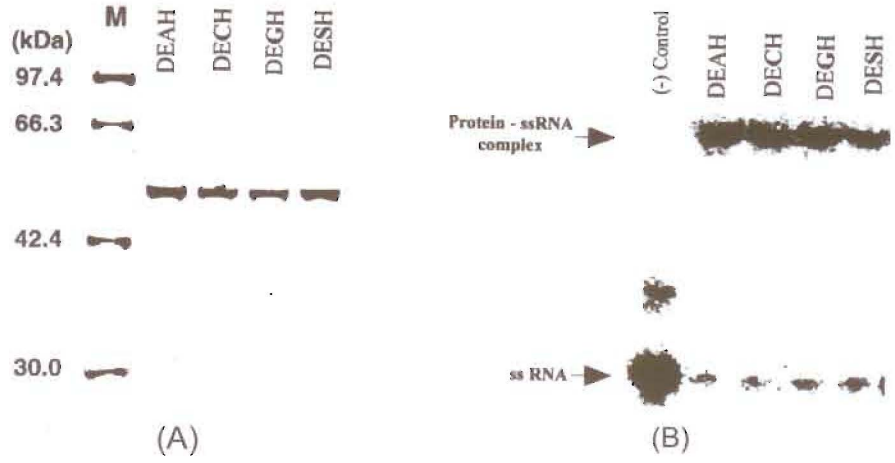

Figure 3. SDS-PAGE analysis (A) and RNA binding activity (B) of wild-type and mutant JEV NS3 (DECH, DEGH and DESH) proteins. (A) Purified JEV NS3 proteins (1 μg/lane) were subjected to 10%-PAGE and stained with Coomassie Blue. (B) After RNA binding reaction, 10 μl of mixture was analyzed by 10% native-PAGE.

activity. The residue was substituted with Cys, Gly, or Ser. DECH, DEGH and DESH mutants (boldface represents mutation position) showed RNA binding activity similar to that of the wild type protein (Fig. 3B, Table 1). It is suggested that A-287 is also not essential for RNA binding acitivity.

Mutagenesis of His in the DExH motif of JEV NS3 protein

To elucidate the significance of histidine residue in motif II ($DEAH_{288}$) of JEV NS3 protein, His-288 was substituted with all other amino acids (Fig. 4A). The resultant mutant proteins demonstrated different level of RNA

binding activity, ranged from 54%-100% of that of wild-type protein (Fig. 4B, Table 1). However, no proteins lost the RNA binding activity, suggesting that the His residue had effect on the RNA binding activity, but was not essential for the activity. Thus, all residues in the motif II is thought to be not related to RNA binding activity.

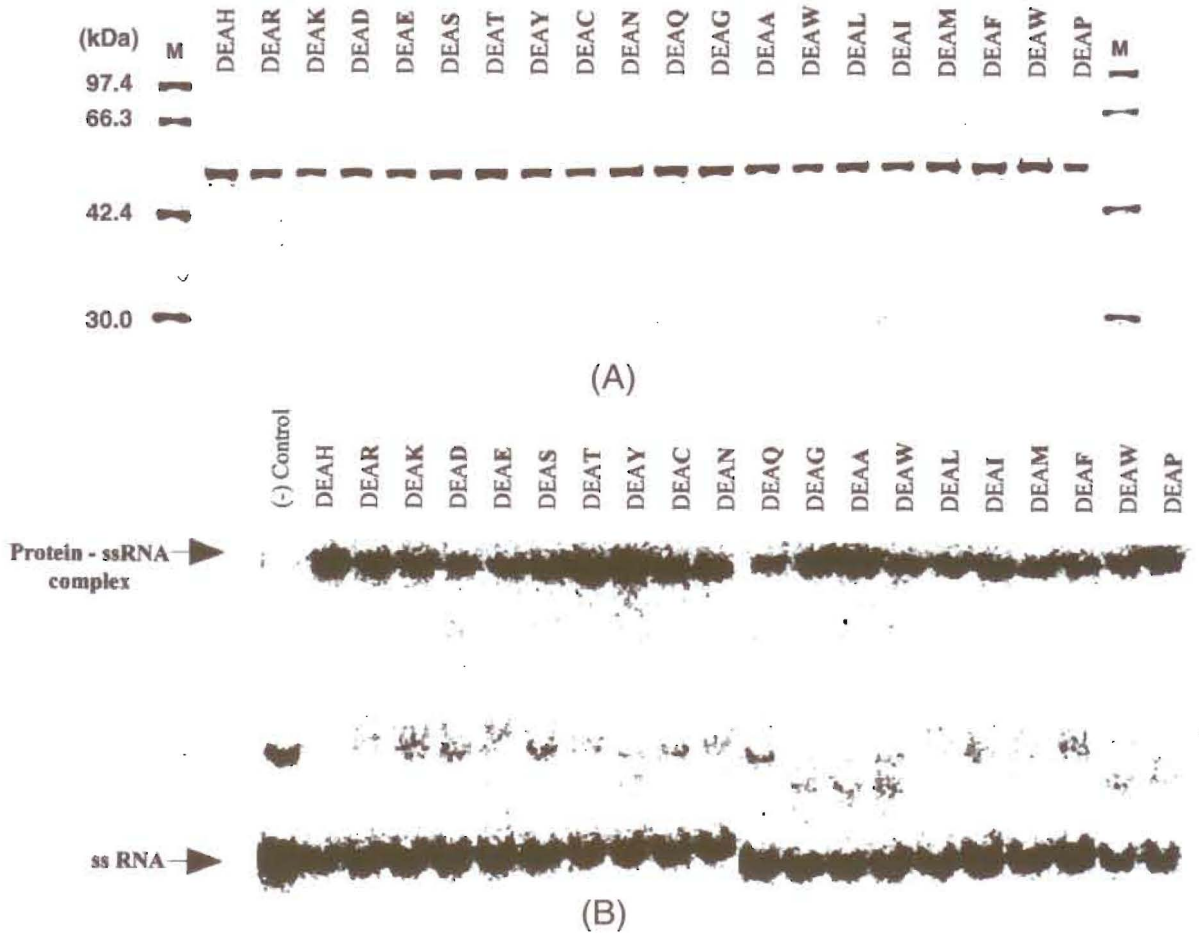

Figure 4. SDS-PAGE analysis (A) and RNA binding activity (B) of wild-type and mutant JEV NS3 (DEAX) proteins. (A) Purified JEV NS3 proteins (1 μg/lane) were subjected to 10%-PAGE and stained with Coomassie Blue. (B) After RNA binding reaction, 10 μl of mixture was analyzed by 10% native-PAGE.

Table 1. Effect of mutagenesis in motif II of JEV NS3 protein on RNA binding activity

Motif II of JEV NS3	RNA binding activity (%)*
DEAH	100
AEAH	100
DAAH	110
DECH	100
DEGH	100
DESH	100
DEAR	75
DEAK	75
DEAD	57
DEAE	69
DEAS	72
DEAT	80
DEAY	86
DEAC	74
DEAN	54
DEAQ	86
DEAG	87
DEAA	74
DEAV	89
DEAL	61
DEAI	82
DEAM	66
DEAF	100
DEAW	97
DEAP	88

*The activity of wild-type JEV NS3 was set as 100%.

Discussion

Based on sequences analysis, NTPase/RNA helicase domain of JEV NS3 protein is a member of DExH family RNA helicase, which have at least seven conserved motifs (I, Ia, II, III, IV, V, and VI) (Fig. 1) (Gorbalenya and Koonin, 1993). The importance roles of the conserved motifs in the enzymatic activities have been demonstrated for some DExH proteins. Particularly, HCV NS3 protein is the most investigated among DExH proteins, either by mutational analysis or crystallographic analysis (Wardel *et al.*, 1999; Cho *et al.*, 1998; Heilek and Peterson, 1997, Kim *et al.*, 1997; Yao *et al.*, 1997). However, study on JEV NS3 has not extensively carried out. In this paper, we present the study on the significance of motif II (DEAH) of the JEV NS3 protein for RNA binding activity. We found out that all amino acids in the motif were not essential for RNA binding activity.

HCV NS3 protein crystallographic analysis showed that His in motif II (DECH) is located at the rim of the interdomain cleft between domain 1 and 2, facing a side chain of the Gln in the motif VI (QREGRTGR) across the cleft (Dumont *et al.*, 2006; Caruthers and McKay, 2002; Cho *et al.*, 1998; Yao *et al.*, 1997). Based on this structure, histidine residue appears to be essential for coupling the ATPase activity to polynucleotide binding. Consequently, mutation of His residue of HCV NS3 (Wardel *et al.*, 1999; Heilek *et al.*, 1997; Kim *et al.*, 1997) and vaccinia virus NPH-II (Gross and Shuman, 1995) proteins resulted in a functional ATPase and RNA binding with RNA unwinding activity, similar to our result. In conclusion, the function of His residue in motif II of DExH protein may be affected by many factors or may be context dependent.

Although a number of studies on DExH protein have been performed, this is the first extensive mutational study on JEV NS3 protein. Our result demonstrated that all amino acids in

motif II of JEV NS3 protein were not essential for RNA binding activity. These findings are different with other DExH protein; cystein residue in DECH motif of HCV is not essential for RNA unwinding activity (Heilek and Peterson, 1997). In this study, however, we performed mutational analysis only in motif II. Since the function of amino acids in the motif, as well as functional roles of conserved motifs, may not be independent, it is important to analyze the effect of other motifs substitution of JEV NS3 protein to elucidate the overall enzymatic functions of the protein.

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