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Effects of Point Mutations at Positions 79, 85 and 91 of the Nipah Virus Leader Sequence to Its Minigenome Expression

(Kesan Mutasi Titik pada Posisi 79, 85 dan 91 dalam Jujukan Pemimpin ke Atas Pengekspresan Minigenom Virus Nipah)

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ABSTRACT

Nipah virus has been identified as the causative agent responsible for an outbreak of fatal human viral encephalitis in Malaysia and Singapore in 1998 to 1999. In vitro *replication assays with Nipah virus minigenome carrying CAT gene (chloramphenicol acetyltransferase) has been developed without the use of infectious virus to allow further study of the replication of Nipah virus in vitro. It has been reported that the viral RNA replication and transcription activity of paramyxoviruses are controlled by essential sequences located in the terminal regions of viral genomic and antigenomic RNAs. In this study, single base substitution was carried out on the Nipah virus minigenome separately at the three guanine residues (G) located in positions 79, 85 and 91 of the leader promoter within the 5' non-translated region (NTR) of the nucleocapsid gene (N) mRNA region. The guanine residues of these positions were substituted with the cytosine (C) or adenine (A) residue, respectively by using the overlapping PCR-mediated mutagenesis method. The resultant mutants containing the desired point mutation were confirmed by sequencing. The mutants were analyzed to determine the effect of substitution mutation on the viral transcription activity of the minigenome. It was found that the substitution of G at positions 79 and 85 decreased the efficiency of transcription of Nipah virus minigenome while the substitution of G at position 91 did not. Our findings also showed that the effect of transition mutation gave more impact than the transversion mutation in term of suppression effect upon the transcription activity of minigenome.*

Keywords: Leader sequence; minigenome; mutagenesis; Nipah virus

ABS TRAK

Virus Nipah telah dikenalpasti sebagai agen yang menyebabkan letusan jangkitan ensefalitis yang boleh membawa maut di kalangan manusia di Malaysia dan Singapura dalam tahun 1998 hingga 1999. Asai replikasi secara in vitro *yang terdiri daripada minigenom virus Nipah yang mengandungi penanda gen CAT (chloramphenicol acetyltransferase) telah dibangunkan tanpa melibatkan virus berjangkit, dengan tujuan untuk mengkaji replikasi virus Nipah secara* in vitro. *Aktiviti replikasi RNA dan transkripsi paramyxovirus telah dilaporkan dikawal oleh jujukan penting yang terkandung dalam kawasan hujung RNA genom dan antigenom virus. Dalam kajian ini, mutagenesis secara penggantian bes tunggal telah dilakukan ke atas minigenom virus Nipah secara berasingan dalam kawasan promoter pemimpin, yakni tiga residu guanina (G) yang terletak di posisi 79, 85 dan 91 dalam kawasan 5' NTR mRNA gen nukleokapsid (N). Residu G tersebut telah digantikan dengan residu sitosina (C) dan adenina (A) masing-masing dengan menggunakan kaedah mutagenesis berasaskan tindak balas berantai polimerase (PCR) bertindih. Mutan-mutan terhasil yang mengandungi mutasi titik yang dikehendaki telah dikenalpastikan melalui penjujukan DNA. Mutan-mutan tersebut telah dianalisis untuk menentu kesan mutasi penukargantian tersebut ke atas aktiviti transkripsi minigenom virus Nipah. Didapati bahawa penukargantian residu G pada posisi 79 dan 85 menurunkan kecekapan aktiviti transkripsi minigenom virus Nipah manakala penukargantian residu G pada posisi 91 tidak memberikan kesan tersebut. Penemuan ini juga menunjukkan kesan mutasi transisi memberikan kesan yang lebih ketara daripada kesan mutasi transversi daripada segi kesan penahanan ke atas aktiviti transkripsi minigenom virus.*

Kata kunci: Jujukan pemimpin; minigenom; mutagenesis; virus Nipah

INTRODUCTION

In September 1998, a fatal viral encephalitis outbreak in peninsular Malaysia was first reported. Consequently, a new virus, termed Nipah virus, was identified as the causative agent of the fatal viral encephalitis outbreak in human (Chua et al. 1999). Nipah virus was first emerged in Kinta district, Perak which then spread to Sikamat and Bukit Pelandok in Negeri Sembilan and Sungai Pelandok in Selangor. In 5 March 1999, Nipah virus was first isolated in Malaysia by the researchers from the Faculty of Medicine, University of Malaya (Ministry of Health Malaysia 2001). Nipah virus is named in accordance with the location where

it was first detected in Malaysia, which is Kampong Sungai Nipah in Bukit Pelandok.

Nipah virus is an enveloped, non-segmented, negative-stranded RNA paramyxovirus belonging to the new genus, *Henipavirus* within the subfamily *Paramyxovirinae* of the family *Paramyxoviridae* (Harcourt et al. 2000; Harcout et al. 2001; Mayo 2002; Wang et al. 2001). The genome of Nipah virus comprised of 18,246 nucleotides has been assigned as the largest genomes within the family *Paramyxoviridae* (Harcout et al. 2001). The genome of Nipah virus consists of six transcription units encoding six major structural proteins, the nucleocapsid (N), phophoprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G) and large protein (L) or RNA polymerase that are ordered in 3'- N-P-M-F-G-L -5'. The P gene of Nipah virus is predicted to encode the multiple proteins namely P, V, W and C protein (Harcout et al. 2000; Wang et al. 2001).

In the case of genome of Nipah virus, it is non-segmented. Therefore, the viral genomic RNA first is transcribed into different multiple mRNA, which is termed as sub-genomic mRNA after the entry of ribonucleoprotein (RNP) into the cytoplasm. The synthesis of multiple mRNA occurs via stop-start mechanism at the intergenic region of viral genome. As a result, each of the six viral genes is transcribed in one by one into six viral mRNA, respectively which are then translated to produce the six major viral proteins in the cytoplasm. The viral transcription process is switched to RNA replication process under the control of viral nucleocapsid protein (N). As the protein synthesis proceeds, the level of N protein is increased and level of N protein become high enough to associate with the nascent positive-sense strand RNA through interaction with the P protein at specific site within the leader region, suppressing the stop-start mechanism. Thus the polyadenylation and mRNA cleavage are blocked. This will allow the synthesis of full-length positive-sense strand RNA which is known as antigenomic RNA. The encapsidated full-length positive-sense strand RNA then serves as template for progeny genomic RNA synthesis (Flint et al. 2000; Wagner & Hewlett 2004).

The terminal regions of the genomic and antigenomic RNAs of the paramyxovirus are known to contain essential sequences for directing viral RNA replication as well as the transcription activity. The 3' end of genomic RNA is known as leader (Le) promoter which directs the viral transcription activity as well as synthesis and encapsidation of antigenomic RNA, whereas the 3' end of the antigenomic RNA is known as trailer complementary (Trc) promoter which directs the synthesis of progeny genomic RNA (Hoffman & Banerjee 2000; Kawaoka 2004; Marcos et al. 2004; Peeples & Collins 2000; Walpita & Peters 2007). There are two essential elements found in the genomic and antigenomic RNA replication promoter, the conserved region I which located at 3' end of leader promoter and the conserved region II which located in 5' NTR of the N gene mRNA region of viral genome (Marcos et al. 2004; Tapparel et al. 1998). Walpita and Peters (2007) proposed that the sequence elements located in the non-coding region within the 5'-end of trailer promoter (antigenomic promoter) of Nipah virus genome control the genome synthesis, and they reported that there was a conserved region at nucleotide 1 – 12 and 79 – 91 in the antigenomic promoter of Nipah virus which appeared to be critical.

The *in vitro* replication assays with Nipah virus minigenome carrying CAT gene has been developed without the use of infectious virus to allow further study on the replication of Nipah virus *in vitro* (Figure 1). In this study, the conserved region II of the leader promoter (genomic promoter) was focused in which the three guanine residues (G) located in positions 79, 85 and 91 of the leader promoter within the 5' NTR of the N gene mRNA region were substituted with the cytosine (C) or adenine (A) residue, respectively (Figure 2). Here, we investigated whether the G residues at positions 79, 85 and 91 of the leader promoter play any role in the transcription of Nipah virus. As for this, we carried out the mutational analysis on these positions where the point mutations were generated. Besides, we wanted to compare the effects of transversion and the transition mutations generated at these positions in efficiency of viral transcription as well.

Materials and Methods

Overlapping PCR-mediated mutagenesis

The point mutations were generated on negative-sense minireplicon of Nipah virus, pMGNiVR6, by overlapping PCR-mediated mutagenesis in which the degenerate primers were used. The overlapping PCR-mediated mutagenesis involved three sets of PCR, that were PCR phase I, PCR phase II and PCR phase III. The primers used to amplify the whole Nipah virus minigenome inserted in pUC19 vector were forward primer 5'- A<u>CCCGGG</u>GATCCACTAGAGTCAAAA -3' which encoded a *Sma*I restriction site (in underlined) and reverse primer 5'- TAATACGACTCACTATA GGGACCGAACAA -3'. Mutations at positions 79, 85 and 91 of the leader promoter were generated using designed primers which contain the desired single base substitution. To obtain the altered Nipah virus minigenome fragments, the primers used in PCR phase I to amplify the downstream sequence of Nipah virus minigenome containing the mutation site were reverse primer and mutated forward primer 5'- CACTTTTG**(C/A)** TCTTGGTATTGGATCCTCA -3' (position 79), 5'- CACTTTT GGTCTTG**(C/A)**TATTGGATCCTC -3' (position 85) or 5'- CACTTTTGGTCTTG GTATTG**(C/A)** ATCCTC -3' (position 91), while the primers used in PCR phase II to amplify the upstream sequence containing mutation site were forward primer and mutated reverse primer 5'- GAGGATCCAATACCAAGA**(T/G)** CAAAAGTG -3'(position 79), 5'- GAGGATCCAATA**(T/G)** CAAGACCAAAAGTG -3' (position 85) or 5'- CTTGAGGAT**(T/G)**CAATACCAAGACCAAAAG -3'

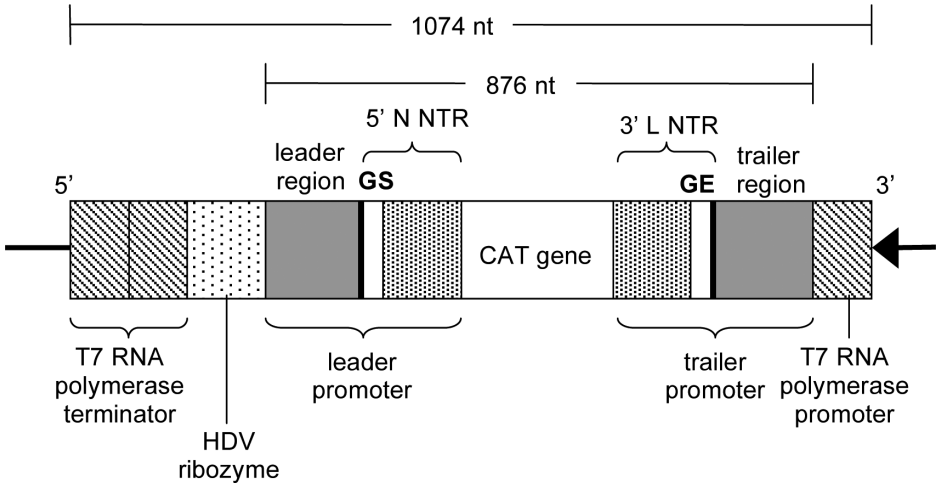


FIGURE 1. Schematic diagram of structure of the Nipah virus minireplicon, pMGNiVR6. In this minigenome system, all of the viral genes were replaced with a reporter gene, the CAT gene flanked by transcription start sequence of N gene, the 5' NTR of the N gene mRNA, the 3' NTR of the L gene mRNA and the transcription stop sequence of L gene. Additional four bases were added after the 3' end of CAT gene to make the length of minigenome evenly divisible by six. Transcription of minigenome from the plasmid is directed by a T7 RNA polymerase promoter. When the minigenome is transcribed by the T7 RNA polymerase in 3' to 5' direction, the negative-sense of primary transcripts are synthesized, which is contain sequence encoding the viral trailer region, a gene end signal (GE), part of 3' NTR of the L gene mRNA, the CAT gene, part of 5' NTR of the N gene mRNA, a gene start signal (GS), the viral leader region, the hepatitis delta virus ribozyme, and the T7 RNA polymerase termination signal

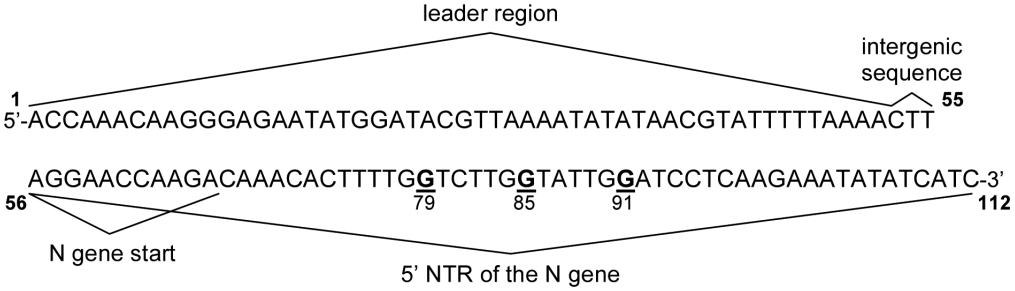


FIGURE 2. The cDNA sequence of leader promoter of Nipah virus located at the 5' end of the minigenome. The underlined and boldfaced nucleotides were substituted with C or A residue

(position 91). The boldface nucleotides (in bracket) in mutated primers represent the substitution of G with C or A residue, respectively. In PCR phase III, the purified overlapping DNA fragments resulted from PCR phase I and phase II were then combined via overlapping, extended and amplified using the forward primer and reverse primer to produce the full-length of altered Nipah virus minigenome. All of the PCR were carried out by using the *Taq* DNA polymerase (Vivantis). The PCR products were purified by using the PCR Products Purification QIAquick kit or Gel Extraction QIAquick kit (Qiagen).

CONSTRUCTION OF MUTANT PLASMIDS

The PCR product resulted from the PCR phase III was a 1095 bp fragment encoded a *Sma*I restriction site at the 5'end of fragment. The altered minigenome fragments were cloned into pTZ57R cloning vector according to the InsT/Aclone™ PCR Product Cloning kit's protocol (Fermentas) and subsequently were transformed into *E. coli* DH5α host cells as previously described (Sambrook & Rusell 2001). The recombinant plasmids were extracted from the positive clones by using the Extraction QIAprep Spin Miniprep kit (Qiagen), and were then analyzed using agarose gel electrophoresis and restriction enzyme digestion. The recombinant plasmids were digested with *Sma*I in single digestion or *Sma*I and *Kpn*I in double digestion. The sequence of altered minigenome fragments finally were confirmed via DNA sequencing.

TRANSFECTION

Approximately 1 × 10^5 cells/mL of Vero cells were plated into 18 mL of Dulbecco's Modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (PAA) and grown to 90-95% confluence in T-75 culture flask

by incubating the cells at 37°C in 5% carbon dioxide condition. The cells in each flask were transfected with 4.5 µg pNiV-N, 3.0 µg pNiV-P, 1.5 µg pNiV-L, 7.5 µg pTriEX-T7 and 13.5 µg minigenome plasmid (pMGNiVR6) by using the Lipofectamine 2000 Transfection reagent (Invitrogen) according to the manufacturer's protocol. The pMGNiVR6, pNiV-N, pNiV-P, pNiV-L and pTriEX-T7 were kindly provided by Dr. Shahreza from Universiti Malaysia Terengganu. For experimental controls, the wild-type minigenome plasmid was co-transfected with the pNiV-N, pNiV-P or pNiV-L alone as indicated above. The plates were incubated at 37°C in the presence of 5% carbon dioxide. Medium was replaced with new Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum after 5 h of incubation period. The cells were subsequently incubated at 37°C in the presence of 5% carbon dioxide for a total of 48 h. The cells were then harvested for the CAT activity.

WESTERN BLOTTING AND CAT ELISA ASSAY

The cells were scraped into the medium and pooled. The harvested cells were then lysed by using CAT lysis buffer which provided by CAT ELISA kit (Roche). After incubation of 30 min at room temperature, the lysate was centrifuged at 4,000 rpm for 15 min at 4°C. The supernatant was then analyzed via Western blotting. The bound CAT protein was detected by primary antibody anti-CAT-DIG followed by secondary antibody anti-DIG-POD, which were provided by CAT ELISA kit (Roche). The band detection was carried out according to the manufacturer's protocol. The CAT ELISA assay was performed by using the CAT ELISA kit in accordance with the manufacturer's protocol, to determine the CAT concentration in cytoplasmic extracts.

RESULTS AND DISCUSSION

All of the viruses belonging to the subfamily *Paramyxovirinae* have genomes which are divisible by six, conforming to the "rule of six". The RNA replication of those viruses is efficient only if the genome is multiple of six (Calain & Roux 1993). The *in vitro* replication assays with Nipah virus minigenome carrying CAT gene has been developed to study the replication of Nipah virus *in vitro*. Therefore, the Nipah virus minigenome that used in this study is divisible by six, in which the four bases were added after the 3' end of CAT gene, extending the length of minigenome to 876 nucleotides.

Transfection of Nipah virus minigenome plasmid together with the support plasmids and plasmid encoding T7 RNA polymerase, pTriEX-T7 into the Vero cells resulted in RNA replication and transcription of the minigenome. When the described minigenome system is transfected into host cells, the naked minigenomic RNA was synthesized from the transfected plasmid carrying the Nipah virus minigenome by T7 RNA polymerase expressed from the co-transfected T7-encoded plasmid. The plasmid-derived minigenomic RNA was a negative-sense of primary transcript with 876 nucleotides in size. The 3' end of the minigenomic RNA was self-cleavaged by a hepatitis delta virus ribozyme sequence located adjacent to the leader region of the minigenome, whilst the 5' end of the minigenomic RNA was created correctly mediated by a T7 promoter sequence that placed adjacent to the trailer region (Figure 1). Thus result in the precise ends of the minigenome transcript.

The other co-transfected support plasmids carrying N, P and L gene of Nipah virus individually were transcribed by T7 RNA polymerase and then translated into the support proteins. This process continues until sufficient levels of support proteins are retained to form the ribonucleoprotein (RNP), in which the N and P are involved in encapsidation of minigenomic RNA. The encapsidated plasmid-derived minigenomic RNA then serves as template for the synthesis of antigenomic RNA and the transcription of viral genes. The synthesized full-length of antigenomic RNA will then be encapsidated and served as template for the synthesis of progeny genomic RNA. As in this minigenome system, the viral genes were replaced with a reporter gene namely chloramphenicol acetyltransferase (CAT) gene (Figure 1). Therefore, CAT gene mRNA strands will be synthesized and subsequently were translated into the CAT protein.

In our initial studies, we focused on the step of viral transcription activity. The three G residues located in the 5' NTR of the N gene mRNA region at the positions 79, 85 and 91 of the leader promoter were substituted with the C or A residue, respectively. The resultant mutants containing desired point mutation were confirmed by sequencing (Figure 3). The mutants were then analyzed by measuring the CAT protein levels relative to wild-type, in order to determine the effect of point mutation on the efficiency of viral transcription activity of the minigenome. Thus the CAT activity could be indirectly measured for the transcription activity of minigenome.

To analyze the minigenome transcription, the transfected cells were harvested after 48 h posttransfection and then were lysed to obtain the cellular total protein. The lysate were analyzed using Western blotting to detect the presence of CAT protein. Detection of CAT protein reflected the synthesis of CAT mRNA from the minigenome, showing that the minigenome system is functioning and be activated in the host cells. Further analysis of CAT expression was conducted through the CAT ELISA assay in order to determine the level of expressed CAT protein in each sample. Differences of CAT protein levels were interpreted to reflect the efficiency of viral transcription from the mutated leader promoter.

From the results gained from Western blotting analysis (Figure 4), there was no CAT protein detected in non-transfected cells and the sample omitting of wild-type minigenome plasmid but it was detected in the sample with wild-type minigenome, as expected. There was a very little or no CAT protein detected from mutant MGNiV79C, MGNiV79A, MGNiV85C and MGNiV85A. The results of CAT ELISA assay showed that the level of CAT protein

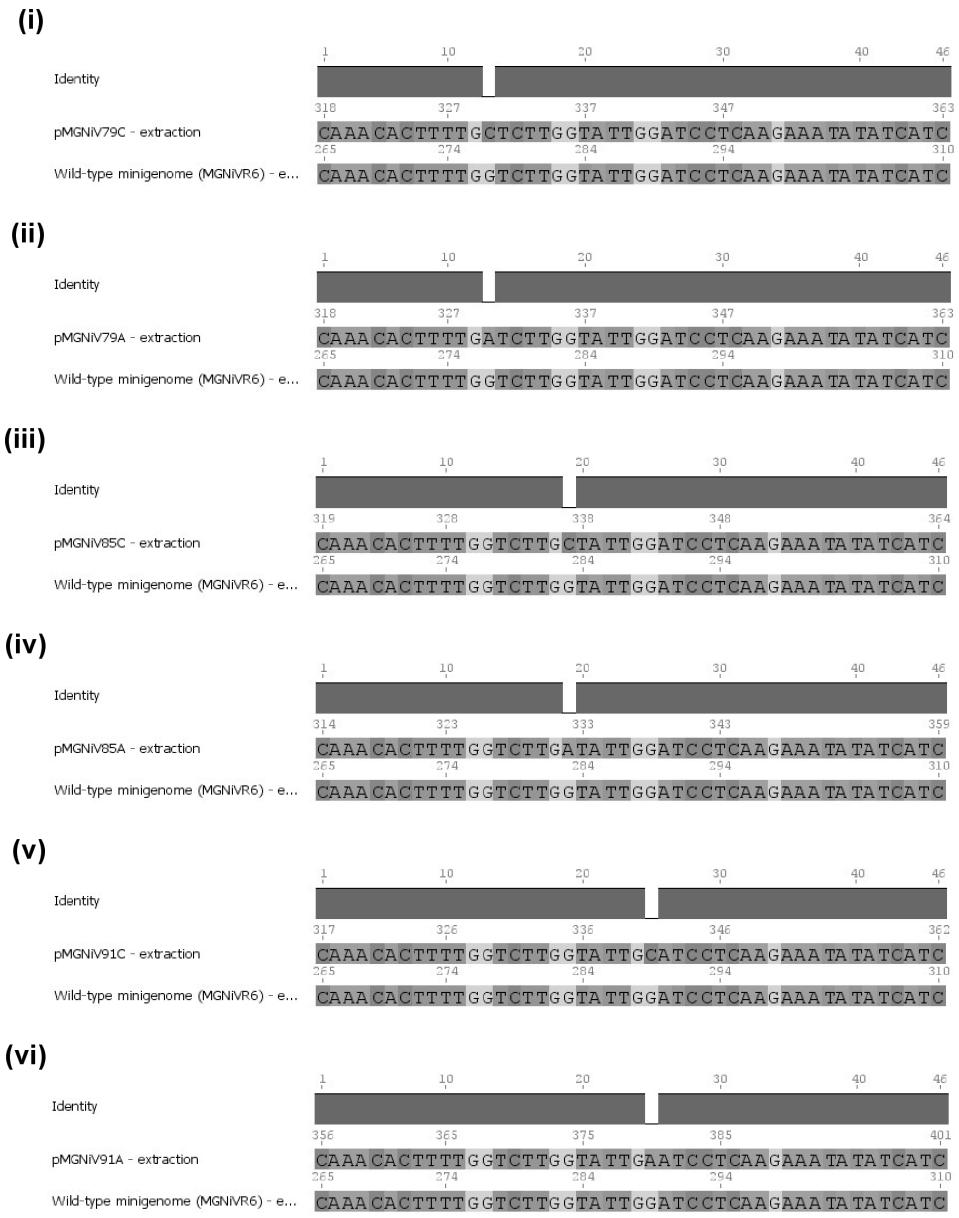


FIGURE 3. Sequence analysis of single base mutations in pMGNiVR6 at positions 79, 85 and 91. The target sites within the leader promoter of minigenome were substituted correctly with the desired bases. (i): pMGNiV79C; (ii): pMGNiV79A; (iii): pMGNiV85C; (iv): pMGNiV85A; (v): pMGNiV91C; (vi): pMGNiV91A

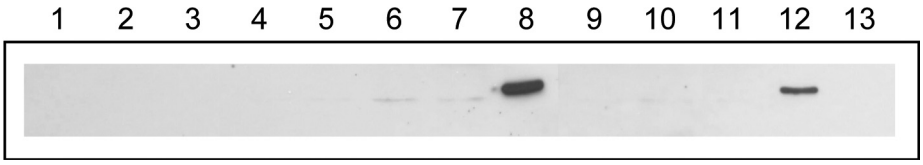
expressed in mutant MGNiV79 and MGNiV85 were lower than the CAT protein level of wild-type minigenome plasmid (Figure 4). The mutant MGNiV85A gave the highest decrease level of CAT protein among the mutants, with a decrement of 37.15% if compared to that of the wild-type. The other mutants MGNiV79C, MGNiV79A and MGNiV85C gave decreased level of CAT protein with 1.54%, 20.07% and 20.16% respectively with respect to the CAT protein level of wild-type. However, a detectable level of CAT protein was observed in mutant MGNiV91C and MGNiV91A. There was a difference in mutation effect upon the CAT expression between transition and transvertion mutations. The transition mutation generated at positions 79 and 85 results in a higher decrement of CAT protein if compared to that of the transversion mutation. But the substitution of G to A residue at position 91 showed the highest expression of CAT protein among the mutants with 60.31% higher than the wild-type, whilst the substitution of G to C residue gave the CAT protein level with 26.25% higher than the wild-type.

Halpin et al. (2004) have demonstrated that no CAT protein was detected in the absence of the plasmid encoding N gene, P gene or L gene of Nipah virus. Nevertheless, in our experimental studies, There was low CAT protein level detected from sample in which the N plasmid were omitted (Figure 4). We have shown that there was a detectable level of CAT protein in the absence of L protein (data not shown). This result indicated that the viral genome may

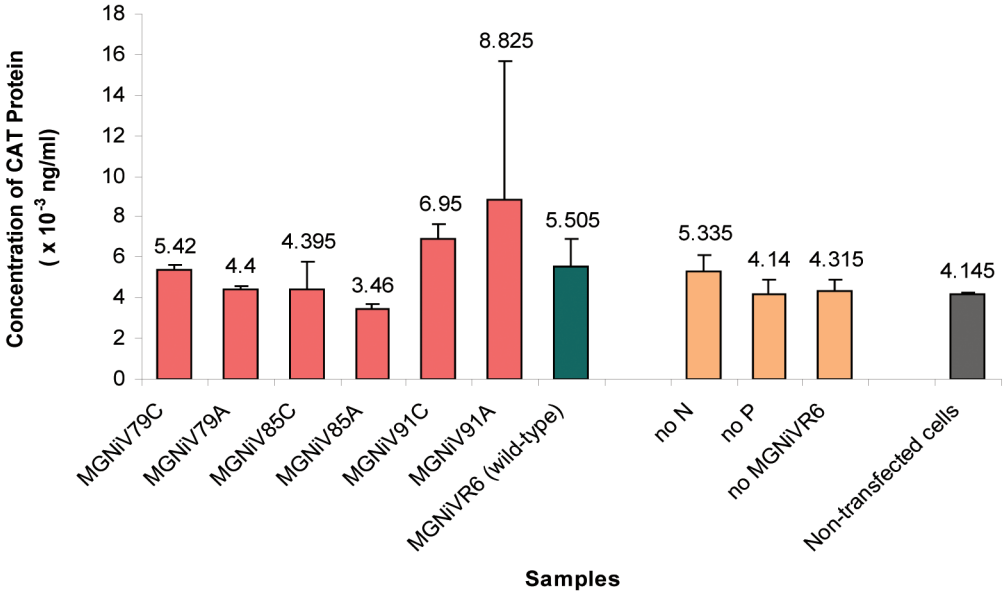
still be able to assemble into the nucleocapsid by solely N and P protein supplied in *trans*.

The N protein plays an important role in encapsidation of viral RNA genome and thus regulates the viral transcription and replication (Eshaghi et al. 2005). The "rule of six" has been proposed as a requirement for N protein to bind exactly six nucleotides to the viral genome (Calain & Roux 1993). The binding of N protein together with P protein to the genomic RNA forms the ribonucleoprotein (RNP) complex that is associated with the viral RNA polymerase (L protein), which serves as the functional template for both viral transcription and RNA replication (Hoffman & Banerjee 2000; Kawaoka 2004). The encapsidated genomic RNA serves as template for synthesis of positive-sense RNAs, namely the sub-genomic viral mRNAs and the antigenomic RNA. The synthesized full-length antigenomic RNAs serve as efficient templates for RNA replication only when they are encapsidated with the N protein.

Tapparel et al. (1998) proposed the existence of three N protein binding sites at the bases 79 – 84, 85 – 90 and 91 – 96 located in the 5' NTR of the N gene mRNA region of Sendai virus, which may serves as a nucleation site for encapsidation of the RNA by the N protein. The first base within each hexamer must be a G residue and therefore only the positions 79, 85 and 91 appear to be critical. These three positions of the Sendai virus replication promoter have been shown to be critical bases in three N binding hexamers. The mutations at these G residues were also critical for RNA replication of Human Parainfluenza virus type 3 as well (Hoffman & Banerjee 2000) (Figure 5).



(a)



(b)

FIGURE 4. Expression of CAT protein by the mutants. Vero cells were transfected with Nipah virus minigenome plasmid containing point mutation together with the helper plasmids (pNiV-N, pNiV-P and pNiV-L) and plasmid encoding T7 RNA polymerase as previously described. The lysate of cells transfected with pMutNiV79C (lane 1), pMutNiV79A (lane 2), pMutNiV85C (lane 3), pMutNiV85A (lane 4), pMutNiV91C (lane 5), pMutNiV91A (lane 6) and wild-type minigenome (lane 7) were prepared after 48 hours posttransfection. The lysates were then analyzed for their CAT expression via Western blotting and CAT ELISA assay. Lane 9, 10 and 11 represented the samples in which the pNiV-N, pNiV-P and wild-type minigenome were omitted in transfection mixtures, respectively. Sample in lane 8 and 12 represented the positive control, in which the CAT protein provided by CAT ELISA kit was used. Lysate of non-transfected cells (lane 13) was also assayed for CAT expression. (a) Western blotting analysis of CAT expression of mutant samples. (b) The concentration of CAT protein in cytoplasmic extracts was measured by using CAT ELISA and expressed as a concentration relative to the wild-type minigenome with the standard error indicated by the vertical bars

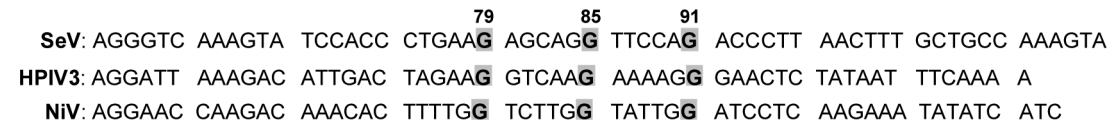


FIGURE 5. Sequence comparison of the 5' NTR of the N gene mRNA region of the Sendai virus (SeV), Human parainfluenza virus type 3 (HPIV3) and Nipah virus (NiV). The 5' NTR of the N gene mRNA sequence of three paramyxoviruses which come from the same subfamily are aligned to emphasize the site of point mutations that correspond to each other

From our result of mutational analysis, we found that the nucleotides at the positions 79 and 85 could be critical in the genome encapsidation process, where the transition or transversion mutation was generated at these positions, respectively. The low CAT protein level relative to wild-type observed in mutant MGNiV79 and MGNiV85 give rise to an initial interpretation that the substitution of G with C or A residue at these positions might block the formation of functional template for viral transcription by preventing the N protein from binding to the 5' NTR of the N gene mRNA region of plasmid-derived minigenomic RNA. In contrast to mutant MGNiV79 and MGNiV85, the substitution of G residue at position 91 had not given any significant effect on CAT expression level. However, this interpretation have to be confirmed and further analyzed by examining the levels of encapsidated minigenomic RNA in future.

We assumed that there were certain nucleotides located in 5' NTR of the N gene mRNA region which might play a role in encapsidation of viral genomic RNA as well as antigenomic RNA. We would expect that nucleotide located at positions 79, 85 and 91 might also affect the viral RNA replication as well. Hoffman and Banerjee (2000) have shown that mutations at these G residues are critical for RNA replication of Human Parainfluenza virus type 3 (HPIV3), where the mutation at positions 85 and 91 completely abrogated the antigenomic RNA synthesis, but mutation at position 79 still allows some antigenomic RNA synthesis (Figure 5). Further studies will be carried out to determine the effects of these mutations on the RNA replication of minigenome.

It has been reported that the N, P and L proteins are sufficient to promote efficient viral replication as well as transcription of virus belonging to subfamily *Paramyxovirinae* (Durbin et al. 1997; Halpin et al. 2004; Kawaoka 2004). However, our experimental results showed that the CAT protein was detected in the absence of L protein. Since the plasmid-derived genomic RNA serves as template for the viral transcription only when it is encapsidated by the N protein together with the P protein, this observation suggests that the viral transcription of CAT gene occurred as the plasmid-derived genomic RNA was encapsidated and functioned as a template for the viral transcription and the synthesis of antigenomic RNA as well. Hence, the plasmid-derived minigenome of Nipah virus synthesized in the presence of N and P could be encapsidated independent of functional viral polymerase. Nevertheless, the levels of encapsidated minigenomic RNA have to be examined to firmly conclude this conclusion. The encapsidated plasmid-derived minigenomic RNA of Respiratory Syncytial virus (RSV) was detected in the presence of N and P proteins together with non-functional L protein, thus the plasmid-derived minigenomic RNA was capable to assemble into nucleocapsid by N and P proteins, the so-called encapsidation process (Peeples and Collins 2000). The support plasmid bearing N and P gene are necessary for achieving the efficient minigenome transcription of Nipah virus while the effect of L gene is less vital. However, no conclusiom can be made clearly on the effect of L gene as it is not the scope of our study.

As a conclusion, substitution of G residue at positions 79 and 85 may comprise the suppression effect upon the transcription of Nipah virus based on the results obtained from our experiments. Substitution of G at position 91 did not suppress the viral transcription. The other nucleotides located in conserved region II of Nipah virus will be studied as well, in order to find out the conserved sequences involved in the encapsidation as well as the RNA replication of Nipah virus. Since the constructed minigenome system could be used to produce the full-length of infectious recombinant virus from a cDNA of Nipah virus genome or antigenome, this will allow the future analysis of leader mutation generated in full-length of Nipah virus genome to be conducted. Thus, the correlation between the replication activity of the mutated minigenome and the ability of recombinant virus carrying that mutation to replicate could be observed. The recombinant virus with reduced replicative ability could be essential for development as a vaccine candidate.

ACKNOWLEDGMENTS

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REFERENCES

Calain, P & Roux, L. 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *Journal of Virology* 76: 4822-4830.

Chua, Kaw Bing, Goh, Khean Jin, Wong, Kum Thong, Kamarulzaman, A., Tan, P.S.K., Ksiazek, T.G., Zaki, S.R., Paul, G., Lam, Sai Kit & Tan, Chong Tin. 1999. Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. *The Lancet* 354: 1257-1259.

Durbin, A.P., Siew, J.W., Murphy, B.R. & Collins, P.L. 1997. Minimum protein requirements for transcription and RNA replication of a minigenome of Human Parainfluenza virus type 3 and evaluation of the rule of six. *Virology* 234: 74-83.

Eshaghi, M., Tan, Wen Siang, Ong, Swee Tin & Khatijah Yusoff. 2005. Purification and characterization of Nipah virus nucleocapsid protein produced in insect cells. *Journal of Clinical Microbiology* 43(7): 3172-3177.

Flint, S.J., Enquist, L.W., Krug, R.M., Racaniello, V.R. & Skalka, A.M. 2000. Genome replication and mRNA production by RNA viruses. In *Principles of Virology: Molecular Biology, Pathogenesis, and Control*, Washington: ASM Press.

Halpin, K., Bankamp, B., Harcourt, B.H., Bellini, W.J. & Rota, P.A. 2004. Nipah virus conforms to the rule of six in a minigenom replication assay. *Journal of General Virology* 85(3): 701-707.

Harcourt, B.H., Tamin, A., Halpin, K., Ksiazek, T.G., Rollin, P.E., Bellini, W.J. & Rota, P.A. 2001. Molecular characterization of the polymerase gene and genomic termini of Nipah virus. *Virology* 287: 192-201.

Harcourt, B.H., Tamin, A., Ksiazek, T.G., Rollin, P.E., Anderson, L.J., Bellini, W.J. & Rota, P.A. 2000. Molecular characterization of Nipah virus, a newly emergent paramyxovirus. *Virology* 271: 334-349.

Hoffman, M.A. & Banerjee, A.K. 2000. Precise mapping of the replication and transcription promoters of Human Parainfluenza virus type 3. *Virology* 269: 201-211.

Kawaoka, Y. 2004. *Biology of Negative Strand RNA Virus: The Power of Reverse Genetics*. Germany: Springer-Verlag Berlin Heidelberg.

Marcos, F., Ferreira, L., Cros, J., Park, M.S., Nakaya, T., García-Sastre, A. & Villar, E. 2004. Mapping of the RNA promoter of Newcastle disease virus. *Virology* 331(2005): 396-406.

Mayo, M.A. 2002. A summary of taxonomic changes recently approved by ICTV. *Arch Virology* 147: 1655-1663.

Ministry of Health Malaysia 2001. Outbreak of JE/Nipah in Malaysia. In *JE/Nipah outbreak in Malaysia*, pg. 7-10. Vol. 1. Kuala Lumpur: Disease Control Division, Department of Public Health, Ministry of Health Malaysia.

Peeples, M.E. & Collins, P.L. 2000. Mutations in the 5' trailer region of a Respiratory Syncytial virus minigenome which limit RNA replication to one step. *Journal of Virology* 74(1): 146-155.

Sambrook, J. & Rusell, D.W. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd Ed. Vol. 2. New York: Cold Spring Harbor Laboratory Press.

Tapparel, C., Maurice, D. & Roux, L. 1998. The activity of Sendai virus genomic and antigenomic promoters requires a second element past the leader template regions: a motif $(GNNNNN)_3$ is essential for replication. *Journal of General Virology* 72(4): 3117-3128.

Walpita, P. & Peters, C.J. 2007. *Cis*-acting elements in the antigenomic promoter of Nipah virus. *Journal of General Virology* 88: 2542-2551.

Wagner, E.K. & Hewlett, M.J. 2004. Replication strategies of RNA viruses requiring RNA-directed mRNA transcription as the first step in viral gene expression. In. *Basic virology*, pg. 258-262. 2nd Ed. Malden: Blackwell Science Ltd.

Wang, Lin Fa, Harcourt, B.H., Yu, Meng, Tamin, A., Rota, P.A., Bellini, W.J. & Eaton, B.T. 2001. Molecular biology of Hendra and Nipah viruses. *Microbes and Infection* 3: 279-287.

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