Cis-acting elements in the antigenic promoter of Nipah virus

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INTRODUCTION

Nipah virus (NiV) is one of several newly emergent zoonotic viruses that cause severe and rapidly progressive febrile encephalitis in humans. Disease outbreaks have been reported in Malaysia, Singapore, Bangladesh and India, with up to 75% mortality (Chua et al., 1999; Hsu et al., 2004; ICDDRBR, 2004a; Chadha et al., 2006). In addition, nosocomial transmissibility (Chadha et al., 2006) and person-to-person transmission (ICDDRBR, 2004b; WHO, 2004) have been reported. NiV may well be infectious by aerosol, which would facilitate its delivery as an agent of bioterrorism. In addition, its ability to infect swine makes it a potential agent of agro-bioterrorism with the resultant economic impact (Garner et al., 2001). Concerns about outbreaks in Malaysia (Chua et al., 2000, 2005). Reporting of the virus comprises six genes, 3'-N-P-M-F-G-L-5', each flanked by gene-end and gene-start signal sequences (Harcourt et al., 2000, 2005). Bordering the genes at the 3' and 5' viral termini are short non-transcribed sequences, the leader (52 nt) and trailer (30 nt). The non-coding 112 nt at the 3' end of the genome (genomic promoter, GP) include the leader sequence and the proximal non-coding nucleotides of the N gene. The 5'-terminal non-coding 100 nt (the complement of the antigenic promoter, AGP) include the trailer and the adjacent untranslated region (UTR) of the L gene. In paramyxoviruses, including NiV, the cis-acting sequence elements that direct mRNA transcription, replication, encapsidation and possibly packaging are embedded in the promoter nucleotides (Lamb & Kolakofsky, 2001; Halpin et al., 2004); mRNA transcription is exclusively initiated from the GP, whilst replication is controlled from both the GP and AGP to make the full-length antigenome and genome copies, respectively. Promoter–polymerase recognition and interaction in most of the viruses in the subfamily Paramyxovirinae is contingent upon N-protein-encapsidated, polyhexameric length (6n+0) genomes and antigenomes (the ‘rule of six’; Calain & Roux, 1993; Pelet et al., 1996; Kolakofsky et al., 1998), where each N protein subunit binds to precisely 6 nt (Egelman et al., 1989), known as a hexamer. The viral polymerase recognizes the template in this encapsidated form to initiate the life cycle under the control of functional cis-acting regulatory elements. The encapsidated (mini)genomic/antigenomic RNA template and transacting factors N, P and L are required for replication (Lamb & Kolakofsky, 2001).
In NiV and related viruses in the subfamily Paramyxovirinae, the 3’-terminal 12–18 nt of the genomic and antigenic RNAs are conserved and are thought to comprise the polymerase-binding site. In addition, there is an internal region in the GP and AGP of these viruses that has been attributed a regulatory role (Blumberg, et al., 1991; Tapparel et al., 1998). However, experimental evidence for minimal promoter length and organization is limited for many paramyxoviruses and is absent in NiV. Understanding the promoter structure and promoter–polymerase interactions at the molecular level should facilitate the development of targeted strategies to combat NiV disease. In this study, using a plasmid-driven minigenome system, we dissected the NiV AGP nucleotides to identify the location and boundaries of the cis-acting signals contained in them.

**METHODS**

**Cells and viruses.** A constitutively expressing T7 polymerase cell line, BHK-T7, was used for all transfections. Cells were grown in minimum essential medium alpha, supplemented with 10% fetal bovine serum and 10 μg puromycin ml⁻¹ (Sigma).

**NiV minigenome system and mutant constructs.** The vaccinia virus-driven NiV minigenome system has been described previously (Halpin et al., 2004) and was kindly provided by Dr Paul Rota (CDC, GA, USA). NiV-CAT (the wild-type minigenome construct) is a genome analogue (Fig. 1a) in which the chloramphenicol acetyl transferase (CAT) open reading frame is flanked by the promoter nucleotides of a NiV isolate from the Malaysian outbreak (GenBank accession no. AF212302). The truncated T7 promoter and the hepatitis delta ribozyme sequences are placed immediately adjacent to the trailer and leader nucleotides, respectively, so that the 876 nt T7-driven antisense minigenome transcript generated from it has precise viral ends. The construct is also made to obey the rule of six (Calain & Roux, 1993; Kolakofsky et al., 1998). The NiV N, P and L expression plasmids have been cloned into a pTM1 backbone containing an internal ribosome entry site (Halpin et al., 2004). For the present study, this system was optimized to function in the cell line BHK-T7 constitutively expressing T7 polymerase.

Using NiV-CAT as template, a series of mutant minigenomes with nucleotide substitutions at specified positions within the promoter region was created by primer-mediated site-directed mutagenesis (QuickChange kit; Stratagene). Each mutation involved replacement of the nucleotide with its complementary base. Mutants were named to denote either the mutated hexamer (e.g. agpH1) or the mutated nucleotides (e.g. agp19–36). The presence of the planned substitutions and the absence of unintended changes were verified by sequencing all of the mutants. Substitutions that disrupted a control element resulted in altered minigenome activity.

**Transfections and CAT rescue.** CAT rescue was achieved by transfecting semi-confluent monolayers of BHK-T7 cells in 60 mm dishes with equal concentrations of NiV-CAT and the mutant minigenome plasmids, co-transfected with the support plasmids N, P and L, using Lipofectamine 2000 (Invitrogen). A reaction with omission of the plasmid expressing the L or N protein served as a negative control. Transfected cells were incubated at 37 °C for 5 h and then supplemented with serum-containing medium and further incubated. Following replication (~48 h post-transfection), the cells were harvested to quantify CAT enzyme levels and minigenome-specific RNA species.

**CAT assays.** A fluorogenic substrate [FAST CAT (deoxy) Chloramphenicol Acetyl Transferase Assay kit; Molecular Probes] was used according the manufacturer’s instructions and allowed accurate quantification over a wide linear range. CAT assays were performed at 37 °C for ~3.5 h using ~5% of the total cell lysate to measure

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**Fig. 1.** NiV minigenome structure. (a) Schematic showing the NiV minigenome cassette and essential flanking sequences. Le, 3’ non-transcribed leader region of the NiV genome; N-GS, N gene transcription start; N-5’UTR, 5’ UTR region of the N gene; CAT ORF, CAT gene open reading frame; L-3’UTR, 3’ UTR region of the L gene; L-GE, L gene transcription stop; Tr, 5’ non-transcribed region of the NiV genome; T7TP, truncated T7 promoter, which does not transcribe the three non-viral G residues; T7Tx2, tandem T7 terminators; HDR, hepatitis delta ribozyme sequence. (b) Sequence of the 5’ terminus of the NiV genome (complement of the 3’ terminus of the NiV AGP). The 5’-terminal 96 nt of the untranslated 100 nt of the NiV genome (complement of the NiV AGP) are written as hexamers and annotated as agpH1 to agpH16. The evolutionarily conserved G residues at position 1 of hexamers 14, 15 and 16 are indicated in bold and underlined.
enzyme levels in the linear range. The separation and detection of acetylated product from unutilized substrate was achieved by thin layer chromatography. Modulation of CAT activity was quantified using the Bio-Rad Quantity One Image Analysis software package (Bio-Rad Laboratories) and expressed as a percentage relative to that from NiV-CAT. The reproducibility of CAT enzyme levels in mutants was verified by testing the mutants in two to four independent experiments.

**Transfection and genome-specific RNA levels.** Transfections were carried out as described above and after 24 h, actinomycin D (actinomycin D-mannitol; Sigma) was added to each dish to a concentration of 5 μg (ml medium)^-1. At 48 h post-transfection, cytoplasmic extracts were treated with micrococcal nuclease (S7 nuclease; Roche Applied Science) as described previously (Bankamp et al., 2002) with minor modifications. Briefly, cells were treated with 200 μl nuclease buffer at 30 °C for 1 h. Total cell RNA was extracted using the TRIZOL-LS method (Invitrogen). The RNA was treated with DNase Turbo (Ambion) to digest the input plasmid DNA and then used in a thermostable rTth RT-PCR assay (Two-step rTth RT-PCR kit; Applied Biosystems) for quantitative analysis of genome levels in mutants relative to wild type as described below.

**rTth RT-PCR assay and densitometry measurements for genome-specific RNA levels.** Strand-specific RT-PCR was undertaken at 70 °C using a thermostable polymerase according to the manufacturer’s recommendations. Equal concentrations of each of the RNA samples and a leader-specific sense primer were used to detect genome-sense replication product. The concentration of input RNA was measured by UV absorbance and their equivalence was confirmed by agarose gel electrophoresis; gel analysis also confirmed the integrity of the input RNA. Following cDNA synthesis, equal volumes of each reverse transcriptase reaction were used for PCR amplification with leader- and trailer-specific primers. The number of PCR cycles for the amplification was limited to 22–25 cycles so that genome levels could be analysed during the exponential phase of amplification (Applied Biosystems User Bulletin #2) to ensure meaningful quantitative comparison of levels. A reaction with omission of template RNA and another with omission of polymerase during cDNA synthesis served as negative controls. PCR products were visualized by agarose gel electrophoresis and analysed for relative levels by densitometry measurements using ONE-DSCAN software (Scanalytics Inc.). Experimental variability was controlled by repeating each analysis in two to four independent experiments. Primer sequences used for cDNA synthesis and PCR amplification are available upon request.

**RESULTS**

Mutational analysis limited to substituting individual nucleotides or individual hexamers may not indicate whether combinations of nucleotides at multiple positions may be responsible for controlling function (Hoffman & Banerjee, 2000; Harmon & Wertz, 2002; Walpita 2004). For this reason, the promoter nucleotides in this study were first scanned to enable broad mapping of the AGP regions required for reporter gene activity; mutational analysis on the basis of RNA synthesis was also undertaken to identify the specific regions that controlled replication. This was followed by a fine-mapping strategy to define in more detail the sequence elements contained within them and to demarcate their boundaries. Reproducibility was verified in two to four repeat experiments and the CAT activity of the mutants presented in Figs 2, 4 and 5 was expressed as a percentage (± SD) relative to that in NiV-CAT.

![Fig. 2. Functional regions in the NiV AGP based on broad mapping. The experimental design and methods are as described in the text. In the CAT assay, the separation of the unutilized deoxy substrate (left band) and the single acetylated CAT enzyme product (right band) was achieved by thin layer chromatography. Neg. C, negative control comprising all the components except the NiV N or L expression plasmid. Each mutant name denotes the substituted nucleotides. CAT activity in the wild-type NiV-CAT and the mutant constructs, expressed as a percentage (± SD) relative to the wild type, is shown to the right of each reaction.](image)

**Broad-mapping studies**

Ninety-six of the 100 nt of the 5′ NiV AGP were substituted sequentially to their respective complementary bases, mainly in blocks of 18, to generate a series of mutant minigenomes. The effect of substitutions in these mutants (agp1–18, agp19–36, agp37–54, agp55–72, agp73–78 and agp79–96) was assayed by measuring CAT levels relative to wild type. In the optimized system, CAT activity in NiV-CAT was high; background activity in the negative control was minimal to none and was factored into the calculations for CAT measurement. The results presented in Fig. 2 showed that mutating nt 1–54 and 79–96 (agp1–18, agp19–36 and agp37–54, and agp79–96) eliminated virtually all activity. Substitutions at positions 73–78 (agp73–78) resulted in less but consistent reduction (52±21 % loss) in function. Only one block of substitutions, which spanned nt 55–72 (agp55–72), had no effect on CAT expression levels. The same mutants were then analysed for minigene-specific RNAs to identify the sequences that control replication. In addition, the two functional regions (nt 1–54 and 79–96) were fine mapped by reporter gene expression levels to define in more detail the cis-acting elements contained within them.

**Replication control elements in NiV-AGP**

Mutations in the AGP can affect CAT activity if the designed substitutions disrupt a replication or possibly an encapsidation control element; both of these functions are
tightly coupled, are initiated in the same region and may be under the control of the same conserved nucleotides. Alternatively, substitutions in the 3' or 5'UTR of the L and N gene (which effectively form the 3' and 5'UTRs of the CAT mRNA respectively, Fig. 1) can impact on mRNA stability and hence modulate CAT protein expression levels (Jacobson & Peltz, 1996). To differentiate between these two possibilities, RNA levels were measured specifically to identify the cis-acting elements that control replication. The rTth RT-PCR method used for genome detection has been used previously in several studies for strand-specific detection of viral RNA and has been reported to have a 10 000- to 100 000-fold differential in the identification of correct over incorrect strand detection (Lanford et al., 1995). Self-priming and mispriming appeared to be avoided or minimized by carrying out the reverse transcriptase step at 70 °C and by using a minimal amount of RNA for amplification. The procedural and other steps taken to ensure accurate quantification are described in Methods. Although the mechanisms of genome synthesis and gene expression and regulation are inherently different (Lamb & Kolakofsky, 2001), and the assays have differing sensitivities and limitations, the results presented in Fig. 3 showed that the genome levels (876 bp fragment) were in general agreement with those obtained on the basis of CAT enzyme levels (Fig. 2). Substitutions in agp1–18, agp19–36 and agp79–96 had a profound effect on replication competence as well as on gene expression. Mutations in agp73–78 also reduced CAT expression (48 ± 21 %) and genome levels (76 ± 7 %), but to a much lesser degree. The one clear difference was in agp37–54, which contained a disrupted L gene-end signal; genome synthesis was unaffected in this mutant but CAT activity was virtually eliminated. Thus, in NiV, genome synthesis appears to be controlled by nt 1–36 and 73–96 from the 5' genome end. However, because of the broad-map approach thus far, the boundary of the terminal element was not clear. Therefore, a further limited analysis of the region between nt 25 and 42 was carried out on the basis of one-hexamer changes (in mutants agpH5, agpH6 and agp7, respectively; Fig. 3b, d) to define the border. RNA synthesis was compromised in agpH5 and agpH6, but not in agpH7, suggesting that this control element was contained within the terminal 36 nt.

**Fine mapping of the 5'-terminal 54 nt**

The functional region comprising the 5'-terminal 54 nt, deduced on the basis of the broad-map approach, was fine mapped by making a set of nine additional mutants with smaller, one-hexamer substitutions (mutants agpH1 to agpH9). CAT levels in these mutants (Fig. 4) demonstrated that the stringency of requirement for these nucleotides
varied, with alterations in the terminal two hexamers (nt 1–12) eliminating virtually all CAT activity, whilst mutations in agpH3 to agpH7 (nt 13–42) resulted in a loss of activity of between ~50 and 80 %. Changes in agpH8 and agpH9 (nt 43–54) had no effect on function. Based on a previous report, it is possible that substitutions in the T7 promoter-proximal nucleotides influence T7 polymerase-mediated transcription efficiency (Peeples & Collins, 2000) or result in suboptimal transcription initiation so that the rule of six is compromised. To exclude the possibility that these considerations may have contributed to the reduction in CAT activity in agpH1 and agpH2, an additional experiment was carried out in which an equal concentration of T7-driven in vitro-generated (according to the MEGAScript T7 kit instructions; Ambion) minigenome RNA transcript was transfected instead of minigenome plasmid DNA. The integrity and equivalence of each RNA transcript was verified (Fig. 4b) for transfection and CAT rescue as described in Methods. The results (Fig. 4c) were the same as described above (Fig. 4a), indicating that this technical consideration did not result in false interpretation of the data. Thus, only the terminal 42 nt (agpH1 to agpH7) were required to retain optimal CAT activity, and not the 54 bases as indicated by the broad-mapping approach. A comparison of the results of broad- (Fig. 2) and fine-mapping strategies (Fig. 4) also showed that the disruptive effect of the substitutions in hexamers 3–7 was cumulative. For example, changing the 18 nt in the mutant agp19–36 resulted in an inactive construct (Fig. 2). The same nucleotides, when changed one hexamer at a time, also compromised function but to a lesser degree (Fig. 4a, agpH4, agpH5 and agpH6).

Thus, the results of fine mapping of the terminal 54 nt of NiV-AGP showed that the 42 trailer-end nucleotides were required for retaining minigenome function and of these, the terminal 36 nt contained sequence elements that controlled genome synthesis. Changes in the L gene-end motif compromised CAT activity in agpH6 and agpH7 (Fig. 4a). Substitutions in agpH6 perturbed the intergenic trinucleotide GAA in addition to the L gene poly(A) tail and also modulated RNA synthesis (Fig. 3b, d).

**Fine mapping of nt 79–96 from the 5’ genome end**

The control region comprising nt 79–96 from the 5’ genome end (hexamers 14, 15 and 16; Fig. 1b) is at an equivalent position to that seen in the majority of paramyxoviruses and has a G residue at the first position (G1) of each of its three hexamers (Tapparel et al., 1998). The regulatory role of G1 residues has been predicted previously (Tapparel et al., 1998) and has been established in several paramyxoviruses, but the function of the remaining nucleotides in these hexamers remains untested (Hoffman & Banerjee, 2000; Marcos et al., 2005) or is at variance (Tapparel et al., 1998; Walpita, 2004). NiV is like other paramyxovirinae in that the importance of G1 is clearly evident from the results presented in Fig. 5(a): a mutant...
minigenome with these residues replaced simultaneously in the three hexamers was virtually inactive (~90% reduction in activity). The results of three additional mutants in which this nucleotide was changed one by one showed an obvious necessity for the G1 of the 14th and 15th hexamers, and these changes downregulated CAT expression by ~83 and ~74%, respectively. However, the loss of G1 from the 16th hexamer (Fig. 5a, lane 6) was less detrimental: it reduced CAT activity by approximately 50%.

The role of the remaining five nucleotides of the three hexamers (at positions 2–6) was tested in a mutant in which all of these nucleotides were changed simultaneously to their complementary base whilst keeping the G1 nucleotides unchanged. Such alterations had a crippling effect on function (95% reduction in CAT gene expression; Fig. 5b, lane 3), indicating that, in NiV, these other nucleotides are also important for function. To examine the role of the nucleotides at positions 2–6 in individual hexamers, three more mutants were made with substitutions at these positions in individual hexamers. CAT enzyme levels in mutants with substitutions in the 14th and 15th hexamers (Fig. 5b, lanes 4 and 5) was only ~6 and 28% relative to that in the wild-type NiV-CAT, whilst a similar alteration to the 16th hexamer (Fig. 5b, lane 6) appeared to have no effect on function. Further mutants were then made to assess the contribution of the individual nucleotides present at positions 2–6 by making simultaneous point mutations at equivalent positions in all three hexamers (Fig. 5c). Substitutions at positions 5 or 6 resulted in elimination of ≥98% of CAT activity, whilst such changes at positions 2, 3 or 4 did not cause any reduction in reporter gene levels.

Thus, mutational analysis of hexamers 14, 15 and 16 showed a requirement for the nucleotides at positions 1, 5 and 6 of the first two hexamers, and the first position of the remaining hexamer. The sequence characteristics of this element could therefore be described as a 5′- (GNNNUG)_{14-15}(GNNNNN)_{16} motif. The redundancy of the nucleotides at positions 2–6 of hexamer 16 for minigenome function defined the minimal AGP length as 91 nt from the 5′ end of the genome.

In summary, genetic analysis of the NiV AGP identified two discrete replication control elements separated by a 36 nt putative spacer sequence. The internal regulatory region spanned four hexamers, and three of these were characterized by a distinct sequence motif. Perturbation of the nucleotides in the L gene-end signal compromised CAT activity.

Fig. 5. Fine mapping to determine the sequence characteristics of the NiV AGP internal control element (hexamers 14, 15 and 16). The experimental strategies and methods are as described in the text. The sequence of the wild-type and mutant minigenomes in the three hexamer regions is written as the nascent strand (5′→3′) and all substitutions are shown in bold. (a) Analysis of the role of the G1 residues of hexamers 14, 15 and 16. (b) Analysis of the role of the nucleotides at positions 2–6 of hexamers 14, 15 and 16. (c) Analysis of the role played by individual nucleotides at hexameric positions 2–6. WT, wild-type minigenome NiV-CAT.
**DISCUSSION**

NiV and Hendra virus (HeV) have several distinctive genetic features (Harcourt et al., 2005), which merited the creation of a new genus, *Hemipavirus*, within the subfamily *Paramyxovirinae*. The results of this study showed that, whilst the overall make-up of the NiV AGP was bipartite, similar to that seen in other paramyxoviruses (Tapparel et al., 1998; Hoffman & Banerjee, 2000; Mioulet et al., 2001; Marcos et al., 2005), it also had some distinct differences: each of the two NiV AGP control elements was bimodal, characterized by a conserved region that was critical (nt 1–12 and 79–91) and an adjacent non-conserved region (nt 13–36 and 73–78), which appeared to be relatively less important for function. The regulatory role of these less critical regions was emphasized by the cumulative negative effect of substitutions in this part of the terminal element. The internal control element was also distinct in that it spanned four hexamers instead of the three in other paramyxovirinae (Tapparel et al., 1998; Murphy & Parks, 1999) and was characterized by a distinct sequence motif, 5’-(GNNUG)_{14-15}(GNNNNN)_{16-3’}. These findings may provide further support for the classification of *Hemipavirus* as a separate genus.

The stringent requirement for the terminal 12 nt of NiV AGP is consistent with the highly conserved nature of the 3’- and 5’-terminal ~12 nt of paramyxoviruses, and their regulatory role in RNA synthesis has been confirmed experimentally (Tapparel & Roux, 1996; Murphy & Parks, 1998; Hoffman & Banerjee, 2000; Mioulet et al., 2001; Marcos et al., 2005). The importance of the contiguous non-conserved nucleotides of the terminal element (nt 13–36) was indicated by the one-hexamer changes, which showed a loss of CAT activity varying between ~50 and 80% of that of wild type. The requirement for these nucleotides in controlling replication was underscored further by two additional supporting observations. First, changes in the bases in the same region in multiple hexamers, for example in mutant agp19–36, seemed to have a cumulative negative effect, resulting in elimination of virtually all activity (compare the results in Fig. 4a, lanes 6–8, with Fig. 2, lane 4). Secondly, the nucleotides that did not appear to have a control function, i.e. nt 43–72, could be replaced in individual hexamers as in agpH8 and agpH9 (Fig. 4), or in combination in multiple hexamers as in agp55–72 (Fig. 2, lane 6), without any apparent disruption in function. These results suggested that nucleotides in multiple hexamers may be interacting with each other and/or with polymerase proteins, to control function collectively.

The functional significance of non-conserved nucleotides adjacent to the conserved 3’- and 5’-terminal core promoter has been reported before for several segmented as well as non-segmented negative-stranded RNA viruses (Calain & Roux, 1995; Tapparel & Roux, 1996; Zheng et al., 1996; Hoffman & Banerjee, 2000; Keller et al., 2001; Lowen & Elliott, 2005; Hoffman et al., 2006). A previous study of the human parainfluenza virus 3 (HPIV3) GP also demonstrated the bimodal nature of its terminal region where the 3’-end 12 nt were essential for function whilst the contiguous bases at nt 13–55 were important but not critical in promoting replication (Hoffman & Banerjee, 2000). In contrast, in the Newcastle disease virus (NDV) GP and AGP, and in the AGP of the paramyxovirus simian virus five (SV5) (Murphy & Parks, 1999), a regulatory role was assigned only to the terminal 18 nt and internal 18 nt at positions 73–90 (conserved regions CRI and CRII, respectively). A direct comparison of the promoter structures of different paramyxoviruses may not be valid, however, as results are often deduced on the basis of different experimental designs. Given the fact that many nucleotides across hexamers appear to control function cooperatively (Hoffman & Banerjee, 2000; Walpita, 2004), the choice of strategy may affect outcome. Subjectivity in data interpretation is another aspect that may confound comparisons among studies: promoter substitutions resulting in similar degrees of modulation have been interpreted variously in different studies (Hoffman & Banerjee, 2000; Marcos et al., 2005).

The length as well as the sequence make-up of the second replication control element in the NiV AGP was different from that seen in related viruses (Tapparel et al., 1998; Murphy & Parks, 1999). The 13th hexamer (mutant agp73–78) appeared to be important but not critical on the basis of CAT activity (Fig. 2), as well as RNA levels (Fig. 3a). The three remaining hexamers were crucial for maintaining replication competence and were characterized by the distinct 5’-(GNNUG)_{14-15}(GNNNNN)_{16-3’} motif. This sequence composition appeared to be more strictly conserved than in all other previously described analogous motifs: the (5’-GNNNNN-3’)₃ motif of Sendai virus (SeV) was the first to be described (Tapparel et al., 1998). The 5’-(GNNAN)₂GNNNCN-3’ motif in the closely related measles virus (MV) (Walpita, 2004) is unlike that in NiV, even when both were analysed using the same experimental approach. The motif in the AGP of SV5 (Murphy & Parks, 1999) in the nascent-strand RNA is (5’-NNNCG-3’); it is like the NiV motif with respect to position 6, but is different from the NiV motif and from SeV and MV motifs with respect to the G groups nucleotide. As the overall promoter structure in viruses of the subfamily *Paramyxovirinae*, including NiV, is similar, their differences with respect to their internal control motifs is intriguing. The significance, if any, of such differences needs to be evaluated experimentally. The properties of the virus promoter may have a role in disease pathogenesis (Banyard et al., 2005).

Analysis of the GP and AGP sequences in hexamers 14, 15 and 16 of two NiV isolates (one each from the Malaysian and Bangladesh outbreaks) with that of other related viruses (Fig. 6) highlights several salient features: there were several base differences between the two NiV isolates, and between NiV and HeV, but the bases at positions 1, 5 and 6 of the 14th and 15th hexamers, and position 1 of the 16th hexamer, which were essential for maintaining
minigenome activity (Fig. 5), remained invariant in all of them. A comparison of the henipavirus AGP and GP sequences with other paramyxoviruses has demonstrated conservation of the G1 residues (Tapparel et al., 1998). Conservation of the nucleotides at position 5 has also been described previously (Walpita, 2004) although the henipaviruses have a U instead of an A as found in the morbilli- and respiroviruses. A further review of the alignment in this study showed the invariable presence of a purine at position 6 of the 14th and 15th hexamers in all three groups of viruses and in all 18 promoters.

In NiV, the intergenic trinucleotides GAA between the L gene end and the trailer nucleotides are at nt 31–33 and the L gene-end polyadenylation signal is at nt ~34–42, and in the minigenome they form the 3'-end of the CAT message (Fig. 1). These nucleotides were perturbed in agpH6 and agp7, resulting in reduced CAT levels. The 3'-end non-coding nucleotides and poly(A) sequence of mRNAs are known to play important roles in maintaining mRNA stability, and mutating them may have altered CAT mRNA expression by destabilizing the CAT mRNA (Jacobson & Peltz, 1996). Genome synthesis was also inhibited in agpH6 (Fig. 3b) and may also have contributed to the reduced CAT activity in this mutant. Surprisingly, in NDV (Marcos et al., 2005), mutating the L gene-end polyadenylation signal appeared to have no effect on reporter gene activity.

The mechanism of interaction between the cis-acting control elements and the role of the conserved and non-conserved nucleotides in controlling replication is not clear. Changes in the AGP may modulate replication by disrupting polymerase binding, RNA encapsidation and/or the required interactions of the virus and/or host proteins. The terminal conserved nucleotides are thought to be important for polymerase recruitment and promoter activity, and they may also be needed to initiate encapsidation of the newly synthesized RNA strand (Blumberg et al., 1983; Moyer et al., 1991; Smallwood & Moyer, 1993; Kouznetzoff et al., 1998; Cowton et al., 2006). The importance of sequence specificity in promoter–polymerase interactions has been reported previously (Keene et al., 1981; Bae et al., 2001), and the recently solved crystal structure of vesicular stomatitis virus and rabies virus N–RNA templates (Albertini et al., 2006; Green et al., 2006) have provided further insights for base recognition in encapsidated RNA templates. The non-conserved regions adjacent to both the terminal and internal conserved elements in the NiV AGP were important for function, where each may contribute to binding affinity (Xu et al., 1997) and enhance the stability of promoter–polymerase interactions to initiate RNA synthesis. Alternatively, a promoter that is defective in this region may prevent the necessary interactions between the template RNA and the viral transacting proteins and/or host protein(s) (Wilusz et al., 1983; De et al., 1996; Zheng et al., 1996; Hoffman & Banerjee, 2000).

Several models have been proposed for the signalling of paramyxovirus replication. The bipartite structure of the NiV AGP and other paramyxovirus promoters is in general agreement with the model proposed for initiation of replication, where apposition of the terminal and internal control elements together form the polymerase recognition site (Lamb & Kolakofsky, 2001). However, based on data presented here, and in a previous study (Walpita, 2004), our working model proposes that the RNA template and the polymerase proteins make multiple contacts for correct and stable positioning on the template to initiate replication: the conserved nucleotides are required for making the sequence-specific contacts, whilst the less critical nucleotides are required for the energetics of binding (Xu et al., 1997). The internal control region has been proposed as a nucleation site to initiate encapsidation (Tapparel et al., 1998). As encapsidation is tightly coupled with replication, and as both of these functions begin in the same area, it is conceivable that the initiation of replication and encapsidation is controlled by the same promoter nucleotides.

### Table 1

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### Fig. 6

Sequence alignment of AGP and GP hexamers 14, 15 and 16 of representative henipaviruses, morbilliviruses and respiroviruses. Sequence alignment (written as the nascent strand, 5'→3') of two NiV isolates (Malaysia, GenBank accession no. AF212302; Bangladesh, GenBank accession no. AY988601), HeV, three morbilliviruses [MV, canine distemper virus (CDV) and rinderpest virus (RV)] and three respiroviruses [SeV, bovine parainfluenzavirus type 3 (BPIV3) and HPIV3]. A total of 18 promoters is shown. Positions 1, 5 and 6 of the 14th and 15th hexamers and position 1 of the 16th hexamer are boxed and in bold to highlight the sequence conservation at these positions.
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