Nipah virus conforms to the rule of six in a minigenome replication assay

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To study the replication of Nipah virus (NiV), a minigenome replication assay that does not require the use of infectious virus was developed. The minigenome was constructed to encode a NiV vRNA analogue containing the gene for chloramphenicol acetyltransferase (CAT) under the control of putative NiV transcription motifs and flanked by the NiV genomic termini. CAT protein was detected only when plasmids encoding the NiV minigenome, nucleocapsid protein (N), phosphoprotein (P) and polymerase protein (L) were transfected into CV1 cells. To determine whether NiV conforms to the rule of six, a series of plasmids encoding minigenomes that differed in length by a single nucleotide was tested in the replication assay. CAT production was detected only with the minigenome whose length was an even multiple of six. The replication assay was also used to show that the N, P and L proteins of NiV recognize cis-acting sequences in the genomic termini of Hendra virus (HeV) but not measles virus. While these results suggest that NiV uses a replication strategy that is similar to those of other paramyxoviruses, they also support the inclusion of NiV and HeV in a separate genus within the subfamily Paramyxovirinae.

INTRODUCTION

In late 1998, Nipah virus (NiV), a new zoonotic paramyxovirus that can infect humans and pigs, emerged in Malaysia. NiV caused 265 cases of encephalitis among humans, resulting in 105 deaths. Ninety-three per cent of the infected humans had occupational exposure to pigs. In pigs, the virus was responsible for a highly infectious respiratory disease with low mortality. To contain the outbreak, over 1 million pigs were culled. Molecular characterization of NiV showed that it was very closely related to another zoonotic paramyxovirus, Hendra virus (HeV), which emerged in Australia in 1994 (Chua et al., 2000; Harcourt et al., 2000, 2001).

HeV and NiV have been assigned to a new genus, Henipavirus, within the subfamily Paramyxovirinae of the family Paramyxoviridae (Mayo, 2002). The number and order of genes in HeV and NiV (3’-N-P-M-F-G-L-5’) are identical to those found in the respiroviruses and morbilliviruses. HeV and NiV have six transcription units encoding six structural proteins, the nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G) and polymerase (L). Like the morbilliviruses and respiroviruses, HeV and NiV encode multiple proteins, designated C, V and W, from the P gene (Harcourt et al., 2000). The genome of NiV is a single-stranded, negative-sense RNA that is 18 246 nt in length and the predicted gene-start, gene-end, RNA editing site and intergenic sequences are conserved compared with the respiroviruses and morbilliviruses (Vidal & Kolakofsky, 1989; Harcourt et al., 2000, 2001; Wang et al., 2000; Yu et al., 1998a, b). The genomic termini of NiV and HeV are highly conserved and complementary, as in other paramyxoviruses (Rima et al., 1995).

All the viruses in the subfamily Paramyxovirinae have genomes whose lengths are multiples of six (Hausmann et al., 1996). Genomes whose lengths deviate from ‘the rule of six’ do not replicate efficiently (Calain & Roux, 1993) and it has been proposed that the templates for transcription and replication are nucleocapsids in which each nucleoprotein subunit is associated with six nucleotides of genomic RNA. Both NiV and HeV have genomes whose lengths are multiples of six, but it was not known whether these viruses conform to the rule of six.

Transcription and RNA replication in NiV are thought to follow the same strategy as in other, related, paramyxoviruses, although to our knowledge no studies have been conducted to address this question directly. Laboratory studies of NiV must be conducted at biosafety level (BSL)-4. Since this biocontainment classification restricts the type and frequency of research projects, alternatives to the use of infectious virus are highly desirable. Reverse genetics systems allow the study of the replication of negative-strand RNA viruses in vitro without the use of...
infectious NiV. The present study describes the development of an in vitro replication system for NiV that does not require infectious virus and can be used at BSL-2. Here, we use this in vitro replication system to determine the minimum subset of viral proteins required for NiV transcription and RNA replication, and to show that NiV obeys the rule of six and that the N, P and L proteins of NiV recognize cis-acting sequences in the genomic termini of HeV.

METHODS

Cells and viruses. CV-1 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% foetal bovine serum (HyClone) and antibiotics (Gibco). The NiV strain used in this study was isolated directly from human brain tissue in Vero E6 cells (Harcourt et al., 2001). MVA-T7, the vaccinia virus recombinant that expresses bacteriophage T7 RNA polymerase, was provided by B. Moss, NIH, Bethesda, MD, and was propagated in primary chicken embryo fibroblasts.

Construction of plasmids. NiV RNA was extracted from infected cells as previously described (Harcourt et al., 2001). RT-PCR for the N and P genes was carried out using AMV reverse transcriptase and Taq polymerase (Roche), as described previously (Harcourt et al., 2001). The L gene was amplified using AMV reverse transcriptase (Roche) and the Elongase enzyme mix (Invitrogen) according to manufacturer's recommendations for long RT-PCR. All primers were based on the published sequence of the NiV genome (Harcourt et al., 2001; GenBank accession no. AF212302). Restriction sites included in primers are given in parentheses; primer sequences are available upon request. The N open reading frame (ORF) was amplified with primers NF (SpeI and NR (SalI) and ligated between the SpeI and SalI sites of the expression plasmid pTM1. The P ORF was amplified with primers PF (Sacl) and PR (Xhol) and cloned into pTM1. In the plasmid expressing NiV P, the C ORF was silenced by mutating the two consecutive start codons to ACG. The NiV L gene was amplified as two fragments. The 5' half of the L gene was amplified with primers LF1 (Ncol) and LR1 (XmaCI). The 3' half of the L gene was amplified with primers LF2 (Nhel) and LR2 (XmaCI). The 5' fragment was cloned into pTM1 using the Ncol and XmaCI sites and then the 3' fragment was added, using the internal Nhel site at nt 14724 and XmaCI sites. The sequences of all plasmids were determined and sequence data were analysed with version 10.0 of the sequence analysis software package of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984). Where necessary, nucleotides that deviated from the consensus sequences of NiV were repaired using the ExSite mutagenesis kit (Stratagene).

A measles virus minigenome, pMV107-CAT, a gift from M. Billetter (Sidhu et al., 1995), was used as a template for the plasmid containing the NiV minigenome. A DNA fragment containing the two T7 terminator sequences and the hepatitis delta virus ribozyme sequence was amplified from pMV107-CAT with primers NGF1 (Sacl) and NGR1. NGR1 included nt 1–41 of the 3' end of the NiV genome. Two additional rounds of PCR, each using the product of the previous amplification as a template, were used to extend the NIV sequence to nt 95. Primer NGR3 included a BamHI site (nt 90 in the NiV genome) allowing this fragment to be inserted into pUC19 using SacI and BamHI (pUC fragment 1). A second fragment was generated by amplification of the chloramphenicol acetyltransferase (CAT) gene from pMV107-CAT with primers NGF2 (BamHI) and NGR4. NGF2 included nt 90–115 of the NiV genome, overlapping with pUC fragment 1, and NGR4 included nt 18147–18187 of NiV. Two rounds of amplification were used to extend the NiV sequence to nt 18246, and the T7 promoter sequence was added to this fragment by reverse primer NGR6. NGR6 contained a HindIII restriction site, allowing the fragment to be inserted into pUC fragment 1 with BamHI and HindIII to produce pNiV-CAT. Additional nucleotides were added to primer NGR4 to generate plasmids encoding minigenomes with lengths ranging from 873 to 878 nt (see Fig. 3). To generate the plasmid containing the HeV minigenome, the CAT gene was amplified from pNiV-CAT with primers HGF1 and HGR1. HGF1 included nt 61–115 of the HeV genome and HGR1 included nt 18134–18184 of the HeV genome. Two additional rounds of PCR, each using the product of the previous amplification as a template, extended the HeV sequence to include nt 1–115 from the 3' genomic terminus and nt 18134–18234 from the 5' genomic terminus by adding nucleotides to primer pairs HGF2 and HGR2, and HGF3 and HGR3. Primer HGF3 included a BsiRI restriction site and primer HGR3 a HindIII site. The DNA fragment was cloned into pNiV-CAT, replacing the NiV termini and the CAT ORF. Additional nucleotides were added to primer HGR3 to generate minigenomes with various lengths as above. The DNA concentrations of the purified plasmid preparations were determined with the fluorochrome Hoechst 33258 (Gallagher, 1996).

Transfection and CAT ELISA. CV-1 cells in 35 mm dishes were infected with MVA-T7 at an m.o.i. of 5 and transfected 45 min later. To determine the ratio of plasmid concentrations that produced the greatest amount of CAT, a series of titration experiments were conducted. For the standard assay, a mixture of 1–25 µg pNiV-N, 0·8 µg pNiV-P, 0·4 µg pNiV-L and 3·5 µg minigenome plasmid in Opti-MEM (Gibco) was transfected with Cellfectin reagent (Invitrogen) according to the manufacturer's instructions. For negative controls, pNiV-N was replaced with pTM1. For each minigenome, three plasmid preparations were tested in duplicate and every experiment was carried out at least three times. At 42–45 h after transfection, cells were harvested and CAT production was measured by ELISA (Roche). The protein concentration of the cytoplasmic extracts was measured using a BCL kit (Pierce).

Northern blot analysis. Transfections were carried out as described above and 24 h after transfection, 10 µg actinomycin D (actinomycin D-mannitol; Sigma) ml−1 of medium was added to each well. RNA was purified 42–45 h after transfection. Poly(A)+ RNA was isolated directly from cell lysates using Oligotex particles and buffers (Qiagen) according to the manufacturer's protocol, which was modified for batch procedure and followed by ethanol precipitation. For the analysis of minigenome replication, cytoplasmic extracts were treated with micrococcal nuclease (S7 nuclease; Roche) as previously described (Bankamp et al., 2002). Samples were additionally treated with RNase-free DNase (Ambion) to remove residual plasmid DNA.

One half of the RNA extracted from a 35 mm dish was separated by electrophoresis on a 3–7% formaldehyde, 1·5% agarose gel and transferred to a nylon membrane by vacuum blotting, then fixed by UV cross-linking. In vitro transcription of CAT gene-specific riboprobes (Bankamp et al., 2002) and hybridization and detection of the bands were carried out using the digoxigenin system (Roche) according to the manufacturer's recommended protocol. Hybridization signals were visualized by autoradiography.

RESULTS

For viruses in the subfamily Paramyxovirinae, the N, P and L proteins are necessary and sufficient for both transcription and genome replication. Therefore, the N, P and L genes of NiV were amplified by RT-PCR from RNA isolated from NiV-infected cells and cloned into the expression vector pTM1. The sequences of the cloned N, P and L genes were identical to the previously published
sequences for these genes (Harcourt et al., 2001) except for two positions in the P gene. To study the function of P in the absence of C, the P cDNA was modified at two positions to eliminate both predicted translation start sites of the C ORF (Harcourt et al., 2000; GenBank accession no. AF212302). Successful expression of the N and P genes in Vero cells transfected with pNiV-N and pNiV-P was confirmed by Western blot assays, which were performed using a monospecific antiserum to NiV P or a monoclonal antibody to NiV N (data not shown). Specific antiserum to the NiV L protein is not available.

When transcribed by T7 polymerase, the plasmid containing the minigenome of NiV, pNiV-CAT, generated an 876 nt RNA containing exact copies of the 3' and 5' non-coding regions of the genomic RNA of NiV (Fig. 1). The 3' terminus of the minigenome RNA was created by self-cleavage mediated by a hepatitis delta virus ribozyme sequence. In the minigenome, all the genes of NiV were replaced with a negative-sense copy of the CAT gene, which was flanked by the predicted N gene transcription start sequence, the 5' non-translated (NTR) region of the N gene mRNA, the 3'NTR of the L gene mRNA and the L gene transcription stop sequence (Fig. 1). The CAT translation start codon replaced the N start codon and the CAT translational stop codon replaced the L stop codon. pNiV-CAT contained a 4 nt insertion between the CAT stop codon and the start of the 3’NTR of the L gene to make the length of the minigenome RNA evenly divisible by six.

When pNiV-CAT was transfected into CV-1 cells that had been infected with MVA-T7, CAT expression was only detected when the plasmids encoding the N, P and L proteins were co-transfected. No CAT protein was detected in the absence of minigenome plasmid or in the absence of plasmid encoding NiV N (Fig. 2a). The optimum ratios and amounts of the minigenome and support plasmids were determined on the basis of CAT enzyme expression. No CAT was detected when pNiV-N, pNiV-P or pNiV-L was omitted from the transfection mix and the background signals on these negative controls were equivalent (data not shown). In subsequent experiments, pNiV-N was routinely replaced with pTM1 in the negative control samples.

RNA from CV-1 cells transfected with pNiV-CAT and the plasmids encoding NiV N, P and L was analysed using Northern blot analysis and hybridized to either positive- or negative-strand-specific riboprobes (Fig. 2b and c). When poly(A)^+ selected mRNA was hybridized with a negative-sense CAT-specific riboprobe, CAT mRNA was detected in cells transfected with pNiV-CAT and all three support plasmids but not when pNiV-N was replaced by pTM-1 (Fig. 2b). To analyse minigenome replication, cell lysates were treated with micrococcal nuclease before Northern blot analysis with a positive-sense CAT-specific probe to detect genomic-sense RNA (Fig. 2c). When pNiV-CAT was transfected with the three support plasmids, a nuclease-resistant RNA that migrated with an approximate molecular size of 800 nt was detected. No nuclease-resistant RNA was detected when pNiV-N was replaced with pTM1. Therefore, the detection of CAT protein correlated with the presence of both CAT mRNA and encapsidated minigenome RNA. These results showed that the termini of the NiV minigenome contained all of the sequences required for transcription and replication and that the proteins encoded by the support plasmids were functioning properly.

To determine whether NiV obeys the rule of six, five additional minigenomes were constructed, which each differing in length by a single nucleotide (Fig. 3). One to six nucleotides were inserted at the junction between the CAT gene translational stop signal and the 3’NTR of the L gene mRNA (Fig. 1), resulting in minigenome RNAs with sizes ranging from 873 to 878 nt (–3, –2, –1, +1 and +2 nt in length relative to pNiV-CAT). pNiV-CAT encoded the only minigenome with a size that was evenly divisible by six. Expression of CAT protein was detected only when pNiV-CAT was co-transfected with the plasmids encoding

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**Fig. 1.** Schematic representation of the RNA minigenome produced from pNiV-CAT drawn as a single strand of negative-sense RNA. Lengths (nt) of the leader, trailer, gene start (GS), gene end (GE) and non-translated regions (NTR) of N and L gene mRNAs, the CAT ORF and the positions of the T7 promoter and hepatitis delta virus ribozyme cleavage site (HDV) are shown. Underlined bases in the insert are the four bases added to correct the length of the minigenome to be evenly divisible by six.
NiV N, P and L proteins (Fig. 3). No CAT protein was
detected when the other minigenome plasmids were used
in the replication assays. The results showed that efficient
CAT expression was only achieved with pNiV-CAT and
strongly suggest that NiV, like other members of the
subfamily Paramyxovirinae
(Mayo, 2002), obeys the rule of six.

NiV and HeV have been placed in a new genus within the
subfamily Paramyxovirinae (Mayo, 2002). Within this
subfamily, it has been shown that it is possible to drive
replication of a minigenome from one virus with support
proteins derived from another virus from the same genus
(Pelet et al., 1996). To test whether the NiV N, P and L
proteins could support replication and transcription of a

Fig. 2. Levels of transcription and replication in the plasmid-based minireplicon assay. (a) Expression of the CAT reporter
gene in a plasmid-based minireplicon assay. CV-1 cells were infected with MVA-T7 and transfected with pNiV-N, pNiV-P,
pNiV-L and pNiV-CAT as indicated. CAT concentration in cytoplasmic extracts was measured by ELISA 48 h after
transfection. Results from four separate experiments are shown, with the standard error indicated by the vertical bars. (b, c)
CV-1 cells were infected with MVA-T7 and transfected as described above. pTM1 was used in place of pNiV-N to serve as a
negative control (lane 1). At 48 h after transfection, poly(A)+ RNA was purified (b) or cell lysates were treated with
micrococcal nuclease before RNA extraction (c). Poly(A)+ or nuclease-resistant RNA samples were separated by
electrophoresis in formaldehyde-agarose gels, transferred to nylon membranes and hybridized with CAT gene-specific,
digoxigenin-labelled riboprobes. Hybridization of poly(A)+ RNA to a negative-sense probe is shown in (b); (c) shows
hybridization of micrococcal nuclease-treated RNA to a positive-sense probe. Hybridization signals were visualized by
chemiluminescence as described in Methods. Digoxigenin-labelled molecular size markers (MW; nt) are shown on the left
of each gel.

Fig. 3. NV obeys the rule of six. CV-1 cells were infected with
MVA-T7 and transfected with pNiV-N, pNiV-P, pNiV-L and one
of a series of minigenome plasmids containing from one to six
nucleotides inserted at the site indicated in Fig. 1. The insertion
sequences and lengths of the RNAs produced from the mini-
genomes are shown in the figure. CAT concentration in cytoplasmic
extracts was measured by ELISA 48 h after transfection. Results
from four separate experiments are shown, with the standard error
indicated by the vertical bars. Omitting any one of the three
support plasmids reduced the background to no detectable
expression of CAT (not shown).
minigenome containing the genetic termini of HeV, two minigenomes for HeV were constructed, pHeV-CAT and pHeV-CAT(-4). The lengths of the 3' leader, 5' trailer, 5' NTR of N and 3' NTR of L are identical in NiV and HeV. For HeV and NiV, the leader and trailer regions share 80% and 90% nucleotide identity, respectively, while the NTRs are more heterogeneous (5' NTR of N, 67%; 3' NTR of L, 58%). pHeV-CAT contained the same four nucleotides between the CAT protein stop codon and the 3' NTR of the L gene as pNiV-CAT (Fig. 1) and its length was evenly divisible by six. pHeV-CAT(-4) lacked the four inserted nucleotides.

The replication efficiencies of the HeV mini-genomes were compared with those of pNiV-CAT and pMV107(-)CAT, a minigenome with the termini and non-coding regions of measles virus, a member of the Morbillivirus genus (Sidhu et al., 1995). In assays using the same ratio of support plasmids and minigenome plasmid as the experiments described above, the pHeV-CAT minigenome was efficiently transcribed by the NiV support plasmids (Fig. 4a) and produced levels of CAT that were slightly higher than those produced with pNiV-CAT. No CAT expression was detected in cells transfected with pHeV-CAT(-4), the minigenome that did not conform to the rule of six. There was no detectable CAT in the cells transfected with pMV107(-)CAT indicating that the N, P and L proteins of NiV did not transcribe this minigenome. Northern blot analysis of poly(A)+-selected mRNA and micrococcal nuclease-resistant RNA demonstrated that the NiV N, P and L proteins supported transcription and replication of the minigenome RNA encoded by pHeV-CAT (Fig. 4b). These results support the previous observations that the N, P and L proteins of one virus can support replication of other viruses within a genus and lend additional support for inclusion of HeV and NiV as a separate genus within the subfamily Paramyxovirinae.

**DISCUSSION**

*In vitro* replication assays with minigenomes expressing reporter genes have been used to study the replication of a number of paramyxoviruses. Here, we report the application of this approach to develop an *in vitro* replication assay for NiV. This is significant because NiV and HeV have been assigned to BSL-4, so studies with infectious virus can only be performed in the few laboratories that have adequate containment. The availability of a minigenome replication assay for NiV will allow us to study aspects of the replication of this important pathogen under BSL-2 conditions.

In this report, we used *in vitro* replication assays to demonstrate that, as for the other paramyxoviruses, the NiV N, P and L proteins are necessary and sufficient for both transcription and RNA replication of NiV. Replication was not affected by the C protein because the sequence of the P gene on the expression plasmid was modified to silence the C ORF. Of course, this conclusion that the N, P and L proteins are necessary and sufficient for transcription and

![Fig. 4. CAT production from minigenomes containing the genomic termini of HeV. (a) CV-1 cells were infected with MVA-T7 and transfected with pNiV-N, pNiV-P, pNiV-L and one of four minigenomes. For the negative control, pNiV-N was replaced with pTM1. CAT concentration in cytoplasmic extracts was measured by CAT ELISA 48 h after transfection and expressed as a percentage relative to pNiV-CAT. Four separate experiments are shown, with the standard error indicated by the vertical bar. (b) Cell extracts were prepared from cells that had been infected and transfected as described in (a). Cell extracts were either treated with micrococcal nuclease prior to RNA extraction (left panel) or subjected to poly(A)+ RNA selection (right panel). Northern blots were performed and hybridized as described in Fig. 2. Both panels show the negative control (NC), cells transfected with the support plasmids plus pNiV-CAT (lanes 1) and cells transfected with the support plasmids plus pHeV-CAT (lanes 2). Digoxigenin-labelled molecular mass markers are shown on the left.
replication must be stated with the caveat that other viral proteins, especially C, V and W, might play a role in virus replication. There is a growing body of evidence that the C and V proteins of paramyxoviruses regulate replication (Tapparel et al., 1997; Curran & Kolakofsky, 1999). For example, the C protein of Sendai virus has been shown to inhibit both transcription and replication by down-regulation of the promoter of genomic RNA (Curran et al., 1992; Cadd et al., 1996), while the C and V proteins of measles virus inhibit transcription (Tober et al., 1998; Reutter et al., 2001). The V proteins of both Sendai virus and rinderpest virus inhibit replication (Horikami et al., 1996; Baron & Barrett, 2000). The minigenome replication assay described in this report will help to define the roles that the V, C and W proteins of NiV and HeV play in regulation of replication. So far, the V proteins of both HeV and NiV have been detected in infected cells (Shiell et al., 2003; B. H. Harcourt, unpublished results), but the C and W proteins have not.

It was assumed that, like other paramyxoviruses, NiV and HeV adhered to the rule of six (Calain & Roux, 1993) because the lengths of their genomes are multiples of six. The results obtained from the minigenome replication assay support this hypothesis. Also, the hexameric phasing positions of the transcription initiation sites are conserved within a paramyxovirus genus (Kolakofsky et al., 1998). NiV and HeV have a hexameric phasing pattern that is unique among the paramyxoviruses and the hexameric phasing positions of the RNA start sites for the P, G and L genes and for the P editing site are not found in any other paramyxovirus (Wang et al., 2000; Harcourt et al., 2001). The biological implications of these phasing patterns have not been explored.

NiV and HeV are the only members of the genus Henipavirus and these viruses have identical genome structures and a high level of nucleotide and amino acid similarity. The amino acid sequence identity between the N, P and L proteins of NiV and HeV is 92, 71 and 87%, respectively. Overall, these viruses share a greater level of nucleotide similarity in their protein encoding regions (70–80%) than in the 5' and 3'NTRs of each gene (40–67%). However, the genomic leader and trailer sequences have 80% and 90% similarity, respectively (Harcourt et al., 2001). Given this level of similarity in the region predicted to contain cis-acting regulatory sequences, it is not surprising that the NiV N, P and L proteins supported the replication of the HeV minigenome. The NiV replicate proteins did not support replication of a minigenome from the more distantly related morbillivirus, measles virus. Previous reports have shown that a Sendai virus defective-interfering genome and minigenome could be rescued by the N, P and L proteins from the respiroviruses, human parainfluenza viruses 1 and 3, but not with the corresponding proteins from measles virus or vesicular stomatitis virus (Curran & Kolakofsky, 1991; Pelet et al., 1996). The ability of the replicate proteins from one virus to recognize the genomic RNA of another virus appears to be restricted to viruses in the same genus. Therefore, the results of our experiments with the NiV minigenome replication assay lend additional support to the designation of NiV and HeV as a separate genus within the subfamily Paramyxovirinae.

While the results presented in this report suggest that NiV employs a replication strategy that is similar to those of other paramyxoviruses, the minigenome replication assay can now be used to address some of the unique genetic characteristics of the henipaviruses. These features include the large size of the 5'- and 3'NTRs in most genes, hexameric phasing and unique predicted functional motifs in the L protein (Wang et al., 2000; Harcourt et al., 2001). Also, RNA editing in the P genes of HeV and NiV is unusual in that more P gene mRNAs with two or more G insertions are found in cells infected with HeV and NiV than in cells infected with other paramyxoviruses (B. H. Harcourt, unpublished results). The availability of the NiV minigenome and plasmids encoding functional replicate proteins also paves the way for the development of an infectious clone for NiV, which could be used to study the pathogenesis of this important viral pathogen.

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REFERENCES


