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Characterization of Maguari orthobunyavirus mutants suggests the nonstructural protein NSm is not essential for growth in tissue culture

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Abstract

Maguari virus (MAGV; genus *Orthobunyavirus*, family Bunyaviridae) contains a tripartite negative-sense RNA genome. Like all orthobunyaviruses, the medium (M) genome segment encodes a precursor polyprotein (NH_2-Gn-NSm-Gc-COOH) for the two virion glycoproteins Gn and Gc and a nonstructural protein NSm. The nucleotide sequences of the M segment of wild-type (wt) MAGV, of a temperature-sensitive (ts) mutant, and of two non-ts revertants, R1 and R2, that show electrophoretic mobility differences in their Gc proteins were determined. Twelve amino acid differences (2 in Gn, 10 in Gc) were observed between wt and ts MAGV, of which 9 were maintained in R1 and R2. The M RNA segments of R1 and R2 contained internal deletions, resulting in the removal of the N-terminal 239 residues of Gc (R1) or the C-terminal two thirds of NSm and the N-terminal 431 amino acids of Gc (R2). The sequence data were consistent with analyses of the virion RNAs and virion glycoproteins. These results suggest that neither the N-terminal domain of Gc nor an intact NSm protein is required for the replication of MAGV in tissue culture.

Keywords: Bunyavirus; Maguari virus; Nonstructural protein; Glycoprotein

Introduction

The family Bunyaviridae contains over 300 enveloped viruses that have tripartite single-stranded negative-sense RNA genomes. The viruses replicate in the cytoplasm, and virion maturation usually occurs by budding at membranes of the Golgi apparatus. The family is classified into five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus* (Nichol et al., 2005). Bunyamwera virus is the prototype of the family and is contained in the *Orthobunyavirus* genus. Orthobunyaviruses encode four structural and two nonstructural proteins in their genomes: the L (large) RNA segment encodes the L protein, an RNA-dependent RNA polymerase; the M (medium) RNA segment encodes the two virion glycoproteins, Gn and Gc, and a nonstructural protein, NSm, as a polyprotein precursor that is co-translationally cleaved; and the S (small) RNA segment encodes the nucleocapsid (N) protein and a second nonstructural protein, NSs, in overlapping reading frames (reviewed by Bishop, 1996; Nichol, 2001; Schmaljohn and Hooper, 2001).

Viruses in the *Orthobunyavirus* genus are divided among 18 serogroups (Calisher, 1996). Temperature-sensitive (ts) mutants of three representatives of the Bunyamwera serogroup, Batai (BATV), Bunyamwera (BUNV), and Maguari (MAGV) viruses were isolated by Iroegbu and Pringle (1981) following treatment with 5-flourouracil and have been used to investigate genetic reassortment between different viruses (reviewed by Pringle, 1996). Mutants assigned to group II were shown to have the ts lesion in the M segment (Iroegbu and Pringle, 1981; Pringle et al., 1984). In screening progeny from a recombination experiment, it was observed that reversion from the ts to non-ts phenotype was frequently associated with a change in the electrophoretic mobility of the Gc protein of the MAGV parent. (Note that in older literature the Gc protein is called G1 and Gn is called G2.) Further work showed that 35 of 36 non-ts revertants of MAG ts8 showed this phenotype, and

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electrophoretic mobility changes in Gc were also observed for non-ts revertants of two other group II mutants, MAG ts14 and ts45. In contrast, no changes in Gc mobility were observed in non-ts revertants of BATV or BUNV group II mutants (Elliott et al., 1984). The non-ts revertants of MAG ts8 fell into two classes, those whose Gc protein had an apparent molecular weight of 80 kDa (designated R1) and those whose Gc protein had an apparent molecular weight of 60 kDa (designated R2); the Gc protein of wild-type (wt) MAGV is about 110 kDa (Elliott et al., 1984; Murphy and Pringle, 1987). Murphy and Pringle (1987) further showed that there were differences in the degree of glycosylation of the faster migrating forms of Gc synthesized by MAG R1 and R2, compared to that of the wt Gc, but these differences were insufficient to account for the observed electrophoretic mobility differences.

To characterize these MAG viruses further, we have cloned and sequenced the M genome segments of wt MAGV, MAG ts8, and two non-ts revertants, MAG R1 and MAG R2. The sequence data show that MAG R1 and MAG R2 contain internal deletions in their M segments that result in deletion of the N-terminal region of Gc and further that in MAG R2 the NSm coding region is severely truncated.

Results

Analysis of Maguari virus proteins

Stocks of the MAG viruses for use in this study were grown in BHK cells at 33 °C following low multiplicity infection, and titers of virus released into the supernatant ranged from 3.3×10^7 (MAG R1) to 1.2×10^8 (wt MAGV) pfu/ml. The temperature-sensitive phenotype of MAGts was confirmed by measuring the yields of virus from BHK cells, infected at 5 pfu/cell, 48 h after infection at either 33 °C or 38 °C; similar yields for all viruses were obtained at 33 °C, but at 38 °C, the titer of MAG ts8 was at least 1000-fold lower than that of wt MAGVV or the revertant viruses MAG R1 and MAG R2 (data not shown). The protein profiles of the MAG viruses were analyzed by polyacrylamide gel electrophoresis of immunoprecipitated radiolabeled cell extracts (Fig. 1A). Antisera were raised in two rabbits (designated 628 and 629) against purified wt MAGV particles, and hence only the structural proteins would be detected; both antisera reacted similarly in immunoprecipitation. It was difficult to observe the L protein band, but, on longer exposure of the gel (data not shown), no differences in its electrophoretic mobility were observed between the different MAG viruses; likewise, the Gn and N proteins of the four viruses migrated similarly. Both wt MAGV and MAG ts8 had Gc proteins of about 110 kDa, but the non-ts revertant viruses MAG R1 and MAG R2 had Gc proteins of 80 kDa and 60 kDa, respectively, in agreement with previous observations (Murphy and Pringle, 1987). Radiolabeled virion preparations were also examined by PAGE (Fig. 1B). The four structural proteins were detected, and, again, the mobilities of the L, Gn, and N proteins were similar. However, the revertant viruses R1 and R2 contained the shorter 80 kDa and 60 kDa Gc proteins, respectively, compared to wt and ts8 viruses which contained the full-size Gc of 110 kDa. This indicated that the shorter Gc proteins observed in infected cell extracts are indeed incorporated into released virus particles.

Cloning and sequence determination of Maguari virus M segment cDNAs

The M RNA segments of the MAG viruses were cloned as cDNA via a reverse transcription-PCR protocol as described in Materials and methods. Preliminary sequence analysis of the wt MAGV M segment (data not shown) enabled the design of primers for specific reverse transcription and subsequent nested PCR for the full-length M segment as described. Using this

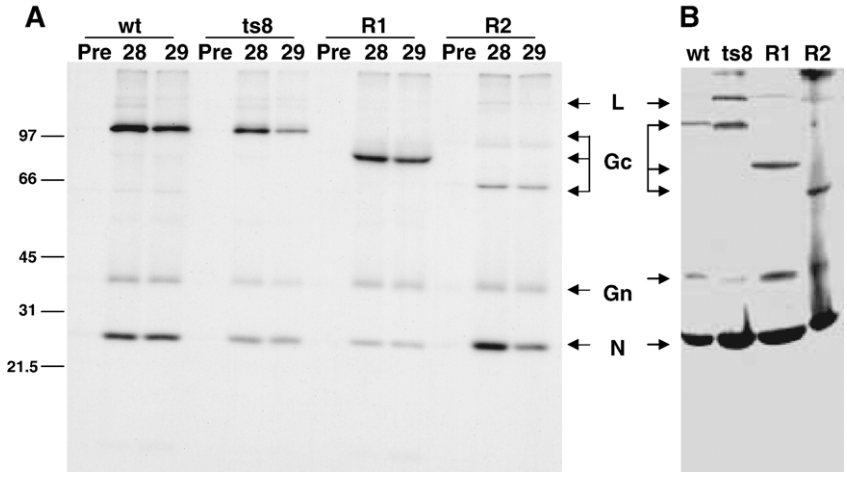

Fig. 1. Analyses of Maguari virus proteins. (A) Immunoprecipitation of radiolabeled infected cell extracts. BHK cells were infected with the different MAG viruses and radiolabeled cell extracts prepared as described in Materials and methods. The extracts were reacted with pre-immune rabbit serum (Pre) or immune serum from rabbits 628 (28) or 629 (29) and precipitated proteins fractionated by SDS–PAGE. The positions of the viral structural proteins L, Gc, Gn, and N are shown. The positions of molecular weight standards are indicated on the left. (B) Radiolabeled virion preparations. The supernatants containing virus particles from radiolabeled infected cells were collected by centrifugation as described in Materials and methods and analyzed by SDS–PAGE. The positions of the viral proteins are indicated. Note that more material was loaded on the MAG ts8 track, giving rise to artefactual faster migration of Gc and Gn compared to wt virus.

protocol, apparently full-length cDNAs about 4.5kb in length (i.e. similar in size to the BUNV M segment; Lees et al., 1986) were obtained for wt MAGV and MAG ts8. Shorter RT-PCR products of 3.7 kb and 2.8 kb were consistently obtained when using RNA extracted from MAG R1 and MAG R2 virus particles respectively. The primers contained *Xho*I and *Xba*I restriction enzyme sites to facilitate cloning because these enzymes do not cut the BUNV M segment cDNA. Indeed, digestion of the PCR products showed no evidence of internal sites, enabling the full-length cDNAs to be ligated into similarly digested pBluescriptSK vector. The nucleotide sequences of the clones were determined as described in Materials and methods and have been deposited in the Nucleotide Sequence Databases under accession numbers AY286443–AY286446.

RNA analysis

The sizes of the RT-PCR products from MAG R1 and MAG R2 suggested that their M segment genomic RNAs were smaller than those of wt MAGV and the parental ts8 virus. Therefore, viral RNAs were analyzed by denaturing agarose gel electrophoresis and Northern blotting. Three RNA species corresponding to the L, M, and S segments were identified in RNA extracted from purified wt MAGV and MAG ts8 virions following agarose gel electrophoresis and staining with ethidium bromide (Fig. 2A). Virion RNA preparations from MAG R1 and MAG R2 showed RNA species that co-migrated with the L and S segments of wt and ts8 viruses, but no RNA equivalent in size to wt M segment was observed. Instead, shorter RNAs were seen. To confirm the identity of these RNAs, Northern blot hybridization was performed using the full-length wt M segment cDNA as a probe (Fig. 2B). Analysis of virion RNA, and also of total infected cell RNA, showed that a single RNA species was detected for each virus, estimated to be 4.5 kb for wt and ts8 viruses, 3.7 kb for R1, and 2.7 kb for R2. These data suggest, therefore, that the M RNA segments of the non-ts revertants of MAG ts8 contain deletions compared to the parental RNA.

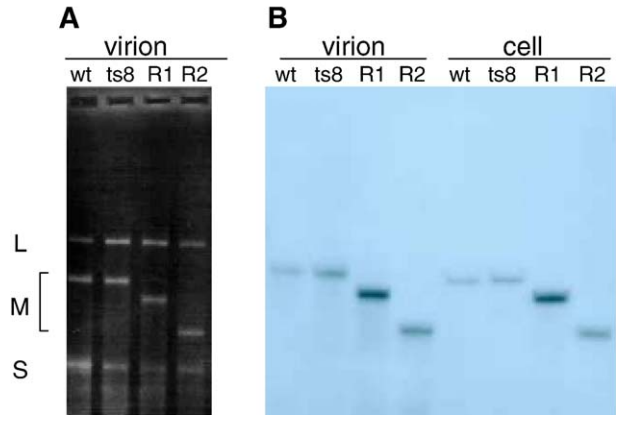

Fig. 2. Analyses of viral RNAs. RNA extracted from virions or infected cells (as indicated) was denatured with glyoxal and fractionated on an agarose gel. The gel was stained with ethidium bromide and photographed under UV light (A) before the RNAs were blotted onto a membrane and hybridized with a radiolabeled cDNA to the wt MAGV M segment (B). The positions of the 3 viral genomic RNAs are indicated.

Nucleotide sequence analysis of M segment cDNAs

The wt MAGV M segment is 4467 nucleotides (nt) in length and, like other bunyavirus M segments, contains a single ORF in the positive sense representing a polyprotein of Gn, NSm, and Gc. The ORF comprises 1434 amino acids, from the AUG codon at nt 50–52 to the stop codon at nt 4352–4354. The positive-sense RNA thus has untranslated regions of 49 nt (5′) and 112 nt (3′), and 17 of the 18 terminal nt are complementary.

Full-length M segment sequences are available for three other members of the Bunyamwera serogroup, BUNV (Lees et al., 1986), Cache Valley (CVV; Brockus and Grimstad, 1999), and Germiston virus (GERV; Pardigon et al., 1988), as well as a number of viruses in the California and Simbu serogroups (see Campbell and Huang, 1999; Yanase et al., 2003; Briese et al., 2004; and references therein). The predicted amino acid sequence of wt MAGV M segment ORF shows approximately 40% identity with that of viruses from heterologous serogroups, but with the characteristic extremely high conservation of cysteine residues (Elliott, 1990). Within the Bunyamwera serogroup, the sequences are more closely related, with MAGV showing 54% identity and 73% similarity to GERV, 63% identity and 73% similarity to BUNV, and 84% identity and 93% similarity to CVV. Comparison of the available Bunyamwera and California serogroup virus M segment sequences allows prediction of the cleavage sites in the precursor. Thus, the N-terminus of Gn is Ser at residue 16, and cleavage at the C-terminus occurs at residue 301 after the highly conserved motif KSLRxAR found in other orthobunyaviruses (Fig. 3). The NSm–Gc junction is less well conserved when comparing different viral sequences, but we suggest that cleavage occurs between Ala475 and Ala476 as predicted for BUNV, CVV, and California serogroup virus glycoprotein precursors (Lees et al., 1986; Brockus and Grimstad, 2001; Fazakerley et al., 1988; Campbell and Huang, 1999).

The wt MAGV ORF sequence has a potential N-linked glycosylation site (N-x-S/T) in the predicted ectodomain of Gn (N59) that is shared with BUNV, CVV, and GERV. There are five potential N-linked glycosylation sites in Gc, with those at amino acids 623 and 1170 shared by all Bunyamwera serogroup viruses, whereas those at amino acids 664, 703, and 764 are shared only with CVV.

The M segment of MAG ts8 is also 4467 nucleotides long and encodes a similar 1434 amino acid polypeptide. There are 12 amino acid differences between wt MAGV and ts8 M segment proteins, 2 in Gn, and 10 in Gc (Fig. 3; Table 1).

Amplification of the M segments of MAG R1 and MAG R2 consistently produced smaller PCR products, suggesting that the terminal sequences to which the primers hybridized were present and that the RNAs contained internal deletions. Cloning and sequencing of these RT-PCR products revealed that the M segment of MAG R1 is 3750 bases long and encodes a single ORF of 1196 amino acids and that the M segment of MAG R2 is 2754 bases long and encodes a single ORF of 864 amino acids.

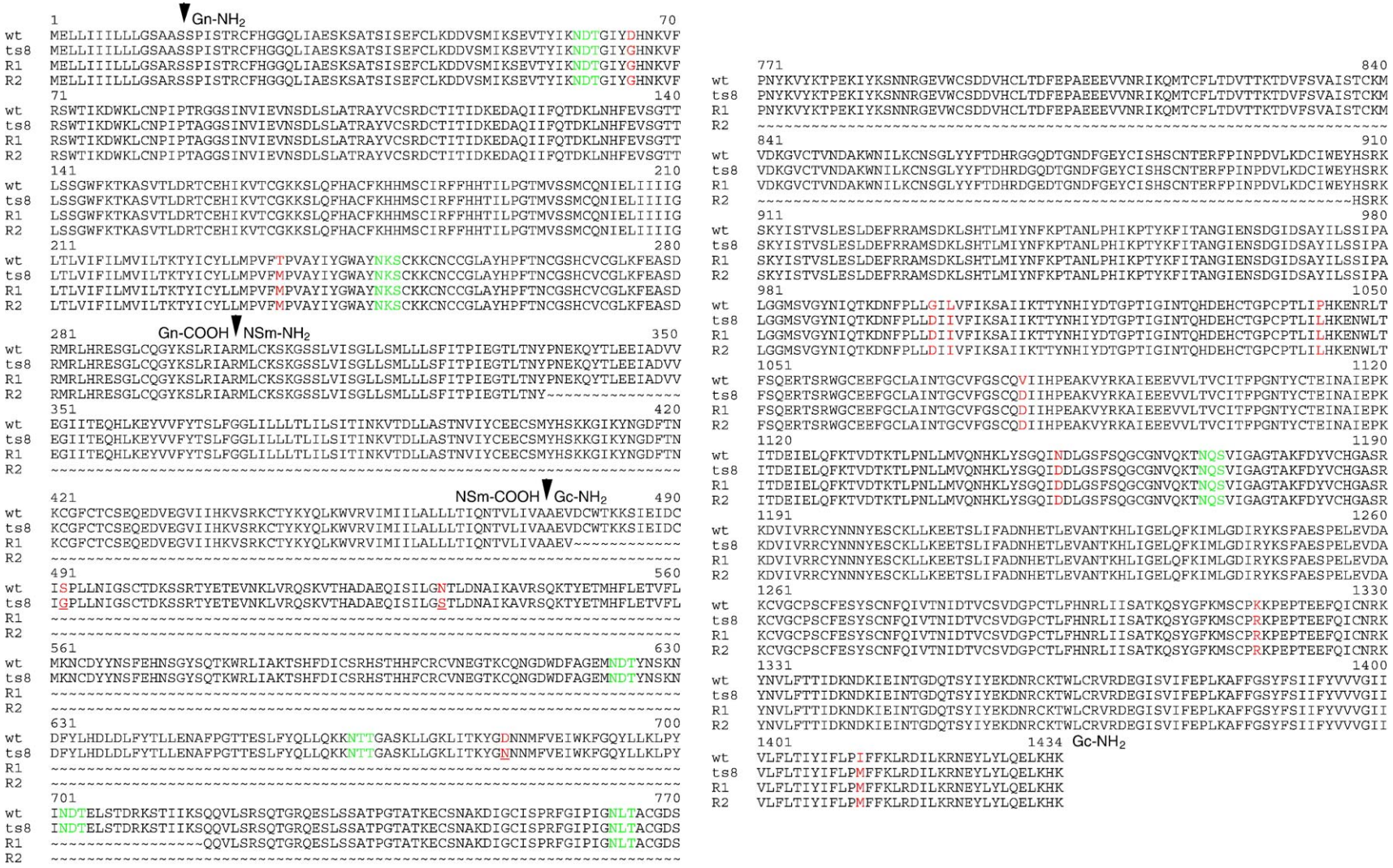

Fig. 3. Alignment of the M segment predicted amino acid sequences of wt MAGV, MAG ts8, MAG R1, and MAG R2. The analysis was performed using PILEUP of the Wisconsin GCG package. Amino acid changes in between the viruses are colored red, and the changes that are exclusive to MAG ts8 at positions 492, 534, and 681 are underlined. The deletions between amino acids 479 and 717 in MAG R1 and between amino acids 336 and 906 in MAG R2 are indicated by ~~. Presumptive polypeptide precursor cleavage sites are marked ▼, and potential glycosylation sites are marked in green.

Table 1
Comparison of the M segments and gene products of wt MAGV, MAG ts8, MAG R1, and MAG R2 viruses

Virus	Size of RNA (nucleotides)	Size of ORF (amino acids)	Potential glycosylation sites in Gc	Amino acids at positions 492, 534, and 681	Size of deletion in ORF (amino acids)
wt	4467	1434	5	S, N, D	–
ts8	4467	1434	5	G, S, N	–
R1	3750	1195	2	deleted	239
R2	2754	863	1	deleted	571

Alignment with the wt MAGV and ts8 sequences (Fig. 3) showed that MAG R1 M segment has a single deletion of 717 nt (comprising nt 1484 to 2200) that corresponds to a deletion of 239 amino acids (amino acids 479 to 717 deleted) of the wt MAGV M segment polypeptide precursor. Thus, the Gc protein of MAG R1 has a deletion comprising residues 4 to 243 in the N-terminal region, though the presumptive cleavage site between NSm and Gc is maintained (Fig. 4).

MAG R2 M segment has a single deletion of 1713 nt (comprising nt 1055 to 2767) that corresponds to a deletion of 571 amino acids (amino acid 336 to 906 deleted) of the wt MAGV M segment polypeptide precursor. This deletion is predicted to result in the removal of the C-terminal two-thirds of NSm (leaving just 34 amino acids) and the N-terminal 431 amino acids of Gc (Figs. 3, 4). The predicted junction between the remaining NSm and Gc sequences does not resemble the authentic cleavage site.

The molecular weight of the deleted Gc proteins predicted from the sequence data is consistent with the molecular weights of the viral proteins estimated from polyacrylamide gel electrophoretic analyses of infected cell lysates or virion preparations (Fig. 1). The deleted regions of both MAG R1 and MAG R2 encompass the conserved potential N-linked glycosylation site at amino acid 623, as well as the sites only shared by MAGV and CVV. This is consistent with the results of Murphy and Pringle (1987) who demonstrated that there was less carbohydrate on the Gc proteins of MAG R1 and MAG R2 compared to the Gc protein of wt MAGV.

Comparison of the predicted protein products of MAG ts8, MAG R1, and MAG R2 M segments demonstrates that, of the 12 amino acid mutations in the M segment of MAG ts8, only three are not present in the revertant viruses and thus could be responsible, individually or in combination, for the ts phenotype of MAG ts8. These three mutations in MAG ts8 are at amino acid positions 492, 534, and 681 in the N terminal domain of Gc (Table 1) and are in the region of the M segment that is deleted in MAG R1 and MAG R2.

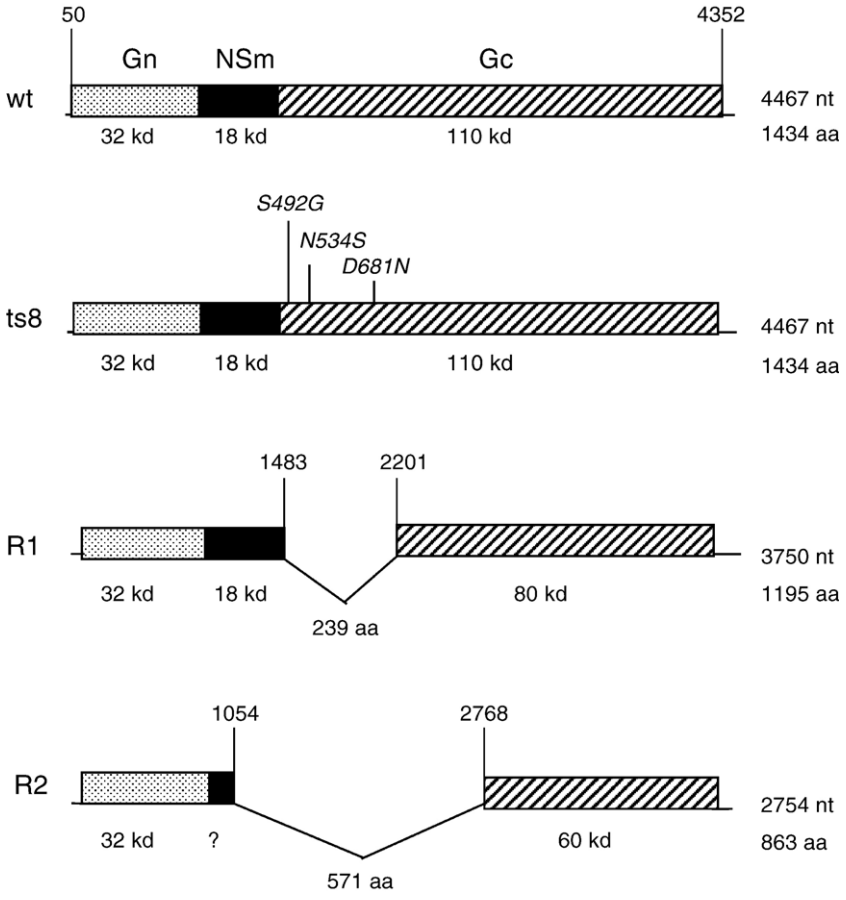

Fig. 4. Schematic diagram showing the genetic organization of the Maguari virus M segments. Numbers above the lines indicate nucleotide coordinates. The positions of the three amino acid changes exclusive to MAG ts8 are shown in italics.

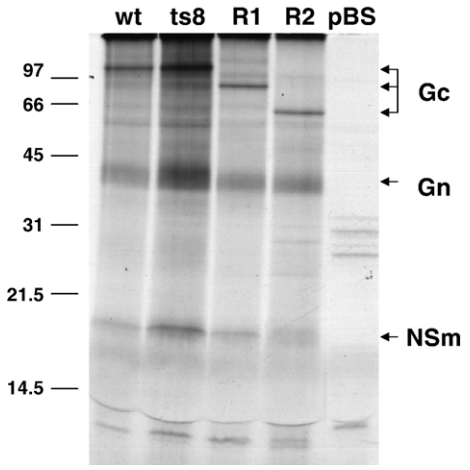

Fig. 5. Expression in vitro of Maguari virus M segment encoded proteins. Coupled cell-free transcription–translation systems were programmed with pBluescriptSK plasmids containing cDNAs to the different MAGV M segments or with empty plasmid (pBS) as indicated. The reactions contained microsomal membranes to allow processing on the polyprotein precursor. The positions of GC, Gn, and NSm bands are shown. No band corresponding to an NSm fragment could be detected in the reaction programmed with the cDNA from MAG R2.

In vitro expression

To further confirm the pattern of proteins expressed by the different MAGV M segments, in vitro translation experiments were performed. The M segment cDNAs had been cloned under control of a bacteriophage T7 RNA polymerase promoter in pBluescriptSK and thus could be used to program coupled cell-free in vitro transcription/translation systems in the presence of microsomal membranes. As shown in Fig. 5, Gc protein approximately 110 kDa in size was synthesized from wt MAGV and MAG ts8 M segment clones. A Gc protein approximately 80 kDa in size was produced from the MAG R1 clone, while that from the MAG R2 M segment clone was approximately 60 kDa. The molecular weights of the in vitro expressed Gc proteins were thus consistent with the molecular weights of the viral proteins seen in infected cells (as estimated from polyacrylamide gels) and concur with the sequencing data. All of the MAGV clones expressed a Gn protein of approximately 32 kDa. The wt MAGV, MAG ts8, and MAG R1 M segment cDNA clones expressed an NSm protein of approximately 18 kDa. We were unable to identify a small protein consistent with the truncated NSm encoded by MAG R2, although it is possible that the NSm/Gc cleavage site is lost in MAG R2, leaving the 34 remaining amino acids of NSm attached to the shortened Gc protein.

Discussion

Iroegbu and Pringle (1981) isolated a number of ts mutants of MAGV following passage of virus in the presence of 5-fluorouracil, and the ts lesion of mutants assigned to group II mapped to the M RNA segment. Analysis of non-ts revertants of these group II mutants revealed a striking instance of gene-specific mutation. Electrophoretic mobility changes in the Gc protein were frequently observed in revertants of three independent group II mutants of MAGV, but not in revertants of BATV or BUNV group II mutants (Elliott et al., 1984). To our knowledge, this phenomenon has not been reported for other bunyavirus ts mutants, of which over 200 have been described in detail (Pringle, 1996). These results were originally interpreted that reversion of ts mutation in the Gc coding region of MAGV occurred by suppression, whereas reversion in BATV or BUNV mutants occurred by same-site mutation. To investigate this further, we determined the nucleotide sequences of the M RNA segments of parental and mutant MAG viruses.

The M RNA segment of wt MAGV shows a similar genetic architecture to other orthobunyaviruses, and the encoded glycoprotein precursor contains the characteristic high percentage of cysteine residues that are largely conserved between the M segment polyproteins of orthobunyaviruses. Both from overall amino acid similarity and conservation of specific potential N-linked glycosylation sites (Brockus and Grimstad, 1999, 2001) MAGV appears most closely related to CVV. This, together with the overall high amino acid identity observed between the N proteins of MAGV and CVV (95%; Dunn et al., 1994), correlates with the close serological relationships of MAGV and CVV (Berge, 1975; Hunt and Calisher, 1979), though the viruses were isolated from geographically distinct regions (MAGV from South America, CVV from North America).

The M genome segment of MAG ts8 contains 12 amino acid differences compared to the wt sequence, and 9 of these changes are retained in the revertant viruses R1 and R2. Further experiments are necessary to determine which of the 3 amino acid mutations, or combination of mutations, that are unique to ts8 (Ser492Gly, Asn534Ser and Asp681Asn) is/are responsible for the ts phenotype. The availability of an efficient reverse genetic protocol to recover infectious orthobunyavirus from cDNA (Lowen et al., 2004) makes it now feasible to address this issue.

The sequence data showed that the mutations in the M segment of MAG ts8 that are apparently responsible for the ts phenotype have been overcome in the revertant viruses by deletion rather than by back mutation. Our data reveal that the smaller (with respect to wt) Gc proteins expressed by MAG R1 and MAG R2 are a result, perhaps unexpectedly (Murphy and Pringle, 1987), of deletions in the coding region rather than changes in protein folding, introduction of novel protease cleavage sites, or aberrant translation of the viral mRNA. Deletions in the L segment of defective interfering (DI) particles of BUNV, tomato spotted wilt tospovirus, and Toscana phlebovirus have been reported (Patel and Elliott, 1992; Resende et al., 1992; Marchi et al., 1998) and are thought to arise when the polymerase jumps from one short sequence repeat to another, brought into proximity by RNA secondary structure (Hacker et al., 1990; Marchi et al., 1998; Resende et al., 1992). While it seems likely that a similar mechanism has been involved in the production of the M segments of MAG R1 and MAG R2, DI RNAs derived from the M segment have not previously been identified (Marchi et al., 1998; Patel and Elliott, 1992; Scallan and Elliott, 1992).

The deletions in the revertant viruses also demonstrate that the N-terminus of Gc and the C-terminal two-thirds of NSm are not essential for the virus to replicate in cell culture. Studies on California serogroup viruses such as La Crosse (LACV) and California encephalitic (CEV) viruses have shown Gc to be a major target for neutralizing antibodies and the principal determinant for tissue tropism, virulence, and vector specificity (reviewed by Gonzalez-Scarano et al., 1992; Nichol, 2001; Schmaljohn and Hooper, 2001). The role of the orthobunyavirus Gc in binding and infection of mammalian and insect cells has been the subject of some debate (Gonzalez-Scarano et al., 1984; Hacker and Hardy, 1997; Hacker et al., 1995; Ludwig et al., 1989, 1991; Pekosz and Gonzalez-Scarano, 1996; Pekosz et al., 1995). Ludwig et al. (1989) suggested that LACV Gc was the ligand for binding to vertebrate cells and Gn the ligand for mosquito cells. They reported that after treatment with protease (that selectively digests Gc but leaves Gn intact) binding of LACV to vertebrate cells was diminished while binding to mosquito cells was enhanced. However, others have reported that protease-treated viruses were equally impaired for binding to either invertebrate or mosquito cells. Trypsin treatment of CEV resulted in virions containing an approximately 60 kDa Gc fragment, and these particles were non-infectious for both vertebrate (BHK-21) and mosquito (*Aedes albopictus* C6/36) cells (Hacker et al., 1995). MAG R2, which contains a similarly sized fragment of Gc, is not impaired for infection of BHK-21 or *A. albopictus* C6/36 cells (unpublished observations) and suggests therefore that the domain that interacts with the cellular receptor is either different between these viruses or else very close to the site of deletion in MAG R2 Gc. Recently, Plassmeyer et al. (2005) provided definitive proof that for California serogroup viruses Gc is the principal determinant for cell fusion and virus entry and that amino acids 860–1442 (numbering on the polyprotein) are critical for these processes. Our data are in accord, in that the truncated Gc encoded by MAG R2 lacks only about 60 amino acids from the N-terminus of this region. The MAGV variants described in this paper provide a useful tool with which to study further the role of Gc in binding and adsorption of virus to mammalian and insect cells.

Processing of the MAG R2 polyprotein occurs normally at the Gn–NSm junction and produces a correctly sized Gn protein. However, we do not yet know what happens at the junction between the remaining 34 residues of NSm and the truncated Gc; experiments are in process to determine whether cleavage occurs or whether the 60 kDa Gc protein represents a fusion of the two truncated products. The role of the orthobunyavirus NSm remains unclear. NSm is able to localize by itself to the Golgi and accumulates there (Nakitare and Elliott, 1993) but is not able to target Gc to this organelle (Lappin et al., 1994; Shi et al., 2004). Insertion of a foreign gene into the NSm region of a LACV M segment cDNA had no effect on the processing, targeting, or transport of Gn–Gc protein complexes expressed from the cDNA when transiently expressed in cells (Bupp et al., 1996). Together, these data suggest that NSm (or at least the C-terminal two thirds) is unlikely to play a role in viral assembly in the Golgi. The reverse genetic system alluded to above will no doubt help in illuminating the function of this nonstructural protein in the future.

Materials and methods

Viruses and antisera

Maguari viruses were grown at 33 °C in BHK-21 cells in GMEM (Glasgow modified MEM; Gibco BRL), supplemented with 10% tryptose phosphate broth and 10% newborn calf serum, essentially as described previously for Bunyamwera virus by Watret et al. (1985). Antisera to wt MAGV were prepared in two rabbits (designated 628 and 629) by intramuscular injection of heat-inactivated purified virus (100 μg per injection in complete Freund's adjuvant) as described by Watret et al. (1985).

Characterization of virus proteins

Monolayers of BHK cells in 35 mm diameter Petri dishes were infected with viruses at a multiplicity of infection (moi) of 5, and proteins were radiolabeled with [^{35}S]methionine at 24 h p.i. as described by Watret et al. (1985). Cytoplasmic extracts were prepared for immunoprecipitation by lysing the cells in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 5 mM EDTA, 0.5% NP-40) and reacted with rabbit anti-MAG sera. Immunoprecipitates were collected on protein-A–sepharose beads (Sigma) and washed three times with RIPA buffer. Proteins were separated on SDS–12% polyacrylamide gels.

To examine proteins in purified virions, the radiolabeled supernatants from infected cell monolayers were clarified by centrifugation at 3000 × *g* for 10 min, and virus particles collected by pelleting at 200,000 × *g* for 3 h in the AH-629 rotor. The pellets were resuspended in protein dissociation buffer, and proteins were separated by SDS–PAGE.

Cloned M segment cDNAs were expressed in vitro using the Quick TNT Coupled Reticulocyte Lysate System supplemented with canine pancreatic microsomal membranes (Promega). Reactions consisted of 10 μl Quick TNT, 10 μCi [^{35}S] methionine, 1.25 μl microsomal membranes, and 1 μg plasmid DNA. The reactions were incubated at 30 °C for 2 h and stopped by addition of protein dissociation buffer. The samples were incubated at 37 °C for 10 min prior to analysis by SDS–PAGE.

Reverse transcription-PCR

Culture fluids from 10^9 infected BHK cells were clarified by centrifugation at 3000×*g* for 10 min, and virus particles collected by pelleting at 200,000×*g* for 3 h in the AH-629 rotor. The pellet was resuspended in 500μl TRIzol reagent (Invitrogen) and the RNA extracted according to the manufacturer's instructions. The viral RNA was dissolved in 20 μl of sterile water. Reverse transcription reactions were carried out with 2 μg MAGV RNA, 50 pmol primer MAG1 (CCGCTCGAGCGGAGTAGTGTAC-TACCGATACA), 1× PCR buffer (Promega), 1 mM dNTPs, 0.5 U/μl RNasin, and 16 U AMV reverse transcriptase (Promega) at 42 °C for 1 h. Nested PCR reactions were carried out with Pfu

DNA polymerase (Stratagene), according to the manufacturer's instructions. Briefly, a first round PCR was carried out with 5 μl cDNA, 1 μM primer MAG1, 1 μM primer MAG4 (GCTCTAGAGCAGTAGTGTGCTACCGATAACA), 200 μM dNTPs, 1× PCR reaction buffer, and 0.5 U/μl Pfu DNA polymerase. The reactions were incubated at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min for 39 cycles, a final cycle of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 10 min. The second round PCR was carried out with primer MAG6 (GCTCTAGAGCAGATTGGTTAGTTATTTGTGCTTC) and primer MAG5 (CCGCTCGAGCGGATTTTGAAATATGGAGTTACTT). The amplified DNA products were digested with *Xho*I and *Xba*I and ligated into similarly digested pBlueScriptSK plasmid.

Nucleotide sequencing

MAGV M segment cDNA clones were sequenced using a combination of the dideoxy-chain termination method of Sanger et al. (1977) and automated cycle sequencing using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc.). The sequences were analyzed using the Wisconsin GCG package.

Characterization of viral RNAs

Viral RNAs from virions and infected cells were characterized by agarose gel electrophoresis and Northern blotting. BHK cells were infected with MAG viruses at an moi of 5 and incubated for 4 days at 31 °C. RNA was extracted from infected cells or released virions with TRIzol as described above. RNAs were denatured by treatment with glyoxal and DMSO prior to running in a 1% agarose gel buffered with 10 mM sodium phosphate pH 7.0 (Kingston, 1997). The RNA was blotted onto a nylon membrane and hybridized with wt MAGV M segment cDNA, [^{32}P]-labeled by nick translation.

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