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The aims of the contract were to elucidate the coding and replication strategies of nairoviruses, and determine whether reagents derived from Dugbe (DUG) virus can be used to detect Crimean Congo hemorrhagic fever (CCHF) viral infections. The small (S) segment of DUG virus was shown to comprise 1712 nucleotides and encode a single protein, the 49.9K nucleoprotein (N), in viral complementary (vc) RNA. No sequence similarity was detected with representatives of the Hanta-, Bunya-, or Phlebovirus genera of the Bunyaviridae family. Approximately 75% (i.e. 4953 nucleotides) of the sequence of the M segment of DUG virus was derived and revealed a single open reading frame (ORF) in the vc RNA. The gene encoding the G1 structural glycoprotein appears to be located at the carboxy-terminal end of the M segment ORF. Cloned cDNA derived from the S and M genomic RNA segments of DUG virus was used to prepare $^{32}$P-labelled RNA probes. Under conditions of low stringency, the DUG S RNA probe hybridized to the S segments of CCHF and Hazara (HAZ) viruses (members of the CCHF serogroup). No hybridisation was detected with members of the other five nairovirus serogroups. The

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19. Abstract (continued)

diagnostic potential of biotinylated probes was demonstrated by their ability to detect DUG viral RNA in infected cell cultures and in sections of infected ticks.

Sequence data derived for the S segment of DUG virus was also used to produce oligonucleotide primers for PCR. Using this technique, DUG viral RNA was detected in the organs and hemolymph of infected ticks, and in the brain and blood of infected mice. A result was obtained within 48 h using PCR whereas biological assays took at least 8 days to diagnose the virus infection. The potential use of baculovirus recombinant proteins as diagnostic reagents was also examined. Using ELISA, a strong cross-reaction was demonstrated between DUG expressed N protein and mouse or human polyclonal antibodies to CCHF virus. Infected ticks used for viral diagnosis were infected *per os* using virus-containing capillaries that were placed over the mouthparts. By this means of infection, *Amblyomma variegatum* was shown to be a competent vector of DUG virus whereas *Rhipicephalus appendiculatus* was non-competent. The observation that DUG virus replicated in *per os* infected *R. appendiculatus* but did not survive trans-stadially suggested a gut-release barrier to infection. These data demonstrate that infection of ticks via inoculation, i.e. by-passing the gut, is not a valid means of identifying natural tick vectors of a virus. The anatomy of DUG virus infection in *Amblyomma variegatum* was examined using light and electron microscopy techniques. Tick hemocytes appeared to be the main site of virus replication and maintenance within the tick vector.

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## CONTENTS

# INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) virus (family, Bunyaviridae; genus, *Nairovirus*) causes significant human morbidity and mortality in Africa, Asia, and Europe (Casals, 1978; Watts *et al.*, 1988; Swanepoel *et al.*, 1989). The virus is maintained in enzootic foci by ticks (Hoogstraal, 1979). Humans are most commonly infected by tick bite, but animal-human and human-human transmission can occur. The exceptionally high morbidity (17%) and mortality rates (13-50% and as high as 80% in intrahospital outbreaks) in humans present a serious threat to the health of the public and military personnel (Goldfarb *et al.*, 1980; Swanepoel *et al.*, 1989). Natural levels of protection are poor among human populations in CCHF foci, probably because infection rates are low. However, few serosurveys have been carried out. The disease is probably underdiagnosed, particularly in poorly developed parts of the world (e.g. Middle East and Central Africa). This is partly because of the lack of commercially available diagnostic kits. Rapid diagnostic tests for CCHF virus infections are not available, and serological tests are hazardous (because live virus is used) and frequently give ambiguous results (Casals, 1978; Shepherd *et al.*, 1989).

Dugbe (DUG) virus is antigenically related to CCHF virus (Casals & Tignor, 1980) but is not a significant human or animal pathogen (reviewed by Steele & Nuttall, 1989). To overcome the problems associated with handling CCHF virus, DUG virus has been used to generate new reagents (DNA and RNA probes, expressed proteins, monospecific sera) for diagnosing nairovirus infections.

The replication strategy of nairoviruses has not been elucidated (Elliott, 1990). Like other members of the Bunyaviridae, the genome of nairoviruses comprises three segments of single-stranded, negative sense RNA, however,

the large- (L) and medium- (M) sized RNA segments are considerably bigger than those of members of the other genera. Furthermore, studies with Hazara virus indicate that there may be three glycoproteins rather than the two commonly found in the Bunyaviridae (Foulke *et al.*, 1981). Determination of the coding strategy of DUG virus will provide important insights into the molecular biology of nairoviruses, and consequently aid in the development of diagnostic reagents and viral vaccines for protecting against nairovirus infections.

## MATERIALS AND METHODS

### Viruses and cells

Six different isolates of DUG virus were used. The prototype, DUG IbAr 1792, was originally isolated from *Amblyomma variegatum* ticks collected in Ibadan, Nigeria in 1964, and obtained from Dr. J.S. Porterfield as an 18th mouse brain passage; DUG IbH11480 was isolated from the blood of a human patient in Nigeria (1966) and obtained as a 5th mouse brain passage from Dr. R.E. Shope, Yale Arbovirus Reference Centre; and KT281/75 was isolated from *A. variegatum* (1975) and obtained from Dr. N.J. Dimmock as infected cell culture supernatant. Three isolates were kindly supplied by Dr. J.-P. Digoutte, Institut Pasteur, Senegal: ArD 16095 isolated from *A. variegatum* (1972) and supplied as a 5th mouse brain passage; ArD 16769 isolated from *Hyalomma rufipes* (1973) and supplied as a 4th mouse brain passage; and ArD 44313 isolated from *A. variegatum* (1985) and supplied as a 3rd mouse brain passage. In addition, the following viruses were used in cross-hybridization experiments: Abu Mina (AM) Eg Art 430 from *Argas persicus* collected at the Dahla Oasis, Egypt (1970) and obtained as an 8th mouse brain passage; Avalon (AVA) Can Ar 173 from *Ixodes uriae* collected on Great Island, Newfoundland (1972) and obtained as a 9th mouse brain passage; Bandia (BDA) IPD/A 611 from *Mastomys* sp. in Bandia forest, Thies region, Senegal (1965) and obtained as a 6th SMB passage; Dera Ghazi Khan (DGK) PAK JD 254 from *Hyalomma dromedarii* larvae collected in the Dera Ghazi Khan district of Pakistan (1966) and obtained as a 17th SMB passage; Erve isolate Brest/An221 (obtained from Prof. C. Chastel, University of Brest, France); Ganjam (GAN) isolate IG619 from *Haemaphysalis intermedia* collected in Poona, India (1954); Hazara (HAZ) isolate JC280 from *Ixodes redikorzevi* collected in Lahore, Pakistan (1964); Hughes (HUG) virus from *Ornithodoros*

*maritimus* collected on the Dry Tortugas of Florida (1962) and obtained as a 17th SMB passage; and Thiafora isolate AnD11411 (obtained from Prof. C. Chastel, University of Brest, France).

Viruses were grown in either PS or BHK-21 cell cultures in the presence of Leibovitz L15 medium supplemented with 10% tryptose phophate broth and 3% heat-inactivated foetal calf serum (FCS), and incubated at 35°C. Plaque assays were carried out in PS cell cultures grown in Linbro plates and overlain with L15 medium containing 0.75% carboxymethyl cellulose. Hazara virus was grown in SW13 cells and Ganjam virus in Vero cells, and the SDS lysates were supplied for testing.

**Infection studies with vertebrates**

The following animals were inoculated subcutaneously with 4.6 to 6.6 $\log_{10}$ PFU DUG virus and then screened for development of a viremia: 6 adult bank voles (*Clethrionomys glareolus*); 3 Long Evans strain rats (*Rattus norvegicus*); 2 PVG nu+nu+ rats; 2 Wistar strain rats; 6 rats (*R. norvegicus*), 3 generations from wild caught; 8 cotton rats (*Sigmodon hispidus*); 4 Dzungarian hamsters (*Phodopus sungorus*); 2 Dunkin Hartley strain guinea pigs (*Cavia porcellus*); 5 DSNO strain Syrian hamsters (*Mesocricetus auratus*); 9 adult house mice (*Mus musculus*); 5 New Zealand rabbits (*Oryctolagus cuniculus*); 10 multimammate rats (*Praomys natalensis*); 5 wood mice (*Apodemus sylvaticus*). Blood samples were taken either from anaethetised animals by cardiac puncture, or from the marginal ear vein (rabbit), or the animal was sacrificed (newborn mice, voles). Samples were collected daily up to 14 days after inoculation (depending on the number of individuals and the method of sampling). The blood (usually 0.1 ml) was diluted 1/2 in medium following collection, frozen at -70°C, and then screened for virus by plaque assay in PS cells and (in the case of multimammate rats) by intra-cerebral

inoculation of 2 day-old mice. Serum was collected on day 14 to test for neutralizing antibody (Steele and Nuttall, 1989). Additional studies were carried out in mice, and Syrian and Dzungarian hamsters inoculated with differing viral doses by intracerebral, intraperitoneal, and subcutaneous routes.

### Tick rearing procedures

Colonies of *Amblyomma variegatum* and *Rhipicephalus appendiculatus* ticks were maintained as described by Jones *et al.* (1988).

### Capillary feeding of ticks

Capillary feeding of ticks was accomplished by a method modified from Burgdorfer (1957). Strips of double-sided tape were stuck onto a glass slide and questing nymphs attached to them. The ticks were orientated so that they were lying on their backs with their hypostomes pointing anteriorly at an approximate angle of 45°. Drawn-out micropipettes were filled with newborn calf serum mixed with DUG virus (cell culture supernate), final titer 3.3 $\log_{10}$ PFU/ml. The capillary tubes were then placed over the hypostome of the ticks, each tube being held in place by a block of plasticine placed in front of the tick. The nymphs were left overnight to feed then removed from the plasticine and immediately placed on a guinea pig to complete engorgement. Preliminary experiments showed that, if completion of feeding was delayed, a large number of the ticks desiccated and died. After engorgment and drop-off, the ticks were held at 28°C and 85% relative humidity. Samples of capillary fed ticks were assayed for virus immediately after capillary feeding, and at 3 time points during their subsequent development: (i) 12 days after engorging and dropping-off, (ii) immediately after moulting and, (iii) 8 days after moulting.

Serum was collected from the hosts on which the capillary fed ticks engorged, and assessed for DUG neutralizing antibodies.

### Tick inoculation

Engorged nymphs were inoculated with DUG virus on the same day that they completed engorgement on guinea pigs (previous experiments had shown that delay in the time of inoculation increased tick mortality dramatically). The engorged ticks were stuck onto plasticine strips on glass slides and, through the posterior margin of the abdomen, injected via a microsyringe with approximately 20 ul of DUG virus diluted in PBS, final titer 3.3 $\log_{10}$ PFU/ml. The ticks were detached from the plasticine and stored in petri dishes at 28°C and 85% relative humidity. Using these methods survival rates of over 90% were recorded. After inoculation, samples of the ticks were collected at appropriate time intervals, frozen at -70°C, and then assayed for DUG virus. Serum was collected from the guinea pigs on which inoculated ticks engorged, and tested for neutralizing antibodies.

### Light and electron microscope immunocytochemistry

Conventional electron microscopy was carried out using the standard glutaraldehyde and osmium tetroxide double fixation technique for embedding in epoxy resin and ultrathin sectioning. Preparations of DUG virus were negatively stained with a mixture of phosphotungstic acid and ammonium molybdate (2:1). For immunohistochemistry, DUG virus infected PS cells or cryostat sections (6 µm) of infected tissues were acetone-fixed and stained with DUG antibody using a biotinylated secondary antibody with either a streptavidin-conjugated fluorescein isothiocyanate detection system (Amersham) or a streptavidin-peroxidase conjugate with amino-ethylcarbazole as the substrate (Sigma). Methylene blue was used as a

counterstain for the red peroxidase reaction product; for fluorescence, slides were mounted in 'Citifluor' (Agar scientific) to enhance specific fluorescence and to minimise and differentiate autofluorescence of some tick cell components.

Initial electron microscopic (EM) investigations were carried out on DUG virus infected PS cells. Cultures were glutaraldehyde-fixed at 48 h p. i. for surface immunogold labelling and processed for electron microscopy as previously described (Booth *et al.*, 1989). For post-embedding labelling of DUG virus antigens for EM, tissues and cells were fixed for 1 h at 20 °c with 0.1 % glutaraldehyde in 0.1 M phosphate buffer pH 7.4, dehydrated in an ethanol series and embedded in LR White resin (London Resin Co.) as described by Newman *et al.* (1983).

Ultrathin sections were cut and mounted on uncoated nickel grids and screened for DUG virus antigen by immunogold labelling. For gold labelling experiments, grids were pre-incubated in 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 10 min followed by 2-3 h with antibody diluted in PBS/BSA. The dilution of antibody that gave the maximum specific labelling with the minimum of non-specific labelled background was determined. After washing three times by floating grids on PBS, followed by another 5 min blocking step with PBS/BSA, sites of antibody binding were detected by incubating with 10nm colloidal gold particles conjugated to goat anti-rabbit IgG (Bio Cell). Controls included omitting the primary antiserum or substituting an irrelevant one, and using uninfected cells or tissue as the substrate. Immunogold labelling of ultrathin cryosections of DUG virus infected PS cells was carried out according to the methods of Griffiths *et al.* (1984).

**Viral growth curves and intracellular polypeptide synthesis in vertebrate cell cultures**

In preliminary labelling experiments, confluent monolayers of PS or BHK cell cultures were inoculated with various input multiplicities of infection (m.o.i.) of the different DUG viral isolates and then incubated at 35°C in the presence of Eagle's Minimum Essential Medium (EMEM) supplemented with 3% FCS. At various times post-infection cells were starved in methionine-free EMEM for 1 h, then pulse-labelled with $^{35}S$ methionine in methionine-free EMEM for 1 h. Monolayers were washed in PBS then lysed in cold lysis buffer (0.15M NaCl, 0.05M Tris/HCl pH 7.4, 0.002M EDTA, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS) prior to subsequent analysis by SDS-PAGE. Infected, unlabelled cell cultures were frozen and thawed, and the supernatant assayed for virus by plaque titration in PS cell cultures.

For radioimmune precipitation, proteins were labelled with $^{35}S$ cysteine in cysteine-free L15 medium, using a 2 h starvation period followed by a pulse of 1 to 2 h. Washed monolayers were lysed in 4% zwittergent 3-14, 10mM Tris HCl pH 8.0, 1mM EDTA. 0.5M NaCl, 0.25 mg/ml aprotinin, and clarified by brief centrifugation. Following incubation with antiserum (preabsorbed against PS cell acetone powder) at 4°C overnight, antigen-antibody complexes were precipitated by incubation with protein A sepharose CL-4B for 2 h. After 3 washes in 2% zwittergent buffer, precipitated proteins were analysed by SDS-PAGE.

**Viral growth in tick cell culture**

Monolayers of the continuous *Rhipicephalus appendiculatus* tick cell line, RA 257 (supplied by Prof. M.G.R. Varma, London School of Hygiene & Tropical Medicine), were inoculated with various input m.o.i. of DUG virus, and then incubated at 28°C in L15 medium containing 10% foetal bovine

serum. At 1 to 14 days post-infection, cells were harvested in a lysis buffer (20mM Tris pH 7.5, 50mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate) and analysed by Western blotting.

### Characterization of viral proteins

Infected PS cell culture supernatant was clarified by centrifugation at 2,000 x g for 15min and then pelleted through a 10% (w/w) sucrose cushion by ultracentrifugation at 100,000 x g for 45min at 4°C in a Beckman SW28 rotor. The virus pellet was resuspended in 0.3ml TNE buffer [0.01M tris-hydrochloride (pH7.5)- 0.1M NaCl- 1.0mM EDTA] and used as a source of DUG viral structural proteins.

Virus surface proteins were investigated by protease digestion. Virus pellets were incubated with 0.1mg/ml α-chymotrypsin (Sigma) for 30min at room temperature, boiled for 2min in sample buffer (see below) and then loaded onto a 10% SDS-polyacrylamide gel.

The enzyme N-glycanase (Genzyme) was used to hydrolyse asparagine-linked oligosaccharides from glycoproteins. The glycoprotein sample was boiled for 3min in the presence of 0.5% SDS and 0.1M 2-mercaptoethanol and then diluted ½ in 0.55M sodium phosphate buffer (pH 8.6). Nonidet P40 was added to a final concentration of 1.25%. The enzyme was added (10 units/ml final concentration) and the mixture incubated overnight at 37°C before analysis by SDS-PAGE.

For the detection of sugars in glycoproteins, a glycan detection system (Boehringer Mannheim) was used. Adjacent hydroxyl groups in saccharides were oxidized to aldehyde groups by mild periodate treatment. The spacer linked steroid hapten digoxigenin was then covalently linked to these aldehydes via a hydrazide group. The samples were resolved by SDS-PAGE and electro-blotted onto nitrocellulose as described below. Digoxigenin

labelled glycoproteins were subsequently detected in an enzyme immunoassay using an antibody to digoxigenin conjugated to alkaline phosphatase.

**Electrophoresis of viral proteins and Western blotting**

Protein samples were electrophoresed in a 10% acrylamide/SDS gel with a 3% stacking gel at 200V using an Atto minigel apparatus. Samples were boiled in 4% 2-mercaptoethanol and 2% SDS for 90 sec before loading onto the gel. For autoradiography, gels were stained in 0.1% kenacid blue and destained in 15% methanol, 7% acetic acid. Gels were then dried at 80°C under vacuum and exposed to Kodak X-Omat S film. For Western blotting, protein bands were electro-blotted onto nitrocellulose membranes in the Sartorius semi-dry trans-blot apparatus at 150mA for 2h. The nitrocellulose membrane was blocked with 5% skimmed milk powder for 20 min or with 0.5% Tween 20 in PBS (PBS-0.5% $T_{20}$) for 5 min at room temperature on a rocking tray, then probed with the appropriate antiserum for 2h at room temperature. Polyclonal antisera were diluted 1/1000 in PBS containing 0.05% Tween 20 (PBS-0.05% $T_{20}$) and monoclonal ascitic fluid was diluted 1/500 in the same diluent. The nitrocellulose membrane was washed twice in PBS-0.05% $T_{20}$ and then treated with the appropriate anti-species antibody conjugated to alkaline phosphatase (AP) (Sigma). The conjugate was diluted 1/3000 in PBS-0.05% $T_{20}$, incubated for 2h at room temperature, and washed off with two changes of PBS- 0.05% $T_{20}$. The AP substrates 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) were prepared according to the manufacturer's instructions (BRL) and added to the nitrocellulose membrane. The bands were allowed to develop in the dark for 10 min, and the reaction stopped by addition of 20 mM Tris-HCl (pH 7.5), 5 mM EDTA in water.

**Extraction of viral RNA**

Monolayers of PS cells in 32 oz glass bottles were infected at an input m.o.i. of 0.05 PFU/ml with DUG isolate 44313, and incubated at 35°C for 3 days. The cultures were then washed with PBS and incubated for 1 h at room temperature in 2 ml of 10 mM Tris pH 7.4 containing 1% Triton N101. The cell lysates were harvested, clarified by centrifugation, and CsCl added to a final concentration of 0.38 g/ml. The gradients were centrifuged in a Beckman SW41 rotor at 30,000 r.p.m. for 24 h at 20°C, and the resulting nucleocapsid band harvested, diluted with 10 mM Tris pH 7.4, and centrifuged at 60,000 r.p.m. for 2 h in a Beckman Ti70 rotor. Resulting nucleocapsid pellets were resuspended in 0.5 ml of 10 mM Tris pH 7.4, 1 mM EDTA (TE) containing 0.4 M NaCl and 1% (w/v) SDS, then extracted twice with phenol, once with chloroform, and the RNA precipitated with ethanol.

**Synthesis and cloning of cDNA**

DUG viral RNA was polyadenylated in a reaction containing 250 µg/ml RNA, 50m*M* Tris-HCl pH7.9, 10m*M* $MgCl_2$, 250m*M* NaCl, 2.5m*M* $MnCl_2$, 0.1m*M* ATP, and 2 units of polyadenylate polymerase (GIBCO-BRL). After 10 min at 37°C, the RNA was precipitated with 2*M* LiCl. The polyadenylated RNA was denatured with 10m*M* methylmercury hydroxide at room temperature for 10 min, and then transcribed to cDNA in a 50µl reaction containing 10m*M* DTT, 50m*M* Tris-HCl pH8.3, 8m*M* $MgCl_2$, 70m*M* KCl, 0.2m*M* each dNTP, 400 units/ml reverse transcriptase (Amersham), and 100µg/ml oligo $(dT)_{12-18}$, at 42°C for 90 min. After extraction with phenol and precipitation with ethanol, the second strand of cDNA was synthesised and the product made blunt-ended as described by Gubler (1988). *Bam*HI linkers (Pharmacia) were added using T4 DNA ligase (Amersham), and, after cleavage with *Bam*HI and removal of excess linkers, the cDNA was ligated

into *Bam*HI digested and alkaline phosphatase (Boerhinger) treated pUC19. Transformation of *E. coli* JM107 was by standard protocol (Maniatis *et al*, 1982). Recombinant clones were determined to contain DUG S, M or L RNA by Northern hybridization analysis.

In addition, two plasmid libraries were made from viral RNA without using polyadenylation. cDNA was synthesised essentially as described above, but the oligo (dT) primer was replaced with the synthetic 15-mer, pr15 (see below) at 80 µg/ml, or random hexamers (Amersham) at 15 µg/ml. Cloning with *Bam*HI linkers was as described above, but plasmid pUC19 was replaced by pBS (Stratagene) and *E. coli* strain XL1-Blue was used.

### Cloning of the 3' end of viral S RNA using PCR

The 3' end of viral S segment RNA was cloned using the polymerase chain reaction (PCR) and two DUG specific primers, pr15 and p.rev. The pr15 primer is a 15-base oligonucleotide complementary to the 3' end of viral sense (v) RNA (Clerx-van Haaster *et al*, 1982), and p.rev is a 17-base oligonucleotide complementary to nucleotides 1189-1205 of viral-complementary (vc) RNA (numbered from the 5' end; see Fig. 4). A 20µl first strand cDNA reaction was carried out as described above using both primers and RNA which had been extracted from infected PS cells by the method of Birnboim (1988). A 5µl aliquot of the first strand cDNA reaction was then submitted to 30 cycles of amplification of 95°C for 1 min, 55°C for 1 min, and 70°C for 3 min, in a 50 µl reaction containing 20µg/ml pr15 and p.rev, 67m*M* Tris-HCl pH8.8, 16.6m*M* ammonium sulphate, 6.7m*M* $MgCl_2$, 10m*M* 2-mercaptoethanol and 2 units of *Taq* DNA polymerase (Anglian Biotec, UK). The subsequent PCR product was end filled using Klenow polymerase and extracted with phenol. After ethanol precipitation, the product was cleaved internally with *Pst*I. The resulting fragments were cloned into the plasmid vector pBS (Stratagene) which had

been digested with *Pst*I and *Sma*I. The presence of cloned DUG cDNA was determined by dot-blot hybridization of resulting colonies using a cDNA probe representing nucleotides 45-420 of vcRNA, followed by analysis of positive clones by restriction endonuclease digestion and agarose gel electrophoresis.

### Northern hybridisation

Viral RNA was glyoxalated (Carmichael and McMaster, 1980) and electrophoresed on 1% agarose in 10m*M* phosphate buffer pH7.0. RNA was transferred by capillary blotting to Hybond-N membrane (Amersham), and then fixed and hybridized according to the manufacturers instructions. Cellular RNA was extracted from DUG virus infected or uninfected PS cells by the method of Birnboim (1988).

### Sequencing of DUG cDNA

Cloned cDNA was subcloned into M13 vectors, mp10, mp11, mp18 and mp19 (Messing, 1983), and sequenced by the dideoxy method (Sanger *et al*, 1977) using a universal primer (Amersham) or synthetic oligonucleotide primers. For some M segment subclones, the ABI Automatic DNA Sequencer was used in conjunction with fluorescent primers. All sequences were determined in both directions except for the terminal 30 bases of the S segment and 20 bases of the M segment 5' ends. To sequence the 5' end of the viral S RNA a 17-base oligonucleotide, pr17, complementary to nucleotides 1632-1648 of vRNA (numbered from the 3' end), was synthesised and then 5' end labelled with polynucleotide kinase and [$\gamma$-$^{32}$p] ATP. Labelled pr17 was annealed to DUG S RNA and extended by reverse transcriptase. The extension product was purified by extraction from a 6.5% polyacrylamide - urea gel, and sequenced by the chemical method of Maxam and Gilbert (1980). The terminal 30 bases were also sequenced by extension of pr17 using reverse transcriptase

and dideoxynucleotides in a modification of the method of Zimmern and Kaesberg (1978). The terminal 20 bases of the M RNA 5' end were sequenced by the method of Maxam and Gilbert and by dideoxynucleotide RNA sequencing as above. A 22 nucleotide primer (BPAL13), complementary to a sequence 121 bases from the 5' end, was synthesised and end-labelled using polynucleotide kinase. The terminal 5' nucleotide was deduced by extending further the gel purified cDNA product using terminal transferase and dTTP. This final product was also determined by the chemical method of Maxam and Gilbert.

**Sequence analysis**

Analysis of the cDNA and derived amino acid sequences was accomplished using the programs of Staden (1982, 1984) on a DEC VAX computer.

**Production of β-galactosidase fusion proteins**

The cDNA inserts of two clones, pDUG16 and pDUG36 (representing nucleotides 44-1682 of vcRNA), were ligated together at the *Sac*I restriction site of S segment cDNA to produce a cDNA clone containing all of the 49.4 kDa ORF determined by sequence analysis. The product was subcloned into the *Bam*HI restriction endonuclease site of the β-galactosidase fusion vector pUEX1 (Amersham), and transformed into *E. coli* XL1-blue cells (Stratagene) using a heat shock of 37°C. Fragments of M segment cDNA were subcloned into similar vectors, pUEX2 and pUEX3. Recombinant clones were identified by hybridization and subsequent restriction endonuclease digestion and agarose gel electrophoresis. The recombinant clones were grown at 30°C overnight and then incubated at 42°C for 2 h to inactivate the temperature sensitive repressor of β-galactosidase and hence express the fusion protein.

The *E. coli* cells were harvested and the presence of a fusion protein determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

### Nucleic acid probes

DNA probes were made by nick-translation (Promega Nick Translation Kit) or "oligo-labelling" (Amersham Multiprime Kit) using [$^{32}$P]-dATP. RNA probes were made by subcloning the inserts of pDUG36 (S-specific) and pDUG61 (M- specific) into the transcription vector pBS (Stratagene) to give pBS-36 and pBS-61. Transcription of *Sac*I- or *Bam*HI-linearised pBS-36 with T3 RNA polymerase yielded viral complementary transcripts, and transcription of *Xba*I-linearised pBS-36 with T7 RNA polymerase yielded viral-sense transcripts. Similarly, transcription of *Kpn*I- linearized pBS-61 with T3 RNA polymerase yielded viral complementary transcripts. $^{32}$P-UTP was incorporated to give [$^{32}$P]-labelled probes for Northern blot and dot blot hybridization. Biotin-11-UTP (Gibco-BRL) was incorporated to give biotin-labelled probes for *in situ* hybridization. Biotinylated RNA was detected using the streptavidin-alkaline phosphatase system (Gibco-BRL DNA Detection System).

### *In situ* hybridization

For *in situ* hybridization, monolayers of PS cells grown on glass slides, or sections of infected ticks, were fixed with 4% paraformaldehyde, treated with 2.5 µg/ml proteinase-K for 10 min at room temperature, and post-fixed in 4% paraformaldehyde. The cells were prehybridised in 50% formamide, 2 x SSC, 200 ug/ml salmon sperm DNA, 10% dextran sulphate, 5 mM VRC, and 2 x Denhardt's solution, at room temperature for 1 h. Hybridization with biotinylated T3 transcripts of pBS-36 was in 50% formamide, 2 x SSC, 10% dextran sulphate, 200 µg/ml salmon sperm DNA, 10 mM VRC, 1 x Denhardt's

solution, at 50°C for 16 to 20 h. Unbound probe was washed off using 2 washes of 2 x SSC, 50% formamide at 37°C for 10 min, 2 washes of 2 x SSC at 37°C for 10 min, and one wash in 0.1 x SSC at room temperature for 10 min. After blocking with PBS containing 3% w/v BSA, biotin was visualized as described above.

**Dot blots**

Dot blots were carried out with suckling mouse brain (SMB) or culture fluids from cells infected with one of the six DUG isolates, or with AM, AVA, BDA, DGK, or HUG viruses. Four 1 µl aliquots of 10% SMB or cell culture fluid were treated with 1% SDS, 100 µg/ml proteinase-K at 50°C for 30 min and then spotted onto Hybond-N membrane. After fixing with UV light, the blots were washed in 2 x SSC, 0.1% SDS at 65°C for 30 min before hybridization.

**Baculovirus expression studies**

Wild type and recombinant AcNPV were grown and plaque assayed in *Spodoptera frugiperda* cells essentially as described by Brown and Faulkner (1977).

**Construction of baculovirus recombinant clones: DUG S segment**

Two cDNA clones, pDUG16 and pDUG36, cloned into the *Bam*HI restriction site of pUC19 using *Bam*HI linkers, and representing nucleotides 44-1682 of viral-complementary DUG S segment RNA, have previously been shown to encode all of the 49.4kDa DUG N protein (nucleotides 50-1375) (Ward *et al.*, 1990a). The two clones were ligated together at the *Sac*I restriction site present in both clones as shown in Fig. 10. Clone pDUG36 was cleaved with *Sac*I plus *Bam*HI and the 810bp fragment produced was subcloned into the plasmid vector pUC19, which had been digested with

*Bam*HI and *Sac*I, to produce plasmid clone pDUGBS36. Clone pDUGBS36 was then linearised with *Sac*I and treated with alkaline phosphatase to prevent recircularisation. Clone pDUG16 was digested with *Sac*I, to generate an 830bp fragment containing the ATG start codon for DUG N protein. This 830bp fragment was ligated into the *Sac*I digested dephosphorylated pDUGBS36 clone, to produce clone pDUG1636, representing all of the N protein open reading frame (ORF), including the ATG start codon present at nucleotides 50-52 of viral-complementary S RNA and the termination codon TGA, present at nucleotides 1373-1375 of viral-complementary S RNA. Clone pDUG1636 was screened for correct orientation of the cDNA fragments by digestion with *Pst*I (see Fig. 10), and could be excised from pUC19 by digestion with *Bam*HI.

The 1639bp cDNA insert of clone pDUG1636 was removed from pUC19 by digestion with *Bam*HI and subcloned into the *Bam*HI site of the AcNPV baculovirus transfer vector, pAcYM1 (Matsuura *et al*, 1987). The orientation of the cDNA insert was determined by digestion with *Hin*dIII, and clone pAcYM1636, containing the DUG N protein ORF in the correct orientation for expression directed by the AcNPV polyhedrin promoter, was selected after screening for the correct restriction fragment pattern.

## Co-transfection and selection of baculovirus recombinants: DUG S segment

Co-transfection of *S. frugiperda* cells with mixtures of infectious AcNPV DNA and the recombinant pAcYM1636 transfer vector DNA was carried out essentially as described by Overton *et al* (1987). One microgram of AcNPV DNA was mixed with 3µg of pAcYM1636 DNA in (final concentrations) 20m*M* HEPES buffer pH 7.5, 1m*M* $Na_2HPO_4$, 5m*M* KCl, 140m*M* NaCl, and 10m*M* glucose (total volume, 935µl). The DNA was precipitated by the addition of 2*M* $CaCl_2$ to a final concentration of 0.125*M* and the DNA suspension inoculated

onto a monolayer of $10^6$ *S. frugiperda* cells in a 35mm tissue culture dish. After adsorption for 30 min at room temperature, 2ml of fresh medium was added and the cells allowed to incubate for a further 2 h, followed by replacement of media with fresh media and incubation at 28°C for 3 days. Success of the transfection was determined by the presence of polyhedra within *S. frugiperda* cells when examined by light microscopy. The cells and media were harvested and used to produce plaques in *S. frugiperda* cell monolayers. Plaques containing recombinant virus were identified by the lack of polyhedra when examined by light microscopy. Polyhedra negative plaques were further screened by repeated plaque assays and virus stocks of AcNPV recombinant clone AcYM1636a were produced.

**Construction of baculovirus recombinant clones: DUG M segment**

For baculovirus expression of a portion of the M segment, 2 oligonucleotide primers were synthesised for use in a PCR reaction. One 58 base primer (BAC1ALI) was located 186 bases from the viral 5' terminus, ending with the stop codon of the ORF of the M segment. A *Xba*I site was engineered next to the stop codon. The other primer (BAC2ALI) was 33 bases long and was located within the ORF, 2120 bases from the stop codon. This second primer was engineered to include a start codon and a *Xba*I site. The nucleotide sequence from the first to the second primer was between two hydrophobic regions and was sufficient to code for approximately 80K of protein. A PCR reaction was performed using 0.2µM each of BAC1ALI and BAC2ALI in the presence of 1.5mM $MgCl_2$ and one unit *Taq* polymerase (Promega). Ten nanograms of the plasmid clone pDUG61, containing the appropriate portion of the M-ORF, was used as template. Twenty-five cycles of 95°C for 90sec, 60°C for 60 sec, and 72°C for 3 min were performed. The PCR

product was digested with *Xba*I and the 2.1kb fragment electro-eluted and inserted into *Nhe*I cut transfer vector, pJVP10Z.

**Co-transfection and selection of baculovirus recombinants: DUG M segment**

The procedure used for the M segment differred from that used for the S segment. A polyhedrin negative baculovirus (AcRP6-SC) was used. One hundred nanograms of *Bsu*36I digested viral DNA (Kitts *et al.*, 1990) was mixed with 2µg transfer vector DNA containing the insert in the correct orientation and in the presence of an equal volume of lipofectin (BRL). After 15min incubation at room temperature, the mixture was layered onto $10^6$ *S. frugiperda* cells in a 35mm dish which had been washed twice in TC100 with no serum, and overlayed with 1ml TC100. Cells were incubated at 28°C for 5h, then the medium was discarded and 2ml TC100 containing 10% FBS were added. The cells were incubated for 2 days at 28°C and then harvested for plaque analysis. The presence of the *lac*Z gene (from the pJVP10Z vector) was determined by staining plaques overnight with BCIG. Blue plaques were picked and plaque purified a further 2 times.

**Western blot analysis of *S. frugiperda* expressed proteins**

*S. frugiperda* cells were infected with recombinant baculoviruses at a multiplicity of infection of 5-10 pfu/cell, and incubated at 28°C for 24 to 72h. The cells were washed with PBS and prepared for SDS-PAGE by resuspension in 5% SDS, 20% glycerol, 120m*M* Tris-HCl pH 6.8, 10% 2-mercaptoethanol, and boiled for 3 min. SDS-PAGE and Western blotting were as described above. Monoclonal antibodies to CCHF N protein (supplied by Dr J.F. Smith, USAMRIID, Frederick, MD) or DUG N and G1 proteins (supplied by Dr S. Higgs, IVEM, Oxford) were used at 200-fold dilutions. Lysates of cells

similarly infected with non-recombinant viruses (AcNPV or AcRP6-SC, as appropriate) were used as negative controls.

### ELISA antigens and antisera

*S. frugiperda* cells expressing DUG N protein or Punta Toro (PT) (*Phlebovirus)* N protein (negative control antigen; donated by M-K Min, IVEM, Oxford) were washed in PBS and disrupted by sonication. The concentration of N protein in each sample was estimated by comparison to BSA standards on stained acrylamide gels. CCHF N protein, used as a positive control antigen, was donated as a sonicated preparation of purified nucleocapsids by Dr J. Smith (USAMRIID, Frederick, MD). A crude antigen was prepared by Prof. A. Antoniadis (Aristotelian University of Thessaloniki, Greece) from Vero E-6 cells infected with the IbAr 10200 strain of CCHF virus. The cells were harvested when approximately 95% cells were infected (as determined by immunofluoresence), and then washed in PBS and disrupted by sonication. Negative control antigens for the expressed DUG N protein and CCHF crude antigen preparations were produced as above but using uninfected *S. frugiperda* and Vero E-6 cells, respectively. Polyclonal rabbit antiserum was prepared by hyperimmunisation with gradient pruified DUG virus. Polyclonal mouse ascitic fluid (MIAF) was produced by hyperimmunisation of mice with partially purified CCHF virus (IbAr 10200 strain). The MIAF raised against HAZ virus was supplied by Dr D. Watts (USAMRIID, Frederick, MD). Human sera (CCHF and control) were supplied by Prof. A. Antoniadis (Aristotelian University of Thessaloniki, Greece).

### ELISA Protocol

DUG and PT N protein samples were diluted to 50ng/100µl in coating buffer (19m*M* $NaHCO_3$, 27m*M* $Na_2CO_3$ pH 9.6) and the CCHF nucleocapsid

preparation was diluted 1/500 in coating buffer. 100µl was added to each microtitre well and allowed to adsorb overnight at 4°C before being removed, and the wells were washed twice with PBST. Sera diluted from 1/100 to 1/51,200 were added to appropriate wells and incubated at 37°C for 1h, then removed, and the wells were washed three times with PBST. Anti-species IgG-HRPO conjugates, diluted in PBST, were added to each well and incubated at 37°C for 30 min before removal and washing of the wells three times with PBST. OPD substrate was added and the colour reaction was allowed to develop for 15 min at 37°C. The reaction was stopped by the addition of 50µl of 1N $H_2SO_4$, and the absorbance was read at 492nm.

**Polymerase chain reaction for viral diagnosis**

Purification of total RNA from infected tick organs and haemolymph samples was essentially as described by Birnboim (1988). The samples were homogenised in 0.5 M LiCl, 1 M urea, 0.25% SDS, 20 mM sodium citrate pH 6.8, 2.5 mM EDTA (RES buffer), and proteinase K was added to 10 µg/ml before incubation of the samples at 50 °C for 30 min. After cooling on ice, the samples were precipitated with ethanol and the resulting precipitate was resuspended in 450 µl of RES buffer. Proteinase K was added to 50 µg/ml and the samples were incubated at 50 °C for 30 min., followed by two extractions with phenol/chloroform. The RNA in the aqueous phase was precipitated by the addition of 3.5 µl of 2 M acetic acid and 0.5 ml of LiCl/ethanol solution (3 vol. 5 M LiCl:2 vol. 95% ethanol), and incubation on ice for 1 h. The RNA was pelleted for 5 min in a microfuge and resuspended in 200 µl of 1 mM sodium citrate pH 6.8, 1 mM EDTA, 0.1% SDS. The RNA was ethanol precipitated, washed with 70% ethanol, and resuspended in 50 µl of $H_2O$. In addition to the RNA extraction procedure described above, RNA was prepared from infected mouse samples by homogenisation in 1% SDS followed by incubation at 65 °C

for 30 min. Yeast RNA was added to a final concentration of 1 µg/ml and the samples were phenol extracted until no interface remained. The crude nucleic acid extract was then precipitated with ethanol and resuspended in $H_2O$.

DUG viral RNA was converted to cDNA in a 20 µl reaction; 11 µl of DUG RNA preparation was mixed with 1 µg each of two 17-mer oligonucleotide primers, PCRDUG1 (5' TCCTCTAGCAGCTACTG 3') and PCRDUG2 (5' TCATTCGACCAGGAGTC 3') which are complementary to nucleotides 733-749 of DUG viral S segment RNA (numbered from the 3' end of viral RNA), or to nucleotides 1189-1205 of viral-complementary (vc) S RNA (numbered from the 5' end of vcRNA), respectively (see Fig. 4). The primers were annealed to DUG S RNA by boiling for 1 min then cooling to room temperature, before conversion of the DUG S RNA to cDNA in a 20 µl reaction containing 10 mM DTT, 50 mM Tris-Cl pH 8.3, 8 mM $MgCl_2$, 70 mM KCl, 1 mM each dNTP, 1 U/µl reverse transcriptase (Amersham) and 1mM vanadyl-ribonucleoside complexes, at 42°C for 30 min.

PCR amplification of DUG cDNA was carried out in a 50 µl reaction containing 10 µl of DUG cDNA from the reverse transcription reaction, 1 µg of each primer PCRDUG1 and PCRDUG2, 0.5 mM of each dNTP, 67 mM Tris-Cl pH 8.8, 16.6 mM ammonium sulphate, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 6.7 µM EDTA, and 2U of *Taq* DNA polymerase (Anglian Biotec or Cambio, UK). The reaction solutions were overlaid with 100 µl of paraffin and then the amplification cycles performed in a Cambio thermal cycler. An initial cycle of amplification of 95 °C for 2 min, 55°C for 2 min, and 70°C for 5 min was performed, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 70 °C for 3 min. The last cycle was 95 °C for 1 min, 55 °C for 1 min, and 70 °C for 5 min.

PCR products were detected by agarose gel electrophoresis or by dot-blot hybridisation. Agarose gel electrophoresis was carried out on 1% agarose and

the presence of DUG virus in the original samples was determined by the presence of an approximately 480 base pair (bp) PCR product predicted from the sequence of DUG S-RNA (Fig. 4). Confirmation that the 480 bp PCR product was DUG specific was obtained by Southern blotting of the gel to Hybond-N nylon membrane (Amersham) and probing of the blot with DUG S segment cDNA clone pDUG36 (see Fig. 3) as described below.

For dot-blot analysis, the PCR products were precipitated with ethanol and resuspended in 5 µl of $H_2O$. The PCR product was applied to Hybond N membrane (Amersham) and the blot was treated with 0.5 M NaOH, 1.5 M NaCl for 10 min, then neutralised with 0.5 M Tris-HCl pH 7.4, 1.5 M NaCl, as previously described (Abbott *et al.*, 1989). The blots were UV fixed for 2 min and prehybridisation, hybridisation and subsequent washes were carried out as described for GeneScreen hybridisation membrane (DuPont). The probe used to detect the PCR products was a 300bp *Sac*I-*Pst*I fragment of cDNA from clone pDUG36 (Ward *et al.*, 1990a) (see Fig. 3), which had been purified from low gelling temperature agarose and labelled with $^{32}$P-dATP using a random-primed labelling kit (Amersham). The cloned *Sac*I-*Pst*I cDNA fragment did not contain either primer sequence. Detection of hybridised PCR products was by autoradiography.

**Determination of the detection limits of PCR**

Mouse blood containing $10^4$ PFU/ml DUG virus was used to identify the limits of PCR and dot-blotting for the detection of DUG virus. For PCR, 1, 10, and 100 µl aliquots of infected mouse blood (equivalent to 10, 100 and 1000 PFU) were used for RNA extraction by the SDS/phenol method described above. Prior to extraction, the 1 and 10 µl samples were made up to 100 µl with uninfected mouse blood. The extracted RNA was resuspended in 11 µl of $H_2O$ and used in the reverse transcriptase reaction. The resulting cDNA

products were precipitated with ethanol, then resuspended in 10 µl of $H_2O$ for use in the PCR reaction. PCR amplification was carried out for 32 cycles as described above, then fresh *Taq* polymerase added, and a further 32 cycles of amplification performed.

DUG viral RNA was extracted from infected tissues by the method of Birnboim (1988). Reverse transcriptase was used in a 20 µl first strand cDNA reaction primed by the two DUG specific primers, PCRDUG1 and PCRDUG2, which anneal 450 bases apart on the S segment. Two µl of the first strand cDNA reaction were used in a 50 µl polymerase chain reaction (PCR) containing 1 µl PCRDUG1, 1 µl PCRDUG2, 1 mM dNTPs, 2 U *Taq* DNA polymerase (Anglian Biotec, UK), and 1 x amplification buffer as supplied with the *Taq* polymerase. 15 cycles of amplification consisting of 95°C for 1 min, 55°C for 1 min, and 70°C for 3 min were carried out. After the 15 cycles, another 2 U of *Taq* DNA polymerase was added and a further 15 cycles of amplification carried out. The 450 bp amplification product was detected by agarose gel electrophoresis, and the specificity confirmed by hybridization with DUG viral RNA.

# RESULTS

## Experimental vertebrate host range of DUG virus

Of the 13 types of mammal tested, viraemia was detected only in mice, and Syrian and Dzungarian hamsters. Neutralizing antibody directed against DUG virus was detected in hamsters and guinea pigs, but not in rabbits and the several rat strains and species tested (Steele & Nuttall, 1989).

Twenty-four 5 week-old Syrian hamsters were inoculated subcutaneously with 1.6 to 6.7 $\log_{10}$ PFU DUG virus. Two hamsters developed a low level of viremia with a maximum titer of 2.5 $\log_{10}$ PFU on day 5. Nine 3 to 4 week-old Dzungarian hamsters were inoculated subcutaneuosly with 4.4 to 6.4 $\log_{10}$ PFU of DUG virus and then 3 animals killed on days 4 to 6 post-inoculation. Viremia was detected in 2 hamsters on days 4 and 5 (maximum titer 3.3 PFU/ml blood on day 5).

## *Per os* infection of ticks

Ticks were infected *per os* either by feeding on inoculated animals or by capillary feeding.

Twelve 6 to 8 week-old Dzungarian hamsters were each infested with fifty *A. variegatum* larvae, and one day later the hamsters were inoculated subcutaneously with 6.5 $\log_{10}$ PFU/ml DUG virus. A total of 112 engorged and semi-engorged ticks were collected. After moulting, the ticks were screened for DUG virus by feeding them together with 100 uninfected nymphs on a total of 10 uninfected Syrian hamsters: no viremia was detected although the animals seroconverted. None of the uninfected ticks acquired virus (Steele & Nuttall, 1989).

After capillary feeding on DUG virus, 8/10 *A. variegatum* and 10/14 *R. appendiculatus* nymphs were infected (Steele & Nuttall, 1989). Following

moulting, DUG virus was detected in 9/12 adult *A. variegatum* but in none of 42 adult *R. appendiculatus*. These results indicate that *A. variegatum*, but not *R. appendiculatus*, is a competent vector of DUG virus.

### Virus replication in inoculated ticks

DUG virus replicated and persisted trans-stadially in both *A. variegatum* and *R. appendiculatus* following virus inoculation. The virus persisted in the adult ticks for long periods: over 140 days in moulted adult *R. appendiculatus* by when the titer had fallen to 2.1 $\log_{10}$ PFU/tick, and over 200 days in moulted adult *A. variegatum* at which time the mean titer was 2.2 $\log_{10}$ PFU/tick (Steele & Nuttall, 1989).

### Horizontal and vertical virus transmission

Attempts to demonstrate "non-viremic" transmission of DUG virus have been unsuccessful. Engorged larvae and nymphs of *A. variegatum* were inoculated with DUG isolate 44313 on the day of drop off. Three months later, after moulting, the emergent nymphs were fed on guinea pigs and hamsters together with uninfected ticks. None of the uninfected ticks became infected although the guinea pigs and hamsters seroconverted.

Egg batches from 6 and 18 engorged females of *A. variegatum* and *R. appendiculatus*, respectively, were assayed for virus either directly or after hatching to larvae. There was no evidence of vertical transmission of DUG virus (Steele & Nuttall, 1989).

### Anatomical basis of virus infection in ticks

A total of 96 specimens were examined by light microscope histochemistry (Table 1). In non-feeding adult ticks, the distribution of DUG virus antigen was essentially the same in ticks infected at the preceding

nymphal stage by either intra-coelomic inoculation or capillary feeding. During feeding of the infected adults, several different organs and tissue types that appeared to be uninfected in the non-feeding, questing stage, became infected. Most notably, the salivary glands and ovary only became infected after the commencement of feeding; the results from both histochemistry and infectivity titration of dissected organs gave the same results, summarised in Table 1 and recorded in detail in Booth *et al.* (1990). The results of this study showed that DUG virus infection in *A. variegatum* predominates in the hemocytes (Fig. 1). Since many tick hemocytes are motile, or loosely attached in the haemocoel, these cells probably play an important role in the internal dissemination of the virus within the tick. Ixodid ticks feed only once per stadium, hence the trans-stadial survival of infected hemocytes, during moulting, is essential for the tick to be a competent vector of the virus. The salivary glands, which undergo necrosis and regeneration during moulting, were not invaded by DUG virus until the commencement of feeding on a host. The close presence of infected haemocytes to uninfected tick salivary gland secretory cells suggests that virus may be transfered via haemocytes by cell to cell contact or by invasion of salivary tissue by a process of diapedesis (the passage of cells through an epithelial layer without its rupture). This conclusion is supported by the fact that hemolymph from infected ticks contained no infectivity once the hemocytes were removed by low speed centrifugation, indicating that most virus was cell-associated rather than released freely in the haemolymph. Moreover, DUG viral RNA could not be detected by polymerase chain reaction in acellular haemolymph whereas it could be detected by this method in tick tissues.

**Viral growth curves** *in vitro*

Growth of the 6 DUG isolates in BHK or PS cells resulted in a peak titer in the culture supernatant within 48 h post-infection, followed by a drop in titer. The peak titers for the different isolates ranged from 1 x $10^5$ to 1.5 x $10^7$ in BHK cell supernatant, and 1 x $10^5$ to 2 x $10^7$ PFU/ml in PS cell supernatant, with highest titers in both cell types obtained with the ArD44313 isolate. The different isolates showed different responses in BHK and PS cells. DUG isolates ArD44313, 16095, 16769, and KT281/75 reached higher titers in BHK cell supernatants than in PS cell supernatants, whereas IbH11480 isolate showed little difference between cell types, and IbAr1792 reached a higher titer when grown in PS cells. Titers in the tick cell line, RA257, reached only 10 to 100 PFU/ml (titrated in PS cells at 36°C). As judged by Western blotting, the maximum yield of intracellular viral polypeptides was obtained 10 days p.i.

**Viral polypeptides**

Polyacrylamide gel electrophoresis of pulse labelled DUG virus infected cells revealed 3 intracellular viral induced polypeptides of $M_r$ approximately 105, 85, and 49 K. The 49 K protein has been demonstrated to be the viral nucleoprotein (N) (see below). The N protein was detected as early as 12 h post-infection, and the 2 larger proteins were apparent at 18 h post-infection or later. Shut-off of host cell protein synthesis was not observed.

SDS-PAGE of semi-purified DUG virus revealed 3 main protein bands of 73, 49 and 35 K that were not present in the uninfected cell control (Fig. 2). These bands corresponded to the G1, N and G2 structural proteins of DUG virus, respectively. When this gel was electro-blotted onto a nitrocellulose sheet and probed with rabbit polyclonal antiserum to DUG virus, only virus specific proteins were detected. Proteins were not detected in the uninfected

cell control whereas several polypeptides, in addition to G1, G2 and N, were consistently detected in the virus pellet. The major, additional proteins were 210, 45, 40, 33 and 30 K (Fig. 2).

Protease treatment of DUG virus pellet with α-chymotrypsin resulted in the removal of the G1 and G2 proteins from the virus while the N protein was unaffected. This indicated that the G1 and G2 proteins were present on the outside of the virus while the N protein was internal to the viral envelope.

Using the glycan detection technique, three glycoprotein bands were observed in DUG virus infected cells. In addition to the 73 K G1 and the 35 K G2 proteins, a 40kD glycoprotein was also observed.

When the enzyme N-glycanase was used to digest asparagine linked oligosaccharide side chains, the G1 glycoprotein migrated differently on SDS-PAGE. A shift of 5 K was observed for the G1 protein but no detectable difference was observed in the migration of N or G2. Additionally, no shift was detected in the 40kD protein on Western blots after treatment with this enzyme. The G1 protein was therefore heavily glycosylated while the G2 and 40 K proteins were only lightly glycosylated or not asparagine linked. The N protein was not glycosylated since it did not react in the glycan detection system (El-Ghorr *et al.*, 1990).

Immunoprecipitation of $^{35}S$ cysteine-labelled proteins with polyclonal serum (raised against purified DUG virus) resulted in precipitation of the 105, 85, 73 (G1) and 49K (N) proteins. This indicated that the 105 and 85K nonstructural proteins shared epitopes with the structural proteins. The 85K protein was also precipitated by a G1-specific monoclonal antibody. Results obtained using $^{3}H$-mannose indicated that G1 and the 85K are glycosylated. G2 has not been detected in cell lysates. Chasing $^{35}S$-cysteine-labelled proteins with unlabelled cysteine resulted in an increase in the amount of G1 as the amount of 85K protein decreased, indicating a precusor-product

relationship. Labelling in the presence of tunicamycin at 10 µg/ml resulted in a shift in the migration of G1 and gp85 equivalent to a reduction in $M_r$ of 6K.

In order to raise monospecific polyclonal antisera, the G1 and G2 protein bands were separately excised from a 10% polyacrylamide gel and homogenised. These preparations were mixed with aluminium hydroxide adjuvant and used to hyperimmunise guinea pigs. The antiserum to G1 reacted by Western blotting with the G1 protein of semi-purified DUG virus preparations, and with the β-galactosidase fusion protein, DEX512. The G2 antiserum showed only non-specific cross-reactions.

### Viral RNA preparation

When RNA extracted from nucleocapsid preparations was run on 1% agarose gels, 3 bands were observed. Comparison with whole cell ribosomal RNA indicated that the S segment was approximately 1.8 kb in length, M of 6.5 kb, and L of 13 kb. The yield of RNA was estimated to be 25 µg from eight 32 oz flasks of infected cell monolayers.

### Cloning and sequencing

cDNA clones were identified as S, M or L segment specific by Northern hybridization with infected whole cell RNA, and by the absence of hybridization with RNA from uninfected cells.

Three overlapping cDNA clones were produced to DUG S RNA in the vectors pUC19 and pBS, and confirmed as S RNA specific by Northern hybridization (Fig. 3). Dideoxy sequencing of these clones provided 1682 nucleotides of sequence including the 3' end of viral S RNA. Clone pDUGd8 was confirmed as representing the 3' end of viral RNA by the presence of the primer sequence (pr15) used to prepare the clone. This primer was complementary to the first 15 3' bases of DUG S RNA (Clerx-van Haaster *et al*,

1982). The final 30 nucleotides at the 5' end of viral RNA were obtained by chemical sequencing of the pr17 primer extension product and by direct RNA sequencing using reverse transcriptase and dideoxynucleotides.

Three overlapping cDNA clones to DUG M RNA provided 4953 bp of sequence. The final 20 nucleotides at the 5' end of the viral RNA were obtained as described for the S segment, using primer BPALI3 (Fig. 15).

### Properties of DUG S RNA

DUG S RNA was found to be 1712 nucleotides in length with a base composition of 30.6% A, 20.1% C, 23.4% G. and 25.9% U (Fig. 4) (Ward *et al.*, 1990a). Comparison of the 3' and 5' end sequences indicated that 12 of the first 13 nucleotides are complementary (Fig. 5). No sequence homology was found between DUG S RNA and the S RNA segments of snowshoe hare (SSH) and La Crosse (LAC) bunyaviruses, Punta Toro (PT) and Sandfly fever Sicilian (SFS) phleboviruses, and Hantaan (HTN) virus. Northern blotting using strand specific RNA transcript probes, synthesised using the vector pBS and an RNA transcription kit (Stratagene), confirmed that viral RNA is negative sense. No subgenomic mRNAs were detected by Northern blotting.

### Predicted gene products of the S RNA

Computer analysis of the DUG S RNA sequence identified one large open reading frame (ORF) of 1326 nucleotides with a single methionine start codon at nucleotides 50-52 of vcRNA. The methionine start codon is contained within a near optimal consensus translation start sequence for eukaryotic mRNA (Kozak, 1986) with an adenine nucleotide at position -3 and a guanine nucleotide at position +4, ie. AAGAUGG. The ORF potentially codes for a protein of 49.4 kDa which is comparable in size to that described for DUG N

protein. No amino acid sequence homology was found by DIAGON analysis (Staden, 1982) to the N proteins of LAC, SSH, PT, SFS, and HTN viruses.

Western blotting of a β-galactosidase/DUG fusion protein, containing the 49.4 kDa ORF, with a monoclonal antibody specific to DUG N protein, indicated that the 49.4 kDa ORF codes for DUG N protein (Fig. 6). Computer analysis of the DUG N protein amino acid sequence indicated the presence of a potential asparagine-linked glycosylation site, however, comparison of DUG N protein from tunicamycin treated PS cells, with DUG N protein from untreated cells, by SDS-polyacrylamide gel electrophoresis, produced, as expected for a viral nucleocapsid protein, no evidence of glycosylation. A short ORF of 150 nucleotides (5.9 kDa), with two methionine start codons at nucleotides 1473-1475 and 1482-1484, was observed in a +1 reading frame, 3' to the N protein ORF in vcRNA . Northern analysis of DUG infected PS cell RNA extracts using clone pDUG 36 as a probe provided no evidence of a subgenomic message which could code for a 5.9 kDa protein (Fig. 7).

**Properties of DUG M RNA**

The sequence has been determined for 4953 nucleotides extending from the viral 5' end (Fig. 16). This sequence has a composition of 32.2% U, 18.4% G, 21.9% C and 27.4% A, in the viral strand. An estimated 1500 nucleotides at the 3' end remain to be sequenced. Comparison of the 5' terminal sequence with the 5' end of the S segment revealed sequence identity between the first nine nucleotides of both segments, but the M segment has an extra adenosine residue at the 5' end. No sequence homology was found between the DUG M RNA sequenced to date and the M RNA segments of any other members of the Bunyaviridae recorded in the EMBL database.

**Predicted gene products of the M RNA**

Analysis of the 5.0 kb of sequence obtained so far reveals a single ORF in the vcRNA (i.e. negative sense coding strategy) with the coding capacity of 185K. The predicted protein contains several putative membrane-spanning regions and at least 9 potential N-linked glycosylation sites. The estimated coding capacity of the M segment is for a polypeptide of 240K. By analogy with other members of the Bunyaviridae, this very large protein is presumably a precursor of the structural glycoproteins and one or more nonstructural proteins. The viral 5' end 2000 bases of the M-ORF appears to code for the G1 structural glycoprotein (see below).

**Cross-hybridization of DUG nucleic acid probes with other nairoviruses**

cDNA clones representing parts of the S and M RNA segments of DUG virus, isolate ArD44313, were tested for their ability to hybridise with 6 other DUG virus isolates, and with representatives of all six other nairovirus serogroups (Marriott *et al.*, 1990): Avalon virus (Sakhalin serogroup), Bandia virus (Qalyub serogroup), Dera Ghazi Khan virus (DGK serogroup), Ganjam virus (NSD serogroup), Hazara virus (CCHF serogroup), Hughes virus (HUG serogroup), and Thiafora and Erve viruses (TFA serogroup). CCHF isolate IbAr10200 (CCHF serogroup) was grown in SW13 cells and the total cellular RNA was kindly donated by C.S. Schmaljohn (USAMRIID, Ft. Detrick, U.S.A). In each case, growth of the virus was confirmed by immunofluorescence or immunophosphatase assays performed on cell monolayers. Hybridization was at 42°C in 50% formamide, 5xSSC, 1% SDS, 50 mM Tris-HCl, pH 7.5, and 0.2 mg/ml denatured salmon sperm DNA (1xSSC contains 0.15 M NaCl, 15 mM sodium citrate, pH 7.0). Washes were in 2xSSC, 0. 1% SDS (2 x 15 min at 42°C) followed by 0.1xSSC, 0.1% SDS (2 x 15 min at 42°C).

As shown in Fig. 8, under the stringency of hybridization used the S-specific DNA probe hybridised to the S RNAs of all six DUG virus isolates but not to the S RNAs of any other nairovirus investigated, nor to the mock-infected control. Similarly, the M-specific DNA probe failed to hybridize to the M RNAs of the other nairoviruses. Although the M-specific DNA probe hybridized to the M RNA of all six DUG isolates, the hybridization to DUG KT281/75 M RNA was weak compared to that seen with the S-specific probe. This may reflect a lower level of homology between the M RNAs of ArD44313 and KT281/75, or a lower copy number of the M RNA of KT281/75 in the cell extract relative to the S RNA of that strain.

Hybridization and washing for the RNA probes were as described for the DNA probes, but an extra wash of 10 µg/ml RNase-A in 2xSSC at 37°C for 15-30 min was used to reduce nonspecific background.

The patterns of hybridization were the same as those obtained with the DNA probes, except with GAN, HAZ and CCHF RNAs (Fig. 9). Although the DNA probes did not apparently hybridize to GAN viral RNA, both S- and M-specific RNA probes clearly detected the respective RNA species of GAN virus. Similar results were obtained for the S RNAs of CCHF and HAZ viruses, however the M RNAs of these two viruses did not hybridize to the M-specific RNA probe under the conditions used (Fig. 9). The difference in hybridization patterns between a DNA probe and its corresponding RNA probe may be attributed to the difference in thermal stability of DNA:RNA and RNA:RNA duplexes in solutions containing formamide (Wahl *et al.*, 1987).

### *In situ* hybridization

Using the biotinylated RNA probe, S RNA was visualised in PS cells infected with DUG 44313 (Fig. 1c). The stain was mainly seen throughout the cytoplasm of infected cells, although in some cells it was confined to the

perinuclear region. There was no background staining of uninfected cells. A 250 nucleotide biotinylated riboprobe derived from DUG S segment (T3 transcripts of pBS-36 cleaved with *Sac* I) was found to give optimum results. When this probe was used on infected tick (*Amblyomma variegatum*) sections (Fig. 1e), viral RNA was detected in gut cells and haemocytes of partially fed inoculated ticks, and in hemocytes and testis of unfed *per os* infected ticks.

**Construction of recombinant baculovirus clones**

In order to express the N protein of DUG virus, two overlapping cDNA clones, pDUG16 and pDUG36, were used to construct a cDNA clone (pDUG1636) representing nucleotides 44-1682 of viral-complementary S segment RNA. The cDNA clone pDUG1636 was then transferred to the baculovirus transfer vector pAcYM1 to produce clone pAcYM1636 (Fig. 10). Transfection of *S. frugiperda* cells with pAcYM1636 and AcNPV DNA, and subsequent plaque assay, produced several putative recombinant viruses with polyhedrin-negative phenotypes. These viruses were recovered and purified by successive plaque assays. One virus was found to have a stable polyhedrin-negative phenotype, and a high titred stock was made of this virus (AcYM1636a) by passage in *S. frugiperda* cells.

**Expression of DUG N protein in *Spodoptera frugiperda* cells**

Monolayers of *S. frugiperda* cells were infected at a high multiplicity (>5 pfu/cell) with the recombinant virus AcYM1636a and incubated at 28°C for 24-72h before analysis by SDS-PAGE. A protein with a molecular weight of 49kDa was observed as the major protein in infected cells (Fig. 11) (Ward *et al.*, 1990c). This protein was not observed in uninfected *S. frugiperda* cells nor in *S. frugiperda* cells infected with wild type AcNPV. To determine if this protein was the 49.4kDa N protein of DUG virus (Ward *et al*, 1990a), the gel was

blotted to nitrocellulose and probed with monoclonal antibody (H28-89) specific to DUG N protein. The expressed protein was found to react specifically with the H28-89 monoclonal antibody and to co-migrate with DUG N protein from pelleted DUG virus particles (Fig. 12), thus confirming the expression of DUG N protein by recombinant virus AcYM1636a. A time course of DUG N protein expression in *S. frugiperda* cells showed the recombinant N protein to be the major protein within cells at 24h post-infection, increasing to a maximum of approximately 50% of total cell protein by 72h post-infection.

### Cross-reaction of expressed DUG N protein with CCHF monoclonal antibody by Western blotting

The possibility of an antigenic cross-reaction between DUG and CCHF viruses was investigated by Western blotting the recombinant DUG N protein against 2 monoclonal antibodies to CCHF N protein. Monoclonal antibodies AC06 and CC02 were found to react weakly to expressed DUG N protein. These cross-reactions, while very weak, confirmed the observations of Casals and Tignor (1980) and indicated the possibility of using the expressed DUG N protein to detect CCHF infection.

### Cross-reaction of expressed DUG N protein with CCHF and HAZ viruses by ELISA

The ability of the expressed DUG N protein to detect CCHF antibodies was demonstrated using ELISA (Fig. 13). Rabbit anti-DUG serum reacted strongly with the expressed DUG N protein and gave a low but significant reaction with the crude CCHF viral antigen (Fig. 13A). The CCHF MIAF reacted strongly with the crude CCHF viral antigen and gave highly significant results with the expressed DUG N protein at antibody dilutions of

1:800 and less (Fig.13B). Using HAZ MIAF, a significant reaction was observed with DUG N protein whereas the reaction with CCHF viral antigen was borderline (Fig. 13C). Similar results were obtained using CCHF nucleocapsid antigen in place of the crude CCHF antigen, and using mouse monospecific serum raised to expressed DUG N protein in place of the rabbit antiserum. The PT N protein did not give a significant reaction with the anti-DUG serum or with the CCHF and HAZ MIAFs.

The human serum reacted strongly to CCHF nucleocapsid antigen, less strongly to DUG N antigen and poorly to PT N antigen when the antiserum was diluted to 1/500 or more (Fig.14). At high serum concentrations, both CCHF serum samples reacted with the PT recombinant N protein negative control.

**Expression of the M RNA ORF**

Three regions of the M RNA ORF (8 K, 27K and 65K) (Fig. 15), which computer analysis indicated lacked strong hydrophobic regions (Fig. 17), were expressed as β-galactosidase fusion proteins (DEX62, DEX71, and DEX512, respectively) to high levels (approximately 50% of total protein) in *E. coli* cells. Mouse ascitic fluid raised against the 3 fusion proteins did not contain specific antibodies as determined by Western blotting and radioimmune precipitation. However, the DEX71 and DEX512 fusion proteins did react with polyclonal anti-DUG serum (raised against purified virus) on Western blots, indicating that this part of the ORF encodes one or more structural proteins, and DEX512 also cross-reacted to a polyclonal anti-G1 serum indicating that G1 is processed from the C-terminal region of the M polyprotein.

The baculovirus expressed M segment product was detected as a 73K band on Coomassie blue stained SDS-polyacrylamide gels but the level of expression was low (Fig.18a). The expressed product cross-reacted with

polyclonal DUG antiserum (Fig. 18b) and polyvalent monospecific G1 antiserum (Fig.18c) by Western blotting. This result supports the above observation that G1 is processed from the C-terminal region of the M polyprotein. However, 2 monoclonal antibodies to G1 did not react with the recombinant protein by Western blotting.

**Diagnosis of DUG viral infections by PCR**

PCR amplification of DUG RNA (Ward *et al.*, 1990b) extracted from infected tick organs produced a 480bp product when analysed by agarose gel electrophoresis (Fig. 19). The PCR product shown in Fig. 19A, lane O, represents the amplified product from 1/200 of the total RNA obtained from 10 infected ticks. Analysis of tick hemolymph for the presence of DUG virus did not produce a detectable PCR product (Fig. 19A, lane H). Southern blotting of the gel, and subsequent probing with a pUC19 sub-clone of the 300bp *Sac*I-*Pst*I fragment from clone pDUG36 (see Fig. 3), confirmed the product as DUG virus-specific (Fig. 19B). The Southern blotting of this agarose gel did not detect any PCR product in the hemolymph sample. However, subsequent use of dot-blotting of PCR products did detect DUG RNA in the hemolymph although the signal was very weak. Virus was not detected in infected tick hemolymph by plaque assay and only rarely detected by mouse inoculation.

PCR amplification was also carried out with RNA extracted from DUG virus infected mouse blood and brain. A positive result was obtained with a mouse brain sample that contained 1000 PFU DUG virus (Fig. 20). However, the blood samples were negative despite the virus being repeatably detectable in mouse blood at a titer of approximately $10^4$ PFU/ml when analysed by plaque assay. The negative result shown in Fig. 20 was obtained with a mouse blood sample containing 200 PFU DUG virus. Further experiments with mouse brain samples using the crude SDS/phenol RNA extraction procedure

indicated a limit of approximately 500 PFU of DUG virus for detection of the virus by PCR and agarose gel electrophoresis (data not shown). Increasing the number of cycles of amplification failed to improve the sensitivity of the assay.

The sensitivity of the assay for viral RNA was significantly improved by dot-blot hybridisation of the PCR product with a DUG specific probe that did not contain any primer sequence. A $^{32}$P-dATP labelled 300bp *Sac*I-*Pst*I cDNA probe detected the presence of DUG specific PCR products in 100 µl of mouse blood containing 10 PFU of DUG virus (Fig. 21). The probe did not react to uninfected mouse blood or the pUC19 DNA, indicating that the hybridisation was specific to DUG sequences. DUG RNA was not detected when the samples were diluted to less than 10 PFU per 100 µl of blood. When 1ml of infected blood was used as the starting material, the signal was weaker (data not shown), indicating that the blood had an inhibitory effect at some stage(s) of the protocol.

### Comparison of infectivity assays and PCR for virus diagnosis

Infected mouse blood used in PCR reactions was titrated in cell culture and in mice. Countable plaques were obtained 5 days after inoculation. Clinical signs of infection were first observed in mice 7 days after inoculation. The titers of the sample were $6.5 \times 10^3$ PFU/ml and $1.4 \times 10^5$ $LD_{50}$ /ml, i.e. a PFU/$LD_{50}$ ratio of 0.05. Thus mouse inoculation was approximately 200 times more sensitive than PCR in detecting DUG virus in viremic mouse blood. The main advantage of PCR was found to be the speed in which a positive identification of the virus as DUG could be achieved, in addition to good sensitivity.

# DISCUSSION

Despite the importance of nairoviruses as causes of disease in humans (CCHF) and domestic animals (NSD), little is known of their molecular biology (Elliott, 1990). Owing to the difficulties of handling CCHF and NSD viruses, DUG virus, a member of the NSD serogroup, was selected for studies of the coding and replication strategies of nairoviruses. An additional aim of these studies was to determine whether reagents derived from DUG virus can be used to detect CCHF viral infections.

## Replication strategy of nairoviruses

The complete sequence of the S segment of DUG virus has been obtained and shown to comprise 1712 nucleotides and contain one large open reading frame (ORF) of 1326 nucleotides coding for a 49.4K protein on viral complementary (vc) RNA (Ward *et al.*, 1990a). The product was confirmed as DUG N protein by Western blotting with DUG N specific monoclonal antibody following expression in pUEX or baculovirus vectors. An additional ORF of 150 nucleotides coding for a possible 5.9 kDa protein is present in the +1 reading frame, 3' to the N protein ORF on vcRNA. DUG S segment mRNA was found to be essentially full length. No evidence was obtained for the existence of a smaller mRNA species that could code for a 5.9 kDa protein. Comparisons of the DUG S RNA sequence and predicted N protein amino acid sequence, with the respective sequences of snowshoe hare, La Crosse (bunyaviruses), Punta Toro, Sandfly fever Sicilian (phleboviruses) and Hantaan (hantavirus) viruses, failed to detect any sequence similarity, although the genomic structure of DUG S RNA is similar to that of the S RNA segment of Hantaan (HTN) virus.

Approximately 75% (i.e. 4953 nucleotides) of the sequence of the M segment of DUG virus has been derived and several clones are currently being examined which extend this sequence even further. Analysis of the derived sequence reveals a single ORF in the vc RNA, indicating that the primary product of this genomic segment is a polyprotein. The detection of virus-induced proteins that are considerably larger than the putative viral glycoproteins supports this conclusion (El-Ghorr *et al.*, 1990). The gene encoding the G1 structural glycoprotein appears to be located at the carboxy-terminal end of the M segment ORF.

### Diagnosis of nairovirus infections

Elucidation of the replication strategy of DUG virus generated reagents that were tested for their diagnostic potential in identifying nairovirus infections.

Cloned cDNA derived from the S and M genomic RNA segments of DUG virus was used to prepare $^{32}$P-labelled DNA and RNA probes (Marriott *et al.*, 1990). The S and M segments of six isolates of DUG virus all hybridised to both DNA and RNA probes, although the M segment of isolate KT281/75 reacted only weakly. Of nine other nairoviruses tested, representing all the six other serogroups within the *Nairovirus* genus, none hybridised to the DNA probes. However, under conditions of low stringency, the DUG S and M RNA probes hybridised to the respective S and M segments of Ganjam (GAN) virus (a fellow member of the NSD serogroup). The DUG S RNA probe also hybridized to the S segments of CCHF and Hazara (HAZ) viruses (members of the CCHF serogroup). The indicated sequence relationships between DUG, GAN, CCHF and HAZ viruses show that the NSD and CCHF serogroups are more closely related to each other than to members of other serogroups within the *Nairovirus* genus. The hybridisation data are consistent with antigenic

data: the only cross-reactions that have been detected between members of different serocomplexes of nairoviruses using complement fixation or immunofluorescent antibody tests are between members of the CCHF and NSD serogroup (Casals, 1978; Casals and Tignor, 1980). These data also demonstrate that cloned probes derived from DUG virus may be used to detect CCHF viral RNA, and thus provide useful diagnostic tools in the absence of cloned probes derived from CCHF virus. The diagnostic potential of biotinylated probes was demonstrated by their ability to detect DUG viral RNA in infected cell cultures and in sections of infected ticks.

The sequence data derived for the S segment of DUG virus was also used to produce oligonucleotide primers for PCR (Ward *et al.*, 1990b). Using this technique, DUG viral RNA was detected in the organs and hemolymph of infected ticks, and in the brain and blood of infected mice. The PCR was as sensitive as a plaque assay for detecting DUG virus, but not as sensitive as intracerebral inoculation of mice. The sensitivity of the technique was greatest using crude RNA extracts combined with dot-blot analysis of the resulting PCR products using a DUG specific cDNA probe. A result was obtained within 48 h using PCR whereas biological assays took at least 8 days to diagnose the virus infection.

The potential use of recombinant proteins as diagnostic reagents has also been examined. A recombinant baculovirus for the expression of DUG N protein was constructed and used to produce DUG N protein as the major constituent of infected *S. frugiperda* cells (Ward *et al.*, 1990c). The cDNA clones used to construct the recombinant baculovirus were produced without extensive precautions, due to the relative safety of DUG virus compared to CCHF virus. The baculovirus recombinant is safe to handle and production of non-infectious DUG N protein is a simple procedure. To investigate the potential of the expressed DUG N protein as a diagnostic reagent for CCHF,

Western blotting analysis was performed using monoclonal antibodies to CCHF N protein. The weak cross-reaction this produced indicated that the use of DUG N protein to detect CCHF viral infections should be pursued. Using ELISA, a significant cross-reaction was demonstrated between DUG expressed N protein and mouse or human polyclonal antibodies to CCHF virus. This was substantiated by the cross-reaction between DUG N protein and HAZ MIAF, and the absence of cross-reactions between expressed PT N protein and CCHF or HAZ antibodies. DUG expressed N protein was more successful in detecting HAZ antibodies than either the crude or nucleocapsid antigens derived from CCHF virus. The results indicate that the recombinant DUG N protein has the potential to fulfill a rapid diagnostic role for CCHF, and may be of use in studying the epidemiology of CCHF infections. Tests utilising the DUG N protein will have limited application in areas where cross-reacting nairoviruses are prevalent but in human cases of CCHF the antigen may provide a safe means of confirming a clinical diagnosis.

### Tick-transmission cycle

The aim of developing a tick-transmission cycle was to examine the interactions between nairoviruses and their tick vectors and vertebrate hosts, and to provide specimens for testing potential diagnostic reagents and procedures. Screening of 13 different mammals failed to identify a vertebrate host that was susceptible to DUG virus infection and was suitable for tick infestation studies. To overcome this problem, ticks were infected *per os* using virus-containing capillaries that were placed over the mouthparts. By this means of infection, *Amblyomma variegatum* was shown to be a competent vector of DUG virus whereas *Rhipicephalus appendiculatus* was not (Steele and Nuttall, 1989). In ticks infected by direct inoculation into the hemocoel, DUG virus replicated and was subsequently transmitted by both tick species.

The observation that DUG virus replicated in *per os* infected *R. appendiculatus* but did not survive trans-stadially suggested a gut-release barrier to infection. These data demonstrate that infection of ticks via inoculation, i.e. by-passing the gut, is not a valid means of identifying natural tick vectors of a virus. The data also raise important questions concerning the epidemiology of tick-borne viruses. For instance, CCHF virus has been isolated from 28 tick species but most isolates were from engorged or partially engorged ticks, therefore making it impossible to distinguish between the disseminated infection of a competent vector and the presence of virus in the gut or bloodmeal of the ticks.

The anatomy of DUG virus infection in *Amblyomma variegatum* was examined using immunohistochemistry and hybridocytochemistry (Booth *et al.*, 1990). For immunolabelling with light microscopy, immunofluorescence was the most sensitive method although the immunoperoxidase technique had the advantage that phase contrast and counterstaining could be used and autofluorescence problems avoided. The immunoperoxidase method had the disadvantage that some tick tissues contain endogenous peroxidase which cannot be fully blocked by preincubation in hydrogen peroxide. Hybridisation was the least sensitive method but a positive reaction shows that a cell contains replicating virus and is not merely presenting antigen produced, for example, in a different cell. A combination of all three methods on serial sections of the same specimen was ideal. When tick organs from infected *A. variegatum* were examined by EM, no clearly defined intracellular vacuoles containing virus particles or viral inclusion bodies (such as are present in infected cell culture) were observed. Viral antigen and RNA were readily detected in tick cells, and infectious virus was isolated from tick tissues. Whether the lack of detectable virions in tick sections is simply a function of the amount of virus present or reflects a difference in viral morphogenesis

compared with that observed in lytic infections of cell cultures, remains to be determined.

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## CONCLUSIONS and FUTURE WORK

The *Nairovirus* genus of the Bunyaviridae family includes Crimean-Congo hemorrhagic fever (CCHF) virus. This tick-borne virus is an important human pathogen in eastern Europe (particularly Bulgaria, Albania, and the USSR), the Middle East (including Saudi Arabia, Iraq, Iran and Afghanistan), Asia (including China, India, Pakistan and Soviet Middle Asia) as well as most of Africa. The threat posed to human health is reflected by the fact that CCHF virus is classed as a category 4 pathogen and requires the highest degree of containment for the handling of most virus strains. Through the support of Contract DAMD17-87-C-7176, we have demonstrated the feasibility of using Dugbe virus, a nairovirus closely related to CCHF virus, to develop diagnostic procedures for identifying nairovirus infections. Dugbe virus does not require high levels of containment for experimentation. We have cloned and sequenced the S segment and 75% of the M segment of Dugbe virus. Reagents derived from the S segment of Dugbe virus (oligonucleotide probes, monospecific anti-N antibody, and a recombinant N protein) were shown to be capable of detecting CCHF virus (viral RNA, antigen, or antibody in human sera). Furthermore, the application of a PCR for rapid diagnosis of virus infections in ticks and in vertebrates was demonstrated.

Having evaluated the diagnostic potential of new reagents produced under Contract DAMD17-87-C-7176, we now propose to produce type and group specific probes, primers and antigens that can distinguish between different nairovirus infections. This will be achieved by completing the sequence data for Dugbe M segment, and obtaining sequence data for the S and M segments of Hazara virus (a member of the CCHF serogroup) and for one or more CCHF virus strains (e.g. the Greek and Chinese isolates). The sequence data will facilitate the selection of probes that detect conserved (group specific)

or variable (type specific) regions of the nairovirus genome, and oligonucleotides primers for PCR that detect all strains of a virus but distinguish between different viruses (e.g. all strains of CCHF virus but not Hazara virus). Emphasis will be placed on the development of rapid diagnostic techniques that detect infections at the onset of symptoms. In addition, studies on the genetics and replication strategy will facilitate the production of nairovirus vaccines. These studies would be performed in conjunction with USAMRIID personnel who would test the reagents in order to confirm their application to the diagnosis of CCHF.

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## PERSONNEL RECEIVING PAY UNDER USAMRIID CONTRACT DAMD17-87-C-7176

| | |
|---|---|
| Marriott, A.C. | (Dec. 1989- Sept. 1990) |
| Clerx, J.P.M. | (Sept. 1987-June 1988) |
| Ward, V.K. | (Sept. 1987-Nov. 1989) |
| El Ghorr, A.A. | (July 1988- Sept. 1990) |

**TABLE 1** Distribution of Dugbe virus in infected ticks examined by light microscopy

| Stage examined | Number of ticks examined | | Virus distribution |
|---|---|---|---|
| | Infected | Control | |
| Non-feeding adult (inoculated as nymphs) | 12 | 9 | hemocytes, integument, basement membranes, connective tissue, motile digestive cells,testis and accessory gland, egg wax gland |
| Capillary-fed adult (24 h post-infection) | 5 | 5 | approx. 10% digestive cells in gut |
| Non-feeding adults after moulting (orally infected by capillary feeding as nymphs) | 13 | 9 | hemocytes, integument, basement membranes, connective tissue, motile digestive cells, testis and accessory gland, egg wax gland |
| Feeding adults (inoculated as nymphs ) | 26 | 17 | in addition to above: salivary gland secretory cells (acini II and III), ovary, 50% of gut secretory and digestive cells |

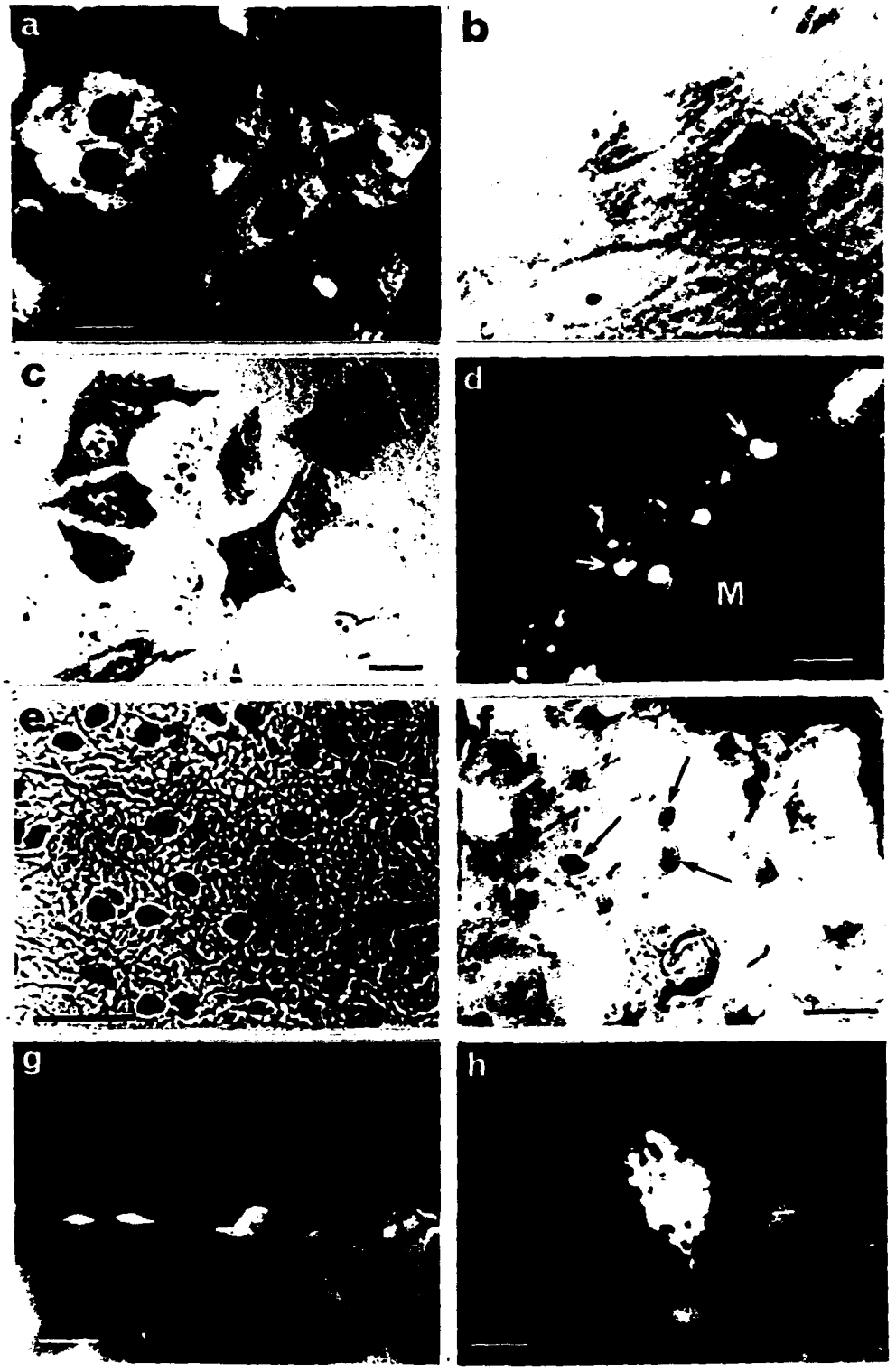


**FIG. 1.** (a-c) DUG virus-infected porcine kidney cells stained by (a) immunofluorescence 48h p.i., (b) immunoperoxidase technique, (c) *in situ* hybridisation with biotinylated DUG S segment-specific riboprobe (infected cells contain dark-coloured alkaline phosphatase reaction product). Scale = 10 µm (d-h) Unfed adult ticks infected with DUG virus at the preceding nymphal stage. (d) Immunofluorescence showing hemocytes (arrows) attached to the surface of a muscle (M). Antigen is also present in the connective tissue surrounding the muscle. Scale = 10 µm. (e,f) *In situ* hybridisation (e) or immunoperoxidase staining (f) showing a positive reaction with tick hemocytes (arrows) within tick connective tissue. Scale = 20µm. (g) Epidermal cells of an infected tick showing positive anti-DUG virus immunofluorescence. Scale = 10 µm. (h) High magnification of a DUG virus infected hemocyte showing amoeboid morphology. Scale = 5 µm.

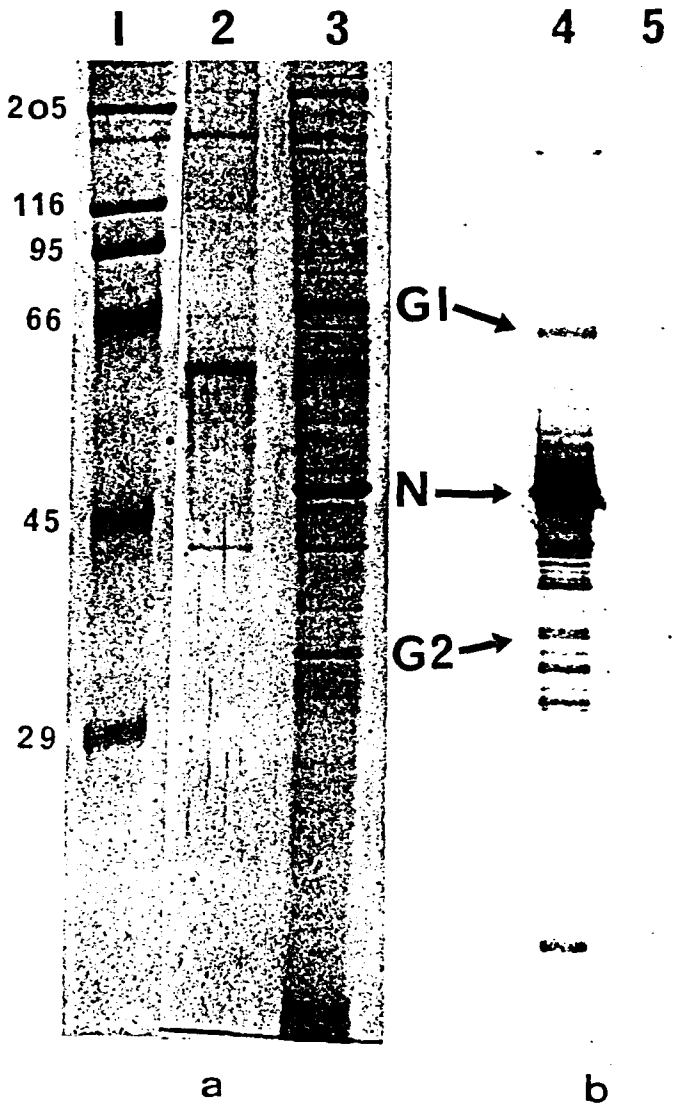


FIG. 2 a) A Coomassie stained 10% polyacrylamide gel of molecular weight markers (lane 1), control mock infected PS cells pellet (lane 2) and DUG virus pellet (lane 3). The G1 , N and G2 proteins were readily detected in the virus pellet. b) On Western blotting and probing with a polyclonal rabbit antiserum to DUG virus, the G1, N and G2 proteins were still detected in the virus pellet in addition to 5 other viral proteins which appeared at 210, 45, 40, 33, and 30kD (lane 4). This rabbit antiserum did not react with any protein bands in the mock infected control (lane 5).

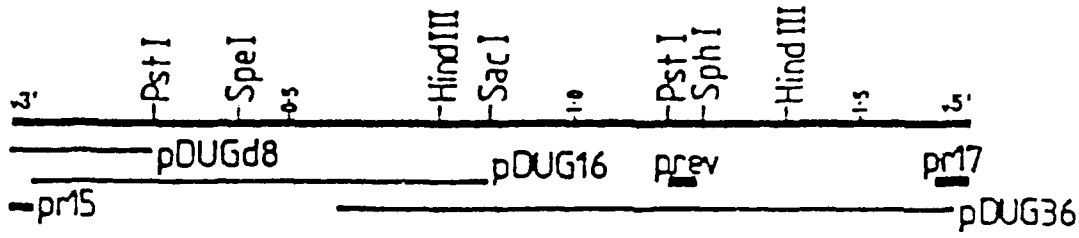


**FIG.3.** Restriction map of DUG S segment cDNA showing clones sequenced and location of the oligonucleotide primers. pr17, oligonucleotide used to sequence viral 5' end of S RNA; pr15 and p.rev, oligonucleotides used for PCR amplification of DUG cDNA prior to cloning of viral 3' end as clone pDUGd8. Nucleotides are numbered from the 3' end of vRNA in kilobases.

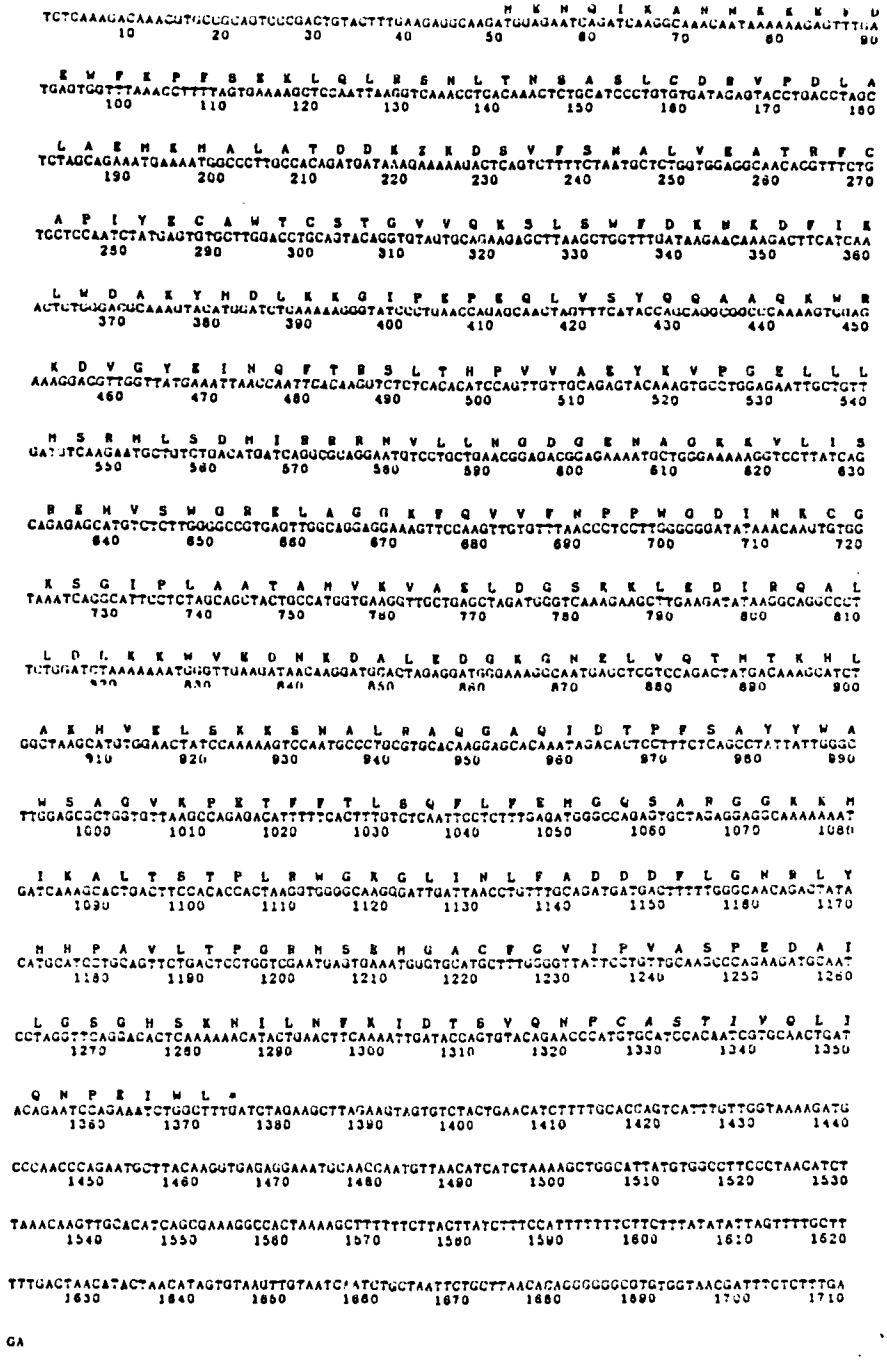

FIG.4. DUG viral-complementary S segment RNA sequence and predicted gene product. The predicted gene product of vc RNA is encoded above the nucleic acid sequence, using the single letter amino acid convention. Nucleotides are numbered from the 5' end of vc RNA.

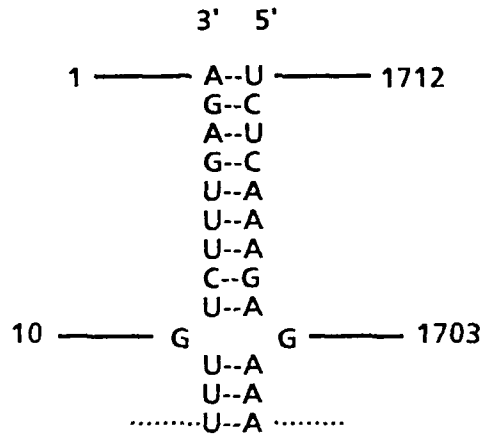


FIG. 5. Comparison of the 3' and 5' terminal sequences of DUG S RNA. Nucleotides are numbered from the 3' end of viral RNA with possible hydrogen bonding indicated by dashes.

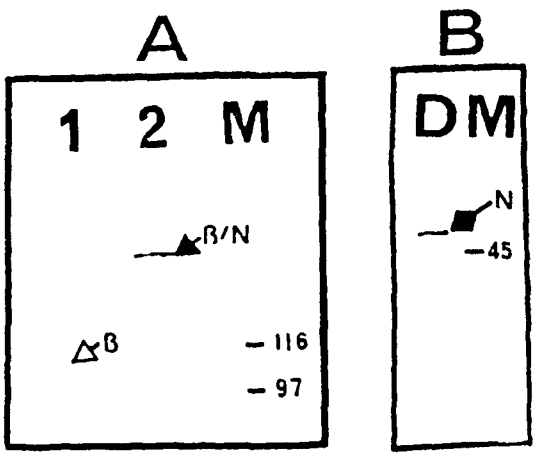


FIG.6. Western blotting of β-galactosidase/DUG fusion protein containing the S segment 49.4 kDa large open reading frame, using monoclonal antibody specific to DUG N protein. (A), Western blot of 5% SDS-polyacrylamide gel; lane 1, *E.coli* XL1- blue cells expressing β-galactosidase and lane 2, *E.coli* XL1-blue cells expressing β-galactosidase/DUG fusion protein; β, β-galactosidase; β/N, β-galactosidase/DUG fusion protein. (B), Western blot of 10% SDS-polyacrylamide gel of; lane D, DUG viral proteins; N, DUG nucleocapsid protein; lane M, markers. The relative positions of β and β/N were determined by comparison with the markers following coomassie blue staining of the gel.

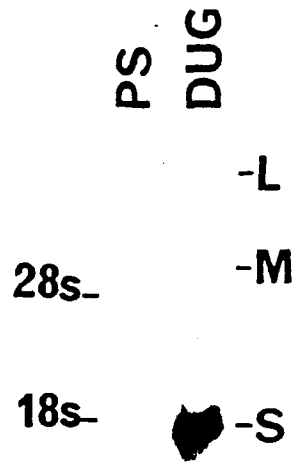


FIG.7. Northern hybridization analysis of DUG S segment using cDNA clone pDUG36 as the probe. PS, whole uninfected PS cell RNA showing the relative position of 28S and 18S ribosomal RNA; DUG, whole DUG infected PS cell RNA: L, M, and S represent the relative positions of DUG viral RNA segments visualised under UV illumination of the ethidium bromide stained gel prior to northern blotting.

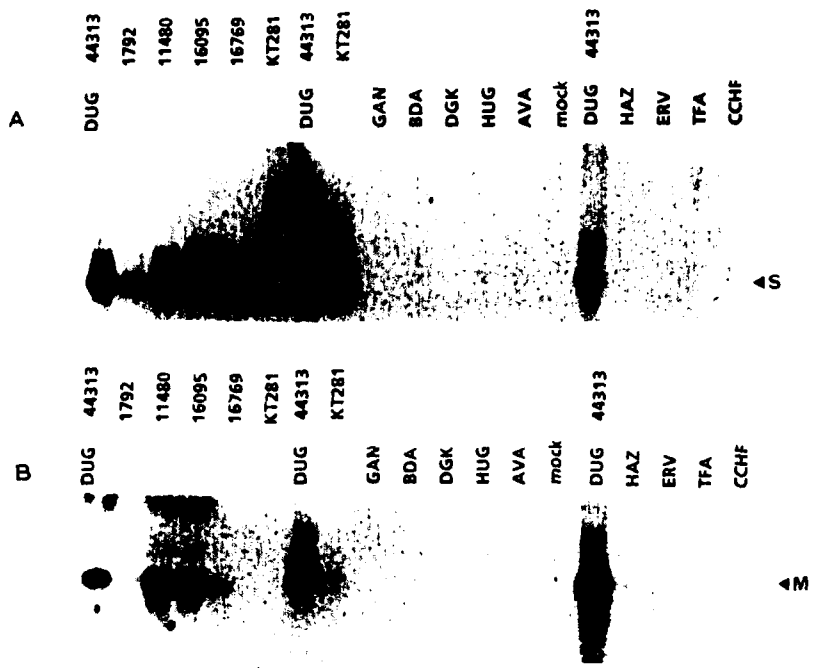


**FIG 8.** Northern blot hybridisation using DNA probes. The probes used were random-primed $^{32}$P-DNA from (A) clone BS-36, (B) clone BS-61. The viruses are identified by the abbreviations and/or isolate numbers described in the text; "mock" refers to mock-infected cellular RNA. Arrows mark the positions of the DUG S and M RNA segments.

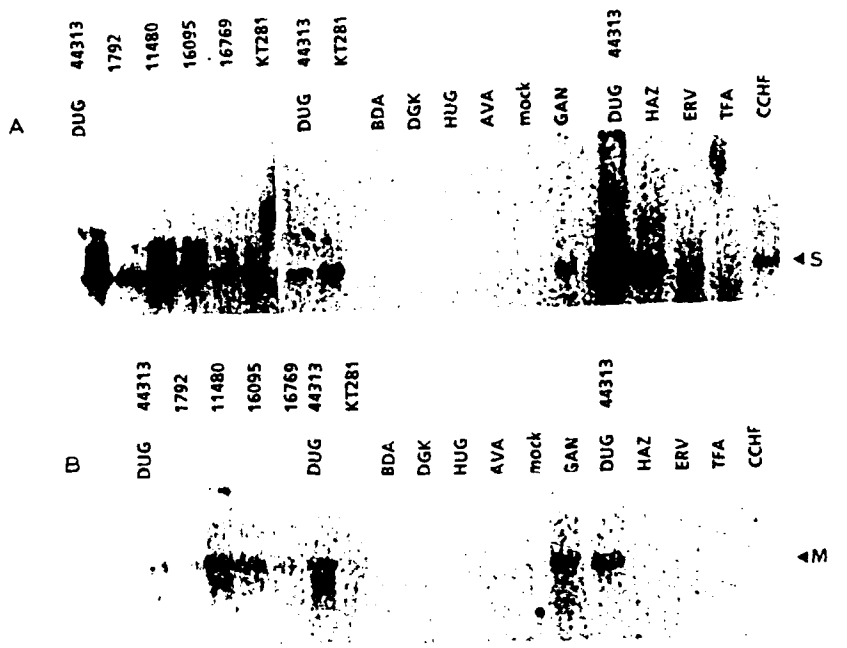


**FIG 9.** Northern blot hybridisation using RNA probes. The probes used were T3 RNA polymerase transcripts of linearised DNA from (A) clone BS-36, (B) clone BS-61. The viruses are identified by the abbreviations and/or isolate numbers described in the text; "mock" refers to mock-infected cellular RNA. Arrows mark the positions of the DUG S and M RNA segments.

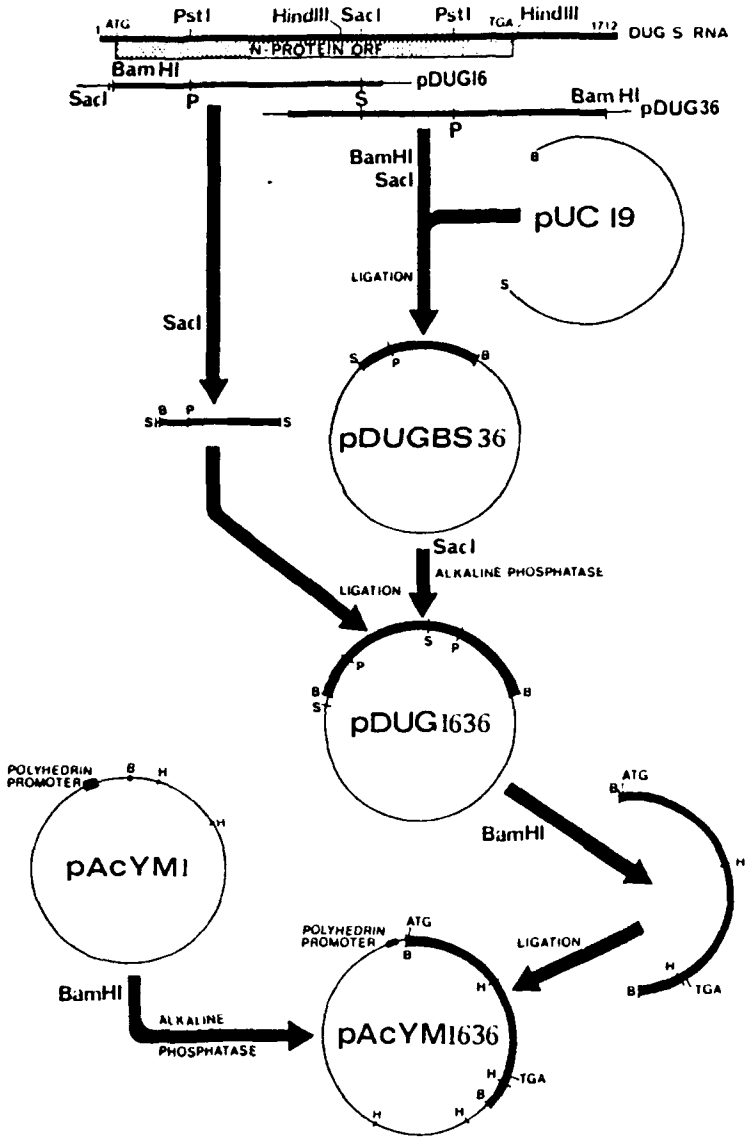


**FIG. 10.** Schematic diagram of the construction of the transfer vector pAcYM1636. Two overlapping cDNA clones, pDUG16 and pDUG36, representing nucleotides 44-1682 of viral-complementary DUG S RNA, were ligated together in pUC19, as described in Materials and Methods, to produce a full length ORF for DUG N protein. The complete clone was used to construct transfer vector pAcYM1636 as described in Materials and Methods. The restriction enzymes used were: *Bam*HI (B), *Hind*III (H), *Pst*I (P), and *Sac*I (S).

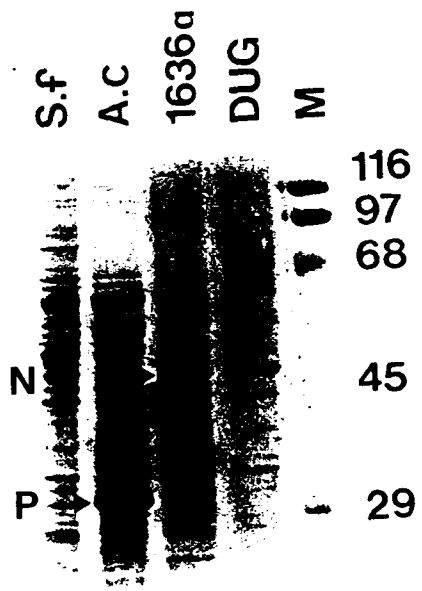


FIG. 11. Expression of DUG N protein by recombinant baculovirus AcYM1636a. *S. frugiperda* cells were infected with wild type AcNPV (lane A.c), or recombinant baculovirus AcYM1636a (lane 1636a). Cellular proteins were recovered at 62h post-infection, and an aliquot of each sample resolved by SDS-PAGE. Uninfected cells were treated similarly (lane S.f). DUG viral proteins (lane DUG) were obtained from virus particles concentrated by centrifugation of media from DUG infected PS cells. Expressed and viral DUG N protein (N) and polyhedrin protein (P) are indicated. Size markers and their molecular weights in kilodaltons, are shown in lane M.

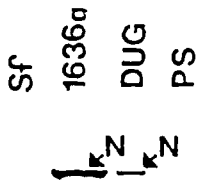


FIG. 12. Western blotting of expressed and viral DUG N proteins. *S. frugiperda* cells were infected with recombinant baculovirus AcYM1636a, and cellular proteins recovered at 62h post-infection for resolution by SDS-PAGE (lane 1636a). Uninfected cells were treated similarly (lane S.f). DUG viral proteins (lane DUG) were obtained from virus particles concentrated by centrifugation of media from DUG infected PS cells. Uninfected PS cells were washed in PBS and prepared for SDS-PAGE as described in Materials and Methods. Blotting of the nitrocellulose membrane and immunological detection of expressed and viral DUG N proteins (N) with monoclonal antiboby to DUG N protein, was as described in Materials and Methods.

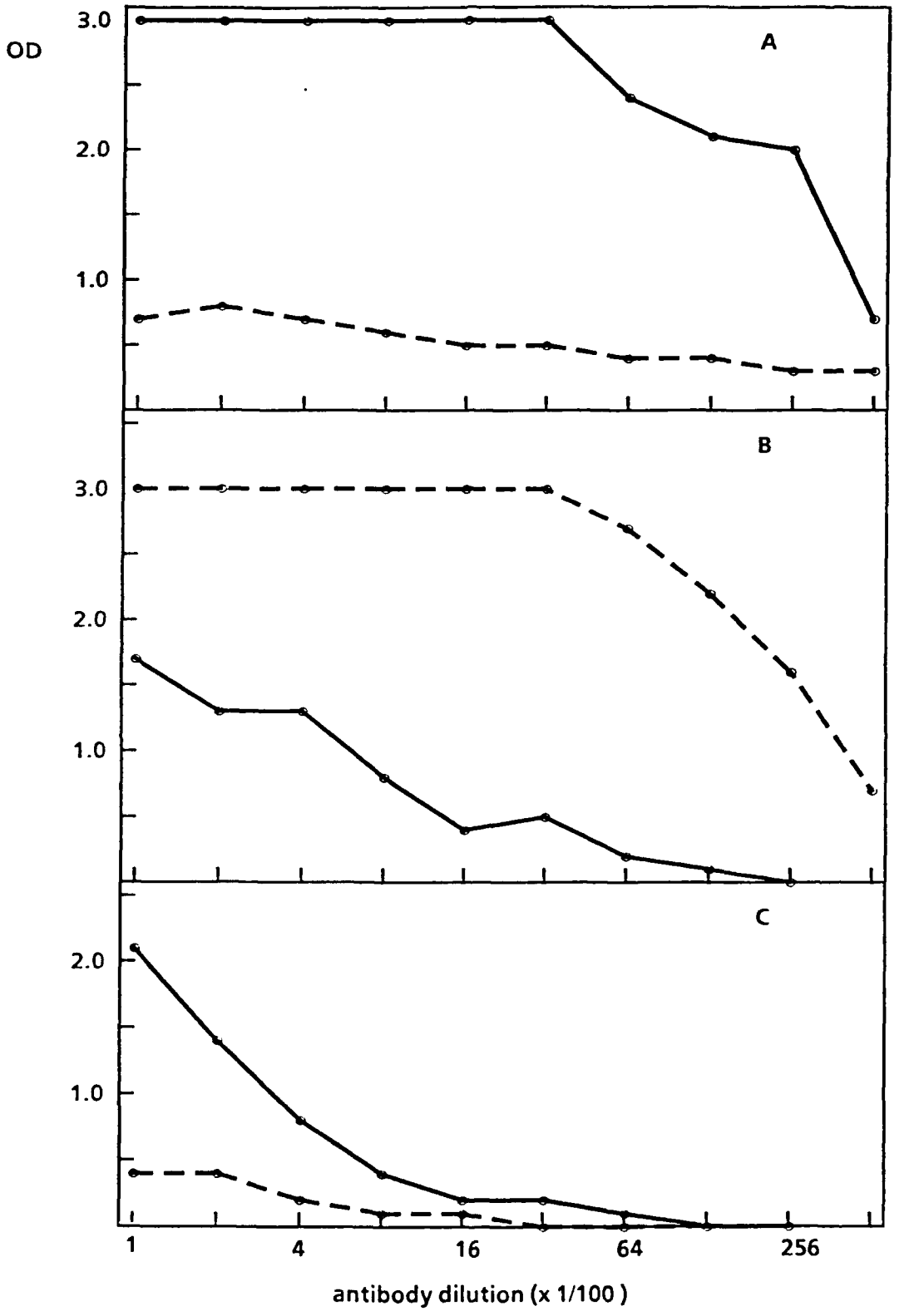


**FIG. 13.** Reaction of antibody with baculovirus expressed DUG N protein (o——o) or CCHF nucleocapsid antigen (o- - - -o). (A) rabbit anti-DUG virus serum; (B) mouse anti-CCHF virus polyclonal immune ascitic fluid; (C) mouse anti-HAZ virus polyclonal immune ascitic fluid. Optical density (OD) results were determined by subtracting values for the relevant control antigens from those obtained using DUG or CCHF antigen (see Materials and Methods).

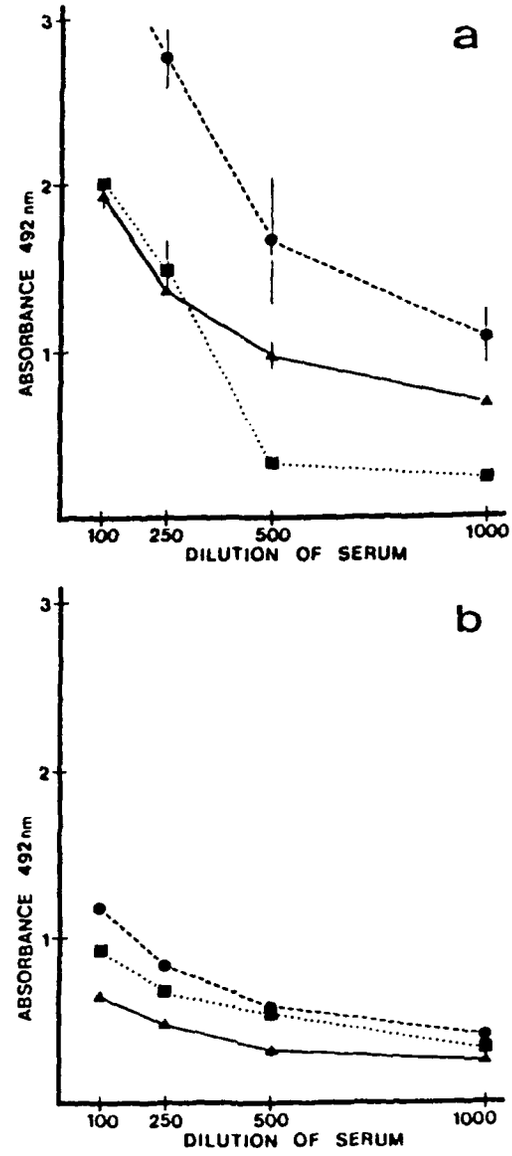


FIG. 14. Reaction of human anti-CCHF virus to CCHF, DUG N, and PT N antigens by ELISA. CCHF nucleocapsid antigen (●----●) and cell extracts of baculovirus expressed DUG (▲—▲) and PT (■···■) N proteins, were absorbed to the solid phase and tested using 1:100 to 1:1000 dilutions of human anti-CCHF virus (a) or human non-immune serum (b). All reactions were performed in duplicate. The range of absorbance values obtained is indicated by a vertical bar when greater than the size of the data point marker.

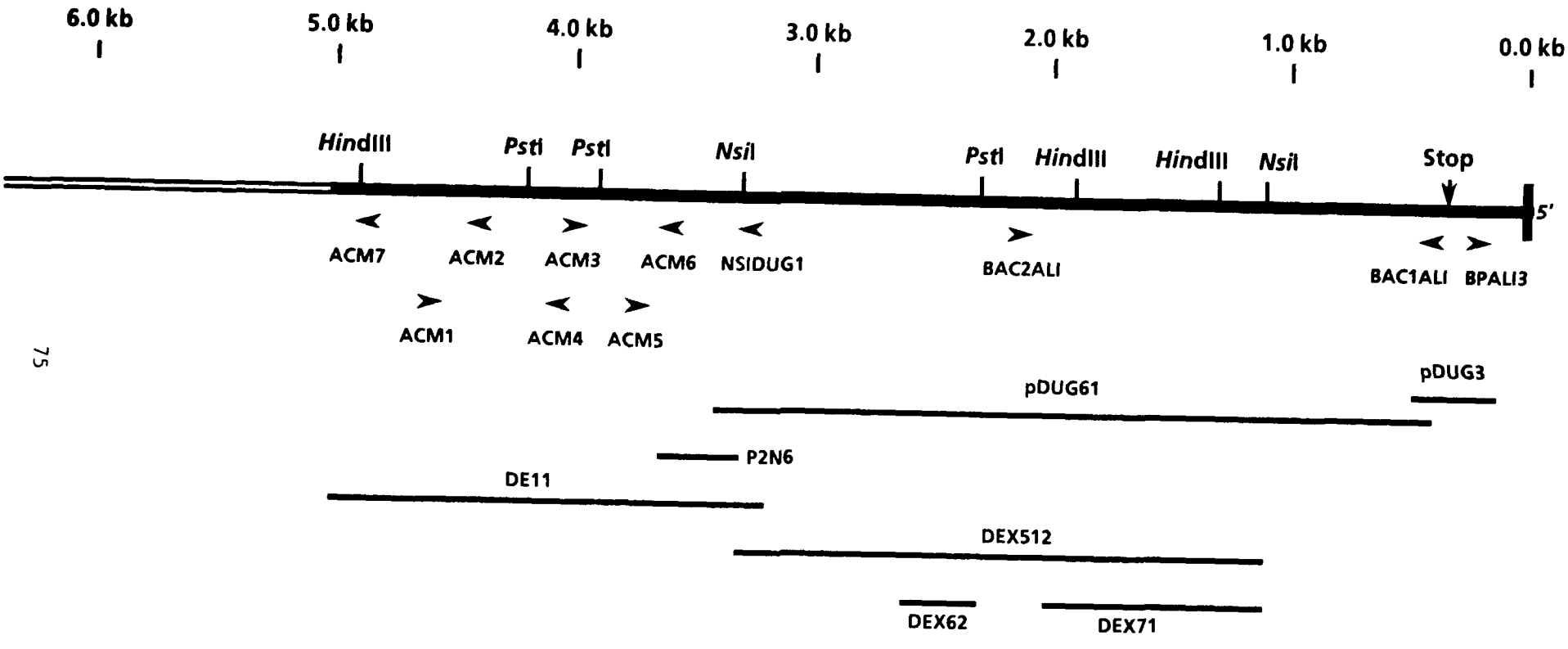




**FIG. 15.** Restriction map of DUG M segment cDNA showing clones sequenced and location of oligonucleotide primers. Nucleotides are numbered from the viral 5' end in kilobases. Primer BPALI3 was used to sequence to the viral 5' end. Primers ACM1 to ACM7 were used to sequence off M13 subclone templates. DUG3, DUG61, P2N6 and DE11 are cDNA clones used to construct the sequence. DEX512, DEX62 AND DEX71 are regions expressed as lacZ-fusion proteins. BAC1ALI and BAC2ALI were used to construct a baculovirus recombinant. The position of the stop codon of the long ORF is also shown.

```
CCTTTTAAGA GGGACATGTT CTTATCGTAA AGACAGCAGA GCATGTTTTC TTTTTTATTT 4380
GATGGCAGTT TTATTGATAA GTTGGTGTCG CAGTGTCTCA CCTTTACCAG TTTAATTCCT 4440
GATCTACTAA ACTCTGTACA ACATCTCAAA AAAGTTTCAC AGATCTTGCT GTATAAGACT 4500

ACCTCCTGAA ACCAACCAAA TGTGCACAAA AGTTGATGAG TTTTACTATT GTTTCTGGCA 4560
CACCTTCATA GGGAGTACTG TAGTACCTCC TGAGAGCTTC CGCAATAACC TCTTTGAAAA 4620
GTATACAATC AGATGAAAAG ATTTTATCAG AGGCCTTTTC TGACAACAAA TTTTTCCTTT 4680

TAGTTTCTAA GTAATCAGCA ATTTCCTTAT TACTTCTGAT AATGAGTTTA TCATTATTTC 4740
TGATGAAGTT GGTCTTACTT TGCATACTTG TGCGTATGTT GAGAAGAATC TCCTCATCCT 4800
TTGTAGGAAC CCTCAAGTCT CTGATTAGTG TTTCTAGCCA CAACCTTGGT ACAGCACACT 4860

TAGAACCTGC TTGCTTGTCT GTTTTTTTTA ATGAACTTGT GATTGTCTTC CTTACATGAT 4920
CTGAGTAGGG CTTAAGCTTC TGCTCTGATA ATT 4953
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FIG. 16. DUG M segment RNA sequence extending from the 5' end of the vira RNA and representing 75% of the complete sequence.

TTCTCAAAGA TAGCGTGGCG GCACAGCAAG TTTTAAAACT AGAGTTAGCA AAAGCAGGCA 60
TAGTGGATTA ATAGAGTAAG TAAGTTAAAA GAACTTTAAA CTGTGAGCAA GTTAGAACTA 120
TATAAAATTC ACTAGGTCTT CTTGATTTAG GTGGTTGTGG TGAGTACACC TAGCTTTTAG 180

TACCTGTTAA GCAATTTCAG AAAGTTTTTT TTCCTTTTTT AATTTTGGGT TGTCAGACAT 240
GAATAGTCTT GCAAGAGTTC TCTTATCTTT TGCATCTTTG TTGATAAGTT CACCACTTTT 300
ACTGAACTTT CTAATTATGT CTCTCAGCTC TTCTTCTTCA GCCAGTTTGT TGTATCCTTC 360

ATTTTTTCTA CAGCACCTTC TACATGGCCT ACAAAAAACT TTGAAAGGCC AAAGCATTTT 420
GTCACCGTAT ATGAAAAAAA GAATTAAAAG GGCGAAAGGA AGAACCGTGC CAAGTACTCC 480
GACAGCAATG CTTCCAATGT AATGCTTAAA AAAATCTCCT ATACCAGATG CGAATGAACT 540

TATAGAATTT GACCAACAGT CAATCTTAGA TTTGCAAGTG TCGTTTTGGT GCTGTACTAT 600
TGTTCCTCTA TGTTCTAACA ATATGTCTTT GGTAGGTTCT AACTCAACAC CAGTGCATGT 660
AGTGAGTTCA TCAGGAGTAC AATCTTTGCA GTATTCTCTT TCCACTACCT CAAAGCAAAT 720

TTTCTTTGTA TCACGAACAG AGAACACTTT AAACCTGCTA GTTGTGGCAC TTGGACCCTT 780
TCTTGCAATT ATGCTAGTTT CTGCCACTAC CACATTGGGA CTGGTGCTCC TCAGATGCAG 840
TGTAAATTCG TCTGGAGAAG TCAACTTGGC ATTGACGCTG CATGATATGC CAGAAGTGCA 900

ACTAAAACAA CCACTGCATT TCAGTCCAGT TAGTCTTATA CCACTTAGCC TTATTGTCTT 960
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TACGGTCTTG CCTTCAAGTC TAACTGAAGT GTGTGACCTT TTGCTGAGAT TCTTTTTGAG 1080

TGAAAATGAA AGCGTTGTGT GTAATCTTTT TCTATGTTGA TCAAGTTTGA GAAACTCTCT 1140
GAGTTTTTGA GTCACTACAC CAGTGTAGGT ACAGGTTGGC CAATCGCCAA CATTACAGTA 1200
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TAAACTTTCT GGAAGCTTAC TTCCAATGTT TTGGGGATCA GAAATCGTTA TTGTTATAGG 1500
GCCAATGTTG AATCTGGTTC CTGCCTCGAC AATTTCACAG TGCCTCTCCT CCTCCGTGAC 1560
CTCCACGCAG ACCAACACCT CAGTTCTAAG ATATTCTGTT GACCACTTAA CACCAAGATA 1620

TTTATTAAAT GGCCTTTCCA CATCCATACC ACAGCAGGTG CAACCAGTAC CAATTCCCAA 1680
CACCATGTGG GTTGACACGC ATTCTTGTGT GTGGCACTCT TTGGCCATAC AAGTTGATGT 1740
TTGGCAGCCA CATCTATGAG GACAATCTCC TGTGCAGACG GCTTTTGGCC ATTCACTTAG 1800

TAGTCTATCA CCTGTTATGT ACTGGAATAC TGAATTGTAG ACTTGGGCAA AATCCATTAT 1860
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CACACCAGTT TTTTCTTTAA GCTTTATTAT GGATGTGCTC CTTCCTGATA GTATGACCTT 1980

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AGAATCAAAA GCACTTGCGT TATGTCCTGT TAACAGTAGC AAGAAGCACA TAATGAGAGC 2340

AAAGACGACA ACATTTGTTT TATTTATGAA GAGCTGCAGT CCTGTTGTGA ACAGTTTTTC 2400
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TGCAACTTTG TAGCATTCTT TGACATGTTC TTCAAGGGTC ACCAGATCAA GAGCCTGTTT 2520

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CAAATAGTGC TGACGGCCTC CTGAGCAGTC CACCATTATA TCCCCTTTTC CAGTTGGTAC 3360
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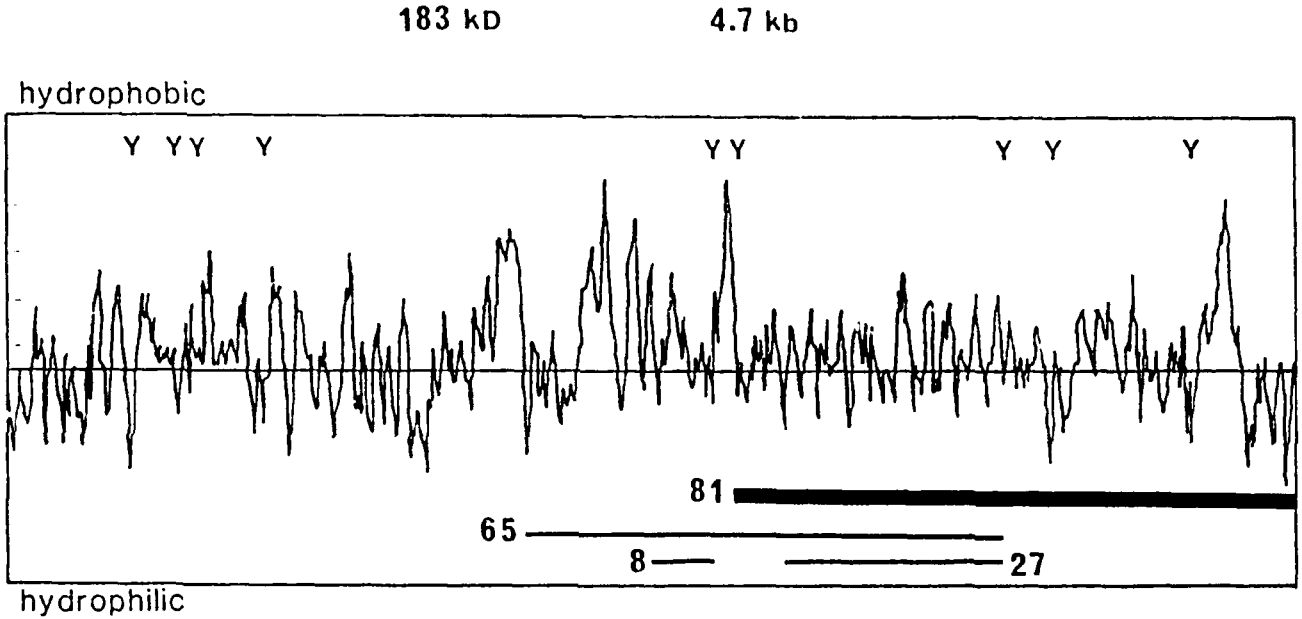


**FIG. 17.** The hydrophobicity plot of the Dugbe M segment ORF utilising 4.7kb of sequence and representing 183kD of protein. Nine potential N-linked glycosylation sites (Y) were present. The pUEX fusion proteins were DEX62 at 8kD, DEX71 at 27kD and DEX512 at 65kD. The baculovirus expressed protein consisted of 81kD of protein sequence shown by the bold line.

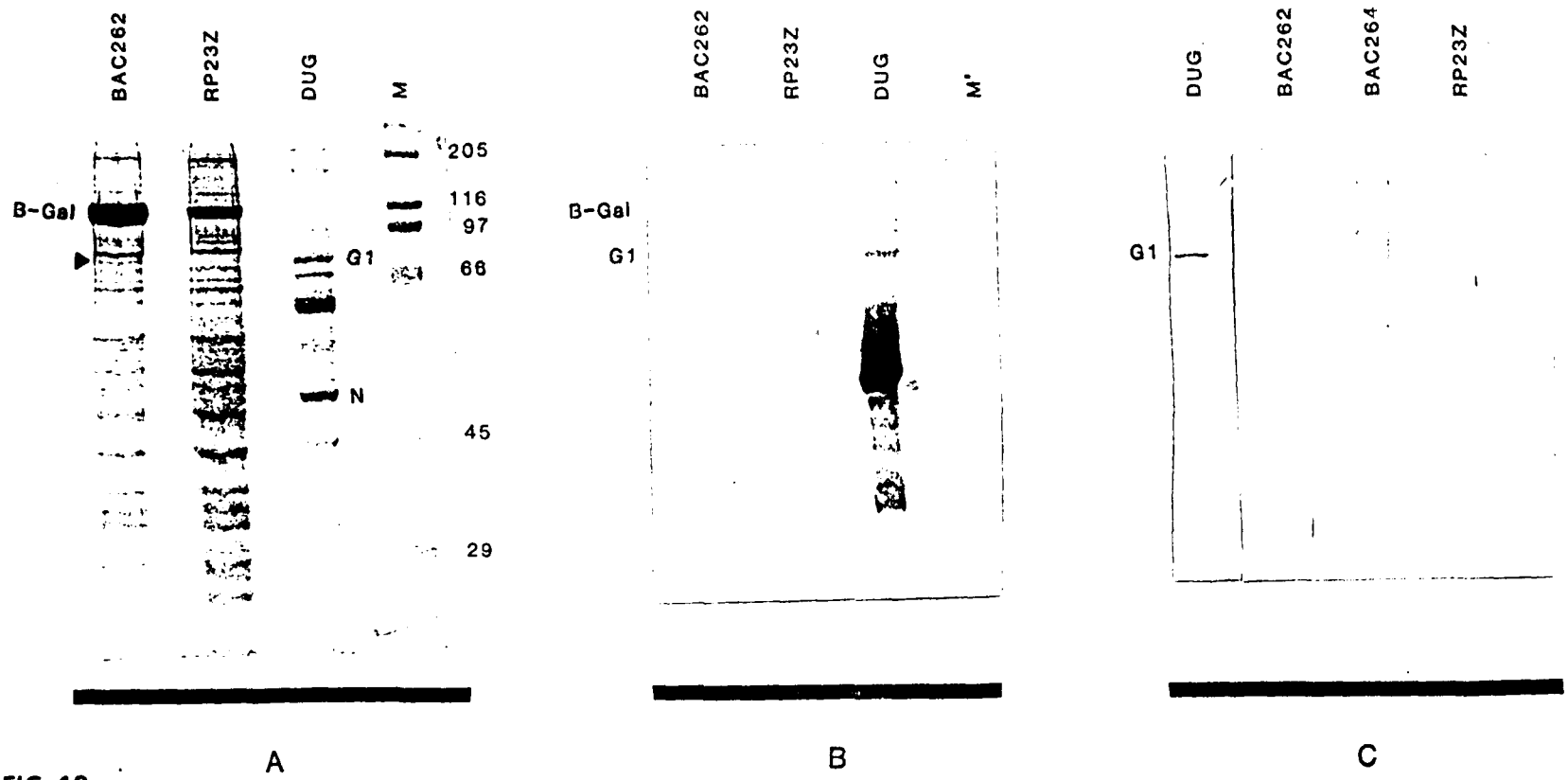


**FIG. 18.**

**A)** A coomassie stained gel of: BAC262 the recombinant baculovirus expressing a portion of the M segment of DUG virus; RP23Z was the negative control baculovirus with the Lac Z gene expressing β-Galactosidase in place of the polyhedrin gene; and DUG virus pellet for comparison. β-Gal was expressed strongly by both *baculoviruses but only the recombinant virus* (BAC262) expressed a protein of the same molecular weight as G1, although the level of expression was very low.

**B)** A Western blot of the above gel probed with polyclonal rabbit anti-whole DUG virus antiserum. In the BAC262 lane, the expressed M segment product as well as the β-Gal cross reacted with this serum. In the RP23Z control only the β-Gal reacted. The M segment product was again of the same molecular weight as the G1 protein. A DUG virus lane was used as a serum control and pre-stained markers (M') were used to check the transfer of the proteins.

**C)** This Western blot was probed with polyclonal anti-G1 antiserum. A reaction was observed with the two recombinant viruses BAC262 and BAC264 but no reaction was observed with the negative control virus RP23Z, indicating that the expressed product was the G1 glycoprotein.

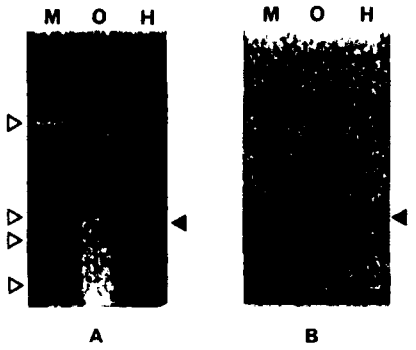


**FIG. 19** Agarose gel electrophoresis (A) and Southern blotting (B) of the PCR product (closed triangle) obtained by amplification of DUG RNA extracted from dissected tick organs (O) or hemolymph (H) using primers PCRDUG1 and PCRDUG2. Marker lane (M) contains *Hin*fI digested pUC19; sizes of markers (open triangles) are 1419, 517, 396, and 214 base pairs, from top to bottom. A subclone of the 300bp *Sac*I-*Pst*I fragment from pDUG36 (see Fig. 3) in pUC19 was used as the probe.

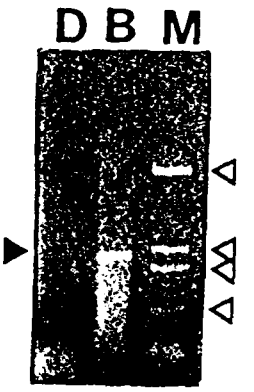


FIG. 20. Agarose gel electrophoresis of the PCR product (closed triangle) obtained by amplificiation of DUG RNA extracted from dissected mouse brain (B) or blood (D) samples using primers PCRDUG1 and PCRDUG2. Marker lane (M) contains *Hin*fI digested pUC19; sizes of markers (open triangles) are 1419, 517, 396, and 214 base pairs, from top to bottom.

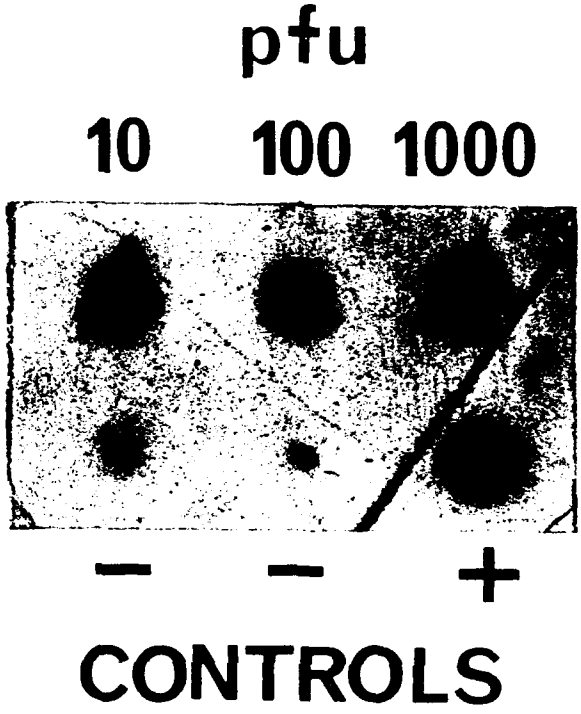


**FIG. 21** Dot-blot hybridisation of PCR products obtained by 64 cycles of amplification of DUG RNA extracted using SDS and phenol from 100 µl of infected mouse blood containing either 10, 100, or 1000 PFU of DUG virus. The 300bp *Sac*I-*Pst*I fragment of cDNA clone pDUG36 was used as the probe. Positive hybridisation control ( + ) was pDUG36 DNA. Negative controls (-), reading from left to right, were uninfected mouse blood RNA amplified by PCR using primers PCRDUG1 and PCRDUG2, and plasmid pUC19.