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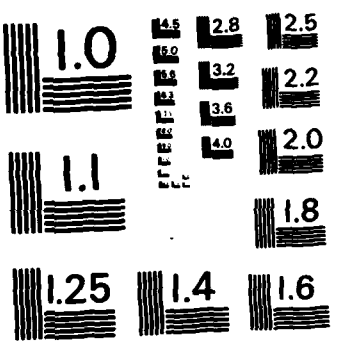

GENETIC AND MOLECULAR STUDIES OF THE PHLEBOTOMUS FEVER GROUP OF VIRUSES

ANNUAL REPORT

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AUGUST 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND
DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-78-C-8017

University of Alabama in Birmingham
Birmingham, Alabama 35294

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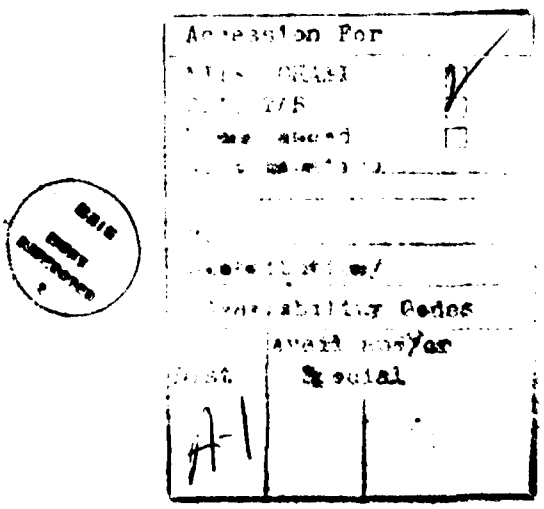

I. SUMMARY

The previous reports of this project have described analyses we have undertaken concerning the biochemical and genetic characterization of members of the Phlebovirus genus of the negative sense RNA virus family Bunyaviridae (Bishop et al., 1980). This genus of viruses include members that are known human pathogens and that are of consequence to military and civilian personnel in particular regions of the world (Bishop and Shope, 1979). Rift valley fever (RVF) virus has caused epidemics of infection including several hundred deaths in a single outbreak in Egypt in 1977-1978. The sandfly fever virus isolates, Sicilian and Naples (SFN, SFS), originally were recovered from American troops in 1943-1944 during epidemics of sandfly fever among military personnel. Isolates representing New World phleboviruses have been obtained from military personnel stationed in Panama (Punta Toro, PT). Other New World isolates some of which are the etiologic agents of human infection include Aguagate (AGU), Anhanga (ANH), Bujaru (BUJ), Icoaraci (ICO), Chagres (CHG), Buenaventura (BUE), Candiru (CDU) and Itaporanga (ITP) viruses. Other Old World isolates include Karimabad (KAR) virus.

Our analyses have been directed towards developing a strategy for phlebovirus vaccine development. Initial studies were therefore aimed at delineating the structural components of member viruses. With the demonstration of a tripartite RNA genome, genetic analyses were also undertaken in order to determine the coding stategies of the 3 RNA species and determine if recombinant viruses could be obtained and used for vaccine purposes. From analyses of intertypic reassortant PT viruses we showed that the viral $7x10^5$ dalton small (S) RNA species codes for the viral $20-25x10^3$ dalton nucleocapsid (N) protein. Although not proven, based on the precedent of the bunyavirus coding strategy, it is probable that the middle (M) size phlebovirus RNA ($2x10^6$ daltons) codes for the 2 viral glycoproteins ($50-70x10^3$ daltons) leaving the $3x10^6$ dalton large (L) RNA species to code for the $200x10^3$ dalton protein (putative transcriptase component) found in viruses. The genetic studies, including interference assays we established, indicated that although intertypic reassortant viruses can be obtained, heterotypic virus interactions are not demonstrable (i.e., heterotypic viruses do not interfere with each other and do not reassort their genomes in dual virus infections). Although not all phleboviruses have been tested for genetic interactions (see below), the results do not hold out much hope for using such an approach for vaccine development. Consequently we have taken an alternate approach, that of developing subunit vaccines. Central to this approach is a determination of the coding strategy of the 3 RNA species and the goal of characterizing the epitopes on viral antigens that elicit and interact with neutralizing antigens. Such goals will be realized by cloning and sequencing studies here and antigenic epitope analyses being undertaken at USAMRIID (Drs. J. Smith and J. Dalrymple). The Report of the current year describes the results of cloning and sequencing the S RNA of PT phlebovirus. From the data obtained an open reading frame in the viral complementary RNA species (mRNA?) has been identified (coding for N?) an open reading frame has also been found in the viral sense strand. Correlations of these predicted products to viral gene products remain goals of our next proposal as do cloning and sequencing the PT M and L RNA species.

The reporting period represents the last 12 of the 66 months since the inception of the project. A synopsis of the results of the prior reporting periods is presented below:

(1). Analyses of the major structural components of PHL group viruses have established that KAR, PT, CHG, Candiru (CDU), ICO, PHL 3, Itaporanga (ITP), Buenaventura (BUE), and the Sicilian and Naples sandfly fever (SFS, SFN) viruses, each has a tripartite RNA genome and three major structural polypeptides (two external glycoproteins, G1 and G2, $57-69x10^3$ daltons, and an internal, nucleocapsid

associated, protein N, 20-24x10^3 daltons). Both the mol. wt. of the major structural polypeptides and the virion RNA segments of the different PHL group viruses can be easily distinguished from those of bunyaviruses (e.g. the California serogroup, CAL, viruses) and, to various extents (depending on the virus), from each other. Tryptic peptide analyses of ^{35}S and ^{3}H methionine labeled G1 and G2 polypeptides of KAR have established that these two polypeptides have distinguishable sequences (Robeson et al., 1979). The behaviour of reduced KAR G1 and G2 polypeptides on polyacrylamide gel electrophoresis was found to be aberrant by comparison with unreduced preparations.

(2). Twenty four ts mutants of PT virus were isolated following mutagenesis of the wild-type virus by growth in the presence of 5-fluorouracil. Recombination assays with these mutants have allowed them to be categorized into 3 non-overlapping recombination groups (Group I has 8 ts mutants; Group II has 5 ts mutants; Group III has 1 ts mutant - so strictly is not a group - and 1 ts mutant is probably a double mutant).

(3). Oligonucleotide fingerprint analyses of prototype DEN 1, 2, 3 and 4 viruses involving both single and mixed coelectropherograms of ribonuclease T1 digests of ^{32}P labeled 40S viral RNA samples showed that each has a unique fingerprint that is easily distinguished from that of another prototype DEN virus (Vezza et al., 1980). These procedures have now been adopted by WRAIR personnel for their Dengue virus studies). Evidence was obtained indicating that the 5' sequence of DEN 2 RNA is m7GpppAmpXp... Oligonucleotide fingerprint analyses of alternate RVF virus isolates obtained from different countries of Africa have similarly been used to categorize and relate/distinguish RVF isolates (Cash et al., 1981).

(4). Electron microscopic analyses of the surface structure arrangments of PHL group viruses have been undertaken and indicate that unlike bunyaviruses, but like uukuviruses (2 of the other Bunyaviridae genera) PHL group viruses have a particular "chimney-pot" arrangements of their surface glycoproteins as evidenced by glutaraldehyde fixation prior to staining (Robeson et al., 1979).

(5). Competition RIA assays using iodinated nucleocapsid and glycoprotein preparations of KAR virus, KAR antisera and the competing antigens of KAR, CHG and SFS indicate that the KAR and SFS N polypeptides have more antigenic determinants in common than have the N polypeptides of KAR and CHG. Also the KAR and SFS G polypeptides share more antigenic determinants than the G polypeptides of KAR and CHG (Klimas et al., 1981). No shared antigenic determinants were detected between KAR and vesicular stomatitis virus (VSV), or the bunyaviruses La Crosse (LAC), Oriboca (ORI), or Bunyamwera (BUN) viruses.

(6). ICO virus has been adapted to produce plaques in Vero cell monolayers at 39.8°C. The original virus stock, which gave 10^5 plaques at 35°C, gave none at 39.8°C. By high temperature passaging of the virus stock and cloning at 39.8°C, a stock of ICO virus has been derived which gives 8x10^4 PFU at 35°C and 2.3x10^3 PFU at 39.8°C.

(7). The viral induced polypeptides, (immune precipitated from infected cell extracts by their homologous antisera), have been characterized for KAR, AGU, BUE, CDU, SFS and SFN viruses. Several alternate isolates of PT virus obtained from the eastern, central and western regions of Panama have been cloned and their L, M and S RNA species fingerprinted and shown to be distinct/related to each other (depending on the virus isolate).

(8). By dual wild-type virus crosses, reassortant viruses have been obtained between prototype PT and an alternate PT isolate (PT-ada). The genotypes of 2 such reassortants were shown by fingerprint analyses to be the L/M/S combinations of ada/PT/ada and PT/PT/ada.

(9). The N polypeptides of PT-ada and prototype PT viruses can be distinguished by tryptic peptide analyses allowing the N polypeptides of the reassortants to be analyzed. By such analyses it has been shown that their S RNA codes for the virion N polypeptide. This conclusion has been confirmed by S mRNA in vitro translation analyses.

(10). Dual wild-type virus infections have failed to detect reassortant virus formation between PT and BUE viruses, or BUE and ICO viruses.

(11). An assay of the homologous and heterologous virus interference capabilities by stocks of PT, or ICO, viruses that contain their respective defective interfering (DI) virus has been developed. Using such assays, homologous virus interference has been demonstrated. No heterologous virus interference has been detected from ICO DI and PT coinfections, or ICO DI and BUE, or ICO DI and snowshoe hare (SSH) bunyavirus coinfections.

(12). The stocks of PT and ICO DI virus have been shown to contain new RNA species not found in virus stocks lacking DI virus. Fingerprint analyses have shown that for both viruses the new RNA species represent deletion derivatives of their respective L RNA species.

(13). The 3' end sequence of the 3 RNA species of both PT and BUE viruses have been shown to be like those of the uukuvirus, Uukuniemi, i.e., $_{HO}$UUC.

(14). Heterologous recombination attempts involving dual infections with wild-type PT and SFS viruses, PT and CHG viruses, PT and ICO viruses, or PT and KAR viruses have failed to yield recombinant viruses. Similar studies with different PT virus varieties yielded recombinant viruses.

(15). Further analyses of the homologous and heterologous virus interference capabilities of PT (or ICO) virus preparations that contain DI particles have failed to detect heterologous virus interference with KAR, SFS, SFN, CHG, CDU or ITP phleboviruses under conditions in which homologous PT (or ICO) interference was demonstrated. We conclude that these heterologous viruses are distantly related to PT (or ICO) viruses, and (by this test) not genetically compatible.

(16). Current data indicate that at the structural level the tick-borne uukuviruses (Uukuvirus genus, Bunyaviridae family) closely resemble phleboviruses. The 3' end sequences of their RNA species, and the virion polypeptides and RNA sizes are similar, as are their viral-induced non-structural polypeptides. The structural components of several non-assigned tick-borne members of the family have been characterized and found to conform to neither the phleboviruses/uukuviruses, nor to the mosquito-borne bunyaviruses (Clerx and Bishop, 1981; Clerx et al., 1981). Because of their unique characteristics these other viruses have been constituted into a new genus, the Nairovirus genus. Member viruses have 3' AGAGUUUC.. end sequences (Clerx van-Haaster et al., 1982), in contrast to bunyaviruses (3' UCAUCACAUGA...), or uukuviruses (3' UGUGUUUUCUGG...), or phleboviruses (3' UGUGUUUCG..). The nairovirus viral RNA species and virion polypeptide sizes also differ significantly from those of uukuviruses, phleboviruses, or bunyaviruses.

(17). Characterization of the unassigned tick-borne Dhori (DHO) virus, a virus that heretofore has been considered as a possible member of the family, has been undertaken in order to determine if this virus has the structural attributes of phleboviruses (or members of other defined genera of the family). The data obtained indicate that DHO virus does not conform to any other member of the family, rather it resembles an orthomyxovirus (Clerx et al., 1983).

(18). In order to clone, sequence and thereby determine the complete coding content the RNA species of PHL group viruses (see Proposal), the Principal Investigator spent a 10 month sabbatical in the laboratory of Dr. G.G. Brownlee in Oxford, England. During this time he cloned and sequenced complete copies of influenza A/PR/8/34 RNA segments 2 and 3, as well as bunyavirus SSH S RNA species (Bishop et al., 1982a,b,c).

In the current reporting period we have obtained an almost complete sequence for the PT S RNA species. The data obtained support the conclusion that the coding strategy of this virus differs from any other known RNA virus. In this year's proposal confirmation will be sought for this conclusion by a variety of experimental approaches.

A synopsis of the results of the current reported period is as follows:

(19). Using a PT S RNA 3' end specific viral complementary oligodeoxynucleotide, clones of DNA copies of the S RNA have been obtained and sequenced. The data have been used to produce other viral complementary oligodeoxynucleotides and further, overlapping, clones obtained and sequenced allowing a DNA sequence of the S RNA to be obtained. Open reading frames have been identified in the viral and viral complementary RNA sequences.

II. REPORT

A. Introduction.

The objectives of this contract are to determine the infection strategy of members of the PHL group viruses (Phlebovirus genus, Bunyaviridae) and develop protocols for vaccine development. Since this group of exotic viruses includes agents that cause illnesses in epidemic proportions (e.g., RVF) in different parts of the world, and therefore are of military importance, our objectives relate to the question of deriving vaccines that will be useful in protecting military personnel against virus infections.

To realize these objectives, initial studies involved determining if reassortant PHL viruses could be used for preparing vaccines. This was studied by determining which PHL group viruses are genetically compatible (capable of producing reassortant viruses with other members of the genus, or members of other genera of the family). Studies conducted in our laboratories have demonstrated that certain virus serotypes of the CAL serogroup of the Bunyavirus genus (another genus of the Bunyaviridae) are capable of producing reassortant viruses with other CAL group members (Gentsch & Bishop, 1976; Gentsch, et al., 1977; Gentsch et al., 1979; Rozhon et al., 1981). However, not all CAL group bunyavirus crosses yield reassortants, indicating that their gene pool is limited. Also no crosses involving viruses representing different bunyavirus serogroups (e.g., CAL and the Group C, or Bunyamwera, serogroups) have yet yielded reassortant viruses. Analyses of reassortant bunyaviruses have shown that the S RNA codes for the nucleocapsid (N) protein and a non-structural protein (NS_S) (Gentsch and Bishop, 1978; Cash et al., 1979; Fuller and Bishop, 1982). The M RNA codes for the viral glycoproteins.

Results reported previously from this contract have documented that PHL group viruses have a tripartite RNA genome with RNA segments that are designated L, M and S. From dual PT ts mutant, or wild-type, virus coinfections recombinant intertypic PT viruses have been obtained. However, no heterotypic reassortants have been obtained using different PHL group viruses. Although not all PHL virus combinations have been tested, this negative result suggests (but does not prove) that there are restrictions at the genetic level between different PHL group viruses. DI interference studies also suggest that there are restrictions on genetic interactions between many of the PHL group viruses.

An alternate strategy for vaccine development is to identify and prepare adequate quantities of the viral antigen(s) that elicit protective antibodies. Central to this issue is knowledge of the RNA segment coding assignments and characterization of the viral antigen(s) that interact with such antibodies (see Gentsch & Bishop, 1978, 1979). Our current studies have been directed towards defining the RNA segment coding assignments of phleboviruses. We have already demonstrated that the S RNA segment codes for N protein. Proof that the M RNA codes for the glycoproteins (as shown for bunyaviruses) has not yet been obtained, and remains a goal of our continuing efforts. Studies by USAMRIID personnel with monoclonal antibodies indicate that the glycoproteins interact with neutralizing sera. Therefore it is important to determine which PHL viral RNA species codes for the glycoproteins. Sequencing the phlebovirus S RNA species is reported in this year's Contract Report. Sequencing the other viral RNA species is a goal of next year's proposal.

The results from this reporting period cover item 19 listed in the Summary. It will not detail the results of items 1-18 that were given in previous reports.

B. Results from this Reporting Period.

The strategies employed to obtain the sequence of the S RNA of PT virus followed the protocols detailed in last year's proposal. In brief, they involved: (1) 3' end sequencing of the viral S RNA; (2) synthesizing an oligodeoxynucleotide primer (S1) complementary to the S RNA (3') nucleotides #9-27; (3) using the primer to prepare viral complementary cDNA; (4) backcopying the cDNA using reverse transcriptase; (5) treating the derived double-stranded (ds) DNA with nuclease S1 to digest the hairpin end of the DNA; (6) repairing the 3' ends of the dsDNA to yield flush ends; (7) blunt-end cloning into the unique PVU II site of the E. coli plasmid pBr322; (8) screening the derived clones with short-copy cDNA probes made from viral RNA and the S1 primer under limiting ^{32}P-dATP nucleotide concentration; (9) restriction enzyme mapping the hybridization positive clones; and (10) sequencing the cDNA inserts by Maxam and Gilbert protocols. Oligodeoxynucleotides were synthesized by a modified phosphotriester methodology and purified using HPLC chromatography and the Waters pumps and column purchased under this contract as described in the contract proposal. The derived DNA sequence data was correlated to the 3' end sequence of the S RNA.

Additional clones representing overlapping and further sequences of the viral RNA were obtained similarly using other S oligonucleotide primers representing the following viral complementary sequences: S2: #361-379; S3: #737-755. The clones, their origins and the individual restriction enzyme strands that were sequenced are depicted in Fig. 1. Although clones reaching to the 5' end of the RNA have yet to be recovered (an estimated additional 100-200 nucleotides), most of the sequence has been obtained. A 12 enzyme restriction enzyme map of the sequence is presented in Fig. 2.

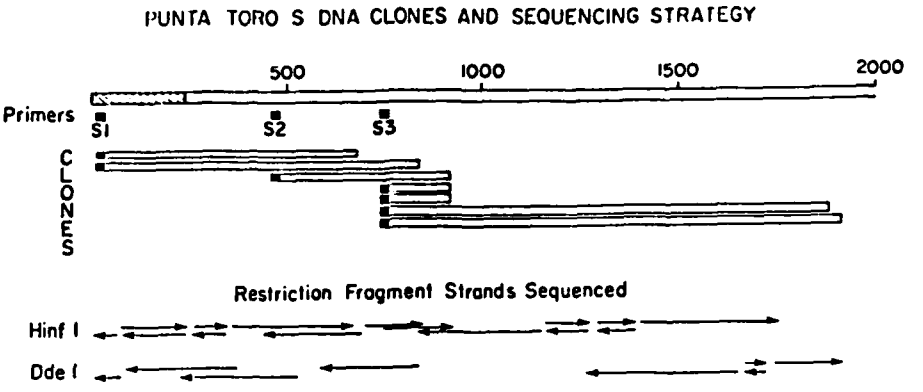

Fig. 1. Sequencing and cloning strategy of Punta Toro S RNA.

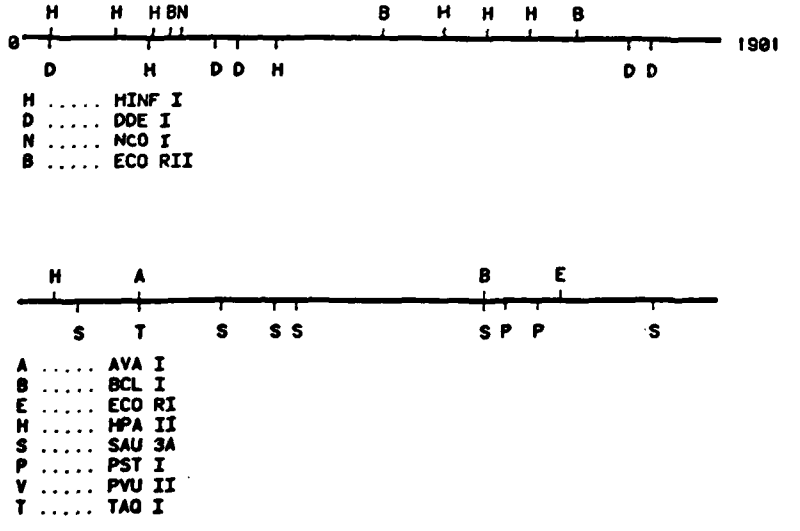

Fig. 2. Restriction enzyme sites in the Punta Toro S RNA sequence

The deduced sequence of the viral/viral complementary RNA species has been analyzed for termination codons in the 3 possible reading frames with the results indicated in Fig. 3.

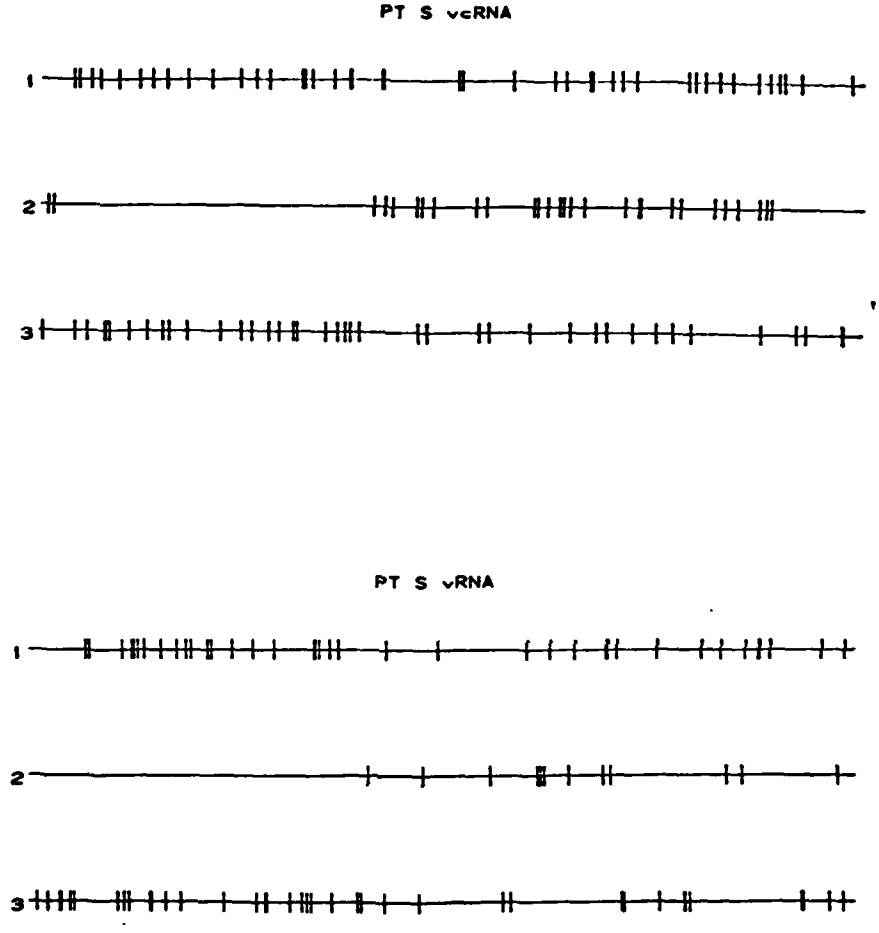

Fig. 3. Positions of translation termination codons (vertical lines) in the PT vcRNA and vRNA sequences. The RNA sequences are arranged left (5' end) to right (3' end).

Vertical lines in Fig. 3 represent the positions of termination codons in the first (1), or second (2), or third (3) reading frame of the viral complementary (vc), or viral (v), sense RNA.

The presence of open reading frames in the vRNA as well as the vcRNA sequences contrasts with the S RNA sequence data of SSH bunyavirus where only the vcRNA has a significant length of open reading (Fig. 4).

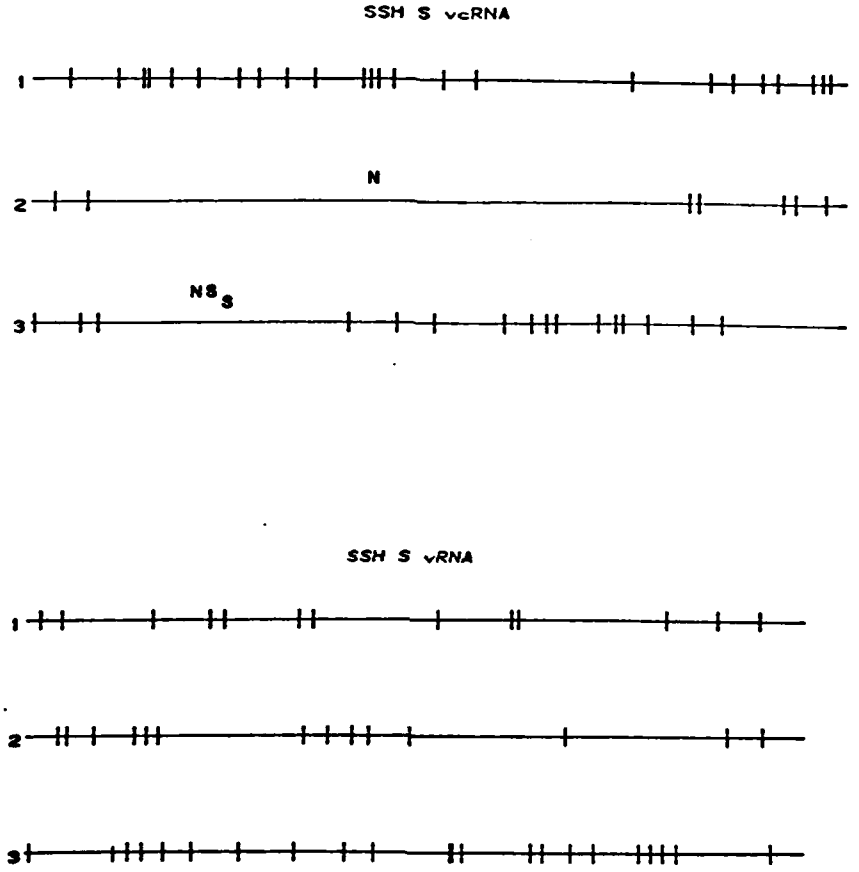

Fig. 4. Positions of translation termination codons (vertical lines) in the SSH vcRNA and vRNA sequences. The RNA sequences are arranged left (5' end) to right (3' end).

As before, vertical lines in Fig. 4 represent the positions of termination codons in the first (1), or second (2), or third (3) reading frame of the viral complementary (vc), or viral (v), sense RN

As shown in Fig. 5, in the PT S viral complementary (vc) RNA sequence there is a large contiguous open reading frame initiated with a methionine codon that extends from vcRNA 5' nucleotide #35 through #763 (243 amino acid residues). In Fig. 5 the encoded amino acids are indicated above the corresponding nucleotide triplets using the single amino acid code. Shorter open reading frames in the viral complementary sequences correspond to amino acid stretches of no more than 60-70 amino acids in length (see Fig. 3). These data indicate that at least 1200 nucleotides of the vc RNA sequence are not involved in coding for a gene product (at least in a contiguous sequence). This is an extremely long non-coding sequence (but see below).

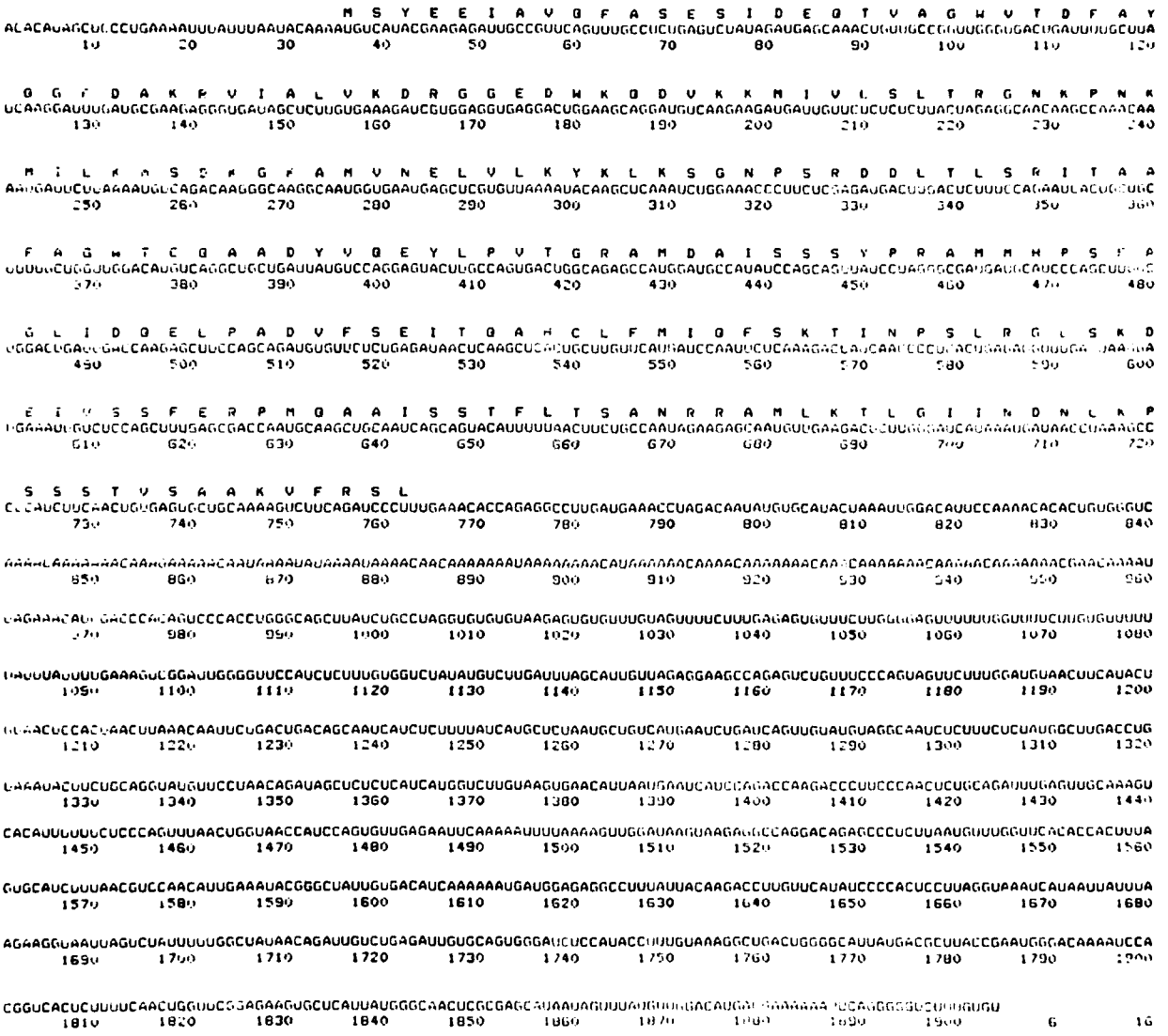

Fig. 5. Sequence and predicted gene product of the largest open reading frame in PT vcRNA.

As demonstrated in Fig. 6, in the PT S viral sequence there is also a large contiguous open reading frame extending from the end of the clone (i.e., corresponding to vRNA 5' nucleotide #2 through #778 (258 amino acid residues). Since the DNA clones obtained do not quite reach the 5' end of the vRNA (see above), presumably this suggested vRNA coding sequence is longer.

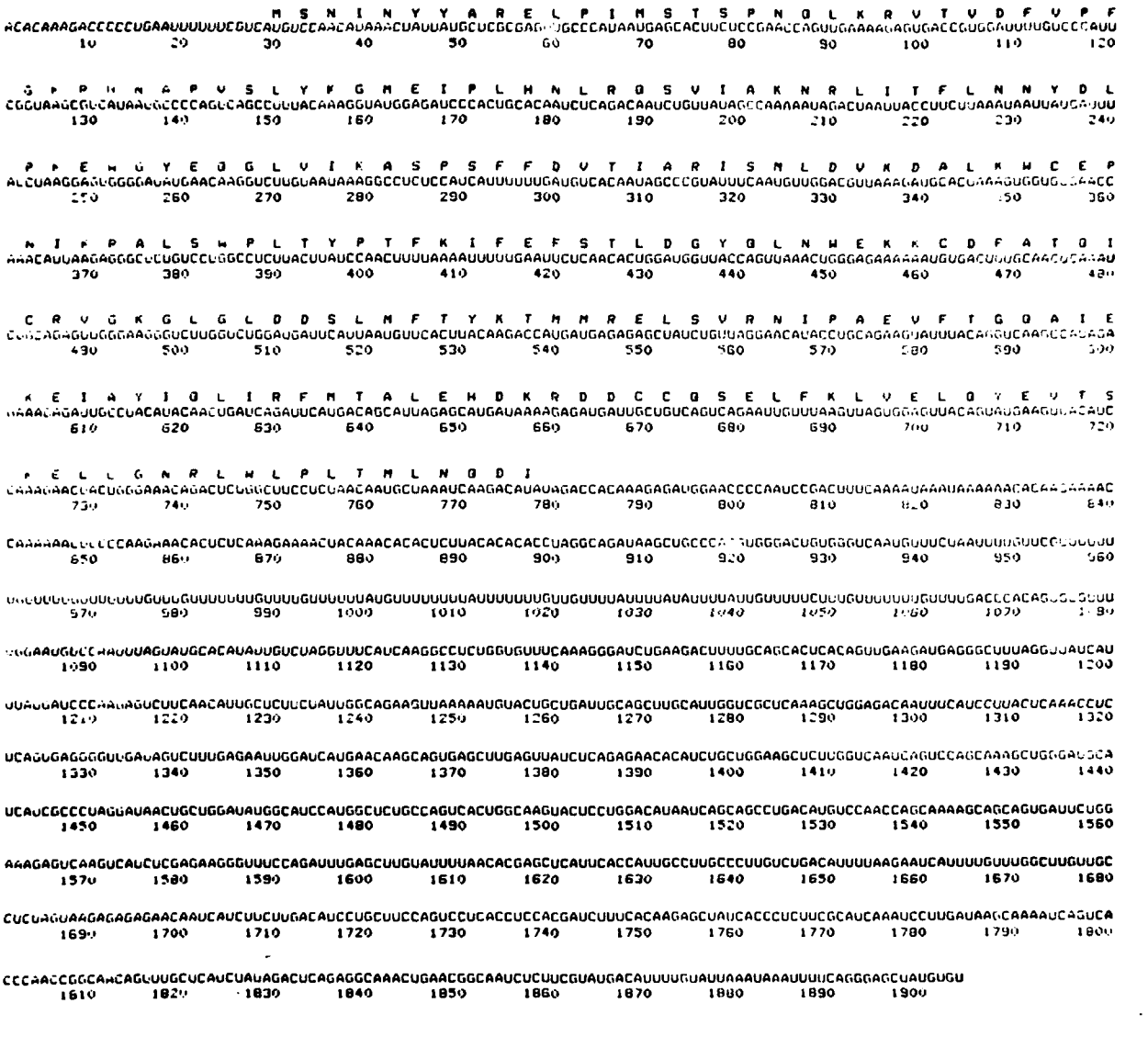

Fig. 6. Sequence and predicted largest open reading frame in PT vRNA.

When the viral L, M and S RNA species were resolved by agarose gel electrophoresis under denaturing conditions (10 mM methylmercury), blotted to Genescreen (NEN, Boston, MA), fixed by heat and used as a substrate for a Northern hybridization using nick-translated DNA representing each of the PT clones shown in Fig. 1, all the probes specifically hybridized to the PT S RNA. This result indicated that the DNA clones used to construct the sequence shown above are all derived from S RNA sequences.

It is possible that the existence of the large open reading frame in the viral sense strand is fortuitous. If so, then it would be unlikely that the viral RNA (or viral sense RNA made in infected cells) could be translated in vitro. Also it would be unlikely that

other phleboviruses would have a similar open reading frame sequence in their viral sense S RNA sequence. Both questions remain to be investigated. However the observation of an open reading frame in the viral sense S RNA strand needs to be followed up in order to define the virus coding strategy. Also, no other RNA virus is known to code for proteins in both the viral and viral complementary RNA species. The question of the relevance of coding information in the viral RNA will be addressed by examining other phleboviruses and translating PT viral S RNA (and cellular viral sense and viral complementary RNA species). In question is whether at any stage in the infection process the viral sense strand serves as a messenger RNA. Experiments are proposed in this year's proposal to investigate these issues.

It should be noted that the potential coding sequences shown in the viral complementary and viral sense strands (Figures 5 and 6) do not overlap each other (i.e., the middle sequences of the RNA, in either sense, are non-coding).

C. Summary of Progress Report.

The research supported by this contract has shown that representative PHL group viruses (Phlebovirus genus) have a tripartite RNA genome consisting of 3 unique RNA species (L, M and S). The L and M RNA species have mol. wts. like those of Bunyavirus and Uukuvirus genus members. Like uukuviruses, the S RNA species of phleboviruses is significantly larger than that of bunyaviruses even though the N proteins coded by the S RNA species of both virus types are similar in size ($20\text{-}24 \times 10^3$ daltons).

The sequencing data from PT S RNA postulate that the viral-complementing RNA codes for a 22×10^3 dalton protein. This protein may be N, however in the viral sense strand there is also a similar size open reading frame. Whether this latter sequence functions as an mRNA is not known.

Our analyses have shown that the size ranges of phlebovirus glycoproteins and their surface arrangement are similar to those of uukuviruses, and unlike those of Bunyavirus genus viruses. Whether the viral M RNA codes for the glycoprotein has not yet to be established.

Genetic studies initiated with Punta Toro (PT) virus have shown that high frequency intertypic genetic recombination can result from certain PT mixed virus infections (ts, or dual wild-type coinfections using alternate isolates of PT virus). From analyses of the reassortants generated from dual infections involving prototype PT and an alternate PT strain, PT-ada, it has been demonstrated that the viral S RNA codes for the N polypeptide. In vitro translation by subgenomic polysome mRNA indicates that N is coded by a small mRNA.

So far no recombination has been detected between PT and BUE, or PT and SFS, or PT and CHG, or PT and ICO, or PT and KAR, or BUE and ICO viruses, either by analyses of the progeny of dual wild-type virus infections, or from dual infections involving PT ts mutants and the wild-type alternate virus (neither of which give plaques at 39.8°) and screening for reassortant progeny at the non-permissive temperature. These results suggest that these phleboviruses do not belong to the same gene pool.

In addition to the heterologous phlebovirus recombination assays, we have screened for genetic interaction between phleboviruses based on heterologous virus interference assays. We have failed to detect heterologous virus interference using PT (or ICO) dI virus preparations and BUE, KAR, SFS, SFN, CHG, CDU or ITP phleboviruses under conditions in which interference of homologous PT, or alternate PT varieties, (or ICO) viruses could be demonstrated.

We conclude from these results that the phleboviruses are genetically diverse. From the vaccine development point of view it now appears unlikely that heterologous phleboviruses could be used to produce reassortants for vaccine development. Evidently, however, intertypic reassortants could be obtained and may be of use for vaccine development.

As an alternate strategy for vaccine development we have initiated a program to characterize the proteins coded by the 3 viral RNA species with the aim of identifying the exact sites on viral antigens that induce and interact with neutralizing antibodies. This objective is being realized by DNA cloning and sequencing the viral RNA species. The goal of vaccine development will then be addressed by expressing cloned viral gene products and, or preparing subunit vaccines of cloned products (or peptides) that protect against virulent virus challenge.

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A139536
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) GENETIC AND MOLECULAR STUDIES OF THE PHLEBOTOMUS FEVER GROUP OF VIRUSES	5. TYPE OF REPORT & PERIOD COVERED 9/1/82-8/31/83 Annual Report
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Dr. David H.L. Bishop	8. CONTRACT OR GRANT NUMBER(s) DAMD17-78-C-8017
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of Alabama Birmingham, Alabama 35294	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS10.AA.059
11. CONTROLLING OFFICE NAME AND ADDRESS Attn: SGRD-RMS US Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701	12. REPORT DATE August, 1983
	13. NUMBER OF PAGES 18
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report) UNCLASSIFIED
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Virus vaccines
Phlebotomus fever viruses
Genetic Recombination
Virus antigen

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

Studies supported by this contract have reported the characterization of the structural components of several Phlebotomus fever serogroup viruses (viral polypeptides and RNA species). The viruses studied include Punta Toro (PT), Karimabad (KAR), Chagres (CHG), Sandfly fever Sicilian (SFS Tesh and Sabin isolates). Sandfly fever Naples (SFN), Rift Valley fever (RVF), Phlebotomus 3 virus (Phl 3), Icoraci (ICO) and Buenaventura (BUE) viruses. In summary, the evidence has shown that the viruses have 3 virion RNA species (large, L, 2.6-2.8x10^6, medium, M, 1.85-2.2x10^6 and small, S, 0.7-0.8x10^6), which are comparable in number to those of other members of the family, but different in size for the S RNA (which is significantly larger than the S RNA of Bunyavirus genus members i.e., 0.4-0.5x10^6). The studies have also shown that, although the nucleocapsid, N, polypeptides of Phlebotomus fever group viruses (20-24x10^3 daltons) are similar in size to those of bunyaviruses, the glycoproteins (G1, G2, 57-69x10^3 daltons) are quite different (e.g., bunyaviruses: G1 115x10^3, and G2 38x10^3 daltons).

With the demonstration of a tripartite RNA genome, genetic analyses were also undertaken in order to determine the coding stategies of the 3 RNA species and determine if recombinant viruses could be obtained and used for vaccine purposes. From analyses of intertypic reassortant PT viruses we showed that the viral S RNA species codes for the viral N protein. Although not yet proven, based on the precedent of the bunyavirus coding strategy, it is probable that the phlebovirus M RNA codes for the 2 viral glycoproteins leaving the L RNA species to code for the 200x10^3 dalton protein (putative transcriptase component) found in viruses. The genetic studies, including interference assays, indicated that although intertypic reassortant viruses could be obtained, heterotypic virus interactions are not demonstrable (i.e., heterotypic viruses do not interfere with each other and do not reassort their genomes in dual virus infections).

The coding strategy of the 3 RNA species are being investigated with the goal of characterizing the epitopes on viral antigens that elicit and interact with neutralizing antigens. Such goals are being realized by cloning and sequencing studies. The Report of the current year describes the results of cloning and sequencing the S RNA of PT phlebovirus. From the data obtained an open reading frame in the viral complementary RNA species has been identified (coding for N?). However another open reading frame has been found in the viral sense strand. Correlations of these predicted products to viral gene products remain goals of our next proposal as do cloning and sequencing the PT M and L RNA species.