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PRINCIPAL INVESTIGATOR: Victor Romanowski

PI ADDRESS: Area de Química Biológica y Biología Molecular
Facultad de Ciencias Exactas, UNLP
Calle 47 y 115 - 1900 La Plata - Argentina

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Junin virus, one of the few human pathogenic arenaviruses, is the etiologic agent of Argentine hemorrhagic fever (AHF). The clinical symptoms of AHF include hematologic, neurologic, cardiovascular, renal and immunologic alterations. The mortality rate may be as high as 30%, but early treatment with immune plasma reduces the fatal cases to less than 2%. In order to control the endemo-epidemics in the richest farming land in Argentina a collaborative effort conducted by US and Argentine Governments led to the production of a live, attenuated Junin virus vaccine. After rigorous biological testing in rhesus monkeys, the highly attenuated Junin virus variant named Candid #1 was used in human volunteers, followed by an extensive clinical trial in the AHF endemic area.

In order to characterize the vaccine strain Candid #1 at the molecular level and initiate studies on the biochemical basis of attenuation of virulence, the glycoprotein precursor (GPC) gene of this attenuated virus was cloned and sequenced. The flanking 5' and 3' untranslated regions of the GPC gene do not differ significantly from the homologous regions in the S RNA of the wild type MC2 strain. However, major changes in the amino acid sequence occur in the amino terminal region of GPC as a result of several insertions and deletions in the nucleotide sequence. After proteolytic cleavage of GPC these alterations appear in the G1 polypeptide, that is thought to be located on the surface of the virion in association with the more internal G2 protein.

Although these findings are strongly suggestive of the genetic changes that lead to an attenuated phenotype, they must be complemented with studies on the direct predecessors of Candid #1, that exhibit gradually more virulent phenotypes, in order to locate precisely the mutations responsible for attenuation.

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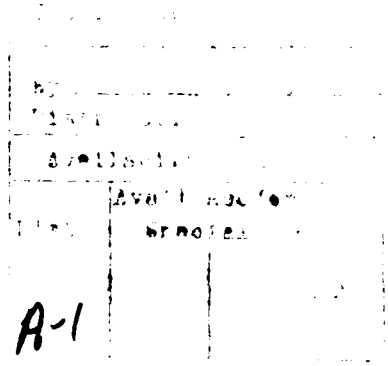

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EXCERPT FROM THE RESEARCH PROPOSAL (June 1988)

The characterization of Junín virus **Candid #1 genome** is the primary goal of the present research proposal. Modern **nucleotide sequencing** technology provides a means of precise description of any genome. However, the sequence analysis of Candid #1 alone will not provide any clue to the mechanisms of virulence attenuation. Therefore, it will be necessary to compare it to sequences of **more virulent ancestors** in the passage history leading from prototype Junin virus to Candid #1 vaccine.

The availability of these genealogically related Junín virus variants (i.e. Candid #1, XJ#44, XJ#13) permits a proper approach to the issue of the molecular basis of arenavirus virulence.

This project focuses mainly on the **changes in the S RNA species**, since a plausible working hypothesis is that changes in tissue tropism affecting neurovirulence could possibly be related to changes in the viral **surface glycoproteins** encoded in this RNA segment. Further progress of this research will provide nucleotide and aminoacid sequence data pointing at molecular changes that will hopefully correlate with differences in virulence. Highly variable regions will also be highlighted.

The comparison of the sequences of the "avirulent" Candid #1 to more than one "virulent" ancestor will also allow for an estimation of the genome stability or the **probability of reversion** to the "virulent" phenotype. This is a question relevant to the theoretical estimation of the risk of using Candid #1 as a vaccine, although the **phenotypic stability** of it has been already assessed empirically.

In addition, the molecular analysis of the viral nucleic acid can be used as a part of a thorough **quality control** of vaccine preparations.

In the frame of the extended AHF vaccine program, a careful evaluation of the clinical and subclinical AHF cases must include the **genotypic identification** of the viruses **isolated from the patients** in the endemic area. Nucleic acid sequence analysis is the unambiguous piece of data that can tell us whether the disease is caused by an indigenous virus or a virus derived by mutation from the vaccine strain. The amplification of nucleic acids using the polymerase chain reaction (PCR) could be employed for this purpose based on the nucleotide sequence data generated in this project. This particular technology is being tested in order to evaluate its potential as a laboratory diagnostic assay.

In summary, the present project shoud be regarded as a first step in the identification of regions in Junin virus genome related to a particular virulence pattern. The other major objective is to provide a molecular description of the AHF vaccine strain.

INTRODUCTION

Junín virus is the etiological agent of Argentine hemorragic fever (AHF). The clinical symptoms of AHF include hematologic, neurologic, cardiovascular, renal and immunologic alterations. The mortality rate may be as high as 30%, but early treatment with immune plasma reduces the fatal cases to less than 2%. (Maiztegui *et al.*, 1979; Enria *et al.*, 1986).

A wide variation in annual number of cases occurs, ranging from 100 to 4000. Since its recognition in the early 1950s, the disease has spread from an area of 16.000 km^2 to an area greater than 120.000 km^2 of the richest farming land in Argentina, with a population of *ca.* 2 million inhabitants (Maiztegui *et al.*, 1986). The major rodent hosts for Junín virus are *Calomys musculinus* and *Calomys laucha* (Maiztegui, 1975), although infection has been detected on occasion in *Mus musculus*, *Akodon azarae* and *Orizomys flavescens* (Sabattini *et al.*, 1977). The human population at risk is composed mainly by field workers. Humans are believed to become infected through cuts or skin abrasions or through airborne dust contaminated with urine and saliva of infected rodents. A high degree of variation in virulence and clinical patterns has been reported for different isolates of Junín virus (Maiztegui, 1975; Mc Kee *et al.*, 1985; Kenyon *et al.*, 1988).

A collaborative effort conducted by US and Argentine Governments led to the production of a live, attenuated Junín virus vaccine (Peters *et al.*, 1987). After rigorous biological testing in rhesus monkeys (Mc Kee *et al.*, 1984), the highly attenuated Junín virus variant named Candid #1 was used in human volunteers, followed by an extensive clinical trial in the AHF endemic area (Maiztegui *et al.*, 1990).

Our interests are directed towards the characterization of the vaccine strain at the molecular level. In addition, the availability of several genealogically related Junín virus variants with an increasing

degree of attenuation (e.g. XJ #13, XJ #44 and Candid #1), derived in the process of developing the AHF vaccine, permits a systematic approach to the issue of the molecular basis of Junín virus virulence.

In the last few years a reasonable amount of information has been accumulating related to the genome structure of some members of the Arenaviridae family, to which the Junín virus also belongs.

All the arenaviruses share morphologic and biochemical properties; they are enveloped and their genome consists of two single-stranded RNA species, designated L (for large, *ca.* 7 kb) and S (for small, *ca.* 3.5 kb). The L RNA codes for two proteins: a large L polypeptide, presumed to be the RNA polymerase, and a small zinc finger-like protein (Salvato and Shimomaye, 1989; Salvato *et al.*, 1989; Iapalucci *et al.*, 1989a, 1989b). The S RNAs of several arenaviruses have been sequenced and shown to have several common features. The nucleocapsid protein gene N and the precursor of the envelope glycoproteins GPC are encoded in an ambisense manner (Bishop, 1988).

The N protein (*ca.* 63 kDa) is translated from a viral-complementary or anti-genome-sense mRNA species complementary to the 3′ half of the viral S RNA (Auperin *et al.*, 1984a, 1984b; Romanowski and Bishop, 1985; Clegg and Oram, 1985; Franze-Fernández *et al.*, 1987). The GPC protein (*ca.* 57 kDa in the unglycosylated form) is translated from a viral or genome-sense mRNA corresponding to the 5′ half of the viral S RNA (Auperin *et al.*, 1984c; Romanowski *et al.*, 1985; Auperin *et al.*, 1986; Franze-Fernández *et al.*, 1987).

Recently, the complete nucleotide sequence of Junín virus S RNA has been determined in our laboratory. The information has been obtained from cDNA clones derived from the MC2 strain, a wild type Junín virus of intermediate virulence (Ghiringhelli et al., 1989; Ghiringhelli et al., submitted). These data were instrumental for the molecular characterization

of the attenuated variant of Junín virus named Candid #1. Here we report the complete sequence of the structural glycoproteins of the attenuated Junín virus vaccine strain, Candid #1, and their relationship to Junín virus MC2 and other closely and distantly related arenaviruses.

MATERIALS and METHODS

Virus and cell lines

The Junín virus vaccine strain, Candid #1 was kindly provided by Drs. J.I. Maiztegui and A.M. Ambrosio from the INEVH (Pergamino, Argentina) and propagated in certified fetal rhesus lung diploid cells (FRhL-2) at passage levels 19 to 28. A "working stock" of Junín Candid #1 virus was produced by infection of FRhL-2 cell monolayers with the master seed (Barrera Oro ARG 8/009, 14.8.81) at an m.o.i. of 0.01 PFU/cell. The supernatant media were harvested 96 h post infection, aliquoted and stored at -70°C. This virus stock was used as inoculum to produce virus particles for biochemical studies.

Preparation of viral RNA

When 50% confluent, FRhl-2 cell monolayers were infected with a multiplicity of infection of 0.1 pfu/cell and incubated at 37°C in Eagle's minimal essential medium with non-essential aminoacids (MEM) and 10% fetal calf serum. The virus was recovered and purified from the supernatant media on day 4 post-infection (Grau *et al.*, 1981, Rosas *et al.*, 1988).

Virions pelleted by ultracentrifugation were disrupted with guanidinium thiocyanate and the RNA was isolated essentially according to the procedure of Chomczynski and Sacchi (1987). The RNA pellet was

resuspended in 0.5% Sarkosyl, phenol extracted and reprecipitated with 0.3M sodium acetate and 2.5 volumes of ethanol. The final pellet was resuspended in water and stored in aliquotes at -70°C.

The RNA was analyzed by agarose gel electrophoresis in the presence of methylmercury(II) hydroxide as denaturant (Bailey and Davidson, 1976).

Synthesis and cloning of cDNA

Synthetic oligonucleotides complementary to nucleotides 1-19, 846-862 and 2300-2316 (nucleotide numbers start at the 3′ end) of Junin virus MC2 S RNA (Ghiringhelli et al., 1989, 1990) were phosphorylated and used to prime cDNA synthesis. The reaction containing viral RNA, primers, deoxynucleoside triphosphates and AMV reverse transcriptase was carried out in a way similar to that described by Gubler and Hoffman (1983).

The double stranded cDNA was blunt-ended using T4 DNA polymerase, size-fractionated by gel electrophoresis and glass powder elution to prevent the generation of a high proportion of clones with short inserts. The ds cDNAs in the range of 1.0 - 4.0 kbp were inserted by ligation in the SmaI site of pBS SK$^+$ (Bluescript SK$^+$, Stratagene, La Jolla, CA) and cloned in *E. coli* DH5 F′α. The colonies containing pBS SK$^+$ with inserts were selected by color on LB-agar plates containing ampicillin, IPTG and X-gal.

Alternatively, Bst XI adapters were ligated to the ds cDNA prior to the size-fractionation step, and the cDNA was inserted in the Bst XI site of the pcDNA II vector (Invitrogen, San Diego, CA).

The screening of the recombinant clones was performed by colony hibridization (Grunstein and Hogness, 1975) using Junin-MC2 S RNA probes. The probes consisted of selected DNA fragments that were obtained from recombinant plasmids containing Junin-MC2 sequences and labeled by nick translation. Plasmid DNA was prepared from the hybridization positive

clones, analyzed by gel electrophoresis after restriction enzyme digestion and tested by Northern blot (Maniatis *et al.*, 1982).

Northern blot analysis

Viral and cellular RNAs were denatured with 10mM CH_3HgOH and electrophoresed on a 1% agarose gel according to Bailey and Davidson (1976).The gel was soaked in 14 mM B-mercaptoethanol and 0.5 μg/ml ethidium bromide, to check the quality of the RNA preparation, and transferred by capillary blotting onto a Gene Screen nylon membrane (NEN, Chicago, IL, USA). The blot was processed according to the manufacturers specifications and hybridized to different cDNA probes. The ^{32}P labelled cDNA probes were prepared by nick translation (Maniatis *et al.*, 1982) or multipriming (Feinberg and Vogelstein, 1983) of the recombinant plasmid DNAs. Northern blots were employed to assess the identity of the cDNA clones that were further analyzed by nucleotide sequencing.

Sequence analysis

The nucleotide sequences of the cDNA inserts were determined by the dideoxy method of Sanger *et al.* (1977) using a modified T7 DNA polymerase (Sequenase™, USB, Cleveland, OH, USA). The original cDNA inserts were subcloned into M13 mp18 and mp19 and the ssDNAs were used as templates for sequencing (Yanish-Perron *et al.*, 1985).

The sequence information was processed on a personal computer using the DNASIS (Hitachi Software Engineering Co.) program and on a VAX computer using the package by Genetics Computer Group (GCG, Madison, WI, USA).

RESULTS

Molecular cloning and nucleotide sequence of Junín-Candid #1 virus S RNA

Although three different oligonucleotides were used simultaneously (primers A, B and C, Fig. 1), the hybridization-positive clones that were recovered contained cDNA inserts beginning with only two of them. So far, no cDNA clones were obtained starting with the oligonucleotide-primer complementary to the 3′ terminal sequence conserved among all the arenavirus S RNAs (5′CGCACAGTGGATCCTAGGC, Auperin *et al.*, 1982). The cDNA clones were shown to anneal specifically to Junín virus S RNA in Northern blot hybridizations (data not shown).

The nucleotide sequences of several overlapping cDNA clones shown in Fig. 1 were determined using the dideoxy chain termination method (Sanger *et al.*, 1977). In this analysis all the overlapping sequences were identical. The nucleotide sequence presented in Fig. 2 comprises the 5′ non-coding region, the GPC gene, the intergenic region and the terminus of the N gene.

The open reading frame (ORF) of viral polarity starts at positions 89-91 and stops at 1544-1546 and codes for the GPC glycoprotein precursor.

Junín virus (Candid #1) glycoprotein precursor GPC

The first AUG codon of the genomic or viral-sense (v) S RNA, found at nucleotides 89-91, initiates a long ORF that remains open down to the in-phase UAA translational termination signal at positions 1544-1546. Seven codons downstream from the first AUG, a second AUG is found in the same frame. However, based upon the observations on most frequent flanking sequences of eukaryotic translation start codons reported by Kozak (1984)

(i.e. CC$\overset{A}{G}$CCAUGG), the first AUG is considered to function as the true initiation codon since a G residue is found at positions +4 and -3, and there is also a C at position -2. The molecular weight calculated for the 485 amino acids long translation product (i.e. 55,700 Da) is smaller than the value estimated by gel electrophoresis for the intracellular glycoprotein precursor that undergoes cotranslational glycosylation (De Mitri and Martínez Segovia, 1985; Rustici, 1984).

Eight potential N-glycosylation sites are found in the GPC sequence (Fig. 3). Whether all are used for the attachment of carbohydrate side chains is not known.

Amino acid sequence homology of arenavirus GPC gene products

Junín virus GPC protein sequence was aligned with the rest of the arenavirus GPCs as depicted in Fig. 4. The best-fit alignment shows that the amino acid sequence homology is concentrated in two regions: the 53 residues at the amino-terminus and the carboxy-proximal half of GPC. Buchmeier *et al.* (1987) demonstrated that, in the infected cell, LCM virus GPC is cleaved at or near the Arg_{262}-Arg_{263} to yield the proteins G1 (amino-terminal half) and G2 (carboxy-terminal half). By analogy, Junín virus GPC should be cleaved at or near the homologous Arg_{243}-Arg_{244} (MC2) or Arg_{247}-Arg_{248} (Candid #1).

The G1 and G2 regions of GPC defined by the dibasic amino acid sequence at positions 243 to 286 —depending on the virus— exhibit quite different degrees of sequence homology. G1 sequences show few clusters of sequence conservation among all the different arenaviruses. These homologous regions concentrate especially at the positions 1 to 53 and 84 to 97.

This sequence comparison shows that Junín and Tacaribe G1 sequences are by far more homologous (50%) than the other pairs analyzed (Table 1). The sequence similarity is even higher for the G2 polypeptide, where 82% of the amino acids are identical in nature and position. Comparison of Junín G2 with the other arenavirus G2 proteins also shows a high degree of homology, in the range of 53-57%, suggesting that there is less sequence flexibility than in G1, compatible with function conservation. The homology figures are higher when conservative amino acid changes are considered in the calculation (Table 2).

Four of the putative N-glycosylation sites of GPC are conserved in all the arenaviruses (one in the G1 region and three in the G2, Fig. 4). This fact, however, is not necessarily linked with the actual use of these or the other less conserved glycosylation sites.

Comparison of the glycoprotein genes of Junín virus strains Candid #1 and MC2

The alignment of the coding sequences of MC2 and Candid #1 GPC genes shows an overall high degree of homology (Fig. 4). Although, all the nucleotide sequence alterations are summarized in Table 3, there are major changes that will be pointed out here. There are several nucleotide insertions and deletions concentrated between the codons 43 and 76 of Junín-MC2 GPC gene that should be formally considered to modify its sequence to that of Candid #1 GPC (codons 43-80, Figs. 3 and 5).

There are six single-nucleotide insertions that change MC2 amino acid sequence $_{43}$CSILD$_{47}$ into $_{43}$LFQFFVF$_{49}$ in Candid #1. Two triplets downstream, there is a six-nucleotide-block insertion resulting in the insertion of two amino acids (LA, codons 52 y 53). Further downstream, one transversion and three single-nucleotide insertions change the amino acid sequence $_{54}$PR$_{55}$ to

to $_{58}$TEE$_{60}$. The other major change results from three different single-nucleotide deletions that shift the reading frame of the coding sequence converting the MC2 sequence $_{65}$VPDCVLLQWWVS$_{76}$ to $_{70}$FQTVSFSMVGL$_{80}$.

Most of the other nucleotide substitutions do not change the coding with the exception of codons 139, 423 and 442 in MC2 or corresponding codons 143, 427 and 446 in Candid #1, that result in three amino acid changes, two of which are conservative according to the considerations of Schwartz and Dayhoff (1979).

As shown in Fig. 6, the distribution of the amino acid sequence changes in MC2 and Candid #1 GPC gene products concentrates in the amino-proximal region of G1 in contrast with the changes in the nucleotide sequence that are scattered through the carboxyl-terminus of G2. However, most of the nucleotide substitutions in G2 do not alter the amino acid sequence.

None of the amino acid residues that are different in both Junín virus strains alters any of the eight potential N-glycosylation sites.

Non coding nucleotide sequences

The untranslated upstream sequence for Junín-Candid #1 virus GPC gene comprises 88 nucleotides, 86 of which are identical to the homologous region in Junín-MC2 S RNA. The two only differences that were found consist of an extra cytosine at the 5′ end of Candid #1 RNA and the substitution of an A for a G at position 38.

The non-coding region dowstream from the translation termination signals for both GPC and N genes (nucleotides 1544-1546 and 1638-1640 of v S RNA) contains two sets of self-complementary nucleotide sequences capable of forming two very stable hairpin-loop structures (Fig. 7).

The hairpin closest to the GPC stop codon is formed by 11 GC and 2 AU pairs (ΔG°= -39.0 kcal/mol, Tinoco *et al.*, 1973) and the one closest to the N stop codon is stabilized by 15 GC and 1 AU pairs (ΔG° = -57.2 kcal/mol. The nucleotide sequences of Junín-MC2 and Junín-Candid #1 are identical but for three nucleotides. Moreover, two of the nucleotide changes (C→U, A→C) fall outside the double hairpin-loop, and the third one (U→C) does not affect the thermodynamic stability of the secondary structure either, since it is located at the top of the first hairpin-loop (Fig. 7). One hairpin-loop structure has been found in the intergenic region of each of the arenaviruses that have been sequenced to date (Auperin *et al.*, 1984; Romanowski and Bishop, 1985; Auperin et al., 1986; Franze-Fernández *et al.*, 1987). In this context Junin virus S RNA has an unusual secondary structure.

DISCUSSION

Changes in the amino acid sequence of the glycoprotein G1 might be responsible for the attenuated phenotype of Junín-Candid #1 vaccine strain

We completed the nucleotide sequence of the glycoprotein precursor gene of Junín-Candid #1 virus and its flanking untranslated regions.

During these studies, special attention was devoted to avoid spurious genetic variations that could possibly obscure the changes relevant to the attenuation of virulence. Taking into account that arenaviruses are notorious for variations in their biologic properties based on passage history (Ahmed *et al.*, 1984), the biochemical studies were conducted on virions that had the same manipulations as the vaccine stock. Junín (Candid #1) virus was passaged in diploid FRhL-2 cells the same limited number of times as the vaccine in order to avoid genotypic variations that could

arise from accumulation of mutations upon repeated passage or a different type of selection posed on the virus if a different cell substrate was used.

The information obtained from cDNA clones of the attenuated Junín virus vaccine has been compared with the homologous regions of Junín-MC2 and other arenavirus S RNAs and their gene products.

The untranslated regions flanking the GPC genes of different arenaviruses are variable in length, sequence and secondary structure. However, when the highly attenuated Junín virus strain, Candid #1, is compared with the MC2 strain, of intermediate virulence, the similarities are notorious. The differences consist of an extra C at the 5′ end of Candid #1 and a G in position 38 instead of the A present in MC2. The secondary structure in the intergenic region downstream of the GPC and N open reading frames of Candid #1 is identical to that of MC2 with only three nucleotide changes, that do not affect the stability of the double hairpin-loop.

In poliovirus type 3, one single base mutation in the 5′ non coding region $C_{472} \rightarrow T_{472}$ has been demonstrated to be responsible of the attenuated phenotype of the vaccine strain P3/Leon 12a,b. On the other hand, another mutation in the VP3 coding sequence that changed a serine to a phenylalanine codon was also shown to contribute to the attenuation of the neurovirulence (Westrop *et al.*, 1986)

In Junín-Candid #1 it cannot be demonstrated directly whether the nucleotide substitutions in the non coding region play a role in attenuation of the virulence. However, the striking differences in the GPC coding sequence of Candid #1 and MC2 suggest that these might be more relevant to the changes in virulence and tissue tropism.

The nucleotide sequence changes found in the GPC ORF when Candid #1 and MC2 S RNAs are compared, concentrate in the NH_2-proximal (20 nucleotide

changes) and the COOH-proximal regions (19 nucleotide substitutions). However, from Fig. 6 it becomes apparent that the major amino acid residue changes resulting from the above mentioned nucleotide sequence alterations are located in the NH_2-proximal region of GPC. This part of the coding sequence corresponds to the G1 protein, which —by analogy with LCM (Burns *et al.*, 1990)— is thought to be exposed on the surface of the virion, in association with the more internal G2 protein.

The amino acid sequence changes occur in a region that contains the proposed signal peptidase cleavage site (Burns et al., 1990). Therefore, the altered G1 amino acid sequence would correspond to the actual amino-terminus of this protein. By contrast, only two isolated amino acid residue substitutions are found in the G2 protein of Candid #1, despite the fact that the number of nucleotide changes in this region of the genome is similar to the one in the G1 sequence.

Although our results that suggest the involvement of the surface glycoprotein in the attenuation of virulence are preliminary, they are consistent with the reports on other viruses.

It should be noted, however, that for each attenuated vaccine strain analyzed (poliovirus types 1 and 3 and yellow fever) suggestive or experimental evidence has been obtained for a role of altered viral surface protein in attenuation (Nomoto *et al.*, 1982; Hahn *et al.*, 1987). As suggested by the literature on different viruses the amino acid substitution can have profound effects on virulence and tissue tropism (Spriggs and Fields, 1982; Spriggs *et al.*, 1983; Coulon *et al.*, 1983; Johnson *et al.*, 1986; Prehaud *et al.*, 1990).

Significant progress has been made in understanding the genetic basis of attenuation of the type 1 poliovirus vaccine strain using the recombinant viruses obtained by *in vitro* manipulation of infectious cDNA (Nomoto *et al.*, 1987; Racaniello and Baltimore, 1981). This approach has

proved to be very productive because the changes at the nucleotide level can be precisely directed and the resulting phenotypes analyzed for changes in the biological properties. Unfortunately, at this time, it is impossible to regenerate infectious viruses from cDNA of segmented RNA genomes. Therefore, we will have to resort to the more classical approach of analyzing the genomes of spontaneously generated mutants with different virulence phenotypes. As a short cut to this end, the variants derived in the process of development of the Candid #1 strain will be analyzed as mentioned elsewhere in this report.

In particular, the literature on viruses such as paramyxo and orthomyxoviruses indicates that tissue tropism and virulence in a particular host are dependent upon the proteolytic activation of a surface glycoprotein (Tashiro *et al.*, 1990; Deshpande *et al.*, 1987). Although, the cleavability of the arenavirus GPC has not been studied in relation to the infection process, we have not found any amino acid sequence changes at or around the cleavage site of Junín Candid #1 GPC. Therefore, it might be concluded that this region plays no role in the attenuation of Candid #1.

The biological significance of the nucleotide changes found in the attenuated and wild type strains of Junín virus may be provisionally sought by evaluation of the secondary structures predicted for the RNAs and the proteins. At this time, we have only compared the sequences of a highly attenuated Junín virus with the data obtained in our laboratory from the cDNA of a wild type virus isolate.

Although these findings are strongly suggestive of the genetic changes that lead to an attenuated phenotype, they must be complemented with studies on the direct predecessors of Candid #1, that exhibit gradually more virulent phenotypes, in order to locate precisely the mutations responsible for attenuation.

Finally, the identification of a highly variable region in the S RNA of Junín virus provides a valuable marker for monitoring the stability of this virus or the identification of different virus isolates recovered from AHF patients.

Confirmation of an unusual secondary structure in the intergenic region

The nucleotide sequence of Junín-MC2 virus S RNA showed a potential secondary structure in the intergenic region consisting of two consecutive hairpin-loops (Ghiringhelli *et al.*, 1987). This unusual structure differed from the single hairpin-loop found in other arenavirus S RNAs but was confirmed by sequence analyses of several independently generated cDNA clones (Ghiringhelli et al., submitted for publication).

A structure identical to the one described for MC2 S RNA has now been determined in the vaccine strain Candid #1. It is interesting to note that, while this report was in preparation, a new corrected version of the Tacaribe virus S RNA, that was made available to us (M.T. Franze-Fernández, personal communication), also showed two potential hairpin-loop structures in the intergenic region. Although, the nucleotide sequence that forms the double hairpin-loop in Tacaribe S RNA is quite similar to the homologous stretch in Junín virus, the complete intergenic region is much longer.

Another difference of Junín virus with respect to the rest of the arenaviruses is located in the very 5′ end sequence. The 5′ termini of Pichindé, Lassa, LCM and Tacaribe virus S RNAs have been reported to contain the conserved sequence 5′ CGCACCGGGGAUCCUAGGC, with an extra G at the 5′ end in the case of Pichindé and Lassa (Auperin *et al.*, 1984c; Romanowski *et al.*, 1985; Auperin *et al.*, 1986; Franze-Fernández *et al.*, 1987). The same portion of the 5′ end sequence of Junín-MC2 virus is slightly different (i.e. UGCAGUAAGGGGAGCCUAGGC; the differences are

underlined) but the base-pairing with the 3′ end of the S RNA yields a panhandle structure similar to the one predicted for the rest of the arenaviruses. The sequence found in Junín-MC2 has been confirmed in independent clones of Junín-Candid #1 cDNA, although an extra C was determined at the 5′ end.

These findings suggest that the non coding regions of the S RNAs could be used to characterize closely related arenaviruses as belonging to the same or different virus "species".

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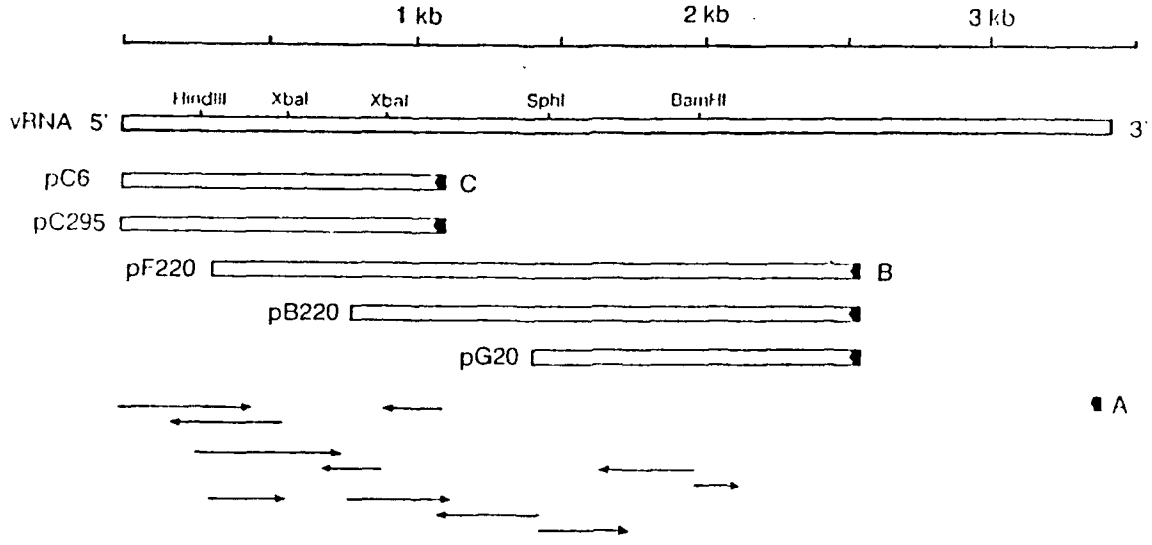

FIGURE 1: Cloning and sequencing strategy of Junín virus (Candid #1) S RNA. The S RNA nucleotide sequence is represented in the viral sense with a size scale in kilobases above. Some of the overlapping clones spanning 75% of the S RNA, are shown as rectangles. Phosphorylated synthetic oligonucleotides (A, B and C) that were used to prime cDNA synthesis are indicated as black boxes at the beginning of each clone. Subcloning into M13 mp18 and mp19, using the restriction sites that are indicated, was performed in order to generate the nucleotide sequence data by the method of Sanger et al. (1977). The arrows indicate the length of the sequences that were obtained using a modified T7 DNA polymerase (Sequenase™, USB) on different M13 subclones.

CUGCAGUAAG GGGAUCCUAG GCGAUUUUGG UAACGCUGUA AGUUGUUACU GCUUUCUAUU UGGACAACAU CAAACCAUCU 80

AUUGUACAAU GGGGCAAUUC AUCAGCUUCA UGCAAGAAAU ACCUACCUUU UUGCAGGAAG CUCUGAAUAU UGCUCUUGUU 160

GCAGUCAGUC UCAUUGCCAU CAUUAAGGGU GUAGUAAACC UGUACAAAAG UGGUUUGUUC CAAUUCUUUG UAUUCCUAGC 240

ACUCGCAGGA AGAUCCUGCA CAGAAGAAGC UUUUAAAAUC GGACUGCACA CAGAGUUCCA GACUGUGUCC UUCUCAAUGG 320

UGGGUCUCUU UUCCAACAAU CCACAUGACC UGCCUCUGUU GUGUACCUUA AACAAGAGCC AUCUUUACAU UAAGGGGGGC 400

AAUGCUUCAU UCAAGAUCAG CUUUGAUGAC AUCGCAGUGU UGUUACCAGA AUAUGACGUU AUAAUUCAGC AUCCGGCAGA 480

UAUGAGCUGG UGUUCUAAAA GUGAUGAUCA AAUUUGGCUG UCUCAGUGGU UCAUGAAUGC UGUGGGGCAU GAUUGGUAUC 560

UAGACCCACC AUUUCUGUGU AGGAACCGUA CAAAGACAGA AGGCUUCAUC UUUCAAGUCA AUACCUCCAA GACUGGUAUC 640

AAUGAAAACU AUGCCAAGAA GUUUAAGACU GGUAUGCACC AUUUAUAUAG AGAAUACCCC GACUCUUGCU UGGAUCGCAA 720

ACUGUGUUUG AUGAAGGCAC AACCCACCAG UUGGCCUCUC CAAUGUCCAC UUGACCAUGU CAACACAUUA CAUUUCCUCA 800

CAAGAGGCAA GAACAUUCAG CUUCCAAGGA GGUCUUUAAA AGCAUUCUUU UCCUGGUCUC UGACAGACUC AUCCGGCAAG 880

GACACCCCUG GAGGCUAUUG UCUAGAAGAG UGGAUGCUCG UUGCAGCCAA AAUGAAGUGU UUUGGCAAUA CUGCUGUAGC 960

AAAAUGCAAU CUGAAUCAUG ACUCUGAAUU UGUGACAUG CUGAGGCUUU UUGAUUACAA CAAAAAUGCU AUCAAAACCU 1040

UAAAUGAUGA AACUAAGAAA CAAGUAAAUC UGAUGGGACA GACAAUCAAU GCGCUGAUAU CUGACAAUUU AUUGAUGAAA 1120

AACAAAAUUA GGGAACUGAU GAGUGUCCCU UACUGCAAUU ACACAAAAUU UUGGUAUGUC AACCACACAC UUUCAGGACA 1200

ACACUCAUUA CCAAGGUGCU GGUUAAUAAA AAACAACAGC UAUUUGAACA UCUCUGACUU CCGUAAUGAC UGGAUAUUAG 1280

AAAGUGACUU CUUAAUUUCU GAAAUGCUAA GCAAAGAGUA UUCGGACAGG CAGGGUAAAA CUCCUUUGAC UUUAGUUGAC 1360

AUCUGUAUUU GGAGCACAGU AUUCUUCACA GCGUCACUCU UCCUUCACUU GGUGGGUAUA CCCUCCCACA GACACAUCAG 1440

GGGCGAAGCA UGCCCUUUGC CACACAGGUU GAACAGCUUG GGUGGUUGCA GAUGUGGUAA GUACCCCAAU CUAAAGAAAC 1520

CAACAGUUUG GCGUAGAGGA CACUAAGACC UCCUGAGGGU CCCCACCAGC CCGGGCACUG CCCGGGCUGG UGUGGCCCCC 1600

CAGUCCGCGG CCUGGCCGCG GACUGGGGAG GCACUGCUUA CAGUGCAUAG GCUGCCUUCG GGAGGAACAG CAAGCUCGGU 1680

GGUAAUAGAG GUGUAGGUUC CUCCUCAUAG AGCUUCCCAU CUAGCACUGA CUGAAACAUU AUGCAGUCUA GCAGAGCACA 1760

GUGUGGUUCA CUGGAGGCCA ACUUGAAGGG AGUAUCCUUU UCCCUCUUUU UCUUAUUGAC AACCACUCCA UUGUGAUAUU 1840

UGCAUAAGUG ACCAUAUUUC UCCCAGACCU GUUGAUCAAA CUGCCUGGCU UGUUCAGAUG UGAGCUUAAC AUCAACCAGU 1920

UUAAGAUCUC UUCUUCCAUG GAGGUCAAAC AACUUCCUGA UGUCAUCGGA UCCUUGAGUA GUCACAACCA UGUCUGGAGG 2000

CAGCAAGCCG AUCACGUAAC UAAGAACUCC UGGCAUGCAU CUUCUAUGUC CUUCAUUAAG AUGCCGUGAG AGUGUCUGCU 2080

ACCAUUUUUA AACCCUUUCU CAUCAUGUGG UUUUCUGAAG CAGUGAAGUC UGCUU 2135

FIGURE 2: Parcial nucleotide sequence of Junin virus (Candid #1) S RNA
A 2135-nucleotide-long sequence S RNA is presented in the viral or genome sense. The translational initiation and termination codons of GPC and the termination anticodon of N (encoded in the viral-complementary sequence) are boxed. Two inverted repeats at the intergenic region are underlined.

FIGURE 3: Amino acid sequence of Junín virus Candid #1 GPC gene products. The regions were Candid #1 GPC sequence differs from that of the wi MC2 strain are identified by letters a through g (for easy cross-reference with table 3) and underlined. The amino acid sequence of the MC2 GPC protein in these regions appears below the lines). The putative N-glycosylation sites are boxed. Two dots (yy) highlight the double basic amino acid sequence identified by Buchmeier et al. (1987) as the putative proteolytic cleavage site that generates G1 (NH_2-terminal half) and G2 (COOH-terminal half of GPC).

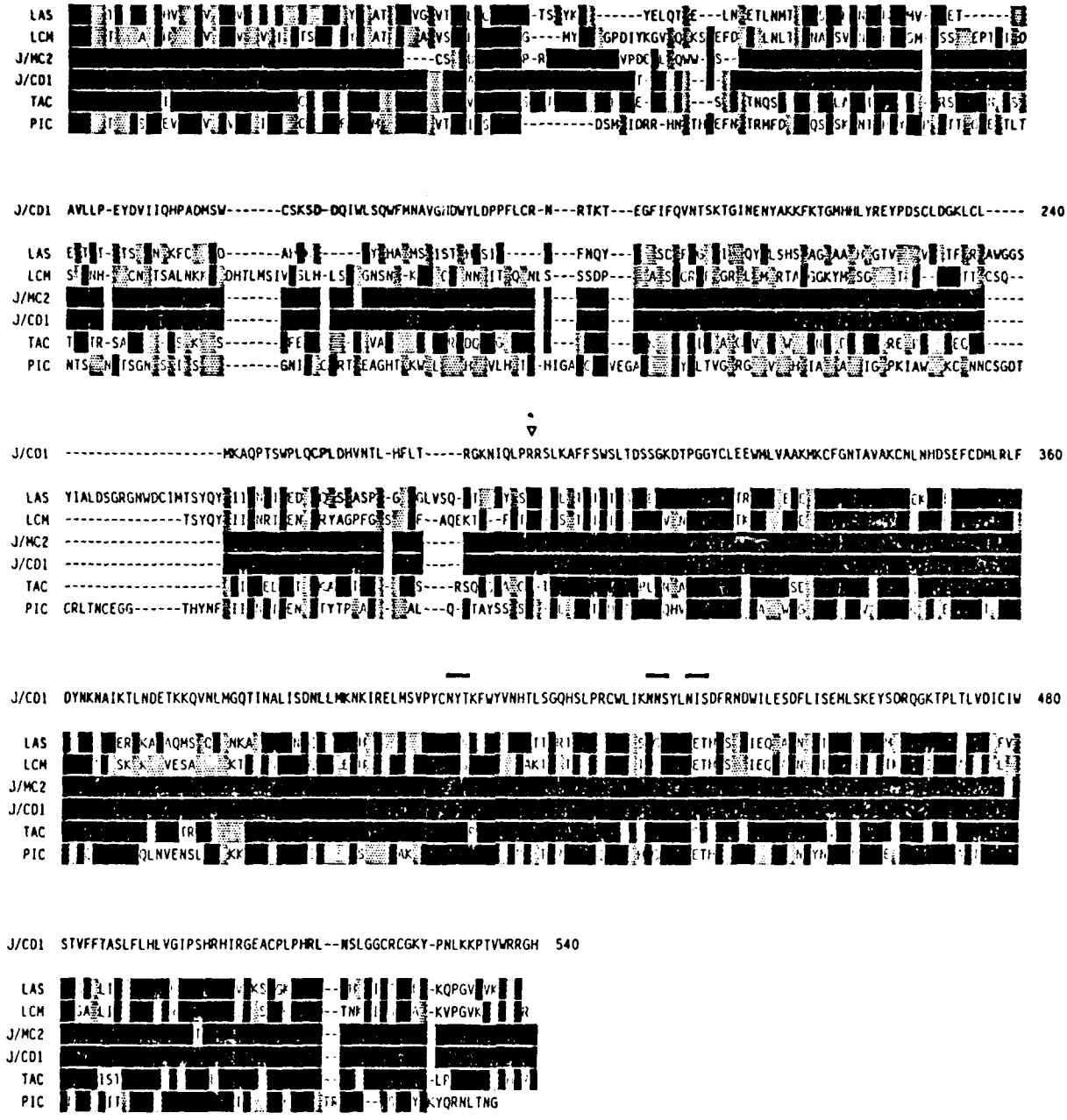

FIGURE 4: Comparison of arenavirus GPC proteins. The predicted amino acid sequences of the GPC proteins of Lassa (LAS, Auperin *et al.*, 1986), LCM-WE (LCM, Romanowski, *et al.*, 1985), Tacaribe (TAC, Franze-Fernández *et al.*, 1987), Pichindé (PIC, Auperin *et al.*, 1984c) and Junín-MC2 (J/MC2, Ghiringhelli et al., submitted) are compared to that of Junín-Candid #1 (J/CD1) GPC.

The amino acid residues identical to those of J/CD1 GPC protein are indicated by black boxes; those that represent conservative changes according to Schwartz and Dayhoff (1979) appear as shaded areas. Gaps were incorporated in the sequences in order to obtain the best fit alignment. The complete amino acid sequence of J/CD1 GPC protein, that was used as reference, is printed above the aligned sequences. In addition, the conserved N-glycosylation sites ($NX\frac{T}{S}$) are indicated by thick lines above the JUN GPC sequence. The position of the proteolytic cleavage site is indicated by a triangle (the amino acid sequence to the left of this site is referred in the text as G1 and that, to the right, as G2).

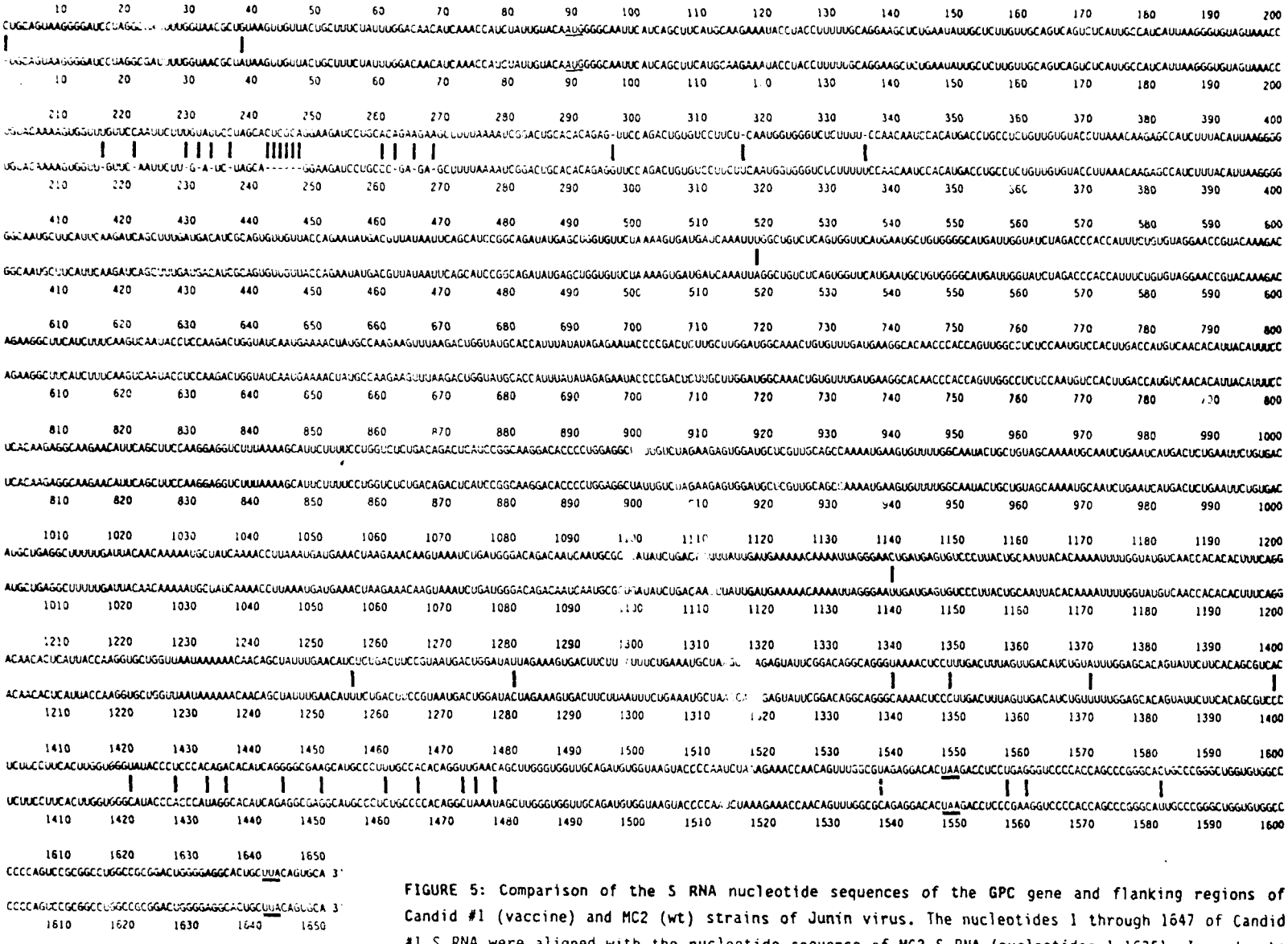

FIGURE 5: Comparison of the S RNA nucleotide sequences of the GPC gene and flanking regions of Candid #1 (vaccine) and MC2 (wt) strains of Junin virus. The nucleotides 1 through 1647 of Candid #1 S RNA were aligned with the nucleotide sequence of MC2 S RNA (nucleotides 1-1635). In order to obtain the best fit alignment 16 gaps were included in the MC2 sequence (J/MC2), and 3, in the Candid #1 S RNA (J/CD1). The numbers that appear in this figure include the gaps as nucleotides and, therefore, do not coincide exactly with the sequence numbers in Fig. 2 and those of Ghiringhelli *et al.* (manuscript submitted for publication).

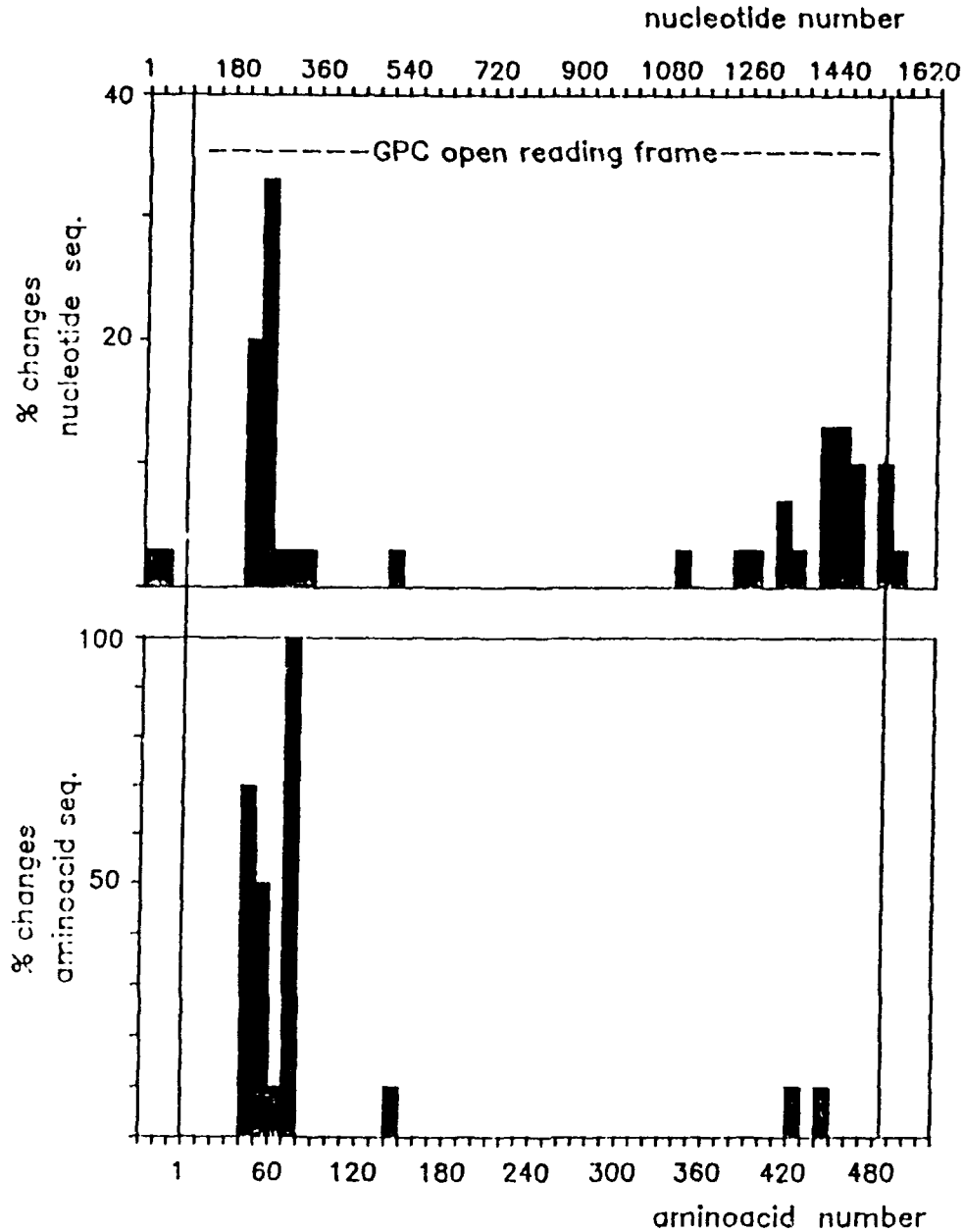

FIGURE 6: Distribution of the amino acid and nucleotide changes in the GPC protein, the corresponding open reading frame and the 5′ and 3′ untranslated regions. The nucleotide and amino acid sequences of Candid #1 and MC2 strains, aligned as in Fig. 5 and 3, were subdivided in fractions of 30 nucleotides and 10 amino acids, respectively, in order to score the differences found while scanning the sequences. The upper panel is a histogram-plot of the nucleotide changes (%) calculated from the pairwise comparison of the homologous 30-nucleotide fragments of Candid #1 and MC2 S RNAs. The boundaries of the GPC ORF are indicated by two vertical lines, that continue into the lower panel. In this panel, the changes (%) in the amino acid sequence of the 10-amino-acid-residue fractions of Candid #1 and MC2 GPC proteins were plotted.

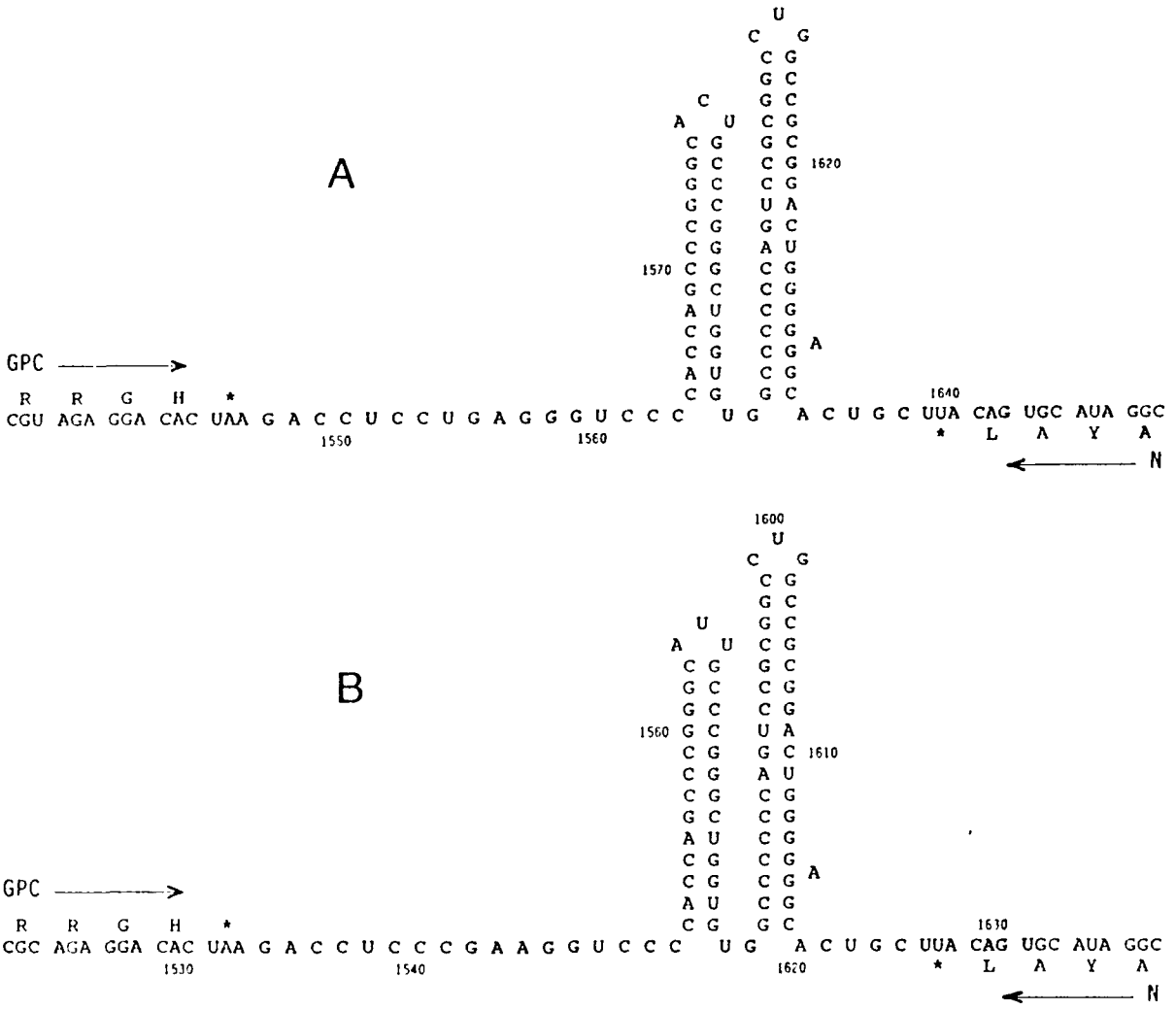

FIGURE 7: Intergenic region of Junin virus S RNA. A portion of the S RNA of Junin-Candid #1 **(A)** virus comprising the ends of the GPC and N genes (sense and anti-sense strand, respectively) and the non-coding intergenic region is shown in the figure. The directions of translation are indicated by arrows and the stop codon for the GPC protein and the stop anticodon for the N gene are marked with asterisks. Two potential hairpin-loop structures are stabilized by 13 and 17 base pairs, respectively (ΔG° = -39.0 and -54.2 kcal/mole). In the lower part of this figure **(B)**, the homologous region of Junin-MC2 virus S RNA is shown for comparison. The nucleotides C_{1541}, A_{1544} and T_{1565} of MC2 have been changed to T_{1554}, G_{1557} and C_{1578} in Candid #1.

TABLE 1

SEQUENCE IDENTITY (%) OF ARENAVIRUS **GPC** PROTEIN

	J/MC2	TAC	PIC	LCM	LAS
J/CD1	90	53	21	20	21
J/MC2		50	20	21	21
TAC			24	16	17
PIC				20	26
LCM					27

G1

	J/MC2	TAC	PIC	LCM	LAS
J/CD1	99	80	57	53	58
J/MC2		82	57	53	57
TAC			51	51	52
PIC				52	54
LCM					69

G2

TABLE 2

SEQUENCE HOMOLOGY (%) OF ARENAVIRUS **GPC** PROTEIN,
AMINO ACID IDENTITY AND CONSERVATIVE SUBSTITUTIONS

	J/MC2	TAC	PIC	LCM	LAS
J/CD1	92	74	43	42	42
J/MC2		69	43	42	44
TAC			41	38	36
PIC				41	47
LCM					44

G1

	J/MC2	TAC	PIC	LCM	LAS
J/CD1	99	89	71	71	69
J/MC2		92	72	70	68
TAC			70	69	67
PIC				68	69
LCM					83

G2

AMINOACID SIMILARITY (Schwartz and Dayhoff,1979)

A	=	G;	P			M	=	V;	F;	L;	I
C	=	S;	Y			N	=	D;	E;	H	
D	=	E;	G;	N;	Q	P	=	A			
E	=	G;	N;	Q;	D	Q	=	E;	D;	H	
F	=	I;	L;	M;	W; Y	R	=	W;	H;	K	
G	=	S;	D;	A;	E	S	=	C;	G		
H	=	N;	Q;	R		T	=				
I	=	L;	M;	V;	F	V	=	I;	L;	M	
K	=	R				W	=	Y;	L;	F;	R
L	=	I;	V;	W;	M; F	Y	=	C;	F;	W	

A	=	Ala	F	=	Phe	K	=	Lys	P	=	Pro	T	=	Thr
C	=	Cys	G	=	Gly	L	=	Leu	Q	=	Gln	V	=	Val
D	=	Asp	H	=	His	M	=	Met	R	=	Arg	W	=	Trp
E	=	Glu	I	=	Ile	N	=	Asn	S	=	Ser	Y	=	Tyr

TABLE 3

Localization of nucleotide and amino acid changes in the glycoprotein genes of wild type MC2 and vaccine Candid#1 strains of Junín virus

position		nucleotide	codon	amino acid
MC2	CD1	MC2 → CD1	MC2 → CD1	MC2 → CD1
--	1	- C	upstream	untranslated
37	38	A → G	non coding	region
214	*216	ins. U	stepwise	
218	*221	ins. C	change of	
225	*229	ins. U	reading	
226	231	ins. U	frames	CSILD → LFQFFVF[a]
228	*234	ins. U	(2 codons	
229	*236	ins. C	extra)	
234	242	6 nt. block	--- CUC	
to	to	insertion		-- → LA[b]
235	247	CUCGCA	--- GCA	
247	260	C → A	stepwise	
248	262	ins. A	change of	
250	265	ins. A	reading	
252	268	ins. A	frames	PR → TEE[c]
			(1 codon	
			extra)	
280	295	del. G	stepwise	
300	314	del. U	change of	
315	*333	del. U	reading	VPDCVLLQWWVS → FQTVSFSMVGL[d]
			frames	
			(1 codon loss)	
502	515	A → U	AGG → UGG	R → W[e]
1123	1136	U → C	UUG → CUG	L → L
1239	1252	U → C	AUU → AUC	I → I
1264	1277	C → U	CUA → UUA	L → L
1323	1336	C → U	GGC → GGU	G → G
1332	1345	C → U	CCC → CCU	P → P
1354	1367	U → A	UUU → AUU	F → I[f]
1383	1396	C → A	UCC → UCA	S → S
1404	1417	C → U	GGC → GGU	G → G
1411	1424	A → U	ACC → UCC	T → S[g]
1416	1429	U → C	CAU → CAC	H → H
1419	1432	G → A	AGG → AGA	R → R
1428	1441	A → G	CAA → CAG	R → R
1434	1447	G → A	CGG → CGA	E → E
1444	1457	C → U	CUG → UUG	L → L
1449	1462	C → A	CCC → CCA	P → P
1456	1469	C → U	CUA → UUG	L → L
1458	1471	A → G	CUA → UUG	L → L
1461	1474	U → C	AAU → AAC	N → N
1521	1534	C → U	CGC → CGU	R → R
1541	1554	C → U	downstream	untranslated
1544	1557	A → G	non coding	region
1565	1578	U → C		

* In insertions or deletions in which a stretch of the same nucleotide is involved, it is not possible to specify the exact position of the mutational change. Therefore, the last nucleotide in the stretch is indicated to identify the sequence position.

a,b,c,d,e,f,g: refer to the regions indicated in Fig. 3

REFERENCES

Ahmed, R., Salmi, A., Butler, L.T., Chiller, J.M., and Oldstone, M.B.A. (1984). Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice: role in suppression of cycotoxic T lymphocyte response and viral persistence. *J. exp. Med.* 60, 521-540.

Auperin, D.D., Compans, R.W., and Bishop, D.H.L. (1982). Nucleotide sequence conservation at the 3′ termini of the virion RNA species of New World and Old World arenaviruses. *Virology* 121, 200-203.

Auperin, D.D., Galinski, M., and Bishop, D.H.L. (1984a). The sequences of the N protein gene and intergenic region of the S RNA of Pichindé arenaviruses. *Virology* 134, 208-219.

Auperin, D., Romanowski, V., and Bishop, D.H.L. (1984b). Genetic analyses of Pichindé and LCM arenaviruses; evidence for a unique organization for Pichindé S RNA. *In* "Segmented negative strand viruses, arenaviruses, bunyaviruses and orthomyxoviruses" (R.W. Compans and D.H.L. Bishop, Eds.), pp. 51-57. Academic Press.

Auperin, D., Romanowski, V., Galinski, M.S., and Bishop, D.H.L. (1984c). Sequencing studies of Pichindé arenavirus S RNA indicate a novel coding strategy, an ambisense viral S RNA. *J.Virol.* 52, 987-904.

Auperin, D.D., Sasso, D.R., and McCormick, J.B. (1986). Nucleotide sequence of the glycoprotein gene and intergenic region of the Lassa virus S genome RNA. *Virology* 154, 155-167.

Bailey, J.M. and Davidson, N. (1976) Methylmercury as a reverside denaturing agent for agarose gel electrophoresis. Analyt. Biochem., 70, 75-85.

Bishop, D.H.L. (1988). The ambisense coding strategy of the S RNA of arenaviruses. *In* "Modern trends in virology" Heidelberg: Springer-Verlag.

Buchmeier, M.J.; Southern, P.J.; Parekh, B.S.; Wooddell, M.K., and Oldstone, M.B.A. (1987). Site-specific antibodies define a cleavage site conserved among arenaviruses GP-C glycoproteins. *J. Virol.*, 61, 982-985.

Burns, J.W., Salvato, M.S. and Buchmeier, M.J. (1990) Molecular architecture of Lymphocytic choriomeningitis. VIII Intl. Congress of Virology (Berlin, Germany) p. 27, W3-001.

Chomczynski, M. and Sacchi, A. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. Analytic. Biochem., 162, 156-159.

Clegg, J.C.S., and Oram, J.D. (1985). Molecular cloning of Lassa-virus RNA: nucleotide sequence and expression of the nucleocapsid protein gene. *Virology*, 144, 363-372.

Coulon, P. Rollin, P.E., and Flamand, A. (1983). Molecular basis of rabies virus virulence. II. Identification of a site on the CVS glycoprotein associated with virulence. *J. Gen. Virol.*, 64, 693-696.

De Mitri, M.I., and Martinez Segovia, Z. (1985). Polypeptide synthesis in Junin virus infected BHK-21 cells. *Acta Virol.*, 29, 97-103.

Deshpande, K.L., Fried, V.A., Ando, M., and Webster, R.G. (1987) Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. *Proc. Natl. Acad. Sci.*, **84**, 36-40.

Enria, D.A., Franco, S.G., Ambrosio, A., Vallejos, D., Lewis, S., and Maiztegui, J. (1986). Current status of the treatment of Argentine hemorrhagic fever. *Med. Microbiol. Immunol.*, **175**, 173-176.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-14.

Franze-Fernandez, M.T.; Zetina, C.; Iapalucci, S.; Lucero, M.A.; Boissou, C.; Lopez, R.; Rey, O.; Daheli, M.; Cohen, G., and Zalein, M. (1987). Molecular structure and early events in the replication of Tacaribe Arenavirus S RNA. *Virus Res.*, **7**, 309-324.

Ghiringhelli, P.D., Rivera Pomar, R.V., Baro, N.I., Grau, O. and Romanowski, V. (1987). Molecular cloning of Junín virus RNA. VII Intl. Congress of Virology (Edmonton, Alberta, Canada). Abstract book, p. 253, OP3.10.

Ghiringhelli, P.D., Rivera Pomar, R.V., Baro, N.I., Rosas, M.F., Grau, O. and Romanowski, V. (1989). Nucleocapsid protein gene of Junín arenavirus (cDNA) sequence). *Nucl. Acids Res.*, **17**, 8001.

Grau, O.; Franze-Fernandez, M.T.; Romanowski, V.; Rustici, S.M., and Rosas, M.F. (1981). Junín Virus Structure. *In* "The Replication of Negative Strand Viruses" (D.H.L. Bishop and R.W. Compans Eds.), pp. 11-14. Elsevier North Holland.

Grunstein, M., and Hogness, D.S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a spefici gene. *Proc. Natl. Acad. Sci. USA*, **72**, 3961-3965.

Gubler, U., and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263-269.

Hahn, C.S., Dalrymple, J.M., Strauss, J.H., and Rice, C.M. (1987). Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. *Proc. Natl. Acad. Sci. USA*, **84**, 2019-2023.

Iapalucci, S., Lopez, R., Rey, O., Lopez, N., Franze-Fernandez, M.T., Cohen, G.N., Lucero, M., Ochoa, A., and Zakin, M.M. (1989a). Tacaribe virus L gene encodes a protein of 2210 amino acid residues. *Virology*, **170**, 40-47.

Iapalucci, S., Lopez, N., Rey, O., Zakin, M.M., Cohen, G.N., and Franze-Fernandez, M.T. (1989b). The 5′ region of Tacaribe virus L RNA encodes a protein with a potential metal binding domain. *Virology* **173**, 357-361.

Johnson, B.J.B., Kinney, R.M., Kost, C.L. and Trent, D.W. (1986) Molecular determinants of alphavirus neurovirulence: nucleotide and deduced protein sequence changes during attenuation of Venezuelan equine encephalitis virus. *J. Gen. Virol.* **67**, 1951-1960.

Kenyon, R.H, Green, D.E., Maiztegui, J.I. and Peters, C.J. (1988) Viral strain dependent differences in experimental Argentine hemorrhagic fever (Junín virus) infection of guinea pig. *Intervirology* 29, 133-143.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start in eukaryotic mRNAs. *Nucl. Acids Res.* 12, 857-872.

Maiztegui, J.I. (1975). Clinical and epidemiological patterns of Argentine haemorrhagic fever. *Bull WHO* 52, 657-575.

Maiztegui, J.I., Fernandez, N.J., and de Damilano, A.J. (1979). Efficacy of immune plasma in treatment of Argentine haemorrhagic fever and association between treatment and a late neurological syndrome. *Lancet* 2, 1216-1217.

Maiztegui, J.I., Feuillade, M., and Briggiler, A. (1986). Progressive extension of the endemic area and changing incidence of AHF. *Med. Microbiol. Immunol.* 175, 73-78.

Maiztegui, J.I., Feinsod, F., Briggiler, A.M., Peters, C.J., Enría, D.A., Lupton, H.W., Ambrosio, A.M., Tiano, E., Feuillade, M.R., Gamboa, G., Conti, O., Vallejos, D., Mac Donald, C., and Barrera Oro, J.G. (1987). Inoculation of Argentine volunteers with a live-attenuated Junín virus vaccine. VII International Congress of Virology, Edmonton, Canada (Abstract book, p. 69, R.3.49).

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual", 1st Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. .

McKee K.T. Jr, Barrera Oro, J.G., Kuehne, A.I., Spisso, J., and Mahlandt, B.G. (1984). Immunogenicity and protective efficacy of a live, attenuated Argentine hemorrhagic fever (AHF) vaccine in primates. XI International Congress for Tropical Medicine and Malaria, Calgary, Canada. Abstract book, p. 29.

McKee K.T. Jr, Mahlandt, B.G., Maiztegui, J.I., Green, D.E., Eddy, G.A. and Peters, C.J. (1985). Experimental Argentine haemorrhagic fever in rhesus macaques: virus strain-dependent clinical response. *J. Infect. Dis.* 152, 218-221.

Nomoto, A., Kohara, M., Kuge, S. et al. (1987). Study on virulence of poliovirus type 1 using *in vitro* modified viruses. In: Brinton M.A., Ruckert, R.R., eds. Positive strand RNA viruses. New York: Alan R. Liss, 437-452.

Nomoto, A., Omata, T., Toyoda, H. et al. (1982). Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. *Proc. Natl. Acad. Sci. USA* 79, 5793-5797.

Peters, C.J., Jahrling, P.B., Liu, C.T., Kenyon, R.H., McKee, K. and Barrera Oro, J.G. (1987) Experimental studies on arenaviral hemorrhagic fevers. *Curr. Top. Microbiol. Immunol.* 134, 5-68.

Prehaud, C., Coulon, P., Lafay, F., Thiers, C., and Flamand, A. (1988) Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. *J. Virol.* 62, 1-7.

Racaniello, V.R., and Baltimore, D. (1981) Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science* **214**, 916-919.

Romanowski, V., Matsuura, Y., and Bishop, D.H.L. (1985). Complete sequence of the S RNA of lymphocytic choriomeningitis virus (WE strain) compared to that of Pichindé arenavirus. *Virus Res.* **3**, 101-114.

Romanowski, V., and Bishop, D.H.L. (1985). Conserved sequences and coding of two strains of lymphocytic choriomeningitis virus (WE and ARM) and Pichindé arenavirus. *Virus Res.*, **2**, 35-51.

Romanowski, V., Ghiringhelli, P.D., Rivera Pomar, R.V., Manzella, E.M., Lozano, M.E., Baro, N.I., Rosas, M.F., and Grau, O. (1989). Junín virus S RNA: nucleotide sequence and expression. Workshop 54, Annual Meeting of the American Society for Virology (London, Ontario, Canada).

Rosas, M.F.; Romanowski, V., and Grau, O. (1988). The phospholipid composition of arenaviruses. *Anal. Asoc. Quim. Argent.* **76**, 269-283.

Rustici, S.M. (1984). Desarrollo "*in vitro*" del virus Junín, proteinas intracelulares. Doctoral Thesis. Facultad de Ciencias Exactas. National University of La Plata.

Sabattini, M.S., Gonzalez de Rios, L.E., Diaz, G.,and Vega, V.R. (1977). Infeccion natural y experimental de roedores con virus Junín. *Medicina (Buenos Aires)* **37** (Suppl. 3), 149-161.

Salvato, M., and Shimomaye, E.M. (1989). The completed sequence of lymphocytic choriomeningitis virus reveals a unique RNA structure and a gene for a zinc finger protein. *Virology* **173**, 1-10.

Salvato, M., Shimomaye, E., and Oldstone, M.B.A. (1989). The primary structure of the lymphocytic choriomeningitis virus L gene encodes a putative RNA polymerase. *Virology* **169**, 377-384.

Sanger, F.; Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5466.

Spriggs, D.R., and Fields, B.N. (1982). Attenuated reovirus type 3 strains generated by selection of haemagglutinin antigenic variants. *Nature* **297**, 68-70.

Spriggs, D.R., Bronson, R.T., and Fields, B.N. (1983). Haemagglutinin variants of reovirus type 3 have altered central nervous system tropism. *Science* **220**, 505-507.

Tashiro, M., Yamakawa, K., Tobita, K., Klenk, H.D., Rott, R., and Seto, J.T. (1990). Organ tropism of Sendai virus in mice: proteolytic activation of the fusion glycoprotein in mouse organs and budding site at the bronchial epithelium. *J. Virol.* **64**, 3627-3634.

Taylor, W.R. (1987). Protein structure prediction. *In* "Nucleic acid and protein sequence analysis, a practical approach" (M.J. Bishop and C.J. Rawlings Eds.), pp. 285-322. IRL Press, Oxford, Washington DC.

Yanish-Perron, C., Vieira, J, and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of M13 mp18 and pUC19 vectors. *Gene*, **33**, 103-119.