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<p>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.</p> <p>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.</p> <p>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.</p>					
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FOREWORD

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EXCERPT FROM THE RESEARCH PROPOSAL (June 1988)

The characterization of Junin 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 Junin 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 should 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 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%. (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 km² to an area greater than 120.000 km² 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 Junin 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 $\mu\text{g/ml}$ 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 (SequenaseTM, 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^ACCAUGG), 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 Arg₂₅₂-Arg₂₆₃ 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 Arg₂₄₃-Arg₂₄₄ (MC2) or Arg₂₄₇-Arg₂₄₈ (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 $_{43}\text{CSILD}_{47}$ into $_{43}\text{LFQFFVF}_{49}$ 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 $_{54}\text{PR}_{55}$ to

to ₅₈TEE₆₀. The other major change results from three different single-nucleotide deletions that shift the reading frame of the coding sequence converting the MC2 sequence ₆₅VPDCVLLQWWVS₇₆ to ₇₀FQTVSFSMVGL₈₀.

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 downstream 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 ($\Delta G^\circ = -39.0$ kcal/mol, Tinoco *et al.*, 1973) and the one closest to the N stop codon is stabilized by 15 GC and 1 AU pairs ($\Delta G^\circ = -57.2$ 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 NH₂-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 Junin-MC2 has been confirmed in independent clones of Junin-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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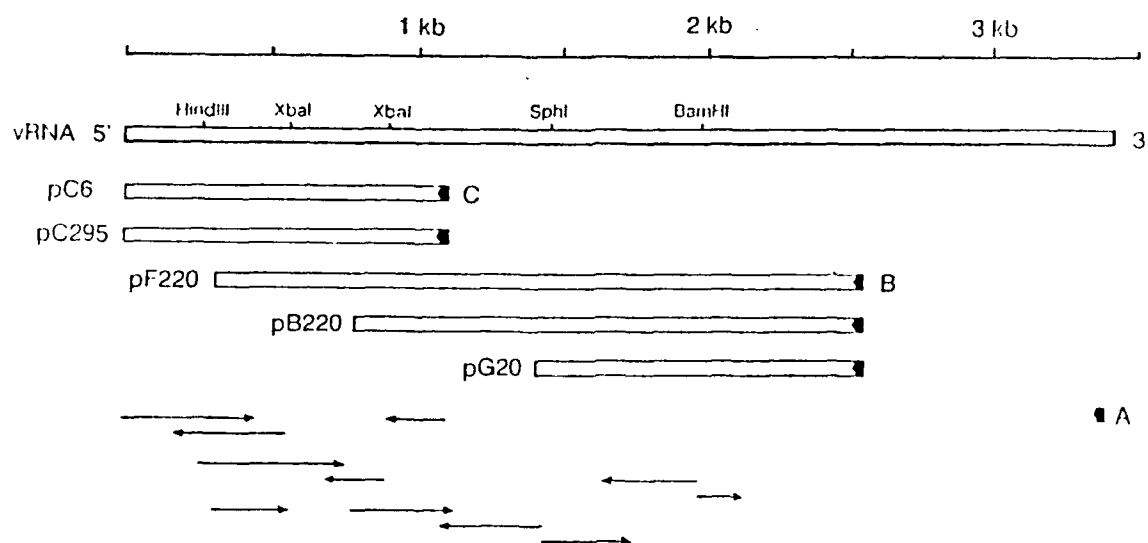


FIGURE 1: Cloning and sequencing strategy of Junin virus (Candid #1) S RNA. The S RNA nucleotide sequence is represented in the viral sense with a size scale in kilobases above. Some of the overlapping clones spanning 75% of the S RNA, are shown as rectangles. Phosphorylated synthetic oligonucleotides (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 (SequenaseTM, USB) on different M13 subclones.

CUGCAGUAAG GGGAUCCUAG GCGAUUUUGG UAACGCUGUA AGUUGUUACU GCUUUCUAAU UGGACAACAU CAAACCAUCU 80
 AUUGUACAAU GGGCAAUUC AUCAGCUUCA UGCAAGAAAU ACCUACCUUU UUGCAGGAAG CUCUGAAUUA UGCUCUUGUU 160
 GCAGUCAGUC UCAUUGCCAU CAUUAAGGGU GUAGUAAACC UGUACAAAAG UGGUUUGUUC CAUUUCUUUG UAUUCCUAGC 240
 ACUCGCAGGA AGAUCCUGCA CAGAAGAAGC UUUUAAAUC GGACUGCACA CAGAGUCCA GACUGUGUCC UUCUCAAUGG 320
 UGGGUCUCUU UUCAACAAU CCACAUGACC UGCCUCUGUU GUGUACCUUA AACAAGAGCC AUCUUUACAU UAGGGGGGC 400
 AAUGCUUCAU UCAAGAUCAU CUUUGAUGAC AUCGCAGUGU UGUUACCAGA AUAUGAGGUU AUAAUUCAGC AUCCGGCAGA 480
 UAUGAGCUGG UGUUCUAAA GUGAUGAUCA AAUUUGGUGU UCUCAGUGGU UCAUGAAUGC UGUGGGGCAU GAUUGGUUUC 560
 UAGACCCACC AUUUCUGUGU AGGAACCGUA CAAAGACAGA AGGUUUAUC UUCAAGUCA AUACCUCAA GACUGGUUUC 640
 AAUGAAACU AUGCCAAGAA GUUAAGACU GGUUAGCACC AUUUAUUAU AGAAUACCCC GACUCUUGCU UGGAUCGCAA 720
 ACUGUGUUUG AUGAAGGCAC AACCCACCAG UUGGCCUCUC CAUUGCCAC UUGACCAUGU CAACACAUUA CAUUUCCUCA 800
 CAAGAGGCAA GAACAUUCAG CUUCCAAGGA GGUCUUUAAA AGCAUUCUUU UCCUGGUCUC UGACAGACUC AUCCGGCAG 880
 GACACCCUG GAGGCUAUG UCUAGAAGAG UGGAUGCUCG UUGCAGCAA AAUGAAGUGU UUUGGCAUA CUGCUGUAGC 960
 AAAUGCAAU CUGAAUCAUG ACUCUGAAU U GUGACAUG CUGAGGCUUU UGUUUACAA CAAAAUGCU AUCAAAACCU 1040
 UAAUGAUGA AACUAAGAA CAAGUAAUC UGAUGGGACA GACAUCAAU GCGUGAUUA CUGACAAUU AUUGAUGAAA 1120
 AACAAAUUA GGAACUGAU GAGUGUCCU UACUGCAAU ACACAAAUU UUGGUUGUC AACACACAC UUCAGGACA 1200
 ACACUCAUA CCAAGGUGU GGUUAUAAA AAACAACAGC UAUUGAACA UCUCUGACUU CCGUAUAGC UGGAUUAUAG 1280
 AAAGUGACUU CUUAAUUCU GAAUUGCUA GCAAAGAGUA UUGGACAGG CAGGGUAAA CUCCUUGAC UUUAGUUGAC 1360
 AUCUGAUUU GGAGCACAGU AUUCUACACA GCGUCACUC UCCUUCACU GUGGGUUAU CCUCCACACA GACACUAGC 1440
 GGGCGAAGCA UGCCCUGUC CACACAGGU GAACAGCUU GUGUGUUGCA GAUGUGGUA GUACCCCAU CUAAAGAAC 1520
 CAACAGUUG GCGUAGAGGA CAUAAGACC UCCUGAGGGU CCCCACCAGC CGGGCACUG CCCGGGCUGG UGUGGCCCC 1600
CAGUCCGGG CCUGGCCGG GACUGGGAG GCACUGUUA CAGUGCAUAG GCUGCCUUG GGAGGAACAG CAAGCUGGU 1680
 GGUAAUAGAG GUGUAGGUUC CUCCUUAUAG AGCUUCCAU CUAGCACUGA CUGAAACAU AUGCAGUCUA GCAGAGCACA 1760
 GUGUGGUUA CUGGAGGCA ACUUGAAGG AGUAUCCUU UCCUCUUUU UCUUAUUGAC AACCAUCCA UUGUGAUUU 1840
 UGCAUAGUG ACCAUUUUC UCCAGACCU GUUGAUCAA CUGCCUGGU UGUUCAGAU UGAGCUAAC AUCAACCAGU 1920
 UUAAGAUUC UUCUCCAUG GAGGCAAC AACUCCUGA UGUCAUCGA UCCUUGAGU GUCACAACCA UGUCUGGAG 2000
 CAGCAAGCG AUACGUAAC UAAGAACUCC UGGCAUGCAU CUUCUUGUC CUUCAUUAAG AUGCCUGAG AGUGUCUGU 2080
 ACCAUUUUA AACCCUUUC CAUCAUGUG UUUUCUGAG CAGUGAAGUC UGCUU 2135

FIGURE 2: Partial 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-comple-
 mentary sequence) are boxed. Two inverted repeats at the intergenic
 region are underlined.


```

1   MGQFISFMQEIPTFLQEALNIALVAVSLIAIIKGVVNLKSGLFQFFVFLALAGRSCTEE
                                     a      b      c
                                     -CSILD-  --    PR-

61   AFKIGLHTEFQTVSFSMVGLFSNNPHDLPLLCTLNKSHLYIKGGNASFKISFDDIAVLLP
      d
      VPDCVLLQWWVS

121  EYDVIIQHPADMSWCSKSDDQIWLSQWFMNAVGHWDWYLDPPFLCRNRTKTEGFIFQVNTS
      e
      R

181  KTGINENYAKKFKTGMHHLRYREYPDSCLDGKLCMKAPTSWPLQCPLDHVNTLHFLTRG

241  KNIQLPRRSLKAFFSWSLTDSSGKDTGGCYCLEEWMLVAAKMKCFGNTAVAKCNLNHDSE
      • •

301  FCDMLRLFDYNKNAIKTLNDETKKQVNLMGQTINALISDNLLMKNKIRELMSVPYCNYTK

361  FWYV[NHT]LSGQHSLPRCWLK[NNSYLNIS]DFRNDWILESDFLISEMLSKEYSDRQGKTPL

421  TLVDICIWSTVFFTASLFLHLVGIPSHRHIRGEACPLPHRLNSLGGCRCGKYPNLKKPTV
      f      g
      F      T

481  WRRGH

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FIGURE 3: Amino acid sequence of Junin virus Candid #1 GPC gene products. The regions where 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 (NH₂-terminal half) and G2 (COOH-terminal half of GPC).

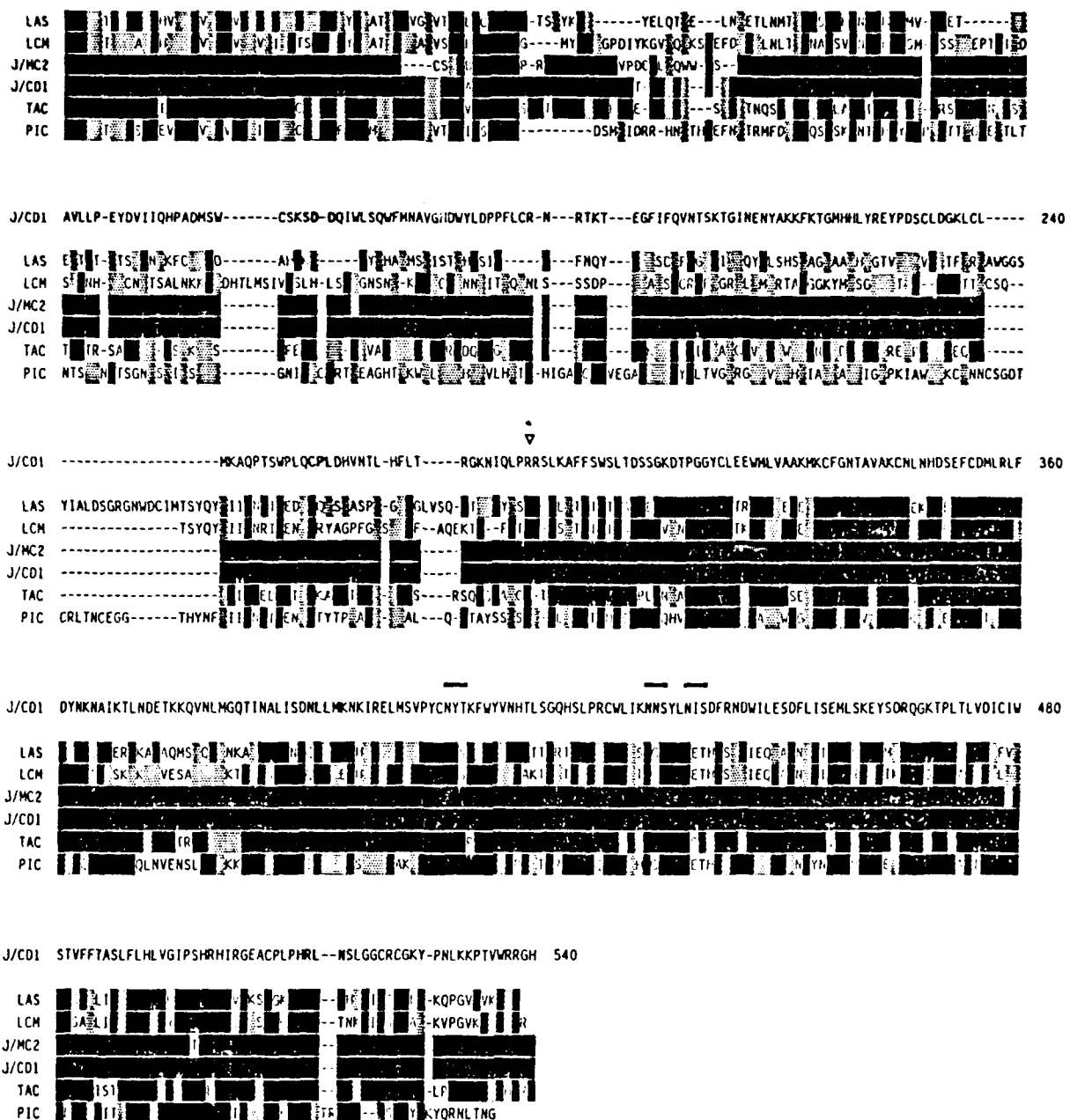


FIGURE 4: Comparison of arenavirus GPC proteins. The predicted amino acid sequences of the GPC proteins of Lassa (LAS, Auperin *et al.*, 1986), LCM-WE (LCM, Romanowski, *et al.*, 1985), Tacaribe (TAC, Franze-Fernández *et al.*, 1987), Pichindé (PIC, Auperin *et al.*, 1984c) and Junin-MC2 (J/MC2, Ghiringhelli *et al.*, submitted) are compared to that of Junin-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 (NX₃) 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).

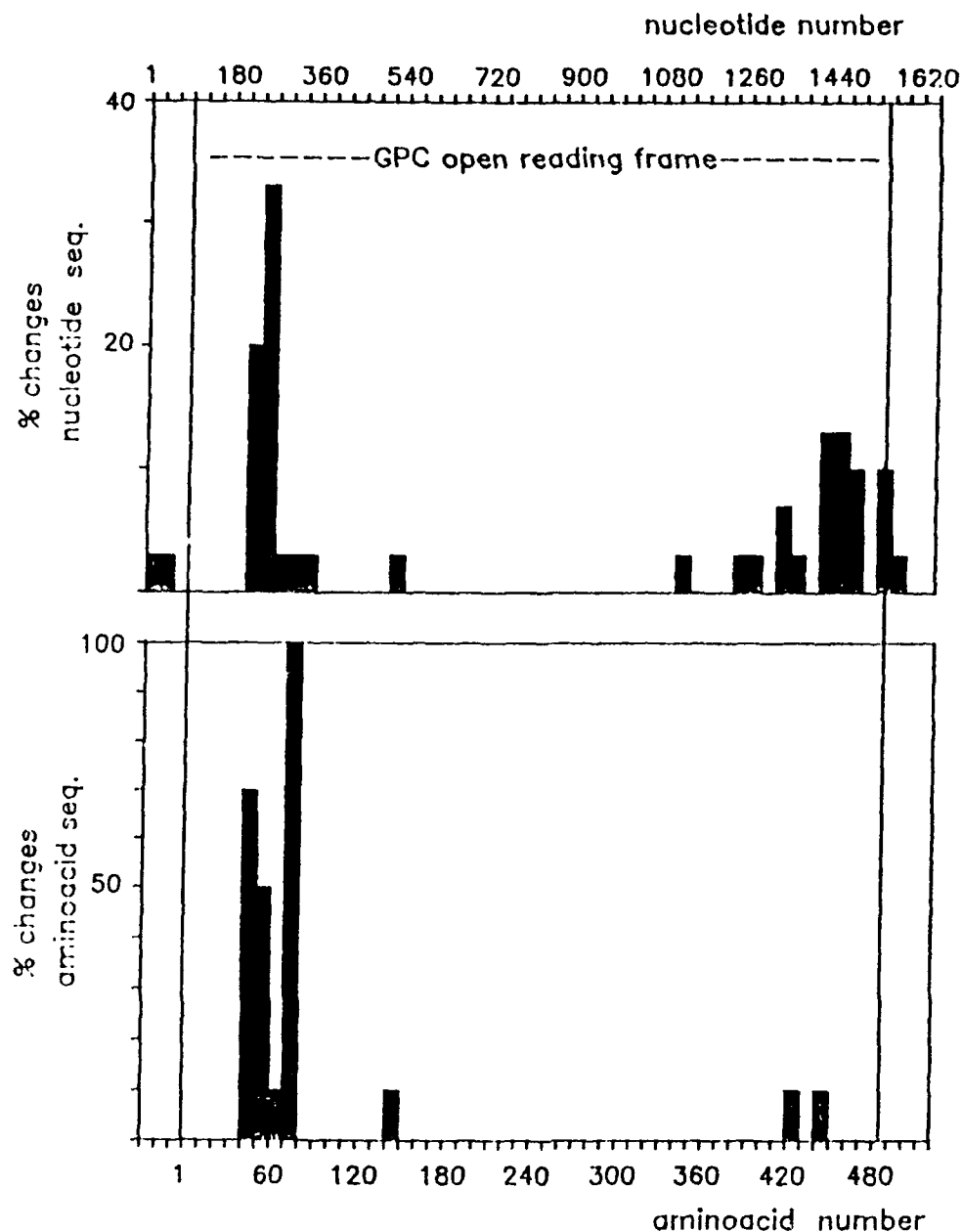


FIGURE 6: Distribution of the amino acid and nucleotide changes in the GPC protein, the corresponding open reading frame and the 5' and 3' untranslated regions. The nucleotide and amino acid sequences of Candid #1 and MC2 strains, aligned as in Fig. 5 and 3, were subdivided in fractions of 30 nucleotides 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.

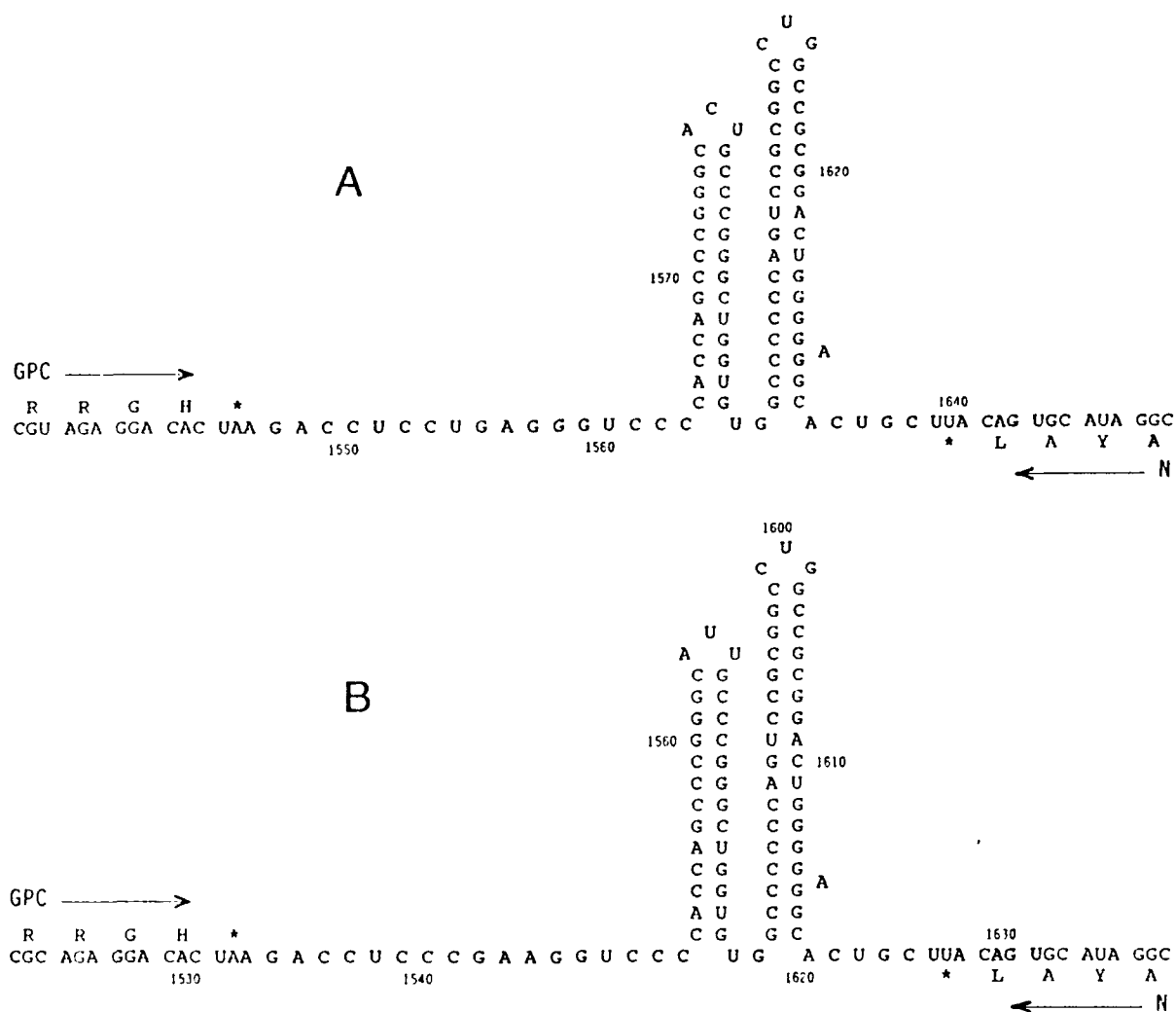


FIGURE 7: Intergenic region of Junin virus S RNA. A portion of the S RNA of Junin-Candid #1 (A) virus comprising the ends of the GPC and N genes (sense and anti-sense strand, respectively) and the non-coding intergenic region is shown in the figure. The directions of translation are indicated by arrows 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 ($\Delta G^\circ = -39.0$ 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 C₁₅₄₁, A₁₅₄₄ and T₁₅₆₅ of MC2 have been changed to T₁₅₅₄, G₁₅₅₇ and C₁₅₇₈ in Candid #1.

TABLE 1

SEQUENCE IDENTITY (%) OF ARENAVIRUS GPC PROTEIN

	J/MC2	TAC	PIC	LCM	LAS	J/MC2	TAC	PIC	LCM	LAS
J/CD1	90	53	21	20	21	99	80	57	53	58
J/MC2		50	20	21	21		82	57	53	57
TAC			24	16	17			51	51	52
PIC				20	26				52	54
LCM					27					69

G1

G2

TABLE 2

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

	J/MC2	TAC	PIC	LCM	LAS	J/MC2	TAC	PIC	LCM	LAS
J/CD1	92	74	43	42	42	99	89	71	71	69
J/MC2		69	43	42	44		92	72	70	68
TAC			41	38	36			70	69	67
PIC				41	47				68	69
LCM					44					83

G1
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

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

* 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.

30

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