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SHORT COMMUNICATION

Molecular characterization of attenuated Junin virus strains

C. G. Albariño,[1,2] P. D. Ghiringhelli,[1,2] D. M. Posik,[1,2] M. E. Lozano,[1,2] A. M. Ambrosio,[3] A. Sanchez[4] and V. Romanowski[1,2]

[1] Instituto de Bioquímica y Biología Molecular, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calles 47 y 115 (1900), La Plata, Argentina

[2] Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, R.S. Peña 180 (1876), Bernal, Argentina

[3] Instituto de Estudios sobre Enfermedades Virales Humanas, Monteagudo 2510 (2700), Pergamino, Argentina

[4] Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA

The Junin virus strain Candid #1 was developed as a live attenuated vaccine for Argentine haemorrhagic fever. In this paper we report the nucleotide sequences of S RNA of Candid #1 and its more virulent ancestors XJ#44 and XJ (prototype). Their relationship to Junin virus wild-type MC2 strain and other closely and distantly related arenaviruses was also examined. Comparisons of the nucleotide and amino acid sequences of N and GPC genes from Candid #1 and its progenitor strains revealed some changes that are unique to the vaccine strain. These changes could be provisionally associated with the attenuated phenotype.

Junin virus, a member of the family *Arenaviridae*, is the etiological agent of Argentine haemorrhagic fever (AHF). The clinical symptoms of AHF include haematological, neurological, cardiovascular, renal and immunological alterations. The mortality rate for AHF may be as high as 30%, but early treatment with immune plasma reduces fatal cases to less than 1%. The population of humans at risk is composed mainly of field workers, who are believed to become infected through cuts or skin abrasions or via airborne dust contaminated with urine, saliva or blood from infected rodents (Maiztegui *et al*., 1986).

All arenaviruses share morphological and biochemical properties. They are enveloped and their genomes consist of two single-stranded RNA species, designated L (ca. 7 kb) and S (ca. 3·5 kb). The open reading frames of both RNA species are arranged in an ambisense manner and are separated by a non-coding intergenic region that folds into a stable secondary structure (Auperin *et al*., 1984; Salvato & Shimomaye, 1989). 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 *et al*., 1989; Franze-Fernandez *et al*., 1993). The complete nucleotide sequences of the S RNA from several arenaviruses have been determined (Clegg, 1993; Bowen *et al*., 1996*a*), and several partial sequences are also available (Griffiths *et al*., 1992; Bowen *et al*., 1996*b*). The S RNA species codes for the nucleocapsid protein, N, and the precursor of the envelope glycoproteins, GPC. The N protein (ca. 63 kDa) is translated from a viral-complementary or anti-genome-sense mRNA, complementary to the 3′ half of the viral S RNA. 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. Proteolytic cleavage of GPC in infected cells produces G1 and G2 polypeptides.

A collaborative effort conducted by the 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 (Maiztegui *et al*., 1990). Molecular characterization of the vaccine strain, Candid #1, and of its more virulent ancestors, XJ (prototype) and XJ#44, permits a systematic approach to determine the basis of Junin virus virulence. Here we describe sequence information of the coding regions in S RNAs obtained from strains Candid #1, XJ#44 and XJ, and comparisons with the wild-type MC2 strain of intermediate virulence and with other closely and distantly related arenaviruses.

The passage history of Junin Candid #1 is depicted in Fig. 1. The original virus isolate that gave rise to the Candid #1 strain was the XJ strain, isolated in Junín City (Buenos Aires, Argentina) from a human AHF patient (Parodi *et al*., 1958).

Author for correspondence: Víctor Romanowski (at Universidad Nacional de La Plata).
Fax +54 21 25 9223. e-mail victor@nahuel.biol.unlp.edu.ar

The GenBank accession numbers of the nucleotide sequences of the N and GPC genes of the Junin virus strains Candid and XJ reported in this paper are U70799, U70800, U70801, U70802, U70803 and U70804.

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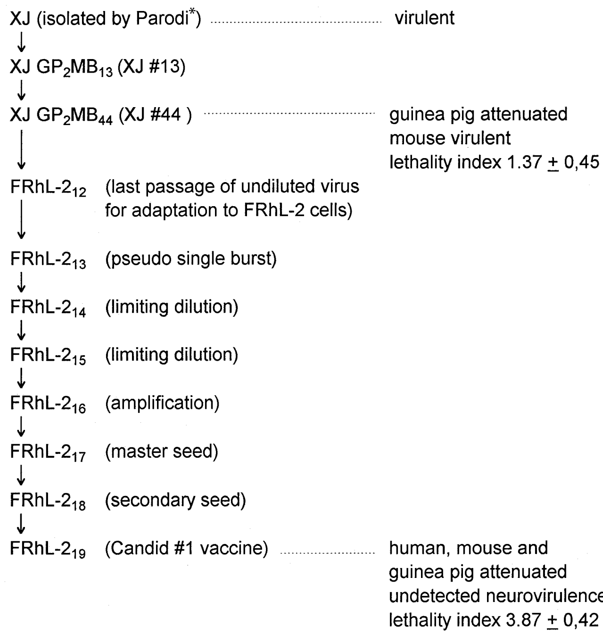

Fig. 1. Passage history of Junin strain Candid #1. The XJ strain was subjected to two passages in guinea-pigs (GP_2) and 43 passages in mouse brain (MB_{43}). Passage number 43 was amplified by one round of mouse brain injection (XJ#44). This brain homogenate was used to infect FRhL-2 cells. After 12 passages, one pseudo single burst growth was carried out, followed by cloning using two limiting dilution steps. After one amplification round, master and secondary seeds were obtained. Finally, the vaccine stock (Candid #1) was obtained by a single amplification of the secondary seed. The lethality index was calculated as $\log_{10}$ p.f.u. that produce one LD_{50} ($\pm$1 SD) by intracerebral inoculation of mice. *, Parodi *et al.*,1958.

Records of the passage history of the XJ strain come from the Yale Arbovirus Research Unit, Connecticut, USA (J. Casals) and USAMRIID, Frederick, Maryland, USA (J. G. Barrera Oro).

A 'working stock' of Junin Candid #1 virus was produced by infection of certified foetal rhesus lung diploid (FRhL-2) cell monolayers with the master seed. The attenuated Junin virus XJ#44 was provided by J. G. Barrera Oro (USAMRIID) and was amplified in our laboratory in BHK21 cells. The more virulent XJ strain (prototype) was provided by C. J. Peters (CDC, Atlanta, Georgia, USA) as a lysate of infected BHK21 cells. Virions were recovered and purified from the supernatant media; viral and total infected cell RNAs were isolated according to procedures described previously (Ghiringhelli *et al.*, 1996).

During molecular cloning of Junin virus S RNAs (Candid #1, XJ#44 and XJ strains), special attention was devoted to avoiding spurious genetic variations that could possibly obscure changes relevant to the attenuation of virulence. Selected regions of the S RNA were amplified by RT–PCR; cDNA synthesis was carried out as reported previously (Ghiringhelli *et al.*, 1991) and the target sequences were amplified using *Pfu* DNA polymerase (Stratagene). Amplified cDNAs were analysed on agarose gels, purified using sodium iodide and glass powder elution (GeneClean, Bio 101) and ligated into linearized pUC19 plasmid DNA.

At least two independent cDNA clones of each region were sequenced by the chain termination method. Additionally, direct sequencing of PCR products with the *fmol* DNA sequencing system (Promega) was used to analyse the 5′ and 3′ non-coding regions and to confirm the sequences of different cDNA clones.

Nucleotide sequences of the following arenavirus S RNAs were obtained from the GenBank database: Junin MC2 strain (MC2), accession number D10072; LCM WE strain (LCM-WE), M22138; LCM Armstrong strain (LCM-Arm), M20869; Lassa Nigeria strain (LAS-Nig), X52400; Lassa Josiah strain (LAS-Jos), J04324; Machupo (MAC), X62616; Mopeia (MOP), M33879; Oliveros virus (OLV), U34248; Pichindé (PIC), K02734; and Tacaribe (TAC), M20304 and M65834.

Sequence information was processed and analysed on a MicroVAX 3100 (Digital) computer using a package from Genetics Computer Group (GCG, Sequence Analysis Software Package, version 7.1, University of Wisconsin, Madison, USA). Sequence alignments were done using the PILEUP program (GCG package) and further processed using a graphics program developed by one of the authors (P. D. Ghiringhelli, unpublished). Secondary structure predictions were done using the Garnier–Osguthorpe–Robson algorithm.

From alignment of the coding sequences of the GPC genes of Junin virus strains it was observed that the closely related strains (Candid #1, XJ#44 and XJ) share a common pattern of insertions and deletions in the amino-terminal region of G1 (amino acid residues 43 to 82, Fig. 2). These changes, compared to the previously published sequence of S RNA from strain MC2, were tentatively correlated with the attenuated phenotype (Ghiringhelli *et al.*, 1996). However, the evidence presented here indicates that this is not the case.

In addition to the aforementioned changes in the GPC sequences, there are 28 silent substitutions and four substitutions that result in four amino acid changes in different strains. One of these changes ($W_{148} \rightarrow R$) is shared by all related strains (i.e. Candid #1, XJ#44 and XJ), two of them are particular to Candid #1 ($I_{473} \rightarrow F$ and $S_{492} \rightarrow T$) and one to XJ#44 ($L_{267} \rightarrow V$). Three of these changes (Fig. 2) are considered semiconservative according to the matrix of Schwartz and Dayhoff (GCG package).

However, Candid #1, the most attenuated strain, has an additional putative attenuation marker. Although most of the nucleotide substitutions do not alter the amino acid sequence, two amino acid changes (at I_{473} and S_{492}) in the hydrophobic region of G2 in Candid #1 result in the loss of four β turns in the predicted secondary structure (data not shown).

Although our results, suggesting the involvement of the surface glycoprotein in attenuation of virulence, are preliminary, they are consistent with reports on other viruses (discussed by Tatem *et al.*, 1992). In some cases, it has been reported that tissue tropism and virulence are dependent upon

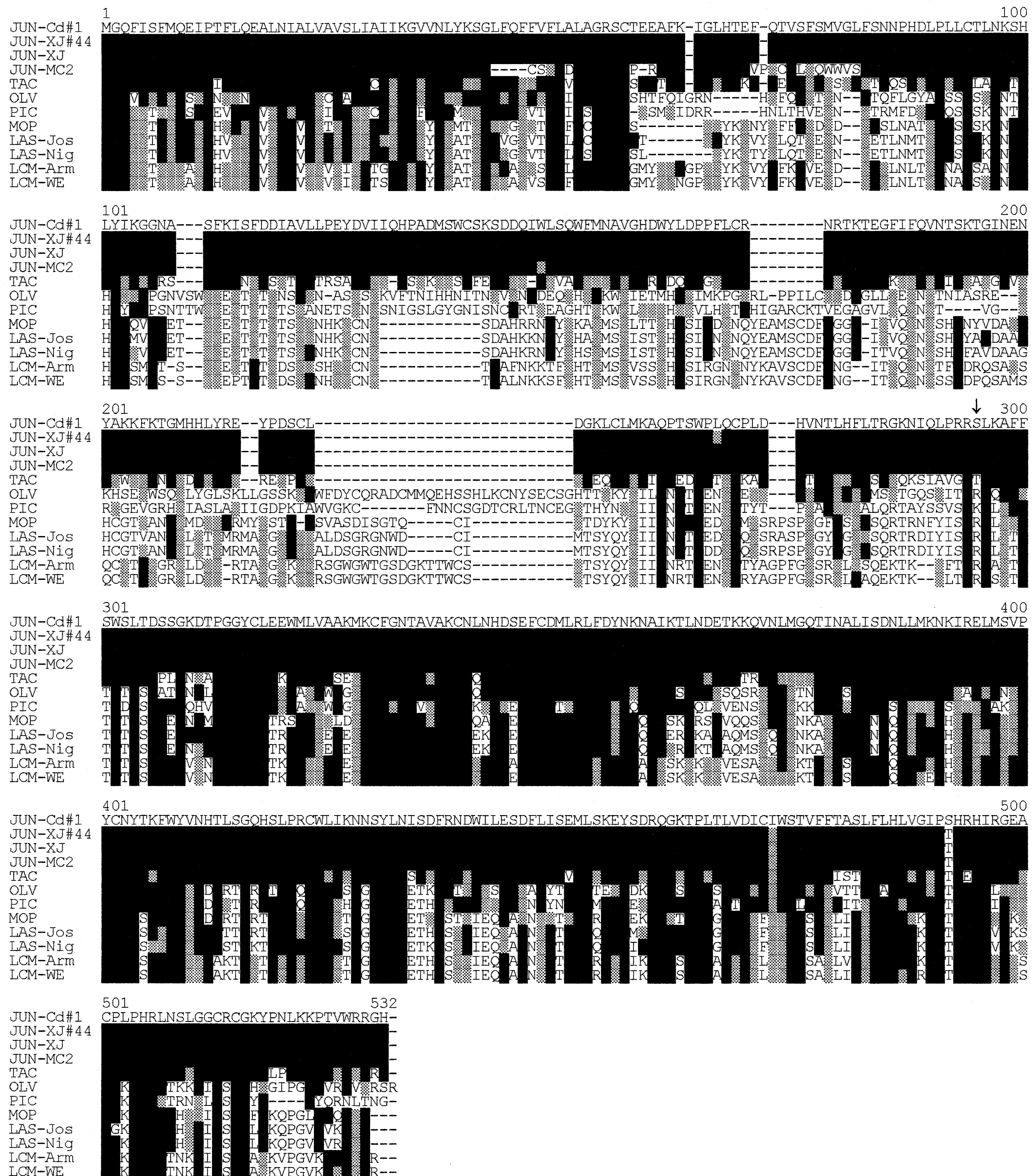

Fig. 2. Comparison of the amino acid sequence of arenavirus GPC proteins. The predicted amino acid sequences of GPC proteins of Junin virus strains (JUN-Cd#1, JUN-XJ#44, JUN-XJ and JUN-MC2) and arenaviruses TAC, PIC, OLV, LCM-WE, LCM-Arm, LAS-Nig, LAS-Jos and MOP are compared using JUN-Cd#1 GPC as reference. The amino acid residues identical to those of the reference sequence are indicated by black boxes, those that represent conservative changes appear as shaded areas and gaps in the alignment are indicated by hyphens. The approximate position of the proteolytic cleavage site is indicated by an arrow (↓). It can be noticed that G1 (amino-terminal half) and G2 (carboxy-terminal half) exhibit quite different degrees of sequence similarity. Whereas G1 sequences show few clusters of sequence conservation, G2 sequences show a high degree of identity among all the different arenaviruses.

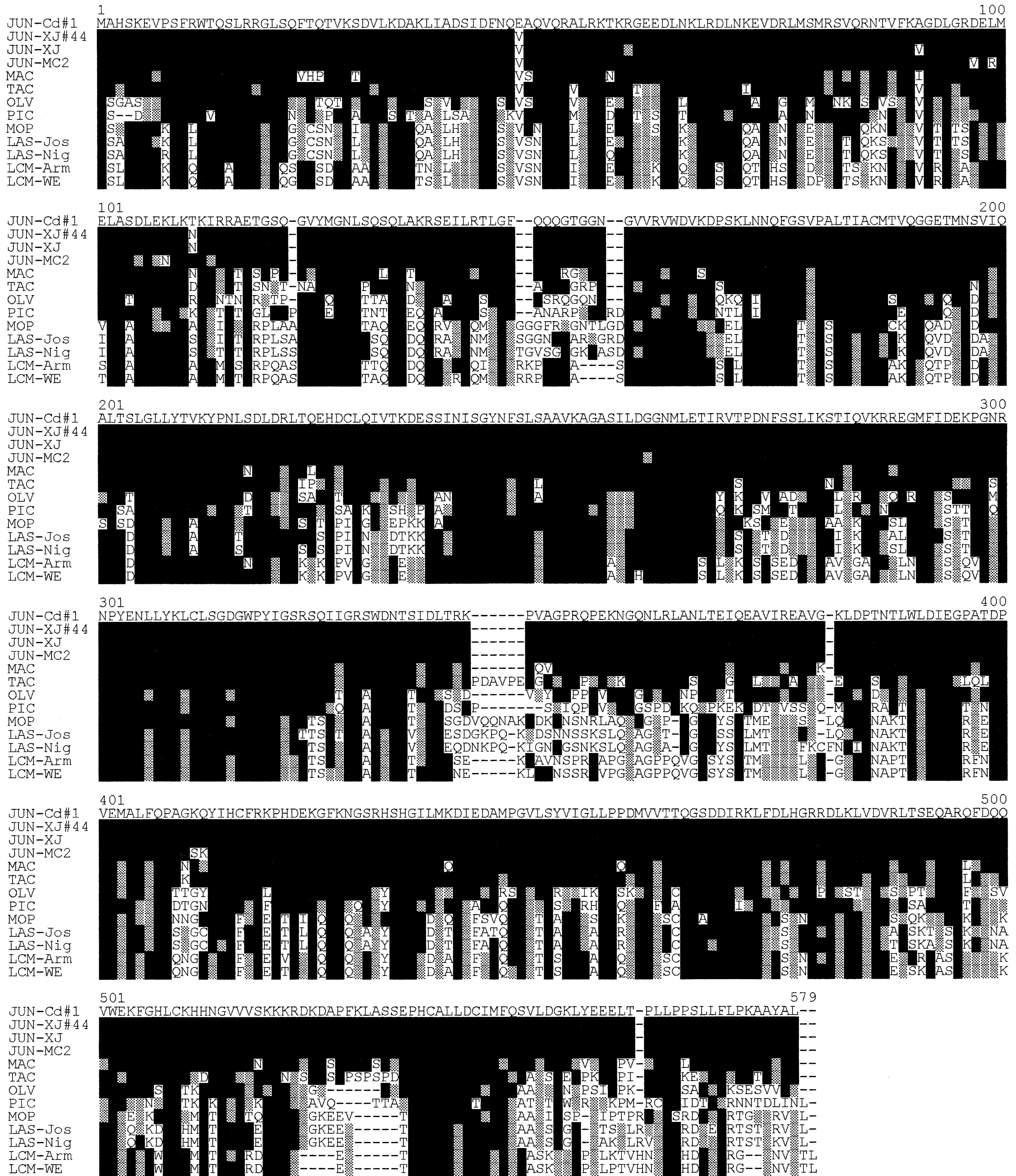

Fig. 3. Comparison of the amino acid sequence of arenavirus N proteins. The predicted amino acid sequences of N proteins of Junin virus strains (JUN-Cd#1, JUN-XJ#44, JUN-XJ, JUN-MC2) are compared with those of different arenaviruses (MAC, TAC, PIC, OLV, LCM-WE, LCM-Arm, LAS-Nig, LAS-Jos, MOP). The amino acid sequence of JUN-Cd#1 N was used as reference. Identities and similarities of amino acid residues are indicated as in Fig. 2. Several regions of well-conserved amino acid sequences can be seen, reflecting the extensive immunological cross-reactivity of the N proteins.

the glycosylation pattern (Deshpande *et al.*, 1987) or the proteolytic activation of a surface glycoprotein (Davis *et al.*, 1995), but a strict conservation of all the potential *N* glycosylation sites and sequences flanking the cleavage site of GPCs have been observed in all Junin virus strains included in this study.

Changes in the amino acid sequence of the N protein are less striking than those observed in G1 and G2. No nucleotide deletions or insertions were detected in the alignment of the N-coding sequences of the four Junin virus strains (Fig. 3). There are 15 silent substitutions and 15 additional substitutions that result in 13 specific amino acid changes. Nine of these changes are shared by the three related strains, two are particular to XJ ($R_{59} \rightarrow K$ and $A_{91} \rightarrow V$), one is shared by XJ#44 and XJ ($T_{111} \rightarrow N$) and one is specific to Candid #1 ($E_{47} \rightarrow V$). Five of these amino acid changes are considered semiconservative according to the matrix of Schwartz and Dayhoff (Fig. 3). The last one, in the amino-terminal region of Candid #1, results in the loss of two β turns in the predicted secondary structure (not shown) for XJ#44, XJ and MC2 strains.

The overall positive charge of the N protein (i.e. $+12$ for the N protein of MC2) is a reflection of the relative abundance of the basic amino acids K and R, which are found scattered throughout the primary structure. On the other hand, net charges at pH 7 change from $+10$ in XJ and XJ#44 to $+9$ in Candid #1. Our laboratory is conducting a study to evaluate the significance of these differences at the protein–RNA interaction level. At this point it might be recalled that N has been proposed to be the transcription anti-terminator and, therefore changes in its interaction with RNA might affect regulation of the transcription/replication process (Romanowski, 1993).

It has previously been suggested that changes in the intergenic region could play a role in the attenuation processes of arenaviruses (Wilson & Clegg, 1991). However, our sequence analysis of the intergenic regions of XJ and Candid #1 revealed 100% conservation. The fact that nucleotide changes are not tolerated in this region suggests that a major constraint is operating, perhaps related to the proposed function of its secondary structure in transcription termination (Franze-Fernandez *et al.*, 1993).

On the other hand, a high degree of sequence variability has been observed in the 5′ non-coding region in independent clones of Candid #1, XJ#44 and MC2 strains. However, the 3′ non-coding region exhibits few differences in clones of each strain and varies only slightly from one strain to another. This heterogeneity could have arisen from different post-transcriptional editing of the subgenomic RNAs, reported previously for arenaviruses (Garcin & Kolakofsky, 1992). However, the involvement of these regions in the attenuation process remains to be evaluated.

In summary, the present work should be regarded as a first step in the identification of regions in the Junin virus genome that are related to a particular virulence pattern. This analysis has been restricted to S RNA, and hence we cannot rule out the role of possible changes in L RNA or its gene products (Riviere *et al.*, 1985; Endres *et al.*, 1991), nor a more complex scheme which may include multiple mutations in S RNA and/or L RNA operating simultaneously in the attenuation processes, as has been reported in different virus systems (Snyder *et al.*, 1988). The information accumulated by sequence analysis of viral genomes with different degrees of virulence will certainly serve as a starting point to study this biological phenomenon, provided that a reverse genetic system for arenaviruses is developed.

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