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ORIGINAL ARTICLE

Genomic features of attenuated Junín virus vaccine strain candidate

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Abstract 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 L RNA of Candid #1 and examine the relationship to its more virulent ancestors Junin virus XJ#44 and XJ 13 (prototype) and other closely and distantly related arenaviruses. Comparisons of the nucleotide and amino acid sequences of L and Z genes of Candid #1 and its progenitor strains revealed twelve point mutations in the L polypeptide that are unique to the vaccine strain. These changes could be provisionally associated with the attenuated phenotype. In contrast, Z ORF was completely conserved among all strains.

Keywords Arenaviridae · Junin · Attenuation · Molecular markers · L RNA · Genomic

The nucleotide sequences data of the of the L RNA of the Junin virus strains reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers: AY819707.

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Introduction

Junín virus, a member of the family *Arenaviridae* [1, 2, virus code: 00.003.0.01.010.], 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 human population 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 [3].

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 in a stable secondary structure [4]. The L RNA codes for two polypeptides: the RNA polymerase (L) and a small zinc, finger-like protein (Z). The complete nucleotide sequences of the S RNA of several arenaviruses have been determined, and several partial sequences are also available [4]. The S RNA species codes for the nucleocapsid protein, N, and the precursor of the envelope glycoproteins, GPC. N and L are translated from anti-genome-sense mRNAs, complementary to the 3′ portion of the viral S or L RNA, respectively. The GPC and Z proteins are translated from viral or genome-sense mRNAs corresponding to the 5′ region of the viral S or L RNA, respectively. Proteolytic cleavage of GPC in infected cells produces a signal peptide and the GI and G2 polypeptides.

A collaborative effort conducted by the US and Argentine Governments led to the production of a live

attenuated Junín virus vaccine, named Candid #1 (Fig. 1). After rigorous biological testing in rhesus monkeys, the vaccine was used in human volunteers, followed by an extensive clinical trial in the AHF endemic area [5]. Molecular characterization of the vaccine strain, Candid #1, and of its more virulent ancestors, XJ 13 (prototype) and XJ#44, permits a systematic approach aimed at studying the basis of Junín virus virulence. Here, we describe sequence information of the L RNAs determined for Junín virus strain Candid #1, compared to XJ#44 and XJ 13 wild-type strains and other closely and distantly related arenaviruses.

Materials and Methods

The passage history of Junín 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 [6]. 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 Junín Candid #1 virus was produced by infection of certified fetal rhesus lung diploid (FRhL−2) cell monolayers with the master seed. The attenuated Junín virus XJ#44 was provided by J. G. Barrera Oro (USAMRIID) and was amplified in our laboratory in BHK21 cells. The more virulent XJ 13 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 [7].

During molecular cloning of Junín virus cDNA (Candid #1, XJ#44 and XJ 13 strains), special attention was devoted to avoid spurious genetic variations that could possibly obscure changes relevant to the attenuation of virulence. Selected regions of the L RNA were amplified by RT-PCR; cDNA synthesis was carried out as reported previously [8] and the target sequences were amplified using high fidelity thermostable DNA polymerases.

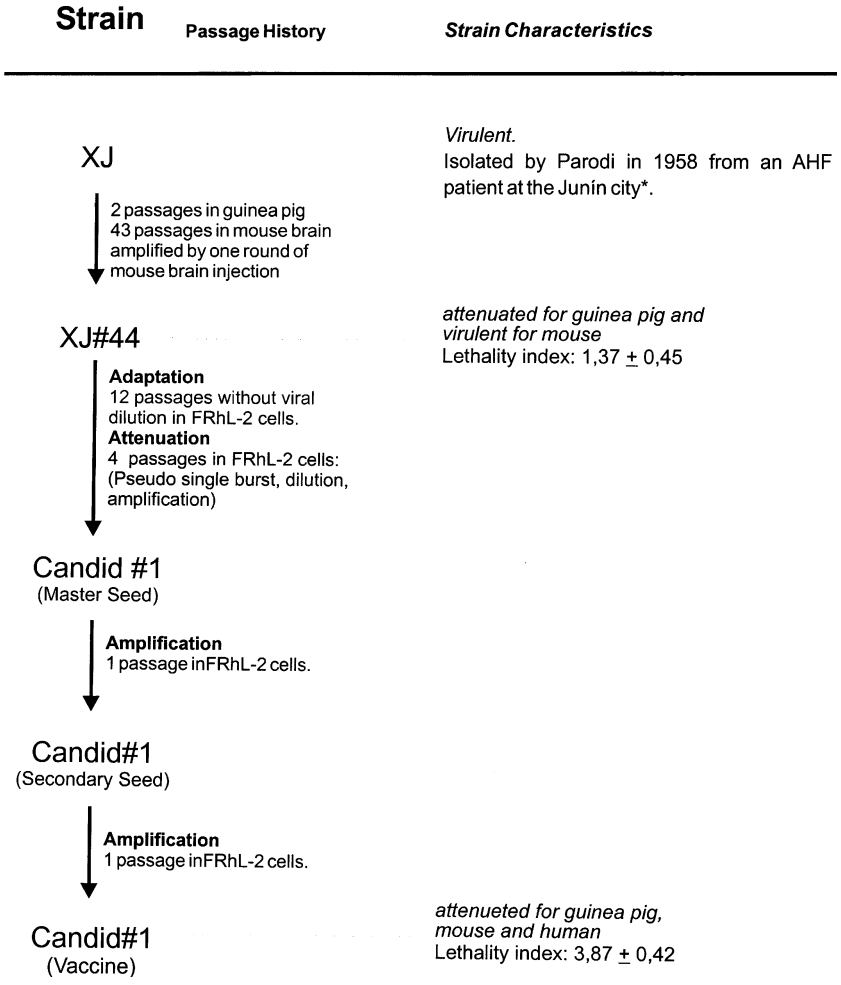

Fig. 1 Passage history of Junín virus, strain Candid #1. The genealogical relationships of the studied Junín virus strains are shown by arrows. The vaccine stock (Candid #1) was obtained by a single amplification of the secondary seed. The lethality index was calculated as $\log_{10}$ p.f.u. that produce one LD_{50} (1 SD) by intracerebral inoculation of mice. (*[6])

Amplified cDNAs were analysed on agarose gels, purified using sodium iodide and glass powder elution (Gene Clean, BIO 101) and ligated into linearized pZErO^{TM}–2 (Invitrogen) plasmid DNA.

At least four independent cDNA clones of each region were sequenced by the chain termination method. Additionally, direct sequencing of PCR products was used to analyse the 5′ and 3′ non-coding regions and to confirm the sequences of cDNA clones. Nucleotide sequences of the following arenavirus L RNAs were obtained from the GenBank database (accession numbers are indicated in brackets): Junín XJ 13 (AY358022); Junín Rumero (AY619640); Guanarito (NP_899221.1, NP_899220.1); Lassa AV, (AAO59509.1, AAO59508.1); Lassa CSF (AAO59515.1, AAO59514.1); Lassa Josiah (NP_694872.1, NP_694871.1); Lassa Macenta (AAT48998.1, AAT 48997.1); Lassa NL (AAO59511.1, AAO59510.1); Lassa Z148 (AAT49006.1, AAT49005.1); LCM Armstrong (NP_694845.1, NP_694846.1); Machupo Carvallo (AAT40450.1, AAT40449.1); Machupo Chicava (AAT 45080.1, AAT45079.1); Machupo Mallele (AAT40454.1, AAT40453.1); Mopeia AN20410 (AAV54107.1, AAV 54106.1); Pichindé (YP_138534.1, YP_138535.1); Pirital (YP_025093.1, YP_025092.1); Sabiá (AAQ55263.1, AAQ55262.1); Tacaribe (NP_694848.1, NP_694847.1). Sequence alignments were done using the CLUSTAL-X program and further processed using a graphics program developed by one of the authors (P. D. Ghiringhelli, unpublished). The neuronal network Jnet [9] (http://www.compbio.dundee.ac.uk/~www-jpred/) was used for secondary structure predictions. Phylogenetic analyses were done using the Phylip package.

Results and Discussion

Alignment of the coding sequences of the L genes of Junín virus strains showed only twelve nucleotide changes between XJ 13 and Candid #1 strains. Six of these changes resulted in amino acid substitutions ($H_{76} \rightarrow Y$; $V_{415} \rightarrow A$; $D_{462} \rightarrow N$; $L_{936} \rightarrow P$; $R_{1156} \rightarrow K$ and $I_{1883} \rightarrow V$; indicated as XJ residue $\rightarrow$ Candid #1 residue). Another virulent strain of Junín virus (Rumero) isolated from a patient has the same nucleotide sequence as the virulent XJ 13 strain at the twelve positions of the nucleotide sequence mentioned above. In view of this, the six amino acid changes might be tentatively correlated with the attenuated phenotype. In contrast, no changes were found in the amino acid sequence of the Z protein, as well as at the nucleotide level, in the four Junín virus strains.

Candid #1, the most attenuated strain, has a set of putative attenuation markers in its RNA polymerase. Even though four of the amino acid changes are semiconservative, according to the Schwartz and Dayhoff matrix, most of them show some incidence on the predicted secondary structure (Fig. 2a). Vieth et al. [10] describe four conserved regions among all arenavirus L proteins. The putative RNA polymerase domains are found in region III,

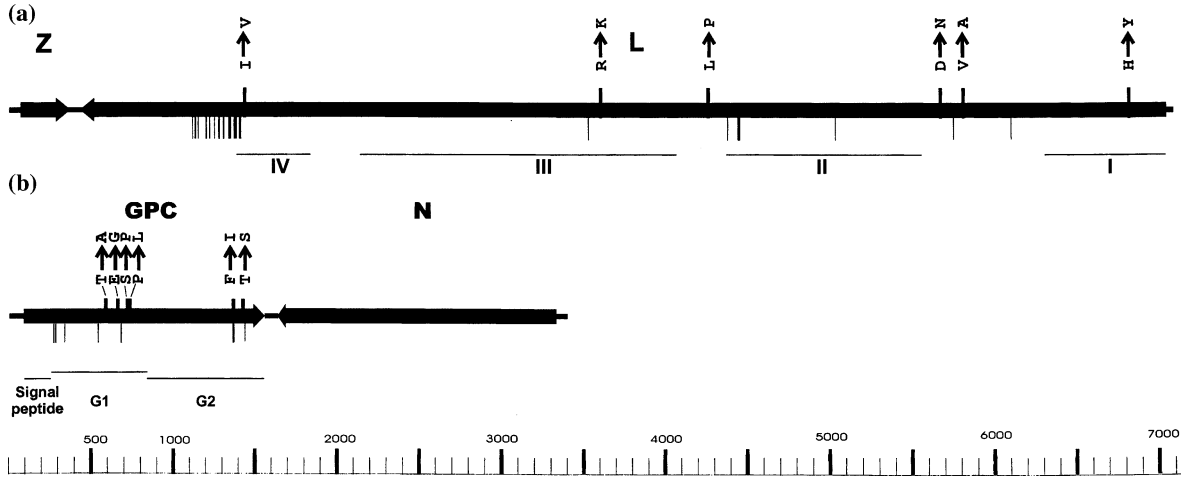

Fig. 2 Schematic of the changes detected in Junín virus proteins (aminoacid sequence and predicted secondary structure) between XJ 13 and Candid #1 strains. (a) L RNA. The open reading frames corresponding to the Z and L genes are shown as closed rectangles with arrowheads indicating the direction of translation. Non-coding sequences are shown as horizontal thick lines, Vertical thick lines over the genes indicate the positions of the changes in the aminoacid sequence (found only in the L protein). Vertical thin lines below the genes indicate the position of changes in the predicted secondary structure of the RNA polymerase. The four conserved regions of the RNA polymerases of arenaviruses as described by Vieth et al. [10] are shown by horizontal lines below the diagram of the L RNA. (b) S RNA. The open reading frames corresponding to the N and GPC genes and the position of the changes in the aminoacid sequence and the predicted secondary structure are shown as in (a). The three cleavage products of GPC protein are shown by horizontal lines below the diagram. (a) Nucleotide rule is depicted below the figure to facilitate the location of each position

where we detect only one conservative change (R → K) associated with a weak change in the secondary structure. Most striking changes in the secondary structure were found in the region IV and at the C terminal region of Junín virus L protein. Although our results, suggesting the involvement of the RNA polymerase in attenuation of virulence, are preliminary, they are consistent with reports on other viruses [11, 12]. In agreement with others authors [10)] we found a lower level of nucleotide sequence conservation in the L RNA than in the S RNA, indicating a faster rate of evolution of the L polypeptides.

In a previous work our group reported a set of few changes between S RNAs from the same Junín virus strains included in this study [13]. However, when we compared the re-sequenced Candid #1 S RNA sequence with the recently sequenced XJ 13 strain, we found most differences that could be associated with the attenuated phenotype. As depicted in Fig. 2b, four of these changes are at the carboxyl terminus of G1 and two more are at the carboxyl terminus of G2. No amino acid changes were detected in the alignment of the N polypeptide of the three genealogically related Junín virus strains. At the level of the predicted secondary structure, changes in the amino acid sequence of the S RNA encoded proteins are less striking than those observed in L protein.

The intergenic region has important roles in different negative and ambisense RNA viruses [14, 15]. It has been suggested that changes in the intergenic region could play a role in the attenuation processes of arenaviruses [16]. However, our sequence analysis of the intergenic regions of XJ 13 and Candid #1 revealed 100% conservation in both genomic RNAs. The absence of nucleotide changes could indicate that they are not tolerated in this region without compensatory substitutions to preserve a secondary structure relevant in the regulation of the transcription/replication processes [15, 16]. On the other hand, a high degree of sequence variability has been observed in the 5′ non-coding region in independent PCR fragments of Candid #1 and XJ 13 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 editing of the viral L RNA-derived molecular species, consistent with previous reports [17, 18]. However, the involvement of

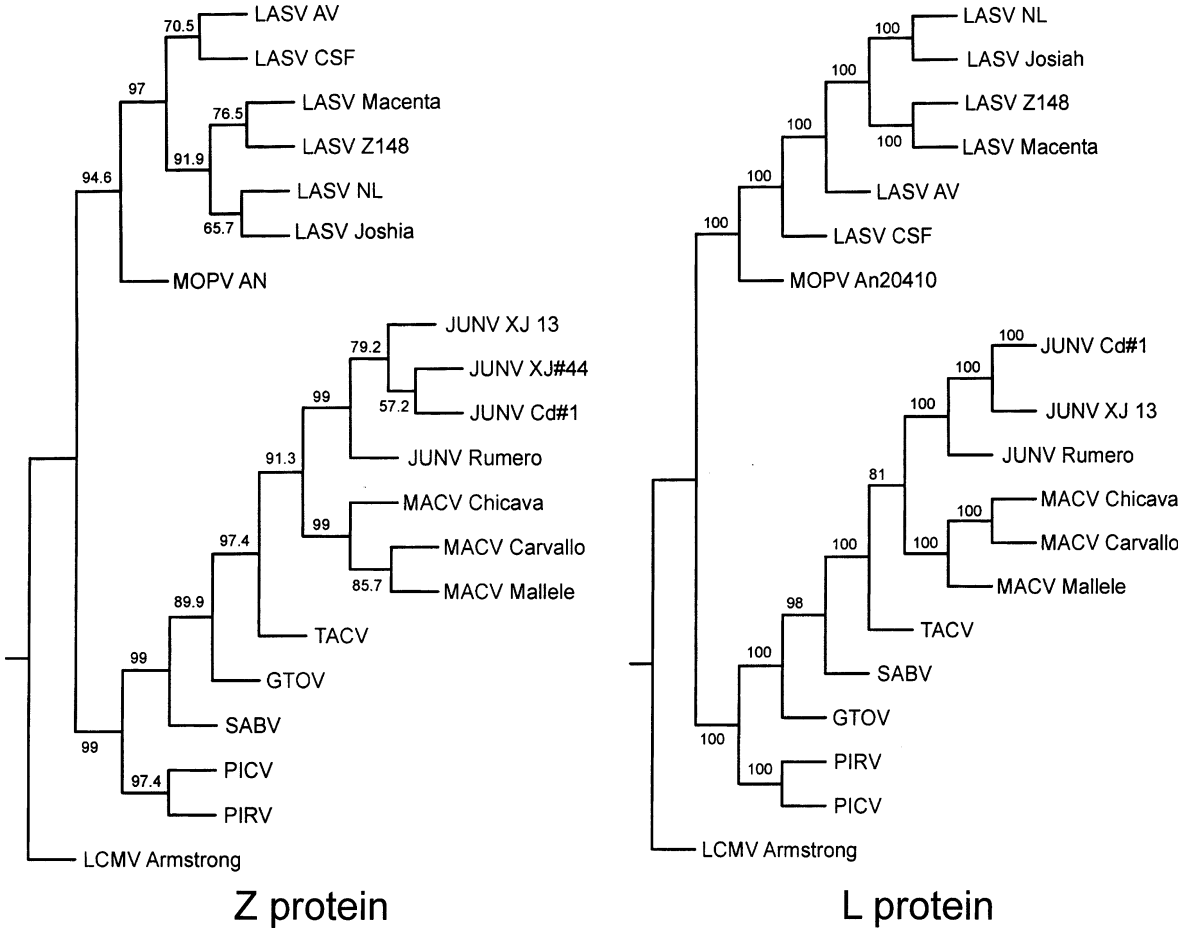

Fig. 3 Phylogenetic study for L RNA proteins. The L and Z proteins of arenaviruses were analyzed to generate a cladogram using Bootstrap (100 replicates) and Protpars routines of Phylip Package. Numbers are percentages and indicates the consistency of that branch disposition

these regions in the attenuation process remains to be evaluated.

Phylogenetic analysis (Fig. 3) using the whole amino-acid sequences of the-arenavirus L RNA polypeptides, shows that all Junín virus strains (Candid #1, XJ 13, XJ#44 and Rumero) grouped together with other haemorrhagic New World arenaviruses. Clades and subclades of Old World and New World arenavirus are in accord to Charrell et al. [19] and it is important to note that, Candid #1 (non-haemorrhagic strain) formed a strong clade (Bootstrap support number of 100 for L and 80 for Z polypeptide) with its haemorrhagic progenitor strains, XJ 13 and XJ#44. Thus, apparently the phylogeny does not correlate necessarily with the virulent phenotype, as was suggested by Bowen et al. [20], who used a more restricted number of sequences available at that time. On the contrary, a small set of sequence changes within a virus species seems to be central to define the phenotypic variation from attenuation to virulence. If this is confirmed in a more extensive study, any surveillance program designed to monitor the natural vaccine variations should search for possible point mutations at those positions related with attenuation.

In summary, the present work should be regarded as a first step in the identification of regions in the Junín virus genome that are related to a particular virulence pattern. We do not rule out the involvement of the S RNA in the attenuation process, 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–host systems [21, 22]. 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 genetics system for Junín virus is being developed to allow the generation of infectious virions with specific mutations.

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