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# Genetic Diversity of the Junin Virus in Argentina: Geographic and Temporal Patterns

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RNA was purified from 39 strains of cell-cultured Junin virus (JUN) from central Argentina, which included both human- and rodent-derived isolates (a total of 26 and 13, respectively), as well as from 2 laboratory JUN strains, XJ Cl3 and XJ #44. JUN-specific primers were used to amplify a 511-nucleotide (nt) fragment of the nucleocapsid protein gene and a 495-nt fragment of the glycoprotein 1 (GP1) gene. Genetic diversity among JUN strains studied was up to 13% at the nt level and up to 9% at the amino acid (aa) level for the GP1 gene and up to 9% (nt) and 4% (aa) for the NP gene. Phylogenetic analyses of both genes revealed three distinct clades. The first clade was composed of the JUN strains from the center of the endemic area and included the majority of JUN strains analyzed in the current study. The second clade contained 4 JUN strains isolated between 1963 and 1971 from Cordoba Province, the western-most edge of the known endemic area. The third clade contained 4 JUN strains that originated from *Calomys musculinus* trapped in Zarate, the northeastern edge of the known endemic area. Certain JUN sequences, which were obtained from GenBank and identified as XJ, XJ #44, and Candid #1 strains, appeared to form a separate clade. Over 400 nt of the GP1 and GP2 genes were additionally sequenced for 7 JUN strains derived from patients with different clinical presentations and outcomes of Argentine hemorrhagic fever. Analysis of the corresponding aa sequences did not allow us to attribute any particular genetic marker to the changing severity or clinical form of the human disease. © 2000 Academic Press

## INTRODUCTION

Argentine hemorrhagic fever (AHF), first described in 1955 (Arribalzaga, 1955), is characterized by vascular, renal, hematological, neurological, and immunological alterations, with a case fatality rate of 15–30% in untreated individuals. The etiological agent of AHF is Junin virus (JUN), which was initially isolated in 1958 (Parodi *et al.,* 1958) and confirmed in 1959 (Pirosky *et al.,* 1959). Subsequently, JUN was serologically assigned to the Tacaribe group of viruses (Mettler *et al.,* 1963), which belongs to the family *Arenaviridiae,* genus *Arenavirus*. Arenaviruses are rodent-borne, enveloped, single-stranded, ambisense RNA viruses with a segmented genome (Southern, 1996). The arenavirus genome is composed of two RNA species. The large (L) segment (about 7200 nucleotides [nt] in length) encodes the virus polymerase (L protein) and a small zinc binding protein. The small RNA segment (S) (ca. 3400 nt in length) encodes the nucleocapsid (NP) protein and the glycoprotein precursor, GPC, of two viral glycoproteins, GP1 and GP2. Recent phylogenetic analysis of the arenaviruses confirmed the existence of two major subdivisions of the viruses within this genus, specifically, the lymphocytic choriomeningitis virus (LCMV)–Lassa complex, which is composed of known Old World arenaviruses, and the Tacaribe complex, which includes the New World arenaviruses (Bowen *et al.,* 1997). Within the Tacaribe complex, the viruses form three phylogenetically distinct clades (Bowen *et al.,* 1996). JUN appears to be most closely related to Machupo virus (the etiological agent of Bolivian hemorrhagic fever), Guanarito virus (the etiological agent of Venezuelan hemorrhagic fever), Sabiá virus (known to cause hemorrhagic fever in Brazil), and Tacaribe virus. Most of the Tacaribe complex arenaviruses are associated in nature with indigenous New World rodents of the subfamily *Sigmodontinae,* in which they establish asymptomatic persistent infections, usually with continuous production of virus for the lifetime of the rodent. Although JUN has been isolated from several rodent species, the natural host of this virus is a member of the genus *Calomys, Calomys musculinus* (a field mouse) (Sabattini *et al.,* 1977; Sabattini and Contigiani, 1982), which is widely distributed in the Argentine plains.

Since the emergence of AHF, a progressive geographic expansion of epidemic outbreaks has been observed (Maiztegui and Sabattini, 1977; Maiztegui *et al.,* 1986). In 1958, human cases were limited to an area of

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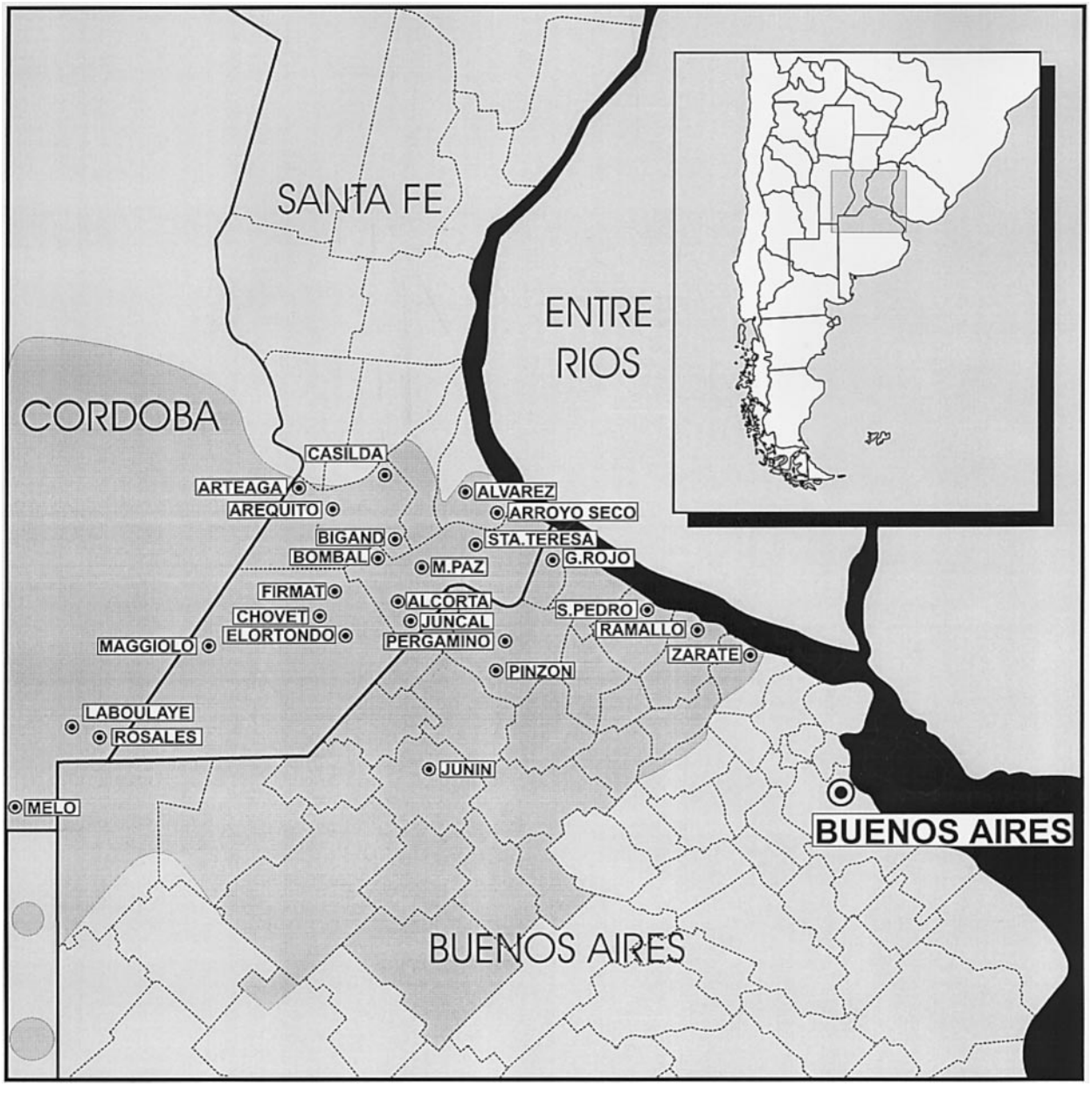


FIG. 1. Map of central Argentina showing the AHF endemo-epidemic area and the locations of origin of Junin virus strains examined. The currently known AHF endemo-epidemic area (according to Maiztegui *et al.,* 1986; Enria and Feuillade, 1998) is shown in dark gray.

16,000 km$^2$ in the north of Buenos Aires province. Currently, the endemo-epidemic area covers approximately 150,000 km$^2$, reaching north of Buenos Aires, south of Santa Fé, southeast of Córdoba, and northeast of La Pampa provinces (Enria and Feuillade, 1998) (Fig. 1). At present, the human population at risk is estimated to be around 5 million. During the 1960s and through the 1990s, annual outbreaks of AHF were detected in many locations (east, northeast, and northwest) surrounding the focal point of the original outbreak. In general, AHF incidence remained high in the new areas for 5 to 10 years and then gradually declined. Though sporadic cases may occur in the "historic" areas, the significant decrease in the number of human cases in such areas has been documented (Sabattini, unpublished results). Several hypotheses have been considered to explain the geographic expansion of the endemo-epidemic area and changing incidence of AHF (Enria and Feuillade, 1998). These include an increase in JUN seroprevalence in humans, lower virulence of JUN strains circulating in the "historic" areas, changes in human agricultural practices, natural fluctuations in JUN prevalence in the population of its rodent host, population dynamics, and patterns of migration of *C. musculinus.* None of these factors (for

TABLE 1

Nucleotide and Amino Acid Sequence Identities between Typical Junin Virus Strains (%)[a]

| | Clade | | | | | |
|---|---|---|---|---|---|---|
| | I. PAn19221 (nt/aa) | | II. Pan19103 (nt/aa) | | III. CbaIV4454 (nt/aa) | |
| | NP | GP1 | NP | GP1 | NP | GP1 |
| Clade | | | | | | |
| I. PAn10004 | 95.1/98.2 | 94.4/96.9 | 94.9/97.6 | 91.0/93.1 | 91.3/98.8 | 91.0/96.2 |
| II. PAn19109 | 93.7/97.1 | 91.5/93.1 | 99.4/100.0 | 99.2/98.5 | 91.8/98.2 | 88.2/92.3 |
| III. CbaFHA5069 | 92.3/98.2 | 89.7/94.6 | 92.5/98.2 | 87.4/93.1 | 98.2/100.0 | 96.9/100.0 |

[a] Two typical JUN strains representative of each identified phylogenetic clade were chosen for comparison. For Clade I, two representative strains were selected from two distinct local subclades (see Fig. 2).

instance, low JUN seroprevalence observed in the local human populations several years after the original detection of AHF [Weissenbacher *et al.,* 1983]) satisfactorily explains the changing incidence of AHF in "historic" areas. Most likely, the endemo-epidemic situation in a particular locality in any particular year depends on a combination of the different factors mentioned above.

The clinical spectrum of the human disease ranges from mild to severe and includes patients presenting with neurologic manifestations, hemorrhagic signs, or both hemorrhagic and neurologic manifestations. In accord with clinical observations, a broad spectrum of virulence has been demonstrated among JUN strains of both human and rodent origin using various animal models, such as adult guinea pigs, 14-day-old mice, primates, and neonatal rats (de Guerrero *et al.,* 1977; Contigiani and Sabattini, 1977; Weissenbacher *et al.,* 1979; Avila *et al.,* 1981; McKee *et al.,* 1985; Kenyon *et al.,* 1986). However, a molecular basis for these differences has not been described. Albariño *et al.* (1997) determined the nucleotide sequence of the entire S RNA of the JUN vaccine strain Candid #1 and its more virulent ancestors, XJ #44 and XJ prototype. By comparing these sequence data with the corresponding sequence of the wild-type MC2 strain, changes were detected in the GPC gene that were unique to the vaccine strain, but their relationship with virulence has not been shown.

The present study was undertaken to compare the genetic variability of the wild-type JUN strains of rodents and human isolates in central Argentina and to reveal possible patterns of their geographic and temporal genetic diversity. Based on the sequence data obtained, JUN strains, which were involved in epidemics across a span of 35 years, were classified genetically. Preliminary conclusions were drawn about the relationship of phylogenetic placement of a JUN strain, its virulence, and its association with unique clinical manifestations of AHF.

## RESULTS

### Sequence divergence and phylogenetic relationships between JUN strains

As mentioned, fragments of both NP and GP1 genes of numerous wild JUN strains from central Argentina were analyzed in this study. A 511-nt fragment of the NP gene and a 495-nt fragment of the GP1 gene (adjacent to the 5′ end of the virus S genome segment) were amplified by PCR and sequenced. The percentage nt and aa sequence identities of the both NP and GP1 gene fragments between typical JUN strains, which originated either from the same locations or from different locations and time points, are shown in Table 1. Genetic diversity among JUN strains in central Argentina was found to be up to 13% at the nt level and up to 9% at the aa level for the GP1 gene and up to 9% (nt) and 4% (aa) for the NP gene. As expected, JUN strains from the same locations display a higher degree of genetic similarity, with percentage nt and aa differences for the GP1 gene not exceeding 5.6% and 3.1%, respectively, and 4.9% and 1.8% for the NP gene.

Phylogenetic analyses were based on the 390-nt fragment of the GP1 coding region (corresponding to the 5′ end of the virus S genome segment) and the entire amplified 511-nt fragment of the NP gene. Both neighbor-joining (NJ) (see Figs. 2A, 2B) and maximum parsimony (MP) (data not shown) analyses of both NP and GP1 gene fragments rendered similar branching patterns for the phylogenetic trees. Use of different algorithms in NJ, as well as application of different weighting schemes in MP, did not change phylogenetic placement of three major clades comprising sequences of the wild JUN strains determined in this study. The first clade comprised the majority of the strains obtained throughout the central and northern parts of the known endemic area of AHF (see Fig. 1). Within this clade, further grouping of the wild JUN strains can be observed, although few strains are positioned differently in the NP and GP1 tree. The


FIG. 2. Phylogenetic relationships among wild Junin virus strains examined in the current study. Phylogenetic trees were generated using the NJ method on the basis of nt sequence differences of the 511-nt fragment of the JUN NP gene (A) and those of the 390-nt fragment of the JUN GP1 gene (B). NJ analysis was performed with MEGA (Kumar *et al.,* 1993) using the Kimura two-parameter algorithm, with options of using both transitional and transversional changes and complete deletion of gaps. Bootstrap confidence levels (BCL) were obtained by 1000 replicates of the analysis (Hillis and Bull, 1993; Kumar *et al.,* 1993). Confidence probabilities (CP) were calculated using the standard error test (Kumar *et al.,* 1993). The percentage of bootstrap support exceeding 60% and CP for corresponding branches are indicated by numbers (BCL/CP) near the appropriate branching points. Statistically significant CP values are marked with asterisks. Lengths of the horizontal branches are proportional to the Kimura two-parameter distances between corresponding taxa. Vertical branches are for visual clarity only. Three phylogenetic clades of wild JUN strains are marked with roman numerals: I, the "major" clade; II, "Zarate" clade; III, "Cordoba" clade. JUN strains are described as follows: strain designation/year/locality of origin/source (human or rodent)/(clinical form of AHF [for humans]). Abbreviations: H, human; Cm, *Calomys musculinus;* Cl, *Calomys laucha;* HS, hemorrhagic severe; NS, neurological severe; MS, mixed severe.

second clade included only four JUN strains (Cba IV4454, Cba FHA5069, Cba An9446, and Cba Lye/63), which originated from human and rodent samples collected in southeast Córdoba province from 1963 to 1971. The third clade also included only four rodent-derived strains from Zárate (Buenos Aires province), namely, PAn16160, PAn19107, PAn19109, and PAn19103. Known sequences of the XJ (Parodi) strain, XJ #44, and Candid #1 vaccine strain (Albariño *et al.,* 1997), which were retrieved from GenBank, formed an additional distinct clade. Surprisingly, the sequence of the XJ #44 strain determined in the current study appeared to be clearly different from the sequences mentioned above, although it was identical to the NP sequence of the XJ strain published by Bowen (Bowen *et al.,* 1997). These two sequences fell in the same major phylogenetic clade (referred to as "the first clade" or "the major clade" later on) as most of the other samples analyzed.

### Geographic and temporal patterns of JUN genetic divergence

The JUN strains analyzed in the current study were originally obtained from rodents and human AHF patients throughout the known AHF endemo-epidemic area, from Casilda in the North to Pinzon in the South, and from Melo in the West to Zarate in the East (see Fig. 1). Most of these JUN strains were found to group to-

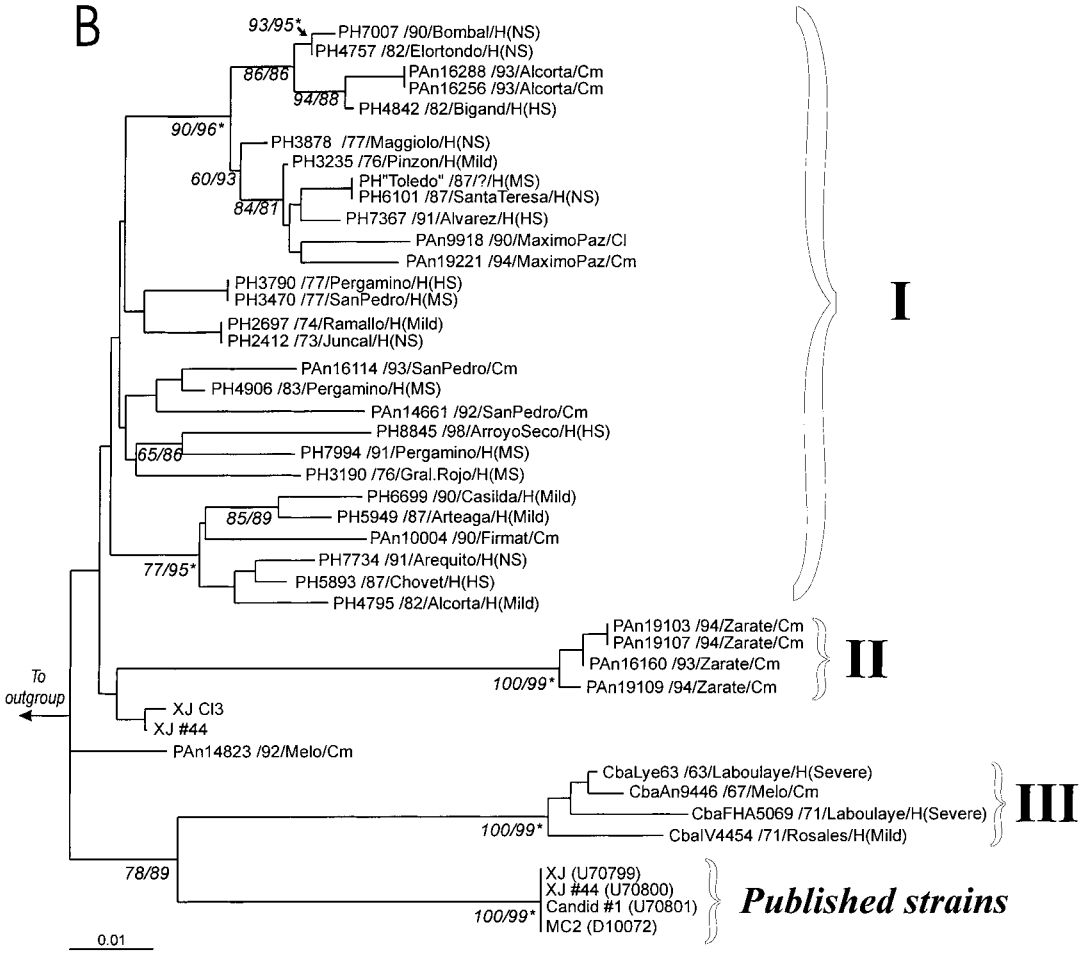


FIG. 2—*Continued*

gether in a single phylogenetic clade. However, a limited number of the JUN strains available from the western-most (Melo, Cordoba province) and eastern-most (Zarate, Buenos Aires province) regions of the AHF endemo-epidemic area appeared to form two additional phylogenetically distinct groups.

As shown in Table 2, the original samples used for isolation of JUN strains were collected during a span of 35 years, from 1963 to 1998. No temporal pattern could be seen in the JUN phylogenetic tree, as most of these strains are positioned randomly within the major clade of JUN sequences (Fig. 2). The only exception found in this study is the group of the four JUN strains from Cordoba (dated 1963–1971), which form a separate clade.

### Phylogenetic relationship of the JUN strains causing distinct clinical forms of AHF in humans

Among 26 human-derived JUN strains analyzed, 24 strains were originally isolated from AHF patients for whom the clinical picture of the disease has been well documented. Of those JUN strains, 7 were associated mainly with severe neurologic manifestations, 5 caused severe hemorrhagic signs, 6 caused both hemorrhagic and neurologic manifestations ("mixed" form of AHF), and the remaining 6 were associated with a mild form of AHF. These strains appear to be positioned randomly within the major clade of JUN sequences, and no clustering of the JUN strains associated with particular clinical manifestations in humans is observed. To identify mutations that could be responsible for the differences in virulence of these JUN strains, sequencing of the GPC gene was extended for 7 JUN strains (PH3235, PH3878, PH4757, PH4842, PH5949, PH7367, and PH7734) associated with different clinical manifestations of AHF. However, comparisons of the nt and deduced aa sequences of the entire GP1 gene and GP2 gene fragment (about 900 nt in length total) did not reveal any particular nt or aa substitution consistently associated with any particular form of AHF in humans (data not shown).

## DISCUSSION

Data presented here represent the first attempt to extensively analyze geographic and temporal genetic variability of JUN strains throughout the AHF endemo-

TABLE 2

Junin Virus Strains Examined

| Strain | Year | Origin | | Clinical disease[a] | Source |
|---|---|---|---|---|---|
| | | Locality | Province | | |
| XJCl3 | [b] | | | | |
| XJ #44 | [b] | | | | |
| Cba Lye/63 | 1963 | Laboulaye | Córdoba | Severe | Human |
| Cba An 9446 | 1967 | Melo | Córdoba | | Cm[c] |
| Cba IV 4454 | 1971 | Rosales | Córdoba | Mild | Human |
| Cba FHA 5069 | 1971 | Laboulaye | Córdoba | Severe | Human |
| PH2412 | 1973 | Juncal | Santa Fe | NS | Human |
| PH2290 | 1974 | Pergamino | Buenos Aires | MS | Human |
| PH2697 | 1974 | Ramallo | Buenos Aires | Mild | Human |
| PH3190 | 1976 | Gral. Rojo | Buenos Aires | MS | Human |
| PH3235 | 1976 | Pinzon | Buenos Aires | Mild | Human |
| PH3470 | 1977 | San Pedro | Buenos Aires | MS | Human |
| PH3790 | 1977 | Pergamino | Buenos Aires | HS | Human |
| PH3878 | 1977 | Maggiolo | Santa Fe | NS | Human |
| PH4757 | 1982 | Elortondo | Santa Fe | NS | Human |
| PH4795 | 1982 | Alcorta | Santa Fe | Mild | Human |
| PH4842 | 1982 | Bigand | Santa Fe | HS | Human |
| PH4906 | 1983 | Pergamino | Buenos Aires | MS | Human |
| PH5893 | 1987 | Chovet | Santa Fe | HS | Human |
| PH5949 | 1987 | Arteaga | Santa Fe | Mild | Human |
| PH6101 | 1987 | Santa Teresa | Santa Fe | NS | Human |
| PH"Toledo" | 1987 | Not known | Not known | MS | Human |
| PH6699 | 1990 | Casilda | Santa Fe | Mild | Human |
| PH7007 | 1990 | Bombal | Santa Fe | NS | Human |
| PAn9918 | 1990 | Máximo Paz | Santa Fe | | Cl[d] |
| PAn10004 | 1990 | Firmat | Santa Fe | | Cm |
| PH7367 | 1991 | Alvarez | Santa Fe | HS | Human |
| PH7734 | 1991 | Arequito | Santa Fe | NS | Human |
| PH7994 | 1991 | Pergamino | Buenos Aires | MS | Human |
| PAn14661 | 1992 | San Pedro | Buenos Aires | | Cm |
| PAn14823 | 1992 | Melo | Córdoba | | Cm |
| PAn16114 | 1993 | San Pedro | Buenos Aires | | Cm |
| PAn16160 | 1993 | Zárate | Buenos Aires | | Cm |
| PAn16256 | 1993 | Alcorta | Santa Fe | | Cm |
| PAn16288 | 1993 | Alcorta | Santa Fe | | Cm |
| PAn19103 | 1994 | Zárate | Buenos Aires | | Cm |
| PAn19107 | 1994 | Zárate | Buenos Aires | | Cm |
| PAn19109 | 1994 | Zárate | Buenos Aires | | Cm |
| PAn19221 | 1994 | Máximo Paz | Santa Fe | | Cm |
| PH8840 | 1998 | Alcorta | Santa Fe | NS | Human |
| PH8845 | 1998 | Arroyo Seco | Santa Fe | HS | Human |

[a] Clinical forms of AHF: NS, neurological severe; HS, hemorrhagic severe; MS, mixed severe.
[b] Strains derived from the XJ prototype strain isolated in 1958 (Parodi *et al.,* 1958).
[c] Cm, *Calomys musculinus.*
[d] Cl, *Calomys laucha.*

epidemic area in central Argentina. The human- and rodent-derived JUN strains analyzed in the current study cover a 35-year period (1963–1998). The geographic region included almost the entire currently known AHF endemic area of approximately 150,000 $km^2$. As expected, JUN strains that originated from the same or closely situated localities display the highest genetic similarity at both nt and aa levels (see Table 1 and Fig. 2). Maximum nt sequence divergence between JUN strains from distant localities observed in the analyzed fragment of the NP protein (~9%) is slightly higher than that between different known strains of any particular virus within the Tacaribe complex (6%), reported by Bowen *et al.* (1997). However, this fact may be merely due to the higher number of JUN strains sequenced in the current study. As expected, nt and deduced aa sequences of the GP1 gene showed even greater divergence (up to 13 and 9%, respectively). Higher genetic variability of the surface glycoproteins, when compared with the nucleocapsid protein, has been reported for all negative-sense single-

stranded RNA viruses, including (but not limited to) rhabdoviruses, paramyxoviruses, hantaviruses, and arenaviruses. For JUN in particular, Albariño *et al.* (1997) reported a higher genetic variability of GPC versus NP among closely related XJ, XJ #44, and Candid #1 strains. However, these authors did not determine the nucleotide sequence corresponding to the first 130 aa of GP1 (i.e., the region sequenced in the current study); thus, direct comparisons with our data are not possible.

Phylogenetic analyses of the fragments of the NP and GP1 genes were performed using both MP and distance-based (NJ) methods and resulted in phylogenetic trees of essentially similar topology. Basically, three phylogenetically distinct clades, composed of the wild JUN strains analyzed in the current study, can be seen (see Results and Fig. 2). The largest phylogenetic clade comprises the majority of the JUN strains analyzed and also includes sequences of two laboratory JUN strains maintained in Pergamino, XJ Cl3 and XJ #44, derived from the XJ prototype strain. Sequences of the XJ strain (Parodi strain), XJ #44 strain, and attenuated Candid #1 vaccine strain, reported by Albariño *et al.* (1997), were also included in the phylogenetic analysis. Surprisingly, these published sequences appear to differ significantly from the sequences of the XJ #44 and XJ Cl3 strains determined in our study and form an additional distinct clade on the phylogenetic tree. However, NP gene sequences of the XJ #44 and XJ Cl3 strains obtained in our laboratory are 100% identical to the corresponding sequence of the XJ prototype strain independently determined by Bowen *et al.* (1997). These three JUN strains have undergone a significantly different number of passages in laboratory animals and cell culture (see Materials and Methods and Albariño *et al.,* 1997). In light of these data, it seems highly unlikely that the passage history of these particular JUN strains could significantly affect their phylogenetic placement through adaptive changes during passages. Further investigation of the laboratory history of the previously published JUN strains could clarify this observed discrepancy.

Not surprisingly, phylogenetic analysis of the JUN strains reveals a pattern of geographic clustering of genetic variants previously observed for some other negative-sense single-stranded RNA viruses, including, in particular, vesicular stomatitis virus (*Rhabdoviridae*) (Nichol *et al.,* 1993a), an enzootic virus transmitted by mosquitoes, and rodent-borne Sin Nombre virus (*Bunyaviridae*) (Nichol *et al.,* 1993b; Monroe *et al.,* 1999). Although we wished to include in this investigation wild JUN strains from the entire endemo-epidemic area, most of the available strains originated from the northeastern quarter (Table 2, Fig. 1). All these strains fell into the single major phylogenetic clade of JUN sequences, apparently irrespective of the time of isolation (see Fig. 2). Within this clade, further geographic clustering can be observed, although few taxa (for instance, PH4795, Fig. 2) appear in different subclades in the NP and GP1 trees. One possible explanation for this phenomenon could be recombination; however, our current sequence data are not sufficient for further investigation of this possibility. Samples from the western-most (Melo, Cordoba province) and the eastern-most (Zárate, Buenos Aires province) locations formed two additional distinct phylogenetic clades. It is likely that further sampling of JUN strains from a wider geographic area will reveal additional divergent JUN genotypes.

As with the other vector-borne negative-sense single-stranded RNA viruses mentioned above, no clear temporal pattern of virus genetic divergence can be seen among the wild JUN strains in our study. The only visible exceptions are four JUN strains from Córdoba province dated 1963–1971. We were able to obtain only one more recent JUN strain, PAn14823, from the same locality (Melo; see Fig. 1), but it does not seem to be closely related to the older samples and is positioned rather within the major clade of JUN sequences (Fig. 2). More extensive studies of the contemporary JUN strains in Cordoba province were precluded because of the low incidence of infected *C. musculinus* (Garcia *et al.,* 1996) and the absence of reported human cases from the Melo area during the last 20 years. One possible explanation for the separate phylogenetic placement of these four JUN strains could be their high passaging in suckling mice. However, this scenario does not seem likely, since, according to our data (see above), highly passaged laboratory strains XJ #44 and XJ Cl3 did not accumulate a significant number of nucleotide changes compared with their progenitor, JUN strain XJ. Alternatively, the separate phylogenetic positioning of these JUN strains from Cordoba may be explained by invoking biogeographic events such as rodent migrations and/or reintroduction of new JUN strains into the resident rodent populations.

Studies of JUN virulence conducted in animal models (McKee *et al.,* 1985) suggested that intrinsic variation between JUN strains could be responsible for different clinical manifestations in humans. In an attempt to clarify this question, JUN strains isolated from patients with different clinical courses of the disease (i.e., hemorrhagic versus neurological forms, mild or severe) were analyzed. As seen in Fig. 2, no clustering of the JUN strains associated with clinically different forms of AHF was observed in the current investigation. Therefore, it is not likely that the different clinical manifestations of AHF observed in humans are the result of a change in JUN virulence due to the accumulation of multiple mutations during long-term evolution of the corresponding JUN strains. For example, two JUN strains from Córdoba province, Cba FHA5069 and Cba IV4454, belong to the same phylogenetic clade (Fig. 2). Of these strains, Cba FHA5069 was originally isolated from the blood of a fatal human case and was proven to be virulent for white albino mice and guinea pigs. Cba IV4454 was isolated

from a mild human case and had low virulence for both guinea pigs and mice (Medeot *et al.,* 1990). Despite the difference in virulence, only one aa change was detected in the analyzed fragments of the virus genome (0/130 aa for GP1; 1/170 aa for NP). Recently, a similar pattern has been observed with Venezuelan equine encephalitis virus (Powers *et al.,* 1997; Wang *et al.,* 1999), where epidemic/epizootic and enzootic strains occur together in the same phylogenetic clade and have sequences that are almost 100% identical on the nucleotide level.

Sequencing of an additional 400 nucleotides of the GPC gene of seven JUN strains displaying different levels of virulence was conducted in an attempt to reveal possible aa sequence changes associated with different profiles of pathogenicity. However, subsequent analysis did not reveal any particular aa change in GP1 and the analyzed part of GP2, which could be associated with altered virulence. Envelope glycoproteins are potentially good candidates for explaining differential virulence because of their variability, both in aa sequence and in glycosylation pattern, and their role in viral entrance into the host cell. In particular, it has been demonstrated that a single aa substitution at glycoprotein position 260 of LCMV, a well-studied Old World arenavirus, is responsible for change of the organ tropism (Ahmed *et al.,* 1991), cytotoxic T-lymphocyte response, and establishment of persistence (Salvato *et al.,* 1991). Another aa substitution in the glycoprotein of this virus has been associated with the ability to cause growth hormone deficiency syndrome (Teng *et al.,* 1996). However, it is possible that other viral proteins, especially viral polymerase, could be involved in altered patterns of pathogenicity. In addition, one still cannot exclude other possibilities. For instance, alteration of pathogenicity and/or cell tropism of different JUN strains could be due to a few different combinations of 2–3 aa substitutions in GP1, a single aa substitution in GP2, or even a single nt substitution in the noncoding regulatory region of the virus S or L genome segment. In fact, altered pathogenicity and/or cell tropism due to a few or a single nucleotide substitution(s) has been demonstrated for Sindbis virus (Dubuisson *et al.,* 1997; Dropulic *et al.,* 1997) and poliovirus (Georgescu *et al.,* 1997). Therefore, further studies, involving sequencing of the larger fragments of the JUN genome, should be done to obtain a definite answer.

## METHODS

### Virus strains

The JUN strains used in this study are summarized in Table 2. In general, JUN strains were isolated by intracranial injection in white albino suckling mice and used after 1 or 2 passages. The Cba IV4454, Cba FHA5069, Cba An9446, and Cba Lye/63 strains were used after 4 to 8 suckling mice passages. The XJ Cl3 strain was originally derived from its prototype XJ strain through 2 guinea pig and 14 mice passages and subsequent plaque purification in rabbit MA-111 cells (de Guerrero *et al.,* 1969). In the current study, XJ Cl3 from 17 suckling mice passages was used. Human isolates were selected from patients with different clinical manifestations, which covered most of the endemic area and different AHF epidemics.

### Total RNA purification

RNA extraction was performed as described elsewhere (Spiropoulou *et al.,* 1994). Briefly, approximately 100 mg of harvested cells was mixed with 300 $\mu$L of cell lysis solution containing guanidine thiocyanate, extracted with phenol/chloroform, and purified with RNA matrix beads (RNAid PLUS kit, Bio 101, La Jolla, CA). RNA was eluted from the matrix with RNase-free water and stored at −80°C until use.

### Oligonucleotide primer design

Standard sets of generic primers to the NP region of the JUN S RNA genome segment described by Bowen (Bowen *et al.,* 1996, 1997; Bowen, unpublished) were used for the original recovery of the corresponding JUN genetic sequences. Additional primers for nested PCR were designed for the regions conserved among published sequences of JUN and other closely related arenaviruses, including published sequences of JUN strains MC2 (GenBank Accession No. D10072; Ghiringhelli *et al.,* 1991), XJ (Parodi strain) (U70799, U70802), XJ #44 (U70800, U70803), Candid #1 strain (U70801, U70804) (Albariño *et al.,* 1997), and Machupo virus strain AA288-77 (X62616; Griffiths *et al.,* 1992). Based on the JUN sequences obtained, a variety of additional primers were designed and used to extend sequencing of the GP1 gene. Primer sequences used in this study are shown in Table 3.

### RT-PCR amplification and sequence analysis

Total RNA was assayed for the presence of JUN genetic sequences using a standard reverse-transcriptase/polymerase chain reaction technique (RT-PCR). A second round of (nested or seminested) PCR was performed on the samples, which appeared to be negative after the initial RT-PCR reactions. Amplifications were performed on an ERICOMP TwinBlock System thermocycler (Ericomp, Inc., San Diego, CA). Typical conditions used in a standard RT-PCR included 1 h at 41°C, followed by 40 cycles of 93°C/30 s, 48°C/30 s, and 72°C/120 s. Synthesized DNA products were separated on agarose gels, and the bands of the correct predicted size were excised from the gel and purified using a MERMaid kit (Bio 101). Direct automated sequencing of the purified fragments was performed on an ABI PRISM 310 Genetic Analyzer using the dyedeoxy cycle sequencing technique (Applied Biosystems, Foster City, CA).

TABLE 3

Oligonucleotide Primers

| Gene | Primer name[a] | Sequence (5′ → 3′) | Notes |
|---|---|---|---|
| Glycoprotein | M13-19C | TGTAAAACGACGGCCAGTGCGCACAGTGGATCCTAGGC | Contains 19C (Bowen *et al.,* 1996) plus M13-21 extension sequence |
| Glycoprotein | 495R | ATCATCACTTTTAGAACA | Designed in current study |
| Glycoprotein | 900R | CCA(T/C)TITTCIAG(G/A)CAGTAICCICCAGG | Designed in current study |
| Nucleocapsid | 2398F (1010C) | TCIGGIGAIGGITGGCC | Bowen *et al.,* 1996 |
| Nucleocapsid | 2350F | ATCA(T/C)AGGCAGGTCATGGG | Designed in current study |
| Nucleocapsid | 1763R | GGCCAACTTAAAGGGAG | Designed in current study |
| Nucleocapsid | 1723R (1696RV2) | AIATGATGCAGTCCAIIAGIGCACA | Bowen, unpublished (Bowen *et al.,* 1997, modified) |

[a] Numbers correspond to the 5′-end nt position in the MC2 Junin strain (Ghiringhelli *et al.,* 1991). Primer names according to Bowen (Bowen *et al.,* 1996, 1997; Bowen, unpublished) are given in parentheses. F, forward; R, reverse; I, inosine.

### Phylogenetic analysis

Sequence comparisons and alignments were performed using Auto Assembler Version 1.4.0 (Perkin Elmer) and various programs (GAP, PILEUP, and LINEUP) of the Wisconsin Sequence Analysis Package, version 8, UNIX (Genetics Computer Group, Madison, WI), followed by fine manual adjustment. The NP sequence alignment appeared to have no gaps; however, several small gaps were introduced into the GP1 sequence alignment to accommodate published sequence of the MC2 strain. In all analyses, these gaps were treated as missing data. Phylogenetic analysis of nucleotide sequence differences between different JUN strains was performed using both the distance-based NJ method (MEGA version 1.02 software [Kumar *et al.,* 1993]) and the MP method (PAUP version 3.1.1 software [Swofford, 1991]). Both methods were applied using various algorithms and weighting schemes. To root phylogenetic trees, additional NJ and MP analyses were conducted on the NP data set, which included corresponding sequences of Machupo virus as an outgroup.

In the NJ method, different distance corrections, including Jukes–Cantor, Kimura two-parameter, and Tamura models were used. Since the rate of transitional substitutions appeared to be higher than the rate of transversional substitutions in the arenavirus genetic sequences, the Kimura two-parameter model, which includes estimation for these differences, was selected for the final phylogenetic analysis of the JUN data set. For the MP analysis, the transition/transversion (ts/tv) ratio was estimated using MacClade software (Maddison and Maddison, 1992) analysis of unweighted trees generated from nucleotide sequence differences detected among JUN strains. Such weighting is predicted to improve the effectiveness of the MP method for estimation of the correct phylogeny (Hillis *et al.,* 1994). To take into account a possible effect of sequential nucleotide substitutions, estimation of the ts/tv ratio was also conducted on the reduced data sets, which included only selected sister taxa in the terminal clades. Based on the results of this analysis, a 4:1 (estimation for the entire JUN data set) and an 8:1 (average estimation among selected taxa) weighting of the transitions over transversions was applied. Phylogenetic trees were obtained using the branch-and-bound or heuristic search methods. Bootstrap confidence limits were calculated by 1000 heuristic search repetitions of the analysis (Hillis and Bull, 1993).

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