Genetic Organization of Junin Virus, the Etiological Agent of Argentine Hemorrhagic Fever

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I. INTRODUCTION

Most of the data on the genome organization of Junin virus presented in this chapter come from our laboratory and include results that are at the stage of manuscript preparation. Molecular cloning and sequencing studies on this human pathogenic arenavirus were focused on the S RNA, which codes for the major structural proteins.

In the first part of this chapter the biochemical structure of Junin virus is reviewed after a short historical background. In some instances, where information specific for Junin virus is ambiguous, references are made to data published for other arenaviruses.

II. HISTORICAL CONSIDERATIONS

The symptoms and clinical forms of Argentine hemorrhagic fever (AHF) were first described by Aribalzaga (1955) after the observations of the 1953 and 1954 epidemic outbreaks in Bragado, Buenos Aires. The viral etiology of the disease was established in 1958 by two groups led by Parodi and Pirotsky, respectively, who isolated the virus from the blood

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of patients and organs obtained from necropsies at the city of Junin Regional Hospital (Parodi *et al.*, 1958; Pirotsky *et al.*, 1959). The etiological agent was named Junin virus after the geographical site of its initial isolation.

At that time the epidemic outbreaks were restricted to an area of 16,000 km². Since then, AHF has spread to the provinces of Cordoba, Santa Fe, and La Pampa, increasing the endemic area to five times its initial size by 1975 (Maiztegui and Sabattini, 1977). In 1986 the progressive extension of the endemic area was reported to cover more than 120,000 km² of the richest farming land in Argentina with more than 2 million inhabitants (Maiztegui *et al.*, 1986). Consequently, the disease has had considerable impact on the welfare and economy of Argentina and has stimulated research on AHF and Junin virus to solve this sanitary problem.

As is obvious, early research on AHF was focused on reproducing the disease in animal models (Boxaca *et al.*, 1961; 1965) and establishing serological relationships with other viruses (Mettler *et al.*, 1963).

The propagation of Junin virus in tissue culture (Mettler *et al.*, 1961; Martinez Segovia *et al.*, 1967; 1974; Murphy *et al.*, 1970; Lascano and Berria, 1970) allowed a better morphological and serological characterization of the virus.

In 1965 Junin virus was found to be related to Tacaribe virus and Machupo virus, the etiological agent of Bolivian hemorrhagic fever, by serological means (Johnson *et al.*, 1973). These viruses and, subsequently, others that were isolated on the American continent, were grouped into what became known as the Tacaribe complex. All members of the Tacaribe complex were found to share the morphological features of LCM and Machupo viruses (Murphy *et al.*, 1970). The relatedness of these viruses was further evidenced by the cross reactivity between antigens of LCM and antisera to viruses of the Tacaribe complex (Rowe *et al.*, 1970a), leading to the establishment of a new taxonomic group known as Arenaviridae (Rowe *et al.*, 1970b; Pfau *et al.*, 1974).

Considerable effort has been devoted to study of the pathobiology of Junin virus both in human patients and animal models, as reviewed by Weissenbacher and Damonte (1983). However, the biochemical characterization of Junin virus and the molecular biology of the infection have received less attention in the same period, as is apparent from the list of published results.

III. STRUCTURAL COMPONENTS OF JUNIN VIRUS

A. Virus Growth and Purification

The biochemical studies on Junin virus were initially precluded by the low titers obtained in tissue cultures (Martinez Segovia *et al.*, 1967).

In 1974 Martinez Segovia *et al.* described a convenient procedure for the production of Junin virus in BHK-21 cell cultures. Daily changes of the supernatant culture medium resulted in maximum virus yields at days 4, 5, and 6 postinfection quantitated as 10^6 to 10^7 LD₅₀/ml by intracerebral inoculation in suckling mice. Several methods have been used to concentrate the viral particles, including an aqueous biphasic polymer system (Martinez Segovia and Diaz, 1968), precipitation with protamine sulfate (Coto and Parodi, 1968) or ammonium sulfate (Help *et al.*, 1970), and ultracentrifugation (Anon *et al.*, 1976). The latter method has been the most popular owing to its simplicity and the good recovery of virus infectivity. Junin virus purification described by Anon *et al.* (1976) included two successive sucrose gradient steps and yielded virus particles sufficiently pure for RNA composition analysis. However, this type of gradient did not eliminate the bovine serum proteins that came from the tissue culture medium. A more efficient purification procedure using a potassium tartrate-glycerol step gradient and a cesium chloride continuous gradient produced virions completely devoid of foreign proteins as judged by Coomassie blue staining or *in vitro* iodination (Grau *et al.*, 1981; Romanowski, 1981). These data suggest that alternative purification procedures should always include a high-salt step to strip off the contaminating serum proteins.

Buoyant densities of virus particles from different sources were reported to range from 1.12 to 1.17 g/cm³ in sucrose gradients (Coto *et al.*, 1972; Johnson *et al.*, 1973; Anon *et al.*, 1976). The reason for this variation could be more related to the different amounts of contaminating proteins than to the pleomorphic nature of the viruses. Purified Junin virus particles float at a density of 1.20 g/cm³ in CsCl (Romanowski, 1981).

B. Proteins

The structural polypeptides of Junin virus have been examined in preparations of virus particles metabolically labeled with ³H or ¹⁴C amino acids. In these studies the MC2 strain of Junin virus was grown in BHK-21 cell monolayers and recovered from the supernatant culture medium. Martinez Segovia and De Mitri (1977) reported six structural proteins with estimated molecular weights of 91, 71, 64, 52, 38, and 25 kDa. The 91-kDa, 71-kDa, 52-kDa, and 38-kDa polypeptides were also labeled with [¹⁴C]glucosamine.

On the other hand, Grau *et al.* (1981) showed a more simple protein pattern for Junin virus particles (MC2 and XJCl3 strains). Three polypeptides with molecular weights of 60, 44, and 35 kDa were labeled with [³⁵S]methionine or [³H]leucine. When [³H]glucosamine was used as radioactive precursor, the 35-kDa polypeptide was intensely labeled and smaller amounts of [³H]glucosamine were found in the 44-kDa protein. In this study the SDS-PAGE pattern of labeled proteins remained un-

changed throughout the purification procedure, rendering coincident patterns of bands in the stained and labeled gels at the last purification step.

The apparent discrepancy on the protein composition of Junin virus published by two laboratories is not very big if one considers that both report two major polypeptides: a 64-kDa or 60-kDa sharp and most prominent band and a 38-kDa or 35-kDa broad glycoprotein band. The 52-kDa glycoprotein in Martinez Segovia and De Mitri's paper (1977) could well be the 44-kDa polypeptide reported by Grau *et al.* (1981), since different polyacrylamide gel electrophoresis systems were employed in both studies. The 91-kDa glycoprotein is thought to be an aggregation artifact in the gel system used by Martinez Segovia and De Mitri (1977). They also report that the proportion of the 25-kDa polypeptide is variable and, sometimes, is not detected. A polypeptide of a similar size has been shown in some preparations of other arenaviruses (Clegg and Lloyd, 1983; Young *et al.*, 1987) and might be a proteolytic cleavage product of the 60-kDa protein. And, at last, the 72-kDa glycosylated polypeptide could reflect the incorporation of the uncleaved glycoprotein precursor GPC (Rustici, 1984; De Mitri and Martinez Segovia, 1985) into virions. Buchmeier *et al.* (1987) have occasionally observed traces of GPC in purified LCM virions.

After all, several laboratories have produced data for other arenaviruses that were also dissimilar to some extent (Gard *et al.*, 1977; Gangemi *et al.*, 1978; Ramos *et al.*, 1972; Vezza *et al.*, 1977; Young *et al.*, 1981; Kiley *et al.*, 1981; Clegg and Lloyd, 1983).

C. Nucleocapsids

Electron microscopic studies have not shown an electron-dense, highly structured nucleocapsid in the arenavirus particles. However, when virions disrupted with nonionic detergents, such as Nonidet P-40, were centrifuged to equilibrium in CsCl gradients, a ribonucleoprotein structure banded at densities of 1.31–1.37 g/cm³. Examination of these structures derived from Tacaribe virus under the electron microscope revealed closed circular structures which appeared as strings of beads (Palmer *et al.*, 1977). The length of the circles fell into two predominant size classes, 640 nm and 1300 nm, which most likely correspond to both genomic RNAs known to be roughly 3.5 kb (S RNA) and 7 kb (L RNA) in length (Franze-Fernandez *et al.*, 1987; Iapalucci *et al.*, 1989 a,b). Vezza *et al.* (1977) also reported circular filamentous structures 10–15 nm in diameter for Pichinde virus ribonucleoproteins. More recently, Young and Howard (1983) were able to reveal a more detailed structure consisting of globular 4- to 5-nm subunits that appeared to fold into helical fiber structures 12–15 nm in diameter.

Martinez-Segovia and De Mitri (1977) reported that Junin virions

disrupted with 2% Triton X-100 and banded in a CsCl gradient contained RNA associated with the major 64-kDa structural protein. When analyzed by ultracentrifugation in sucrose gradients, Junin virus treated with 0.12% Nonidet P-40 was shown to dissociate into a soluble protein fraction, containing only the glycoproteins, and a fast sedimenting (>170 S) nucleoprotein component containing RNA and only the 60-kDa major structural polypeptide (Grau *et al.*, 1981; Romanowski, 1981). This 60-kDa polypeptide will be called N (for nucleocapsid protein) throughout the rest of this text.

The N protein as well as its proteolytic cleavage products occurring in infected cells have been shown to react in complement fixation (CF) tests (De Mitri and Martinez Segovia, 1980). This test was earlier used to examine serological interrelationships between members of the Arenaviridae family (Rowe *et al.*, 1970a; Casals *et al.*, 1975). Viruses belonging to the New World arenavirus group or Tacaribe complex exhibit more extensive cross-reactivities than those shown between members of this group and the Old World arenaviruses LCM and Lassa.

Using immunoprecipitation of radiolabeled virus proteins with homologous and heterologous antisera, it was demonstrated that the N protein was responsible for the antigenic cross-reactivity among the Tacaribe complex arenaviruses (Buchmeier and Oldstone, 1978). Moreover, among the monoclonal antibodies raised against Pichinde virus, several were shown to react with one or more heterologous viruses and one of them cross-reacted broadly with five New World and two Old World arenaviruses (Buchmeier *et al.*, 1981). Although, the epitopes responsible for the serological cross-reactivity among N proteins of different arenaviruses have not been identified, cloning and sequencing of Pichinde and LCM S RNA showed a high degree of conservation in several regions of the N protein amino acid sequence (Romanowski and Bishop, 1985).

D. Viral Envelope

Electron microscopic studies show round or oval particles of Junin virus surrounded by a lipid bilayer with 10-nm surface projections or spikes (Lascano and Berria, 1974). The viral envelope is acquired during virus budding at the cellular plasma membrane (Murphy *et al.*, 1970; Murphy and Whitfield, 1975; Lascano and Berria, 1974). This aspect of Junin virus morphogenesis is supported by comparative data on the phospholipid composition of viruses and host cells (Rosas *et al.*, 1988; Rosas, 1984).

The solubilization of the viral envelope using nonionic detergents removes the 35- to 38-kDa major glycoprotein (Grau *et al.*, 1981; Cresta *et al.*, 1980). This protein is exposed on the surface of the viral envelope as shown by *in vitro* iodination of intact highly purified virions (Roman-

owski, 1981). Removal of glycoproteins by protease treatment of Pichinde virus coincided with the disappearance of spike structures on the virus envelope (Vezza *et al.*, 1977). By analogy, it is deduced that the 35- to 38-kDa glycoprotein builds the spikes on the surface of Junin virions.

Pulse-chase experiments of Junin virus-infected BHK-21 cells have demonstrated that a 34-kDa glycoprotein is formed from a 57-kDa precursor. No other glucosamine-labeled polypeptides have been observed during the processing of the 57-kDa protein (GPC?) (Rustici, 1984). However, in most of the gel electrophoretic analyses of mature virus proteins the major glycoprotein band appears often as a doublet peaking at 34 kDa and 39 kDa (Grau *et al.*, 1981). This observation has been *a priori* attributed to a difference in the glycosylation of a single polypeptide, but has not been confirmed by peptide mapping. In relation to this possibility, it is worth mentioning that only one structural glycoprotein has been reported for Tacaribe virus, a very close serological relative of Junin virus (Gard *et al.*, 1977; Boersma *et al.*, 1982). More recent studies have indicated that the broad glycoprotein band found in Tacaribe virions actually contains two different polypeptide species, one of which is partially lost during some purification procedures using high-salt solutions (Burns and Buchmeier, personal communication, and see their Chapter 2, this volume).

The antigenic potential of Junin virus major glycoprotein has been explored by Cresta *et al.* (1980), who have shown that it elicits neutralizing antibodies. In this respect, this glycoprotein resembles the behavior of LCM virus GP-1 and Lassa virus G1. The inoculation of the purified protein protected guinea pigs from a challenge with 10 LD₅₀ of a pathogenic Junin virus strain. Therefore, it is reasonable to expect that the cloned glycoprotein gene, or a fragment of it, expressed in a suitable host-vector system could be used as a candidate vaccine.

Regarding the 44-kDa minor glycoprotein of Junin virus, no data are available on its localization in the viral envelope or its relationship to the GPC.

E. Viral RNA

Indirect evidence arguing for an RNA genome in Junin virus was published in 1969 (Martinez Segovia and Graziola, 1969; Coto and Vombergar, 1969). Only after a large-scale virus growth was optimized (Martinez Segovia *et al.*, 1974; Anon *et al.*, 1976) was biochemical analysis of the genome possible.

The genome of Junin virus consists of two single-stranded RNA species described in the original report by Anon *et al.* (1976) as 33S and 25S. Their molecular weights were estimated to be 2.4×10^6 and 1.3×10^6 , respectively, from the electrophoretic mobilities in 2.7% polyacrylamide gels. The apparent molecular weights of the two genomic

RNA species, known now as L (large) and S (small), estimated from their mobility in denaturing agarose gels using CH_3HgOH , are very similar to the ones previously described (Romanowski *et al.*, unpublished results).

In addition to the genomic RNA, 28S, 18S, and 4–6S RNAs of cellular origin were found to account for 50% or even more of the [^3H]uridine incorporated into the viruses. These RNAs come from the ribosomes and tRNAs incorporated into the virions in quite variable proportions in different virus preparations (Rawls and Leung, 1979).

Electron micrographs normally show that variable numbers of ribosomal granules are packaged in virions that range in diameter from 50 to 300 nm (Murphy and Whitfield, 1975). Using cells with a *ts* mutation that affected the 60S ribosomal subunit, Leung and Rawls (1977) demonstrated that Pichinde virus-associated ribosomes were not required for virus replication. In these experiments, the virus was first grown in the *ts* cells and its progeny, containing *ts* ribosomes, was subsequently replicated successfully in wild-type cells at either the permissive or the non-permissive temperature.

On the other hand, Mannweiler and Lehmann-Grube (1973) have observed small ribosome-less LCM virus particles and suggested that these were the truly infective units, whereas the larger particles containing the electron-dense granules were not infectious. A large proportion of similar 60-nm particles were observed in tissues from AHF patients in addition to the more classical larger particles with ribosomes (Maiztegui *et al.*, 1975). Consistent with these data, filtration of Junin virus through membranes with different pore-size distributions suggested that infectivity was associated with 40 to 70 nm particles (Coto *et al.*, 1972).

In addition to the variability in the amount of ribosomal RNAs between different virus populations (preparations) and among virions within the same population, a variability in the L RNA to S RNA ratio can be noted in the literature reports (see Table I). In all the arenaviruses studied, S RNA molecules are present in excess relative to the L RNA.

TABLE I. Molar Ratios of L and S RNAs in Purified Virions^a

Virus	Radioactivity ratio (L-RNA/S-RNA)	Molar ratio (S-RNA/L-RNA)	Ref.
Junin	1.3	1.5	Anon <i>et al.</i> (1976)
LCM	0.7–0.4	2.9–5.0	Pedersen (1973)
LCM	0.55	3.6	Romanowski and Bishop (1983)
Pichindé	1.0	2.0	Carter <i>et al.</i> (1973)
Pichindé	1.1	1.8	Farber and Rawls (1975)
Pichindé	<1.0	>2.0	Dutko <i>et al.</i> (1976)
Pichindé	<1.0	>2.0	Vezza <i>et al.</i> (1978)

^a The incorporation of radioactivity precursors ([^{32}P]-orthophosphate or [^3H]-uridine) into the different RNA species of purified virions was used to calculate the relative molar amounts of L and S RNA. To this end, the molecular size ratio of L to S RNA was considered to be roughly 2 and the proportion of uridine in the RNAs was assumed to be 25%.

The nonequimolar proportions of L and S RNAs could be explained through the formation of virions containing multiple copies of viral nucleocapsids, or different amounts of virions that have packaged S or L nucleocapsid only. A not very accurate packaging mechanism for the arenavirus particles would not be surprising since different amounts of ribosomes are included in the virions budding from the infected cells. In fact, the formation of arenaviruses that are genetically diploid for S RNA has been demonstrated in the progeny of cells coinfecting with complementing ts mutants of two LCM virus strains (Romanowski and Bishop, 1983).

The formation of virus particles was also studied from a kinetic perspective using a double isotope label technique (Lopez *et al.*, 1986). In this approach, Junin virus-infected BHK-21 cells were first labeled with [^{14}C]uridine until a constant specific activity was reached in the RNA of the virions recovered from the supernatant medium. Only then the medium was changed to one containing both [^{14}C]uridine (with a specific activity equal to the one used in the previous step) and [^3H]uridine. The variation of the $^3\text{H}/^{14}\text{C}$ ratio in the virus RNA collected at different time intervals after the addition of [^3H]uridine was used to calculate the generation time of a virus particle. This time was estimated to be 5 hr for Junin virus and includes the replication, assembly, and release of a virion to the medium at the stationary phase of virus growth.

The same authors also demonstrated that actinomycin D affects neither arenavirus RNA synthesis nor virus yields in tissue culture, when the times of exposure to the drug are shorter than the time when cell-toxic effects become evident. These results indicate that continuous host transcription is not required for replication of the arenaviruses.

IV. JUNIN VIRUS S RNA

A. Molecular Cloning and Sequence Analysis of Junin Virus S RNA

As most of the biochemical data were derived from the MC2 strain of Junin virus, it was also chosen for the first cDNA cloning and sequencing studies. RNA extracted from plaque-cloned virus grown in BHK-21 cells was used for cDNA synthesis in several rounds. Based on the published 3' end sequence of different arenaviruses (Aupein *et al.*, 1982; i.e., GCGUGUACCUAGGAUCCG), a complementary synthetic oligodeoxyribonucleotide was used to prime cDNA synthesis in a protocol similar to the one described by Gubler and Hoffman (1983). Double-stranded cDNA was inserted into pUC plasmid vectors and cloned in *Escherichia coli* DH5 α . Initial screening of the cDNA library using a short copy cDNA probe (Bishop *et al.*, 1982) yielded clones that belonged to both L and S viral RNAs as shown by Northern blot analysis.

Analogous results have also been observed during the cloning of LCM-WE S RNA (Romanowski and Bishop, 1985). The recovery of L cDNA clones using an S RNA primer was presumably due to the similarity of the L and S 3' end sequences.

Since full-length cDNA clones were not obtained, a primer complementary to the distal end of the first set of S RNA clones was used for a second round of cDNA synthesis and cloning (see Fig. 1). A third round of cDNA synthesis and cloning was necessary to reach the 5' end of Junin virus S RNA.

The DNA sequences of partially overlapping clones were determined according to the method of Sanger *et al.* (1977). Some regions of these clones were also sequenced using the chemical modification method of Maxam and Gilbert (1980). Nucleotide sequences in the overlapping regions of the inserts were identical (Fig. 1). The 5' and 3' terminal nucleotide sequences were carefully confirmed in additional experiments. The 5' end of the S RNA was confirmed by primer extension using an oligonucleotide complementary to a sequence near this

FIGURE 1. Cloning and sequencing strategy of Junin virus S RNA. The sequence of S RNA nucleotide is represented in the viral sense with a size scale in kilobases above. Some of the overlapping clones spanning the entire S RNA are shown as rectangles. Phosphorylated synthetic oligonucleotides that were used to prime cDNA synthesis are indicated as black boxes at the beginning of each clone. Subcloning into M13 mpl8 and mpl9, 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 sequences were obtained using a modified T7 DNA polymerase (SequenaseTM, USB) as shown by the continuous arrows. A region of the sequence (nucleotides 2472–3400) determined by the chemical cleavage method of Maxam and Gilbert (1980) is indicated by dashed arrows representing the restriction fragments involved. The thick arrow indicates the 5' end sequence confirmed by primer extension using a synthetic oligonucleotide complementary to Junin virus S RNA nucleotides 143–194 (Ghiringhelli *et al.*, 1991).

end. The 3' end sequence of the viral RNA was deduced from primer extension products generated using intracellular viral-complementary full-length S RNA as template and was found to be coincident with the cloned cDNA sequence derived from viral genomic RNA. The 19 nucleotides at the 3' terminus were identical to the direct RNA sequence data derived for other arenaviruses by Auperin *et al.* (1982). This analysis showed that the complete S RNA sequence comprised 3400 nucleotides with a base ratio of 26.6% A, 21.0% G, 23.8% C, and 28.6% U (Fig. 2) (Ghiringhelli *et al.*, 1991).

The analysis of the nucleotide sequence indicates the existence of two nonoverlapping long open reading frames of opposite polarities (Fig. 3). They were deduced to code for the N protein and GPC protein, by analogy to the other arenaviruses and confirmed by Western blot analyses of recombinant products using bacterial and eukaryotic expression systems (Rivera-Pomar *et al.*, 1990, 1991; Romanowski *et al.*, unpublished results). One open reading frame, in the viral-complementary

FIGURE 2. Nucleotide sequence of Junin virus S RNA. The 3400-nucleotide-long sequence of the genome or viral S RNA of Junin virus is presented and the orientation of the open reading frames corresponding to the GPC and N genes is indicated by arrows on the sides. The translational initiation and termination codons of GPC and the corresponding anticodons of N (encoded in the viral-complementary sequence) are boxed. The inverted repeats at the intergenic region are underlined.

FIGURE 3. Schematic of the gene arrangement in Junin virus S RNA. The "ambisense" S RNA of Junin virus is shown divided into a negative (–) and positive (+) region encoding the N gene in the (–) or viral complementary (VC) and the GPC gene in the (+) or viral (genomic) polarity RNA (V). The open reading frames corresponding to the N and GPC genes are shown as filled and open rectangles, respectively, with arrowheads indicating the direction of translation. The subgenomic mRNAs (correlating well with size and polarity detected in cytoplasmic RNA of infected cells) are also shown, the asterisks indicating nontemplated nucleotide sequences at their 5' ends (Rivera-Pomar, 1991). The thin curved arrows indicate explicitly the template–transcript relationships among the different RNA species.

(vc) RNA sequence, extends from nucleotides 82–84 (AUG) to nucleotides 1774–1776 (UAA) and codes for the nucleocapsid protein, N (Fig. 4, Ghiringhelli *et al.*, 1989). The other open reading frame, encoding the GPC (glycoprotein precursor) in the viral genome-sense (v) RNA sequence starts at positions 88–90 (AUG; CAU vc sequence residues 3310–3312) and stops at positions 1531–1533 (UAA, UUA vc sequence residues 1867–1869, Fig. 7).

B. The Nucleocapsid Protein Gene (N)

The first AUG in the vc S RNA at positions 82–84 is probably the true initiation codon for the N protein. Examination of the surrounding nucleotide sequences indicates that this AUG is placed in a favorable context (CTGGCAUGG) according to the consensus nucleotides identified by Kozak (1978, 1984) as the most frequent flanking sequence for eukaryotic translation initiation codons (i.e., CC_ACC AUG G). The 1692-nucleotides-long open reading frame specifies a polypeptide of 564 amino acids with a molecular weight of 63,033 daltons, in good agreement with the estimates based on electrophoretic mobility (Martinez Segovia and De Mitri, 1977; Grau *et al.*, 1981). Assuming, for simplicity, the charge of amino acids at neutral pH for K or R as +1, for H as +0.5, and for D and E as –1, the N protein has a net charge of +14.5. Although most of the positively charged amino acid residues are scattered throughout the protein sequence, some clustering of Ks and/or Rs may be noted. A strongly positive KKKR center is located at amino acid

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1  MAHS[K]EVPSF [K]WTQSI[R]RGL SQFTQTV[K]SD V[K]DA[K]LIAD SIDFNQVAQV
51  Q[RAI]R[K]K[K]G EEDLN[K]L[R]DL N[K]EVD[R]LMSM [R]SVQ[R]NTVF[K] AGDLG[R]VERM
101 ELASGLGNL[K] T[K]F[R]R[RA]ETGS QGVYMGNLSQ SQLA[K]R[SE]IL [R]TLGFQQQGT
151 GGNGVV[R]VWD V[K]DPS[K]LNNQ FGSVPALTIA CMTVQGETM NSVIQALTSL
201 GLLYTV[K]YPN LSDLD[R]LTQE HDCLQIVT[K]D ESSINISGYN FLSAAV[K]AG
251 ASILDDGNML ETIR[V]TPDNF SSLI[K]STIQV [K]R[RE]GMFIDE [K]PGN[R]NPYEN
301 LLY[K]LCLSGD GWPYIGS[R]SQ IIG[R]SWDNTS IDLT[R]K[P]VAG P[RO]P[R]E[K]NGQN
351 I[R]LANLTEIQ EAVI[R]EAVG[K] LDPTNTLWLD IEGPATDPVE MALFQPAGS[K]
401 YIHCF[R]KPHD E[K]G[F]KNGSRH SHGILM[K]DIE DAMPGVLSYV IGLLPDMVV
451 TTQGSDDI[R]K LFDLHG[R]RDL [K]LVDV[R]LTSE QARQFDQQVW E[K]FGHLC[K]HH
501 NGVVVS[K]K[K]K[R] D[K]DAPF[K]LAS SEPHCALLDC IMFQSVLDG[K] LYEEELTPLL
551 PPSLLFLP[K]A AYAL*

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FIGURE 4. Amino acid sequence of Junin virus N protein. Translation product of the N ORF from Fig. 2. The basic amino acid residues K and R are boxed and clusters of two, three, and four such residues are shaded.

residues 507–510, a triple basic KRR sequence at amino acid positions 281–283, and nine groups of two basic K and/or R residues.

The relatively high frequency of positively charged amino acid residues in the N protein sequence conforms well to the idea of interaction with the negatively charged RNA in the viral nucleocapsids. However, no homology to the eukaryotic RNP consensus sequence was found (Dreyfuss *et al.*, 1988). It has also been suggested that N could play a role in the regulation of the transcription/replication process during arenavirus infection (Ghiringhelli *et al.*, 1991; Iapalucci *et al.*, 1991). On the other hand, there is some evidence that N-derived polypeptides can enter the cell nucleus (Young *et al.*, 1987). The essence of a nuclear targeting sequence appears to be a short run of basic amino acids such as those found in Junin virus N residues 507–510 or 55–59 (Hunt, 1989). However, this sequence, while probably necessary, is not sufficient to ensure nuclear entry. At the present time, the nuclear targeting of N requires further investigation.

The alignment of the amino acid sequences of the N gene products of Junin and Tacaribe viruses reveals a sequence identity as high as 76%. If the same comparison is made with other New World and Old World arenaviruses the sequence homologies fall within the range of 53–47%

FIGURE 5. Comparison of amino acid sequence of the N proteins. Sequences of the Old World arenaviruses Lassa [LAS, Clegg and Oram, 1985] and LCM-WE [LCM, Romanowski and Bishop, 1985] and the New World arenaviruses Tacaribe [TAC, Franze-Fernandez *et al.*, 1987] and Pichinde [PIC, Aupein *et al.*, 1984] with that of Junin N polypeptide [JUN, Ghiringhelli *et al.*, 1989]. The amino acid residues identical to those of JUN N 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 to obtain a best-fit alignment. The complete amino acid sequence of JUN N protein, which was used as reference, is printed above the comparison of the five N proteins (Ghiringhelli *et al.*, 1991).

TABLE II. Amino Acid Sequence Homology of Arenavirus Structural Polypeptides

	TAC	PIC	LCM	LAS	TAC	PIC	LCM	LAS	TAC	PIC	LCM	LAS	
JUN	76	54	49	47	50	20	21	21	82	57	53	57	A
TAC		54	48	48		24	16	17		51	51	52	
PIC			49	49			20	26			52	54	
LCM				60				27				69	
JUN	87	70	66	65	69	43	42	44	92	72	70	68	B
TAC		69	64	64		41	38	36		70	69	67	
PIC			65	66			41	47			68	69	
LCM				73				44				83	
	N				G1				G2				
	GPC												

(A) The figures represent the percentages of amino acid identities obtained by the comparison between the different arenavirus proteins. Gaps were introduced in the amino acid sequences of pairs of homologous proteins to produce a best-fit alignment, but conservative changes were not considered for the calculation of homology percentages (the names of the different arenaviruses are abbreviated as in Fig. 5). N: nucleocapsid protein. The amino acid sequence of the glycoprotein precursor (GPC) was formally divided into the amino-terminal half G1 and the carboxy-terminal half G2. The cleavage site demonstrated for LCM virus GPC and the homologous sequences in the other arenaviruses were used to formally define the G1 and G2 regions (see Fig. 8).

(B) Sequence homology (%) including conservative changes according to Schwartz and Dayhoff (1979).

(Fig. 5 and Table II). These homology figures do not take into account substitutions representing amino acid residues with similar biochemical characteristics (e.g., R-K, D-E, V-I, etc.). In addition, the conservation of many cysteine (C), glycine (G), and proline (P) residues suggests that the three-dimensional structures of the arenavirus N proteins are more conserved than indicated by simple comparison of their primary structures. The amino acid sequence similarities between the N proteins are in agreement with the monoclonal antibodies cross-reactivities reported by Howard *et al.* (1985), Sanchez *et al.* (1989), and Buchmeier *et al.* (1981). In particular, the outstanding degree of cross-reactivity between Junin and Tacaribe viruses is highlighted by the percentage of sequence homology in Table II.

C. Expression of the Nucleocapsid Protein Gene in Transfected Cells

When the cloned genes for the nucleocapsid proteins of Junin and LCM viruses were inserted into an SV40-derived expression vector and transfected into BHK-21 and CV-1 cells, the transient expression yielded a polypeptide biochemically and immunologically identical to the N protein synthesized during viral infection (Rivera-Pomar *et al.*, 1991). The N polypeptide was detected by indirect immunofluorescence

in 5–20% of the cells, so that the rest of the cells could be used as internal negative controls. The level of expression of Junin and LCM virus nucleocapsid protein in these transfected cells was found to be roughly similar to that observed in infected BHK-21 and CV-1 cell lines. Therefore, one would expect the N protein synthesized in the transfected cell to behave in a manner similar to that observed in virus-infected cells (Weissenbacher and Damonte, 1983).

A cytoplasmic localization was determined for the N polypeptide expressed in cells transfected with Junin as well as LCM virus N genes. There are some reports of the nucleocapsid protein found in the nucleus of Pichinde virus-infected cells (Young *et al.*, 1987) and in the membrane of L cells infected with LCM virus (Zeller *et al.*, 1986, 1988). However, Rivera-Pomar *et al.*, (1990, 1991) could not demonstrate these localizations either for the transfected cells or for the infected ones. The patterns of immunofluorescence are variable and match those found in cells infected with these arenaviruses, i.e., varying from diffuse cytoplasmic staining to granules either distributed throughout the cytoplasm or concentrated in the perinuclear region. In particular, a speckled pattern of immunofluorescence has been observed coincident with previous reports on arenavirus-infected cells (Weissenbacher and Damonte, 1983).

These studies suggest that N protein is associated with basophilic granules. Such basophilic particles have previously been described in infected cells as part of the cytopathic effect (Bruno-Lobo *et al.*, 1968; Weissenbacher and Damonte, 1983). The above mentioned association is possibly related to the biochemical properties of the polypeptides, exhibiting clusters of basic amino acids (Romanowski and Bishop, 1985; Ghiringhelli *et al.*, 1989 and 1991). These polypeptides might interact with cytoplasmic RNA to form ribonucleoprotein complexes.

The association of N protein with cytoplasmic granules that undergo rearrangements at different stages of the cell cycle suggests a behavior similar to that of a newly described group of ribonucleoprotein particles. These large virus-like structures named "vaults" have been found in the cytoplasm of higher and lower eukaryotes and reported to associate with cytoskeletal elements (Kedersha and Rome, 1990). The regular appearance of extensive granulation in the cytoplasm of N-expressing cells suggests a possible link between the synthesis of the N protein and the development of the cytopathic effect (CPE) during arenavirus infection.

The use of SV40-derived expression vectors bearing the *E. coli* neomycin resistance gene permitted the isolation of BHK-21 and CV-1 cell lines stably expressing N. CV1 and Vero cells that expressed LCM or Junin virus N protein showed a marked CPE, similar to that observed in infected cells. The CPE led to cell lysis after few days, thus preventing the isolation of stably transformed clones. On the contrary, it was possible to isolate G418-resistant clones from transfected BHK-21 cultures.

FIGURE 6. Transfected BHK-21 cells stably expressing Junin virus N protein. Cell monolayers were transfected with either the SV40-derived vector pKG4 or the recombinant expression plasmid pKG4-NJUN containing the N ORF of Junin virus in the appropriate orientation. Stable transfectant clones were selected using the neomycin analog G418 and propagated in selective medium. The BHK-21 cells transfected with pKG4-NJUN yielded clones where all or none of the cells expressed N, depending on the mode of integration of the plasmids. Shown are G418-resistant BHK cells that do not express N (N-) and those that do (N+). The phase-contrast micrographs (200 $\times$ magnification) were taken without prior fixation of the cell monolayers to prevent artifactual distortion of the cell morphology. Examination of fixed cells using indirect immunofluorescence detected N in 100% of the cells of the G418-resistant clones that exhibited altered cell morphology, such as shown in (N+), whereas the other G418-resistant clones that did not express N (such as the one in N-), derived from pKG4 or pKG4.N JUN, had no signs of CPE (Rivera-Pomar *et al.*, 1991; Rivera-Pomar, 1991).

BHK-21 clones expressing the N gene showed cytoplasmic granulation, vacuolization, polykaryosis, and altered cell shape (Fig. 6; Rivera-Pomar *et al.*, 1990, 1991). Further studies will improve our understanding of the mechanisms leading to these dramatic changes in cell morphology.

D. The Glycoprotein Precursor Gene (GPC)

The first AUG codon of the viral-sense S RNA of Junin virus is found at 88–90 nucleotides from the 5' end. Its flanking nucleotides (i.e., GUACAAUGG) match the consensus purine residue at position –3 and the G at +4, observed in all arenavirus genes, and also contain one of the C residues identified in the upstream region of Kozak's most frequent flanking sequence to eukaryotic translation initiation codons (Kozak, 1984). A second AUG is found within the next seven codons in frame with the first one, but lacking the G at position +4 and the A or G at position –3. Therefore, the first AUG triplet is deduced to be the initiating codon for a 481-amino-acids long polypeptide.

The calculated molecular weight of the GPC gene product (i.e., 55,126 daltons) is smaller than the molecular weight determined by gel electrophoresis for the intracellular glycoprotein precursor (De Mitri and Martinez Segovia, 1985; Rustici, 1984). The cotranslational glycosylation renders an obvious explanation to this experimental observation. Eight potential N-linked glycosylation sites are found in the GPC sequence (Fig. 7). Whether all are actually used for attachment of carbohydrate side chains is not known. However, one should note that four of the potential glycosylation sites are conserved among all the arenavirus GPC genes analyzed to date (Fig. 8), suggesting their functional relevance. When all the available amino acid sequences of arenavirus GPC genes are compared, it is found that the region proximal to the carboxyl-terminus contains a high proportion of homologous stretches, while the amino-terminal sequences diverge considerably (see Fig. 8, Table II). However, if Junin virus GPC gene product is compared to the homologous protein of Tacaribe virus, it is noticed that the first 40 amino acids from the amino-terminus are conserved with just five amino acid substitutions, only two of which could be considered nonconservative changes (i.e., Ala for Cys, Thr for Ile).

Recently, a live Junin virus vaccine, named Candid 1, was developed to control AHF. The GPC gene of this highly attenuated virus was cloned and sequenced, and its ORF was compared to the homologous regions in the S RNA of the wild-type MC2 strain. The nucleotide changes concentrate in the NH₂-proximal (20 nucleotide changes) and the COOH-proximal (19 nucleotide substitutions) regions. However, major changes in the amino acid sequence occur only in the amino-terminal region of GPC as a result of several insertions and deletions in the nucleotide sequence affecting codons 43–80. After proteolytic cleav-

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1   MGQFISFMQE IPTFLQEALN IALVAVSLIA IIKGVVNLYK SGCSILDLAG
51  RSCPRAFKIG LHTEVPDCVL LQWVVSFSNN PHDLPLLCTL NKSHLYIKGG
101 NASFKISFDD IAVLLPEYDV IIQHPADMSW CSKSDQIRL SQWFMNAVGH
151 DWYLDPPFLC RNRTKTEGFI FQVNTSKTGI NENYAKKFKT GMHHLRYEYP
201 DSCLDGKLCL MKAQPTSWPL QCPLDHVNTL HFLTRGKNIQ LPRSLKAFF
251 SWSLTSSGK DTPGGYCLEE WMLVAAKMKC FGNTAVAKCN LNHDFEFCDM
301 LRLFDYNKNA IKTLNDETKK QVNLMGQTIN ALISDNLLMK NKIRELMSVP
351 YCNVTKFWYV NHTLSGQHSL PRCWLIKNNS YLNNISDFRND WILESDFLIS
401 EMLSKEYSDR QGKTPLTLVD ICFWSTVFFT ASLFLHLVGI PTHRHIRGEA
451 CPLPHRLNSL GGCRCGKYPN LKKPTVWRRG H*

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FIGURE 7. Amino acid sequence of Junin virus GPC gene product. The putative N-glycosylation sites are boxed; those that are also conserved among the rest of the arenavirus GPC proteins are indicated by the shaded boxes. The double basic amino acid sequence identified by Buchmeier *et al.* (1987) as the putative proteolytic cleavage site is underlined.

age of GPC, these alterations appear in the G1 polypeptide, which is thought to be located on the surface of the virion in association with the more internal G2 protein. Although these findings are strongly suggestive of the genetic changes that lead to an attenuated phenotype, they must be complemented with studies on the direct predecessors of Candid 1, which exhibit gradually more virulent phenotypes, in order to locate precisely the mutations responsible for attenuation.

E. Proteolytic Cleavage Site for the Processing of GPC

Most of the arenaviruses have been shown to exhibit a similar glycoprotein-processing pattern, differing only in the apparent molecular weights of the GPC precursor and the processed viral glycoproteins G1 (or GP1) and G2 (or GP2) (Buchmeier and Oldstone, 1978; Compans and Bishop, 1985; Saleh *et al.*, 1979; Grau *et al.*, 1981; Harnish *et al.*, 1981; Lukashovich *et al.*, 1985; De Mitri and Martinez Segovia, 1985; Clegg and Lloyd, 1983). However, only one structural glycoprotein (G) has been reported for Tacaribe and Tamiami virions (Gard *et al.*, 1977; Boersma *et al.*, 1982). This apparent discrepancy is due to the selective loss of GP1 during virus purification (see Chapter 2, this volume).

In LCM virus a mannose-rich precursor, GPC (75 kDa) is posttrans-

lationally modified by proteolytic cleavage and oligosaccharide processing to yield the glycoproteins GP1 (44 kDa) and GP2 (35 kDa). Buchmeier *et al.* (1987) have mapped the cleavage site to be a paired basic amino acid group Arg-Arg corresponding to amino acids 262-263. This Arg-Arg pair is followed by a predominantly hydrophobic stretch of some 23 amino acids. These two features are highly conserved among the LCM (strains WE and ARM), Lassa, Pichinde, and Junin virus GPC proteins (Fig. 8). In Pichinde virus GPC the Arg-Arg pair is replaced by Arg-Lys, of similar characteristics.

The hypothesis, confirmed for LCM-ARM GPC, is that a protease of cellular origin cleaves the GPC at or near this Arg-Lys or Arg-Arg sequence generating two structural glycoproteins designated GP-1 (for LCM) or G1 (for Pichinde and Lassa) and GP-2 (for LCM) or G2 (for Pichinde and Lassa). Immunoprecipitation with antibodies raised against synthetic peptides (Buchmeier *et al.*, 1987) and limited microsequencing of G1 and G2 tryptic peptides (Auperin *et al.*, 1984) demonstrated that GP-1 or G1 comprises the amino-terminal half of GPC. It is also known that this protein is the antigen that elicits the synthesis of antiviral neutralizing antibodies. On the other hand, GP2 or G2 corresponds to the most conserved carboxy-terminal half of GPC. The amino terminus of GP2/G2, which would be liberated by cleavage at the Arg-Arg or Arg-Lys site, contains the above mentioned consensus hydrophobic sequence.

In this scenario, Junin virus major 35-kDa glycoprotein, which elicits a neutralizing antibody response, should be homologous to LCM, Lassa, and Pichinde virus G1 protein, whereas the 44-kDa protein could be the counterpart of G2. At this point, the functional or biological properties as well as the unambiguous sequence data should prevail over the electrophoretic mobility to designate the Junin virus glycoproteins as G1 and G2.

A particular consideration should be devoted to the comparison of Junin and Tacaribe virus glycoprotein genes. A best fit alignment of the GPC genes shows that 66% of the amino acids are conserved in identity and position. When the G1- and G2-homologous regions are analyzed separately, the percentages of amino acid sequence identity are 50% and 82%, respectively. These figures are by far higher than any of the others presented in Table II.

The putative proteolytic cleavage site in Tacaribe virus GPC does not contain a doublet of basic, amino acid residues. This pair of residues is replaced by one basic amino acid (Arg) followed by a Thr and the consensus 23-amino-acids-long hydrophobic sequence. An alignment of this hydrophobic region with that of Junin virus places the above-mentioned Arg₂₅₈-Thr₂₅₉ pair in register with an Arg₂₄₄-Ser₂₄₅ of Junin virus GPC. The fact that Tacaribe virus GPC is proteolytically processed, together with the existence of other dibasic amino acid sequences in non-conserved regions of arenavirus GPC and N proteins, which are not

known to be cleaved intracellularly, suggests that the double basic amino acid cluster is not an essential feature of the proteolytic target. Most likely, the protease involved in the processing of GPC requires only one basic amino acid in a sequence context that makes the particular peptide bond accessible. The sequence requirements might include all or part of the predominantly hydrophobic 23-amino-acid-long sequence conserved in all the arenaviruses, including Tacaribe (Figure 9).

F. Noncoding Sequences in S RNA 5' and 3' Ends

The N and GPC open reading frames do not overlap and account for 3135 of the total 3400 nucleotides of Junin virus S RNA. The noncoding regions upstream from the N and GPC translation initiation codons comprise 81 and 87 nucleotides, respectively. The lengths of the same regions in different arenavirus S RNAs vary, but are generally shorter than the ones described here for Junin virus.

The first 19 nucleotides at the 3' end of the viral S RNA are known

FIGURE 9. Arenavirus GPC sequence region surrounding the cleavage site (RR). The polypeptides G1 (amino-terminal half) and G2 (carboxy-terminal half) are predicted to flank the cleavage site (Buchmeier *et al.*, 1987). The figures above the sequences indicate the amino acid residue numbers starting at the first met codon of the GPC ORF and including the gaps introduced to obtain the best-fit alignment. A consensus amino acid sequence was derived from the comparison of the aligned arenavirus GPC sequences, grouping the amino acid residues, according to Taylor (1987): hydrophobic (h), hydrophobic-aliphatic (l), charged-acidic (a), charged-basic (b), polar (p); no observation is indicated by dots (.) and gaps are shown using dashes.

to be conserved in the Arenaviridae family. This information was obtained by direct RNA sequencing of Pichinde (prototype and Munchique strain), LCM (Armstrong strain), and Tacaribe virus genomic RNA (Auperin *et al.*, 1982), and by Maxam and Gilbert sequencing of cDNA clones derived from *in vitro* polyadenylated LCM (WE strain) virus S RNA (Romanowski and Bishop, 1985). The characteristic 19-nucleotide-long 3' terminal sequence was also found to be conserved in Junin virus by sequencing the primer extension products complementary to the 5' end of the antigenomic copy of the full-length S RNA (Rivera-Pomar, 1991).

In general, the sequence homology for the 3' termini of arenavirus S RNA does not extend beyond the 19 nucleotides consensus sequence. In this context, the homology between Junin and Tacaribe stands out, extending through nucleotide 53 with only six mismatches. Lassa and LCM viruses are the only other pair of arenaviruses that exhibit a sequence homology beyond the first 19 nucleotides, reaching nucleotide 34 with three mismatches (Clegg and Oram, 1985; Romanowski and Bishop, 1985).

In contrast with the conserved 3' terminus, the 5'-terminal-most sequence of Junin virus S RNA is slightly different from the sequence conserved in Pichinde, LCM, Lassa, and Tacaribe virus (Fig. 10) (Auperin *et al.*, 1984, 1986; Romanowski *et al.*, 1985; Raju *et al.*, 1990). Nevertheless, both ends can be aligned to produce a partially double-stranded secondary structure with a stabilization free energy of ca. -58.0 kcal/mole calculated according to Tinoco *et al.* (1973). The fact that other arenavirus S RNA ends can be also aligned to form a homologous secondary structure is most likely related to the observation of circular forms in preparations of RNAs and nucleocapsids of Pichinde and Tacaribe virions (Vezza *et al.*, 1978; Palmer *et al.*, 1977). Recently, the actual existence of similar panhandle structures has been demonstrated in cross-linking experiments of influenza and La Crosse virus RNAs and nucleocapsids (Hsu *et al.*, 1987; Raju and Kolakofsky, 1989).

On the other hand, the unexpected difference found in a region of the S RNA that was presumed to be more conserved might indicate that the viral RNA polymerase does not recognize an invariant nucleotide sequence at the 3' end when viral or viral-complementary RNA is used as template. In turn, the sequence specificity could be less stringent, or the conserved panhandle secondary structure could be recognized as the RNA polymerase docking signal.

G. Intergenic Region

As mentioned in the previous sections, the coding sequences and part of the noncoding upstream regions exhibit varying degrees of nucleotide sequence homology among the different arenaviruses. How-

FIGURE 10. Predicted panhandle structure in Junin virus S RNA. The noncoding sequences at the 5' and 3' ends were aligned. The first 19 nucleotides at the 3' end base-pair with the complementary sequence at the 5' end forming a secondary structure that is conserved among the arenaviruses (boxed, $\Delta G^\circ = -37.8$ kcal/mole). Complementary nucleotides, indicated by asterisks, hold the 5' and 3' ends of this RNA molecule with a stabilization free energy of -58.0 kcal/mole (Tinoco *et al.*, 1973). G-U pairs are also indicated.

ever, no such sequence conservation can be inferred from the comparison of the noncoding region downstream from the translation termination signals of the N and GPC genes. Conversely, this intergenic region can be arranged in a relatively stable hairpin-loop structure. It is this type of secondary structure that has been conserved throughout the evolution of the arenaviruses, pointing to its functional significance (Auperin *et al.*, 1984, 1986; Romanowski and Bishop, 1985; Franze-Fernandez *et al.*, 1987).

Junin virus exhibits two consecutive very stable hairpin structures ($\Delta G^\circ = -57.2$ and -39.0 kcal/mole) separated by a hinge of two nucleotides instead of a single hairpin loop reported for most of the other arenaviruses (Ghiringhelli *et al.*, 1987, 1991) (Fig. 11). However, more recently a corrected version of Tacaribe virus S RNA and that of the African arenavirus Mopeia were shown to contain also two potential hairpin-loop structures in the intergenic region (Iapalucci *et al.*, 1991; Wilson and Clegg, 1991).

Northern blot analyses indicate that Junin virus-infected cells contain genomic and antigenomic copies of S RNA as well as subgenomic mRNAs (Fig. 3). Nevertheless, when cells are infected in the presence of protein synthesis inhibitors, only the subgenomic N mRNA is transcribed from the S RNA template (Franze-Fernandez *et al.*, 1987; Rivera-Pomar, 1991). This observation and the mRNA size indicate that an antiterminator function is required for the viral RNA polymerase to synthesize a full-length copy of the S RNA, and the antiterminator function is probably supplied by the N protein binding to and destabilizing the intergenic hairpin structures. Although other viral or cellular proteins with high turnover rate could be alternative antiterminators, Junin

FIGURE 11. Intergenic region of Junin virus S RNA. A portion of the S RNA (nucleotides 1519–1639) comprising the ends of the GPC and N genes (sense and anti-sense strand, respectively) and the noncoding intergenic region is shown. 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 ($\Delta G^\circ = -39.0$ and -54.2 kcal/mole).

virus N protein contains a basic amino-acid-rich sequence (RALRKRGE) similar to those found in several RNA-binding proteins such as N anti-terminators of phage lambda, P21 and P22, some ribosomal proteins, the HIV Tat, and others (Lazinski *et al.*, 1989).

The proposed mechanism of transcription termination consists in the formation of a hairpin loop at the 3' end of N mRNA, when the intergenic region is copied, leading to the release of RNA polymerase. The binding of N to the RNA would partially melt the RNA structure, allowing the polymerase to continue the transcription process. In this perspective, RNase protection experiments indicated that the 3' termini of the subgenomic mRNAs are complementary to the first hairpin-loop-forming sequence encountered by the RNA polymerase during transcription of the intergenic region. This information correlates with the proposed model, and the role of N is currently being evaluated using mammalian expression systems (Rivera-Pomar, 1991).

V. CONCLUDING REMARKS

Figure 12 shows a scheme of the organization of Junin virus S RNA based on the information presented in this chapter. Our knowledge of the molecular biology of Junin virus is still fragmentary, but cDNA cloning and sequencing of the genomic RNA have given some clarifying

FIGURE 12. Molecular organization of Junin virus S RNA. The schematic summarizes the conclusions and predictions of the nucleotide sequence data. The nucleotide numbering starts at the 5' end of the ambisense S RNA. The S RNA molecule is shown to form a circle closed by a panhandle structure (5' and 3' terminal complementary regions are not drawn to scale). The positive-sense GPC gene and the negative-sense N gene regions of this ambisense RNA are indicated (coding regions are shown as arrows). The double hairpin structure in the intergenic region is not drawn to scale.

insights, raised new questions, and yielded some solutions to diagnostic problems.

The insertion of the N and GPC open reading frames in prokaryotic and eukaryotic expression vectors provides safe-to-handle antigens for immunoassays to be used in confirmatory AHF diagnosis or epidemiological screening studies. On the other hand, making use of the sequence data, a simplified nucleic acid amplification assay has been developed for the early and rapid detection of Junin virus RNA in clinical samples, a critical issue considering that the only effective therapy that reduces AHF mortality rate from 30% to 1% consists of treatment with immune plasma in the first few days after the onset of symptoms that are not always clear (Romanowski *et al.*, 1991).

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