
Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV

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ABSTRACT

We have determined the nucleotide sequence of the 3' region of the rabies genome (PV strain). This work is a first step in a project aimed at establishing the complete primary structure. From the 3' nucleotide sequence of the RNA genome, an octadecanucleotide complementary to the 3' extremity was constructed and used to prime cDNA synthesis. Two overlapping recombinant cDNA clones hybridizing with the nucleoprotein mRNA (NmRNA) were isolated and sequenced. The 1500 first nucleotides of the rabies genome cover two transcriptional units: the leader RNA and the NmRNA which was shown to be initiated around residue 59 by S1 nuclease protection experiments. Comparison between rabies PV and CVS strains up to residue 180 suggests a rapid evolution in the leader region. Studies of the sequence relationships between the 3' regions of two Rhabdoviruses, rabies virus and Vesicular Stomatitis Virus (VSV), demonstrate that there is a segmented homology. Stretches of highly conserved amino acids possibly involved in the interaction with the RNA genome were observed in the N protein, despite a wide divergence in the remaining sequence. In addition, the high homology between the transcription start and stop signals reflects the conservation of a similar transcriptional mechanism in these two non segmented negative strand RNA viruses.

INTRODUCTION

Rhabdoviridae family is divided into two main genus, Lyssavirus and Vesiculovirus. Rabies virus, the prototype member of the Lyssavirus genus, is a bullet shaped virus containing a non segmented negative strand RNA genome. Rabies virion consists in a nucleocapsid core surrounded by a host derived lipidic envelope. The nucleocapsid core is thought to contain all elements necessary for the viral transcription (1). It is composed of the genomic RNA (about 12 kb) associated with the nucleoprotein N (MW=58500), the polymerase L (MW=170000) and the phosphoprotein M1 (MW=39500) whose position has been reassigned from the envelope to the nucleocapsid core (2). Two membrane proteins are found in the envelope: the protein M2 (MW=25000) on the inner-side and the transmembrane glycoprotein G (MW=70500) which presents antigenic spikes on the outside (3). As for the Vesicular Stomatitis Virus (VSV), the prototype member of the Vesiculovirus genus, transcription of the rabies

genomic RNA produces sequentially one leader RNA (4) and five polyadenylated monocistronic mRNAs (5, 6) defining the gene order along the genome from 3' to 5': leader, N, M1, M2, G and L (7).

Despite their analogical genome organization and similar transcriptive and replicative strategy (6), rabies virus and VSV exhibit notable differences in their biological activity. While rabies virus has a slower infectious cycle and releases lower yield of progeny virions (8), VSV has a greater cell killing ability (9) and causes much greater inhibition of host cell macromolecular synthesis, in particular RNA synthesis, by a mechanism involving the leader RNA (10). It was therefore interesting to compare the VSV genome whose nucleotide sequence has been widely documented (11-15), with the rabies virus genome about which relatively little is known. The only rabies sequences reported to date are those of the 3' end of the genome up to nucleotide 180 including the complete leader RNA coding region (4) and of the cDNA copy of the glycoprotein GmRNA (16-18).

We have undertaken the cloning of the rabies genome by priming the cDNA synthesis with an octadeca nucleotide complementary to the 3' end. In this paper we describe cDNA clones corresponding to the 3' extremity of the rabies genome including the leader RNA and the N protein genes. The 5' transcription initiation site of N mRNA was deduced from S1 nuclease protection experiments. The nucleotide sequence is compared with the corresponding region of VSV and with the 3' end of the challenge rabies virus strain (CVS) genome (4).

MATERIALS AND METHODS

Virus growth and RNA purification

Pasteur strain of rabies virus (PV) was grown on Baby Hamster Kidney cells (BHK-21) as previously described (19). Virions were purified from culture supernatants by a method adapted from Arita and Atanasiu (20). To isolate viral genomic RNA, purified virions were incubated with 100 µg/ml proteinase K (Merck) in 1,5% SDS, 100mM Tris-HCl pH7,5, 100mM NaCl, 10mM EDTA for 30 min at 37°C, followed by two phenol-chloroform extractions (vol/vol) and ethanol precipitation.

Extraction of total cellular RNA and selection of poly(A)⁺RNA by oligo(dT)-cellulose chromatography were performed as described (19, 21).

3' end labelling and sequencing of genomic RNA

Genomic RNA was 3' end labelled with (³²P)Cp and T4 RNA ligase (22). Exhaustive hydrolysis occurred in 15% piperidin and the end nucleotide was determined by thin layer chromatography on cellulose (23). Partial hydrolysis

was performed in distilled water for 2.5 hours at 90°C before two-dimensional electrophoresis homochromatography (23). Chemical RNA sequencing was carried out according to Peattie (24). Cleavage fragments were separated by 20% acrylamide-urea gel electrophoresis.

Cloning of the genomic RNA

1 μ g (about 0,25 picomoles) of purified genomic RNA was annealed to a 10 fold excess (mole/mole) of synthetic primer and transcribed into cDNA as described (25) with the following modifications: actinomycin D was omitted, 5U/ml RNAsine (genofit) was added and incubation occurred for 2.5 hours at 42°C. The (³²P) single stranded cDNA (10⁶ cpm/ μ g) was either transcribed in double stranded cDNA (26), or submitted to alkaline hydrolysis and gel filtration (G 75 sephadex), before rehybridized with freshly purified genomic RNA (ratio 1/5) during 2 hours at 65°C in Tris-HCl 20mM pH7.4, NaCl 300mM, EDTA 1mM, SDS 0.1%. Hybrids cDNA-RNA or double stranded cDNA were treated with S1 nuclease (25), phenol extracted and fractionated on 1.5% low-melting point agarose gel or 5-20% sucrose gradient. Each class size was recovered and inserted in the PstI site of pBR322 plasmid vectors by the dC/dG tailing method (25). The transformation of the HB101 strain of Escherichia coli cells was performed as described (25).

DNA sequencing, S1 protection experiments

Endonuclease cleavage fragments of pRb43 and pRb28 plasmids were sub-cloned into M13 vectors (27) before enzymatic sequencing by the chain-terminating inhibitors method (28). The 18-mer primer sequence was checked using the chemical sequencing method of Maxam and Gilbert (29).

S1 nuclease protection experiments are described elsewhere (30).

Hybridization of cDNA with viral mRNAs

Total cellular RNA was electrophoresed through a 1.2% agarose gel containing formaldehyde and transferred to nitrocellulose filters (Schleicher & Schuell) according to Thomas (31). Filters were hybridized with high specific activity (³²P)nick-translated cDNA probes (32) overnight at 42°C in 50% formamide.

For hybridization selection experiments, filters were saturated with cDNA plasmids and hybridized with poly(A)⁺RNA from infected cells. Complementary viral mRNA was eluted and translated as described below.

In vitro translation of poly(A)⁺RNA

Preparation of mRNA dependent rabbit reticulocyte lysates, translational conditions and analysis of translational products are described elsewhere (21). Immunoprecipitations with monoclonal antibodies directed against the nucleoprotein were carried out according to Shih (33).

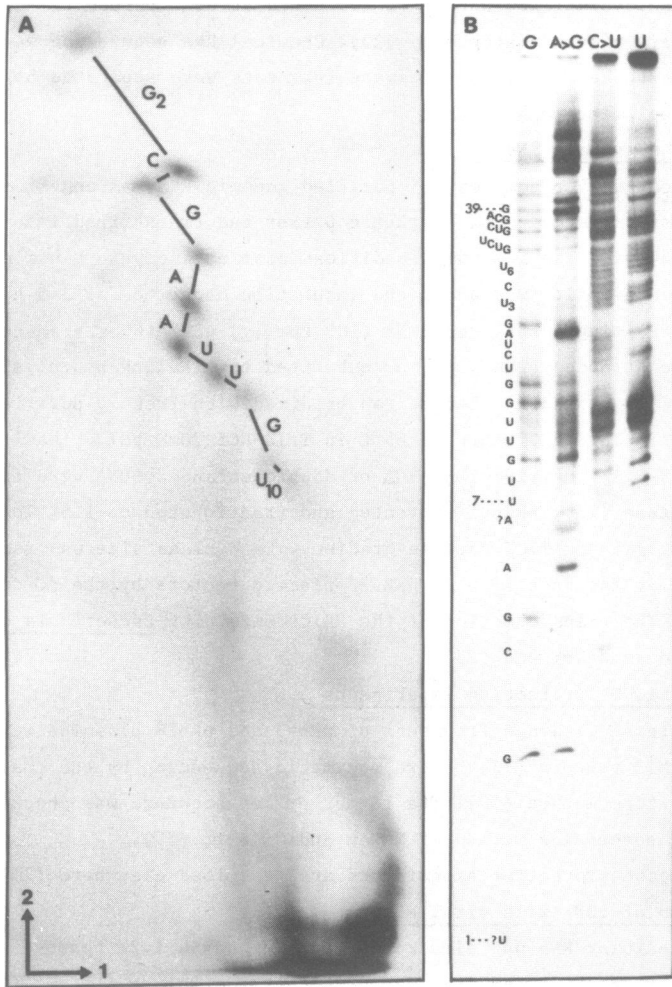
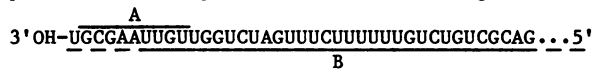


Figure 1: 3'-labelled genomic RNA was analysed (A) by two-dimensional electrophoresis homochromatography after random hydrolysis and (B) by 20% polyacrylamide gel electrophoresis after specific chemical cleavages. The resulting sequence is:



Computer analysis

National Biomedical Research Foundation (NBRF) and PGtrans (34) protein data banks were searched using the program of Wilbur and Lipman (35) with the following parameters: K-tuple size=2, window size=40 , gap penalty=2.

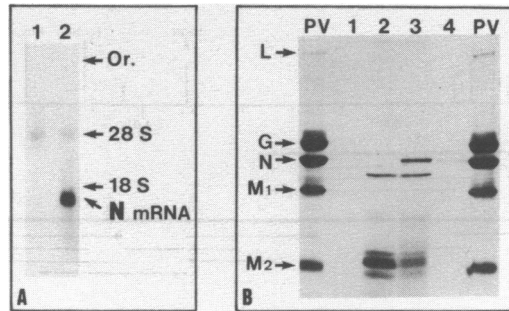


Figure 2: (A). ^{32}P nick-translated pRb43 insert was hybridized to a Northern blot of total RNA from rabies PV infected (lane 2) or non-infected (lane 1) BHK-21 cells.

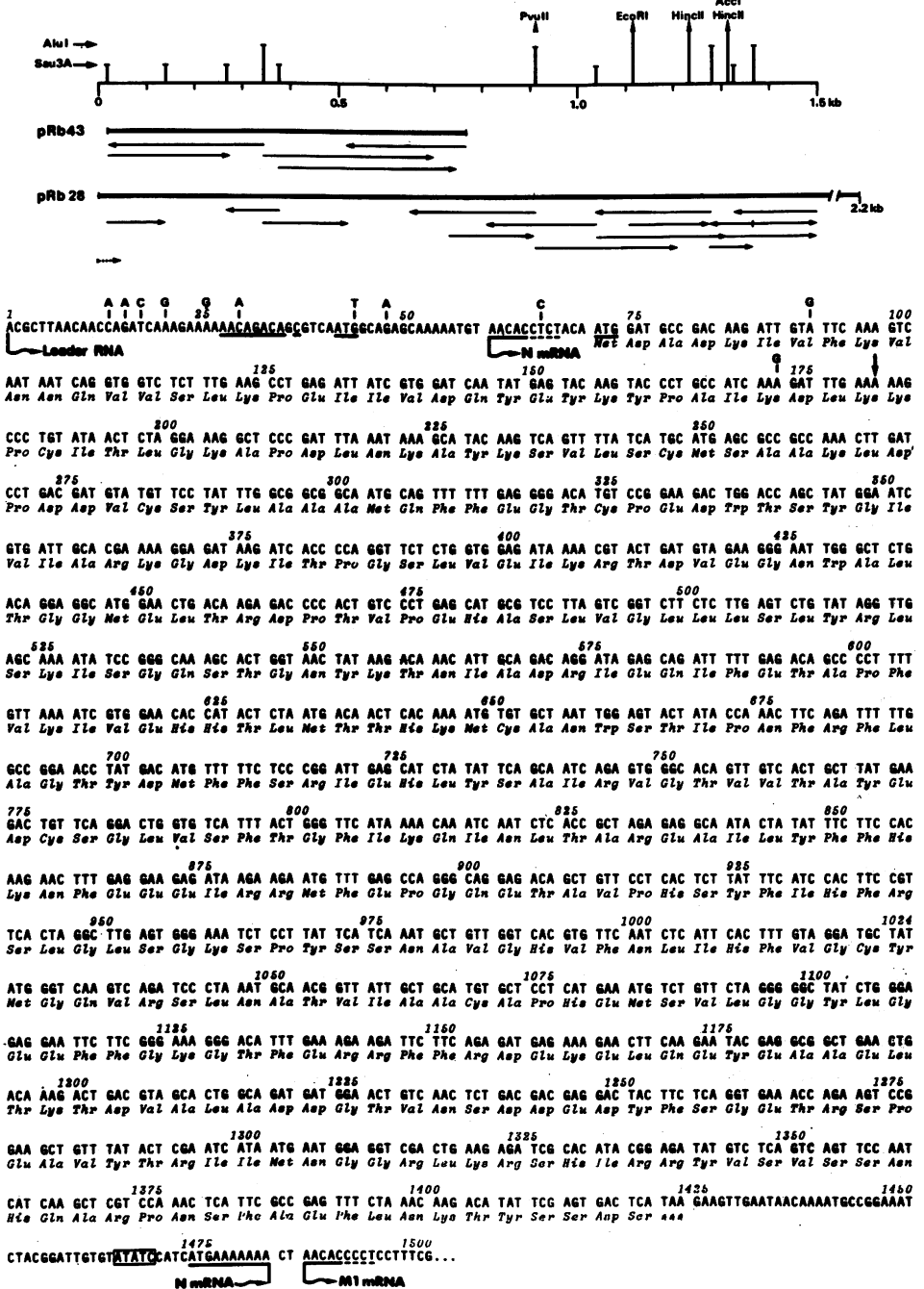
(B). 12% SDS polyacrylamide gel separation of *in vitro* translation products of mRNA selected by hybridization with pRb43 insert among poly(A)⁺RNA of infected (lane 3) or non-infected (lane 2) BHK-21 cells. Lanes 1 and 4 represent the immunoprecipitable products from lanes 2 and 3 respectively, using a mixture of 3 monoclonal antibodies directed against N protein. Lanes PV show proteins of purified rabies virus (PV strain).

RESULTS

Cloning strategy of the 3' region of the rabies genomic RNA

With the aim to establish the primary structure of the rabies genome, we have determined the 3' terminal sequence of the genomic RNA. The 12,000 nucleotide long RNA was isolated by electrophoresis on low-melting point agarose gel and 3' end-labelled as described in Methods. Sequence determination was performed using three different techniques: (1) the 3' terminal nucleotide was identified as Up by thin layer chromatography (data not shown); (2) the sequence up to nucleotide 10 was determined by two-dimensional electrophoresis homochromatography (Figure 1A); (3) the sequence from nucleotide 7 to nucleotide 39 was determined by RNA chemical sequencing (Figure 1B). From the RNA sequence, we have synthesized a 18-mer DNA complementary to the 3' end to prime cDNA synthesis. cDNA-RNA hybrids or double stranded cDNA were inserted in pBR322 plasmid vectors.

To identify rabies recombinant clones, cDNA inserts were hybridized with RNA from infected or non infected BHK-21 cells. Figure 2A shows the results of Northern blot hybridization experiments with the clone pRb43 (800 bp). The blot of infected cell total RNA (lane 2) gave a single additional hybridization band corresponding to a size of 1600 nucleotides when probed with (^{32}P)nick-translated insert. This is in agreement with the length expected for the rabies N mRNA (6). In order to show that this mRNA effectively



encodes the N protein, pRb43 plasmid DNA was used in hybridization selection experiments. Hybridized mRNA was eluted and in vitro translated in a rabbit reticulocyte lysate. Comparison of lanes 2 and 3 of Figure 2B shows that the pRb43 insert selects an mRNA in infected cell extracts which directs the synthesis of a protein comigrating with the rabies N protein of PV strain (lanes PV). Furthermore, the translation product is immunoprecipitated by a mixture of 3 monoclonal antibodies directed against N protein (lane 4).

Sequencing studies of cDNA clones, mapping of the 5' start of NmrRNA

Two overlapping recombinant clones were used for sequence determination according to the strategy outlined in Figure 3. The resulting sequence, presented as DNA(+) sense, was determined until residue 1500. The 39 terminal nucleotides, complementary to the 3' extremity of the viral RNA, allow us to define the polarity of the insert.

Only one open reading frame uninterrupted until residue 1421 could be obtained, the other two contain multiple stop codons. Two ATG codons at position 41 and 71 respectively are potential initiation codons. In order to determine which was used, we carried out S1 nuclease protection experiments to define the transcription initiation site of NmrRNA. As shown in Figure 4, the protected probe maps around residue A₉₀ (genomic sense). Because the first 32 nucleotides of each sequencing reaction belong to the cloning system (universal primer and M13mp701 cloning sites) and are sensitive to nuclease S1 digestion, the transcription of NmrRNA begins around residue U₅₉ (genomic sense). This result is in agreement with the previous observation that a leader RNA species of 55 to 58 nucleotides long is encoded by the 3' extremity of the rabies genome (4). Thus, the nucleoprotein must be initiated at position 71.

The deduced amino acid sequence (Figure 3) consists of 450 residues. Consistent with the VSV nucleoprotein (12), it has a small excess of basic amino acids and a calculated molecular weight (50550) slightly lower than the

Figure 3: The sequence of 1500 nucleotides at the 3' end of the rabies PV genome (presented as complementary DNA(+) sense) was established after sub-cloning restriction fragments of pRb43 and pRb28 inserts into M13 vector and enzymatic sequencing. The direction and extent of nucleotide reading are indicated by solid arrows. The dotted arrow represents the extent of genomic RNA sequencing. The deduced N protein sequence is presented. Wavy lines indicate the leader RNA, NmrRNA and M1mrRNA transcription start as well as the 3' end of NmrRNA. The two potential N protein initiation codons with their respective transcription initiation site, the NmrRNA polyadenylation signal and the postulated M1mrRNA initiation site are underlined. The putative promoter sequence of the M1mrRNA is boxed. The mismatched nucleotides in the CVS strain relatively to the PV sequence are notified up to residue 180 (vertical arrow) (4).

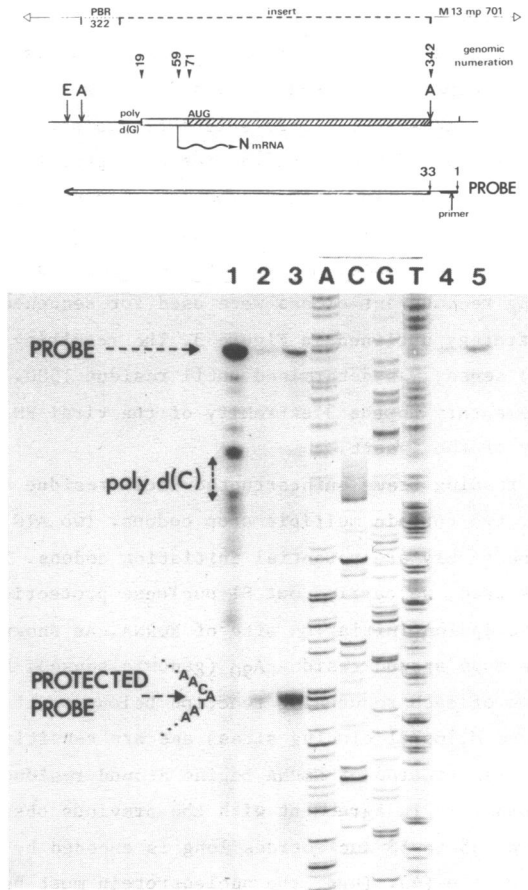


Figure 4: Mapping of the 5' end of N mRNA.

The AluI fragment from nucleotide 342 (genomic numeration) of pRb43 insert to the proximal AluI site in pBR322, was subcloned into the HincII site of M13mp701. Sequence corresponding to the genome is boxed and N protein coding region is hatched. A and E stand for AluI and EcoRI sites respectively. Uniformly ³²P labelled single stranded DNA probe of genomic sense (DNA -) was synthesized. 5 × 10⁴ cpm was hybridized with 1 µg or 4 µg of poly(A)⁺RNA from BHK-21 infected cells (lanes 2 and 3 respectively) or non infected cells (lanes 4 and 5 respectively) and digested with S1 nuclease. 10³ cpm of undigested probe is shown in lane 1. The protected probe position is indicated with respect to enzymatic sequencing tracks (lanes A,C,G,T). Poly d(C) track constitutes the end of the pRb43 insert sequence. The wavy lane designates the transcription start point.

apparent molecular weight estimated from polyacrylamide gel electrophoresis (58500)(5). The deficiency in CG dinucleotides, widely reported among negative strand RNA viruses, is also observed along the rabies N mRNA and results in a clear bias against CGN, NCG and NNC-GNN codons.

DISCUSSION

All non segmented negative strand RNA viruses use the same basic mechanism for the multiplication: the genomic RNA (-) is first transcribed into monocistronic RNAs (+) (leader and mRNAs), then it is replicated into a complete genome (+) which will serve as template for the synthesis of novel genomic RNAs (-). Comparative studies between the VSV and rabies genomes can allow us to define more precisely the sequences involved in the transcription and replication. Since the RNA genome is always found associated with the nucleoprotein in the Rhabdovirus genus, we can expect to characterize in the N protein, the segments which are important for the formation of the nucleocapsid.

The sequence reported here includes two transcriptional units: the leader RNA and the nucleoprotein mRNA.

The leader RNA

The leader RNA is a 3' transcription product which has been known for a long time in VSV (36) and more recently demonstrated in the CVS strain of the rabies virus (4)(Figure 3). The 3' location of the leader region implies that such essential events as the initiation of transcription and replication (37), the switching between these two functions (38, 39) and the initiation of encapsidation (40) take place here. Kurilla has already shown some common features between VSV and rabies leader RNA: they are of similar length (about 50 nucleotides) with a high content of A residues (50%), they both initiate with the same trinucleotide (ACG) which seems to be conserved throughout the Rhabdoviridae family (14) and have a very homologous 3' end. Our results on the rabies PV strain strengthens the idea of such a relationships. Nevertheless, comparison of the PV and CVS strains indicates that certain sequences are less important than had previously been thought. For example, the hexanucleotide 3'₁₄UUUGGU₁₉5' (genomic sense) is present in the CVS strain as throughout the Vesiculovirus genus (one nucleotide upstream) where it is thought to be implicated in the RNA synthesis initiation (14). Since this hexanucleotide is absent from the PV strain due to one U/C change at position 15 and one G/A change at position 17, it is uncertain that it plays the same role in the rabies genome. Furthermore the two rabies strains show 11 mismatches between positions 1 and 180 (6%). It is of interest to observe that this divergence is mainly located in the leader region (8 differences =14%), while only two silent substitutions are found in the nucleoprotein coding region (2.5%). Consequently, the leader region appears to be an area of rapid evolution in the rabies virus genome. Sequence studies of other

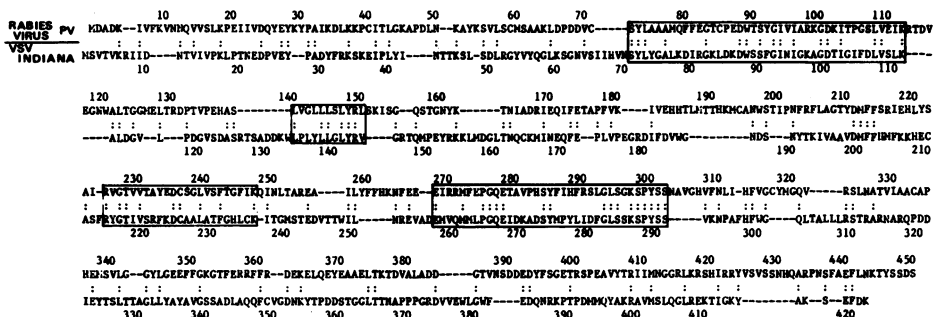


Figure 5: Alignment of the rabies PV and VSV Indiana nucleoproteins using the computer program of Wilbur and Lipman (35) with the K-tuple size=1, window size=40 and gap penalty=1 parameters. Four particularly conserved regions are boxed.

rabies strains should permit us to better elucidate the variation in the leader region and to characterize the essential sequences.

The nucleoprotein

Using the computer program of Wilbur et Lipman (35), we have searched in protein data banks for sequences which may have homology with the rabies nucleoprotein. The only very significant homology (score 4,4) was found with the nucleoprotein of VSV. Using the same program, the previously reported homology between the glycoproteins of the two viruses (41), is lower (score 3,3). Figure 5 shows one optimal alignment between rabies PV and VSV Indiana N protein. The invariant amino acids are not uniformly located along the sequence but some regions seem more conserved, such as between amino acids 72 and 112 (41,5%), 140 and 150 (54,5%), 225 and 247 (39%), 268 and 302 (48,5%). In this last region, a stretch of 10 but one consecutive invariant amino acids is particularly remarkable (293 to 302). It is difficult to assign a function to these conserved regions on the sole consideration of sequencing data. Nevertheless, it is tempting to suggest that they are involved in the interaction of the nucleoprotein with the RNA genome. Since another homology, but at a lower level (score 3,2), can also be observed between the nucleoproteins of rabies virus and Sendai virus, a member of Paramyxovirus genus, the nucleoprotein seems to be relatively conserved throughout the non segmented negative strand RNA viruses. Further comparison with nucleoproteins of such other viruses should allow us to define particularly conserved amino acids that could be essential for the formation of the nucleocapsid structure.

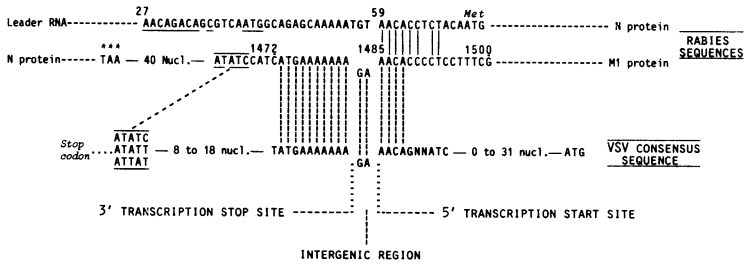


Figure 6: Intergenic and flanking gene regions.

The flanking gene and intergenic sequences

Intergenic and flanking gene sequences have already been determined for VSV (15). Three consensus sequences were characterized in the non protein coding regions: the 5'mRNA initiation site, the intergenic segment whose complement does not appear in the mRNA and the 3'end of mRNA with its polyadenylation signal (A₇) (Figure 6).

S1 mapping protection experiments demonstrate that NmRNA transcription initiates around position 59 with the pentanucleotide 5'AACAC 3'. It differs slightly from the canonical pentanucleotide 5'AACAG 3' of the VSV 5'mRNA initiation site. Interestingly, such a VSV consensus site, but one nucleotide, appears in the middle of the leader RNA of the rabies PV strain (position 27) 14 nucleotides upstream from the ₄₁ATG codon in the open reading frame. Although this structure could be a putative initiation site of NmRNA, S1 mapping experiments do not show any detectable transcription start at position 27 (Figure 4). On the other hand, the pentanucleotide 5'AACAC 3' beginning the rabies NmRNA, has also been involved *in vitro* in the initiation of one VSV intracistronic transcription event (42). Downstream from the N protein coding sequence, this pentanucleotide also appears in position 1485 and we therefore postulate that it represents the transcription initiation site of the M1mRNA. This assumption is strengthened by the finding of the sequence 5'ATG(A)₇3' three nucleotides upstream (position 1473 to 1482), closely similar (one nucleotide less) to the consensus 3'end of VSV mRNAs. This sequence must represent the polyadenylation signal of rabies NmRNA. Furthermore, the sequence 5'ATATC 3' beginning 21 nucleotides upstream from the M1mRNA transcription start site, looks like the promoter sequence preceding each VSV mRNA (15). However, in contrast to VSV, a similar promoter sequence for the NmRNA is absent in the rabies leader region. Finally, the 3'stop of NmRNA and the 5'start of M1mRNA define a two nucleotides long

intergenic region (GA in the genome) identical to most of VSV intergenic segments. All these remarks are summarized in Figure 6.

The major conclusion of these results is that VSV and rabies virus are homologous only in limited regions. Despite a wide divergence of the nucleoprotein sequence, some stretches possibly involved in the interaction with the genomic RNA are highly conserved. Furthermore, as a result of the identical multiplication mechanism of these non segmented negative strand RNA viruses, a high conservation of transcriptional start and stop signals is observed. There is a strong homology between the 3' end of the rabies N mRNA and the corresponding consensus sequence of VSV genes while the 5' start sites are less homologous. Nevertheless, an extensive comparison of rabies N and M1 mRNA 5' start sites, indicates that they both initiate with the sequence 5' AACACCPyCT, Py being T and C respectively. Further sequence analysis of the genome is needed to elucidate a rabies 5' transcription start consensus sequence.

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