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Genomic changes associated with antigenic variation of visna virus during persistent infection

(RNase T1 fingerprints/nucleotide sequence analysis/mapping of altered oligonucleotides)

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ABSTRACT **Visna virus undergoes antigenic change during persistent infection of sheep. Antigenic variants of visna virus were compared by using the genomic RNA and analyzing the large RNase T1-resistant oligonucleotides. Mutants isolated from a persistently infected sheep contained a small number of changes in their oligonucleotide patterns when compared with parental virus. To determine whether the changes in the nucleotide structure were clustered in one region of the genome, we determined the order of the oligonucleotides of the parental and mutant RNAs along the genome with respect to the 3′ polyadenylylated end. All but one difference between the parental strain and the antigenic mutant used for mapping were located within 2 kilobases from the 3′ terminus. Nucleotide sequence analyses showed that several of the oligonucleotides that differed in the parental and mutant RNAs could be accounted for by single base changes.**

Visna virus, a member of the retrovirus family, is the etiologic agent of an inflammatory and degenerative disease of the central nervous system of sheep (1, 2). A remarkable feature of the disease is the protracted time course: a prolonged incubation period, from months to years, followed by a subacute progression of symptoms to death. For this reason visna is a classic model of a slow virus infection.

The unusual aspect of visna virus is the life-long persistence of the virus, which undergoes progressive antigenic variation in a single infected animal (3, 4). In a visna virus-infected animal, some virus isolates obtained months after inoculation are of parental serotype. However, serologically distinct viruses can be isolated later in the course of the disease that are not efficiently neutralized by the sera obtained shortly after inoculation (4). These variants are serologically distinct from one another. The infected animal subsequently develops neutralizing antibody against these variants. These antigenic variants are stable to multiple subcloning and are virulent in sheep (4). Thus, they provide a system in which natural genetic selection and antigenic variation can be studied. To investigate this problem we have studied the genomic RNA of a parental strain of visna virus and three antigenic variants isolated from a single animal.

METHODS

Virus Strains. Virus strain 1514 was purified three times prior to inoculation into a sheep (4). The viruses were plaque purified, grown in sheep choroid plexus cells (4), and purified by equilibrium centrifugation (5).

RNA Isolation and Fingerprinting. Viral RNA was isolated as described (6, 7). RNA (0.1–1 μg) from each virus was incubated in the presence of RNase T1 and bacterial alkaline phosphatase followed by polynucleotide kinase-catalyzed transfer of [^{32}P]P_i from [γ-^{32}P]ATP to the 5′-hydroxyl groups of the RNase T1-resistant oligonucleotides as described (6,7). The oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis (6, 7). The first dimension (10% acrylamide, pH 3.5) was oriented from left to right; the second dimension (22.8% acrylamide, pH 8.3) was from bottom to top.

Sequence Analysis of RNase T1-Resistant Oligonucleotides. The 5′-labeled oligonucleotides were eluted from the second-dimension gel and concentrated by ethanol precipitation (6, 7). The sample was dissolved in 35 μl of 20 mM Na citrate, 1 mM EDTA, 7 M urea, 0.25 μg of yeast carrier RNA per ml, and 0.025% xylene cyanol FF and bromophenol blue adjusted to pH 5.8 with citric acid.

Aliquots of 5 μl were added to microtiter wells with the following additions (7): no addition; 2.5 μg of RNase A; 0.125 μg of RNase A; 0.25 unit of RNase U2; 0.025 unit of RNase U2; an appropriate amount of *Physarum* RNase; and 0.005 unit of RNase T2. Samples were incubated for 1 hr at 50°C. The samples were analyzed by electrophoresis in polyacrylamide gels containing 7 M urea (7).

The 5′-terminal nucleotide was identified by thin-layer chromatography on polyethyleneimine-cellulose after complete alkaline hydrolysis of the oligonucleotides as described (7).

Isolation of Subgenomic Fragments of Viral RNAs. Size classes of subgenomic poly(A)-containing RNA fractions were derived from 70S RNA containing partially fragmented RNA. Approximately 100 μg of 70S RNA was denatured by heating for 5 min at 95°C in 1 ml of 5 mM EDTA (pH 7.0). The poly(A)-containing RNA molecules were isolated by chromatography on an oligo(dT)-cellulose column (6, 7) and separated according to size by velocity sedimentation through linear gradients of 0.44–0.88 M sucrose in 50 mM Tris·HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 2 g of $NaDodSO_4$ per liter by centrifugation for 4.5 hr at 37,000 rpm and 20°C in an SW 41 rotor. A parallel gradient contained sedimentation markers: 28S, 18S, and 4S ribosomal [^{3}H]RNA. The RNA from pooled fractions of the gradient was concentrated by chromatography on an oligo(dT)-cellulose column followed by ethanol precipitations (6, 7). The RNA samples were analyzed by two-dimensional mapping of RNase T1-resistant oligonucleotides.

Order of Oligonucleotides. The location of the oligonucleotides near the 5′ end relative to the poly(A) sequence was determined by visual inspection of RNase T1 fingerprints of isolated subgenomic RNA fractions. The detailed order was determined with the oligonucleotides in the 3′ region of the genome of virus strain LV1-1. The relative content of RNase T1-resistant oligonucleotides in two short poly(A)-containing subgenomic fractions of RNA from virus strains LV1-1 and 1514 was determined by measurement of Cerenkov radiation of 5′-labeled oligonucleotides of second-dimension gels. The values were normalized to the amount of radioactivity in the corresponding oligonucleotides derived from analysis of 70S RNA.

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RESULTS

The viruses used for these studies were the inoculated strain, 1514, and three antigenic variants isolated from the same sheep. Strain 1514 was cloned in three successive steps by selection of single plaques prior to inoculation. Eighteen months after inoculation of a sheep with 1514, LV1-1 was isolated from the peripheral leukocytes. LV1-5 and LV1-7 were isolated from the peripheral leukocytes of the same sheep 3 years after inoculation.

RNase T1 Fingerprints of RNA from Viruses 1514 and LV1-1. RNA isolated from the parental virus 1514 and the antigenic variants LV1-1, LV1-5, and LV1-7 were examined by mapping of RNase T1-resistant oligonucleotides. Oligonucleotides produced by digestion of viral 70S RNA with RNase T1 were radioactively labeled at the 5′ end with ^{32}P. The mixture was fractionated by two-dimensional gel electrophoresis, and the oligonucleotide pattern was analyzed by autoradiography (6, 7). Fig. 1 shows the autoradiograms (termed RNase T1 fingerprints) of the oligonucleotides. Approximately 80 large oligonucleotides of each virus were resolved.

The fingerprint patterns of the viral RNAs were strikingly similar. However, they were not identical. Because more differences were observed between parental strain 1514 and antigenic variant LV1-1, RNase T1-resistant oligonucleotides of these two strains were studied in more detail. To determine whether the majority of the oligonucleotides had the same electrophoretic mobilities, we mixed samples of the labeled oligonucleotides from each virus and analyzed them on the same gel. Fig. 1*E*, schematically diagrammed in Fig. 1*F*, shows that the majority of the oligonucleotides comigrate in the two-dimensional gel system. This indicates that the genomes of the two viruses have similar nucleotide sequences. However, several oligonucleotides unique to each isolate can be identified. These include oligonucleotide spots 2, 21, and 24, which are present only in parental strain 1514. Spots 101–106 represent oligonucleotide sequences that are unique to LV1-1. Oligonucleotide 105 is not completely resolved from the neighboring oligonucleotide. Therefore, it could not be established from the fingerprint of the 70S RNAs whether or not this oligonucleotide was missing in strain 1514. However, studies with subgenomic fragments of the viral RNA demonstrate that oligonucleotide 105 of LV1-1 is absent from the fingerprint of 1514 RNA.

To determine whether the differences in the fingerprint patterns of the two isolates were a stable property of these viruses, we purified the RNA of a subclone of each virus. These subclones were derived from single plaques of each virus. The RNase T1-resistant oligonucleotide fingerprints of the 70S RNA of the newly cloned 1541 and LV1-1 viruses were identical to the original fingerprints obtained (data not shown). Thus, the diversity in the unique oligonucleotides observed for strains 1514 and LV1-1 represents stable genetic characteristics of the two viruses. This is consistent with the stable antigenic differences observed for these viruses upon multiple subcloning (4).

Nucleotide Sequence Analysis of Oligonucleotides. A majority of the unique RNase T1-resistant oligonucleotides of the two isolates comigrated by two-dimensional gel electrophoresis. Comigrating oligonucleotides have the same base composition but not necessarily identical nucleotide sequences. However, the extensive overall similarities in the nucleotide patterns of the two viral strains suggest that the comigrating oligonucleotides are in fact identical. To test this assumption, we analyzed several oligonucleotides of identical mobility derived from the two viral strains. The 5′-labeled oligonucleotides were isolated from the second-dimension gel and their nucleotide sequences were determined by mapping of cleavage sites for sequence-specific ribonucleases (8). The positions of all adenine residues were determined for oligonucleotides 1, 3, 4, 5, and 7. These oligonucleotides from strains 1514 and LV1-1 had identical adenine tracks (Table 1*B*). This observation supports the notion that the 1514 and LV1-1 isolates are identical in sequence over most of the genome.

What is the relationship among the oligonucleotides of the two viruses that do not comigrate? The electrophoretic mobilities of some of the oligonucleotides in the LV1-1 fingerprint suggest that they might be derived by simple mutations from

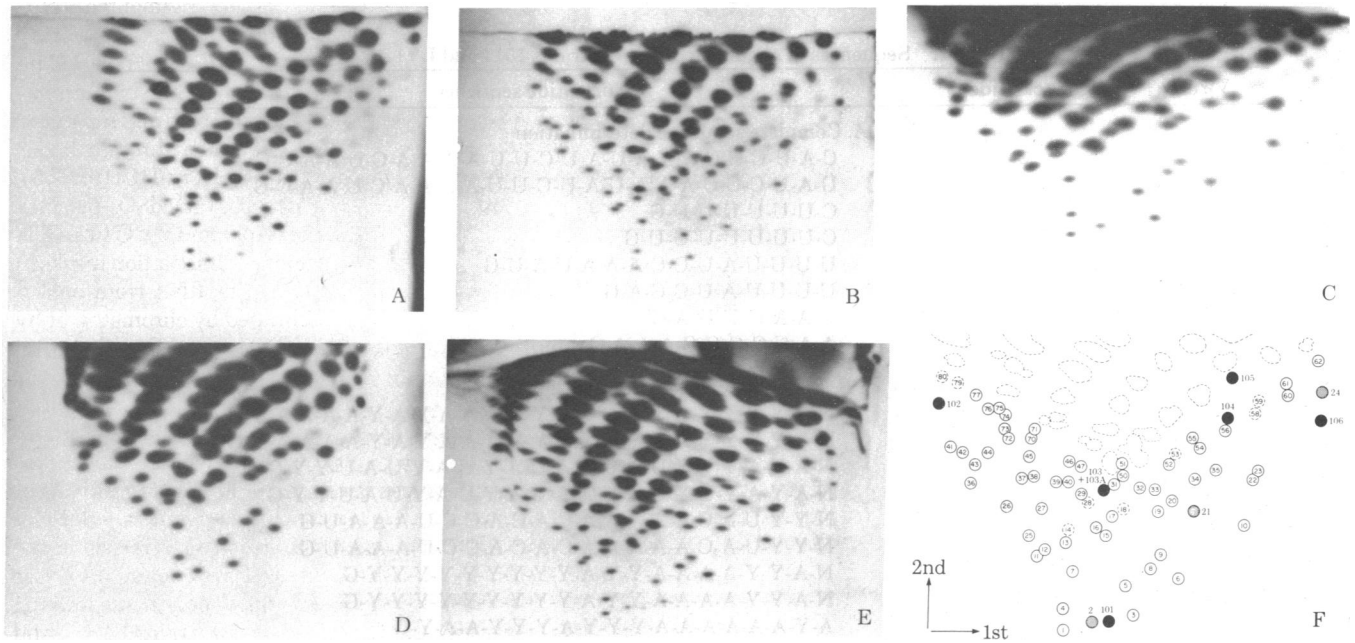

FIG. 1. RNase T1 fingerprints of 70S RNA of visna virus strain 1514 (*A*) and variants LV1-1 (*B*), LV1-5 (*C*), and LV1-7 (*D*). (*E*) Analysis of a mixture of the RNAs of strains 1514 and LV-1. (*F*) Diagram of *E* showing numbers used in identification of the oligonucleotides: ○, single oligonucleotides common to the two strains; ◕, single oligonucleotides unique to strain 1514; ●, single oligonucleotides unique to strain LV1-1. Spots represented by broken lines contain oligonucleotides in more than unimolar amounts. Some of these spots, containing only a few oligonucleotides, have been assigned a number. The first dimension (10% acrylamide, pH 3.5) is oriented from left to right; the second dimension (22.8% acrylamide, pH 8.3) is from bottom to top.

oligonucleotides in the parental strain (7). Therefore, we decided to obtain the complete nucleotide sequence of the oligonucleotides unique to each virus.

The sequences of these oligonucleotides are given in Table 1*A*. The sequence of oligonucleotide 106 differs from that of oligonucleotide 24 by only an additional uridine. The sequence of oligonucleotide 101 is different from that of oligonucleotide 2 only in the 5′-terminal oligonucleotide, a change from a cytidine to a uridine. The 5′-terminal sequence of oligonucleotide 104 is the same as that of the first eight nucleotides of oligonucleotide 21 of 1514. Thus, oligonucleotide 104 of LV1-1 could be derived from oligonucleotide 21 of 1514 by a mutation from an adenine to a guanine located three bases from the 5′ end of the oligonucleotide. Such a change would provide a new cleavage site for RNase T1.

Sequences of oligonucleotides 102 and 105 that were found only in the fingerprint of LV1-1 were determined. No sequences similar to these were apparent in the sequences of the oligonucleotides of 1514. Sequence analysis of the material corresponding to spot 103 of LV1-1 did not lead to the determination of an unambiguous nucleotide sequence. This is most likely due to the presence of more than one oligonucleotide in this sample. Because we were unable to separate individual components of this material by gel electrophoresis, we took a different approach to the analysis of this sample.

The oligonucleotide material corresponding to spot 103 was divided into three aliquots, which were digested to completion with RNase U2, RNase A, and RNase T2. The radioactive cleavage products derived from the oligonucleotide 5′ ends were then analyzed by electrophoresis on DEAE-paper at pH 3.5 (9). A single product, identified as pAp, was detected in the aliquots treated with RNase U2 and T2. The complete RNase A digestion, however, produced two products, which contained an equal amount of radioactivity (experimental value, 1.01). Therefore, we conclude that spot 103 of LV1-1 represents two unique oligonucleotides that are not present in the fingerprint of 1514. These two oligonucleotides, 103A and 103B, were characterized by a partial nucleotide sequence (Table 1).

Order of Unique Oligonucleotides of Strains 1514 and LV1-1. To evaluate the biological significance of the difference in nucleotide sequence between RNAs of the two viral strains, we wished to determine the position on the genome of the unique oligonucleotides. Previous studies have shown that the genome of visna is not permuted relative to the 3′ poly(A)-containing terminus (10–12). Therefore, it should be possible to construct a physical oligonucleotide map of the genome of the two variants by analysis of the relative content of the large RNase T1-resistant oligonucleotides in different subgenomic size classes of poly(A)-containing RNA. For this purpose, 70S RNA was denatured by heating and the poly(A)-containing molecules were isolated by chromatography on oligo(dT)-cellulose. The poly(A)-containing RNA was fractionated according to size by velocity sedimentation in sucrose gradients. RNA samples isolated from different parts of the gradient were analyzed by RNase T1 fingerprinting (Fig. 2). For each RNA fraction, the relative content of the large RNase T1-resistant oligonucleotides was determined by visual inspection of the autoradiogram and by measurement of Cerenkov radiation of gel pieces containing individual oligonucleotides.

This analysis permitted us to construct a precise oligonucleotide map of the 3′ region based on the measurements shown in Fig. 3. Oligonucleotides derived from other parts of the genome, on which the mapping technique is less accurate, were divided into four groups according to their location relative to the poly(A) sequence, as shown in the oligonucleotide map of Fig. 4. Analysis of the oligonucleotides in the simplest fingerprints (see Fig. 2) also permitted the identification of oligonucleotide 105 as unique to LV1-1, but did not reveal any differences between RNAs of the two strains that had not been detected by analysis of the complete genome.

Fig. 4 presents the combined results of analysis of the two variants; we found that comigrating oligonucleotides derived from the two viruses were located in the same region of the genome. We also found that oligonucleotides from the two strains that have related but not identical nucleotide sequences were derived from the same region of the genome, indicating that such oligonucleotides (2 and 101, 21 and 104, and 24 and 106) are allelic.

Table 1. Sequences of oligonucleotides of strains 1514 and LV1-1

Virus	Oligonucleotide	Nucleotide sequence
		A. Complete sequence determination
1514	2	C-A-U-C-C-C-A-A-A-U-A-U-C-U-U-A-A-A-A-C-U-A-A-C-G
LV1-1	101	U-A-U-C-C-C-A-A-A-U-A-U-C-U-U-A-A-A-A-C-U-A-A-C-G
1514	24	C-U-U-U-U-U-U-G
LV1-1	106	C-U-U-U-U-U-U-U-G
1514	21	U-U-U-U-A-U-C-C-A-A-A-U-A-U-G
LV1-1	104	U-U-U-U-A-U-C-C-A-G
LV1-1	105	U-A-A-U-U-U-A-G
LV1-1	102	A-A-C-C-C-C-C-A-A-A-G
		B. Partial sequence determination
1514	1	N-Y-Y-Y-Y-Y-A-Y-Y-A-Y-Y-Y-Y-A-A-A-Y-A-Y-A-A-A-A-Y-G
LV1-1	1	N-Y-Y-Y-Y-Y-A-Y-Y-A-Y-Y-Y-Y-A-A-A-Y-A-Y-A-A-A-A-Y-G
1514	3	N-A-Y-A-Y-Y-Y-U-A-U-A-A-U-A-Y-U-A-Y-U-A-U-Y-Y-G
LV1-1	3	N-A-Y-A-Y-Y-Y-U-A-U-A-A-U-A-Y-U-A-Y-U-A-U-Y-Y-G
1514	4	N-Y-Y-U-A-C-A-A-A-A-A-C-A-C-A-C-C-U-A-A-A-U-G
LV1-1	4	N-Y-Y-U-A-C-A-A-A-A-A-C-A-C-A-C-C-U-A-A-A-U-G
1514	5	N-A-Y-Y-A-A-A-A-Y-Y-A-Y-Y-Y-Y-Y-Y-Y-Y-Y-G
LV1-1	5	N-A-Y-Y-A-A-A-A-Y-Y-A-Y-Y-Y-Y-Y-Y-Y-Y-Y-G
LV1-1	7	A-Y-A-A-A-A-A-A-Y-Y-Y-A-Y-Y-Y-A-A-Y-G
LV1-1	7	A-Y-A-A-A-A-A-A-Y-Y-Y-A-Y-Y-Y-A-A-Y-G
LV1-1	103A	A-A-Y-N-N-N-N-N-N-N-N-N-N-G
LV1-1	103B	A-A-A-N-N-N-N-N-N-N-N-N-N-G

Oligonucleotides were isolated from the gels and their nucleotide sequences were determined as described. N, unknown residue; Y, pyrimidine residue.

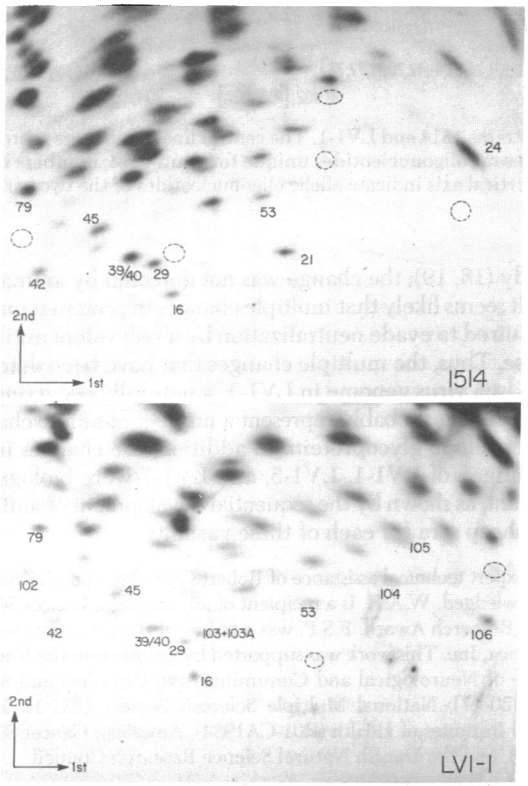

FIG. 2. RNase T1 fingerprints of short subgenomic fractions of poly(A)-containing RNA isolated from virus 1514 (*Upper*) and LV1-1 (*Lower*). The RNA samples analyzed have a sedimentation coefficient of less than 18 S. The more abundant oligonucleotides are identified by numbers. Broken circles indicate the positions corresponding to oligonucleotides unique to the sample derived from the other isolate. Size classes of subgenomic poly(A)-containing RNA fractions were derived from 70S RNA containing partially fragmented RNA.

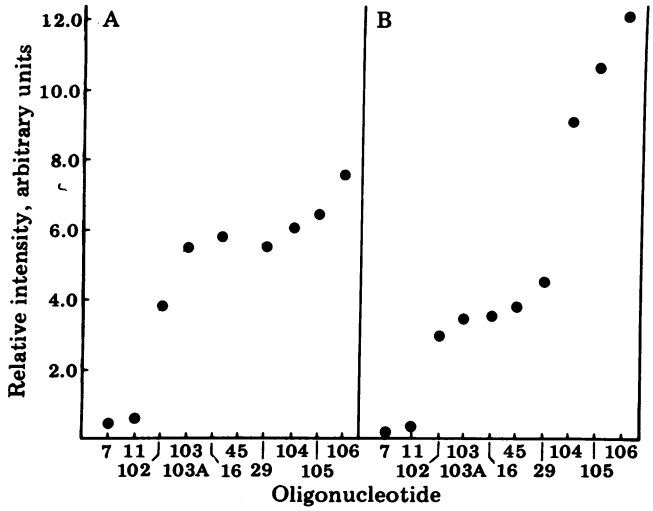

FIG. 3. Detailed order of the oligonucleotides in the 3′ region of the genome of virus strain LV1-1. The relative content of RNase T1-resistant oligonucleotides in two short poly(A)-containing subgenomic fractions of RNA from virus strain LV1-1 was determined by measurement of Cerenkov radiation of 5′-labeled oligonucleotides of second-dimension gels. The values were normalized to the amount of radioactivity in the corresponding oligonucleotides derived from analysis of 70S RNA. (*A*) The experiment of Fig. 2 *Lower* was used. (*B*) Analysis of the smaller subgenomic fraction of poly(A)-containing RNA.

The oligonucleotide map shows that all but one of the differences between the parental strain and LV1-1 are located in the 3′ region of the genome. Oligonucleotides 24 and 21 of strain 1514 and oligonucleotides 102, 104, 105, and 106 of the LV1-1 virus are located in this region. Of the oligonucleotides that differ between the two isolates, only oligonucleotides 2 and 101 map in the 5′ half of the genome. The identity of oligonucleotides 24, 21, 104, 105, and 106 in the subgenomic fragments was confirmed by sequence analysis (Table 1).

Mapping analysis showed that six out of seven alterations in the genome of the antigenic variant LV1-1 are located among the oligonucleotides closest to the poly(A) sequence. This would place them within 2 kilobases from the 3′end, assuming an even distribution of the marker oligonucleotides along the genome. The altered oligonucleotides are located in at least two distinct groups within the region. Oligonucleotides 104, 105, and 106 are located very near the poly(A) tail, and oligonucleotides 102, 103A, and 103B are derived from a sequence farther from the 3′ terminus.

Fingerprint Analysis of RNA from Strains LV1-5 and LV1-7. Inspection of the fingerprints of the viral RNAs of variants LV1-5 and LV1-7 suggested that the differences in the oligonucleotides of these strains from parental strain 1514 were a subset of the changes observed between 1514 and LV1-1. To determine which of the RNase T1 oligonucleotides of the variants comigrated with the oligonucleotides of 1514, we mixed samples of the labeled oligonucleotides of LV1-5 or LV1-7 with those of 1514 and analyzed them on the same gel. The fingerprint of LV1-7 differed from that of 1514 only by the absence of oligonucleotide 21 and the presence of oligonucleotide 104. The mutation from an adenosine to a guanosine in oligonucleotide 21 observed in LV1-1 (Table 1) could account for this difference. The fingerprint of LV1-5 differed from that of 1514 by the absence of oligonucleotides 21 and 24 and the presence of new spots 103 and 104. The new oligonucleotides were also present in LV1-1. Therefore, the changes observed in these variants are probably subsets of the changes observed in LV1-1.

DISCUSSION

Three natural antigenic variants of visna virus isolated from a persistently infected sheep have been compared to the parental virus strain. RNase T1 oligonucleotide fingerprints of the genomic RNA of these viruses revealed only small differences in their nucleotide structure. In an analysis of over 80 unique oligonucleotides in each of the four viruses studied, variant LV1-1 had seven oligonucleotides with altered mobilities when compared to parental strain 1514. Variant LV1-7 contained only one such altered oligonucleotide that was identified in LV1-1. Variant LV1-5 contained two altered oligonucleotides that were also observed in LV-1. Although LV1-1 was isolated from the animal prior to LV1-5 and LV1-7, this does not necessarily reflect the temporal appearance of these variants in the animal. Specific neutralizing antibody in the animal serum against LV1-7 could be detected 1 year prior to that against LV1-5 or LV1-1 (4). The variant viruses probably persist in the peripheral blood leukocytes for extended periods of time; this is supported by the reisolation of parental type virus in this sheep months after the isolation of LV1-1 (4). Thus, one explanation for the similarities between these three visna virus variants is the sequential alterations in the nucleotide sequence of the parental virus, giving rise to LV1-7, then LV1-5, and finally LV1-1. This can be correlated with the animal's developing a specific humoral immune response to each antigenic variant.

A minimum of seven single-base mutations could account for the differences observed between LV1-1 and 1514. However, the unique ribonuclease T1-resistant oligonucleotides

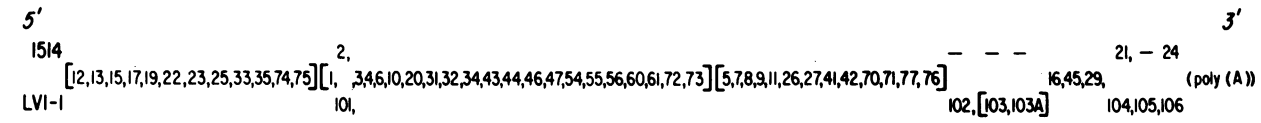

FIG. 4. Order of RNase T1-resistant oligonucleotides along the genome of virus strains 1514 and LV1-1. The central line of numbers represents oligonucleotides common to the two variants. Numbers above the central line represent oligonucleotides unique to strain 1514; numbers below the central line represent oligonucleotides unique to strain LV1-1. Numbers on the vertical axis indicate allelic oligonucleotides of the two variants. No order of the oligonucleotides within the brackets is implied.

comprise only 15% of the total sequence (80 oligonucleotides and an average length of 20 nucleotides gives ≈1600 nucleotides). Therefore, the total number of differences between these two virus strains is probably much higher than the seven differences observed here. Although the unique oligonucleotides represent a portion of the nucleotide structure of the viruses, it is likely that they are representative of the changes that occur throughout the genome.

Change in the nucleotide sequence of selected oligonucleotides can be attributed to single base changes in the primary nucleotide sequence. The other four alterations, which are in the 3′ region, are detected as unique oligonucleotides in LV1-1 for which no related sequences have been identified in the unique oligonucleotides of 1514. The simple explanation of the origin of these four oligonucleotides is that they are derived by mutations from sequences that are not resolved as unique oligonucleotides in the fingerprint of the genome of 1514.

How could such a visna variant arise? One explanation could be selection of pre-existing variants from the population of infecting virus. This seems unlikely for several reasons. When virus used as inoculum was plaque purified three times and examined for the existence of antigenic variants, none could be detected (13). Another possibility is that the changes in the 3′ end of the viral genome represent acquisition or substitution of a block of new sequences in the viruses from the host. No homology has been detected between the genome RNA of visna viruses and sheep DNA. Moreover, the similarity between the variants argues against this, as do experiments that show that the variants have the same genome length (14). Nonetheless, the possibility that small regions of homology exist between the virus and host sequence cannot be completely eliminated. An explanation we favor is the accumulation of multiple mutations within a single genome.

The clustering of the differences between these viral strains in the 3′ region of the genome suggests that changes in this region are a result of selection of variants that are not neutralized by the immune responses of the animal. Therefore, we would expect the viral antigen that elicits neutralizing antibody to be coded for by this region of the genome. Although the genetic organization of visna virus is not known, by analogy with other retroviruses the 3′-terminal region would be expected to contain the genetic information of the envelope glycoprotein. The major envelope glycoprotein is the antigenic target for neutralizing antibodies in other retroviruses (15, 16), and antibody against the envelope glycoprotein of visna virus will neutralize the virus (17). In addition, the glycoproteins of 1514 and LV1-1 can be distinguished by partial protease digestion (B. A. Nexø, personal communication). For these reasons it is likely that some of the changes in the structure of the genomic RNA of the parental and variant strains result in changes in the amino acid sequence of the envelope glycoprotein.

Changes in a single amino acid can change the recognition of a protein by an antibody. Alterations of a single amino acid in the hemagglutinin protein of influenza changed the neutralization properties of the virus when tested with a monoclonal antibody (18, 19); the change was not detected by animal antisera. It seems likely that multiple changes in protein sequence are required to evade neutralization by a polyvalent antibody response. Thus, the multiple changes that have been detected in the visna virus genome in LV1-1, a naturally occurring antigenic mutant, probably represent a number of amino changes in the envelope glycoprotein. In addition, the changes in the viral antigen of LV1-1, LV1-5, and LV1-7 were biologically significant, as shown by the sequential development of antibody in the sheep sera for each of these variants.

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