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Characterization and mapping of RNase III cleavage sites in VSV genome RNA

Gail W.Wertz and Nancy Davis

School of Medicine, University of North Carolina, Department of Bacteriology and Immunology, Chapel Hill, NC 27514, USA

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ABSTRACT

Ribonuclease III cleaves the genome RNA of vesicular stomatitis virus (VSV) to yield an array of fragments which range in size from 3.5 to 0.1 x 10^6 daltons under partial digestion conditions. The locations of the RNase III cleavage sites which give rise to these fragments have been ordered relative to the 3' end of the virion RNA by digestion of 3' end-labeled RNA. Based on a map of the cleavage sites we predicted that fragments having the same size could be generated which contain information from each gene. Annealing of individual VSV mRNA probes to Northern blots of the separated RNase III-generated fragments confirmed that fragments having the same size are, in fact, generated which contain information from each coding region of the VSV genome. Analysis of maps of partial digestion products indicates that fragments having the same size arise repeatedly along the 3' half of the genome.

The cleavage of VSV RNA by RNase III can be detected only if the nuclease treated molecules are denatured. This suggests that the structural features in VSV RNA which signal cleavage involve areas of higher order RNA structure.

INTRODUCTION

The negative-strand genome RNA of vesicular stomatitis virus (VSV) in the form of a nucleocapsid serves as template for two RNA synthetic processes: transcription and replication. Transcription of the genome RNA yields a leader RNA and 5 monocistronic messenger (m)RNAs. Replication is at least a two-step process involving synthesis first of a genome-size virion complementary (VC) RNA, which in turn serves as template for the second step, the synthesis of the progeny negative-strand virion RNAs.

Two models for the mechanism of RNA synthesis have been proposed. One model is based on the UV inactivation studies which show that synthesis of the 5 mRNAs is processive. These data were interpreted to suggest that a single initiation event occurs for transcription of all 5 mRNAs and it was proposed that the individual messages are generated by a cleavage process (1, 2). According to this model the full-length virion complementary RNA which is produced during the first step in replication could be generated by failure to

process the nascent RNA into the mature mRNAs. A second model, that individual initiation and termination events occur for each message, would account for a full-length virion complementary RNA by a process involving failure to terminate and reinitiate.

The variety of RNA synthetic events carried out by the negative strand viruses, presumably by the same template structure, is impressive. Of primary importance to understanding the biology of these viruses is the question of what factors are involved in controlling and signaling these various RNA transcriptive and replicative events. For either of the models of transcription and replication one must postulate signals either for initiation and termination or for processing events. In addition, there must be factors that control the balance between the two RNA synthetic events, transcription of discrete mRNAs or synthesis of the complete genome-size complementary RNA to begin replication.

With regard to the former process, sequence analysis shows that extensive homologies exist at the junctions of the five VSV genes (3, 4). The sequence (3') AUACUUUUUUU NA UUGUCNNUAG (5') is common to all junctions. N represents a variable nucleotide and the dinucleotides NA on the genome are not represented in the mRNA sequences. Therefore, RNA primary sequence appears to be a relevant factor. With regard to the latter process, at present, we can distinguish only that replication, in contrast to transcription, requires concomitant protein synthesis (5).

We have reported previously that the procaryotic RNA processing enzyme endoribonuclease III (EC 3.1.4.24) recognizes and cleaves sites in the VSV virion RNA to yield 20-24 size classes of fragments which range from 3.5 to 0.1 x 10^6 daltons (6). Although the exact specificity by which RNase III recognizes cleavage sites in natural substrates is not clear, it appears that all sites recognized have some double-stranded character, at least 9 base pairs both 5' and 3' to each bond cleaved (7, 8, 9). In addition, a limited sequence homology has been noted which is located a precise distance from several cleavage sites (7, 9).

The finding that RNase III can recognize and cleave sites on VSV virion RNA raises several interesting possibilities. First, this observation provides an indication that previously undetected higher order structure may exist in the single-stranded VSV virion RNA and we demonstrate here a method to probe the location of these structures. Second, RNase III provides a good candidate enzyme for use in generating fragments of the VSV genome. Finally, it raises the possibility that the procaryotic RNA processing enzyme, RNase

III, recognizes sites on a eucaryotic RNA that may be similar to those in procaryotic RNAs. This possibility is interesting since one of the models for generation of VSV mRNAs involves a processing event.

To evaluate these possibilities it was necessary to locate the sites at which RNase III cleaves the VSV virion RNA. We report here the characterization and the mapping of the RNase III cleavage sites on the VSV virion RNA.

MATERIALS AND METHODS

RNA Preparation. (i) Uniformly-labeled VSV Virion RNA. Baby hamster kidney (BHK) cells were infected with VSV at an input multiplicity of 10^{-4} plaque forming units per cell. At one hour post infection actinomycin D (4 μg/ml) and ^{3}H uridine (33 μCi/ml) were added to the culture fluids and incubation was continued for 21 hours at 37°C. The virus was harvested and concentrated as described previously (5). The virion RNA was obtained by two cycles of phenol extraction in the presence of 1% sodium dodecyl sulfate (SDS) and recovered by ethanol precipitation. The RNA was further purified by velocity sedimentation in 15-30% sucrose gradients containing 0.1% SDS. The single peak of RNA sedimenting at 40S was recovered by ethanol precipitation. (ii) 3' End-labeled Virion RNA. Virion RNA prepared as described above was labeled at its 3' end using cytidine 3'-5' (^{32}P) bisphosphate and RNA ligase according to the method described by Keene et al. (10). The labeling reaction was carried out at 4°C overnight. The labeled RNAs were separated from unincorporated label by chromatography over Sephadex G-75. The RNAs were further purified by velocity sedimentation on a 15-30% sucrose gradient containing 0.1% SDS. The 40S virion RNA was concentrated by ethanol precipitation.

Purification of E. coli RNase III. RNase III was purified from E. coli strain MRE-600 as described previously (6).

Digestion of RNA by RNase III. Conditions for digestion of virion RNA with RNase III were as described previously (6). The concentration of NH_4Cl was 0.15M in all reactions.

Gel Electrophoresis. RNAs were analyzed by electrophoresis in 2% acrylamide gels as described previously (11) or in 1.2% or 1.5% agarose gels containing 6 M urea and 0.025 M sodium citrate buffer at pH 3.0. Following electrophoresis, urea was removed from the gels by soaking in water. Gels containing ^{32}P were dried onto Whatman 3MM paper and used to expose du Pont Cronex 2DC x-ray film at -70°C using a du Pont Cronex "Lightning Plus" intensifying screen. Gels containing ^{3}H were dehydrated by soaking for 30 minutes each in two changes of 95% methanol, then immersed for 3 hours in 12.5%

2, 5-diphenyloxazole in 95% methanol. The gel was removed from the PPO, soaked in water for 60 minutes and then dried onto Whatman 3MM paper and used to expose film at -70°C as above.

Two-phase RNA: RNA Hybridization. RNAs that had been separated by electrophoresis in denaturing 1.5% agarose, 6 M urea gels were transferred to diazobenzyloxymethyl (DBM) paper essentially according to the method of Alwine et al. (12) with modifications as described below. This method is referred to as the "Northern" blotting technique.

(i) Preparation of Gel for Blot Transfer. Following electrophoresis, the urea was removed from the gel by soaking in water for 60 minutes at 4°C. The gel (~ 3 mm thick) was then soaked in 50 mM NaOH for 25 minutes at 23°C to break the RNA and facilitate transfer of both large and small RNAs. The gel was next soaked in water twice for 10 minutes and then treated twice for 10 minutes with 0.2M sodium acetate, pH 4.0.

(ii) Transfer of RNA to DBM Paper. RNA was transferred to the DBM paper by blotting as described by Alwine et al. (12) except that the transfer was done using 0.2M sodium acetate buffer. Transfer was carried out for 5-15 hours at room temperature. After transfer, the DBM paper with the RNA covalently linked to it was prepared for hybridization.

(iii) Hybridization of Immobilized RNA to Radioactive Probes. The RNA covalently linked to the DBM paper was prepared for hybridization by treatment for two hours at 65° in a sealed plastic boiling bag with hybridization buffer (0.6M sodium chloride, 0.06M sodium citrate, 0.02% (wt/vol) each of polyvinyl-pyrrolidone and ficoll, 0.05M sodium phosphate pH 6.5, 0.1% sodium dodecyl sulfate (SDS), and 0.2 mg/ml polyadenylate) containing 1% (wt/vol) glycine (for the purpose of hydrolyzing any unreacted diazo groups on the DBM paper that might react with the labeled probe). The pretreatment solution was removed and replaced with fresh hybridization buffer (75-100 $\mu l/cm^2$ of paper) containing the desired concentration of ^{32}P-labeled RNA probe. The bag was then resealed and incubated at 65° with a constant gentle rotating motion. After 12 to 18 hours incubation, the paper strips were removed from the bags and washed thoroughly: twice for 30 minutes at 37°C in 0.3M sodium chloride/0.03M sodium citrate (2 x SSC) plus 0.1% SDS and twice for 30 minutes at 50°C in 0.1 x SSC plus 0.1% SDS. The strips were then air dried and used to expose Cronex 2DC film using a "lightning plus" intensifying screen at -70°C.

Materials. [5-^3H] Uridine (20-25 Ci/mmol) was from Moravek Biochemicals. ^{32}Pi was from New England Nuclear. DBM paper was prepared as described by Alwine et al. (12) or purchased from Schliecher and Schuell.

RESULTS

Characteristics of RNase III cleavage. Ribonuclease III cleaves the 4 x 10^6 dalton VSV virion RNA to generate an array of fragments which range in size from 3.5 to 0.1 x 10^6 daltons. The effect of the enzyme on RNA as a function of time of incubation is shown by the data presented in Fig. 1. Cleavage of the RNA by the endonuclease was assayed by electrophoresis in agarose-urea gels.

The time of incubation of the RNA with the enzyme affects the pattern of fragments generated (Fig. 1). Short periods of incubation result in generation of fragments as large as 3.5 x 10^6 daltons. As the time of incubation increases, the higher molecular weight fragments are no longer discerned in the array of product fragments. At the same time that the decrease

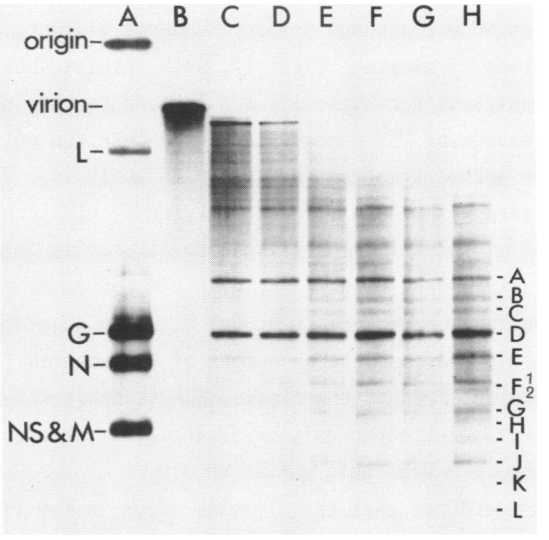

Figure 1. Effect of length of incubation on products generated by RNase III cleavage of VSV virion RNA. ^{3}H-uridine labeled VSV virion RNA (1.2μg) was incubated with 4 units of RNase III for varying lengths of time at 37°C. Reaction products were analyzed by electrophoresis on 1.5% agarose, 6 M urea gels as described in the text. (a) ^{3}H-uridine labeled VSV intracellular RNAs electrophoresed as internal size references; (b) ^{3}H-uridine labeled VSV virion RNA not incubated; (c) ^{3}H-uridine labeled VSV virion RNA incubated with RNase III for 2 min.; (d) 5 min.; (e) 10 min.; (f) 20 min.; (g) 45 min.; (h) 60 min. Molecular weight values for the VSV mRNAs were determined by co-electrophoresis with sequenced Qβ RNA, MS2 RNA, and E. coli 16s and 23s rRNAs as internal markers. The values obtained in this manner are essentially identical to those based on the sequence analysis data for the 4 small mRNAs (plus added value for polyA of 125 residues)(17,18).

in high molecular weight fragments is observed, there is an increase in the amount of smaller molecular weight fragments. These findings suggest that the large fragments represent partial digestion products of the virion RNA which contain additional cleavage sites that can be recognized. Similar results were observed as a function of enzyme concentration (6).

Another characteristic of digestion of VSV RNA by RNase III (also shown by the data in Fig. 1) is that not all of the fragments are present in equimolar amounts. For example, it is apparent that the fragments designated A and D are present in much greater relative concentration than fragments B and C. The explanation for this observation will be derived from experiments that follow.

Effect of Denaturation on Visualization of Cleavage Products. Previous work has indicated that higher order structure in RNA, either hydrogen-bonded secondary structure, or other non-hydrogen-bonded RNA: RNA interactions, as well as some concensus primary sequence elements are involved in RNase III recognition of cleavage sites (7, 9). Results obtained using VSV genome RNA as a substrate for RNase III add support to this hypothesis.

RNase III cleavage of VSV genome RNA can be detected only if the RNA is denatured after enzyme treatment and prior to analysis. VSV virion RNA that has been treated with RNase III and analyzed by electrophoresis without prior denaturation migrates in neutral agarose-acrylamide gels the same as untreated control virion RNA (Fig. 2, a, b). In contrast, virion RNA that has undergone identical enzyme treatment but has been denatured by heating prior to analysis migrates as a large number of RNA species (Fig. 2c). Similar results are obtained if the cleaved RNA is denatured by treatment with glyoxal prior to electrophoresis or if the electrophoresis is carried out in 1.5% agarose, 6 M urea gels run at pH 3.0.

These results indicate that the cleavage sites in the virion RNA may include structural features that involve areas of secondary or tertiary structure. At present, we do not know if these structural features are due to long range interactions in the RNA (with the cleavage site located in between the interacting structures perhaps) or if the structures are due to interactions between adjacent sequences. If due to hydrogen-bonded secondary structure, the regions must be very limited; we find no more that 2% of the VSV virion RNA resistant to pancreatic ribonuclease A in 0.15 M salt (13). Nevertheless, the structures involved are strong enough to hold the 4 million dalton virion RNA intact after enzymatic digestion even throughout procedures such as electrophoresis in non-denaturing acrylamide: agarose gels, ethanol

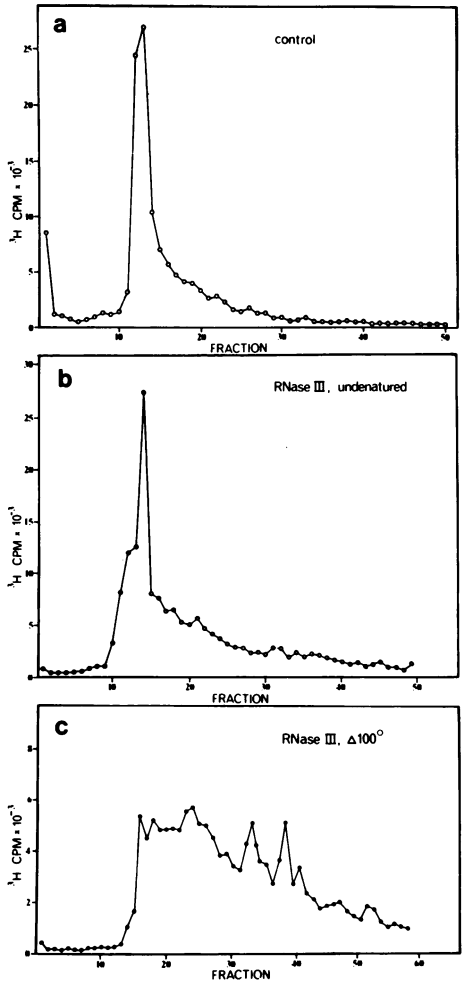

Figure 2. Effect of denaturation on detection of RNase III cleavage products of VSV virion RNA. VSV virion RNA (.6µg) was incubated with RNase III (2 units) for 10 min. at 37°C. The products of the reaction were electrophoresed on non-denaturing 2% acrylamide gels. (a) No enzyme; (b) 2 units of enzyme, RNA untreated before electrophoresis; (c) 2 units of enzyme, RNA heated to 100°C for 2 min. prior to electrophoresis. RNA incubated with no enzyme and then heated to 100°C for 2 min. prior to electrophoresis displayed a profile identical to that shown in (a).

precipitation or velocity sedimentation in sucrose gradients containing 0.1% SDS.

Mapping of the RNase III Cleavage Sites in the Virion RNA. The follow-

ing strategy was used to determine the sites at which RNase III cleaves VSV virion RNA. Virion RNA uniformly labeled with ^{3}H uridine was prepared and one-half of the preparation was then labeled at the 3' end with cytidine 3'-5' (^{32}P)bisphosphate. Equal microgram quantities of RNA labeled in either manner were then digested with RNase III for 25 minutes, a time chosen to yield partial digestion conditions (see Fig. 1, F). The fragments generated from each separate digestion reaction were analyzed by electrophoresis in adjacent lanes of an agarose-urea gel. After electrophoresis the gel was cut in half and the digestion products of the RNA labeled only with ^{3}H uridine were visualized by fluorography. The digestion products of the RNA labeled both uniformly with ^{3}H uridine and with ^{32}P at the 3' end were visualized by autoradiography allowing detection of only fragments containing a ^{32}P label. Since the ^{32}P label was present only on the 3' end of the molecule it was possible by comparing the paired digests to determine which fragments in a display of all the digestion products extended from the 3' end of the molecule. In Figure 3, for example, the bands designated D_2, G_2, J, K, and L in the uniform digest (Lane C) all have ^{32}P end-labeled counterparts (Lane D) and hence extend from the 3' end of the molecule.

The sizes of the fragments having a ^{32}P end-label were determined from a standard curve based on the migration rates of single-strand RNAs of known sequence. These standards were co-electrophoresed in adjacent wells of the analytical gel. The size determinations for the end-labeled fragments designated in Lane D, Fig. 3 are shown in Figure 4. The end-labeled fragments range in size from 3.2 to 0.1 x 10^6 daltons. Having determined the size of the end-labeled fragments it was possible to determine the distance from the 3' end of the virion RNA to the position of the RNase III cleavage site that had generated a particular fragment.

A map of the location of the cleavage sites identified relative to the 3' end of the VSV genome RNA is shown in Figure 5, where the cleavage sites are indicated by the solid arrows. This map has been drawn to scale using values for the sizes of the coding regions of the 4 VSV gense N, NS, M and G as determined from sequence data (17, 18). This 3'-end label mapping technique identifies six RNase III cleavage sites in the N gene, two in the NS gene, one site in the M gene, two in the G gene and at least five sites in the L gene. Due to the size of the VSV RNA, this method identifies primarily sites in the 3' half of the genome. Other methods, including 5' end-label analysis, will have to be used to accurately map sites at the 5' end of the molecule. Specific labeling of the 5' end is technically difficult at present due to impurities in

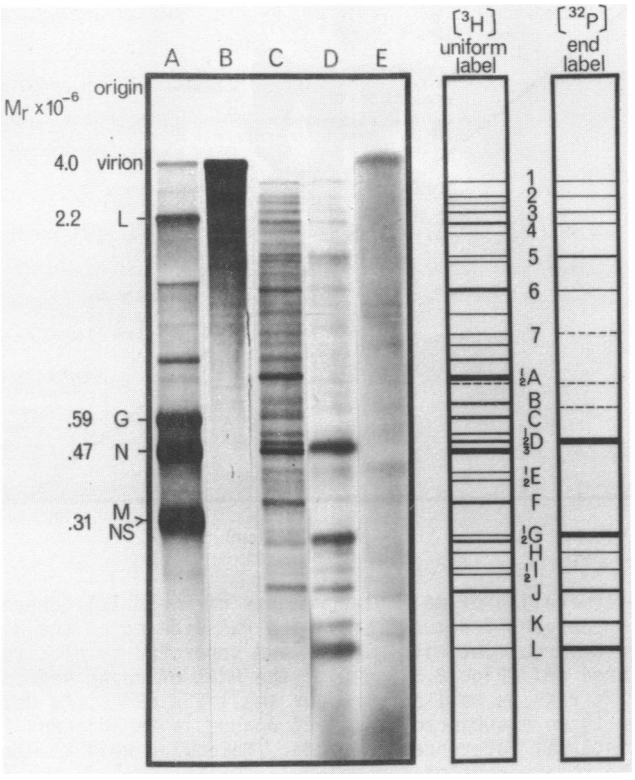

Figure 3. Identification of fragments generated by RNase III digestion of VSV virion RNA that extend from the 3' end of the RNA. VSV RNAs (1.4µg) either uniformly labeled with ^{3}H-uridine or labeled only at the 3' end (using ^{32}pCp and RNA ligase) were digested for 25 min. at 37°C with 4 units of RNase III. The digestion products were denatured by treatment with 1.4 M glyoxal for 60 min. at 23°C and then separated by electrophoresis in adjacent lanes of a 1.2% agarose, 6 M urea gel, pH 3.0. The ^{3}H-labeled digestion products were visualized by fluorography; the ^{32}P products by autoradiography. (A) VSV intracellular RNAs as size markers; (B) ^{3}H uniformly labeled VSV virion RNA, no enzyme; (C) ^{3}H-uridine uniformly labeled virion RNA digested with 4 units of RNase III; (D) ^{32}P-3' end-labeled VSV virion RNA digested with 4 units of RNase III; (E) ^{32}P-3' end-labeled VSV virion RNA, no enzyme.

the kinase used in this method.

Based on the cleavage sites indicated in Figure 5, it is possible to predict and to catalogue the partial cleavage fragments that could arise from within the 3' half of the genome. The array of these predicted partial digestion fragments, drawn to scale, is indicated by open rectangular boxes in the bottom half of Figure 5. The sizes of all the various groups of partial

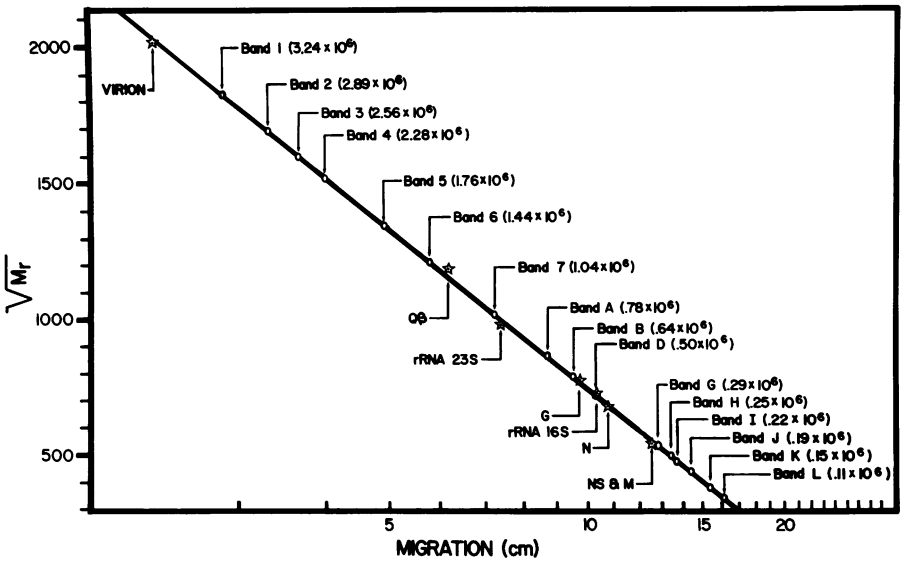

Figure 4. Determination of molecular weights of RNase III-generated fragments. The RNase III-generated fragments of virion RNA labeled at the 3' end with ^{32}P were denatured by treatment with glyoxal and separated by electrophoresis in a 1.2% agarose urea gel (Fig. 3, land D). The RNAs of known sequence, Qβ and E. coli 16s and 23s rRNA as well as the four small VSV mRNAs (MW determined as in Legend to Fig. 1) were subjected to electrophoresis in adjacent lanes of the same gel for internal reference standards. Molecular weights were calculated by plotting the square root of the molecular weight (Mr) vs the log mobility according to Lerach et al. (16).

digestion fragments predicted from the map correspond to the actual size classes of fragments that are generated by digestion of VSV RNA with RNase III (see Figs. 3, 4, 5). This correspondence is indicated by the letters enclosed in the boxes in Fig. 5 and also used to designate fragments in Fig. 1, 3, 4, and 8. For example, band G (the designation of which should not be confused with G mRNA,) contains RNase III digestion products of 0.29 x 10^6 daltons. The map of cleavage sites in Fig. 5 predicts that fragments of this size could be generated from at least 8 regions of the genome. Band G, therefore, could include 8 distinct digestion products representing portions of all 5 VSV genes.

The array of partial digestion fragments shown in Figure 5 predicts that numerous fragments having the same size arise from several different regions on the genome. This observation, in turn, predicts that the various size classes of fragments separated in the agarose-urea gels are not unique but are

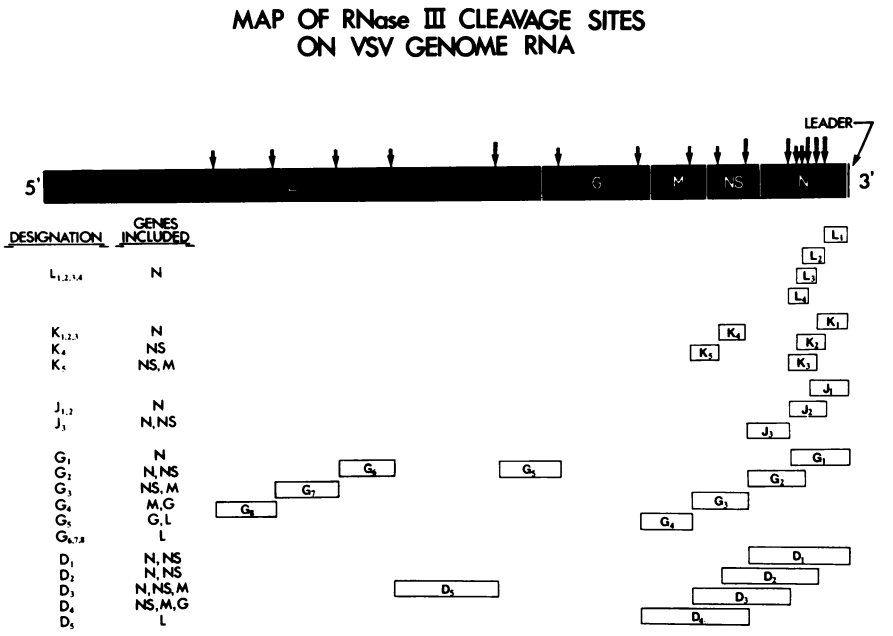

Figure 5. Location of RNase III cleavage sites on the VSV genome. The distance of the RNase III cleavage sites from the 3' end of the virion RNA were determined by analyzing the size (see Fig. 4) of the RNA fragments generated by RNase III cleavage that contained a 3' end-label. The locations of these sites on the genome are indicated by arrows (drawn to scale). Based on these sites a catalogue of possible partial digestion fragments was generated. These predicted fragments, indicated by rectangular boxes, are drawn to scale; they correspond in size to fragments actually generated by RNase III digestion and this is indicated by the letter designation.

composed of similarly-sized fragments that are generated from several locations on the genome.

Genomic Location of RNase III fragments. The hypothesis that fragments having the same size could arise from several different locations on the genome was tested by annealing of the separated RNase III-generated fragments of the VSV genome to individual VSV mRNAs using the "Northern" blotting technique. If the RNA fragments which migrate as apparent discrete bands in the gel are generated by cleavages at several different locations on the genome, then that particular band in the gel should hybridize to more than one of the VSV mRNAs.

Following partial digestion of unlabled VSV virion RNA with RNase III, the fragments generated were separated by electrophoresis in agarose-urea gels

and then transferred from the gel to DBM paper. Replicate lanes containing the array of unlabeled virion RNA fragments immobilized on DBM paper were then hybridized to different ^{32}P-labeled VSV mRNA probes. Four of the VSV mRNAs, N, NS, M and G, were used as individual probes. The mRNA probes were separated from a mix of total VSV mRNAs by electrophoresis in a high resolution preparative gel system (14). The extent to which the mRNAs were separated before use as probes in this experiment is shown in Figure 6.

It was found that each of the individual VSV mRNAs when used separately as probes could anneal to all of the major size classes of RNase III-generated fragments (Fig. 7). These individual hybridizations of fragments with purified N, NS, M or G mRNA demonstrated identical patterns of annealing to all fragments. When a mixture of all five of the VSV mRNAs was used as a probe, it was found, as expected, that they hybridized to all of the size classes of RNase III generated fragments and the pattern for annealing with total mRNA was the same as that displayed by annealing with any one of the individual messages (Fig. 7). The annealing reactions were shown to be specific by negative controls using ^{32}P-labeled virion RNA as a probe (Fig. 7) and by using

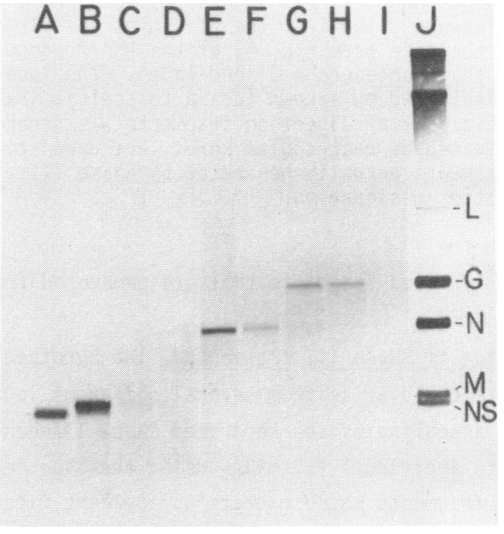

Figure 6. Analytical gel electrophoresis of ^{32}P-labeled VSV mRNAs separated by preparative electrophoresis. Total cytoplasmic RNA from VSV infected cells was separated by preparative electrophoresis in a 1.5% agarose, 6 M urea gel. RNA eluting in successive fractions was collected by ethanol precipitation and aliquots of each fraction were analyzed in a 1.5% analytical agarose, 6 M urea gel. RNA was visualized by autoradiography.

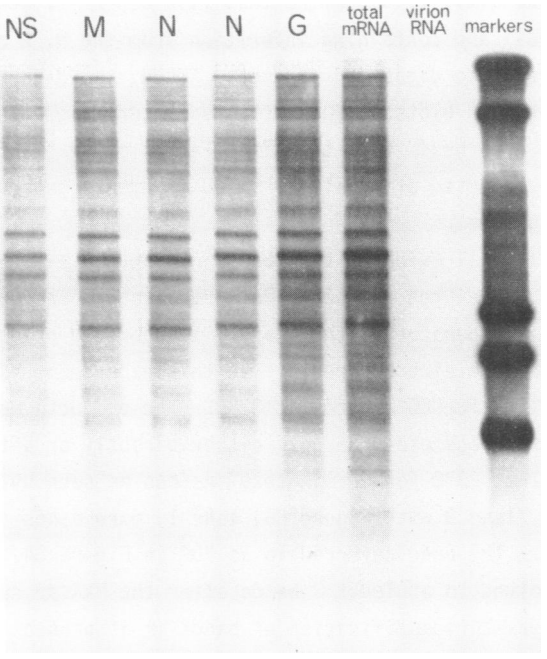

Figure 7. Two-phase hybridization of VSV virion RNA fragments generated by cleavage with RNase III to individual ^{32}P-labeled VSV mRNAs. Unlabeled VSV virion RNA (1.4μg) was treated with 4 units of RNase III for 25 min. at 37°C. The fragments generated by enzymatic cleavage were separated by electrophoresis in denaturing gels, transferred to DBM paper and replicate lanes were separately annealed to the individual ^{32}P-labeled VSV mRNA probes. As indicated, the RNase III generated fragments of virion RNA were annealed to ^{32}P-labeled NS, M, N or G mRNAs individually, or to a mixture of all 5 VSV mRNAs (including L mRNA), or to ^{32}P-labeled virion RNA.

extremely stringent hybridization and washing conditions. All controls, including a pancreatic ribonuclease digestion of the annealed RNAs, indicated that these hybridizations were extremely well matched.

The seeming paradox that a fragment as small as G (0.29 x 10^6 daltons), for example, could anneal to all of the VSV messages, each of which is larger than the fragment, can be explained with reference to Fig. 5. By taking into account the partial digestion products that could be generated from the original single cleavage site in the M gene, for instance, it is possible to see that three fragments, G_1, G_2, and G_3 having information form the N, N and NS, and NS and M genes respectively, could arise extending toward the 3' end of the molecule. Also, in the direction of the 5' end of the molecule from the single

site in M, it is possible to determine that five other fragments having the size of G (G_5 to G_8) and containing informtion from the M, G and L genes could be generated as complete digestion products. Thus, the data provided by hybridization of Northern blots of RNase III-generated fragments to individual mRNAs support the hypothesis that the size classes of fragments generated are not unique but are compsed of similarly sized fragments generated from several locations on the genome.

Finally, additional evidence that bands having the same electrophoretic mobility in the agarose-urea gels contain more than one species of RNA was obtained by extensively denaturing the RNase III-generated products by treatment with glyoxal prior to elelctrophoresis. In this experiment VSV RNA was digested with RNase III for 20 minutes at 37°C. The products were then analyzed by electrophoresis in agarose-urea gels either directly or after denaturation with 1.4 M glyoxal at 23°C for 60 minutes. As can be seen by comparing the data in Figure 8A, lane 3 with Figure 8B, lane 1, more bands are discerned after glyoxal treatment. The band referred to as "D" in Figure 8A, for example, appears to be resolved to at least 3 bands after the RNA products have been denatured with glyoxal (identification of bands is at present tentative based only on relative mobility). Additionally, it is possible to see that the bands referred to as B, C, E, and G also appear to resolve into at least two electrophoretic species each after glyoxal treatment. Thus, these data show that several bands migrating as single species before denaturation are not unique as they can be resolved to electrophoretically distinct species after denaturation with glyoxal.

Taken together, the results presented here show that fragments having similar electrophoretic mobility can be generated that contain information from each coding region of the VSV genome.

DISCUSSION

Labeling of the VSV virion RNA at the 3' end prior to digestion with RNase III has enabled us to map the location of the sites of cleavage by this enzyme relative to the 3' end of the genome. This technique allowed us to map six cleavage sites that lie within the N gene, two each within the NS and G genes, one in the M gene and at least five in the L gene. Within the limits of our ability to determine the size of digestion products (by comparison to the relative mobility of sequenced RNA strands electrophoresed in adjacent wells of the same gel), none of the cleavage sites occurred exactly at intercistronic boundaries.

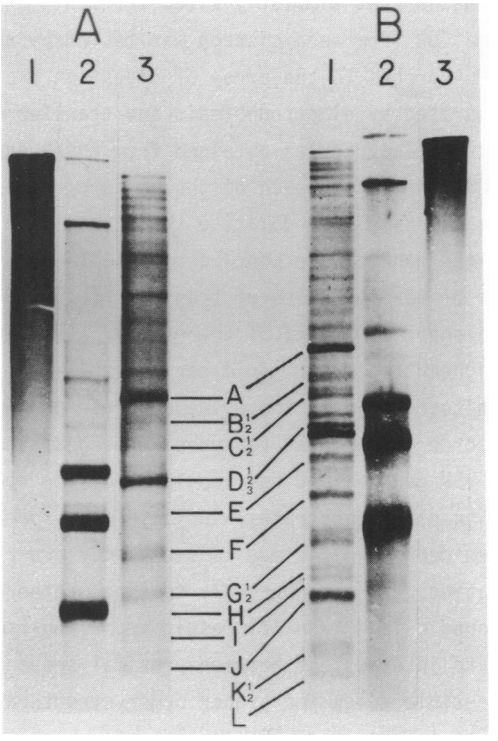

Figure 8. Effect of denaturation with glyoxal on ability to resolve RNase III digestion products by electrophoretic separation. RNA fragments of VSV virion RNA (1.4µg) generated by cleavage with RNase III were analyzed by electrophoresis in 1.5% agarose urea gels. [A] RNAs untreated before electrophoresis; (1) ^{3}H virion RNA, no enzyme, (2) VSV mRNA markers, (3) ^{3}H virion RNA, 4 units RNase III; [B] RNAs treated with 1.4 M glyoxal, 23°C, 60 min. before electrophoresis; (1) ^{3}H virion RNA incubated with 4 units RNase III, (2) VSV mRNA markers, (3) ^{3}H virion RNA control, no enzyme.

Analysis of RNase III digestion products of VSV virion RNA as a function of time of incubation or enzyme concentration demonstrated that partial digestion products occur. Additionally, as shown by the data presented in Figure 1, several size classes of fragments are generated in higher molar yield than others. A map cataloging the various possible partial digestion products was constructed based on the cleavage sites identified by 3' end mapping. Analysis of the informtion provided by this map (1) indicated that fragments having the same size (electrophoretic mobility) could arise repeatedly along the genome; and (2) predicted that fragments having the same size could be generated that contained information from each coding region of

the genome. The hypothesis that similarly-sized fragments could be generated which contained information from each cistron was tested by annealing highly-purified individual mRNA probes to the array of digestion products after the fragments had been separated by electrophoresis and transferred from the gel to DBM paper by blotting. The results obtained from these experiments showed that each purified mRNA annealed to each of the bands of separated fragments. Thus, fragments having the same size do arise that contain information from each coding region of the genome. We conclude from this analysis of digestion products that families of various sizes of fragments (e.g., D, G, Figure 5) can be generated all along the 3' half of the genome. Thus, the presence of fragment classes in higher than molar yield can be explained.

These data, taken together with the evidence presented here indicating involvement of higher order structural features at or near the RNase III cleavage sites on the VSV genome (and our knowledge of the specificity of cleavage by RNase III on other substrates) indicate that specific regions of the VSV virion RNA when deproteinized may assume higher order structures that are non-random in location. We are currently testing whether or not the structures we have probed using RNase III result from long-range interactions in the RNA or are due to interactions between proximal sequences.

In considering possible roles for higher order structural features in VSV RNA, it is important to note that the template for both types of RNA synthesis in the VSV system is the RNA in a nucleocapsid structure. The active template is the RNA covered with N protein such that the phosphodiester backbone is resistant to attack by pancreatic ribonuclease. Additionally, the protein NS must bind to the RNA: N protein complex before polymerase will bind (15). In light of these facts, one possible function for the structural features probed by RNase III may be in the binding of proteins to the RNA in order to construct the active RNA synthetic template. Structure in the nascent RNA may function in the binding of putative controlling proteins or it may function in the maturation of the nucleocapsid, for example, in establishing a putative nucleation site for addition of the N proteins to the RNA. Alternatively, structural features in the virion RNA may have no function, but the structures they confer on the complementary mRNAs may be relevant to the latter's function.

In summary, analysis of the products of digestion of VSV RNA with RNase III has shown that cleavage generates an array of similarly sized fragments that contain information from each coding region of the genome. The fragments generated by cleavage with RNase III can be visualized only if the treated RNA

is subjected to denaturing conditions. These data suggest that small areas of higher order structure may be involved in or located near the cleavage sites. These sites, which we have probed and mapped using RNase III as a tool, are located such that cleavage generates similarly sized fragments from within each of the VSV genes, often in a repeating array.

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