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A Site-Specific Self-Cleavage Reaction Performed by a Novel RNA in Neurospora Mitochondria

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Summary

We describe a novel DNA and RNA found in the mitochondria of the Varkud-1c natural isolate of Neurospora. The majority of the RNA, termed VSRNA, is an 881 nucleotide single-stranded circular molecule complementary to one strand of a low copy, double-stranded circular DNA, VSDNA. VSRNA combines some features of the human hepatitis delta virus, group I introns, retroelements, and plant viral satellite RNAs. VSRNA synthesized in vitro performs a self-cleavage reaction whose products terminate with a 5′ hydroxyl and a 2′,3′ cyclic phosphate. This reaction may be involved in the natural processing pathway of multimeric VSRNA in vivo. VSRNA lacks a hammerhead structure or substantial sequence similarity to any other self-cleaving RNA, suggesting that the RNA structure involved in cleavage may be different from those in previously characterized catalytic RNAs.

Introduction

The discovery that certain RNAs are capable of catalyzing reactions using themselves or exogenous molecules as substrates has prompted renewed interest in the roles of RNA in the function (Cech, 1987) and evolution (Sharp, 1985; Gilbert, 1986; Darnell and Doolittle, 1986) of biological systems. The exploitation of RNA catalysts (ribozymes) as research and therapeutic tools to interfere selectively with gene expression at the RNA level is also being investigated (Haseloff and Gerlach, 1988; Cotten and Birnstiel, 1989; Cameron and Jennings, 1989).

The known catalytic RNAs can be categorized into at least four groups: group I introns (Cech, 1988), group II introns (Michel et al., 1989), RNAase P (Guerrier-Takada et al., 1983), and hammerheads (which include several plant viral satellite RNAs, one viroid, and a transcript of a newt satellite DNA; Symons, 1989). Members of each group share some similarity in secondary structure and primary sequence. The genomic (plus) and anti-genomic (minus) strands of hepatitis delta virus (HDV) RNA (Kuo et al., 1988; Wu et al., 1989) and the minus strand of the satellite of tobacco ringspot virus (STobRV; Buzayan et al., 1986a; Feldstein et al., 1989) may constitute additional groups, since sequences required for their cleavage appear not to share similarities with any of the better characterized RNAs.

Group I and II introns, RNAase P, and the newt RNA are generally thought of as transcripts of a gene or sequence of DNA that is part of the host genome. HDV and the plant pathogenic RNAs replicate without a DNA intermediate by a rolling circle mechanism using polymerases encoded elsewhere to synthesize multimeric RNAs, which then self-process by site-specific, RNA-catalyzed cleavage to produce new monomer RNAs. Many of the pathogenic monomer RNAs are circular, implying a ligation step either during or after cleavage (reviewed by Symons, 1989).

The similarities among group I introns in different species and the sporadic distribution of a given intron within a species suggest that these sequences represent a type of mobile element. Experimental support for this idea comes from the observation that some group I introns encode enzymes that initiate gene conversion events directly involved in intron mobility (reviewed by Dujon, 1989; Lambowitz, 1989; Belfort, 1989). A variety of small catalytic RNAs, including HDV (Makino et al., 1987) and certain plant pathogenic RNAs, also contain some of the conserved group I sequences (Dinter-Gottleib, 1986; Hadidi, 1986).

Some plasmids in fungal mitochondria contain intron-like sequences (Nargang et al., 1984) and, like introns, have a scattered distribution within and among species (Natvig et al., 1984; Collins and Saville, 1990). The best characterized Neurospora mitochondrial (mt) plasmid is present, in slightly different allelic forms, in the Mauriceville-1c (Collins et al., 1981), Varkud-1c (Akins et al., 1988), and other (Collins and Saville, 1990) natural isolates of Neurospora. It has been speculated that this plasmid may be a type of retroelement, since it encodes a reverse transcriptase that may be involved in its replication (Kuiper and Lambowitz, 1988).

We describe here a novel genetic element found in the mitochondria of a natural isolate of Neurospora. It is not closely related in nucleotide sequence to any previously characterized element, although it combines some of the features found in a variety of other mobile elements.

Results

Figure 1 shows the ethidium bromide staining pattern of electrophoretically separated total mt RNAs isolated from Neurospora laboratory strain 74*A* and two natural isolates, Adiopodoume and Varkud-1c. In most strains only the relatively abundant RNAs such as mt rRNAs are detectable (left lane). Some natural isolates also contain detectable amounts of a stable group II intron excised from the transcript of the *col* gene, which encodes cytochrome oxidase subunit I (middle and right lanes; Field et al., 1989). In addition to these RNAs, mitochondria of the Varkud-1c strain contain two abundant small RNAs of ~0.9 kb that are present at very high concentrations (right lane). These small RNAs are designated C-VSRNA and L-VSRNA and will be shown below to be circular and linear forms, respectively, of a novel RNA found in Varkud-1c mitochondria.

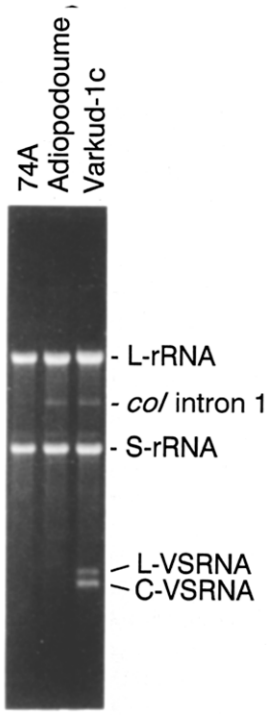

Figure 1. Novel, Abundant RNAs Isolated from Mitochondria of Varkud-1c

Total mtRNAs isolated from laboratory strain 74A and natural isolates Adiopodoume and Varkud-1c were denatured with glyoxal, electrophoresed in an agarose gel, and stained with ethidium bromide. The positions of circular C-VSRNA and linear L-VSRNA are indicated.

The VSRNAs Are Complementary to One Strand of a Low Abundance Mitochondrial DNA

The VSRNAs (either separately or together) were isolated by electroelution from an agarose gel, and a radioactively labeled probe was prepared from them by cDNA synthesis (using reverse transcriptase, random oligonucleotide primers, and [α-^{32}P]dATP). This probe was hybridized to a Southern blot containing total mtDNA from Varkud-1c that had been electrophoresed either undigested or after digestion with one of five restriction enzymes (Figure 2A). If the VSRNAs were transcripts of the mt chromosome, the cDNA probe would be expected to hybridize to the high molecular weight DNA in the undigested lane and to a specific fragment(s) in each of the digests. Instead, we observed hybridization to a multimeric series of bands (Figure 2B) in the lanes containing undigested DNA. This suggests that the VS cDNA probe hybridized to a plasmid DNA, which we designate VSDNA. The same pattern of multimers was observed even in digests with any of several enzymes that digest the mt chromosome (EcoRI is shown in Figure 2B; others are not shown). However, digestion with AvaI, HaeII, HaeIII, or DraI produced a common size monomer length band (0.9 kb; indicated by the arrowhead in Figure 2).

These observations indicate that the VSDNA population is organized as a series of head-to-tail multimers, with each monomer unit containing a single restriction site for each of these four enzymes (confirmed by DNA sequence analysis of the cloned VSDNA; see Figure 4). VSDNA bands on CsCl–ethidium bromide gradients at a position expected for supercoiled DNA, indicating that it is a covalently closed circular DNA (data not shown). This multimeric organization is typical of that previously found for other Neurospora mt plasmid DNAs (Collins et al., 1981; Stohl et al., 1982). Consistent with previous terminology (Nargang, 1985) we will refer to VSDNA as a plasmid, since it is apparently not derived from the mt chromosome; however, we have not investigated whether VSDNA replicates autonomously or whether it is synthesized by reverse transcription from VSRNA.

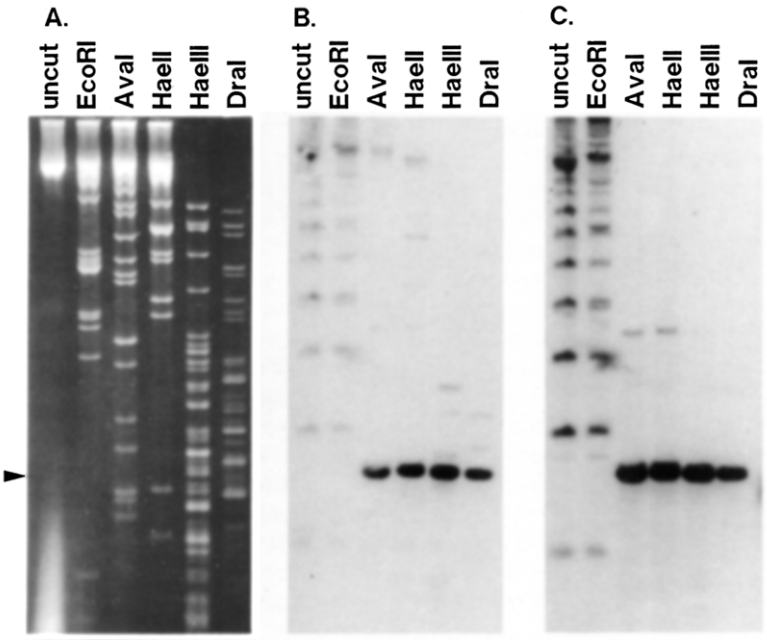

Figure 2. Southern Hybridization Detects a Low Abundance DNA Plasmid Complementary to the VSRNAs

(A) Varkud-1c mtDNA was digested with the restriction endonucleases indicated, electrophoresed in a 1% agarose gel, and stained with ethidium bromide.

(B) and (C) Autoradiogram of a Southern transfer of the gel in (A), hybridized with (B) a VSRNA probe (prepared from electroeluted VSRNAs by cDNA synthesis using reverse transcriptase, random primers, and [α-^{32}P] dATP) and (C) clone pD7, which contains one VSDNA monomer (see Experimental Procedures). The arrowhead indicates the position of linearized VSDNA detected by hybridization. Slower migrating bands in the AvaI and HaeII lanes of Figure 2C are due to incomplete digestion.

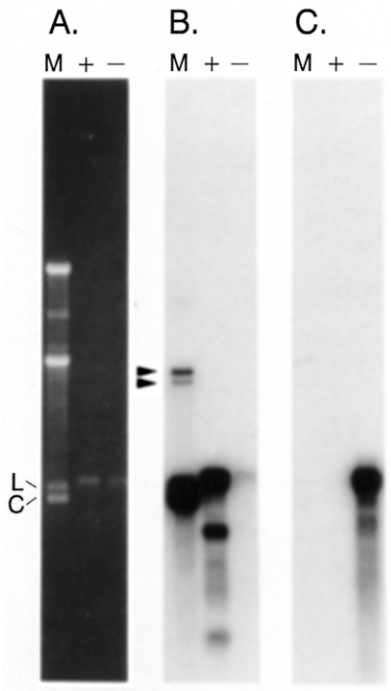

Figure 3. C- and L-VSRNAs Are Complementary to the Same DNA Strand of the VSDNA Plasmid

T7 RNA polymerase runoff transcription of VSDNA clones pD7 or pD7R was used to synthesize unlabeled plus strand (corresponding to the abundant in vivo RNA) and minus strand (complementary to the in vivo RNA) RNAs. Full-length transcripts were purified by electroelution from an agarose gel. These RNAs were electrophoresed through an agarose gel along with total Varkud mtRNA (lane M). Positions of circular and linear VSRNAs are indicated as C and L, respectively. The in vitro transcripts migrate slightly slower than L-VSRNA because they include short regions of vector sequences. Radiolabeled probes complementary to either the minus or plus RNA strands were prepared by T7 transcription of pD7 or pD7R, respectively.

(A) Ethidium bromide–stained gel.

(B) Hybridization of Northern blot of a gel identical to that in (A) with a probe complementary to the plus strand RNA. The faint bands indicated by the arrowheads are dimeric VSRNAs.

(C) As in (B) but hybridized with a probe complementary to minus strand RNA.

Faint hybridization of the cDNA probe to some chromosomal restriction fragments was also observed (Figure 2B). This is likely due to contamination of the gel-purified VSRNAs with chromosomal transcripts (see below). The monomer VSDNA restriction fragment detected by hybridization is not detectable by ethidium bromide staining of this gel, indicating that the copy number of the VSDNA is much lower than that of the mt chromosome.

Using restriction enzymes that cleave once per monomer unit, VSDNA clones were obtained in each orientation (see Experimental Procedures) and used for hybridization and determination of nucleotide sequence (see below). One such clone, pD7, was hybridized to the same Southern blot used in Figure 2B to investigate the nature of the faint hybridization of the VS cDNA probe to some mt chromosomal restriction fragments. The pD7 clone did not hybridize to any chromosomal fragments (Figure 2C), indicating that VSDNA does not contain chromosomal sequences. Therefore, unless the VSRNAs are altered during or after transcription (as observed for transcripts of other Neurospora mt plasmids; Akins et al., 1988), the hybridization observed with the VS cDNA probe is probably due to contamination of the gel-purified VSRNAs with chromosomal transcripts or their breakdown products.

Subcellular fractionation and DNAase I treatments similar to those used previously to determine the localization of other Neurospora mt plasmids (Collins et al., 1981) showed that at least the majority of VSDNA is located in the mitochondria (data not shown). However, slight mtDNA contamination could have obscured the presence of small amounts of VSDNA in other fractions.

Northern hybridizations using strand-specific VSRNA probes showed that both the C- and L-VSRNAs are complementary to the same DNA strand (Figure 3). These in vivo RNAs are arbitrarily designated as the plus strand. Larger RNAs with mobilities expected for C- and L-VSRNAs of dimer (visible in Figure 3B; indicated by the arrowheads) and trimer (visible in longer exposures) lengths were also observed. The smaller RNAs detected in the (+) lane of Figure 3B are self-cleavage products of the in vitro synthesized plus RNA (see Figure 7). No evidence of minus (−) strand RNA was found (Figure 3C), even in longer exposures of these blots; however, upon long exposure, the plus and minus strand probes show some cross-hybridization that could obscure small amounts of minus strand RNA. If no minus strand RNA is present to serve as a template, plus strand VSRNA is presumably transcribed from VSDNA.

The nucleotide sequence of VSDNA corresponding to the plus strand of VSRNA is presented in Figure 4A. The length of a VSDNA monomer is 881 bp, and the sequence shows no substantial primary sequence similarity to other Neurospora plasmids, to any of the sequenced regions of the Neurospora mtDNA, or to any sequence in GenBank (Release 61). The longest methionine-initiated open reading frame on the plus strand is 74 amino acids, from nucleotides 38 to 259. Although this open reading frame shows no statistically significant similarity over its entire length to any sequence in National Biomedical Research Foundation PIR (Release 19) or to a translation of GenBank (Release 61), we did notice short regions of similarity to the surface antigen encoded by mammalian hepatitis B viruses (Figure 4A). This may be interesting in light of similarities between VSRNA and human HDV (see Figure 8).

The plus strand RNA contains potential secondary structures and short regions of primary sequence resembling those of the core of a group I intron flanked by short exons (Figure 4B). Helical regions in the appropriate order and relative position as group I pairings P1, P3, P4, P6, P7, P8, and P9 can be devised from the VSRNA sequence. The structure of a well-characterized group I intron in the Tetrahymena nuclear large rRNA gene (Cech, 1988) is shown in Figure 4C for comparison. The Tetrahymena intron contains several optional helices, for example P9.1 and P9.2, that are not found in all group I introns (Burke et al., 1987) but that are present in VSRNA. In VSRNA, the five contiguous nucleotides in the R and S sequences,

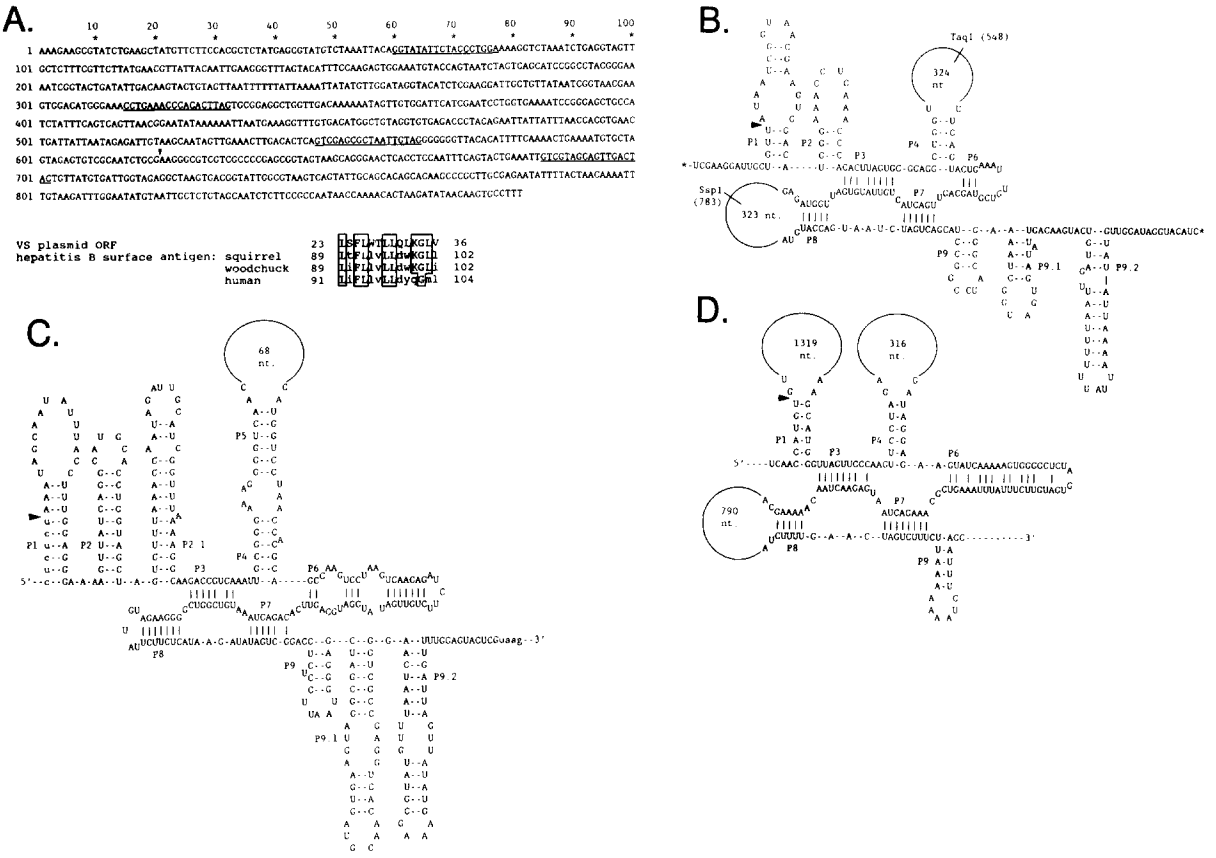

Figure 4. Nucleotide Sequence of VSDNA and Similarities with Group I Introns

(A) The DNA sequence corresponds to that of the plus strand VSRNA. Numbering begins arbitrarily from the single DraI site. Sequences complementary to the oligonucleotides used as primers for hybridizations (Figure 7) and reverse transcriptase dideoxy sequencing of the VSRNAs are underlined (Figures 6 and 8; Experimental Procedures). The site of self-cleavage is marked with an arrowhead. A short region of amino acid sequence similarity between part of a VSDNA open reading frame and a region of the surface antigen encoded by hepatitis B viruses of squirrel (Seeger et al., 1984), woodchuck (Galibert et al., 1982), and human (Valenzuela et al., 1979) is indicated below the DNA sequence. Numbers represent amino acids positions in the corresponding proteins.

(B)–(D) Possible secondary structures drawn according to the conventions of group I introns (Burke et al., 1987). (B) VSRNA: the circular sequence has been linearized for presentation between nucleotides 269 and 270 and is actually contiguous at the asterisks. The location of the TaqI and SspI sites used in construction and/or transcription of subclone pD7TS (Figure 9) are indicated. (C) Tetrahymena large rRNA intron (Davies et al., 1982; Michel and Dujon, 1983). (D) Varkud large plasmid RNA. Arrows indicate positions homologous to upstream exon–intron junctions.

which comprise the P7 pairing, match the group I consensus (Cech, 1988) perfectly, although flanking bases deviate and the unpaired nucleotide found in P7 of real group I introns is missing. The regions of the P and Q sequences comprising the P4 pairing also match the consensus very well, but the unpaired bases do not. A P5 pairing, conserved in all real group I introns, would be disrupted by "extra" nucleotides inserted into the VSRNA group I structure in this region.

Nargang et al. (1984) noted that sequences resembling those in the P4 and P7 pairings are also present in the large, high-copy plasmid found in the Mauriceville-1c (Nargang et al., 1984) and Varkud-1c (Akins et al., 1988) natural isolates of Neurospora. We found that the sequence of the Varkud-1c allele of this large plasmid can be drawn in a manner resembling a group I structure (Figure 4D), but which deviates from real group I introns in the same ways as the VSDNA: the lack of a P5 pairing and poor similarity to the consensus sequences in the unpaired regions of the R and S sequences.

C-VSRNA Is Circular and L-VSRNA Is Linear

Electron microscopy of C-VSRNA isolated from an agarose gel revealed a mixture of linear and circular molecules. Two of the circular molecules are shown in Figure 5A. Length measurements of the circular molecules from micrographs of grids that also contained linear standard RNAs of known size indicated that the circles were ~1.0 ± 0.1 kb. This is in good agreement with estimates of 0.9 kb obtained from agarose gel electrophoresis. C-VSRNA exhibits aberrant mobility during electrophoresis in denaturing polyacrylamide gels, running even slower than the 3.3 kb mt large rRNA; L-VSRNA migrates at 0.9 kb, equivalent to the mobility of glyoxal-denatured L-VSRNA on agarose gel electrophoresis (Figures 5B and 5C). Aberrantly slow mobility on acrylamide gels has been reported

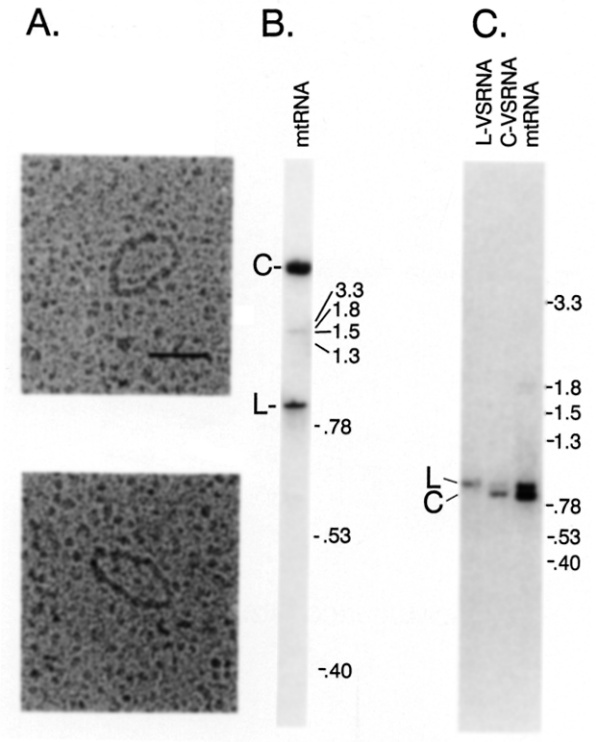

Figure 5. Characterization of C- and L-VSRNAs

(A) C-VSRNA was electroeluted from an agarose gel and spread for electron microscopy as outlined in the Experimental Procedures. The bar represents a length equivalent to 300 nucleotides.

(B) Total Varkud mtRNA was electrophoretically separated in a 4.0% polyacrylamide/8 M urea gel, electroblotted to a nylon membrane, and hybridized with a radioactively labeled VSRNA probe.

(C) C- and L-VSRNAs that had been individually eluted from an acrylamide gel, as well as total Varkud mtRNA, were denatured with glyoxal, electrophoresed in an agarose gel, transferred to nylon membrane, and hybridized as in (B).

The sizes (in kb) and mobilities of RNA size markers (Bethesda Research Laboratories; and Neurospora mt large rRNA [3.3 kb]) are indicated in (B) and (C).

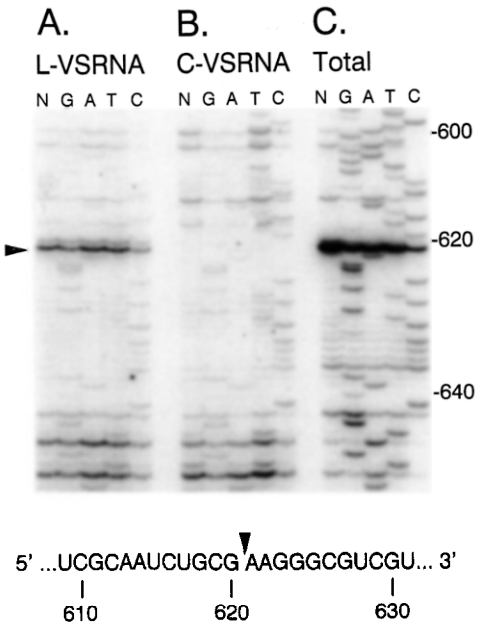

Figure 6. Identification of a Prominent 5′ End of L-VSRNA

L- and C-VSRNAs individually electroeluted from a polyacrylamide gel and total RNA isolated from Varkud mitochondria were used as templates for dideoxy sequencing using AMV reverse transcriptase and ^{32}P end-labeled oligonucleotide primer O4. The sequence of the RNA around the termination site is shown at the bottom.

previously for circular (Sanger et al., 1976; Bruce and Uhlenbeck, 1978; Chu et al., 1986; Grabowski et al., 1981) or lariat (Peebles et al., 1986; Ruskin et al., 1984) RNAs. When C-VSRNA was eluted from a polyacrylamide gel and reelectrophoresed in either a polyacrylamide (data not shown) or agarose (Figure 5C) gel, variable amounts of an RNA with the mobility of L-VSRNA were produced, consistent with the idea that C-VSRNA is circular and some of these molecules are nicked during handling.

The sequence of VSRNA was obtained by dideoxy sequencing of total mtRNA using four specific end-labeled oligonucleotide primers and reverse transcriptase. A continuous sequence equivalent to one strand of the monomer VSDNA was observed, although it was not possible to confirm the identity of all of the nucleotides due to reverse transcriptase termination bands in all lanes at some positions (Figure 6C and data not shown). Some of these termination bands were more pronounced in the sequences of the individual C- and L-VSRNAs eluted from polyacrylamide gels, suggesting that they may result from breakage of the RNAs during handling.

One major termination band corresponding to nucleotide 621 (numbered as in Figure 4A) was observed in the sequence of total mtRNA and of purified L-VSRNA but not C-VSRNA (Figure 6). These data, along with the electron microscopy and electrophoresis, suggest that C-VSRNA is circular and that L-VSRNA is linear and a prominent class of the linear molecules has a 5′ end at position 621.

Self-Cleavage of a Plus Strand VSRNA Transcript In Vitro

During in vitro synthesis of plus strand VSRNA by T7 RNA polymerase transcription of a cloned VSDNA monomer (pD7), two prominent smaller RNAs, P (promoter proximal) and D (promoter distal), were observed in addition to the expected full-length transcript (called Pre, for precursor; Figure 7). Several faint bands whose relative intensities varied in different experiments were also observed, especially when transcripts were resolved by polyacrylamide gel electrophoresis (data not shown); these faint bands appear to result from occasional breakage of the RNA at additional sites (see below). The sum of the sizes of bands P and D is approximately equal to the size of Pre. Northern hybridizations (Figure 7A) with each of the four oligonucleotides used previously as sequencing primers showed that P and D contain 5′ and 3′ portions, respectively, of the full-length transcript. This suggests that site-specific cleavage of some of the Pre RNA occurred in the T7 tran-

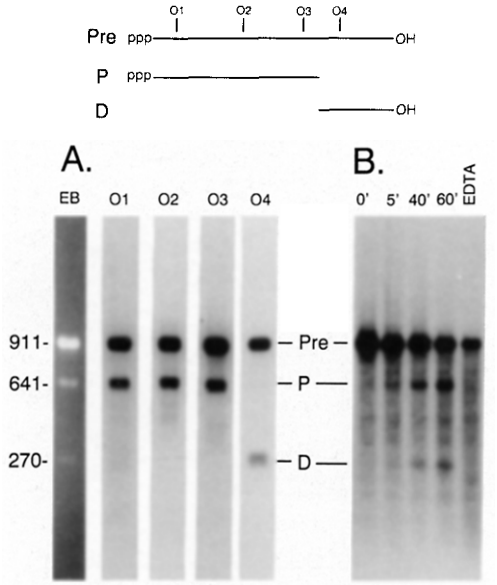

Figure 7. In Vitro Site-Specific Cleavage of Plus Strand VSRNA

(A) Unlabeled plus strand RNA was synthesized by T7 RNA polymerase runoff transcription of linearized monomer VSDNA clone pD7. The RNA was dissolved in water, denatured with glyoxal, and electrophoresed on an agarose gel. One lane was stained with ethidium bromide (EB). Northern transfers of duplicate lanes were hybridized with end-labeled oligonucleotides O1, O2, O3, or O4, which are complementary to regions of the RNA indicated in the diagram (see also Experimental Procedures).

(B) Full-length (Pre) RNA was eluted from an agarose gel similar to the one shown in (A), and aliquots incubated in 40 mM Tris–HCl (pH 8.0), 20 mM $MgCl_2$ at 42°C for the times indicated. One aliquot (EDTA) was incubated for 60 min at 42°C in 40 mM Tris–HCl (pH 8.0), 5 mM EDTA. The incubated samples were precipitated with ethanol, glyoxalated, and electrophoresed as in (A), transferred to Nylon membrane, and hybridized with a ^{32}P-labeled VSRNA probe.

Pre: the full-length plus strand transcript; P: the promoter proximal (upstream) cleavage product; D: the promoter distal (downstream) cleavage product.

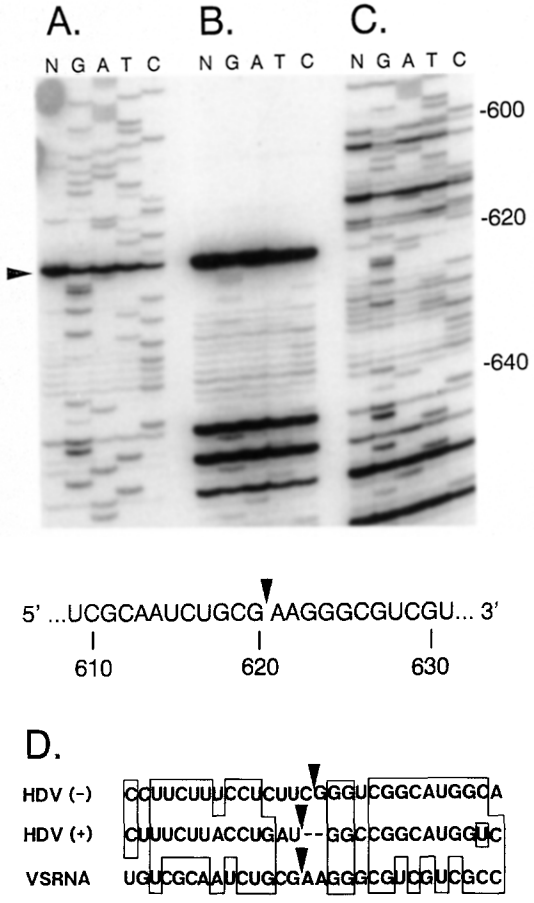

Figure 8. The 5′ End of Self-Cleavage Product D Is at the Same Position as the Major 5′ End of In Vivo L-VSRNA

Full-length in vitro transcript (Pre) of clone pD7 and the downstream self-cleavage product D (see Figure 7) were electroeluted from an agarose gel. Reverse transcriptase dideoxy sequencing of these templates and of total RNA isolated from Varkud-1c mitochondria was carried out as described in Figure 6 and Experimental Procedures using oligonucleotide O4 (see Figure 7) as a primer.

(A) Total Varkud mtRNA; (B) in vitro self-cleavage product D RNA; (C) full-length Pre RNA; (D) nucleotide sequence similarities downstream of the cleavage sites (indicated by the arrowheads) of human HDV genomic (+) and anti-genomic (−) RNAs (Kuo et al., 1988) and VSRNA.

scription buffer. To investigate this reaction, the remaining uncleaved, full-length Pre RNA was isolated by electroelution from an agarose gel and incubated under a variety of conditions. Figure 7B shows the time-dependent production of self-cleavage products P and D in a reaction that requires Mg^{2+} and is inhibited by EDTA.

To determine more precisely the site of self-cleavage, the promoter-distal product (band D) was isolated from an agarose gel and the location of its 5′ end was identified by dideoxy sequencing. A major termination band was observed corresponding to nucleotide 621, indicating that cleavage occurred between G620 and A621 (Figure 8). The location of this cleavage site was further supported by the identification of guanosine as the nucleotide at the 3′ end of the upstream cleavage product and adenosine at the 5′ end of the downstream cleavage product (see below). This site corresponds to the major termination band observed in sequencing of total mtRNA and isolated L-VSRNA (Figure 6), suggesting that the same cleavage may occur in vitro and in vivo. The nucleotide sequence downstream of this self-cleavage site shows some similarity to the sequences downstream of the cleavage sites of the genomic (+) and anti-genomic (−) strands of human HDV (Figure 8D).

The Self-Cleavage Reaction Produces 2′,3′ Cyclic Guanosine and 5′ Hydroxyl Adenosine Termini

More detailed analyses of the cleavage products of the monomer transcript were complicated by additional cleavages that occur during or after elution of the product bands from gels (see below). We found that a transcript of a small subclone (pD7TS) containing nucleotides 549–783 of VSRNA is also capable of self-cleavage after nucleotide 620 (determined by primer extension reverse transcriptase sequencing as in Figure 6; data not shown). Compared with the monomer pD7 transcript, a larger percentage of the pD7TS transcript cleaves after nucleotide 620 to produce the two major products, and fewer of the

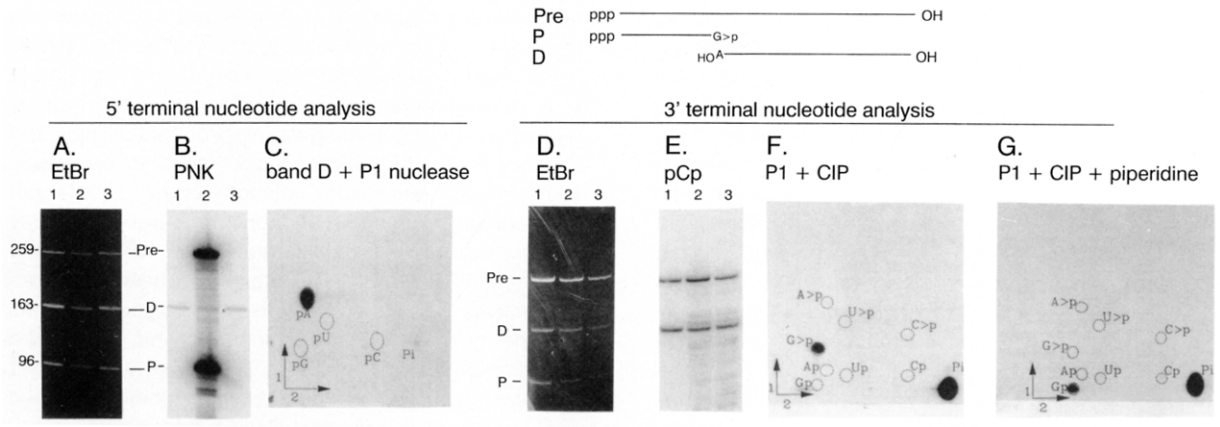

Figure 9. Analysis of the Terminal Nucleotides Formed by In Vitro Self-Cleavage of the Transcript of pD7TS

Unlabeled RNA was synthesized by T7 transcription from clone pD7TS (see Experimental Procedures), which had been linearized at the SspI site. Aliquots were used for 5′ end–labeling (A and B) or 3′ end–labeling (D and E). The runoff RNA contained 25 nucleotides of vector sequence followed by nucleotides 549 through 783 of VSRNA.

(A) and (B) Aliquots of RNA were incubated, directly (lanes 1 and 3) or after treatment with CIP (lane 2), with [γ-^{32}P]ATP and polynucleotide kinase. Reactions in lanes 1 and 2 were performed at pH 7.5, and the reaction in lane 3 at pH 9.0. Aliquots of each reaction were electrophoresed in a 4% polyacrylamide/8 M urea gel, stained with ethidium bromide (A), dried, and autoradiographed (B).

(C) Identification of the nucleotide at the 5′ end of band D. The downstream cleavage product (band D) labeled at its 5′ end was eluted from a gel similar to that shown in (B), lane 1, and digested with P1 nuclease. The pN products were separated by two-dimensional TLC, using solvent A in the first dimension and solvent B in the second dimension, and autoradiographed.

(D) and (E) Aliquots of RNA were incubated, directly (lane 1) or after treatment with CIP (lane 3) or after treatment with buffer but no CIP (lane 2), with 5′-^{32}P-pCp and RNA ligase, electrophoresed as in (A), stained with ethidium bromide (D), dried, and autoradiographed (E).

(F) and (G) Identification of the cyclic nucleotide at the 3′ end of band P. Radioactively labeled transcripts were synthesized by T7 transcription of clone pD7TS in the presence of [α-^{32}P]ATP and separated by electrophoresis. Band P was recovered from the gel and digested with P1 nuclease and then CIP. Aliquots of this material were separated directly (F) or after further treatment with piperidine to open the cyclic phosphate (G) by two-dimensional TLC using solvent C in the first dimension and solvent B in the second dimension. Nucleotide 2′,3′ cyclic monophosphates are indicated as N>p; corresponding 2′ or 3′ (noncyclic) monophosphates, which comigrate in this solvent system, are indicated as Np. See Experimental Procedures for further details.

additional products are found. The nature of the cleavage reaction products was therefore investigated in more detail using this smaller transcript.

RNA synthesized by T7 polymerase runoff transcription of clone pD7TS was incubated with polynucleotide kinase and [γ-^{32}P]ATP. Band D is labeled under these conditions (Figure 9B, lane 1), showing that at least some of the RNAs of this size contain a 5′ hydroxyl group. The full-length precursor (Pre) and band P both have 5′ triphosphate termini and, as expected, are not labeled. Prior treatment of these RNAs with alkaline phosphatase does not increase the extent of labeling of band D but, as expected, does allow labeling of bands Pre and P (Figure 9B, lane 2). In relation to dephosphorylated Pre and P RNAs, the RNA in band D appears to be a poor substrate for polynucleotide kinase. This enzyme has been found to vary widely in labeling efficiency of different substrates (Lillehaug and Kleppe, 1975; Richardson, 1981), and it may be that structural features of the 5′ end of band D inhibit its labeling. It is unlikely that band D contains a phosphatase-resistant, 5′ phosphate terminus that was then labeled by the polynucleotide kinase exchange reaction (Berkner and Folk, 1977), since band D was labeled to the same extent when the kinase reaction was performed at pH 9.0 (Figure 9B, lane 3), a condition that should severely inhibit any exchange reaction (van de Sande et al., 1973). Also, the phosphate between positions 620 and 621 was almost quantitatively recovered in the form of 2′,3′ cyclic guanosine at the 3′ end of the upstream cleavage product (band P; see below). Poor labeling of the 5′ OH on the downstream cleavage product, in relation to dephosphorylated promoter proximal upstream fragment, has also been observed for the minus strand of STobRV (compare the labeling of band M with that of band P in Figure 2b of Buzayan et al., 1986b).

Band D, labeled at its 5′ end, was purified by electroelution from a polyacrylamide gel (similar to that shown in lane 1 of Figure 9B) and digested with P1 nuclease. The resulting mononucleotide 5′ phosphates (pN) were separated by two-dimensional thin layer chromatography and autoradiographed. Figure 9C shows that the 5′ terminal nucleotide is pA, consistent with the primer extension sequencing (Figure 8), which indicated nucleotide 621 (an adenosine) as the 5′ end of band D. Using these same procedures, the 5′ end of the downstream cleavage product of the full-length monomer transcript (from clone pD7) was also found to be adenosine (data not shown).

The 3′ end of the promoter-proximal cleavage product (band P) does not label with 5′-^{32}P-pCp and RNA ligase, even after prior treatment with alkaline phosphatase (Figures 9D and 9E). This indicates that it does not terminate with either a hydroxyl or phosphate monoester. These observations combined with the finding of a 5′ hydroxyl group on the downstream cleavage product (band D; see above) suggest that the upstream product terminates with a 2′,3′ cyclic nucleotide.

To investigate this possibility and to identify the 3′ terminal nucleotide, T7 runoff transcription of pD7TS was performed in the presence of [α-^{32}P]ATP and band P was electroeluted from a polyacrylamide gel. This RNA was digested to mononucleotide 5′ phosphates with P1 nuclease and treated with alkaline phosphatase to remove the 5′ phosphate monoesters. An aliquot of this material was analyzed by two-dimensional thin layer chromatography and autoradiography. Figure 9F shows that the major radioactive nucleotide cochromatographs with unlabeled authentic guanosine 2′,3′ cyclic phosphate (abbreviated G>p). This identification was confirmed by further treatment of another aliquot with piperidine (Figure 9G) or HCl (data not shown) to open the cyclic phosphate. After such treatment the radioactive nucleotide cochromatographed with noncyclic guanosine monophosphate.

Quantitation of the radioactivity in the G>p and inorganic phosphate (Pi) spots by Cerenkov counting of material removed from appropriate regions of the TLC plate (Silberklang et al., 1979) suggests that the majority, and possibly all, of the molecules in band P terminate with G>p. As deduced from the sequence of VSRNA and the portion of the vector downstream of the T7 promoter included in the in vitro transcript, band P would be labeled by [α-^{32}P]ATP at 24 positions and the G>p would have acquired its labeled phosphate from the downstream adenosine (nucleotide 621). Thus, the ratio of radioactivity in the G>p and Pi spots on the TLC should be 1:24. In reasonable agreement with this, we observed a ratio of 0.8:24.

When the 3′ end of the upstream cleavage product of the monomer transcript from clone pD7 was analyzed by the same procedures, the most prominently labeled nucleotide was usually G>p, but substantial and variable amounts of U>p and C>p were also found (data not shown). Longer exposures of the TLCs in Figures 9F and 9G also revealed very small amounts of radioactive U>p and C>p (data not shown). These observations may mean that cleavage at other sites occurs during handling, especially of the self-cleavage product of the pD7 transcript. In parallel experiments (data not shown) in which either the pD7 or pD7TS transcripts were labeled with UTP instead of ATP, no phosphatase-resistant labeled nucleotides were observed. These observations suggest that the other cleavage sites are nonrandom and that one class of such sites involves preferential cleavage at U/A and C/A sequences.

Discussion

We describe novel genetic elements, termed VSDNA and VSRNA, that are found in the mitochondria of the Varkud-1c natural isolate of Neurospora. VSRNA is presumably transcribed from one strand of a low-copy, double-stranded DNA (VSDNA), which is organized as a population of head-to-tail multimers. VSRNA synthesized in vitro by transcription from a cloned VSDNA is capable of performing an in vitro site-specific, self-cleavage reaction similar to those described previously for some plant and animal pathogenic RNAs, but not employing similar catalytic nucleotide sequences. In our laboratory growth conditions, we have not noticed any phenotype in VS-containing Neurospora isolates that is attributable to the VS plasmid.

The majority of the VSRNA isolated from mitochondria is a single-stranded, circular RNA, as determined by nucleotide sequence analysis, electron microscopy, and aberrant mobility in polyacrylamide gel electrophoresis. VSRNA is 881 nucleotides in length, equal to the length and sequence of one VSDNA monomer. Larger molecules whose electrophoretic mobilities are consistent with dimer and trimer VSRNAs are also detectable. These may be intermediates in the processing of even larger transcripts into the monomer RNA: we recently found that in vitro transcription of multimeric VSDNA clones yields RNAs with electrophoretic mobilities expected for monomer and dimer products (Saville and Collins, unpublished data). The RNA isolated from mitochondria also contains significant amounts of a linear monomer VSRNA whose 5′ end is located at the same position (nucleotide 621) as the site of self-cleavage of VSRNA transcripts in vitro. Such a linear VSRNA could have been produced during isolation, for example by site-specific cleavage of C-VSRNA, or it may actually be present in vivo. The latter possibility would suggest that the cleavage site observed in vitro may be relevant to the processing of VSRNA in vivo.

VSRNA is not closely related in nucleotide sequence to any of the previously described catalytic RNAs, to other mt plasmids or mt genes, or to any sequence in Release 61 of GenBank. Despite its distinct sequence, the self-cleavage reaction that it performs is similar to those performed by a variety of other small RNAs (see below), suggesting a similarity in reaction mechanism. This reaction requires magnesium, but no added nucleosides, and yields products with 5′ OH and 2′,3′ cyclic phosphate termini.

A "hammerhead" model, characterized by a conserved secondary structure containing several invariant bases at particular positions, has been proposed for the active structure found in many of the catalytic plant viral satellite RNAs, one viroid RNA and the transcript of newt satellite DNA (Forster and Symons, 1987a, 1987b). Like VSRNA, cleavage by hammerhead RNAs produces 5′ OH and 2′,3′ cyclic phosphate termini. However, the minimal diagnostic hammerhead sequences CUGnnGA and GAAA are not contained within the subfragment of VSRNA produced by transcription of the pD7TS clone, which is nonetheless capable of the self-cleavage reaction (Figure 9). Thus, VSRNA is not a member of the hammerhead family (as it is currently understood) of catalytic RNAs.

Cleavage products terminating with 2′,3′ cyclic phosphate and 5′ OH are also produced by ribozymes that do not involve the conserved sequences found in known hammerhead structures. These include the minus strand of the satellite RNA of STobRV (Buzayan et al., 1986a) and the genomic (plus) and anti-genomic (minus) strands of HDV RNA (Kuo et al., 1988; Wu et al., 1989). We have not found any similarity between VSRNA and minus STobRV. However, the sequence of VSRNA downstream of the cleavage site resembles that found downstream of the cleavage sites of both the plus and minus strands of the HDV RNAs (Figure 8D). Once the higher order structure of the cata-

lytic regions of HDV and VSRNA have been established, it should be productive to determine experimentally how or if the similar sequences near the self-cleavage sites are important for the reaction.

VSRNA does not appear to be related to two of the other well-characterized catalytic RNAs: the RNA component of RNAase P and the self-splicing group II introns. The 5′ OH and 2′,3′ cyclic phosphate termini produced by VSRNA distinguish this reaction from that catalyzed by the RNAase P, which processes tRNA precursors leaving 3′ OH and 5′ phosphate termini (Guerrier-Takada et al., 1983). 2′,3′ cyclic phosphate termini are also not involved in splicing of group II introns. In addition, group II introns contain very characteristic conserved sequences and secondary structures, especially near their 3′ ends (reviewed by Michel et al., 1989); these are not apparent in the VSRNA.

A reasonable approximation of the group I intron core structure (Cech, 1988) flanked by hypothetical exons can be devised from the primary sequence of the VSRNA (Figure 4B). Some conserved group I sequences are present in other nonintron, catalytic RNAs, although such sequences may not be involved in the self-cleavage reaction. This might imply a functional or evolutionary relationship among these RNAs (see below; Lambowitz, 1989; Diener, 1989).

Despite the presence of a group I intron-like structure in VSRNA, there is no indication that VSRNA is or contains a functional group I intron. Neither Northern hybridizations nor dideoxy sequencing of the RNA (data not shown) gives any indication of splicing or even cleavage of VSRNA at the position that would be predicted to be homologous to an upstream exon–intron junction (after nucleotide 285 in VSRNA; marked by the arrowhead in Figure 4B). This is not surprising, since the hypothetical VSRNA structure deviates in significant ways from real group I introns. The most obvious differences include: first, the lack of a P5 pairing—instead, several hundred nucleotides, which contain the site of self-cleavage, are "inserted" into the group I structure in this region; and second, although the five contiguous nucleotides in each of the R and S sequences that comprise the P7 pairing match the group I consensus perfectly, several of the adjacent unpaired bases, which are also highly conserved among group I introns, do not. Also, many of the nucleotides in the group I structure are not present in the transcript of a subclone of VSDNA (containing only sequences between the TaqI and SspI sites marked in Figure 4B) that is nonetheless capable of self-cleavage (Figure 9).

Parts of an apparently nonfunctional group I core structure have also been recognized in some plant pathogenic RNAs (Dinter-Gottleib, 1986; Hadidi; 1986) and in another Neurospora mt plasmid. Different alleles of this Neurospora plasmid are found in the Mauriceville-1c (Nargang et al., 1984), Varkud-1c (Akins et al., 1988), and other (Collins and Saville, 1990) natural isolates of Neurospora. We will refer to this plasmid as the V plasmid to distinguish it from the VSDNA described in the current paper. We found that the sequence of the Varkud-1c allele of the V plasmid can be drawn in a manner resembling a group I structure (Figure 4D) but that deviates from real group I introns in some of the same ways as the VSDNA: the lack of a P5 pairing, a large number of nucleotides "looped out" of the group I structure in this region, and poor similarity to the consensus sequences in the unpaired regions of R and S. These similarities in structure may suggest that the V and VS plasmids share a common evolutionary origin or certain functional properties not yet recognized.

Nucleotide sequence analysis suggests that VSDNA is unlikely to encode any large proteins, suggesting that it depends for its transcription and replication on polymerase(s) encoded elsewhere. We identified five additional natural isolates of Neurospora that contain VSDNA; they all contain the V plasmid as well. This coincidence in plasmid distribution suggests that VSDNA may depend upon the V plasmid for its propagation. The converse dependence is apparently not the case, since some natural isolates (for example Mauriceville-1c) have been found that contain the V plasmid but not VSDNA (Collins and Saville, 1990). In this respect, VSRNA may be analogous to viral satellite RNAs, such as HDV and those of some plant viruses that depend upon a helper virus for their propagation.

The synthesis of VSRNA is nonetheless different from that of plant viral satellite RNAs and HDV. Replication of these other RNAs occurs by a rolling circle mechanism that involves a complementary minus strand or anti-genomic RNA (Branch and Robertson, 1984; Symons 1989). We did not detect any minus strand VSRNA (Figure 3), although we cannot rule out the possibility that very small amounts of such an RNA could have escaped detection. As discussed above, we found instead a double-stranded DNA that could serve as the template for transcription of new VSRNAs. Interestingly, the V plasmid, which is also present in all isolates that contain the VSDNA, has been shown to encode a reverse transcriptase that Kuiper and Lambowitz (1988) have hypothesized to be involved in replication of the V plasmid. We are currently investigating (Kuiper, Lambowitz, B. J. S., and R. A. C., unpublished data) whether this enzyme is able to synthesize the multimeric complementary VSDNA that we have found in mitochondria. If this is the case, the VSRNA/DNA may represent a type of retrovirus-like genetic element. In one model for the life cycle of this element, monomer VSRNA could function as a template for rolling circle reverse transcription, followed by second strand synthesis and then transcription (possibly by the mt RNA polymerase) to yield multimeric VSRNA that would then self-process to produce new monomer VSRNAs.

Experimental Procedures

Strains of Neurospora and Growth Conditions

Neurospora strains were obtained from the Fungal Genetics Stock Center: N. intermedia Varkud-1c (FGSC# 1823), N. crassa strains 74-OR23-1A (FGSC# 987; abbreviated 74A), and Adiopodoume I (formerly North Africa I; FGSC# 430). Mycelia were grown in Vogels N medium according to standard procedures (Davis and de Serres, 1970; Lambowitz, 1979).

Isolation of Mitochondrial RNA and DNA

Total mt nucleic acids were isolated from flotation gradient-purified mitochondria (Lambowitz, 1979) essentially by the UNSET-phenol procedure (Garriga et al., 1984), except that the RNA was not treated with protease K on some occasions, with no deleterious effects. When appropriate, mtDNA was removed by treatment with DNAase I as described below (see In Vitro Synthesis of RNA).

Recombinant Plasmids

Aliquots of Varkud-1c mtDNA were digested separately with enzymes that cut the VSDNA, and libraries were constructed in pTZ18R (Pharmacia). Clones containing VSDNA inserts were identified by colony filter hybridization (Grunstein and Wallis, 1979) using a cDNA probe synthesized from VSRNAs that had been eluted from an agarose gel. The cDNA probe was synthesized, using 15–20 U of AMV reverse transcriptase and random hexamer primers (Palukaitis, 1986), in a 50 μl reaction volume containing 1–10 μg of gel-purified VSRNA, 0.2–0.5 A_{260} U of random oligonucleotides (Pharmacia catalog number 27-2166-01), 50 mM Tris–Cl (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM each dGTP, dCTP, and dTTP, and 75 μCi of [α-^{32}P]dATP (>3000 Ci/mmol, Amersham). After incubation for 2 hr at 42°C, 2 μl of 10 mM ATP was added, and incubation was continued for an additional 1.5 hr. The reaction was stopped by the addition of EDTA to 40 mM and the cDNA was recovered by Sephadex G-50 chromatography.

Plasmid pD7 contains a VSDNA monomer obtained by DraI digestion of mtDNA cloned in the SmaI site of pTZ18R (Pharmacia) oriented such that transcription of this clone from the T7 promoter yields an RNA equivalent to the in vivo plus RNA. pD7R contains the monomer DraI insert in the orientation opposite to that of pD7, constructed by subcloning the entire monomer insert from pD7 into pTZ19R. pD7TS is a subclone of pD7 from the TaqI site at VSDNA position 548 to the XbaI site of the multiple cloning site into the AccI and XbaI sites of pTZ19R. Additional clones used for sequencing were obtained from libraries prepared from either HaeIII- or ScaI-digested mtDNA.

DNA and RNA Sequencing

DNA sequencing was carried out by the dideoxy chain-termination procedure (Sanger et al., 1977), using four complementary oligonucleotide primers (oligonucleotide sequences are written 5′ to 3′, and the numbers in parentheses refer to the regions of the sequence in Figure 4 to which the oligomers are complementary): primer O1 (60–77) TCCAGGGTAGAATATACC; primer O2 (315–332) CTAAGTGTGGGTTT-CAGG; primer O3 (547–564) CTACAATTAGCGCTCGAC; and primer O4 (685–702) GTAGTCAACTGCTACGAC. 7-deaza dGTP (Boehringer Mannheim) was substituted for dGTP in the sequencing reactions (Mizusawa et al., 1986). Single-stranded templates were prepared from pTZ clones (see above) using helper phage R408 (Russell et al., 1986) or by subcloning into m13mp18/19 (Yanisch-Perron et al., 1985).

Dideoxy sequencing of total mtRNA or RNAs eluted from gels was carried out, using the same oligomers as above, essentially as described by Kjems and Garrett (1988) except that 7-deaza dGTP (Boehringer Mannheim) was substituted for dGTP in the sequencing reactions.

Sequence analysis and data base searching were performed using the IBI Pustell Sequence Analysis Software (International Biotechnologies, Inc., New Haven, CT; see Pustell, 1988).

In Vitro Synthesis and Gel Purification of RNAs

Unlabeled RNAs were prepared by T7 RNA polymerase transcription of linearized recombinant plasmids in a 50 μl volume containing 0.2–0.6 μg of DNA template, 1 mM each ATP, UTP, CTP, and GTP (Pharmacia), 5 mM dithiothreitol, 1× T7 polymerase buffer (40 mM Tris–HCl [pH 8.0]; 8 mM $MgCl_2$; 25 mM NaCl; 2 mM spermidine-$(HCl)_3$, Bethesda Research Laboratories), 0.5–1.0 U/μl RNAguard (Pharmacia), and 1.5 U/μl T7 RNA polymerase (Bethesda Research Laboratories) for 1 hr at 37°C or 42°C. Radioactive transcripts were prepared as above except that the volume was 20 μl and the concentration of the unlabeled nucleotide corresponding to that which was radioactively labeled ([α-^{32}P]ATP or [α-^{32}P]UTP, Amersham) was 0.1 mM. RNAs were treated with DNAase I (Pharmacia; 10–30 U/μg DNA template) at 37°C for 10 min, extracted with phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol, brought to 0.3 M sodium acetate, and precipitated with ethanol.

After staining with ethidium bromide or autoradiographic exposure, the desired RNAs were electroeluted from agarose and acrylamide gels into ammonium acetate using an apparatus from International Biotechnologies, Inc. (New Haven, CT) and collected by precipitation with ethanol. Some RNAs were soaked out of acrylamide gels following the procedure of Maxam and Gilbert (1977).

Agarose Gel Electrophoresis and Northern Hybridization

RNAs were resuspended in diethyl pyrocarbonate–treated water, denatured with glyoxal, and electrophoresed in a 1.45% agarose gel (Carmichael and McMaster, 1980). Separated RNAs were transferred to a Nytran membrane following the manufacturer's instructions (Schleicher & Schuell). After binding the RNA to the membrane, either by baking at 80°C (1 hr) or exposure to ultraviolet light (5′ on a Model UV300 transilluminator, Fotodyne, Inc.), the membrane was washed at 65°C in 0.1× SSC (20× SSC = 3 M NaCl, 0.3 M sodium citrate [pH 7.0]), 0.1% w/v SDS before prehybridization.

Hybridizations with oligonucleotide probes (5′ end–labeled with [γ-^{32}P]ATP and polynucleotide kinase; Maniatis et al., 1982) were carried out as suggested by the membrane manufacturer (Schleicher & Schuell): prehybridization for 1–3 hr in 6× SSPE (1× SSPE = 0.18 M NaCl, 10 mM $NaPO_4$ [pH 7.6], 1 mM EDTA), 0.1% w/v SDS, 10× Denhardt's reagent (Denhardt, 1966), 50 μg/ml tRNA, and 50 μg/ml denatured, phenol-extracted, sheared calf thymus DNA; and hybridization in 6× SSPE, 1% w/v SDS at the T_h of the hybrid (calculated according to Suggs et al. [1981] based on the sequence of the oligomer).

Hybridizations with RNA probes (synthesized using T7 RNA polymerase in the presence of [α-^{32}P]UTP, see above) were carried out at 65°C–70°C in 50% formamide, 5× Denhardt's reagent, 5× SSPE, 0.1% w/v SDS, and 100 μg/ml denatured, sheared calf thymus DNA. Blots were washed twice for 15 min at room temperature in 1× SSPE, 0.1% SDS and once for 60 min at 65°C–70°C in 0.1× SSPE, 0.1% SDS.

Polyacrylamide gel electrophoresis of RNA was performed on 4% polyacrylamide/8 M urea gels in 1× TBE (89 mM Tris–borate, 2 mM EDTA). RNAs were electroblotted to Nytran membranes in 0.5× TBE at 70 V for 60–70 min. Hybridizations and washes were carried out as for agarose gel Northern analyses.

Southern Hybridizations

Restriction digests of mtDNA were electrophoresed in 1.0% agarose gels and transferred to nitrocellulose as described by Southern (1975). Hybridizations with the clone pD7 (labeled by the random primer method; Feinberg and Vogelstein, 1983) or with a cDNA probe (see Recombinant Plasmids, above) were carried out for at least 18 hr at 37°C in 50% formamide, 5× SSPE, 4× Denhardt's solution, 0.2% SDS, and 100 μg/ml sheared denatured calf thymus DNA. Washes were carried out as for Northern hybridizations using the RNA probes (see above) except that the higher temperature wash was at 50°C–55°C.

Electron Microscopy

The preparation of single-stranded RNA for electron microscopy was carried out essentially by the Kleinschmidt (1968) technique (see also Davis et al., 1971). The RNA was dissolved in 20% formamide, 10 mM Tris–HCl (pH 8.5), 1 mM EDTA, 50 μg/ml cytochrome c and spread on 50% formamide, 0.1 M Tris (pH 8.5), 0.01 M EDTA.

Analysis of 5′ and 3′ Termini

5′ end–labeling with [γ-^{32}P]ATP was carried out for 30 min at 37°C, using the forward reaction of polynucleotide kinase (PNK) as outlined by Maniatis et al. (1982) with the addition of RNAguard (Pharmacia) to 2–3 U/μl; or, in this manner except at pH 9.0 instead of 7.5. 3′ end–labeling using 5′-^{32}P-pCp and T4 RNA ligase was performed essentially as described by Bruce and Uhlenbeck (1978). Calf intestinal alkaline phosphatase (CIP; Bethesda Research Laboratories) treatment of some aliquots of RNA prior to labeling was performed in 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, using 1 U of CIP/10 μl reaction at 37°C or 50°C for 30 min (Kjems and Garrett, 1988). The reactions were stopped by heating in the presence of EDTA and SDS followed by phenol extraction and ethanol precipitation as outlined by Maniatis et al. (1982). RNAs were dissolved in water, denatured by the addition of 2 vol of sequencing dye (80% formamide, 0.5× TBE, 0.1% xylene cyanol, 0.1% bromophenol blue), heated to 65°C–70°C for 3 min, and

then separated by electrophoresis in 4% polyacrylamide/8 M urea gels in 1× TBE buffer.

To identify 5′ nucleotides, gel-purified polynucleotide kinase–labeled RNAs were dissolved in 9 μl of 50 mM ammonium acetate (pH 5.3) (Buzayan et al., 1986b), digested with 29 U of P1 nuclease (Bethesda Research Laboratories) for 1.5 hr at 37°C, evaporated to dryness, and dissolved in 2 μl of water. The resulting pN mononucleotides, plus each of the four authentic, unlabeled nucleoside 5′ monophosphates (Sigma), were separated by two-dimensional thin layer chromatography on 20 × 20 cm cellulose plates (Merck) using solvent A (isobutyric acid:concentrated NH_4OH:H_2O [66:1:33, v:v:v]) in the first dimension and solvent B (t-butanol:concentrated HCl:H_2O [70:15:15, v:v:v]) in the second dimension (Silberklang et al., 1979). Unlabeled nucleotides were detected by UV illumination and the labeled nucleotides by autoradiography.

To identify the 3′ terminal nucleotide T7, transcription of the pD7TS subclone was carried out in the presence of [α-^{32}P]ATP. Band P was recovered from a 4% polyacrylamide/8 M urea gel, digested with P1 nuclease (2.9 U for 30 min at 37°C) and CIP (60 min at 37°C in 100 mM NH_4HCO_3,34 mM NH_3 buffer; Buzayan et al., 1986a, 1986b). This material along with the four authentic unlabeled 2′,3′ cyclic and 2′ or 3′ noncyclic nucleotide standards (Sigma) were separated as described above except using solvent C (isopropanol:concentrated NH_4OH:H_2O [70:10:20, v:v:v]; Shugar, 1967) in the first dimension and solvent B in the second dimension.

Cyclic nucleotides were converted to mixtures of the corresponding 2′ and 3′ nucleoside (noncyclic) monophosphates by incubation with piperidine (20% [v/v] for 90 min at 95°C; Buzayan et al., 1986b) or 0.2 M HCl (70 min at 30°C; Abrash et al., 1967).

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