The VS catalytic RNA replicates by reverse transcription as a satellite of a retroplasmid

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The mitochondria of certain natural isolates of *Neurospora* contain both the Varkud plasmid, which encodes a reverse transcriptase, and a small unrelated RNA (VS RNA) that performs RNA-mediated self-cleavage and ligation reactions. Here, we show that VS RNA is transcribed from a VS plasmid DNA template by the *Neurospora* mitochondrial RNA polymerase using a promoter located immediately upstream of the RNA self-cleavage site that generates monomeric transcripts. VS RNA is then reverse transcribed by the Varkud plasmid reverse transcriptase to yield a full-length (–) strand cDNA, a predicted replication intermediate. Combined with previous genetic evidence, our results indicate that the VS plasmid replicates by reverse transcription as a satellite of the Varkud plasmid. This mode of replication, unprecedented for a satellite RNA, likely reflects the promiscuity of the Varkud plasmid reverse transcriptase, which does not require a specific primer to initiate cDNA synthesis. Our findings indicate how primitive reverse transcriptases with similar relaxed specificity could have facilitated the evolution of new retroelements.

[Key Words: Mitochondrial plasmid; *Neurospora*; retroelement; reverse transcriptase; ribozyme]

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Satellite RNAs, whose propagation is dependent on an unrelated helper virus, have been found in plants and animals (Roossinck et al. 1992; Taylor 1992). Most satellite RNAs, including virusoids in plants and hepatitis δ virus in animals, are believed to replicate via a complementary RNA intermediate using a rolling circle mechanism in which an RNA self-cleavage domain is used to generate monomer-length RNAs (Symons 1992; Taylor 1992). Different satellite RNAs contain different RNA self-cleaving domains, denoted hammerhead, hairpin or hepatitis δ virus ribozyme, but all use a common cleavage pathway yielding products having 2',3'-cyclic phosphate and 5' OH termini (Symons 1992). In most cases, the polymerase that replicates the satellite RNA has not been conclusively identified. However, recent studies have provided evidence that some plant RNA virus satellite RNAs replicate by using a helper virus RNA-dependent RNA polymerase complex (Wu et al. 1991, 1993; Song and Simon 1994).

The mitochondria of certain *Neurospora* spp. natural isolates contain an abundant noncoding RNA, denoted VS RNA, which performs RNA-mediated self-cleavage and ligation reactions. A less abundant DNA form, VS plasmid DNA, is also present (Fig. 1, left; Saville and Collins 1990, 1991). The ribozyme domain of VS RNA has a novel secondary structure, but like other RNA self-cleaving domains, yields products having 2',3'-cyclic phosphate and 5' OH termini (Saville and Collins 1990; Beattie et al. 1995). The VS RNAs found in mitochondria include linear and circular monomers and concatemers, and the VS plasmid DNAs include closed-circular double-stranded DNA monomers and concatemers.

The *Neurospora* strains that contain the VS plasmid invariably contain a larger mitochondrial (mt) plasmid, the Varkud (V) plasmid, which is not homologous to VS. The V plasmid and its close relative, the Mauriceville (M) plasmid, denoted retroplasmids, are closed-circular DNAs of 3.7 and 3.6 kb, respectively, that encode a reverse transcriptase (RT) (Collins et al. 1981; Akins et al. 1988; Kuiper and Lambowitz 1988; Kuiper et al. 1990). The retroplasmids appear to replicate by synthesizing a monomer-length transcript that is reverse transcribed into DNA (Kuiper and Lambowitz 1988; Kennell et al. 1994). Remarkably, the retroplasmid transcript contains a 3' tRNA-like structure similar to those of certain plant

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Figure 1. Maps of the VS and VS/V1-2 plasmids. The VS/V1-2 plasmid is a deletion derivative of the VS plasmid in which nucleotides 30–239 have been replaced by 10 nucleotides of unknown origin (shaded box). The VS/V1-2 plasmid also has five single-base mutations, which are indicated. The region of VS RNA required for RNA self-cleavage is indicated in black (VS620–773; Guo et al. 1993). The transcription start site and RNA self-cleavage/ligation site are indicated by solid and open arrows, respectively. Nucleotide position numbers for both plasmids refer to the VS plasmid sequence (Saville and Collins 1990). Restriction enzyme sites are abbreviated: (D) *DraI*, (H) *HincII*, (Sc) *ScaI*, (Sp) *SspI*.

RNA viruses, and the retroplasmid RT functions analogously to the viral RNA-dependent RNA polymerases in recognizing this structure and initiating cDNA synthesis *de novo* (i.e., without a primer) opposite the C residue at position 2 (position C2) of the 3' CCA sequence (Kuiper and Lambowitz 1988; Wang and Lambowitz 1993a). In addition to *de novo* initiation, which is unprecedented for a DNA polymerase, the retroplasmid RT can also use various DNA or RNA primers to initiate cDNA synthesis at the 3' end or an internal region of the retroplasmid transcript (Wang and Lambowitz 1993a; Kennell et al. 1994). In contrast to retroviral RTs, however, the primers are not complementary to the RNA template and accurate initiation depends on the ability of the retroplasmid RT to recognize specific sequence or structural features of the RNA template (Wang and Lambowitz 1993a). The characteristics of the retroplasmid RT are expected for a primitive RT that evolved from an RNA-dependent RNA polymerase and was ancestral to other RTs and DNA polymerases (Wang and Lambowitz 1993a). The retroplasmid RT is phylogenetically related to bacterial retromer and group II intron RTs, which are also believed to be primitive RTs (Xiong and Eickbush 1990; Eickbush 1994).

Circumstantial evidence suggested that the VS plasmid might be a satellite of the V retroplasmid. All eleven natural isolates of *Neurospora* in which VS was found also contain the retroplasmid, whereas two other natural isolates, including the Mauriceville strain, contain only the retroplasmid (Guo et al. 1993; E.R.M. Tillier, D.M. De Abreu, and R.A. Collins, unpubl.). In addition, experiments in which the plasmids were transferred between strains via transient hyphal fusions showed that the VS plasmid could be transferred only in association with the V retroplasmid, whereas the V retroplasmid could be transferred independently of VS (Collins and Saville 1990). These findings, together with the absence of long open reading frames (ORFs) in the VS plasmid and the failure to detect (–) strand VS RNAs in mitochondria, raised the possibility that the VS plasmid replicates by reverse transcription as a satellite of the retroplasmid.

In the present work, we provide biochemical evidence that the VS plasmid replicates via reverse transcription.

We show that the VS plasmid DNA is transcribed by the *Neurospora* mt RNA polymerase to yield VS RNA and that VS RNA is reverse transcribed by the retroplasmid RT to yield full-length (–) strand cDNA, a predicted replication intermediate. The ability of the V retroplasmid to support replication of a satellite RNA likely reflects the promiscuity of its RT, which does not require a specific primer to initiate cDNA synthesis. Our findings indicate how primitive RTs with similar relaxed specificity could have led to the evolution of new retroelements.

Results and Discussion

In vitro transcription of VS DNA by mt RNA polymerase

The synthesis of VS RNA was investigated by *in vitro* transcription experiments using plasmid pD7, which contains a monomer clone of VS DNA. Figure 2 shows that when pD7 was digested with *ScaI*, *HincII*, *SspI*, or *XbaI* and incubated with mt lysates from strains that contained or lacked plasmids, runoff transcripts of 78, 99, 185, and 300 nucleotides, respectively, were synthesized. A control showed that no transcripts were synthesized from the linearized vector, pTZ18R/*XbaI* (pTZ/*XbaI*). The sizes of the pD7 runoff transcripts indicate that the major *in vitro* transcription start site is located around VS position 600. Primer extension mapping of *in vitro* transcripts synthesized from pD7/*SspI* showed that the predominant 5' end corresponds to either G601 or T602 (not shown). Because all transcription start sites described previously for *Neurospora* mt RNA polymerase correspond to a G-residue, we infer that the major initiation site is most likely G601 (indicated by a star in Fig. 2).

As shown in Figure 2 (bottom), the sequence around the *in vitro* transcription start site in VS DNA is an 8/11 match to the *Neurospora* mt DNA promoter consensus sequence and is preceded by an upstream AT-rich region characteristic of active promoters in *Neurospora* mtDNA (Kubelik et al. 1990). Notably, the transcription start site is only ~20 nucleotides upstream of the VS

Figure 2. In vitro transcription of VS DNA. One microgram of plasmid pD7, which contains monomer VS plasmid, or the vector pTZ18R (pTZ), was digested with restriction enzymes (indicated) and transcribed with mt lysates from *[poky]* 13-6 (left and middle) or the Varkud strain (right), as described in Materials and methods. In vitro transcripts were analyzed by electrophoresis in a 12% polyacrylamide/8 M urea gel (left) or a 6% polyacrylamide/8 M urea gel (same gel for middle and right). The open arrow indicates a band that likely results from self-cleavage of in vitro transcript pD7/XbaI. Numbers to the left of the autoradiograph indicate positions and sizes of 5' end-labeled *Sau*3AI fragments of Bluescribe (+) vector (Stratagene, La Jolla, CA) used as molecular weight markers. Numbers to the right indicate estimated sizes (nucleotides) of the in vitro transcripts. The schematic shows a partial restriction map of VS DNA and expected sizes of runoff transcripts beginning at position 601. The bottom compares the sequence flanking the transcription start site of the VS plasmid with the *Neurospora* mtDNA promoter consensus sequence (Kubelik et al. 1990). The star indicates the putative initiating nucleotide, G601. Restriction enzymes are abbreviated: (D) *Dra*I; (Sc) *Sca*I; (H) *Hinc*II; (Sp) *Ssp*I; and (X) *Xba*I.

RNA self-cleavage site. The transcripts pD7/XbaI and pD7/SspI contain the minimal ribozyme domain (VS620 to 773; see Fig. 1), and the longer pD7/XbaI transcript appears to be efficiently cleaved at the expected position (prominent band indicated by open arrow). The location of the in vitro transcription start site relative to the 5'

end of linear VS RNA suggests that VS RNA monomers are synthesized by transcription of circular or multicentric VS plasmid DNA followed by RNA self-cleavage.

VS RNA is reverse transcribed

The analysis of VS cDNA synthesis was facilitated by using a mutant Varkud strain, V1-2, which accumulates a suppressive variant of the V retroplasmid (V1-2 plasmid) leading to elevated RT activity (Akink et al. 1989; Kuiper et al. 1990). The V1-2 strain contains a deletion derivative of the VS plasmid, denoted VS/V1-2, in which nucleotides 30–239 of VS are replaced by 10 nucleotides of unknown origin (Fig. 1, right). The VS/V1-2 plasmid also has five single-base mutations compared to VS. We confirmed that the VS/V1-2 plasmid uses the same in vitro transcription start site and RNA self-cleavage site as the wild-type VS plasmid (not shown). In addition to the above plasmids, the V1-2 strain also contains a very small deletion derivative of the retroplasmid (V1-2/Δ1; ~250 bp; S. Mohr, B.J. Saville, R.A. Collins, and A.M. Lambowitz, unpubl.).

To determine whether the VS RNAs are reverse transcribed, mt RNP particles from the Varkud and V1-2 strains were incubated with [³²P]dCTP + other dNTPs, and ³²P-labeled cDNAs synthesized from endogenous RNA templates were hybridized to Northern blots of mt RNAs from *Neurospora* strains that contain or lack the mt plasmids. Figure 3 shows that the ³²P-labeled cDNAs synthesized in both strains hybridized to the full-length transcripts of the M, V, or V1-2 retroplasmids (3.6 or 3.7 kb) and to the related V1-2/Δ1 RNA in the V1-2 strain. Strikingly, the ³²P-labeled cDNAs from both strains hybridized strongly to VS and VS/V1-2 RNAs, whereas no hybridization was observed to most other abundant RNAs, for example, the large rRNA or *cox*I and *cob* mRNAs. The hybridization of the ³²P-labeled cDNAs synthesized in the Varkud strain to the mt small rRNA (SSU rRNA) likely reflects reverse transcription of a Varkud strain-specific, 4.9-kb hybrid RNA, which consists of the 3.7-kb V plasmid transcript with a 1.2-kb 5' leader derived from the mt small rRNA (Akink et al. 1988; Chiang et al. 1994). The 4.9-kb RNA is detected as a weak, diffuse band in this Varkud RNA preparation. In analogous experiments Southern hybridizations to restriction digests of DNA preparations likewise showed that the ³²P-labeled cDNAs hybridized primarily to the retroplasmid and VS plasmid DNAs, with little if any hybridization to mtDNA restriction fragments encoding mRNAs (not shown). Together, these findings show that the VS and VS/V1-2 RNAs are preferred substrates for reverse transcription in mt RNP particles.

For further analysis, ³²P-labeled cDNAs synthesized in ribonucleoprotein (RNP) particles from the V or V1-2 strain were hybridized to dot blots of the M13-cloned (+) and (–) strands corresponding to the V and VS plasmids. The M13 vectors were included as controls. Time course experiments showed that cDNA synthesis for both the large and small plasmids continued for at least 30 min and that the ³²P-labeled cDNAs isolated at all times hy-

Figure 3. Northern hybridization analysis of ³²P-labeled cDNAs synthesized in endogenous reverse transcription reactions in mt RNP particles. ³²P-labeled cDNAs synthesized in Varkud or V1-2 mt RNP particles were hybridized to Northern blots containing 5 μg of glyoxalated whole mt RNA from strains 74A (no plasmids), M, V, or V1-2. (A) A gel stained with ethidium bromide; (B,C) the blots hybridized with ³²P-labeled cDNAs. Prominent RNAs and RNAs that hybridized to ³²P-labeled cDNAs are indicated to the right of the gel and autoradiographs. The gel resolves linear (l) and circular (c) VS and VS/V1-2 RNAs (Saville and Collins 1990). LSU and SSU are the mt large and small rRNAs, respectively. The numbers to the left show positions and sizes of glyoxalated ³²P-labeled *Hind*III–*Eco*RI fragments of phage λ DNA used as molecular weight markers.

bridized preferentially to the (+) strands of the plasmids, indicating that (–) strand cDNAs are synthesized (Fig. 4). The dot blots, which provide a more quantitative comparison than the Northern hybridizations, indicate that the level of reverse transcription of VS RNA is relatively low in the wild-type V strain but much higher for the VS/V1-2 plasmid in the V1-2 strain. Although the dot blots for both strains show some hybridization to the (–) strands of the V and VS plasmids, this mostly reflects cross-hybridization rather than (+) strand synthesis in this experiment, because the ratio of (+) to (–) strand hybridization was unaffected by actinomycin D, an inhibitor of DNA-directed DNA synthesis (Fig. 4, bottom). In other dot blot experiments with V1-2 mt RNP particles, we showed that reverse transcription of VS/V1-2 RNA is inhibited by pretreatment with RNase A + T₁ or with micrococcal nuclease (not shown), confirming that an intact RNA template is required. Additionally, we confirmed that reverse transcription is dependent on all four deoxynucleotides, that ribonucleotides could not substitute for deoxyribonucleotides, and that the ³²P-labeled products are sensitive to DNase and insensitive to RNase (not shown). Comparisons of VS/V1-2 and retroplasmid reverse transcription showed that both have similar optima for Mg²⁺ (1–5 mM), pH (~8.0), and temperature (45–50°C) and were equally inhibited by substitution of Mg²⁺ with Mn²⁺ or 5 mM EDTA (data not shown), as expected for cDNA synthesis by the same enzyme.

Sucrose gradient analysis of RNP particles

Sucrose gradient analysis was carried out to determine whether the large and small plasmid RNAs are reverse transcribed in separate RNP particles. The gradient pro-

file shows that linear and circular VS/V1-2 RNAs sedimented with a peak at 17S–20S (Fig. 5A; fractions 4–8), whereas the monomer-length retroplasmid transcripts detected by Northern hybridization sedimented with a peak at 27S–29S (fractions 7–9; not shown). Lesser amounts of both plasmid RNAs were broadly distributed through the gradient ahead of the peak, possibly reflecting aggregation. To assay RT activity associated with the RNP particles, the sucrose gradient fractions were incubated with [³²P]dCTP plus other dNTPs, and ³²P-labeled cDNA synthesized in endogenous reactions were analyzed by gel electrophoresis and by hybridization to Northern blots of total mt RNAs from the Varkud strain. Figure 5, B and C, shows that the RNP particles in gradient fractions 4 and 5 synthesized VS/V1-2 cDNA extending up to the full length of the monomer plasmid, whereas the RNP particles in later gradient fractions synthesized both VS/V1-2 and the larger retroplasmid cDNAs.

The proteins in gradient fractions were analyzed by SDS-PAGE and immunoblotting with an antibody against the retroplasmid RT (Kuiper et al. 1990). Figure 5D shows that gradient fractions 4–6, which synthesize VS/V1-2 cDNAs, do contain the 81-kD retroplasmid RT. The amount of the RT protein in most of the other gradient fractions was too low to be detected immunochemically, but RT activity in these fractions (Fig. 5B) paralleled the distribution of the retroplasmid transcript detected by Northern hybridization (not shown).

In additional experiments, we confirmed by nondenaturing gel electrophoresis that both wild-type VS and VS/V1-2 RNAs are reverse transcribed in discrete, small RNP particles, separate from the larger retroplasmid RNP particles (J.R. Sabourin, E.J. Vidunas, and A.M. Lambowitz, unpubl.). Furthermore, the synthesis of full-length VS and VS/V1-2 cDNAs in endogenous reverse

Figure 4. Dot blot hybridization of ^{32}P -labeled cDNAs synthesized in endogenous reverse transcription reactions to the (+) and (–) strands of the V and VS plasmids. ^{32}P -Labeled cDNAs were synthesized in Varkud and V1-2 mt RNP particles and hybridized to dot blots of (+) and (–) strands corresponding to the V plasmid (M13 phage M92 and M94, respectively; the M and V plasmids have 97% sequence identity); the (+) and (–) strands of the VS plasmid (M13 phage V18 and V19, respectively); and the single-strand M13mp18 and M13mp19 vector DNAs. In the time course experiments, the blots were hybridized to ^{32}P -labeled cDNAs synthesized in endogenous reverse transcription reactions of 2.5, 5, 10, and 30 min. (Bottom) Blots hybridized with ^{32}P -labeled cDNAs synthesized in V1-2 RNP particles in the absence or presence of actinomycin D (act. D; 100 $\mu\text{g}/\text{ml}$). We confirmed that the actinomycin D preparation completely inhibited DNA-directed DNA synthesis by Sequenase (not shown).

transcription reactions was confirmed by polyacrylamide gel electrophoresis and by restriction enzyme analysis of the gel-isolated, ^{32}P -labeled cDNAs, after hybridization to unlabeled (+) strand DNA or using enzymes that cut single-stranded DNAs (not shown).

Identification of putative cDNA initiation sites in VS RNAs

Because the retroplasmid RT ordinarily initiates cDNA synthesis at the 3' tRNA-like structure of the retroplasmid transcript (Kuiper and Lambowitz 1988; Wang et al. 1992), we suspected that it might likewise use the 3' end of VS RNA as a cDNA initiation site. The 3' end of VS RNA does not appear to have a tRNA-like structure but does have a 3' CG sequence like the (–) strand of CARNA 5 satellite RNA, which is recognized as an initiation site by a cucumber mosaic virus (CMV) replicase complex (Fig. 6, bottom; Wu and Kaper 1994). However, attempts to demonstrate that cDNAs begin at the 3' end

of VS RNA *in vivo* have been unsuccessful. For wild-type VS, primer extension and S_1 -nuclease mapping of 5' cDNA ends in mitochondria or mt RNP particles was not possible because of the low level of reverse transcription of this element. Possibly for the same reason, attempts to clone the 5' ends of wild-type VS cDNAs via anchored PCR gave no indication of a predominant initiation site and instead gave clones having 5' sequences corresponding to scattered internal positions, some or all of which may be artifactually derived from closed-circular VS plasmid DNAs in the preparations (not shown).

Mapping of 5' cDNA ends was possible for VS/V1-2, which is more efficiently reverse transcribed. In this case, however, we were surprised to find that the cDNAs synthesized in RNP particles begin near the 10-bp insertion that replaces the large deletion. As shown in Figure 6, primer extension mapping of the 5' ends of the cDNAs gave a predominant doublet band, as well as lighter bands that may correspond to minor 5' cDNA ends or may be artifactually derived from closed-circular plasmid DNAs. The two components of the predominant doublet band map to the terminal G-residue of the 10-bp insertion and the U-residue at VS position 240. These locations were confirmed by dideoxy sequencing (Fig. 6A, right) and by cloning and sequencing the 5' ends of the cDNAs via anchored PCR (not shown). Both of the latter methods indicated that some cDNAs may also begin within a few nucleotides upstream or downstream of the major primer extension bands. These and other experiments in which the 5' ends of cDNAs were analyzed by primer extension before and after alkali treatment did not detect potential DNA or RNA primer sequences attached to the 5' ends of the cDNAs, but it remains possible that a primer is rapidly removed or is connected to the 5' end of the cDNA via an unusual linkage (e.g., a 2'–5' phosphodiester bond). Although we cannot completely exclude that the 5' cDNA ends result from DNA cleavage rather than initiation, we could not detect such cleavage of a control ^{32}P -labeled monomeric (–) strand DNA incubated with RNP particles under reverse transcription conditions (not shown). Furthermore, the same 5' cDNA ends were found for endogenous cDNAs isolated from mitochondria or synthesized in 5 or 30 min reverse transcription reactions in RNP particles, and no additional 5' cDNA ends were found by using the VSP1 primer (see Materials and methods). Because primer extension mapping gave no indication that VS/V1-2 RNAs are cleaved near position 240 to generate a new 3' end (not shown), these findings suggest that the retroplasmid RT initiates predominantly at an internal position in VS/V1-2 RNA.

The structural basis for the putative internal initiation in VS/V1-2 RNA is unclear. The region around the internal site lacks a CCA sequence and does not otherwise resemble the previously described *in vitro* internal initiation site in the retroplasmid RNA (Wang and Lambowitz 1993a). Examination of sequences within the deleted regions showed that the 10-bp insertion could be derived by the RT skipping between nonadjacent sequences (A-GCC-GACAAG), a mechanism suggested for

Reverse transcription of satellite RNA

Figure 5. Sucrose gradient analysis of V1-2 mt RNP particles. Mt RNP particles were analyzed by centrifugation in a 5–20% sucrose gradient containing 500 mM KCl buffer, as described in Materials and methods. The high salt–sucrose gradients minimize adventitious association of proteins with RNP particles, but the RNPs tend to aggregate under these conditions at loads required to detect RT activity. (A) Gel electrophoretic analysis of nucleic acids from gradient fractions. Gradient fractions were ethanol-precipitated in the presence of 2 M ammonium acetate and 20 μ g of *Escherichia coli* tRNA carrier, and nucleic acids were analyzed by electrophoresis in a 1.5% agarose gel containing 0.1% SDS. The gel stained with ethidium bromide is shown. Fractions are numbered from the top (T) to the bottom (B) of the gradient. Lane P is the pellet recovered from the bottom of the centrifuge tube; lanes D and R show mt nucleic acids treated with DNase I (Pharmacia) or RNase A + T₁ (Sigma), respectively, to distinguish DNA and RNA species. Prominent RNAs are identified at left, and their sizes (kb) are indicated at right. (B) Gel electrophoretic analysis of ³²P-labeled cDNAs synthesized in endogenous reverse transcription reactions in gradient fractions. Two-microliter portions of gradient fractions were incubated with [³²P]dCTP + dNTPs, as described for endogenous reverse transcription reactions in Materials and methods, and cDNA products were analyzed by electrophoresis in a 1.7% agarose gel containing 0.1% SDS, followed by autoradiography of the dried gel. (C) Hybridization of ³²P-labeled cDNAs synthesized in the gradient fractions to a Northern blot of total mt RNA from the Varkud strain. The ³²P-labeled cDNAs synthesized in earlier gradient fractions hybridized to VS RNA, whereas those synthesized in later gradient fractions hybridized to both VS RNA and the V retroplasmid transcript (Varkud RNA). The hybridization of ³²P-labeled cDNAs in gradient fractions 9, 11, 15, and 19 to VS RNA appears greater than expected from the small amounts of VS/V1-2 RNPs present in these fractions. This disproportionate hybridization likely reflects the large amount of VS RNA relative to V RNA in the Northern blot. (D) Immunoblot analysis of proteins in gradient fractions. Portions (250 μ l) of gradient fractions were TCA-precipitated, and proteins were analyzed by electrophoresis in a 1% SDS–10% polyacrylamide gel. The gel was blotted to nitrocellulose and probed with anti-peptide antibody 62, which is directed against the carboxyl terminus of the retroplasmid RT (Kuiper et al. 1990). Shown are immunoblots of proteins in gradient fractions 3–7 compared with the 81-kD RT protein detected in a mt RNP particle preparation (RNP) run in a parallel lane of the same gel. As discussed in Kuiper et al. (1990), the 81-kD RT protein is present in very low concentrations in mitochondrial preparations and cannot be detected by immunoblotting without bringing up background bands due to nonspecific interaction of the primary or secondary antibody with more abundant proteins, particularly ribosomal proteins in the RNP particle preparations. The 81-kD protein in V1-2 mt RNP particle is not detected by preimmune serum (Kuiper et al. 1990). Numbers at left indicate sizes (kDa) and positions of rainbow size markers (Amersham).

similar deletion/addition mutations created by other DNA polymerases (Bebenek and Kunkel 1993). Because the cDNAs begin opposite the terminal G-residue of the 10-bp insertion, an intriguing possibility is that the de-

letion/addition event in the VS/V1-2 plasmid created a sequence or structure required for efficient internal initiation. As discussed above, it is not clear whether an internal cDNA initiation site is also used in wild-type

VS RNA or whether initiation occurs at the 3' end of the linear RNA.

VS plasmid replication cycle

Considered together with previous findings, our results for the wild-type VS and mutant VS/V1-2 plasmids suggest that these genetic elements replicate by the pathway outlined in Figure 7. The first step is transcription of the double-stranded DNA by the mt RNA polymerase starting at a cognate of the *Neurospora* mt DNA promoter consensus sequence. The location of the transcription start site, just upstream of the RNA self-cleavage site, suggests that linear and circular VS RNA monomers result from transcription of circular or multimeric VS DNAs combined with RNA catalyzed cleavage and ligation. This mode of RNA synthesis is analogous to the rolling circle mechanism used by other satellite RNAs, except that VS RNA is transcribed from a DNA, not an RNA, template.

Biochemical experiments showed that VS/V1-2 RNA is associated with the retroplasmid RT in RNP particles and that both VS and VS/V1-2 RNAs are reverse transcribed to yield full-length cDNAs that are presumed replication intermediates. Although the level of reverse transcription of the wild-type VS plasmid is relatively low compared to the mutant VS/V1-2, we detected (–) cDNA synthesis in both cases, whereas (–) strand RNAs could not be detected by hybridization (Saville and Collins 1990). The finding that the putative cDNA initiation site is located internally in VS/V1-2 RNA suggests that

the observed monomer-length cDNAs are synthesized either from a circular or multimeric template or by template switching upon reaching the 5' end of a linear RNA. Such template switching, if it occurs, would have to be very efficient, as we do not detect discrete “strong stop” cDNAs terminating opposite the 5' end of the linear VS/V1-2 RNA. Following (–) strand DNA synthesis, the remaining steps in the replication of the VS plasmid presumably include displacement or degradation of the RNA template, perhaps by the endogenous mt RNase H (Wang and Lambowitz 1993b), synthesis of (+) strand DNA and ligation to generate a closed circular DNA. As is the case for the retroplasmid itself, the mechanisms of (+) strand synthesis and DNA circularization are un-

Figure 6. Primer extension mapping and sequencing of the 5' ends of VS/V1-2 (–) strand cDNAs synthesized in RNP particles. (A) cDNA synthesis was carried out with V1-2 mt RNP particles in the presence (+) of 125 μ M dNTPs. The two left panels show primer extension mapping of 5' cDNA ends performed with Sequenase, using 5' 32 P-labeled DNA oligonucleotide primers VSP2 or VSP3 (see Materials and methods). Control lanes show parallel primer extension analysis with nucleic acids isolated from RNP particles incubated in the absence (–) of dNTPs. The numbers to the left indicate the sizes (nucleotides) and positions of 32 P-labeled *Pst*I fragments of phage λ DNA used as molecular weight markers. Sizes of primer extension bands are indicated at right. The right panel shows dideoxy sequencing of (–) strand cDNAs using Sequenase with the 5' end-labeled VSP2 primer. Lane N shows a primer extension reaction carried out in parallel. The right lanes show dideoxy sequencing of pAD7, using the same 5' end-labeled primer. The sequence around the 5' cDNA ends is shown to the right of the autoradiograph, with the box indicating the 10-bp insertion in VS/V1-2. The schematic beneath the autoradiographs shows the locations of primers VSP2 and VSP3, and the sizes of primer extension bands expected for a cDNA beginning opposite the G-residue at the end of the 10-bp insertion. (B) The top shows alignment of the 3' ends of VS and VS/V1-2 RNAs with the 3' end of CARNA 5 (–) strand RNA (Wu and Kaper 1994). The arrow indicates the site of initiation of (+) strand RNA synthesis in the CARNA 5 RNA. The bottom shows the sequence around the putative internal cDNA initiation site in VS/V1-2. The boxed sequence corresponds to the 10-bp insertion.

Figure 7. Proposed replication pathway of the VS and VS/V1-2 plasmids. Double-stranded VS or VS/V1-2 DNAs are transcribed by the mt RNA polymerase from a cognate of a *Neurospora* mtDNA promoter, with the major in vitro transcription start site corresponding to VS position 601. Linear and circular RNA monomers result from transcription of circular or multimeric DNAs coupled with RNA catalyzed self-cleavage and ligation. VS and VS/V1-2 RNAs are reverse transcribed by the retroplasmid RT to yield full-length (–) strand cDNAs. For VS/V1-2 RNA, the predominant cDNA initiation site is located internally at the site of the deletion/addition (shaded box). Full-length (–) strand cDNAs initiated at this location could result from reverse transcription of circular or multimeric VS/V1-2 RNAs or by the RT switching templates upon reaching the 5' end of a linear RNA. It is not known whether cDNA synthesis in wild-type VS RNA initiates at the 3' end of the linear RNA or at an internal site. Following (–) strand synthesis, the remaining replication steps presumably include displacement or degradation of the RNA template, perhaps by the endogenous mt RNase H (Wang and Lambowitz 1993b), synthesis of (+) strand DNA, and ligation to regenerate closed circular DNA.

clear, and it is possible that a conventional DNA replication mechanism is used for the further amplification of VS DNA in mitochondria.

VS RNA is the first catalytic RNA species that appears to replicate via reverse transcription. Hepatitis δ virus, a satellite of the pararetrovirus hepatitis B, replicates via an RNA intermediate and depends on the helper virus for

packaging (Taylor 1992). The ability of the retroplasmid to support replication of a satellite RNA presumably reflects the relative promiscuity of its RT, which can initiate cDNA synthesis on natural RNA templates without using a complementary primer. Although the retroplasmid RT ordinarily initiates cDNA synthesis at the 3' tRNA-like structure of the plasmid transcript, the solubilized RT also initiates at an internal position in the plasmid RNA (Wang and Lambowitz 1993a) and can use other RNA templates to some degree (Wang et al. 1992). The present results extend these findings by showing that the retroplasmid RT reverse transcribes an unrelated RNA species, VS RNA, in vivo and suggest further that this ability may be enhanced by the mutation in VS/V1-2 to generate a more efficient retroelement. Finally, we note that the ability of primitive RTs to reverse transcribe unrelated RNA species followed by mutational enhancement of the interaction could provide a mechanism for the evolution of new species of retroelements. Such a pathway may have been used for the generation of mobile group II introns, which appear to have acquired a RT that is phylogenetically related to the retroplasmid RT and have adapted it to function in both intron mobility and RNA splicing (Kennell et al. 1993; Lambowitz and Belfort 1993; J. Moran, S. Zimmerly, R. Eskes, J.C. Kennell, A.M. Lambowitz, R.A. Butow, and P.S. Perlman, in prep.).

Materials and methods

Neurospora strains and growth conditions

Strains used in this study were *Neurospora crassa* 74-OR23-1A [designated 74A; Fungal Genetics Stock Center (FGSC) no. 987] and Mauriceville-1c A (FGSC no. 2225) and *Neurospora intermedia* Varkud-1c (FGSC no. 1823) and senescent mutant V1-2 (FGSC no. 6006). *N. crassa* [poky] 13–6, which accumulates mt RNA polymerase (Barath and Kuntzel 1972; Kennell and Lambowitz 1989), was used for in vitro transcription assays. Procedures for growing the strains were as described (Kuiper and Lambowitz 1988; Kennell and Lambowitz 1989).

Recombinant plasmids and phage

Plasmid pD7 contains monomer VS DNA linearized at the *DraI* site and cloned in the *SmaI* site of the vector pTZ18R (Saville and Collins 1990). p Δ D7 contains monomer VS/V1-2 DNA linearized at the *SspI* site and cloned into *SmaI* site of pTZ18R (Pharmacia, Piscataway, NJ). pS1519 contains monomer VS DNA cloned as a *SacI* partial-digest fragment (VS675-674) into the *SmaI* site of pTZ18R. Phage M13mpM92 (M92) and M13mpM94 (M94) contain the M plasmid linearized at the *SacI* site and cloned into the *SacI* site of M13mp18 and M13mp19, respectively (Kuiper and Lambowitz 1988). Phage V18 and V19 contain monomer VS plasmid subcloned from pS1519 between the *HindIII* and *EcoRI* sites of M13mp18 and M13mp19, respectively.

Isolation of mt nucleic acids and RNP particles

Mt RNA and DNA were extracted from flotation gradient-purified mitochondria, as described (Lambowitz 1979; Garriga and Lambowitz 1984). Mt RNP particles were isolated by lysing

mitochondria with 1% NP-40 and centrifuging the lysate through a 1.85 M sucrose cushion containing 0.5 M KCl buffer (Garriga and Lambowitz 1986; Kuiper and Lambowitz 1988). The mt RNP particles were further purified by an additional centrifugation through the sucrose cushion to minimize associated nuclease activity.

In vitro transcription by *Neurospora* mt RNA polymerase

For *in vitro* transcription, DNA templates were incubated with [³²P]UTP plus other NTPs in the presence of mt lysates from [poky] 13-6 or the Varkud strain or with heparin–Sephacrose (HS) column fractions containing partially purified mt RNA polymerase from [poky] 13-6 (Kennell and Lambowitz 1989). Transcription with mt lysates was done in the presence of an antibody preparation against *N. crassa* endo-exonuclease (Chow and Fraser 1983) to inhibit nonspecific nuclease activity. ³²P-labeled transcripts were analyzed by electrophoresis in 6 or 12% polyacrylamide gels containing 8 M urea, 88 mM Tris-borate (pH 8.3), and 2 mM EDTA (TBE), followed by autoradiography. For 5' end mapping, *in vitro* transcription of pD7/SspI with HS-enriched mt RNA polymerase was scaled up fivefold, and primer extension was carried out as described (Kennell and Lambowitz 1989), by using MMLV-RT (Life Science Technologies, Inc., Gaithersburg, MD) with a 5' ³²P-labeled synthetic oligonucleotide primer (5' GTAGTCAACTGCTACGAC, complementary to nucleotide positions VS702-685.)

Endogenous reverse transcription reactions in mt RNP particles

Endogenous reverse transcription reactions were carried out in 20 µl of reaction medium containing 2 µl of mt RNP particles (~0.04 A₂₆₀ units), 100 mM KCl, 6 mM MgCl₂, 50 mM Tris-HCl (pH 8.3), 5 mM dithiothreitol, 125 µM each dATP, dGTP, dTTP, and 20 µCi of [α-³²P]dCTP (3000 Ci/mmole; Dupont/NEN, Boston, MA) (Kuiper and Lambowitz 1988; Kennell et al. 1994). The reactions were incubated for 10 min at 37°C, "chased" for 10 min with 100 µM dCTP, and terminated by extraction with phenol/chloroform/isoamyl alcohol (25:24:1). The products were incubated with 0.2 M NaOH and then were ethanol precipitated (Kennell et al. 1994). For time course experiments, the dCTP chase was omitted.

Hybridization analysis of ³²P-labeled cDNAs

The ³²P-labeled cDNAs synthesized in endogenous reverse transcription reactions were hybridized to Northern or Southern blots of mt RNAs or DNAs, respectively, or to dot blots of M13-cloned (+) or (–) strands of the mt plasmids. ³²P-labeled cDNA probes were dissolved in 20 µl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and boiled for 5 min at 95°C. Southern hybridizations were carried out with gels blotted to Hybond N membranes (Amersham, Chicago, IL), as described (Kuiper and Lambowitz 1988). For Northern hybridizations, mt RNAs were denatured with glyoxal and analyzed by electrophoresis in 1.4% agarose gels containing 10 mM sodium phosphate buffer (McMaster and Carmichael 1977). Gels were blotted to Hybond N membranes and hybridized as described for Southern blots (Kuiper and Lambowitz 1988). Dot blots were done on Hybond N membranes in a Minifold SRC 96 dot blot apparatus (Schleicher & Schuell, Keene, NH) with 0.5 µg of single-stranded DNA in each well. The dot blots were prehybridized for 12 hr at 37°C in 2.5 ml of 40% formamide, 1 M NaCl, 50 mM Na₂PO₄ (pH 7.0), 1% SDS, 0.2 mg/ml salmon sperm DNA, and 5× Denhardt's solution (Denhardt 1966), and then hybridized with the ³²P-

labeled cDNAs (10⁴–10⁵ cpm) in the same solution for 48 hr at 37°C. The blots were washed twice with 2× SSC (SSC = 150 mM NaCl, 15 mM Na citrate), 0.5% SDS for 20 min at room temperature, and then twice with 0.5× SSC, 0.5% SDS for 20 min at 37°C, prior to autoradiography.

Sucrose gradient analysis

Ten A₂₆₀ units of mt RNP particles from the V1-2 strain were resuspended in 91 µl of HKCTD [500 mM KCl, 50 mM CaCl₂, 25 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol] plus 1 mM spermidine and 1 mM phenylmethylsulfonyl fluoride and incubated with 1 mM puromycin (Sigma, St. Louis, MO) for 5 min at 37°C. The RNPs were then centrifuged through a 5–20% sucrose gradient containing HKCTD (Beckman SW41 rotor, 40,000 rpm, 3 hr at 4°C), and fractions of 0.43 ml were collected. MgCl₂ ordinarily used in sucrose gradients was replaced with CaCl₂, which suppresses nuclease activity while preserving the integrity of ribosomal subunits (Lambowitz 1979).

Primer extension mapping of cDNA 5' ends

cDNAs for primer extension mapping were synthesized in 10-fold, scaled-up endogenous reverse transcription reactions containing 125 µM dNTPs and 100 µg/ml of actinomycin D (Boehringer Mannheim, Indianapolis, IN). Primer extension mapping of alkali-treated cDNAs was carried out as described (Kennell et al. 1994), with Sequenase (U.S. Biochemical, Cleveland, OH) with 5' ³²P-labeled oligonucleotide primers (VSP1, 5'-ACATG-GCTGTAGGTGTGAGA, corresponding to nucleotide positions VS450-469; VSP2, 5'-TCTGTAGCAATCTCTCCGCC, corresponding to VS826-846; or VSP3, 5'-GGCTAAGTGACG-GTATTGGCG, corresponding to VS721-740). The primer extension products were analyzed by electrophoresis in 6% polyacrylamide/8 M urea gels containing TBE. Dideoxy sequencing of the cDNAs with the VSP2 primer (Sanger et al. 1977) was carried out in parallel.

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