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The Mauriceville Plasmid of *Neurospora crassa*: Characterization of a Novel Reverse Transcriptase That Begins cDNA Synthesis at the 3′ End of Template RNA

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The Mauriceville and Varkud plasmids are retroid elements that propagate in the mitochondria of some *Neurospora* spp. strains. Previous studies of endogenous reactions in ribonucleoprotein particle preparations suggested that the plasmids use a novel mechanism of reverse transcription that involves synthesis of a full-length minus-strand DNA beginning at the 3′ end of the plasmid transcript, which has a 3′ tRNA-like structure (M. T. R. Kuiper and A. M. Lambowitz, Cell 55:693–704, 1988). In this study, we developed procedures for releasing the Mauriceville plasmid reverse transcriptase from mitochondrial ribonucleoprotein particles and partially purifying it by heparin-Sepharose chromatography. By using these soluble preparations, we show directly that the Mauriceville plasmid reverse transcriptase synthesizes full-length cDNA copies of in vitro transcripts beginning at the 3′ end and has a preference for transcripts having the 3′ tRNA-like structure. Further, unlike retroviral reverse transcriptases, the Mauriceville plasmid reverse transcriptase begins cDNA synthesis directly opposite the 3′-terminal nucleotide of the template RNA. The ability to initiate cDNA synthesis directly at the 3′ end of template RNAs may also be relevant to the mechanisms of reverse transcription used by LINEs, group II introns, and other non-long terminal repeat retroid elements.

The Mauriceville plasmid and the closely related Varkud plasmid are small circular DNAs that propagate as retroid elements in the mitochondria of some *Neurospora* spp. strains (16). The plasmid monomers are circular DNAs of 3.6 and 3.7 kb, respectively, which encode a 710-amino-acid open reading frame (ORF) (Fig. 1) (1, 6, 30). This ORF contains short blocks of amino acids (I to VII) characteristic of reverse transcriptases (27, 30) and has been shown to encode an 81-kDa protein having reverse transcriptase activity (17). The major transcripts of the plasmids are full-length linear RNAs that presumably serve both as mRNAs for the reverse transcriptase and as intermediates in the replication of the plasmids by reverse transcription (1, 16, 30). The plasmids also have characteristics of group I introns and may be related to mobile elements involved in the propagation of these introns (1, 30, 33).

The Mauriceville plasmid reverse transcriptase has been isolated in mitochondrial ribonucleoprotein (mtRNP) particles, in which it is associated with the 3.6-kb plasmid transcript (16). These particles can be considered equivalent to retroviral core particles and have been used similarly to obtain information about the mechanism of reverse transcription. We found that the plasmid reverse transcriptase uses the endogenous plasmid transcript in mtRNP particles to synthesize a full-length 3.6-kb cDNA (minus-strand DNA). Further, the 5′ end of this cDNA begins opposite the 3′ end of the plasmid transcript, which has tRNA-like characteristics similar to those of the 3′ tRNA-like structures of plant RNA viruses (Fig. 2) (16). Although the nature of the primer was not clear, the initiation of minus-strand synthesis at the extreme 3′ end of the plasmid transcript precluded the use of an RNA primer complementary to the template, as in retroviruses (35).

In brome mosaic virus, the 3′ tRNA-like structure has been shown to be a recognition site for the initiation of minus-strand RNA synthesis by a virus-encoded RNA-dependent RNA polymerase (9, 28). We suggested that the 3′ tRNA-like structure of the Mauriceville plasmid transcript might function analogously as a recognition site for initiation of minus-strand DNA synthesis by the plasmid reverse transcriptase (16). In that case, the plasmid 3′ tRNA-like structure may be the remnant of a genomic tag used in the so-called RNA world to mark the ends of self-replicating RNA genomes (36). The characteristics of the plasmid reverse transcription mechanism raise the possibility that it evolved early in evolution, at the time of transition from an RNA to a DNA world, and is ancestral to the mechanism used by present-day retroviruses, in which a host cell tRNA primer has been substituted for the 3′ tRNA-like structure (16).

Phylogenetic comparisons show that the Mauriceville plasmid reverse transcriptase is most closely related to the reverse transcriptase-like proteins encoded by group II introns and to the reverse transcriptases encoded by the LINE1-like or non-long terminal repeat (LTR) family of retroid elements (8, 38). In general, relatively little is known about the mechanism of reverse transcription used by these elements (5, 12). The plasmid's reverse transcriptase is similar to that of other retroid elements in that the region of reverse transcriptase homology is located toward the N terminus of the protein (Fig. 1), but the plasmid does not appear to have a *gag* gene and lacks or has poor matches to sequence motifs characteristic of RNase H, protease, or integrase domains of retroviral *pol* genes (8, 24). It is possible that these latter activities are present, but associated with nonstandard or degenerate sequence motifs (24).

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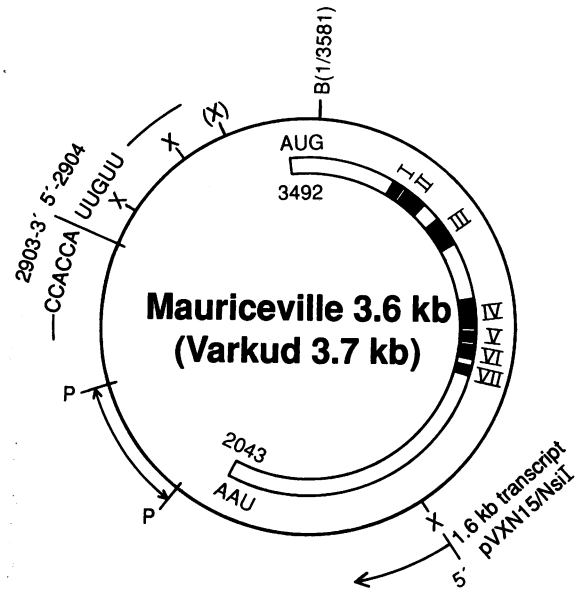

FIG. 1. Map of the Mauriceville and Varkud plasmids. The 710-amino-acid ORF is shown inside the circular map. Boxes I to VII indicate blocks of amino acids characteristic of reverse transcriptases (27, 30, 38). The map shows the locations of the major 5′ and 3′ ends of the 3.6-kb Mauriceville plasmid transcript at positions 2904 and 2903, respectively. The region corresponding to the 1.6 kb in vitro transcript (pVXN15/*Nsi*I) is also shown (see Materials and Methods). Nucleotide positions shown are for the Mauriceville plasmid (30). The *Xba*I site in parentheses is found only in the Varkud plasmid. Abbreviations: B, *Bgl*II; P, *Pst*I; X, *Xba*I.

Alternatively, the activities may not be required as part of the life cycle of the plasmid, or the plasmid may use host enzymes to supply the missing activities. If the plasmid reverse transcriptase is a primitive enzyme, the lack of these other activities would suggest that they were acquired subsequently by other retroid elements (17).

Further insight into the mechanism of replication of the plasmids requires detailed biochemical analysis of the reverse transcriptase and the reconstitution of individual steps of the reaction. As a first step in this direction, we developed methods for releasing active plasmid reverse transcriptase from mtRNP particle preparations and partially purifying it by heparin-Sepharose CL-6B (HS) chromatography. We show here that this HS-enriched reverse transcriptase can use in vitro transcript substrates and mimics key features of the endogenous reverse transcription reaction in RNP particles, including a preference for transcripts having the 3′ tRNA-like structure and the ability to begin cDNA synthesis directly opposite the 3′-terminal nucleotide of the template RNA.

MATERIALS AND METHODS

Strain and growth conditions. The *Neurospora crassa* strain used in this work was Mauriceville 1c-*A* (FGSC 2225). Procedures for maintaining the strain and growing mycelia in liquid media were as described previously (7, 16).

Recombinant plasmids. pVX55 consists of the Varkud plasmid 1,628-bp *Xba*I fragment (1), which contains sequences corresponding to the 3′ end of the major transcript of the plasmid, cloned behind the bacteriophage T3 promoter in the *Xba*I site of pBS(−) (Stratagene, La Jolla, Calif.). pMP24 contains the 3.2-kb *Pst*I-fragment of the Mauriceville plasmid cloned in pBS(−) (16).

pVXN15, which was used to synthesize a 1,575-nucleotide

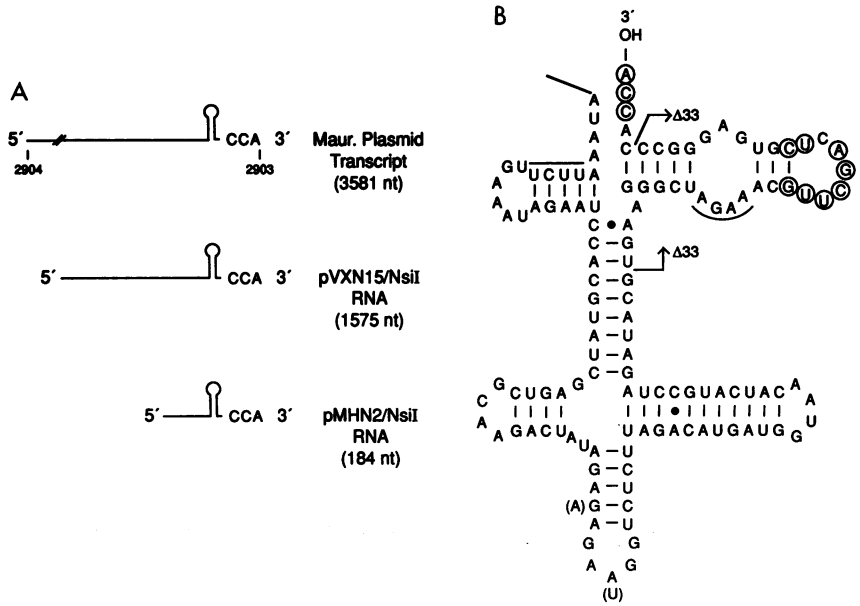

FIG. 2. In vitro transcript templates and 3′ tRNA-like structure of the Mauriceville (Maur.) plasmid transcript. (A) Schematic of Mauriceville plasmid transcript and in vitro transcript templates pVXN15/*Nsi*I and pMHN2/*Nsi*I, which correspond to the 3′ end of the plasmid transcript. (B) 3′ tRNA-like structure of the Mauriceville plasmid transcript adapted from reference 3. Circled bases indicate nucleotides that are conserved in tRNAs. Nucleotides in parentheses indicate differences in the Varkud plasmid. Lines indicate nucleotides involved in a potential pseudoknot suggested by Mans et al. (23). Δ33 indicates a portion of the tRNA-like structure deleted in pVXNd33 (see Materials and Methods).

(nt) in vitro transcript corresponding to the 3′ end of the mitochondrial plasmid transcript, was derived from pVX55 by introducing an *Nsi*I site at a position such that cleavage with *Nsi*I followed by transcription with bacteriophage T3 RNA polymerase results in RNAs ending exactly at the 3′ CCA terminus. To accomplish this, pVX55 was cleaved with *Apa*I and *Xma*I, dephosphorylated, and ligated to the bridging 19-mer 5′-CCGGGATGCATGGTGGGCC-3′. The resulting gapped plasmid was filled in with the Klenow fragment of DNA polymerase I (GIBCO BRL, Gaithersburg, Md.) and used to transform *Escherichia coli* JM109. Putative clones were identified by screening for the presence of the *Nsi*I site and confirmed by sequencing.

pVXS211, which was used to obtain a related 1.6-kb transcript ending in CC instead of CCA, was constructed from pVX55 by the same procedure as that used for pVXN15 (see above) except that an *Sst*II site was introduced by using the bridging 17-mer 5′-CCGGGCCGCGGTGGGCC-3′.

pMHN2 was used to synthesize a 184-nt in vitro transcript corresponding to the 3′ end of the mitochondrial plasmid transcript. This plasmid was derived from pVXN15 by replacing the 1,561-bp *Hin*dIII-*Apa*I fragment with a 170-bp *Hin*dIII-*Apa*I fragment containing sequence from the 3′ end of the Mauriceville plasmid transcript (Mauriceville plasmid sequence 2729 to 2898 from pMP24; see above).

pVXNd33, which contains an internal deletion of part of the 3′-terminal tRNA-like structure, was constructed by BAL 31 digestion of pVXN15 from the *Apa*I site located 9 bp upstream of the CCA terminus (see Fig. 8). To protect the CCA terminus from BAL 31 digestion, a stuffer DNA fragment (1-kb *Apa*I fragment of *N. crassa* wild-type 74*A* mtDNA) was first inserted at the *Apa*I site of pVXN15. The resulting plasmid (pVX8b) was then linearized by partial digestion with *Apa*I and digested with BAL 31 nuclease (slow; IBI, New Haven, Conn.) for various times. Following digestion, the stuffer DNA fragment was deleted by digestion with *Apa*I, 3′ overhangs were removed with mung bean nuclease (New England Biolabs, Beverly, Mass.), and the plasmids were religated to yield a series of deletions upstream of the original *Apa*I site. Screening yielded plasmid pVXNd33, whose transcript corresponds to that derived from pVXN15 with a deletion from −6 to −38 nt from the 3′ end (Fig. 2).

Exogenous substrates for reverse transcriptase assays. In vitro transcripts used as templates in reverse transcription reactions were synthesized from recombinant plasmids linearized with restriction enzymes by using bacteriophage T3 RNA polymerase (32). In vitro transcription was carried out in 50 μl of reaction medium containing 1 to 5 μg of DNA template, 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 50 mM NaCl, 30 mM dithiothreitol (DTT), 2 mM spermidine, 1.25 mM each ribonucleoside triphosphate (rNTP), RNasin (50 U; Promega, Madison, Wis.), and 50 U of bacteriophage T3 RNA polymerase (Stratagene). After incubation for 60 min at 37°C, the DNA template was digested with DNase I (15 U; fast protein liquid chromatography purified; Pharmacia, Piscataway, N.J.) for 15 min at 37°C. Transcripts were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1; phenol-CIA) and ethanol precipitated twice in the presence of 2.5 M ammonium acetate. The final pellet was dissolved in double-distilled H_2O and stored at −20°C. For some experiments, in vitro transcripts were purified by CsCl gradient centrifugation to remove DNA fragments that might potentially be used as primers (32).

A template-primer substrate, consisting of the in vitro transcript pVXN15/*Nsi*I annealed with oligonucleotide MPST (5′-GATCGATTCCAACCTTAGAC-3′), was prepared by incubating 0.1 μM pVXN15/*Nsi*I RNA with 0.1 or 1 μM oligonucleotide in 40 μl of 40 mM Tris-HCl (pH 8.0)–40 mM KCl–4 mM $MgCl_2$–1 mM DTT–40 U of RNasin (Promega) at 65°C for 5 min and then slowly cooling the mixture to 45°C for 30 min.

Artificial template-primer substrates, poly(rC)-oligo $(dG)_{12-18}$ and poly(rA)-oligo$(dT)_{12-18}$ (Pharmacia), were dissolved in double-distilled H_2O at 0.5 mg/ml and stored at −20°C.

Isolation of mitochondria and mtRNP particles. Mitochondria were isolated by the modified flotation gradient method (19). mtRNP particles were isolated from mitochondria by lysis with 1.0% Nonidet P-40 and centrifugation through a 1.85 M sucrose cushion containing 0.5 M KCl buffer (11, 19). For assays of endogenous reverse transcription, RNP particles were purified further by DEAE-Sephacel chromatography to minimize contaminating nuclease activities. In a typical purification, 5 OD_{260} (optical density at 260 nm) units of RNP particles was suspended in 1 ml of buffer A (50 mM Tris-HCl [pH 8.3], 1 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5% glycerol) and loaded on a DEAE-Sephacel column (glass column [0.6 by 3 cm] containing 1 ml of DEAE-Sephacel [Pharmacia] equilibrated with buffer A). The column was reloaded with the flowthrough and then washed with 5 ml of buffer A and 5 ml of 0.25 M KCl in buffer A prior to elution of RNP particles with 10 ml of 0.75 M KCl in buffer A. Fractions of 0.4 ml were collected and assayed for reverse transcription of the endogenous RNA template as described below. Peak fractions were pooled, and RNP particles were concentrated by ultracentrifugation (Beckman 50 Ti rotor, 35,000 rpm, 4 h at 4°C). The final RNP pellets were suspended in 100 mM KCl–50 mM Tris-HCl (pH 8.3)–5 mM DTT–1 mM PMSF–50% glycerol and stored at −20°C.

Micrococcal nuclease digestion of RNP particles. For further purification, the plasmid reverse transcriptase was released from mtRNP particles by degrading the endogenous RNA template with micrococcal nuclease. Just before digestion, the RNP particles were dissolved in 500 mM NH_4Cl–10 mM $MgCl_2$–50 mM Tris-HCl (pH 7.5)–5 mM DTT–1 mM PMSF–1 mM spermidine (16) or in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (10 OD_{260} units per 100 μl) at 4°C. After removal of insoluble material by brief centrifugation in a microcentrifuge, the RNP particles were diluted 10-fold to 1 ml with 50 mM Tris-HCl (pH 8.2)–1 mM $CaCl_2$–5 mM DTT–1 mM PMSF and digested with micrococcal nuclease (1,000 U/10 OD_{260} units; Pharmacia) for 30 min at 37°C. To ensure maximal digestion, 5 mM EDTA was then added to weaken association of the reverse transcriptase with endogenous RNA fragments, and incubation was continued for an additional 5 min at 37°C. The concentration of $CaCl_2$ was then brought to 10 mM, and incubation was continued for another 10 min. The reaction could be terminated at this point by addition of 20 mM EGTA, and the released reverse transcriptase was assayed directly. Alternatively, for further purification of the reverse transcriptase, the digests were loaded directly on an HS or other column without addition of EGTA.

HS chromatography. The plasmid reverse transcriptase was partially purified from micrococcal nuclease-digested RNP particles by HS chromatography at 4°C. HS (Pharmacia) was prepared as recommended by the manufacturer and equilibrated with 0.2 M KCl in buffer B (25 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 1 mM PMSF, 10% glycerol). For the experiment shown in Fig. 3,

50 OD_{260} units of micrococcal nuclease-digested RNP particles was loaded on a glass column (0.8 by 5 cm) containing 4 ml of HS. The flowthrough was reapplied via recirculation to ensure maximum binding of the reverse transcriptase, and weakly bound material was then removed by washing with 6 column volumes of 0.2 M KCl in buffer B. The column was eluted with a linear gradient of 0.2 to 1.2 M KCl in buffer B at a flow rate of 10 ml/h, and conductivity in column fractions was measured by using a YSI conductivity meter (YSI Scientific, Yellow Springs, Ohio). The reverse transcriptase activity eluted at approximately 0.8 M KCl (see Fig. 3). The omission of EDTA from buffer B had no effect on the chromatographic behavior of the reverse transcriptase but resulted in a higher amount of coeluting RNAs. When RNP particles were loaded directly on an HS column without prior micrococcal nuclease digestion, no reverse transcriptase capable of using exogenous RNA template was recovered from the column (data not shown).

After initial experiments using linear gradients, we developed a simple step gradient procedure for routine preparation of the reverse transcriptase. Ten OD_{260} units of micrococcal nuclease-digested RNPs was loaded on a column (0.6 by 3 cm) containing 1 ml of HS preequilibrated with 0.1 M KCl in buffer C (50 mM Tris-HCl [pH 8.2], 5 mM EDTA, 5 mM DTT, 1 mM PMSF, 10% glycerol). The flowthrough was reapplied five times, and the column was washed with 5 ml each of 0.1 and 0.5 M KCl in buffer C prior to elution of reverse transcriptase activity with 1.2 M KCl in buffer C with the EDTA concentration reduced to 1 mM. Column fractions were stored at −20°C in a solution containing 100 μg of acetylated bovine serum albumin per ml plus 35% glycerol and retained activity for more than 1 month.

Reverse transcriptase activity was quantitated by incubating column fractions with the 1.6-kb pVXN15/*Nsi*I transcript template in the presence of [^{32}P]dCTP and other dNTPs and measuring incorporation of [^{32}P]dCTP into material retained on DE81 paper as described below. One unit of reverse transcriptase activity is defined as the amount necessary to incorporate 1 pmol of dCTP at 37°C in 20 min, which is within the linear range of the reactions. In a standard preparation, 2 μl of peak column fractions contained 1 to 2 mU of reverse transcriptase activity.

In other experiments, we evaluated alternative purification protocols that made use of either DEAE-Sephacel chromatography or a DEAE-Sephacel step prior to ion-exchange chromatography (data not shown). The Mauriceville plasmid reverse transcriptase from micrococcal nuclease-digested mtRNP particles bound efficiently to DEAE-Sephacel in column buffers containing either Mg^{2+} or EDTA, presumably reflecting binding of the enzyme to nucleic acids, as in the case of retroviral reverse transcriptases (37). However, the Mauriceville plasmid enzyme eluted from DEAE-Sephacel at salt concentrations similar to those at which nucleic acids eluted (0.25 to 0.5 M KCl). In addition, the coeluting nucleic acids impaired subsequent binding of the plasmid reverse transcriptase to carboxymethyl-Sepharose under low-salt conditions, making such protocols unsuitable for further purification.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Proteins in column fractions were precipitated with trichloroacetic acid (TCA) and analyzed by electrophoresis in sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (18, 22). To detect the 81-kDa plasmid reverse transcriptase protein, gels were blotted to nitrocellulose membranes and probed with antibody 62 directed against the C terminus of the Mauriceville plasmid 710-amino-acid ORF (17). SDS-polyacrylamide gels were silver stained as described previously (26).

Assay of endogenous reverse transcriptase activity in RNP particle preparations. Reverse transcription of endogenous plasmid transcript in DEAE-Sephacel-purified RNP particles was assayed by incubating the particles with [^{32}P]dCTP and other dNTPs (16). Reactions were for times indicated in the text at 37°C in reaction medium containing 1 mM $MgCl_2$ with 125 μM each dATP, dGTP, and dTTP, 5 μM dCTP, and 20 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Boston, Mass.). After extraction with phenol-CIA and ethanol precipitation, ^{32}P-labeled cDNAs were glyoxylated and analyzed by electrophoresis in 1.4% agarose gels followed by autoradiography (25).

Assay of reverse transcriptase activity with in vitro transcript templates. Reverse transcription reactions with in vitro transcript templates were carried out in 20 μl of reaction mixture containing 2 μl of column fraction (generally 1 to 2 mU of reverse transcriptase activity), 0.5 μg of RNA template, 200 mM KCl, 1 or 1.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.2), 10 mM DTT, 125 μM each dGTP, dATP, and dTTP, and 20 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear). Reactions were initiated by addition of enzyme, incubated for 10 min at 37°C, and chased for 20 min with 125 μM unlabeled dCTP to ensure synthesis of full-length cDNA products. Reactions were then terminated by the addition of 30 μl of 0.5% SDS–150 mM NaCl–100 mM Tris-HCl (pH 8.2)–1 mM EDTA plus 20 μg of carrier *E. coli* tRNA (Sigma, St. Louis, Mo.), extraction with phenol-CIA, and ethanol precipitation in the presence of 2.5 M ammonium acetate. For analysis of the HS column fractions in Fig. 3, ^{32}P-labeled cDNAs obtained with the 1.6-kb pVXN15/*Nsi*I template were dissolved in 20 μl of 0.5% SDS–50% dimethyl sulfoxide–1 mM EDTA–44 mM Tris-borate (pH 8.3), incubated at 90°C for 3 min, and analyzed by electrophoresis in a 1.7% agarose gel containing 0.1% SDS, 1 mM EDTA, and 44 mM Tris-borate (pH 8.3). Gels were run for 4 h at 180 V with buffer circulation, dried, and autoradiographed. Otherwise, the cDNAs obtained with the 1.6-kb template were glyoxylated and analyzed by electrophoresis in 1.4 or 1.7% agarose gels (25). In the experiment shown in Fig. 5, shorter ^{32}P-labeled cDNAs obtained with the 184-nt pMNH2/*Nsi*I template were analyzed by electrophoresis in 6% polyacrylamide gels containing 8 M urea. In some experiments, cDNAs were treated with alkali under conditions indicated in the figure legends and then neutralized with 0.2 M ammonium acetate (pH 4.6) prior to phenol-CIA extraction, ethanol precipitation, and gel electrophoresis. ^{32}P-labeled cDNAs were quantitated with a Betascope 603 blot analyzer (Betagen, Waltham, Mass.).

Assay of reverse transcriptase activity with artificial template-primer substrates. Assays with artificial template-primer substrates, poly(rC)-oligo$(dG)_{12-18}$ and poly(rA)-oligo$(dT)_{12-18}$, were done essentially as described previously (34), with 0.1 to 0.5 μg of substrate per reaction. ^{32}P-labeled products were extracted with phenol-CIA, spotted on Whatman DE81 paper (VWR, Cleveland, Ohio), washed four times over 30 min with 2× SSC (SSC is 150 mM NaCl plus 15 mM sodium citrate), dried, and quantitated by Cerenkov counting in a Beckman LS 1801 scintillation counter.

Determination of optimal conditions for reverse transcription with exogenous template. Optimal conditions for the reverse transcription of in vitro transcript template were determined by varying one parameter at a time in 20 μl of reaction medium containing 0.5 μg of pVXN15/*Nsi*I RNA, 200 mM KCl, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.2), 10

mM DTT, 125 μM each dGTP, dATP, and dTTP, and 10 μCi of [α-^{32}P]dCTP (1,500 Ci/mmol; New England Nuclear). Optimal conditions with poly(rC)-oligo(dG)$_{12-18}$ were determined similarly in 20 μl of reaction mix containing 0.5 μg of substrate, 200 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.2), 10 mM DTT, and 10 μCi of [α-^{32}P]dGTP (1,500 Ci/mmol; New England Nuclear). For both types of substrates, reactions were initiated by addition of 2 μl (1 to 2 mU) of HS-enriched reverse transcriptase, incubated at 37°C for 20 min, determined in separate experiments to be within the linear range of the reactions, and terminated by extraction with phenol-CIA. ^{32}P-labeled products were spotted on DE81 filter paper, washed, and counted as described above.

Sequencing of minus-strand cDNA synthesized in vitro. To obtain sufficient minus-strand DNA for sequencing, we carried out a scaled-up reverse transcription reaction in 1 ml of reaction medium containing 1 μg of 184-nt pMHN2/*Nsi*I transcript (CsCl purified [32]), 250 mM KCl, 0.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.2), 10 mM DTT, 100 μM each dNTP, and 100 mU of HS-enriched reverse transcriptase. The reaction was carried out for 1 h at 37°C, and the cDNAs were extracted and concentrated as described above. The minus-strand DNAs were then sequenced by the dideoxy method, using Sequenase (United States Biochemical, Cleveland, Ohio) with a ^{32}P-labeled oligonucleotide primer (VOSI; 5′-CCACGTATCGAGTCGCAAGA-3′) corresponding to a sequence from bases 109 to 90 upstream of the 3′ end of the plasmid transcript. The primer was ^{32}P labeled by using T4 polynucleotide kinase (GIBCO BRL) (32). Sequencing reactions were analyzed on a 6% polyacrylamide gel containing 8 M urea.

Cloning of primer sequences by anchored PCR. Primers at the 5′ end of minus-strand DNAs synthesized by HS-enriched reverse transcriptase from pMHN2/*Nsi*I RNA were cloned by anchored polymerase chain reaction (PCR) (21) and sequenced. Reverse transcription was carried out with approximately 50 mU of HS-enriched reverse transcriptase, 10 μg of gel-purified, DNA-free pMHN2/*Nsi*I RNA, and 125 μM dNTPs for 30 min at 37°C and terminated by extraction with phenol-CIA and ethanol precipitation. The pellet was dissolved in 40 μl of 50 mM NaCl–20 mM $MgCl_2$–40 mM Tris-HCl (pH 7.5) and digested with 25 U of RNase A (Sigma) plus 10 U of RNase T_1 (Boehringer Mannheim, Indianapolis, Ind.) for 15 min at 37°C to degrade the template RNA, which otherwise remains associated with minus-strand DNA. After the RNase incubation, a DNA copy of the 5′ end of minus-strand DNA was synthesized by using Sequenase (United States Biochemicals) with 125 μM dNTPs and primer VOSII (5′-AATGGTTTCACCTAGT TCCC-3′, corresponding to Mauriceville plasmid sequence 2737 to 2756). The synthesized DNA strand was purified by electrophoresis in a 6% polyacrylamide gel containing 8 M urea, eluted by soaking in 10 mM Tris-HCl (pH 8.0)–0.2 mM EDTA for 2 h at 37°C, extracted with phenol-CIA, and ethanol precipitated. The DNA pellet was then resuspended in 10 μl of 0.1 M potassium cacodylate (pH 7.2)–2 mM $CoCl_2$–0.2 mM DTT–1 mM dGTP and incubated with terminal deoxynucleotidyltransferase (15 U, 60 min, 37°C; GIBCO BRL) to add G tails to the 3′ end of the DNA. For PCR amplification, 5 μl of the G-tailed DNA was added to 45 μl of 50 mM NaCl–10 mM $MgCl_2$–50 mM Tris-HCl (pH 9.0)–200 μM each dATP, dCTP, dGTP, and dTTP–200 ng of M28 (5′-CGTGTAAAGCTTCCTAATGGTTTCACCT-3′) and dCBam (5′-ATATAGGATC$_{15}$-3′). After the mixture was heated to 95°C for 3 min and slowly cooled to 55°C, AmpliTaq DNA polymerase (5 U; Cetus, Norwalk, Conn.) was added and the mixture was cycled in a Perkin-Elmer thermocycler (25 cycles of 95°C for 45 s, 55°C for 2 min, and 72°C for 1 min). PCR products were purified by electrophoresis in 2% agarose gels containing 90 mM Tris-borate (pH 8.3)–2 mM EDTA and cloned between the *Hin*dIII and *Bam*HI sites of pBS(−) (Stratagene). The cloned DNAs were sequenced by using Sequenase with the M13 forward and reverse primers.

TABLE 1. Release of reverse transcriptase activity from mtRNP particles[a]

Detergent	Nuclease	cpm
Nonidet P-40		
None	None	3.8×10^3
0.125%	None	3.8×10^3
0.25%	None	4.3×10^3
0.50%	None	3.6×10^3
1%	None	3.9×10^3
None	Micrococcal	4.9×10^4
0.125%	Micrococcal	3.7×10^4
0.25%	Micrococcal	4.5×10^4
0.50%	Micrococcal	5.6×10^4
1%	Micrococcal	5.5×10^4
None	RNase A	4.5×10^4
0.125%	RNase A	5.4×10^4
0.25%	RNase A	4.2×10^4
0.50%	RNase A	5.7×10^4
1%	RNase A	6.2×10^4
0.1% SDS	None	90

[a] RNP particles were suspended in 200 μl of distilled water and diluted to 20 OD_{260} units per ml in buffer adjusted to 100 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 50 mM Tris-HCl (pH 8.2), and 10 mM DTT; 50 μl of this suspension was added to 50 μl of the same buffer containing 0 to 1% Nonidet P-40, with or without micrococcal nuclease (100 U; Pharmacia), or RNase A (0.5 U; Boehringer Mannheim), as specified. The solutions were incubated at 30°C for 15 min, and EGTA was added to all the reaction mixtures to a final concentration of 5 mM. Reverse transcriptase activity was assayed in 10 μl of solution containing 2 μl of the treated RNP suspensions, 0.1 μg of poly(rA)-oligo(dT), 100 mM KCl, 2.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.2), 10 mM DTT, and 10 μCi of [^{32}P]dTTP (3,000 Ci/mmol). Reaction mixtures were incubated for 15 min at 37°C, terminated by extraction with phenol-CIA, spotted on DE81 paper, washed, and counted as described in Materials and Methods. Values are averages of two replicate samples with an average standard deviation of less than 10%.

RESULTS

Solubilization and partial purification of Mauriceville plasmid reverse transcriptase from mtRNP particles. Our first objective was to purify the mitochondrial plasmid reverse transcriptase in order to carry out a biochemical analysis. Initial experiments showed that about 60% of the 81-kDa reverse transcriptase protein detected in immunoblots of mitochondrial lysates could be recovered in the RNP particle preparations obtained by centrifuging the lysates through 1.85 M sucrose cushions containing 0.5 M KCl buffer (data not shown). The RNP particle preparations provide a substantial enrichment of the reverse transcriptase from mitochondrial lysates and were used as the starting material for further purification of the enzyme.

An impediment to biochemical analysis of nonretroviral reverse transcriptases has been the difficulty of releasing active enzymes from core particles (10, 15, 35). Initial experiments showed that the Mauriceville plasmid reverse transcriptase could not be released from mtRNP particles by high salt (e.g., 1 M KCl or 3 M LiCl) or by nonionic detergents (see below) but could be released by digesting the RNP particles with micrococcal nuclease or RNase to de-

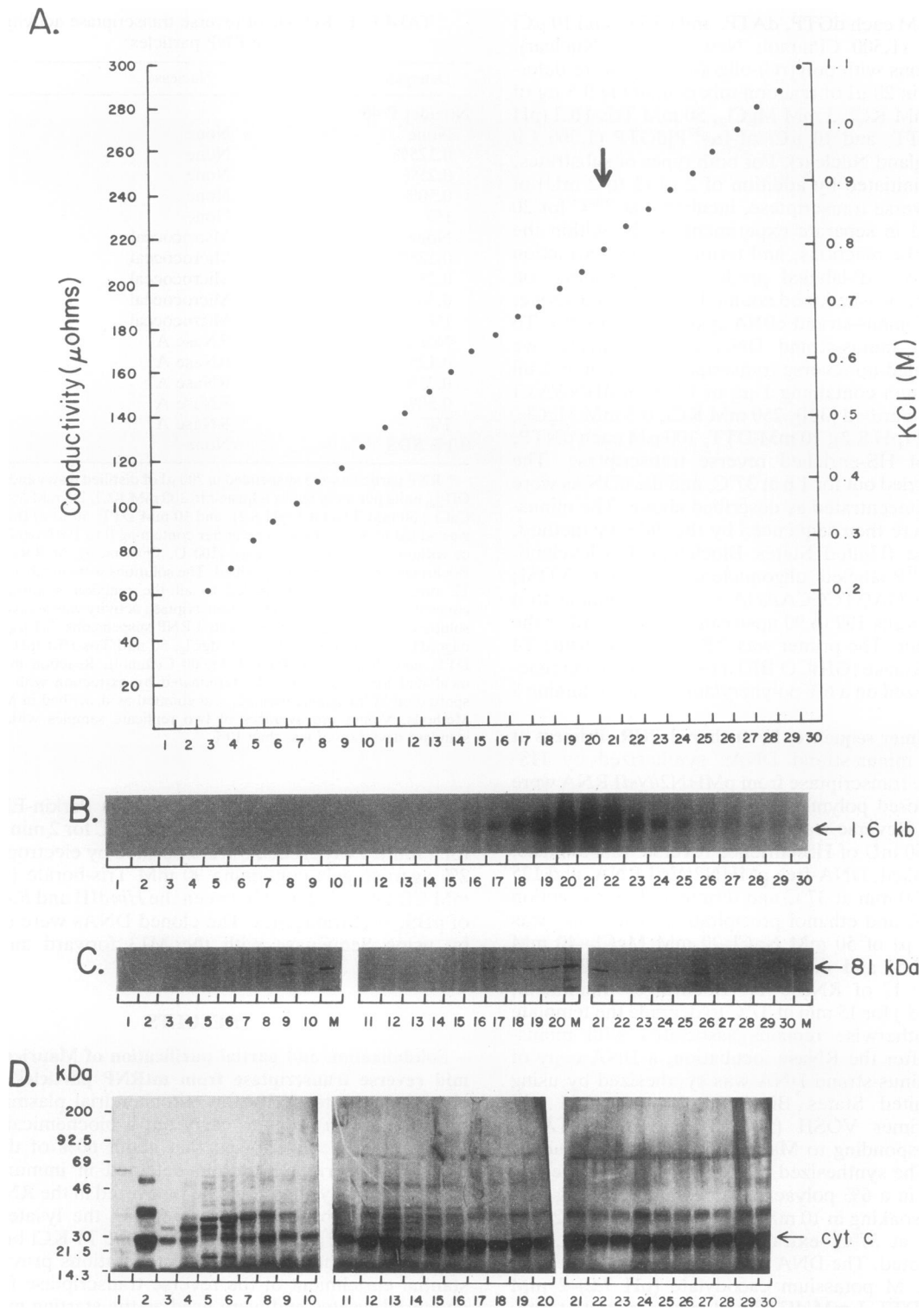

FIG. 3. HS chromatography of the Mauriceville plasmid reverse transcriptase. Micrococcal nuclease-digested mtRNP particles (50 OD_{260} units) from *N. crassa* Mauriceville-1c were analyzed by chromatography in a 4-ml HS column, as described in Materials and Methods. The column was eluted with a linear gradient of 0.2 to 1.2 M KCl, and 2.0-ml fractions were collected. (A) KCl gradient determined by measuring conductivity in 1:500 dilutions of 10-μl portions of column fractions. (B) Reverse transcriptase activity assayed with the 1.6-kb in vitro transcript template (pVXN15/*Nsi*I). Assays were in 20 μl of reaction medium containing 2-μl portions of column fractions, 0.5 μg of in vitro transcript template, and [^{32}P]dCTP plus other dNTPs as described in Materials and Methods. ^{32}P-labeled cDNAs were analyzed by electrophoresis in a 1.7% agarose gel followed by autoradiography. (C) Immunoblots of 81-kDa reverse transcriptase protein in column fractions. Portions (1.3 ml) of column fractions were TCA precipitated in the presence of 10 μg of cytochrome *c* carrier, and proteins were analyzed by electrophoresis in SDS–10% polyacrylamide gels. Gels were blotted to nitrocellulose membranes and probed with antibody 62 directed against the C terminus of the 81-kDa Mauriceville plasmid protein (17). In each gel, a marker lane (M) shows the 81-kDa reverse

grade endogenous template RNA. As shown in Table 1, untreated RNPs had relatively low reverse transcriptase activity with the poly(rA)-oligo(dT) substrate, but the activity increased 10- to 20-fold after digestion of endogenous RNA with micrococcal nuclease or RNase A. Table 1 also shows that the reverse transcriptase activity was not released by treating RNPs with Nonidet P-40 at concentrations ranging from 0.125 to 1% and that Nonidet P-40 did not substantially enhance the release of activity by micrococcal nuclease or RNase. The reverse transcriptase activity was inhibited completely by 0.1% SDS.

After evaluating several possible purification protocols (see Materials and Methods), we focused on HS chromatography, which enabled us to obtain highly active reverse transcriptase that was free of gross nuclease or nucleic acid contamination. Figure 3 shows an experiment in which proteins released from mtRNP particles by digestion with micrococcal nuclease were fractionated on an HS column eluted with a linear KCl gradient. In this experiment, reverse transcriptase was assayed by using a 1.6-kb in vitro transcript, corresponding to the 3′ end of the mitochondrial plasmid transcript ending in CCA (pVXN15/*Nsi*I; Fig. 1 and 2). Portions of column fractions were incubated with in vitro transcript and [^{32}P]dCTP plus other dNTPs and assayed by gel electrophoresis for the synthesis of a 1.6-kb cDNA. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antipeptide antibody 62 to detect the 81-kDa reverse transcriptase protein.

The column profile shows that the reverse transcriptase activity eluted at a relatively high salt concentration (0.8 M KCl) and that the major peak of activity coincided with the peak of the 81-kDa plasmid-encoded protein detected in immunoblots (fractions 20 and 21). Significantly, the reverse transcriptase by itself synthesized a full-length cDNA copy of the in vitro transcript template and did not require an exogenous primer complementary to the template. The column fractions having reverse transcriptase activity contained relatively few proteins detected by silver staining other than cytochrome *c*, which was used as a carrier during TCA precipitation. The 81-kDa protein was apparently present in concentrations too low to detect by silver staining, despite the fact that fractions have high reverse transcriptase activity (each assay represents 1/1,000 of a column fraction). Similar results were obtained for the reverse transcriptase activity from the Varkud strain (data not shown).

After establishing the chromatographic behavior of the reverse transcriptase on HS, we developed a step gradient procedure for routine preparation of the reverse transcriptase activity. The columns were loaded with proteins released from micrococcal nuclease-digested mtRNP particles and washed with buffers containing 0.1 and 0.5 M KCl prior to elution of the reverse transcriptase with 1.2 M KCl. In a typical experiment starting with 10 OD_{260} units of RNP particles (approximately 300 μg of protein), we recovered more than 40% (approximately 1 U) of the total reverse transcriptase activity with about 20-fold purification. Reverse transcriptase preparations obtained when EDTA was included in the column buffers had no gross nucleic acid contamination, as judged by labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase followed by gel electrophoresis and autoradiography (data not shown). Reverse transcriptase activity isolated directly from mitochondrial lysates showed the same chromatographic behavior as did that from mtRNP particles, but the yield was no better than that from the RNP particles, and the fractions contained more contaminating proteins and nuclease activity (data not shown).

Optimal conditions for reverse transcription. Optimal conditions for the HS-enriched reverse transcriptase were determined with two different RNA templates (Fig. 4). With the 1.6-kb in vitro transcript corresponding to the 3′ end of the plasmid RNA (pVXN15/*Nsi*I; CCA transcript), the reverse transcriptase had optima of 300 mM KCl, 1 mM Mg^{2+}, pH 8.0 to 8.5, and 30 to 37°C (closed circles in Fig. 4), whereas with poly(rC)-oligo$(dG)_{12-18}$, optima were 200 mM KCl, 5 to 10 mM Mg^{2+}, pH 8.0 to 8.5, and 20 to 30°C (open circles in Fig. 4). In both sets of assays, activity was dependent on both the substrate and the reverse transcriptase fraction. Further, the activity with poly(rC) alone was about 5% of that with poly(rC)-oligo(dG), indicating that the efficient use of this substrate is dependent on the primer (data not shown).

Previous work showed that all of the mitochondrial reverse transcriptase activity detected in the Mauriceville strain is associated with the 81-kDa plasmid-encoded protein (17). The differences in optimal conditions for the CCA transcript and template-primer substrates may reflect two forms of the plasmid reverse transcriptase, which differ in their ability to use the in vitro transcript or the template-primer substrates. Alternatively, these differences may reflect different mechanisms for initiation of reverse transcription with the two substrates and/or the effect of reaction medium or temperature on the structure of the template. For example, variations in the conditions tested may affect the ability of the CCA transcript to adopt a conformation recognized by the reverse transcriptase.

cDNAs synthesized by the HS-enriched reverse transcriptase retain primers attached to their 5′ ends. The finding that the HS-enriched reverse transcriptase gave full-length cDNA copies of in vitro transcript substrates suggests that the initiation of cDNA synthesis occurs at or near the 3′ end of the plasmid transcript, as found for the endogenous plasmid RNA in RNP particles (16). The first clue as to how this might occur was obtained in the experiment shown in Fig. 5, in which we used a short 184-nt in vitro transcript template (pMNH2/*Nsi*I) and analyzed the ^{32}P-labeled cDNA products by electrophoresis in sequencing gels. Surprisingly, the gels showed that the major ^{32}P-labeled cDNA had a length of 204 nt, about 20 nt longer than the template RNA (lanes 1 and 2). An additional band of approximately 400 nt was present in smaller amounts and had the size expected for a cDNA dimer (lane 3). In lanes 4 to 8, the cDNA preparation was ethanol precipitated, divided into equal portions, and subjected to various treatments. The 204-nt cDNA band was unaffected by incubation with NaOH, RNase, or protease (lanes 6 to 8), but the band was completely sensitive to DNase (not shown). In addition, the recovery of the cDNA was unaffected by phenol extraction (compare lanes 4 and 5). Together, these findings indicate that cDNA does not con-

transcriptase protein detected in mtRNP particle preparations. The bands in lanes 9 and 10 do not comigrate with the reverse transcriptase marker and are due to nonspecific binding of the antibody to other proteins in the blot. (D) Silver-stained SDS-polyacrylamide gels of proteins in column fractions. Proteins in 325-μl portions of column fractions were TCA precipitated in the presence of 10 μg of cytochrome *c* (cyt *c*) and analyzed by electrophoresis in SDS–10% polyacrylamide gels. The arrow indicates the peak of reverse transcriptase activity.

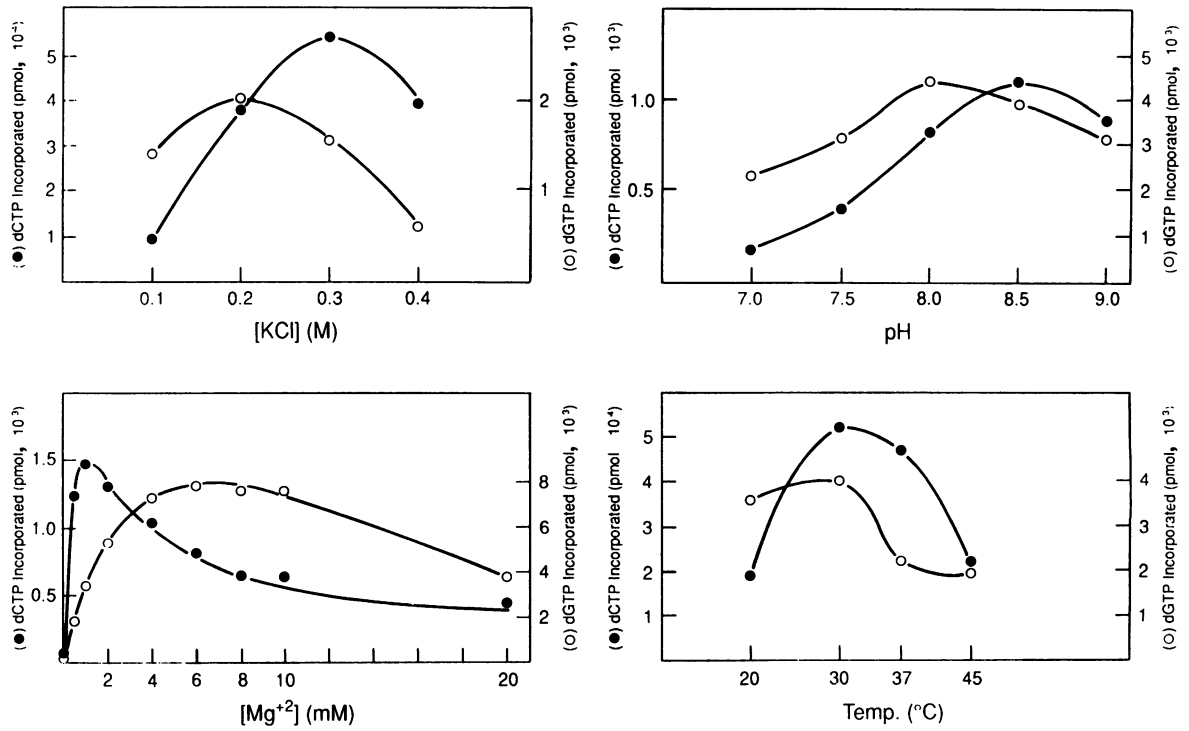

FIG. 4. Determination of optimal conditions for the Mauriceville plasmid reverse transcriptase. Optimal conditions with the 1.6-kb in vitro transcript (pVXN15; closed circles) were determined by varying one parameter at a time in reaction medium containing 2 μl (1 to 2 mU) of HS-enriched reverse transcriptase, 200 mM KCl, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.2), 10 mM DTT, 125 μM each dGTP, dATP, and dTTP, and 10 μCi of [^{32}P]dCTP (1,500 Ci/mmol) as described in Materials and Methods. Optimal conditions with poly(rC)-oligo$(dG)_{12-18}$ (open circles) were determined similarly with 2 μl (1 to 2 mU) of HS-purified reverse transcriptase in reaction medium containing 200 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.2), 10 mM DTT, and 10 μCi of [^{32}P]dGTP (1,500 Ci/mmol).

tain significant amounts of covalently linked RNA or protein, and they suggest that the extra ~20-nt sequences correspond to DNA.

To investigate whether the extra ~20-nt sequences might be primers retained at the 5′ end of minus-strand DNA, the 5′ ends of minus-strand DNAs synthesized in a scaled-up reverse transcription reaction were analyzed by dideoxy sequencing. The in vitro RNA template used in this experiment was purified by CsCl gradient centrifugation to remove any DNA fragments that might potentially be used as primers (32). As shown in Fig. 6, the sequence of the minus-strand DNA can be read up to the GGT copied from the CCA sequence at the 3′ end of the RNA substrate but then becomes unreadable and ends approximately 20 nt beyond the CCA end. Primer extension analysis of the minus-strand DNA gave a single band likewise extending approximately 20 nt beyond the CCA terminus (lane N). The presence of the 20-mer primers was not dependent on unique features of the CCA transcript, as they were also found attached to cDNAs synthesized from other in vitro transcripts (e.g., those terminating at the multicloning site; data not shown).

Cloning of putative primer sequences by anchored PCR. The primer sequences at the 5′ ends of minus-strand DNAs were cloned by anchored PCR and sequenced. The sequences of individual clones showed that short primer sequences (9 to 29 nt) are attached directly to the TGG sequence at the 5′ end of the minus-strand DNA (Table 2). Most of the primer sequences are from the Mauriceville plasmid, but two appear to be mtDNA sequences, and one is an unidentified sequence that is not present in the plasmid, sequenced regions of the mtDNA, or the pBS(−) vector. The findings that the primers are complementary to the plasmid transcript and that most are from regions outside that represented in the in vitro transcript template suggest that they correspond to cDNAs associated with the reverse transcriptase. The primers are tightly bound to the reverse transcriptase and could not be displaced by synthetic DNA oligonucleotides or by *N. crassa* mt tRNA or *E. coli* tRNA (not shown). In several cases, there are deviations from the Mauriceville plasmid sequence at the junction of the primer and the 5′ end of the minus-strand DNA. Significantly, none of the primers has substantial complementarity to the 3′ end of the plasmid transcript. Together, these findings suggest that priming occurs by a template switch mechanism, using the 3′ OHs of short cDNA fragments associated with the reverse transcriptase.

As described elsewhere, template switching from the 5′ end or internal regions of the Mauriceville plasmid transcript to the 3′ end of a new template is also a mechanism for synthesis of minus-strand DNA in vivo (13, 14). In the endogenous RNP particle reaction, the primers at the 5′ end of minus-strand DNA appear to be removed by a cleavage activity that is present in the RNP particle preparations (13, 14). The results presented here show that this cleavage activity is not associated with the purified reverse transcriptase.

The reverse transcriptase in RNP particles does not switch to exogenous RNA template. To determine whether the endogenous reverse transcriptase in RNP particles is capable of diffusing or strand switching to exogenously added

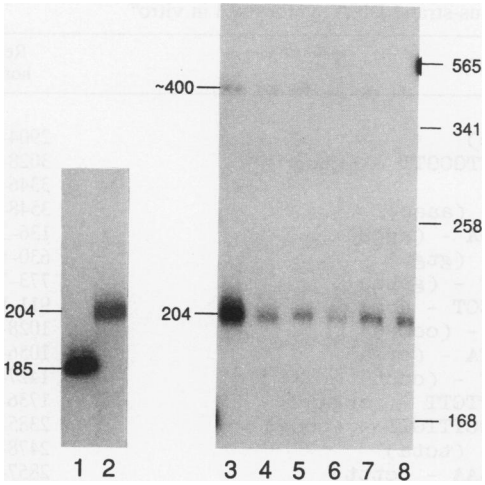

FIG. 5. Characterization of cDNAs synthesized by the HS-enriched Mauriceville plasmid reverse transcriptase from an in vitro transcript template. ^{32}P-labeled cDNAs were synthesized by HS-enriched plasmid reverse transcriptase from the 184-nt pMNH2/*Nsi*I template as described in Materials and Methods and analyzed by electrophoresis in 6% polyacrylamide gels containing 8 M urea. Lane 1, pMNH2/*Nsi*I RNA labeled in vitro with vaccinia virus guanylyl transferase (GIBCO BRL), which adds [^{32}P]GTP to the 5′ end of the 184-nt RNA; lane 2, ^{32}P-labeled cDNA incubated with 0.1 M NaOH–1 mM EDTA overnight at 37°C followed by neutralization. For lanes 3 to 8, ^{32}P-labeled cDNAs from a fivefold-scaled-up reaction with 1 μg of template RNA (CsCl purified) were either untreated (lane 3) or ethanol precipitated, resuspended in distilled H_2O, divided into equal portions, and subjected to the following treatments: lane 4, incubated at 37°C for 30 min; lane 5, incubated at 37°C for 30 min and then extracted with phenol-CIA; lane 6, incubated in 0.1 M NaOH–1 mM EDTA at 50°C for 30 min and then neutralized; lane 7, incubated with 1 U of RNase A (Sigma) plus 25 U of RNase T_1 (Boehringer Mannheim) in 50 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$ for 30 min at 37°C; lane 8, incubated with protease K (1 mg/ml; Merck, Darmstadt, Germany) for 30 min at 37°C. The samples in lanes 5 to 8 were phenol extracted prior to ethanol precipitation in the presence of *E. coli* tRNA carrier and gel electrophoresis. The samples in lanes 3 and 4 were simply ethanol precipitated in the presence of carrier and analyzed by gel electrophoresis. In lanes 4 to 8, a ^{32}P-labeled DNA oligomer (40-mer) was added prior to the final ethanol precipitation step to monitor recovery of ^{32}P-labeled cDNA products (data not shown). Numbers at the right indicate positions (base pairs) of *Sau*3AI fragments of pBS(−) and *Hin*dIII-*Eco*RI fragments of bacteriophage λ DNA used as molecular weight markers.

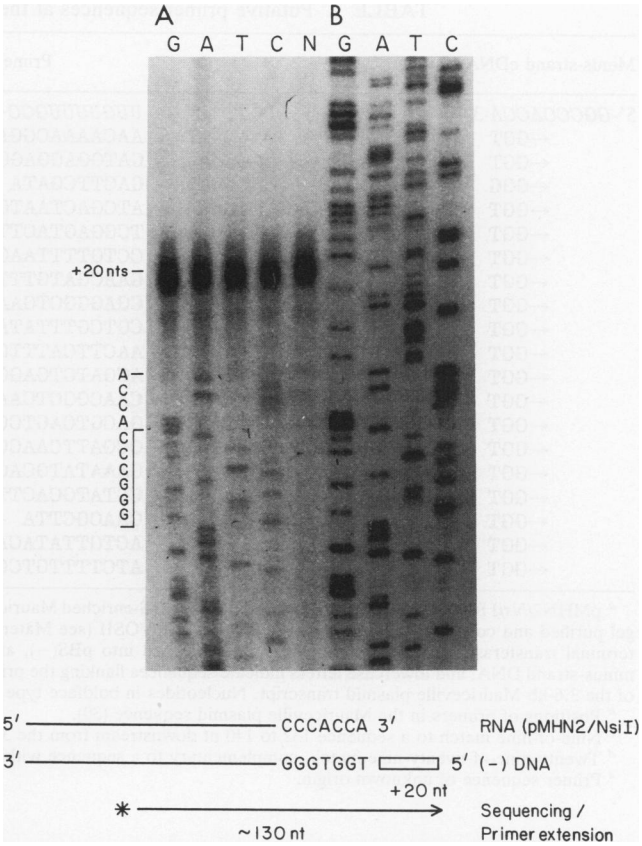

FIG. 6. Evidence that the Mauriceville plasmid reverse transcriptase initiates minus-strand synthesis directly opposite the 3′ end of the RNA template. (A) Minus-strand DNA was synthesized from the 184-nt pMHN2/*Nsi*I RNA template in a large-scale incubation and sequenced by the dideoxy method with ^{32}P-labeled primer VOSI, corresponding to a region from nt 109 to 90 upstream of the 3′ end of the plasmid transcript (see Materials and Methods). Lane N shows primer extension mapping of the 5′ end of minus-strand DNA from the same primer. Control reactions (B) show sequence obtained with the same primer from recombinant plasmid pMP24, which contains the 3.2-kb *Pst*I fragment of the Mauriceville plasmid (16).

substrates, we carried out endogenous reverse transcription reactions using purified RNPs in the presence or absence of exogenous in vitro transcript template. As shown in Fig. 7, the reverse transcriptase in RNP particles used endogenous plasmid transcript to synthesize a 3.6-kb cDNA (lane 1 and 2) but did not utilize the exogenous 1.6-kb CCA template even after prolonged incubation to ensure completion of minus-strand DNAs (lanes 3 to 5). In control reactions (lanes 6 to 8), HS-enriched reverse transcriptase added to the same reaction at different times after initiation of the endogenous reaction efficiently utilized the in vitro transcript to synthesize a 1.6-kb cDNA, indicating that failure of the RNP-bound reverse transcriptase to use this template was not due to degradation of the template, depletion of the deoxyribonucleotide pool, or the presence of a diffusible inhibitor. Mild detergent treatment (0.01 to 0.05% Nonidet P-40) failed to promote utilization of the exogenous template by the RNP-bound reverse transcriptase (not shown). Together with the previous findings, the results indicate that unless it is released by micrococcal nuclease digestion, the reverse transcriptase associated with RNP particles does not reinitiate on exogenous templates under in vitro conditions.

The Mauriceville plasmid reverse transcriptase shows specificity for in vitro transcripts having the 3′ tRNA-like structure and CCA terminus. To investigate whether the plasmid reverse transcriptase could specifically recognize the 3′ end of the plasmid transcript, we tested the ability of the HS-enriched reverse transcriptase to utilize in vitro transcripts having the authentic 3′ tRNA-like structure or lacking all or part of this structure. In initial experiments, we determined optimal conditions for specificity by comparing the ability of the HS-enriched reverse transcriptase to utilize two different in vitro transcript substrates; one was the 1.6-kb RNA corresponding precisely to the 3′ end of the plasmid transcript ending in CCA (pVXN15/*Nsi*I) and the other was truncated by 9 nt and ended in GAG (pVXN15/*Apa*I). As shown in Fig. 8A, the Mauriceville plasmid reverse transcriptase showed considerable preference for the CCA tran-

TABLE 2. Putative primer sequences at the 5′ end of minus-strand DNA synthesized in vitro[a]

Minus-strand cDNA	Primer sequence (3′←5′)	Region of homology[b]
5′-GGCCCACCA-3′	*5′ UUGUUUUGCC→*	
←GGT	3′ AACAAAACGGATT - (atac)	2904–2915
←GGT	(atcc) - GATGGAGGAGGAGGCTAATCTTGCGTT - (gtcg)	3028–3053
←GGG	(ttta) - GACTTCGATA - (cctt)	3346–3354
←GGT	(ttga) - ATCGACTAATGTACCTTAC - (aaac)	3548–3566
←GGT	(atct) - TCGGAGTACTTCTTACTCCGGA - (aggt)	136–157
←GGT	(ggct) - CCTGTTTTAAGGACTTGGA - (gtgt)	630–648
←GGT	(ctgt) - GAACGATGTTTACCTCATGGT - (gttc)	773–792
←GGT	(aggt) - CGAGGGCTGAAGAGACAGCTCCT - (ccgc)	911–932
←GGT	(taag) - CGTCGTTTATAGGGGGGAGA - (cccg)	1028–1046
←GGT	(ttcc) - AACTTCATTTCCTAATAGGATA - (ctga)	1056–1075
←GGT	(ctaa) - ACGATCTGAGGACATGGCATT - (ccct)	1427–1445
←GGT	(tttt) - CAACGGCTCAAGAGACTCCGTTCTT - (ccga)	1736–1759
←GGT	(tcat) - GACGTCAGTCCCAGCTCAATGCTTTGTT - (cccg)	2385–2412
←GGT	(gaat) - CCGATTCAACCTCATTCGG - (tcta)	2478–2496
←GGT	(tacg) - GAAATATGCACTTCCCGATGAAA - (cttt)	2857–2875
←GGT	(gatc) - CGTATGCACTTCCCGATG - (cttt)	2861–2875
←GGT	(cccc) - CAACGCTTA - (attg)	Near *coIII*[c]
←GGT	(tatt) - ACTGTTATACATGTAAACAGAACTGATTA - (atgc)	Small rRNA[d]
←GGT	ATCTTTTGTCGAGCACAA	(?)[e]

[a] pMHN2/*Nsi*I RNA was reverse transcribed by using HS-enriched Mauriceville plasmid reverse transcriptase. The minus-strand DNA synthesized in vitro was gel purified and copied by using Sequenase with primer VOSII (see Materials and Methods). The resulting DNA strand was then tailed with dGTP by using terminal transferase, amplified by anchored PCR, cloned into pBS(−), and sequenced. Uppercase letters indicate sequences of primers at the 5′ ends of minus-strand DNA, and lowercase letters indicate sequences flanking the primer in the Mauriceville plasmid (30). Italics indicate nucleotides at the 5′ and 3′ ends of the 3.6-kb Mauriceville plasmid transcript. Nucleotides in boldface type do not match the plasmid sequence.

[b] Positions of primers in the Mauriceville plasmid sequence (30).

[c] Nine-of-nine match to a sequence 132 to 140 nt downstream from the 3′ end of the *coIII* ORF on the transcribed strand of *Neurospora* spp. mtDNA.

[d] Twenty-nine-of-twenty-nine match, complementary to a sequence within the mitochondrial small (19S) rRNA.

[e] Primer sequence of unknown origin.

script compared with the GAG transcript in reaction media containing high KCl (250 to 375 mM) and low Mg^{2+} (0.5 to 5 mM) concentrations. Additional experiments under these conditions showed that a longer in vitro transcript, which contains all 3.6 kb of Mauriceville plasmid sequence and ends at the correct position, was used with no greater efficiency than was the 1.6-kb pVXN15/*Nsi*I transcript (data not shown).

Structural features required for efficient recognition by the reverse transcriptase were investigated by comparing the ability of the HS-enriched reverse transcriptase to utilize the 1.6-kb pVXN15/*Nsi*I transcript with the authentic 3′ end and a series of related transcripts having deletions or containing additional sequences beyond the CCA terminus. As shown in Fig. 8B, the Mauriceville plasmid reverse transcriptase efficiently utilized the in vitro transcripts having the correct 3′ end or lacking only the terminal A residue, whereas other in vitro transcripts having truncations (−9 to −383 nt) or extra nucleotides (+8 to +198 nt) were used less efficiently (15 to 56% of the pVXN15/*Nsi*I transcript). In the case of RNAs with extensions beyond the normal CCA end, it made little difference whether the extra sequences were derived from the vector (pVXN15 digested with *Sma*I, *Eco*RI, or *Bgl*I) or the Mauriceville plasmid (pVX55/*Sma*I). Significantly, transcript pVXNd33/*Nsi*I, which retains the CCA terminus but has an internal deletion of part of the 3′ tRNA-like structure (Fig. 2), was used inefficiently, suggesting that this structure, in addition to the CCA terminus, contributes to recognition.

We note that the specificity shown by the HS-enriched reverse transcriptase is considerably less than that found in the endogenous reverse transcription reaction, in which the Mauriceville plasmid transcript is used almost exclusively (16). It made no difference whether the transcripts were used

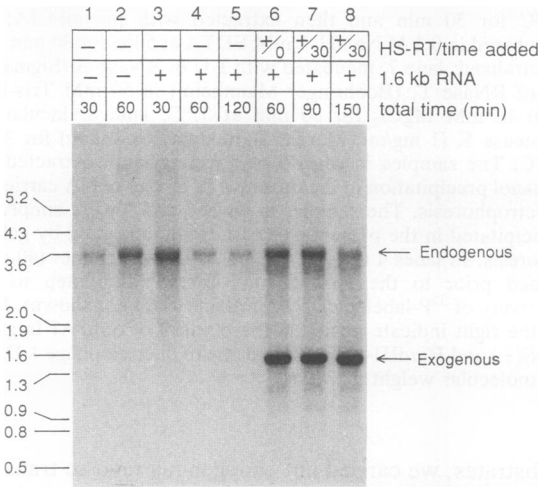

FIG. 7. Evidence that reverse transcriptase associated with RNP particles does not utilize exogenous templates. Endogenous reverse transcription reactions were carried out with Mauriceville mtRNP particles (0.0025 OD_{260} units) that had been purified by DEAE-Sephacel chromatography. Reactions were carried out as described in Materials and Methods for the times indicated. For reactions in lanes 3 to 8, 0.5 μg of pVXN15/*Nsi*I RNA (1.6 kb RNA) was added prior to initiation of the reaction. For reactions in lanes 6 to 8, HS-enriched reverse transcriptase (HS-RT; 1 to 2 mU) was added at the times indicated. ^{32}P-labeled cDNAs were glyoxylated and analyzed by electrophoresis in a 1.4% agarose gel followed by autoradiography. Arrows at the right indicate 3.6-kb cDNA synthesized from the endogenous plasmid transcript (Endogenous) and the 1.6-kb cDNA synthesized from the in vitro transcript template (Exogenous). Numbers at the left indicate the positions (kilobases) of *Hin*dIII-*Eco*RI fragments of bacteriophage λ DNA used as molecular weight markers.

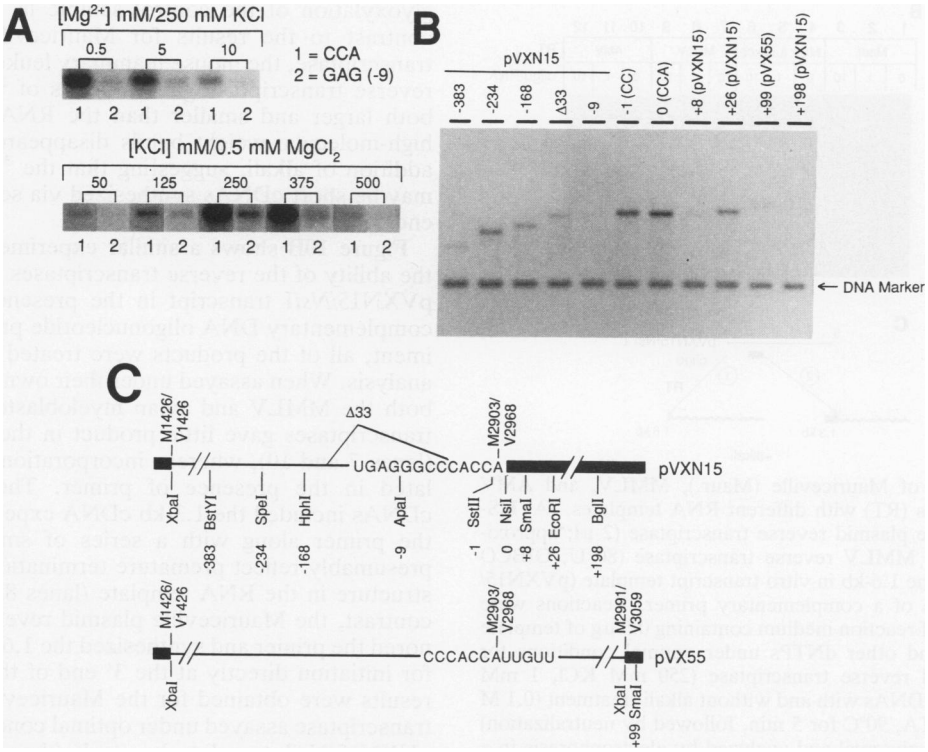

FIG. 8. Evidence that the HS-enriched reverse transcriptase preferentially utilizes in vitro transcripts with 3′ ends corresponding to that of the Mauriceville plasmid transcript. (A) Determination of optimal KCl and Mg^{2+} concentrations for discrimination between two RNA templates. In vitro transcripts of pVXN15/*Nsi*I, which ends at the CCA terminus, and pVXN15/*Apa*I, which ends 9 bp upstream at a GAG sequence, were incubated with HS-enriched reverse transcriptase in the presence of [^{32}P]dCTP and other dNTPs as described in Materials and Methods in reaction media containing different concentrations of Mg^{2+} or KCl. ^{32}P-labeled cDNAs were glyoxylated and analyzed by electrophoresis in a 1.7% agarose gel. (B) Reverse transcription of a series of in vitro transcripts differing at their 3′ ends. In vitro transcripts were synthesized from plasmids pVXN15, pVX55, pVXS211, and pVXNd33, linearized at restriction sites shown in panel C. Assays were carried out in 20 μl of reaction medium containing 0.06 pmol of each in vitro transcript, 2 μl (approximately 1.5 mU) of HS-enriched reverse transcriptase, [^{32}P]dCTP, and other dNTPs as described in Materials and Methods except that the labeling period was shortened to 5 min. Products were glyoxylated and analyzed by electrophoresis in a 1.4% agarose gel followed by autoradiography. A ^{32}P-labeled 0.9-kb DNA (*Nde*I-*Hin*dIII fragment from pMHN2) was added prior to phenol extraction and ethanol precipitation to monitor recovery of cDNA products. The experiment was repeated three times, with similar results. (C) Schematic of plasmids used to obtain in vitro transcripts. Nucleotide positions are shown for both the Mauriceville (M) and Varkud (V) plasmids.

directly or heated and slowly cooled prior to use as templates or whether the reaction was carried out with solubilized reverse transcriptase in RNP particle preparations without purification on HS columns (data not shown). Interestingly, the three nonspecific transcripts used most efficiently by the HS-enriched reverse transcriptase (−234, −168, and +26) had 3′ ends, which could hypothetically fold into a small stem-loop followed by a single-stranded tail of 3 to 5 nt (Fig. 9), similar to part of the Mauriceville plasmid 3′ tRNA-like structure (Fig. 2).

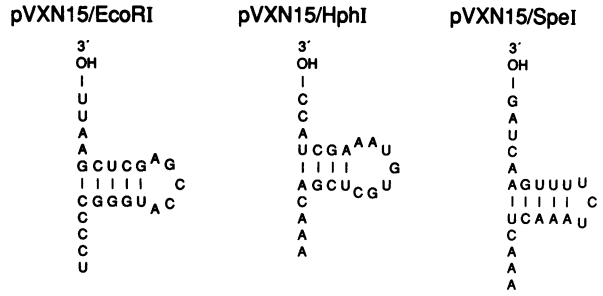

FIG. 9. Hypothetical stem-loop structures within 40 nt of the 3′ end for each of three nonspecific in vitro transcripts for which the Mauriceville plasmid reverse transcriptase shows some preference.

Comparison of the Mauriceville plasmid and retroviral reverse transcriptases with different templates. The mechanism of reverse transcription used by the Mauriceville plasmid differs from that used by retroviruses in that minus-strand DNA synthesis initiates directly at the 3′ end of the plasmid transcript rather than at a tRNA primer complementary to an internal region of the template RNA (16). To determine whether this difference reflects an inherent difference in the reverse transcriptases, we compared the ability of the Mauriceville plasmid and retroviral reverse transcriptases to use different types of templates.

Figure 10A shows results for the 1.6-kb CCA transcript (pVXN15/*Nsi*I) under optimal conditions for the Mauriceville plasmid reverse transcriptase (250 mM KCl, 1 mM $MgCl_2$) in the absence of added primer. As before, the Mauriceville plasmid reverse transcriptase efficiently used the 1.6-kb CCA transcript and gave a single prominent band

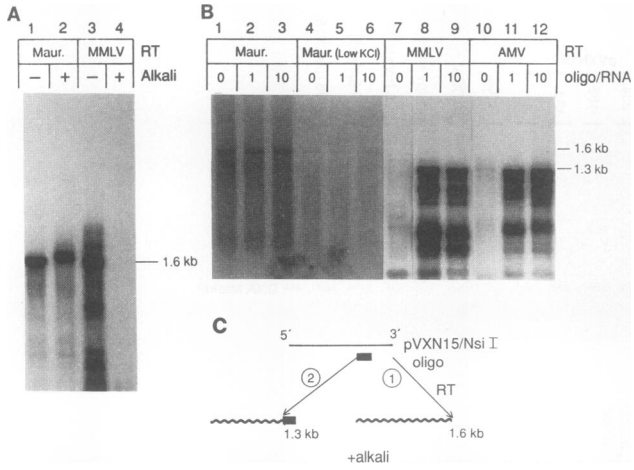

FIG. 10. Activity of Mauriceville (Maur.), MMLV, and AMV reverse transcriptases (RT) with different RNA templates. (A) HS-enriched Mauriceville plasmid reverse transcriptase (2 μl; approximately 1.5 mU) and MMLV reverse transcriptase (80 U; GIBCO BRL) assayed with the 1.6-kb in vitro transcript template (pVXN15/*Nsi*I) in the absence of a complementary primer. Reactions were carried out in 20 μl of reaction medium containing 0.5 μg of template RNA, [^{32}P]dCTP, and other dNTPs under optimal conditions for Mauriceville plasmid reverse transcriptase (250 mM KCl, 1 mM $MgCl_2$). ^{32}P-labeled cDNAs with and without alkali treatment (0.1 M NaOH, 0.5 mM EDTA, 90°C for 5 min, followed by neutralization) were denatured with glyoxyal and analyzed by electrophoresis in a 1.4% agarose gel followed by autoradiography. Lanes: 1 and 2, products obtained with Mauriceville plasmid reverse transcriptase before and after the alkali treatment; 3 and 4, products obtained with MMLV reverse transcriptase before and after the alkali treatment. (B) HS-enriched Mauriceville plasmid reverse transcriptase, MMLV reverse transcriptase, and AMV reverse transcriptase (GIBCO BRL) assayed with the 1.6-kb pVXN15/*Nsi*I transcript in the presence or absence of a synthetic oligonucleotide primer. Reactions were carried out as described above in 20 μl of reaction medium containing Mauriceville reverse transcriptase (2 μl; approximately 1.5 mU), MMLV reverse transcriptase (10 U), or AMV reverse transcriptase (0.2 U), [^{32}P]dCTP, and other dNTPs. Substrates were 0.1 μg of pVXN15/*Nsi*I RNA annealed with indicated molar ratios of a synthetic oligonucleotide primer (MPST) complementary to nt 286 to 305 upstream of the 3′ end of the 1.6-kb in vitro transcript. The reaction with Mauriceville plasmid reverse transcriptase was carried out under optimal conditions for the use of template-primer substrate (250 mM KCl, 5 mM $MgCl_2$, 30°C; lanes 1 to 3) and under conditions comparable to those for retroviral reverse transcriptases (90 mM KCl, 6 mM $MgCl_2$, 50 mM Tris-HCl [pH 8.3] at 37°C; lanes 4 to 6). Similar results were also obtained under optimal conditions for Mauriceville plasmid reverse transcriptase with in vitro transcript substrates (250 mM KCl, 1 mM $MgCl_2$; data not shown). Reactions with MMLV and AMV reverse transcriptases were carried out in 40 mM KCl–6 mM $MgCl_2$–50 mM Tris-HCl (pH 8.3) at 37°C (lanes 7 to 12) or in the same medium containing 90 mM KCl (data not shown) with similar results. Reaction mixtures were incubated for 10 min and chased for 40 min with 1.25 mM dNTP. ^{32}P-labeled cDNA products were treated with alkali (0.1 M NaOH, 0.5 mM EDTA, 90°C for 5 min, followed by neutralization), denatured with glyoxyal, and analyzed by electrophoresis in 1.4% agarose gels followed by autoradiography. (C) Predicted products for (i) cDNA synthesis beginning at the 3′ end of the pVXN15/*Nsi*I transcript and (ii) cDNA synthesis beginning from the oligonucleotide primer.

corresponding to full-length 1.6-kb cDNA (lane 1). The mobility of the band appeared to decrease slightly after alkali treatment (lane 2), but this apparent shift was not observed reproducibly (see Fig. 5) and most likely reflects incomplete glyoxylation of the control sample in this experiment. In contrast to the results for Mauriceville plasmid reverse transcriptase, the mouse mammary leukemia virus (MMLV) reverse transcriptase gave a series of ^{32}P-labeled cDNAs, both larger and smaller than the RNA template, and the high-molecular-weight bands disappeared completely after addition of alkali, suggesting that the ^{32}P-labeled products may be short cDNAs synthesized via self-priming by the 3′ end of the template RNA.

Figure 10B shows a similar experiment, now comparing the ability of the reverse transcriptases to utilize the 1.6-kb pVXN15/*Nsi*I transcript in the presence or absence of a complementary DNA oligonucleotide primer. In this experiment, all of the products were treated with alkali prior to analysis. When assayed under their own optimal conditions, both the MMLV and avian myeloblastosis (AMV) reverse transcriptases gave little product in the absence of primer (lanes 7 and 10), whereas incorporation was greatly stimulated in the presence of primer. The primer-dependent cDNAs included the 1.3-kb cDNA expected for initiation at the primer along with a series of smaller bands, which presumably reflect premature termination due to secondary structure in the RNA template (lanes 8, 9, 11, and 12). By contrast, the Mauriceville plasmid reverse transcriptase ignored the primer and synthesized the 1.6-kb cDNA expected for initiation directly at the 3′ end of the template. Similar results were obtained for the Mauriceville plasmid reverse transcriptase assayed under optimal conditions for use of the pVXN15/*Nsi*I template by itself (data not shown), under optimal conditions for template-primer substrates (lanes 2 and 3), or under conditions similar to those for the retroviral reverse transcriptases (lanes 5 and 6). We note that the MMLV and AMV enzymes did not initiate efficiently at the 3′ terminus even in the presence of excess primer (Fig. 10) or noncomplementary DNA oligonucleotides (data not shown), which could in principle provide a free 3′ OH for initiation directly at the end. Our results suggest, therefore, that the Mauriceville plasmid reverse transcriptase and retroviral reverse transcriptases have inherent differences in their abilities to initiate at the 3′ ends of RNA templates.

DISCUSSION

In this work, we developed procedures for releasing active Mauriceville plasmid reverse transcriptase from mtRNP particles and for partially purifying it by HS chromatography. We confirmed that the reverse transcriptase activity copurifies with the 81-kDa plasmid-encoded protein detected in immunoblots. The solubilized plasmid reverse transcriptase uses in vitro transcript templates and mimics key features of the previously described endogenous RNP particle reaction. It synthesizes full-length cDNA copies of RNA templates beginning at their 3′ ends, it shows specificity for transcripts having the 3′ tRNA-like structure, and it begins minus-strand DNA synthesis directly opposite the 3′-terminal nucleotide of the template RNA.

The plasmid reverse transcriptase begins cDNA synthesis at the 3′ end of in vitro transcripts by using the 3′ OH of a DNA primer that is associated with the HS-enriched reverse transcriptase. These endogenous primers are tightly bound to the reverse transcriptase and could not be displaced by DNA oligonucleotides or *E. coli* or *N. crassa* mitochondrial tRNAs. For unknown reasons, the Mauriceville reverse transcriptase could use simple homopolymer substrates, poly(rC)-oligo(dG) and poly(rA)-oligo(dT), in a primer-dependent manner, but when given an in vitro transcript

template with an annealed oligonucleotide primer, it ignored the primer and initiated exclusively at the 3′ end of the template. Significantly, the endogenous primer DNAs used to initiate at the 3′ ends of RNAs have no substantial complementarity to the template RNA.

In RNP particle preparations, which also contain mtRNAs, the reverse transcriptase is tightly bound to the endogenous plasmid transcript and uses the plasmid RNA template almost exclusively (16). Such high specificity is presumably necessary to minimize promiscuous reverse transcription of mitochondrial RNAs in vivo. Our results show that the specificity of the plasmid reverse transcriptase reflects at least in part recognition of the 3′ tRNA-like structure. In vitro transcripts having this structure with a CCA or CC terminus are used more efficiently than are other template RNAs. Further, mutant templates that either retain the tRNA-like secondary structure but extend slightly (8 nt) beyond the normal CCA terminus or retain the CCA terminus but lack part of the tRNA-like structure are used less efficiently (Fig. 8). These results suggest that both sequence and structure may be important for recognition.

We note that the HS-enriched reverse transcriptase uses other in vitro transcripts to various degrees and its specificity is not as great as that of the endogenous reaction in RNP particle preparations. The decreased specificity of the solubilized in vitro system may be due to loss of a critical protein factor (4) or differences in structure of the RNA template, which may be modified or associated with proteins in vivo. Alternatively, the specificity of the endogenous reaction may simply appear higher because of the inaccessibility of the 3′ ends of mtRNAs. In addition, the reverse transcriptase in vivo may associate preferentially with the mRNA from which it is synthesized, as shown for the bacterial Tn*10* transposase, which associates with DNA ends near its site of synthesis (29). In that case, the higher specificity in vivo may reflect a combination of the ability of the reverse transcriptase to recognize the tRNA-like structure and proximity of the natural template.

Our results provide the first direct evidence that the Mauriceville plasmid 3′ tRNA-like structure is functionally equivalent to the 3′ tRNA-like structures of plant RNA viruses in serving as a recognition site for the initiation of minus-strand synthesis by a polymerase encoded by the element (9, 28). In general, this functional equivalence could reflect either convergent evolution or an evolutionary relationship between reverse transcriptases and viral RNA-dependent RNA polymerases, as proposed by others (20, 31). The characteristics of the Mauriceville plasmid reverse transcriptase are consistent with it being a primitive enzyme of the type that may have first evolved from an RNA-dependent RNA polymerase. We envision that such an enzyme could develop into a retroviral-type reverse transcriptase by acquiring associated RNase H, protease, and integrase coding regions and by adapting its ability to recognize a 3′ tRNA-like structure to bind cellular tRNAs used as primers (16). The previous finding that mutant plasmids preferentially incorporate mitochondrial tRNAs via reverse transcription is consistent with the idea that the Mauriceville plasmid reverse transcriptase has a general affinity for tRNA structures (2, 3).

The primers used by the HS-enriched Mauriceville reverse transcriptase correspond to tightly associated cDNAs synthesized from plasmid RNA or mtRNAs, and as described elsewhere, this appears to reflect a mechanism for initiation of minus-strand synthesis in vivo (13, 14). The experiments presented here give no indication that the Mauriceville plasmid reverse transcriptase initiates minus-strand synthesis de novo in the absence of a nucleic acid primer. However, unlike the situation for retroviral reverse transcriptases, for which specificity is achieved by using tRNA primers complementary to a specific location in the RNA template, the primers used by the Mauriceville plasmid reverse transcriptase have no significant complementary to the RNA template; instead, the specificity appears to be achieved by the ability of the enzyme to specifically recognize the 3′ end of the template RNA.

Finally, the ability to begin minus-strand DNA synthesis at the 3′ end of template RNAs could also be relevant to the mechanism(s) of reverse transcription used by LINEs, group II introns, and other non-LTR elements, whose reverse transcriptases are related to that encoded by the Mauriceville plasmid. In the case of other non-LTR elements, however, recognition of the template RNAs would have to depend on something other than a 3′ tRNA-like structure, which has not been reported in these elements. For some non-LTR elements, this structural feature may be related to a poly(A) tail at the 3′ end of the template RNA (5, 12). Alternatively, our observation that the Mauriceville plasmid reverse transcriptase shows some preference for 3′ ends, which hypothetically fold into stem-loop structures with short, single-stranded tails, may be significant.

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