Relaxed Primer Specificity Associated with Reverse Transcriptases Encoded by the pFOXC Retroplasmids of *Fusarium oxysporum*

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The pFOXC mitochondrial retroplasmids are small, autonomously replicating linear DNAs that have a telomere-like repeat of a 5-bp sequence at their termini. The plasmids are possible evolutionary precursors of the ribonucleoprotein complex telomerase, as they encode an active reverse transcriptase (RT) that is involved in plasmid replication. Using an in vitro system to study reverse transcription, we show that the pFOXC RT is capable of copying in vitro-synthesized RNAs by use of cDNA primers or extension of snapped-back RNA templates. The ability of the pFOXC RT to use base-paired primers distinguishes it from the closely related RTs encoded by the Mauriceville and Varkud mitochondrial retroplasmids of *Neurospora* spp. Reaction products are similar, but not identical, to those obtained with conventional RTs, and differences reflect the ability of the pFOXC RT to initiate cDNA synthesis with loosely associated primers. The pFOXC RT can also copy DNA templates and extend 3′ mismatched DNA oligonucleotide primers. Analysis of pFOXC in vivo replication intermediates suggests that telomeric repeats are added during reverse transcription, and the ability to extend loosely associated primers could play a role in repeat formation by mechanisms similar to those associated with telomerase and certain non-long-terminal-repeat retrotransposons.

Plasmids pFOXC2 and pFOXC3 are 1.9-kb linear DNAs that reside in mitochondria of certain forms specialis of the fungal plant pathogen *Fusarium oxysporum* (17). The plasmids are classified as retroplasmids because they each encode a reverse transcriptase (RT) and replicate via an RNA intermediate (18, 24). To date, retroplasmids have been found only in mitochondria of six species of filamentous fungi and fall into two groups, linear and circular, based on the structure of the plasmid DNA (1, 3, 7, 14, 20).

Plasmids pFOXC2 and pFOXC3 have an interesting structure that is described as a “clothespin,” as it includes a hairpin at one terminus and two to five copies of a pentameric repeat (5′-ATCTA) at the other terminus (24). This structure is unique among plasmids that have been identified thus far, yet it bears some resemblance to eukaryotic chromosomes that contain multiple copies of short DNA sequences at their termini. The association of genetic elements that have telomere-like repeats and replicate via reverse transcription suggests that the pFOXC plasmids may be contemporary descendants of primitive chromosomes and/or have a direct evolutionary relationship to telomerase, the ribonucleoprotein (RNP) complex that protects and maintains the ends of chromosomes. Phylogenetic analysis of the RT encoded by the pFOXC plasmids (pFOXC RT) indicates that it is most closely related to the RTs encoded by the Mauriceville and Varkud circular retroplasmids of *Neurospora* spp. (24). As a group, mitochondrial retroplasmids are deeply rooted within the RT phylogenetic tree and are thought to be ancestral to other retroelements, including telomerase RTs (TERTs) (9, 10). The ancestral nature of mitochondrial retroplasmids was further supported by the finding that the RT encoded by the Mauriceville retroplasmid (Maur-RT) can initiate cDNA synthesis de novo (without a primer) (28), which suggests that it may be mechanistically related to RNA-dependent RNA polymerases—nucleotide polymerases with the greatest sequence and structural similarity to RTs (11).

Studies of RT activity associated with pFOXC2 and pFOXC3 plasmids have revealed similarities between the replication mechanisms of linear and circular retroplasmids. Like the Maur-RT and TERTs, the pFOXC RT is associated with its RNA template in an RNP complex. Mitochondrial RNP (mtRNP) particles isolated from pFOXC2- and pFOXC3-containing strains contain RT activity that can generate full-length minus-strand cDNA products from endogenous plasmid RNAs (24). The length of the minus-strand cDNA products synthesized in the reverse transcription reactions suggests that cDNA synthesis starts at the 3′ end of the transcript (i.e., the region containing the pentameric repeats). Unlike the transcripts of the Mauriceville and Varkud retroplasmids of *Neurospora* spp. that terminate in a tRNA-like structure (2), the pFOXC RNAs lack an identifiable 3′ secondary structure. Sequence analysis of the pFOXC2 and pFOXC3 DNAs showed that the plasmids are 85% identical and contain the same pentameric repeat at their 3′ termini. The number of terminal repeats was found to vary for the two plasmids, ranging between two and five copies, which indicated that the 3′ ends of the plasmid DNAs are in flux and that specific mechanisms may be involved in the generation and maintenance of the repeats. The nature of the primer for minus-strand cDNA synthesis is unknown, yet once initiated, reverse transcription proceeds to the 5′ end of the transcript. Based on the structural organization of the plasmid DNAs and the analysis of RT assays by using mtRNP particles, a hypothetical model for the replication of pFOXC plasmids was fashioned (Fig. 1A) (24).
Here we describe the development of an in vitro system to study pFOXC reverse transcription. We show that the pFOXC RT has clear mechanistic differences from the related Maur-RT and is able to initiate cDNA synthesis by use of base-paired DNA or RNA primers. The pFOXC RT was also found to have DNA polymerase activity and could extend weakly associated primers, including those that had 3′ mismatches with the template. Characterization of in vivo retroplasmid replication intermediates suggests that the 3′ telomere-like repeats are generated during reverse transcription, and together with the findings from the in vitro studies, we propose a model for repeat addition (Fig. 1B) that has similarities to telomere addition catalyzed by telomerase as well as mechanisms associated with the retrotransposition of non-long-terminal-repeat retrotransposons.

MATERIALS AND METHODS

F. oxysporum strains and growth conditions. F. oxysporum strains used in this study were pFOXC2-containing strain 699, f. sp. raphani, pFOXC3-containing strain 725, f. sp. matthioli, and plasmid-free strain 9129, f. sp. cubense. These strains are maintained at the USDA-ARS Cereal Disease Laboratory (St. Paul, Minn.). Strains were grown on potato dextrose agar plates, and conidia were used directly in vegetative cultures or preserved in 50% glycerol and stored at −80°C. Conidia were germinated for 7 days in 750 ml of 1× Vogel’s medium (8) at 25°C for mycelial isolation.

Isolation and partial purification of mtRNP particles. Mitochondria were prepared from mycelial pads by a modified flotation gradient method developed for isolation of Neurospora mitochondria (19). mtRNP complexes were isolated by procedures described in the work of Kennell et al. (15). RNP pellets were resuspended at −80°C and resuspended in TE (10 mM Tris-HCl [pH 7.0], 1 mM EDTA) at a concentration of 1 to 10 A260 optical density (OD) U/ml. Where specified, nuclease-free RNP particles were obtained by partial purification by using DEAE-Sepharose column chromatography as described in the work of Walther and Kennell (24).

Isolation of mtDNA and mtRNA. Mitochondrial pellets were resuspended in 50 mM Tris-HCl (pH 8.0)–62.5 mM EDTA–2% sodium dodecyl sulfate, and resulting lysates were incubated with proteinase K (20 μg/ml) at 55°C for 45 min. Mitochondrial nucleic acids were precipitated with ethanol following two to four extractions with a mixture of phenol and chloroform-isooamyl alcohol (CIA; 25:24:1). For DNA isolation, pellets were resuspended in TE and digested with RNase A for 15 min at 37°C. Following digestion, mtDNA was extracted with phenol-CIA and precipitated with ethanol. mtRNA was isolated via the Trizol method (Invitrogen, Carlsbad, Calif.), and following precipitation, pellets were resuspended in TE.

Endogenous reverse transcription reactions and analysis of cDNA products. Reverse transcription reactions with endogenous RNAs associated with the mtRNP particles were carried out in a 20-μl volume that contained 50 mM Tris-HCl (pH 8.2); 5 mM MgCl2; 50 mM KCl; 5 mM dithiothreitol (DTT); 125 μM dATP, dGTP, and TTP; and 20 μCi of [α-32P]dCTP. In most cases, 0.4 A260 OD U of mtRNP particles or −0.1 A260 OD U of DEAE-RT preparations were used directly in reverse transcription reactions.
added to initiate the reactions. Reaction mixtures were incubated at 37°C for 15 min and then chased by the addition of dCTP to 100 μM and incubated for an additional 5 min at 37°C. Reactions were stopped by the addition of an equal volume of 0.25 M EDTA, and the products were precipitated with ethanol. Where indicated, reaction mixtures were preincubated with actinomycin D (100 μg/ml; Sigma, St. Louis, Mo.) or 0.1 U of RNase A for 5 min prior to the addition of [3P]dCTP. For posttreatment analysis, DEAE-purified mTRNPs were preincubated with actinomycin D or used directly in scaled-up endogenous reactions. Reaction products were precipitated with ethanol, resuspended in TE, aliquoted into equal portions, and subjected to the following treatments: mock incubation at 37°C for 15 min, incubation with 0.1 U of RNase A in a reaction buffer containing 10 mM MgCl2 for 10 min at 37°C, incubation with 10 U of DNase I (Invitrogen) in a reaction buffer supplied by the manufacturer for 10 min at 37°C, incubation in 0.1 M NaOH at 50°C for 15 min, incubation with proteinase K (0.2 mg/ml) at 50°C for 15 min and then extraction with an equal volume of phenol:CHCl3, or incubation at 37°C for 15 min and then extraction with an equal volume of phenol:CHCl3. In addition, products from a twofold-scaled-up reaction with the use of DEAE-purified mTRNP were divided and incubated at 37°C for 30 min either with or without 0.1 U of α-amanitin (Invitrogen) in a reaction buffer containing 67 mM glycerol (pH 9.4) and 2.5 mM MgCl2. Previous studies demonstrate that pFOXC2 preparations isolated from bisbenzamide CsCl gradients are resistant to α-amanitin digestion following incubation periods of up to 3 h (17). Following posttreatment, cDNAs were precipitated and denatured with glyoxal (23) prior to electrophoresis in a 1.4% agarose gel. DNA standards were dephosphorylated PstI restriction fragments of bacteriophage λ that had been 5′-end labeled with [γ-32P]ATP by using polynucleotide kinase (New England Biolabs, Beverly, Mass.). The probe was generated from a 3-kb HindIII restriction fragment of bacteriophage λ containing 10 mM MgCl2 for 10 min at 37°C. Reactions were treated with proteinase K and precipitated with ethanol. Yeast tRNA (2 μg/ml) was included as a carrier. Products of the endogenous reactions were denatured with glyoxal, and minus-strand DNAs were separated from full-length products on a 1.4% agarose gel containing 10 mM phosphate buffer. Products migrating at <1 kb were isolated by electroelution and used in primer extension reactions with end-labeled primer C2/C3 Sac, which is complementary to a region approximately 150 nucleotides (nt) from the 5′ end of the minus-strand cDNA. Primer extension products were isolated from a 6% polyacrylamide gel and visualized by autoradiography. The probe was amplified by anchored PCR with primer cDNA2/C2 Sac, which is complementary to the tailed region (dCBam; 5′-CCGGATCCAATGTAGATTAGATTAGATCTAGACTAATTGG-3′) and amplified in an anchor primer (C2/C3 Sac) and a primer complementary to the tailed region (dTBam; 5′-ATTATAGGATCAAAAC-3′). Amplified products were digested with BamHI and SacI and cloned into the appropriate sites of pBluescript. Plasmid DNA was digested with digestion with BglIII and sequenced.

Synthesis of in vitro RNA templates. The terminal EcoRI fragment of pFOXC3 DNA was amplified by anchored PCR following tailing with dGTP by using terminal deoxynucleotidyl polymerase (24). Primers C3-Eco (5′-GGGAA TTCCTAACTATTTGAAGAACAC) and CDbam were used for amplification, and the products were cloned into the BamHI site of pBluescript (Stratagene, La Jolla, Calif.). The clones were used for further amplification by PCR with primer 2R (5′-GGATCTTAACTCTTACGACCTCATTCTG-3′) and primer 3R (5′-GATCATGTTAGATTAGATTAGATCTAGACTAATTGG-3′), which would create DNAs having two or three copies of the 5-bp repeat, respectively. Primer ATC (5′-CCGGATCCTGAGGATTAGATTAGATCTAGACTAATTGG-3′) was used to create DNA templates having two repeats plus sequence ATC at the 3′ end, and primers GoN (5′-CCGGATCCTGAGGATTAGATTAGATCTAGACTAATTGG-3′) was used to create an in vitro RNA that had two G-to-C mutations at the 3′ end of the transcript. An NlaIII restriction site was engi-neered into the primers so that digestion of the cloned DNAs would produce linear DNAs having repeats at the 3′ terminus. DNAs used as templates also included BglII or BamHI restriction fragments of the 2R and 3R clones; the former lacks the 3′-terminal repeats, while the latter terminates within the pBluescript vector. Other RNAs synthesized had specific 3′ modifications that were obtained from DNA templates generated directly from PCR products. In this case, the primers for PCR amplification included oligonucleotide pBS-326 (5′-CACATTCACTCCTCATTCTGGC-3′) and pBS-327 (5′-ATATAGGATCCT17) and amplified in an anchored PCR by using an internal primer, C2/C3 Sac (5′-CCGGATCCTGAGGATTAGATTAGATCTAGACTAATTGG-3′) and an internal primer, C2/C3 Sac and a primer complementary to the tailed region (dTBam; 5′-ATTATAGGATCAAAAC-3′). Amplified products were digested with BamHI and SacI and cloned into the appropriate sites of pBluescript. Plasmid DNA was digested with digestion with BglIII and sequenced.

Southern hybridization with endogenous reaction products. Total mtRNA from strain 725 was glyoxylated and subjected to electrophoresis through a 1.4% agarose gel containing 10 mM phosphate buffer. Plasmid DNA (~1.9 kb) was isolated from the gel and eluted in an ISCO electrophoretic apparatus. Eluted products were extracted with phenol:CHCl3, precipitated with ethanol, and resuspended in 10 μl of TE. Precipitated products were tiled with A residues by using poly(A) polymerase (Invitrogen). Tiled products were used as templates for cDNA synthesis with MMLV RT (Promega, Madison, Wis.) and primer dTbam (5′-CCGGATCCT17) and amplified in an anchored PCR by using an internal primer, C2/C3 Sac (5′-GCACTATAACGATGAGCTC-3′) and dTBam. Amplified products digested with BamHI and SacI were cloned into the appropriate sites of pBluescript. Positive clones were screened by digestion with BglII and sequenced by the dideoxy chain termination method with Sequenase (Amersham, Piscataway, N.J.).

Isolation and characterization of the 5′ terminus of the pFOXC3 minus-strand cDNA. DEAE-purified mTRNPs from strain 725 were used in endogenous reactions to synthesize minus-strand cDNA products. Endogenous reaction mixtures containing actinomycin D (100 μg/ml) and 125 μM (each) dATP, dCTP, dGTP, and dTTP in a 50- to 100-μl volume were incubated for 30 minutes at 37°C. Reactions were treated with proteinase K and precipitated with ethanol. Yeast tRNA (2 μg/ml) was included as a carrier. Products of the endogenous reactions were denatured with glyoxal, and minus-strand DNAs were separated from full-length products on a 1.4% agarose gel containing 10 mM phosphate buffer. Products migrating at <1 kb were isolated by electroelution and used in primer extension reactions with end-labeled primer C2/C3 Sac, which is complementary to a region approximately 150 nucleotides (nt) from the 5′ end of the minus-strand cDNA. Primer extension products were isolated from a 6% polyacrylamide gel and visualized by autoradiography. The probe was amplified by anchored PCR with primer C2/C3 Sac and a primer complementary to the tailed region (dTBam; 5′-ATTATAGGATCAAAAC-3′). Amplified products were digested with BamHI and SacI and cloned into the appropriate sites of pBluescript. Plasmid DNA was digested with digestion with BglIII and sequenced.
were incubated at 37°C for 15 min and then chased by the addition of dCTP or dATP to 100 μM and incubated for an additional 5 min at 37°C. Optimal magnesium and salt conditions were determined by varying one component at a time. In reactions lacking DNA primers, the magnesium optimum was found to be 30 mM, as determined from the intensity of full-length cDNA products observed in denaturing polyacrylamide gels. The addition of spermidine (2 mM) and KCl (10 to 500 mM) did not enhance the activity. DNA oligonucleotides complementary to the in vitro RNAs were initially added at 100 molar excess (50 nM), and reaction mixtures were incubated at 37°C for 10 min prior to addition of the RT. To diminish base pairing between oligonucleotides and prevent snapback of RNA templates, subsequent experiments used equimolar amounts of RNA and primer (100 and 25 ng, respectively). In this case, RNAs and oligonucleotides were annealed in buffer having 50 mM Tris-HCl (pH 8.3), 7 mM MgCl₂, and 10 mM DTT at 70°C for 2 min and then slowly cooled to 25°C prior to being added into the exogenous RT assays. Reaction conditions with in vitro RNA-oligonucleotide substrates were optimized using the C3-2R RNA with the INT primer, and the optimal magnesium concentration was found to be 10 mM. Template-primer substrates tested included 2R and 3R RNAs annealed with oligonucleotides, INT (5’-TGCTGAATTGGAGGACTACTCA), 2R (5’-C15TAGATTAGATAGATC), 1R (5’-C15TAGATTAGATG), or 1Rc (5’-C15TAGATTAGACT). Parallel reactions were conducted using MMLV RT (Promega) in reaction mixtures having 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, and 10 mM DTT. Product cDNAs were treated with 0.1 M NaOH for 15 min at 50°C where indicated and precipitated. The pellets were resuspended in 10 mM EDTA and heated for 5 min at 95°C prior to electrophoresis in 6% polyacrylamide gels containing 8 M urea. Gels were dried and exposed to X-ray film for autoradiography or analyzed by a Storm Phosphorimager (Amersham).

Oligonucleotide extension experiments. Oligonucleotides used in reactions that lacked RNA templates were purified from high-percentage polyacrylamide gels to ensure that the DNAs were of homogeneous length. Typically, 50 nM oligonucleotides were included in a reaction buffer having 50 mM Tris-HCl (pH 8.2); 30 mM MgCl₂; 5 mM DTT with 15 μM of [α-32P]dATP; 20 μM dCTP, GTP, and TTP; or 100 μM dCTP. The solution was heated to 95°C for 3 min and 37°C for 10 min, prior to the addition of MN-treated pFOXC RT. Reactions were carried out for 15 min at 37°C and stopped by the addition of 0.25 M EDTA. Products were precipitated with ethanol and 2 μg of yeast tRNA as carrier. Following precipitation, products were resuspended in 95% formamide, boiled for 5 min, and loaded onto a 10% polyacrylamide gel containing 8 M urea. Gels were exposed to X-ray film for autoradiography. Markers were 5’-end-labeled oligonucleotides that ranged in size from 16 to 50 nt. Oligonucleotides used in these experiments included 2R, 3R, ATC, NoG, and Nla (5’-CGGGATCCATGAGATTAGATTAGATTAGC). Analogous reactions carried out with 200 U of MMLV RT (Invitrogen) failed to produce detectable products, even under conditions that were optimal for MMLV RT activity. Free energies of the primers were determined using the nearest-neighbor method of Breslaur et al. (4), with software from Premier Biosoft International (Palo Alto, Calif.).

RESULTS

Minus-strand cDNA products do not contain remnants of large RNA or protein primers. Partially purified mtRNPs particles isolated from pFOXC-containing strains have RT activity that is capable of generating full-length minus-strand cDNA products from endogenous RNA templates (24) (Fig. 2). As expected for reverse transcription reactions, pretreatment of mtRNPs particles with actinomycin D (an inhibitor of DNA-dependent DNA polymerase activity) had little effect on the reactions (Fig. 2, lanes 2 and 6 to 10), whereas pretreatment with RNase A abolished the activity (lane 3). Conversely, post-treatment with RNase A did not significantly affect the reaction products, while they were degraded by DNase I (Fig. 2, lanes 4 and 5, respectively). As described previously, hybridization of reaction products to single-stranded minus-sense RNAs indicated that plus-strand DNAs are not produced in these assays (24).

The finding that the size of the major minus-strand product is equal to the length of the plasmid transcript suggests that cDNA synthesis likely starts at or near the 3’ end of the endogenous RNA templates. To assess if RNA or proteins serve as primers for cDNA synthesis, minus-strand cDNAs synthesized in reactions with nuclease-free mtRNPs that had been partially purified by DEAE-Sepahcyl chromatography were digested with alkali (Fig. 2, lane 7) and proteinase K (Fig. 2, lanes 8 and 9). Following treatments, 32P-labeled cDNAs were denatured with glyoxal and separated by electrophoresis in a 1.5% agarose gel. No difference in the migration of the minus-strand cDNAs was observed among the treated products; extraction of the products with phenol-CIA, with and without proteinase K treatment, also had no obvious effect on their migration (Fig. 2, lanes 9 and 10). The lack of an apparent shift in the migration of the cDNA products following treatment with alkali, RNase A, and proteinase K indicates that nascent minus-strand cDNA products do not contain remnants of large attached RNAs or proteins. The mature 1.9-kb pFOXC2 plasmid DNA contains a 5’-covalently linked protein, as it is insensitive to λ exonuclease, a 5’→3’ exonuclease, and is retained in the organic phase when extracted with phenol (17, 24). To assess if the 5’ termini of minus-strand cDNAs contain remnants of a covalently linked
peptide, labeled products were incubated with λ exonuclease. As shown in Fig. 2 (lane 12), the minus-strand cDNA products of endogenous reactions are completely degraded, indicating that the 5’ ends of cDNAs are not blocked. Therefore, it is likely that the putative modification occurs at a later step in plasmid replication.

Liberation of the pFOXC RT from endogenous RNAs. To directly study reverse transcription, we sought to purify the pFOXC RT and conduct reactions using exogenous template-primer substrates (described hereafter as exogenous reactions). Following steps that proved successful in the partial purification of the closely related RTs encoded by the Mauriceville and Varkud retroplasmids of Neurospora spp., the pFOXC RT was liberated from mtRNP particles by nuclease treatment with RNase A or MN to degrade endogenous template RNAs. RT activity of RNase A-treated mtRNPs was measured using artificial poly(rA)-oligo(dT) template-primers, and MN-treated mtRNPs were assayed using both poly(rA)-oligo(dT) and poly(rC)-oligo(dG) (see Materials and Methods). Nuclease-treated mtRNPs from pFOXC-containing strains were effective in incorporating [32P]labeled dNTPs into high-molecular-weight products, whereas little or no activity was detected in mtRNPs isolated from a strain that lacked the plasmid (data not shown). No significant difference in RT activity was detected between pFOXC2- or pFOXC3-containing strains, and reactions having poly(rA)-oligo(dT) were found to be comparable to those having poly(rC)-oligo(dG) artificial template-primer substrates.

Optimal conditions for the exogenous reactions with MN-treated pFOXC RT preparations were determined by varying one component at a time ([pH], [Mg2+], [Mn2+], [salt], and temperature) and were established as pH 8.2, 20 mM MgCl2, no salt, and incubation at 42°C. Figure 3A shows the results of a set of reactions that plot the activity under various MgCl2 concentrations with poly(rC)-oligo(dG) templates and shows that the MN-treated pFOXC RT has a broad magnesium optimum. In general, conditions for optimal RT activity with pFOXC-containing Fusarium MN-treated mtRNPs with homopolymeric template-primer substrates were similar to those for previously characterized reactions with Neurospora crassa mtRNPs containing the Mauriceville retroplasmid. Two exceptions were that the magnesium optimum range was greater (15 to 30 versus 5 to 10 mM), and the salt optimum was lower (no salt versus 200 mM KCl) for reaction mixtures containing pFOXC RT and Maur-RT, respectively (27). mtRNPs that were not pretreated with MN were unable to use defined template-primer substrates (described below), suggesting that there is not an appreciable amount of free (or unbound) RT in the mtRNP preparations that is able to access exogenous substrates.

The pFOXC RT utilizes exogenous templates and retains specificity for the plasmid transcript. The specificity of pFOXC RT for RNA templates was initially assessed by using MN-treated pFOXC3 RT with total mtRNA isolated from pFOXC3-containing strains in place of artificial homopolymeric template-primer substrates. Southern hybridization experiments were carried out with MN-treated pFOXC3 mtRNPs with poly(rC)-oligo(dG) template-primer substrates. The ability to incorporate [32P]dGTP into high-molecular-weight products was measured as described in Materials and Methods, and counts per minute represent an average of two or more reactions having specified amounts of MgCl2. (B) Products of MN-treated mtRNP reactions containing total mtRNA isolated from a pFOXC3-containing strain used as a probe for a Southern blot containing HindIII-digested mtDNA from the same strain. The ethidium bromide (EtBr)-stained gel is shown on the left, and the autoradiogram is on the right. An arrow identifies the band that corresponds to the 1.9-kb pFOXC2 plasmid DNA. The size of DNA markers (lambda DNA digested with PstI) is indicated to the left in kilobases. (C) Partial restriction map of pFOXC2 and pFOXC3. Restriction sites for BglII (Bg), EcoRV (E5), XmaI (X), and EcoRI (E) of pFOXC2 are shown above the “clothespin” DNA, and the sites that are also found in pFOXC3 are indicated by a vertical line below the hairpin structure. The long open reading frame is indicated by diagonal lines, and the location of highly conserved amino acids characteristic of RTs is shown (boxes 1, 2, 2a, and A to E). DNA constructs used to generate clones for the synthesis of in vitro RNAs (dashed horizontal lines) are shown below the hairpin DNA. The terminal EcoRI fragment of pFOXC3 was cloned into pBluescribe and engineered to have two or three copies of the 3’ pentameric repeat (small boxes). DNA templates were digested with NlaIII and used in in vitro transcription reactions with T7 RNA polymerase to synthesize 93-nt (C3-2R) and 98-nt (C3-3R) RNAs having two or three copies of the 5’-hp repeat, respectively. Additional RNAs were synthesized from DNAs that were digested with BglII (Bg) and BamHII (B). (D) Determination of optimal MgCl2 and KCl concentrations for cDNA synthesis with C3-2R RNAs. Reactions were carried out with MN-treated pFOXC3 RT and 0.5 μg of RNA in standard exogenous reaction conditions having variable concentrations of MgCl2 or KCl (in millimolar concentrations), as indicated. The prominent cDNA product of 173 nt is shown.
mid DNA (Fig. 3B). These data indicate that the pFOXC RT has an apparent specificity for plasmid RNAs following treatment with MN and is amenable for use in an in vitro system that utilizes exogenous RNA substrates.

Identification of the 3′ end of the retroplasmid transcript. To identify the 3′ termini of in vivo plasmid transcripts, full-length RNA transcripts of pFOXC3 were denatured and separated by electrophoresis in an agarose gel. RNAs of ~1.9 kb were isolated from the gel, tailed with A residues by using poly(A) polymerase, and copied using an oligo(dT) primer with MMLV RT. Complementary cDNAs were amplified by anchored PCR, cloned, and sequenced. Analysis of 28 clones revealed that the 3′ termini were variable in length and averaged about three copies of the 5-bp repeat (Table 1, column labeled “3′ RNA”). Although the average length of the RNAs is slightly less than the average length of the pFOXC3 DNAs (Table 1, column labeled “3′ DNA”) (24), the results suggest that transcription proceeds to the end of the DNA template rather than terminating at an internal site.

MN-treated pFOXC RT can copy in vitro RNAs corresponding to the 3′ end of the retroplasmid transcript. DNA clones containing the terminal ~100 nt of the pFOXC3 plasmid were engineered, and the two most frequently used constructs are shown in Fig. 3C. When these DNAs are linearized and used as templates in in vitro transcription reactions, RNAs of 93 and 100 nt of the pFOXC3 plasmid were isolated from the gel, tailed with A residues by using poly(A) polymerase, and copied using an oligo(dT) primer with MMLV RT. Complementary cDNAs were amplified by anchored PCR, cloned, and sequenced. Analysis of 28 clones revealed that the 3′ termini were variable in length and averaged about three copies of the 5-bp repeat (Table 1, column labeled “3′ RNA”). Although the average length of the RNAs is slightly less than the average length of the pFOXC3 DNAs (Table 1, column labeled “3′ DNA”) (24), the results suggest that transcription proceeds to the end of the DNA template rather than terminating at an internal site.

Once optimal conditions were established, MN-treated pFOXC RT preparations were used in a series of reactions to characterize reverse transcription by using in vitro RNA templates corresponding to the 3′ end of the pFOXC3 retroplasmid. For each set of experiments, the effectiveness of the MN treatment to degrade endogenous RNAs in the mtRNPs was assessed by examining reactions that lacked exogenous RNA (Fig. 4, lane 1). For comparative purposes, parallel reactions with MMLV RT were also performed using reaction conditions defined to be optimal by the supplier. As indicated above, in reaction mixtures containing the 93-nt C3-2R RNA, the major cDNA product was approximately 173 nt, which is 80 nt longer than the RNA template (Fig. 4, lane 2). To identify the source of the additional sequences, 32P-labeled cDNA products were digested with RNase A, alkali, or proteinase K. Proteinase K treatment had no effect on the migration of the 173-nt band (data not shown), whereas both RNase A and alkali treatment resulted in bands of approximately 84 to 85 nt (Fig. 4, lane 3, and data not shown). This indicated that the 173-nt product represents an RNA-cDNA hybrid molecule that was generated by the elongation of the 3′ end of the RNA that had snapped back upon itself. Similar but slightly smaller products (~166 nt) were observed when reactions were carried out using 4 U of MMLV RT (Fig. 4, lane 7). Posttreatment of MMLV RT cDNA products with RNase A also indicated that the larger bands represent an RNA-cDNA hybrid. Interestingly, the major cDNA products obtained with MMLV RT were significantly shorter (73, 76, and 82 nt) than those obtained with the MN-treated pFOXC RT. This suggests that template RNAs had either snapped back in alternative ways in the two reactions or that only specific forms were extended by the different RTs. It is also possible that other mechanisms were involved. Additional experiments were carried out with in
shown at the bottom, with the incorporated nucleotides indicated in box, 2R oligonucleotide). Potential base-pairing interactions of the 2R products observed (dashed line, C3-2R RNA; solid line, cDNA; open interactions of snapped-back C3-2R RNAs that may correspond to the well as a schematic drawing of the most favorable base-pairing inter-

FIG. 4. Exogenous reverse transcription reactions with MN-treated pFOXC RT and MMLV RT. Reactions with mtRNP particles isolated from pFOXC3-containing strains digested with MN (lanes 1 to 5) or MMLV RT (lanes 7 to 10). Lane 1, no exogenous RNA. Lanes 2 to 5 and 7 to 10, reaction mixtures containing 93-nt C3-2R RNA that corresponds to the 3’ terminus of the pFOXC3 plasmid transcript. Reactions were carried out with (lanes 4, 5, 9, and 10) or without (lanes 2, 3, 7, and 8) a 34-nt oligonucleotide that is complementary to the 3’ end of the RNA template. Following cDNA synthesis, products were incubated with RNase A (lanes 3, 5, 8, and 10) or left untreated (lanes 1, 2, 4, 7, and 9), prior to electrophoresis in a 6% polyacrylamide gel containing 8 M urea. Numbers on the left indicate the sizes (nucleotides) of a 100-bp marker and Sau3AI fragments of pBS(−) molecular weight markers (M, lane 6). Numbers on the right indicate the sizes (nucleotides) of the 32P-labeled DNA products as well as a schematic drawing of the most favorable base-pairing interactions of snapped-back C3-2R RNAs that may correspond to the products observed (dashed line, C3-2R RNA; solid line, cDNA; open box, 2R oligonucleotide). Potential base-pairing interactions of the 2R oligonucleotide self-dimer that is extended by the pFOXC RT are shown at the bottom, with the incorporated nucleotides indicated in lowercase letters.

vitro RNAs that had sequence modifications near the 3’ end in hopes of interfering with the ability to snap back. Reactions with RNAs that lack the terminal repeats, as well as those in which two G residues were replaced with C residues to limit potential base-pairing interactions (see schematic in Fig. 4 and Materials and Methods), also produced cDNA products that resulted from snapped-back RNAs (data not shown), and we were unable to establish reaction conditions that completely prevented the extension of snapped-back RNAs without compromising RT activity.

The pFOXC RT uses base-paired DNAs as primers of minus-strand cDNA synthesis. When a 34-nt oligonucleotide (2R) having 25 nt of complementarity to the 3’-terminal sequence of the C3-2R RNA was included in exogenous reactions, a 102 nt product was obtained, indicating that the pFOXC RT is capable of extending a DNA primer. The product obtained in reactions having the C3-2R RNA template-primer substrates was very near the size predicted (102 nt) and was found to comigrate with analogous products observed in reactions with MMLV RT (lanes 9 and 10; data not shown). An unexpected result was also found in MN-treated pFOXC RT reactions that included DNA oligonucleotides. In addition to the expected cDNA products, labeled products were also observed that migrated at approximately 40 to 50 nt. As shown in lanes 4 and 5 of Fig. 4, a prominent product of 42 nt is detected in reactions containing the 34-nt 2R oligonucleotide. In reactions that used other oligonucleotides as primers, the size of the smaller products varied slightly, yet they were consistently produced. Significantly, these products were absent from reactions with MMLV RT (Fig. 4, lanes 9 and 10) and were found in reactions lacking RNA templates (see below).

pFOXC RT has DNA polymerase activity and can extend mismatched primers. The anomalous labeled DNA products were readily detected in exogenous reactions containing oligonucleotide 2R as well as in reactions that included other oligonucleotides that had complementarity to the in vitro RNA templates (Fig. 5 and data not shown). Significantly, identical products were produced in reactions that lacked RNA templates, indicating that the small products did not represent complementary DNAs. To better understand this anomalous activity, reaction products were analyzed in high-percentage denaturing polyacrylamide gels, and labeled DNA products were found to be 4 to 16 nt longer than the oligonucleotides. Based on the size of the products and sequence of the oligonucleotides, it was concluded that the small products result from self-priming of oligonucleotides that had snapped back or had formed self-dimers, despite highly unfavorable predicted base-pairing interactions (Fig. 4 and 5).

To confirm that particular alignments were being extended, reactions were carried out with specific combinations of dNTPs and ddNTPs to assess if predicted extension products could be obtained. An example of reactions with the 37-nt oligonucleotide 3R is shown in Fig. 5. Reactions having [32P]dATP with dCTP, dGTP, and TTP resulted in a major product of 41 nt, as well as bands that migrated at 42, 46, and 51 nt (Fig. 5, lane 1). The length of the 41-nt product suggested that the 3R oligonucleotide had been elongated by 4 nt, which matches the length of an extended product from the most favorable base-pairing interaction of the 3R oligonucleotide (Fig. 5, top alignment). Reactions carried out with progressively lower concentrations of the 3R oligonucleotide demonstrated that the synthesis of the labeled products was concentration dependent (i.e., the intensity of the band was not linear with concentration) and thus involved a bimolecular reaction (data not shown). The alignment that produced the major 41-nt product
FIG. 5. Extension of 3R oligonucleotide in reactions lacking RNA templates. (A) Unlabeled 3R oligonucleotide was used in reverse transcription reactions with MN-pFOXC RT containing 0.33 mM [32P]dATP and either 20 μM dCTP, dGTP, and TTP (lane 1); no additional nucleotides (lane 2); [32P]dATP plus 100 μM dideoxy-TTP (lane 3); or [32P]dATP plus 20 μM dCTP or TTP and 100 μM dideoxy-GTP (lane 4). Lane 5 contains 5′-end-labeled oligonucleotide 3R. Sizes are indicated on the left (in nucleotides) and are based on DNA size standards (not shown). (B) The sequence and length of predicted extension products for reactions with the three most favorable base pairings. The predicted ΔG for the self-dimer in the reaction conditions used in these experiments (30 mM MgCl2, no salt, 37°C) is −4.7 kcal/mol (versus −1.6 kcal/mol for a snapback), as determined using the nearest-neighbor method of Breslauer et al. (4). For comparison, the ΔG of a primer that has been modified to correct the mismatches is four- to fivefold more favorable, depending on whether mismatches are replaced by A/T or G/C base pairs, respectively.

The 46-nt product seen in lane 1 most likely represents the addition of a single nontemplated nucleotide, a reaction that commonly occurs with many polymerases (6) and has been shown to be associated with cDNA synthesis by the Maur-RT (16). In reactions that generate products that terminate with dideoxynucleotides, bands representing nontemplated nucleotide addition were not observed. The origins of the 46- and 51-nt products were less evident. Analysis of reactions carried out with or without the addition of specific deoxy- or dideoxynucleotides indicated that the longer products likely derive from two additional base-pairing alignments that are shown in Fig. 5. For example, in the reaction containing [32P]dATP, dCTP, TTP, and ddGTP (lane 4), the synthesis of the 46-nt product is limited degree of complementarity to in vitro RNA templates. The ability of the pFOXC RT to extend DNA primers that have a pentameric repeat, having complementarity to the terminal 10 nucleotides, equimolar amounts of RNA and primer were preannealed with an appreciable amount of nuclease activity (data not shown). Significantly, in no case were analogous products observed when MMLV RT was used in equivalent reactions, even when used under the same reaction conditions. Taken together, the results indicate that the pFOXC RT can copy DNA templates and has a remarkably loose specificity for primers.

The pFOXC RT uses minimally base-paired primers more readily than conventional RTs do. Based on the findings that the pFOXC RT can extend minimally base-paired, snapback RNAs and unstable primer duplexes, we assessed the ability of the pFOXC RT to extend DNA primers that have a limited degree of complementarity to in vitro RNA templates. A DNA oligonucleotide (2Rc, containing two copies of the same 23-nt internal region of the pentameric repeat) having complementarity to the terminal 10 nt of the C3-2R and C3-3R RNA templates was synthesized and used in reverse transcription reactions. In these experiments, equimolar amounts of RNA and primer were preannealed to diminish base pairing between oligonucleotides and to prevent snapback of the RNA templates. As a control, an oligonucleotide complementary to a 23-nt internal region of the in vitro RNAs (INT) was annealed to the templates under the same conditions. These template-primer substrates were then used in reactions having either AMV, MMLV, or pFOXC RT, each with the appropriate optimal reaction conditions. In experiments using the C3-3R RNA (having three copies of the pentameric repeat), all three RTs efficiently extended the in-
The sizes (in nucleotides) of products that derive from the panel, and they are end-labeled Sau3AI and AluI pBS restriction size (in nucleotides) of the DNA markers is shown on the left of each lane. For comparison, reactions were carried out under standard reaction conditions with AMV, MMLV, or pFOXC RT. All reactions contain the 98-nt C3-3R RNA (dashed line) having three copies of the terminal 5-bp repeat. (Left panel) Lanes 1, 4, and 7 lack primer 1Rc (1c) that is complementary to an internal region of the template; and lanes 3, 6, and 9 contain the 2Rc (2c) primer having 10 nt of complementarity to the 3’ end of the template. A band of the same size as the RNA template was detected in lanes 1 to 6 and may represent partial end-labeling activity in reactions having AMV and MMLV RT. Reaction products were not posttreated with alkali, and the 175-nt band in lanes 7 and 8 is RNA-cDNA hybrids formed by extension of snapped-back RNA templates. (Right panel) Primer extension reactions with three oligonucleotides having diminishing amounts of complementarity to the 3’-terminal sequences of the C3-3R RNA template. Primer 1R+ATc (175) has 7 nt of complementarity, and primer 1Rc (1c) has 5 nt of complementarity with the template. Reactions were carried out under standard reaction conditions with 10 mM MgCl2, and products were subjected to alkaline hydrolysis. The sizes (in nucleotides) of products that derive from primers bound to the 3’ end of the RNAs are shown below the gel. The size (in nucleotides) of the DNA markers is shown on the left of each panel, and they are end-labeled Sau3AI and AluI pBS restriction fragments.

FIG. 6. Comparison of cDNA synthesis with internally and terminally annealed DNA primers with AMV, MMLV, or pFOXC RT. All reactions contain the 98-nt C3-3R RNA (dashed line) having three copies of the terminal 5-bp repeat. (Left panel) Lanes 1, 4, and 7 lack DNA oligonucleotides; lanes 2, 5, and 8 contain the 23-nt INT (I) primer (open box) that is complementary to an internal region of the template; and lanes 3, 6, and 9 contain the 2Rc (2c) primer having 10 nt of complementarity to the 3’ end of the template. A band of the same size as the RNA template was detected in lanes 1 to 6 and may represent partial end-labeling activity in reactions having AMV and MMLV RT. Reaction products were not posttreated with alkali, and the 175-nt band in lanes 7 and 8 is RNA-cDNA hybrids formed by extension of snapped-back RNA templates. (Right panel) Primer extension reactions with three oligonucleotides having diminishing amounts of complementarity to the 3’-terminal sequences of the C3-3R RNA template. Primer 1R+ATc (175) has 7 nt of complementarity, and primer 1Rc (1c) has 5 nt of complementarity with the template. Reactions were carried out under standard reaction conditions with 10 mM MgCl2, and products were subjected to alkaline hydrolysis. The sizes (in nucleotides) of products that derive from primers bound to the 3’ end of the RNAs are shown below the gel. The size (in nucleotides) of the DNA markers is shown on the left of each panel, and they are end-labeled Sau3AI and AluI pBS restriction fragments.

The sequences listed in Table 1 include several that contain single-base mismatches with the previously reported plasmid DNA sequence. In many cases, mismatches are detected at the extreme 3’ end (or 5’ end of the corresponding minus-strand cDNA) and could represent a nontemplated nucleotide added during transcription. It is possible that the addition occurs during the cloning procedure; however, nucleotides that deviated from the ATCTA repeat sequence were not identified among plasmid DNA products that were cloned by the same procedures. All but two of the mismatches are associated with cDNA products, suggesting that they could be due to errors that occur during in vivo reverse transcription. Surprisingly, when downstream sequences of the minus-strand cDNAs were examined (up to 100 nt from the 5’ end), additional changes were seen in 17 of the 27 nascent cDNA products and all occurred in a region that is approximately 15 to 25 nt downstream from the 5’ end of the cDNA products (Table 2). As these changes were seen only among minus-strand cDNAs and were detected at a similar frequency in endogenous products that were derived from two independent mtRNP preparations, it appears that the changes are introduced during minus-strand cDNA synthesis and are not an artifact of the cloning procedure. Thus, it is possible that the events that created these additions are relevant to mechanisms used to maintain the terminal repeats.

not shown). These experiments demonstrate that the pFOXC RT is capable of extending primers that have minimal base-pairing interactions with RNA templates.

5’ ends of in vivo minus-strand cDNA replication intermediates contain additional copies of the pentameric repeat. To assess if the activity seen in the in vitro reactions has relevance to mechanisms used to maintain the telomere-like repeats, the 5’ termini of minus-strand cDNA replication intermediates were analyzed. Minus-strand cDNAs were generated from endogenous RNA templates by using DEAE-Sephacyl-purified mtRNP particles, and reaction products were gel isolated to eliminate plasmid DNAs and mtDNAs that could potentially be associated with the RNP particles. The denatured plasmid DNAs, having a hairpin structure at their upstream terminus, migrate at a size of approximately 3.8 kb, whereas the nascent minus-strand cDNAs are only 1.9 kb (Fig. 2 and data not shown). Once isolated, the 5’ ends of the minus-strand cDNAs were copied via primer extension, and the products were tailed and amplified by anchored PCR (see Materials and Methods). Twenty-seven separate clones that derive from reactions with two independent mtRNP preparations were sequenced, and the results are included in Table 1. As with the plasmid DNAs and plasmid transcripts, the minus-strand cDNAs contained a variable number of pentameric repeats, and similar results were found from both cDNA reactions analyzed. A comparison of the number of repeats among the three plasmid molecules reveals that the cDNAs are, on average, longer than the plasmid RNAs and as long as or longer than most plasmid DNAs. The increased length of the minus-strand cDNA sequences relative to the plasmid RNAs suggests that additional sequences are added during the initial steps of reverse transcription.

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TABLE 2. Mismatched and inserted nucleotides detected in clones of in vivo minus-strand cDNAs

| Sequence of the 5' terminus of in vivo minus-strand cDNAs | a | b | c |
|-----------------------------------------------------------|---|---|---|
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGATA-5'       |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TA                    |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGA            |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGG            |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGGg           |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAg           |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAA           |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAAa          |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAAb          |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAAb          |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAAa          |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAAb          |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAAb          |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAAa          |   |   |   |
| AAATGTCGTTCAGGTTAAATCAGATC TAGAT TAGAT TAGAAb          |   |   |   |

a Nucleotides mismatched with the plasmid DNA sequence are indicated in boldface lowercase, and insertions are underlined.

b Three clones having this sequence were recovered.

c Insert shows sequence similarity to upstream sequences (in italics).

DISCUSSION

We demonstrate that the pFOXC RT can be liberated from mtRNP particles and can copy exogenous RNA templates by use of DNA and RNA primers. The pFOXC RT is found complexed with the plasmid RNA in vivo and, in reactions with mtRNP particles, synthesizes full-length 1.9-kb minus-strand cDNAs that begin at or near the 3' end of the plasmid RNA. Further characterization of endogenous reaction products indicates that long RNAs or proteins do not appear to serve as primers for cDNA synthesis and suggests that the modification found at the 5' end of the mature plasmid DNA occurs at a subsequent step of plasmid replication.

The pFOXC RT can access and copy exogenous RNA templates following treatment with nuclease s that digest endogenous RNAs associated with mtRNP particles. MN-treated pFOXC RT preparations were found to efficiently copy homopolymeric RNA and RNA template-DNA primer combinations. When provided with total mtRNAs from a plasmid-containing strain, the pFOXC RT retained specificity for the plasmid RNA; however, in reactions with short in vitro-synthesized RNA templates, the pFOXC RT did not demonstrate a strong preference for specific templates. Full-length in vivo plasmid transcripts may have specificity determinants not found in the in vitro-synthesized transcripts, such as structural features of upstream regions necessary for recognition and binding, or the 3' end of the plasmid RNAs could be more accessible than nonplasmid mitochondrial transcripts. Alternatively, the lack of template specificity in reactions with in vitro RNAs (without cDNA primers) may reflect the mechanism used to initiate cDNA synthesis that involves self-priming of snapped-back RNA templates.

The ability to extend snapped-back RNAs is a common characteristic of retroviral RTs, and analogous RNA-cDNA hybrid products were also formed in reactions containing MMLV RT. However, the length of cDNA products synthesized by MMLV RT suggests that it requires more substantial base-pairing interactions at the 3' end of the snapped-back RNAs in order to initiate cDNA synthesis compared to reactions with pFOXC RT. It is not clear whether this disparity reflects differences in recognition and binding of templates or potential structural constraints involved in coordinating the 3' OH group of the terminal nucleotide within the catalytic site of the RT.

To various degrees, MN-treated pFOXC RT preparations were able to copy all in vitro RNAs provided in exogenous reactions, which confounded efforts to determine whether the pFOXC RT has a preference for templates with specific sequences, as was shown for the closely related RT encoded by the Mauriceville mitochondrial retroplasmid of N. crassa (5, 28). The Maur-RT can initiate cDNA synthesis in a primer-independent manner (28), or when treated with MN, Maur-RT preparations initiate cDNA synthesis at the extreme 3' end of RNA templates by using preexisting cDNAs as primers (27). Importantly, the Maur-RT exhibits the same high degree of specificity for RNAs having a 3' CCA whether cDNA synthesis initiates de novo or is DNA primer mediated (5, 28).

Based on the results presented here, the most apparent differences between the RTs encoded by the Fusarium and Neurospora retropiasmids concern the ability to utilize base-paired primers to initiate cDNA synthesis and the ability to copy DNA templates. The Maur-RT is not able to use internally annealed DNA primers (27) and inefficiently uses RNAs as primers (28), while the MN-treated pFOXC RT is able to efficiently extend DNA oligonucleotides that are complementary to RNA templates and, in certain reactions, displays an even greater capacity to extend DNA primers than conventional RTs (see below). Furthermore, in reactions containing excess DNA oligonucleotides, the MN-treated pFOXC RT unexpectedly extended oligonucleotides that had formed self-dimers via exceptionally weak base-pairing interactions. In some cases, DNA oligonucleotides were extended that contained up to three terminal mismatches with the template. Since the oligonucleotide extension and snapped-back RNA experiments were performed under conditions having a high magnesium concentration (30 mM), it is possible that the elevated level of magnesium ions stabilized template-primer interactions or helped coordinate the positioning of the 3' OH of the primers in the catalytic site of the RT. However, extension products from snapped-back RNAs were also readily detected under conditions that were optimal for in vitro RNA template-primer substrates having a magnesium concentration of 10 mM, and under no conditions were oligonucleotide self-dimers used as substrates by MMLV RT.

In a direct comparison with AMV RT and MMLV RT, the pFOXC RT was significantly more proficient at extending a DNA primer that had only 10 nt of complementarity to the 3' termini of the RNA templates. Since the template-primer substrates used in these reactions were identical, differences in the ability to extend the minimally base-paired primers likely relate to binding site requirements (i.e., the length of the heteroduplexed region) and/or the ability to position the primer within the active site of the RT. The pFOXC RT was also able to use primers having 7 and 5 nt of homology, albeit at much lower efficiencies. Interestingly, in each of the experiments using DNA oligonucleotides having homology to the 3'-terminal re-
pept, the length of the major primer extension product was approximately 113 nt, indicating that the oligonucleotides had been extended from the extreme 3’ end of the RNA template, rather than from upstream binding sites. For example, the 2Re primer could potentially base pair at two positions of the 3R RNA template, resulting in cDNA products of 108 or 113 nt. The cloning and sequencing of cDNA products from all three primer extension reactions confirm that the pFOXC RT has a strong preference for initiation from primers bound at the 3’ end of the template RNAs (Marchetti and Kennell, unpublished). It is possible that the primers preferentially bind to the terminal binding site of the RNA templates, perhaps due to interference of the snapped-back RNA. Alternatively, the pFOXC RT may exhibit a preference for primers that are bound at the 3’ terminus of the templates, similar to the mechanism of minus-strand cDNA initiation associated with the Mau-RT (5). Taken together, the ability to use snapped-back RNAs and extend terminally bound DNA oligonucleotides—including loosely base-paired DNA oligonucleotides as primers for DNA synthesis—demonstrates that the pFOXC RT has an unusually relaxed specificity for primers and has unique capabilities compared to other RTs.

The ability of the pFOXC RT to copy DNA templates was unexpected and suggests that the RT has flexibility with regard to templates, as well as primers. Although we have not found evidence that plus-strand synthesis occurs in the endogenous reverse transcription reactions, our results suggest that it may play a role in the synthesis of the retroplasmid plus-strand DNA. The propensity of the pFOXC RT to use snapped-back RNAs could also support the proposed mechanism for initiation of plus-strand synthesis via the snap-back of a full-length minus-strand DNA (Fig. 1A).

**Relationship to plasmid replication and other RTs.** The use of minimally base-paired and/or mismatched primers to initiate minus-strand cDNA synthesis may be important for the generation and maintenance of the 3’ telomere-like repeats. Our analysis of the 3’ termini of pFOXC3 RNAs and 5’ termini of minus-strand cDNAs revealed that both molecules contain a variable number of pentameric repeats, and the degree of heterogeneity matched that found at the 3’ end of retroplasmid DNAs. The majority of RNAs analyzed were found to be slightly shorter than the corresponding plasmid DNAs, which indicates that the 3’-terminal repeats are not added during transcription. In contrast, the 5’ termini of minus-strand cDNAs were, on average, longer than the plasmid RNAs and as long as or longer than plasmid DNAs. The increased length of the cDNA sequences relative to the plasmid RNAs suggests that additional sequences are added during minus-strand cDNA synthesis. While the evidence is indirect, our results support models in which the synthesis of repeats occurs during the initiation of reverse transcription.

We previously speculated that the generation of 5-bp repeats of the pFOXC plasmids could involve a “DNA slippage” mechanism (24) that has been shown to be involved in 3’-terminal repeat addition of linear DNAs that initiate replication via a protein primer (22). Analysis of cDNA products from endogenous reactions does not support the involvement of a protein primer; however, the relaxed primer specificity associated with the pFOXC RT could be indicative that analogous mechanisms are occurring with nucleic acid primers. Based on our findings to date, we favor a self-priming model for the initiation of minus-strand cDNA synthesis that involves the extension of snapped-back plasmid RNAs, coupled with DNA slippage events to generate additional pentameric repeats (Fig. 1B). While we show that the pFOXC RT is able to initiate cDNA synthesis by extension of the 3’ end of snapped-back in vitro RNAs, our analysis of minus-strand cDNA products from endogenous reactions failed to find evidence that RNAs are used as primers in vivo. It is possible that RNA primers are cleaved or degraded immediately after initiation and thus escape detection, and it is noteworthy that a nucleolytic cleavage activity has recently been reported to be associated with telomerase (12). If initiation of minus-strand cDNA synthesis is self-primed in vivo, it is likely that the pFOXC RT preferentially binds its own message following translation in order to maintain specificity for the plasmid transcript. This “cis” preference has been shown for human L1 non-long-terminal-repeat retrotransposons (30) and other transposable elements (21).

A report by Kajikawa and Okada (13) showed that 5-bp repeats (5’-TGTTA) found at the 3’ end of certain LINE and SINE elements of eel (Anguilla japonica) are added during retrotransposition. By measuring the frequency of retrotransposition in a HeLa cell transposition assay, they demonstrated that the repeats are necessary for transposition, and the recovery of specific insertions revealed that the LINE RT generates additional repeats during target-primed reverse transcription via a DNA slideback mechanism. While we have yet to find direct evidence that the pFOXC RT undergoes a slideback during initiation, it is noteworthy that we detect minor higher-molecular-weight products in vitro reactions with terminally bound DNA primers, which could be indicative of cDNAs having additional sequences. In addition, the majority of in vivo minus-strand pFOXC cDNA products recovered contained insertions and mismatches with the DNA sequence. Since most in vivo changes occurred in a region approximately 15 to 25 nt downstream from the 5’ terminus, it is not clear if they occur during initiation; however, most changes included insertions that, in some cases, appear to have been created via polymerase slippage. For example, one clone contained a 7-nt insertion directly upstream of the pentameric repeats that shows high similarity to upstream flanking sequences (Table 2). Although the significance of these changes is difficult to assess at this time, it is noteworthy that no deletions were detected among the clones; thus, it is possible that these modifications are created by the same activity that maintains the terminal pentameric repeats.

It is highly unusual for DNA polymerases to efficiently extend mismatched 3’-terminal nucleotides, and, to our knowledge, this has been described only for the Tetrahymena TERT (25, 26, 29). The Tetrahymena TERT was shown to add telomeric repeats to noncomplementary (nontelomeric) DNAs. This “de novo” addition was found to be position dependent, occurring at the 3’ boundary of the RNA template region, and an increasing degree of complementarity between the 3’ end of the primer and the template was required for extension to occur at downstream sites. Since the 3’ ends of the pFOXC RNAs are heterogeneous, the ability to initiate with a mismatched 3’ nucleotide of a snapped-back RNA would ensure that all plasmid RNAs could be copied. The permissive nature
of the pFOXC RT may be indicative of a potential mechanistic connection to de novo telomere addition associated with TERTs and could provide additional support for an evolutionary relationship between the pFOXC retroplasmids and telomerase.

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