In Vitro Activities of the Multifunctional RNA Silencing Polymerase QDE-1 of Neurospora crassa*

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QDE-1 is an RNA- and DNA-dependent RNA polymerase that has functions in the RNA silencing and DNA repair pathways of the filamentous fungus Neurospora crassa. The crystal structure of the dimeric enzyme has been solved, and the fold of its catalytic core is related closely to that of eukaryotic DNA-dependent RNA polymerases. However, the specific activities of this multifunctional enzyme are still largely unknown. In this study, we characterized the in vitro activities of the N-terminally truncated QDE-1ΔN utilizing structure-based mutagenesis. Our results indicate that QDE-1 displays five distinct catalytic activities, which can be dissected by mutating critical amino acids or by altering reaction conditions. Our data also suggest that the RNA- and DNA-dependent activities have different modes for the initiation of RNA synthesis, which may reflect the mechanism that enables the polymerase to discriminate between template nucleic acids. Moreover, we show that QDE-1 is a highly potent terminal nucleotidyltransferase. Our results suggest that QDE-1 is able to regulate its activity mode depending on the template nucleic acid. This work extends our understanding of the biochemical properties of the QDE-1 enzyme and related RNA polymerases.

Gene expression of most eukaryotic organisms is regulated by an immense assortment of small RNAs and proteins that associate with them. These various components form networks known as RNA silencing pathways, most important of which employ small interfering RNAs (siRNAs), microRNAs, or piwi-interacting RNAs to achieve sequence specificity (1–3). RNA silencing associated cell-encoded RNA-dependent RNA polymerases (RdRPs) are found commonly as components of the RNA silencing associated cell-encoded RNA-dependent RNA polymerases (RdRPs) are found commonly as components of the RNA silencing pathways of plants, fungi, and nematodes (1, 4).

The functions of cellular RdRPs have been largely elusive, but recent studies have shed some light on their enigmatic character. Caenorhabditis elegans RdRPs have been shown to synthesize secondary siRNAs that are important for amplifying the initial silencing signal (5–7). Tetrahymena termophila RdRP (Rdr1) is known to interact with Dicer to produce endogenous siRNAs, whereas the RdRP (Rdp1) of Schizosaccharomyces pombe is critical for heterochromatic gene silencing (2, 8, 9). Arabidopsis thaliana has six genes that code for RdRPs, but only a few of these have been studied in detail (10, 11). Most of the above studies imply that the main function of cellular RdRPs is in synthesizing siRNAs directly or making double-stranded RNA (dsRNA) from single-stranded RNA (ssRNA) templates to be used as Dicer substrate. For a long time, it was thought that cellular RdRPs are absent in insects and mammals, but recently, robust RdRP activities have been detected in Drosophila melanogaster and humans (12, 13) suggesting that cellular RdRPs may have crucial functions throughout the eukaryotic domain.

Neurospora crassa is a filamentous fungus that displays remarkable genomic stability (14, 15). One of the cellular mechanisms that affect this stability is an RNA-silencing pathway known as quelling (16). Quelling is initiated by repetitive genetic elements and is dependent on three genes: qde-1 (quelling defective) encoding an RdRP, qde-2 (a member of the Argonaute family), and qde-3 (a RecQ-like DNA helicase) (17–19). It has been shown that overexpression of QDE-1 results in increased silencing and that expression of hairpin dsRNA molecules abolishes the need of QDE-1 activity, suggesting that the primary function of QDE-1 is to synthesize dsRNA to be used as substrates for the two Dicers (DCL-1 and DCL-2) of Neurospora (15, 20–25). The Argonaute protein QDE-2 has slicer activity and interacts with an exonuclease known as QIP (25). The expression of QDE-2 is induced by dsRNA, and its steady-state levels are regulated by the DCLs (15). The biochemical roles of QDE-3 largely are unknown, but it has been suggested to have roles in both DNA repair and quelling (18, 26, 27).

The classical model of transgene quelling in Neurospora begins by RNA polymerase II and QDE-3 synthesizing an aberrant RNA molecule, which is recognized by QDE-1 and converted into dsRNA (24). This dsRNA is digested to double-stranded siRNAs by the DCLs. These associate with QDE-2, which nicks the passenger strand of the siRNA that is degraded subsequently by the QIP exonuclease (25). QDE-2, now containing a single-stranded siRNA guide strand, finds its complementary mRNA (or aberrant RNA) targets, which leads to
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the silencing of both transgenic and endogenous transcripts. Recently, this model has been challenged by the discovery that quelling components have essential roles in the nucleus associated with DNA repair (27, 28). QDE-1 was shown to co-purify with ssDNA binding replication protein A (RPA), and DNA damage was shown to induce QDE-2 expression. Immunoprecipitation of QDE-2 from DNA damaged Neurospora cultures revealed a novel type of small RNAs known as QDE-2-interacting small RNAs that are mostly derived from the ribosomal DNA (rDNA) locus (27). qiriRNA production is dependent on QDE-1, QDE-3, and the DCLs but not on QDE-2. Notably, QDE-2-interacting small RNAs are derived from aberrant RNAs that are synthesized by QDE-1 and not by any of the canonical RNA polymerases. QDE-1 was shown to have a robust DNA-dependent RNA polymerase (DdRP) activity, generating a DNA/RNA hybrid from an ssDNA template (27).

Much insight into the structure and function of cellular RdRPs has come from the studies of a recombinant QDE-1 and its catalytically active C-terminal portion QDE-1ΔN (residues 377–1402 of the wild-type) (29–31). The recombinant polymerase is able to initiate RNA synthesis without a primer and convert heterologous ssRNAs into double-stranded molecules. In addition to making full-length dsRNA copies of ssRNA templates, QDE-1 was observed to synthesize small 9–21-nt RNAs scattered along template RNAs (29). The crystal structure of QDE-1ΔN showed that the molecule is a dimer and that the catalytic core has a fold that is related to those in eukaryotic DdRPs (31).

In this study, we demonstrate that QDE-1ΔN displays five distinct in vitro activities. We use structure-based mutagenesis to show that the activities can be dissected by mutating critical amino acid residues and suggest that RdRP and DdRP activities have different initiation mechanisms and pH optima. The biochemical data presented in this study imply a recognition mechanism that discerns a DNA template from an RNA template. These results have broader ramifications in eukaryotic RNA- and DNA-dependent RNA polymerases associated with RNA silencing pathways.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The expression vectors for QDE-1ΔN point mutants were generated by site-directed mutagenesis using PCR. The mutagenic primers are listed in supplemental Table S1. Plasmid pEM69 (30) encoding for a His-tagged QDE-1ΔN (missing amino acids 1–376) was used as a template in 50-μl PCR reactions each containing sense and antisense primers, and 2.5 units of Pfu Turbo DNA polymerase (Stratagene). After completion, the reactions were treated with 10 units of DpnI (Fermentas) and transformed into CaCl2 competent Escherichia coli XL1-Blue cells (Stratagene). The correct constructs were verified by restriction enzyme analysis and sequencing.

Yeast Expression and Protein Purification—The recombinant proteins were expressed and purified as described previously for QDE-1ΔN (pEM69; 30)). Briefly, the plasmids were introduced into Saccharomyces cerevisiae strain INVSc1 (Invitrogen), the recombinant proteins were expressed at +28 °C for 22 h and purified to near homogeneity. The yeast cells were first harvested and disrupted by a French press. The cell lysates were then cleared by centrifugation, and the supernatants were loaded onto nickel-nitrilotriacetic acid affinity columns (Qiagen), washed with 5 mM and 25 mM imidazole-containing buffers, and eluted with 200 mM imidazole. Subsequently, the proteins were purified by HiTrap™ heparin HP and Q HP columns (GE Healthcare) and eluted by increasing NaCl gradients. The purified proteins were stored in 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.13% Triton X-100, 100 mM NaCl, and 62.5% glycerol at −20 °C. The oligomeric status of the recombinant polymerases was analyzed by size-exclusion chromatography using a Superdex 200 16/60 gel filtration column (GE Healthcare) with appropriate control proteins (Sigma).

Template RNAs and DNAs—Plasmid pLM659 (32) contains a cDNA copy of the S segment of bacteriophage φ6 under a T7 promoter. For the production of ssRNA, pLM659 was linearized by Smal digestion, purified using a PCR purification kit (Qiagen), and used as a template for run-off transcription by T7 RNA polymerase. The template DNA was degraded with DNAse I (Promega) and the ssRNA purified by chloroform extraction and LiCl precipitation. To generate a ssDNA molecule of the same length and sequence, Smal-digested pLM659 was used as a template in PCR reactions containing primers AO49 and AO50 (see supplemental Table S4) and Phusion® DNA polymerase (Finnzymes). AO49 contains a 5′-biotin. The biotinylated PCR product was immobilized onto Dynabeads® MyOne™ streptavidin C1 magnetic beads (Invitrogen) according to the manufacturer's instructions. The immobilized PCR product was dissolved by treating the DNA briefly with fresh 0.1 M NaOH. The ssDNA was precipitated with sodium acetate and ethanol and gel-purified through agarose gel electrophoresis. Prior to 5′-labeling, the ssRNA was treated with alkaline phosphatase (Finnzymes). Both ssRNA and ssDNA were 5′-labeled with [γ-32P]ATP (NEN Radiochemicals, PerkinElmer Life Sciences) and T4 polynucleotide kinase (Fermentas). M13mp18 ssDNA was purchased from New England Biolabs. The oligonucleotides (AO49–52) were purchased from biomers.net or Eurofins MWG Operon.

Polymerase Activity Assays—Polymerase reactions were performed essentially as described (29, 33). The standard QDE-1 reaction mixture contained 50 mM HEPES-KOH (pH 7.8), 20 mM ammonium acetate, 1 mM MgCl2, 1 mM MnCl2, 6% (w/v) polyethylene glycol 4000, 0.1 mM EDTA, 0.1% Triton X-100, 0.2 mM of each NTP, 1 unit/μl RNasin® ribonuclease inhibitor (Promega), and 0.01–0.02 μg/μl QDE-1ΔN. In some of the pH experiments, HEPES-KOH (pH 7.2–7.8) was replaced by Bis-Tris (pH 6.0–6.9) or Tris-HCl (pH 8.0–8.9). The ladder reactions were programmed with 5 mM MgCl2. Reactions were supplemented with 0.1 μCi/ml of [α-32P]UTP (GE Healthcare or NEN Radiochemicals, PerkinElmer Life Sciences) or other radioactive NTPs where indicated. The reactions were incubated at +30 °C for 1 h and quenched with U2 (8 M urea, 10 mM EDTA, 0.2% SDS, 6% (v/v) glycerol, 0.05% bromphenol blue, and 0.05% xylene cyanol) loading buffer. Some reaction products were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), precipitated with NH4OAc and ethanol, and dissolved in milli-Q water.
Some samples were treated with RNase T1 (Fermentas) for 15 min at +37 °C.

The samples were subjected to standard Tris-Borate-EDTA or Tris-Acetate-EDTA agarose gel electrophoresis or denaturing, formaldehyde-containing agarose gel electrophoresis (34). The gels were visualized by ethidium bromide staining and dried, and radioactivity was detected by phosphorimaging (Fuji FLA-5000) and analyzed by densitometry with AIDA software (Raytest Isotopenmessgeräte). Some samples were subjected to denaturing PAGE by urea-containing 20% sequencing gels. These were either desalted by Zeba Spin Desalting Columns (Thermo Scientific) or purified by phenol extraction and ethanol precipitation. Prior to loading, the samples were mixed with Gel Loading Buffer II (Ambion) and heated to +95 °C for 5 min.

RESULTS

QDE-1 Displays Five Distinct Activities—To elucidate the different catalytic activities of QDE-1ΔN, standard polymerization reactions were carried out with ssRNA or ssDNA templates in different conditions (Fig. 1A). Mixing QDE-1ΔN with ssRNA, all four NTPs and [32P]UTP resulted in dsRNA synthesis as well as decreased mobility (shifting) and labeling of the ssRNA template (lane 2). In addition, labeled RNA products were detected that varied in size from tens of nucleotides to several hundreds. Some of these products did not enter the agarose gel and remained in the wells. No labeled products were detected when the catalytically inactive QDE-1ΔNDA was used in the reaction mix (lane 3) (29). When the reactions were carried out with only UTP and trace amounts of [32P]UTP, the template ssRNA was efficiently labeled without dsRNA synthesis (lane 4), indicating that QDE-1ΔN is a potent terminal nucleotidyltransferase (TNTase). QDE-1ΔN displays also a strong DNA-dependent RNA polymerase activity (lane 6) (27). None of the template ssDNA migrates as template-sized but is very efficiently converted to the DNA/RNA hybrid form (lane 6). Again, substituting the ΔN polymerase with QDE-1ΔN DA abolishes this activity (lane 7). The TNTase activity also is very prominent with an ssDNA template (lane 8). Using only UTP as the substrate, the ssDNA template migrates at its normal position (upper panel) but is extensively labeled (lower panel).

The above experiments show that QDE-1ΔN displays five distinct activities (Fig. 1, A and B): (i) RNA-dependent RNA polymerase activity (Fig. 1A, lane 2) (29), (ii) DNA-dependent RNA polymerase activity (Fig. 1A, lane 6) (27), (iii) ssRNA template shift and labeling activity (Fig. 1A, lane 2), (iv) TNTase activity (Fig. 1A, lanes 4 and 8), and (v) ladder activity (Fig. 1A, lanes 2, 6, and 9) (supplemental Fig. S1). Activities (i) and (ii) have been described previously (27, 29). Activity (iii) has been suggested previously to result from the synthesis of 9–21-nt small RNAs that are scattered across the ssRNA template, as well as to be the main in vitro reaction product of QDE-1ΔN (29). However, in this work, the intensity of the ssRNA labeling was not significantly more extensive than the labeling of the dsRNA product (Fig. 1A, lane 2), suggesting that the “small RNAs” are not the main reaction product. The identity of this activity is further discussed below. TNTase activity (iv) (see below) has been detected previously in both viral and eukaryotic RdRPs, where nucleotides are added to the 3’-ends of the templates in a template-independent fashion (11, 35). Activity (v) (Fig. 1A and supplemental Fig. S1) was assigned as a ladder activity because it generates RNA products of all sizes. When the reaction products of a reaction without a template were analyzed on a denaturing sequencing gel, they migrated at one-nucleotide increments (starting from ~8 nts) forming a “ladder” (supplemental Fig. S1A). This activity is template-independent because omitting the template does not affect the formation of the ladder (Fig. 1A, lane 9). However, the sensitivity of the ladder activity to varying reaction conditions suggests that it is an in vitro side reaction occurring at high enzyme and substrate conditions (supplemental Fig. S1). The in vitro activities of QDE-1ΔN are summarized schematically in Fig. 1B.

QDE-1 Uses Different Initiation Mechanisms on ssDNA and ssRNA Templates—As QDE-1 displays both RNA- and DNA-dependent RNA polymerase activities, we further studied the nature of these reactions. We programmed polymerization

FIGURE 1. QDE-1 displays five distinct activities. A, QDE-1 reactions were programmed with the same amounts of ssRNA or ssDNA, NTPs or UTP, and QDE-1ΔN or QDE-1ΔNDA as indicated. All reactions contained trace amounts of [32P]UTP. The control nucleic acids were labeled in the 5’-end with [γ-32P]ATP and polynucleotide kinase. Reaction mixtures were incubated for 1 h at +30 °C, quenched with U2 loading dye, and analyzed by native agarose gel electrophoresis. Upper panel, ethidium bromide-stained gel; lower panel, autoradiogram of the same gel. Positions of templates (ss) and products (ds) are indicated. The band migrating in between ss and ds on lanes 5, 7, and 8 is a conformer of the ssDNA template. B, a schematic presentation of different QDE-1 in vitro activities. See text for details.
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**FIGURE 2.** QDE-1 has different initiation mechanisms for DNA and RNA templates. A, QDE-1ΔN reactions were programmed as described in Fig. 1 and incubated for 1 h at +30 °C. The reactions were purified by phenol extraction and ethanol precipitation, and the samples were subjected to denaturing agarose gel electrophoresis. On control (CTL) lanes are the template nucleic acids labeled in the 5′ end. Shown is the autoradiogram of the gel. B, QDE-1ΔN reactions were programmed with ssRNA or ssDNA with the same length and sequence. Both templates end with CC-3′. Nucleic acids labeled in the 5′ end. Shown is the autoradiogram of the gel. Lower panel, DdRP reactions were carried out with an initiating nucleotide [γ-32P]ATP. The products were analyzed by native agarose gel electrophoresis, and shown are EtBr-stained gel (upper panel) and autoradiogram of the same gel (lower panel).

reactions with ssRNA or ssDNA templates of the same length and sequence, purified the reaction products, and analyzed them by denaturing formaldehyde-containing agarose gel electrophoresis. As controls we labeled the templates at the 5′-end with [γ-32P]ATP and polynucleotide kinase (Fig. 2A). As has been shown previously (29), most of the product that accumulated using an ssRNA template migrated more slowly than the template, indicating that the strands of the dsRNA molecule are covalently linked together (“back-priming”). In contrast, the products of the reaction using an ssDNA template migrated as template-sized or smaller, indicating that the mode of RNA synthesis initiation differs between these two templates. This result is further supported by an experiment where RdRP and DdRP reactions were carried out with an initiating nucleotide that was 32P-labeled at the γ-phosphate (Fig. 2B). In this experimental setup, the product RNA can be labeled only if the γ-phosphate remains within the first nucleotide of the new strand. Conversely, if RNA synthesis is initiated by back-priming, the γ-phosphate is removed from the product RNA. As expected, radioactivity was detected only in the double-stranded products of the ssDNA template (DNA/RNA hybrids) and the template-sized products of the ssRNA template (resulting from abortive initiation, see below). dsRNA was not labeled. In addition, we performed QDE-1ΔN activity assays with both ssRNA and ssDNA templates in the same reaction mixture simultaneously (supplemental Fig. S2). The RdRP or DdRP activities did not inhibit each other, but both templates were processed into products. The ssDNA template in this experiment was circular, indicating that QDE-1ΔN is able to initiate RNA synthesis without the need for a free 3′-end (supplemental Fig. S2). All in all, these data suggest that QDE-1 is able to discriminate between ssRNA and ssDNA templates. **Mutant Polymerases Display Altered Activities**—The crystal structure of QDE-1ΔN has been solved previously (31). Using the structural information, we designed eight point mutations that were predicted to functionally disrupt QDE-1ΔN (Fig. 3A and supplemental Fig. S3 and Tables S1 and S2). The constructs were transformed into *S. cerevisiae*, and the recombinant proteins were expressed. All of the mutant enzymes were soluble and purified to near homogeneity and behaved like the wild-type during purification (data not shown). Initial screening of the RdRP and DdRP activities of the point mutants revealed that they possessed characteristics that differed from the wild-type polymerase (supplemental Fig. S4A). As expected (as these were assumed to be catalytically essential aspartic acids), QDE-1ΔN[D1011A] (29) and D1007A were catalytically completely inactive. Of the active point mutants, five (R738A, R944E, K1119W, M1357D, and M1357C) were chosen for more extensive studies due to their catalytic properties. Arg738 lies within a channel that is predicted to accommodate the reaction product of QDE-1ΔN and direct it away from the active site (Fig. 3A). The R944E mutation is predicted to partly block the communication tunnel that links the two active sites in a QDE-1ΔN dimer (Fig. 3A). The K1119W mutation was designed to occlude a pore in QDE-1ΔN that apparently allows substrate nucleotides to enter the active site. The M1357D and M1357C mutations are predicted to respectively weaken and lock together the dimeric interface of the QDE-1ΔN head domains (Fig. 3A). However, the interface of the entire QDE-1 dimer is so extensive that mutating Met1357 should not affect the oligomerization state of the enzyme. To confirm this, we performed analytical gel filtration chromatography with QDE-1ΔN WT in different conditions, some of the point mutant enzymes, and control proteins of various sizes (Table 1). Dimeric QDE-1ΔN is predicted to be ~230 kDa in size, whereas a monomer would have the predicted size of ~120 kDa (31). Our results establish that all QDE-1ΔN enzymes are dimeric, regardless of the surrounding pH (Table 1). This has been deduced previously from the crystal structure, as each of the subunits has >2000 Å2 of contact area with the neighboring subunit (31).

All of the five point mutants under closer scrutiny were catalytically active on both ssRNA and ssDNA templates (Fig. 3B). The catalytic activity of R738A is reduced to approximately half of that of the wild-type regardless of the template (Fig. 3C), in accordance with a nonspecific charge steering role for this residue. In addition, R738A is incapable of shifting and labeling the ssRNA template (Fig. 3B and supplemental Fig. S4A). The DdRP activity of R944E is close to that of the native polymerase. However, its RdRP activity is only ~20% of the wild-type (Fig. 3C). It shifts the ssRNA template and labels it efficiently (Fig. 3B and supplemental Fig. S4A). These results are consistent with a role for the tunnel bridging the active sites of the dimer to initiate RNA-templated polymerization (see below). The RdRP and DdRP activities of K1119W are close to those of the wild-type, the former activity even being slightly higher (Fig. 3C). However, this mutant shows dramatically reduced activities (iii) and (iv) (shifting of the template ssRNA and TNTase activity) (Fig. 3B and supplemental Fig. S4, A and B), which may reflect a slight weakening of nonspecific charge stabilization of a product complex by this residue close to the active site. Both M1357D and M1357C mutants display catalytic activities that are very similar to wild-type (Fig. 3, B and C). The ssRNA tem-
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Mutations Affecting TNTase Activity Suggest a Mechanism for Template Recognition—Terminal nucleotidyltransferase activity has been described in both viral and cellular RdRPs (11, 35). QDE-1ΔN WT displays a strong TNTase activity (iv) on linear ssRNA or ssDNA of the same length and sequence (Fig. 4B). The RdRP activity was, however, always higher than the DdRP activity. We also performed reactions with all the QDE-1ΔN point mutants varying the pH (Fig. 4C and data not shown). Interestingly, the K1119W mutant was catalytically inactive at pH 6.3 with both ssRNA and ssDNA templates, regaining its activity as the pH increased (Fig. 4C). These data indicate that pH might be one of the factors regulating RdRP and DdRP activities.

pH Has a Differential Effect on RdRP and DdRP Activities of QDE-1ΔN—Point mutations to QDE-1 structure introduce internal variations to the protein. To study external factors on the catalytic properties of the enzyme, we assayed the pH dependence of QDE-1ΔN on RdRP and DdRP activity by using a linear ssRNA or a circular ssDNA as templates in reactions with QDE-1ΔN WT (Fig. 4A). To our surprise, we observed that the RdRP activity was high at a low pH (optimum at approximately pH 6.3) and decreased as the pH increased. In contrast, the DdRP activity was low at a low pH and increased with the increasing pH (optimum at approximately pH 7.4). Similar results were obtained when the QDE-1ΔN WT reactions were programmed with linear ssRNA or ssDNA of the same length and sequence at pH 6.3, 7.4, and 8.3 (Fig. 4B). The DdRP activity was, however, always higher than the RdRP activity. We also performed reactions with all the QDE-1ΔN point mutants varying the pH (Fig. 4C and data not shown). Interestingly, the K1119W mutant was catalytically inactive at pH 6.3 with both ssRNA and ssDNA templates, regaining its activity as the pH increased (Fig. 4C). These data indicate that pH might be one of the factors regulating RdRP and DdRP activities.

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FIGURE 4. pH has an effect on RdRP and DdRP activities. A, a linear ssRNA and a circular ssDNA were used as templates in QDE-1 WT reactions containing 25 mM Bis-Tris (pH 6.0 to 6.9), 50 mM HEPES-KOH (pH 7.2 to 7.8) or 50 mM Tris-HCl (pH 8.0 to 8.9) in addition to all the standard components. The maximum (max.) activity of each reaction set (RdRP or DdRP) was set as 100%. The experiment was repeated independently three times. Error bars indicate S.E. B, equal amounts of ssRNA or ssDNA templates with the same length and sequence were used as templates in QDE-1 ΔN WT reactions in the indicated pH values. The maximum activity of each reaction set (RdRP or DdRP) was set as 100%. The experiment was repeated independently three times. Error bars indicate S.E. C, equal amounts of QDE-1 ΔN WT or K1119W were used in reactions similar as above. Shown are the autoradiograms of native agarose gels.

DISCUSSION

In this study, we have shown that QDE-1 displays five distinct activities on RNA or DNA templates (Fig. 1) and that these activities can be dissected by altering the reaction conditions (Fig. 4) or utilizing mutant polymerases (Fig. 3). Interestingly, both DdRP and TNTase activities also have been described for RDR6 of Arabidopsis (11), suggesting that such biochemical activities may be conserved evolutionally among cellular RdRPs. Our results indicate that QDE-1 is most

this oligonucleotide was labeled at the 5′-end with [γ-32P]ATP and polynucleotide kinase and subsequently digested with RNase T1 (that specifically cleaves ssRNA at 3′ of G residues), the labeled product was 10-nt-long (Fig. 5A, lanes 1 and 2). When an unlabeled oligonucleotide was incubated with QDE-1 WT and [α-32P]UTP, the reaction product migrated at a position corresponding to ~31 nt. As this product was digested with RNase T1, the position of the label corresponded to ~21 nt (Fig. 5A, lanes 3 and 4). These results indicate that QDE-1 transfers approximately one nucleotide to the 3′-end of the template. In addition to UTP, the other NTPs (ATP, GTP, and CTP) were accepted as substrates as well (data not shown).

To further investigate the TNTase activity, we used all eight point mutants in a TNTase assay with both ssRNA and ssDNA templates (supplemental Fig. S4B). QDE-1ΔNDA and D1007A were completely inactive, suggesting that the TNTase activity resides in the same catalytic site as the other activities. The point mutants labeled the templates with different efficiencies, with R738A and K1119W showing very little activity. To our surprise, we noticed that the P964A mutant was able to label the ssRNA but not the ssDNA. When QDE-1ΔN structure (31) was superimposed with yeast RNA polymerase II elongation complex (based on the conserved double-psi β-barrels) the incoming DNA template could be modeled very close to Pro964 (36 and data not shown). Mutation P964A introduces changes to the course of the polypeptide chain in its proximity but is far enough from the active site (11 Å) to produce only a subtle effect. When QDE-1ΔN WT and P964A polymerases were combined with both template types and NTPs or UTP, the P964A mutant differed from the ΔN WT only in being unable to add a terminal UTP to the 3′-end of the ssDNA (Fig. 5B). We also performed the TNTase assays with 30-nt-long ssRNA or ssDNA oligonucleotides and analyzed the reaction products on a denaturing polyacrylamide gel (Fig. 5C). The ΔN WT and P964A enzymes both added ~1 nucleotide on the 3′-end of the ssRNA. The ΔN WT also was capable of adding ~1 nt on the 3′-end of the ssDNA template, albeit less efficiently, whereas the P964A mutant was inactive when the ssDNA template was applied. These results indicate that QDE-1 is able to distinguish between ssRNA and ssDNA. Although the ssRNA and ssDNA oligonucleotides have the same sequence, the ssRNA migrated more slowly on the denaturing gel than the ssDNA due to its higher molecular weight (9189 g/mol versus 8821 g/mol). As the molecular weight of uridyl monophosphate is ~324 g/mol, this result shows that in these conditions QDE-1 adds only one nucleotide to the 3′-end of the template. However, in higher NTP concentrations, the number of added nucleotides may increase (data not shown).
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It was suggested previously that the template shift (iii) seen in the RdRP reaction of QDE-1 results from the synthesis of small RNAs of 9–21 nt that are scattered across the template ssRNA (29). Subsequent studies have, however, shown that the main in vivo functions of QDE-1 are aberrant RNA (on a DNA template) and dsRNA (on an RNA template) synthesis, and the "small RNAs" may not be relevant biologically (15, 20, 27). Indeed, when dsRNA is produced from hairpin constructs in Neurospora, the requirement for QDE-1 is abolished, but RNA silencing is not compromised (15, 20). Our results suggest that the template shift is not required for efficient dsRNA synthesis (see, for example, R738A, R944E, and K1119W in Fig. 3B), and it is not detected when using an ssDNA template. Thus, it seems plausible that this is not an essential step in the reaction pathway leading to dsRNA production but rather a side reaction resulting from abortive RNA polymerization initiation.

Based on the biochemical and genetic evidence obtained here, we suggest a model for QDE-1 activity in vitro. It is plausible that the dimeric nature of the polymerase is crucial for its activity; QDE-1ΔN is a functional dimer, and the two subunits, tightly associated with each other, have slightly different conformations (31). There also is a distinct communication tunnel connecting the catalytic sites of the two subunits (31; see also Fig. 3A). Mutation R994E partly blocks this tunnel resulting in significant reduction in the RdRP activity (i) but with no effect on the DdRP activity (ii) (Fig. 3B). We therefore propose that the DdRP activity of QDE-1 is a "monomeric" feature of the enzyme, whereas for RdRP activity, the ssRNA template would have to be guided through the communication tunnel to the active site of the other subunit for dsRNA synthesis, making the RdRP activity a "dimeric" property of the enzyme. Indeed, in the structurally similar yeast DdRP elongation complex, there is no 2-fold symmetry (36) supporting the idea that the DNA-dependent reaction of QDE-1 would occur independently at the two active sites. The different pH optima (Fig. 4), the different initiation modes (Fig. 2), as well as the observation that the two activities may occur simultaneously (supplemental Fig. S2) further support the idea that the RdRP and DdRP activities are uncoupled.

DdRP activity is likely the primary activity of QDE-1 as it is constantly considerably higher than RdRP activity (Figs. 1 and 3B). Apparently, the RNA molecules also are directed via the "DdRP" site through the communication tunnel to the "RdRP" site of the dimer. However, not all ssRNA molecules would reach the RdRP site at the other subunit under the in vitro conditions applied. They would be labeled erroneously by the DdRP active site, which would result in an abortive product that then appears as activity (iii) (as exemplified by R944E in Fig. 3B and labeling of ssRNA in Fig. 2B). Such small RNAs that were predicted previously to cause silencing are not observed in vivo (15, 20, 27).

It is possible that the TNTase activity (iv) would target an ssRNA template on the other subunit for dsRNA synthesis. This is supported by the notion that the TNTase activity in the P964A mutant labels only ssRNA but not ssDNA. The mutated proline lies close to the active site and might convey the template in proper directions. The incoming DNA template would be guided through the communication tunnel connecting the catalytic sites of the two subunits of the enzyme. However, not all ssRNA molecules would reach the RdRP site at the other subunit under the in vitro conditions applied. They would be labeled erroneously by the DdRP active site, which would result in an abortive product that then appears as activity (iii) (as exemplified by R944E in Fig. 3B and labeling of ssRNA in Fig. 2B). Such small RNAs that were predicted previously to cause the shifting of the ssRNA template (29) have not been observed in vivo (15, 20, 27).

As pH has a clear differential effect on the RdRP and DdRP activities of QDE-1, it may be that this would reflect pH differences in various cellular compartments (e.g. nucleus and cyto-

**FIGURE 5.** The terminal nucleotidyl transferase activity of QDE-1. A, sequence and RNase T1 cleavage products of the 30-nt-long ssRNA (upper panel). Control and QDE-1ΔN reactions were purified by phenol extraction and ethanol precipitation and analyzed on a denaturing 20% urea-PAGE (lower panel). The band migrating at ∼20 nt on lane 1 is a conformer of the ssRNA. B, standard QDE-1 reactions were programmed with ssRNA or ssDNA, NTPs or UTPs, [α-32P]UTP and equal amounts of QDE-1ΔN enzymes as indicated. The reaction products were analyzed by native agarose gel electrophoresis, and shown is the autoradiogram of the gel. Positions of single- and double-stranded nucleic acids are indicated.

<table>
<thead>
<tr>
<th>Products</th>
<th>ssRNA</th>
<th>ssDNA</th>
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<tbody>
<tr>
<td>RNAase T1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RNase T1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NTP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UTP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5'-32P[UTP]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>QDE-1+α-32P[UTP]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ssRNA 30 nt</td>
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The terminal nucleotidyl transferase activity of QDE-1 is a DdRP and that the initiation mechanism of RNA synthesis is different with ssRNA and ssDNA templates (Fig. 2 and supplemental Fig. S2).
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plasm). However, because the QDE-1 dimer is known to associate with several proteins and other cellular factors (15, 27) it is obvious that there are many regulatory mechanisms that control the activity of this multifunctional polymerase.

This work lays the biochemical foundations for the properties of QDE-1. Consequently, it is possible to apply this knowledge in future in vivo studies, allowing us to probe the effect of amended polymerases in quelling and other activities.

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