Mutations in the middle domain of yeast poly(A) polymerase affect interactions with RNA but not ATP

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ABSTRACT

The eukaryotic poly(A) polymerase (PAP) is responsible for the posttranscriptional extension of mRNA 3′ ends by the addition of a poly(A) tract. The recently published three-dimensional structures of yeast and bovine PAPs have made a more directed biochemical analysis of this enzyme possible. Based on these structures, the middle domain of PAP was predicted to interact with ATP. However, in this study, we show that mutations of conserved residues in this domain of yeast PAP, Pap1, do not affect interaction with ATP, but instead disrupt the interaction with RNA and affect the enzyme's ability to process substrate lacking 2′ hydroxyls at the 3′ end. These results are most consistent with a model in which the middle domain of PAP interacts directly with the recently extended RNA and pyrophosphate byproduct.

Keywords: poly(A) polymerase; nucleotidyl transferase; polyadenylation

INTRODUCTION

Polyadenylation of eukaryotic mRNA is an essential step in gene expression and regulation. In this two-step process, a large multiprotein complex refines mRNA in the nucleus by precisely cleaving primary transcripts and extending the new 3′ end by addition of an adenosine tract (reviewed in Zhao et al. 1999; Edmonds 2002). Poly(A) polymerase (PAP) is the subunit of the polyadenylation machinery directly responsible for synthesis of this poly(A) tail. When separated from this complex, the activity of PAP is no longer regulated. It still acts as an efficient terminal adenylyltransferase and maintains specificity for ATP and RNA, but almost any RNA will serve as substrate. Furthermore, the size of the poly(A) tail is no longer constrained to the species-specific lengths observed when PAP is part of a holoenzyme.

PAP belongs to the nucleotidyl transferase superfamily, which contains enzymes such as DNA polymerase β, terminal deoxynucleotide transferase, and the tRNA CCA-adding enzymes (Holm and Sander 1995; Yue et al. 1996). Analysis of the three-dimensional structure of the yeast and bovine PAPs (Bard et al. 2000; Martin et al. 2000) showed three domains in a U-shaped configuration. The N-terminal domain has strong structural homology to the catalytic domains of other members of this superfamily, with an active site of three aspartates that coordinate two metal ions necessary to position the phosphates of ATP for catalysis. The unique C-terminal domain, with a topology similar to that found in RNA recognition motifs (RRMs), is thought to hold the RNA primer. In support of this function, this domain contains an unstructured region called C-RBS that has been demonstrated to bind to the body of the RNA (Zhelkovsky et al. 1998). This C-RBS/RNA interaction contributes to enzyme processivity but is not necessary for catalysis, and thus is distinct from the site contacting the 3′ end of the RNA.

The N-terminal and C-terminal domains of PAP are connected by a domain with no obvious structural homology to other characterized proteins. The orientation of the middle domain relative to the palm differs in the two crystallographically independent molecules within Pap1/3′dATP cocrystals (Bard et al. 2000), suggesting the presence of a hinge and the possibility that PAP utilizes the induced fit mechanism known for other polymerases (Doublie et al. 1999). The region of the active site of Pap1 in these two configurations is illustrated in Figure 1. Both structures show the incoming 3′dATP as well as a second nucleotide that occupies a position expected for the 3′ end of the primer based on the structure of DNA polymerase β. In one Pap1 structure (Fig. 1A), the K215 and Y224 residues, which

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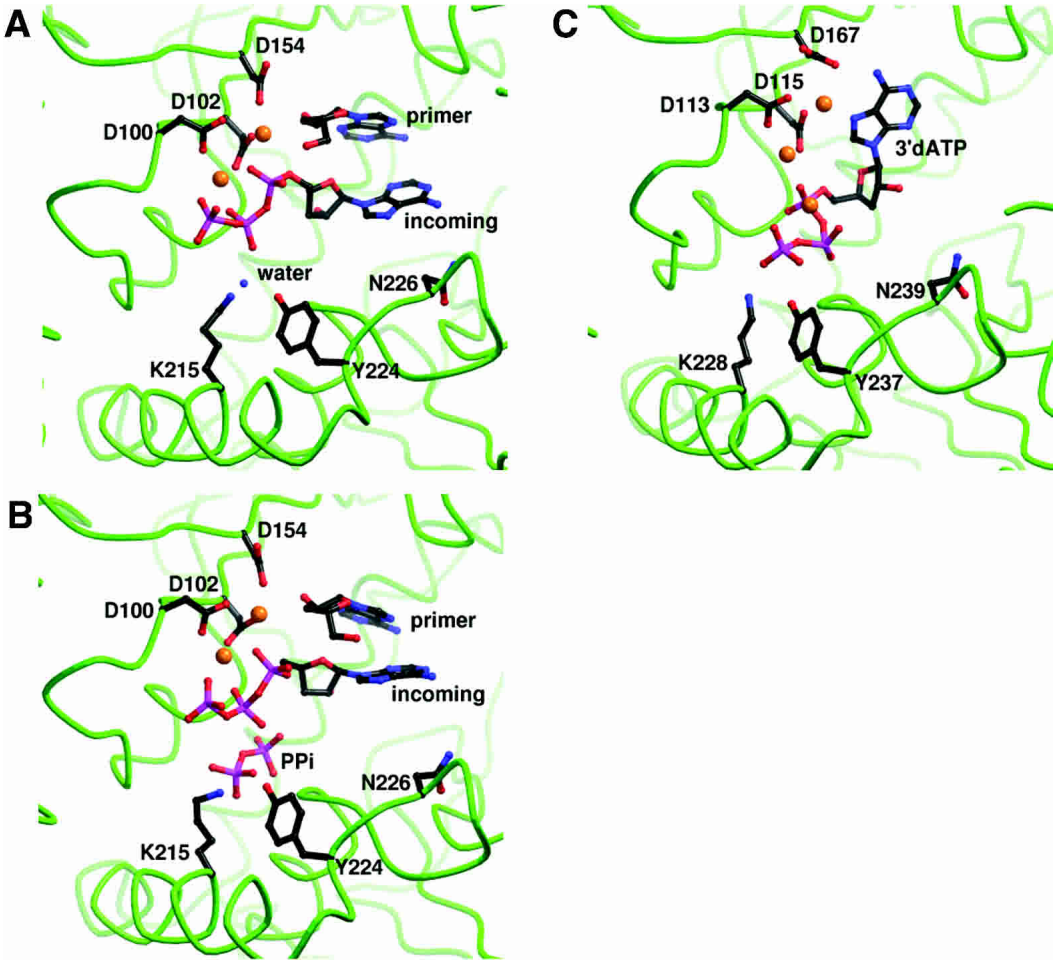

FIGURE 1. Comparison of the structures derived for the active sites of the yeast (*A*,*B*) and bovine PAP (*C*). The positions of the catalytic aspartates (D100, D102, and D154), divalent cations (yellow), 3′dATP, and the first nucleotide of the primer are based on the published structures (Bard et al. 2000; Martin et al. 2000). Conserved amino acids in the middle domain are also indicated.

are invariant in the known eukaryotic PAPs, are close to the triphosphate of the incoming 3′dATP, and K215 coordinates a water molecule with the β-phosphate. In the second, more open conformation (Fig. 1B), Y224 and K215 interact with pyrophosphate, suggesting an additional function in removing this reaction by-product.

Although the structure of PAP in complex with RNA has not yet been determined, a hydrophobic pocket located above the catalytic site shows extensive interactions with the base of the primer mononucleotide and has been proposed to form part of a site involved in positioning the 3′ end of the primer (Bard et al. 2000). The structures of yeast and bovine PAP solved in the presence of 3′dATP show different orientations and interactions with the base of the incoming nucleotide (Bard et al. 2000; Martin et al. 2000), leaving the mechanism of adenine specificity unclear. For yeast PAP, it was proposed that rotation of the middle domain could place residue N226 of this domain within hydrogen bonding distance of the adenosine-specific N6 atom of the incoming nucleotide. In bovine PAP, a third cation was found coordinating N7, and one of the catalytic aspartates formed a hydrogen bond with N6 of the 3′dATP (Fig. 1C). Neither the yeast nor the bovine structures adequately explain the strong preference of PAP for ribose-containing substrates. However, our previous biochemical analysis demonstrated that although Pap1 is highly sensitive to the lack of 2′OH groups at the end of the RNA primer, it is much less affected by their absence on the incoming nucleotide triphosphate (Zhelkovsky et al. 1998).

To gather biochemical support for interactions revealed by the structural analysis of PAP, we have examined the consequences of mutations in K215, Y224, and N226 of the middle domain of Pap1. Surprisingly, substitution of these conserved residues affects primarily interactions with the RNA but not ATP, as was predicted by the crystal structures. Furthermore, an interaction of the 2′-hydroxyl group of the 3′ end of the RNA and Y224 may be important for ribose/deoxyribose discrimination by the mechanism of product RNA translocation. We discuss models of enzyme mechanism that would be consistent with this data.

RESULTS AND DISCUSSION

Because of the important roles for K215, Y224, and N226 suggested by the structural analysis described above, these amino acids of the middle domain of Pap1 were chosen for mutagenesis. The mutants were expressed in *Escherichia coli*, and kinetic parameters determined with purified protein (Table 1). Nonconservative mutations to alanine were made for K215 and N226, whereas Y224 was changed to phenylalanine (Y224F) or to serine (Y224S). The first Y224 mutation preserves the aromatic ring of the amino acid whereas the second one maintains the hydroxyl group. The mutations only moderately affected the K_m for ATP. This is in contrast to what might be predicted from the crystal structure, and from the fact that alanine substitutions of similarly positioned residues in the DNA polymerase I motif B greatly increase the K_m for dNTP (Astatke et al. 1995). Of all the Pap1 mutants examined in this study, the greatest change in the K_m for ATP was the 2.4-fold increase observed with K215A, which is similar to the fourfold increase caused by mutation of the equivalent lysine in the bovine PAP to alanine (Martin et al. 1999). This small change suggests that the primary ATP contacts are mediated by the two metal ions at the active site, and that the interaction of K215 with the β-phosphate makes a minor contribution.

As described in the Introduction, N226 had been proposed to have a role in selection of adenosine as the base of the incoming NTP. However, when adjusted by activity to wild-type Pap1 and presented with a mixture of ATP and radioactive UTP, CTP, or GTP, the N226A mutant misincorporated nonadenosine nucleotides into poly(A) tail to the same extent as the wild-type enzyme (data not shown). Thus, it is unlikely that N226 participates in base discrimination.

The middle domain mutations, however, caused striking alterations in the interaction with RNA. For example, the mutations K215A and N226A caused a 74- and 56-fold increase in the K_m for primer, respectively (Table 1). Furthermore, elimination of the hydroxyl group by the mutation Y224F caused a 30-fold increase in K_m for primer in comparison with wild-type enzyme. Mutation to serine is less detrimental, with a 12-fold increase in K_m for primer. For K215A, Y224F, and Y224S, there is a 2.8- to 3.6-fold decrease in V_{max}, but no change for N226A. Not all mutations in the middle domain have such dramatic effects on Pap1 activity. A change of the conserved phenylalanine 230 to tyrosine, located in the same loop as Y224 and N226, has no effect (data not shown), supporting the conclusion that the other mutated amino acids are functionally important.

TABLE 1. Kinetic analysis of Pap1 mutations

	K_m primer, μM	K_m primer, (Fip1), μM	K_m ATP, μM	V_{max} primer, μmole/min mg
Wt PAP	0.5	10	40	2.5
K215A	37	>500[a]	96	0.8
Y224F	15	56	60	0.7
Y224S	6	11.5	31	0.9
N226A	28	500	62	2.5

The value represents the average obtained from at least three independent reactions. The standard deviations are all within 20% of the average.
[a]This value is between 500 μM and 1 mM and could not be determined more precisely because it is beyond the range of substrate concentrations used in the assay.

Considering the distance between the proposed 3′ RNA-binding pocket and mutated amino acids in the crystallographic structure, this result was unexpected. However, in Pap1 the poly(A) substrate (primer) and poly(A) product are essentially identical, and only differ in positioning within the active site. We further explored the possibility that the mutations described above might affect interaction with poly(A) product and/or its repositioning to a productive complex.

Until now, the only reported mutations that significantly affect the interaction of Pap1 with RNA, as reflected by an increase in K_m for primer, are a V141F replacement in the putative 3′-end-binding site and truncation of the C-RBS (Zhelkovsky et al. 1995; Bard et al. 2000). Because the C-RBS mutation also adversely affects the ability of the enzyme to act processively and to be cross-linked to RNA by ultraviolet light, we tested the new mutants for these functions. The assay for processivity used approximately equal units of enzyme activity at standard reaction conditions (Zhelkovsky et al. 1998). Aliquots of the reaction mixture were removed after different times of incubation and the pattern of products was analyzed by denaturing gel electrophoresis. Long, slowly migrating poly(A) product is visible by 5 min for both of the Y224 mutants and the wild-type Pap1 (Fig. 2A), and for the K215A and N226A mutants (data not shown). Cross-linking experiments with the Y224 mutants showed that they were covalently coupled to radiolabeled RNA with the same efficiency as seen for the wild-type enzyme (Fig. 2C, lanes 5–7). Even though cross-linking is not a quantitative assay, this result does suggest that the Y224 mutations do not perturb the RNA-binding site at the C terminus, which binds the body of the RNA but not the 3′ end.

To investigate how interactions with the 3′ end of the RNA might be affected by middle domain mutations, we examined kinetic parameters when the Pap1 is working distributively. This is accomplished by performing the assays in the presence of Fip1, a component of yeast Polyadenylation Factor I, which binds to Pap1 in a region adjacent to C-RBS. Because Fip1 competes with RNA for access to C-RBS, the enzyme releases the RNA after each round of adenylation (Zhelkovsky et al. 1998), and produces primarily a spectrum of short products characteristic of a distributive mechanism, as shown in Figure 2B. The Y224 mutants give a pattern similar to wild-type enzyme. Even though experimental variability in the amounts of enzyme used and in the

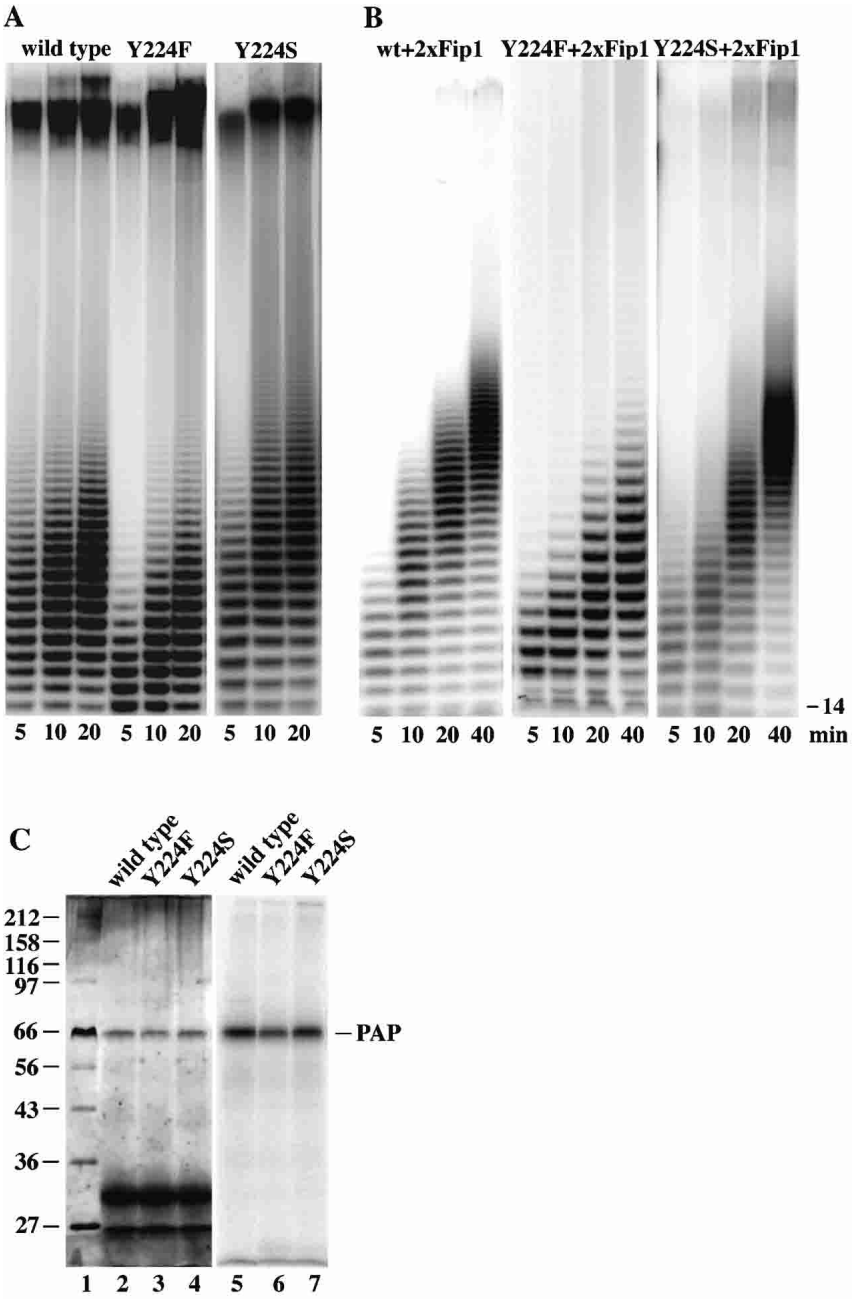

FIGURE 2. Mutations in Y224 of Pap1 do not affect interactions of Pap1 and RNA mediated by the C-RBS. (*A*) Processive synthesis of poly(A). Equal units of wild-type, Y224F, or Y224S enzymes were incubated with radioactive ATP and unlabeled oligo$(A)_{14}$ for the indicated times, followed by separation of the reaction products on a denaturing polyacrylamide gel and visualization by PhosphorImager analysis. (*B*) Distributive synthesis by wild-type, Y224S, and Y224F Pap1 in the presence of Fip1. Analysis was performed as in Panel *A* except that Fip1 was added to the samples in a twofold molar ratio to Pap1. (*C*) UV cross-linking of Pap1 and Y224 mutants to RNA. GAL7–9 RNA, which had been randomly labeled with [^{32}P]UMP, was mixed with the indicated enzymes, and the samples were exposed to UV light and digested with RNase A. Proteins were separated by SDS-PAGE and visualized by silver staining (lanes *2–4*) or with the PhosphorImager (lanes *5–7*). The molecular weights of markers (lane *1*) are indicated on the *left* side.

ratio of Fip1/Pap1 cause some differences in the appearance of products, these results show that the mutants are inhibited by Fip1 in a manner similar to wild-type enzyme.

For normal Pap1, loss of C-RBS interaction also increases the K_m for primer from 0.5 μM to 10 μM (Zhelkovsky et al. 1998; Table 1). This value represents the affinity for the 3′ end of the RNA. For the K215A and N226A mutants, the K_m for primer in the presence of Fip1 was 50-fold higher compared to wild-type PAP with Fip1 (Table 1). Curiously, the increase was much smaller for Y224F (5.6-fold), and for Y224S, it was equal to the K_m(+Fip1) of wild-type Pap1. This analysis suggests that the K215A and N226A mutations affect 3′ end binding. However, the Y224 mutants are less defective when acting distributively. A possible explanation for the Y224 results is that these mutants have difficulty in translocating the RNA within the active site during processive poly(A) elongation.

Our kinetic analysis suggested a critical role for the hydroxyl of Y224 in Pap1 function. In T7 RNA polymerase, Y639 of motif B is involved in 2′-OH recognition of NTP (Sousa and Padilla 1995), and a Y639F mutation relaxes the bias against dNTPs without losing activity or affinity for NTP. Because its position with respect to the active site is similar to that of Y639 of T7 RNA polymerase, Y224 of Pap1 might also be a good candidate for discriminating the 2′-OH of incoming ATP. To test this possibility, we examined whether mutation of the Pap1 Y224 affected its ability to utilize dATP in a reaction using unlabeled oligoA_{14} and radioactive dATP. Equal units of wild-type or mutant enzyme, as determined in standard reactions using ATP and RNA, were added to each reaction.

As we had reported previously (Zhelkovsky et al. 1998), Pap1 could readily incorporate one to three molecules of dAMP onto the end of an oligo(A) primer, but had difficulty with further extension of oligomers possessing deoxyadenosines at the 3′ end (Fig. 3A). The Y224S mutation could add one dAMP but was less efficient than the wild-type Pap1 in adding a second one. Y224F Pap1 could also readily extend the primer by one dAMP, but unlike wild-type and the Y224S enzyme, it was barely able to incorporate the second one (Fig. 3A).

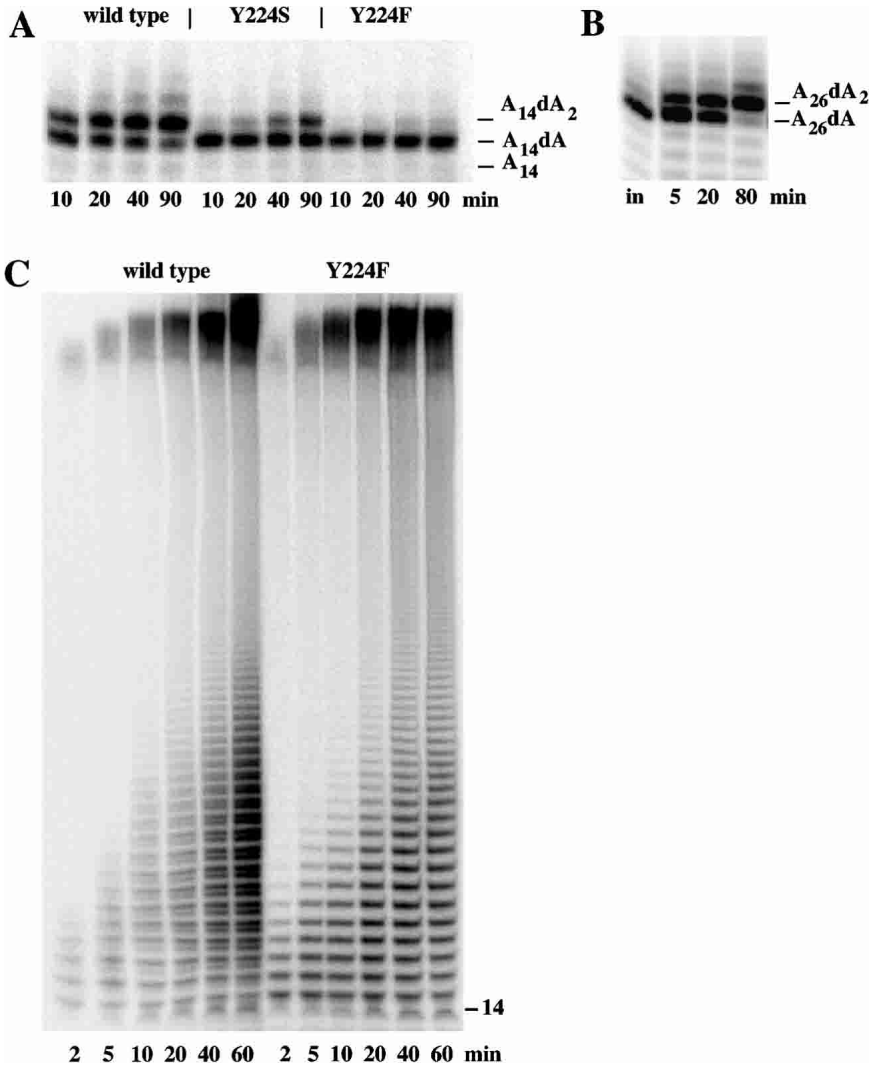

FIGURE 3. Utilization of dATP substrates by Pap1 and Y224F and Y224S mutants. (*A*) Incorporation of dAMP residues. Equal units (based on ATP polymerization) of the different forms of Pap1 were incubated with radioactive dATP and unlabeled oligo$(A)_{14}$ for the indicated times, followed by separation of the reaction products on a denaturing polyacrylamide gel and visualization by PhosphorImager analysis. The positions of substrate and products are indicated on the right. (*B*) Utilization of primer with a single dAMP at the 3′ end. Oligo$(A)_{26}$dA terminating in dAMP was synthesized with Y224F Pap1 and radioactive dATP, purified, and used as substrate in reactions with Y224F Pap1. The lane marked "in" shows the input material. (*C*) Misincorporation of dAMP into poly(A) tail. Reaction conditions are the same as for Figure 2A except that a mixture of radioactive dATP and unlabeled ATP was used.

A difference in wild-type Pap1 and Y224F in the ability to extend dAMP-terminated RNA was also observed in the misincorporation of dAMP into poly(A) tail in a reaction using radioactive dATP, unlabeled ATP, and oligo$(A)_{14}$ primer (Fig. 3C). When the products of these reactions are analyzed by denaturing gel electrophoresis, the smaller products in the case of wild-type Pap1 can be resolved into doublets. This pattern of products was reproducible and reflects the different gel mobility of oligo$(A)_n$ with dAMPs located internally and/or at the 3′ end. In contrast, Y224F produced single poly(A) bands. Partial digestion of reaction products with snake venom phosphodiesterase showed that radioactivity could be removed much more quickly from poly(A) formed by Y224F than from that made by wild-type enzyme (data not shown), confirming that the Y224F products contained radioactive dAMP primarily at the 3′ ends, and not in the body of the poly(A) like the wild-type PAP products.

One interpretation of these data with dATP is that Y224 is not involved in recognition of the hydroxyls of the incoming ATP, but instead in discerning the presence of 2′ hydroxyls at the 3′ end of a primer RNA. However, Y224F PAP when adjusted by specific activity could add ATP to single-stranded DNA to the same extent as wild-type polymerase (data not shown; Zhelkovsky et al. 1998). To further test the ability of Y224F to utilize substrate with a terminal dAMP, oligomers with these ends were synthesized using Y224F PAP and radioactive dATP, purified (Fig. 3B, input lane), and added to new reactions in the presence of dATP. In such an assay, Y224F could add a second dAMP to these substrates but again slowed after the first nucleotide addition (Fig. 3B). Taken together, these results suggest that the Y224F enzyme can interact productively with a substrate possessing terminal dAMP if the substrate is presented exogenously, but not if that substrate was created by the same enzyme molecule. Whereas wild-type Pap1 is capable of slow recycling in this reaction, the "increased specificity" of the Y224F mutant is most likely due to a decreased ability to recycle dAMP-terminated RNA.

In summary, our mutational analysis has shown that amino acids in the middle domain of Pap1 (K215, Y224, and N226) predicted to interact with ATP have instead a role in how the enzyme handles RNA. The increased K_m for primer, especially that seen with the K215 and N226 mutants when acting distributively in the presence of Fip1, and the wild-type levels of C-RBS cross-linking of the Y224 mutants, implies a defect in interaction with the 3′ end of the RNA. However, based on a common mechanism of polymerization among the members of the nucleotide transferase family, it is not sterically possible for the 3′ end of the RNA to be in an appropriate position for catalysis and simultaneously con-

tact the region of the middle domain containing K215, Y224, and N226 (Fig. 1A).

How then can these observations be interpreted in terms of enzyme mechanism? One possibility is that these substitutions cause a defect in removal of the pyrophosphate product, which in turn may prevent a conformational change that is needed to firmly bind and position the 3′ end, but does not interfere with ATP binding. This explanation would be consistent with the more open structure seen in Figure 1B, in which K215 and Y224 interact with pyrophosphate, yet ATP is correctly oriented for catalysis. The nucleotide occupying the primer site also appears to be appropriately placed for catalysis; however, interaction with a true primer end may require additional contacts only formed in a more closed conformation.

A second possibility is that these amino acids in Pap1 interact not with the primer, but with the RNA product. The recently published structure of terminal deoxytransferase (TdT; Delarue et al. 2002) lends support to this interpretation. TdT, like Pap1, belongs to the nucleotidyltransferase superfamily, and adds nucleotides to single-stranded DNA in a template-independent fashion. Figure 4 shows a superimposition of the binary complexes of Pap1/3′dATP and TdT/ssDNA, using the catalytic aspartate triad to align the two structures. The alpha phosphate of 3′dATP in the Pap1 complex overlaps with the last phosphodiester bond of the ssDNA, which in the TdT complex is thought to occupy the product position. If the RNA product of Pap1 is in a similar orientation, Y224 of Pap1 would be about 2.9 Å from the hydroxyl of the last primer nucleotide and capable of interacting with this moiety on the RNA.

This model would also explain our results with the Y224 mutants, if these substitutions decrease the ability of Pap1 to remove RNA product from the active site. Our data with Y224F indicate that this function depends on the presence of a hydroxyl group at position 224, and to a lesser extent, the correct positioning of this hydroxyl, in the case of Y224S. It is also facilitated by a 2′ hydroxyl on the recently extended RNA. Even though Y224 does not appear to be involved in discriminating the 2′ hydroxyls of RNA in the primer site, it may provide a different level for discrimination of ribose-containing substrates through slower translocation of a dAMP-terminated RNA from the product position. This might also increase the likelihood of pyrophosphorolysis and removal of the 2′-deoxyadenosine through the reverse reaction. In conclusion, our results are most consistent with the middle domain of PAP interacting directly with products of the reaction, the RNA and pyrophosphate, as a transition stage in reorganizing the active site for catalysis. However, a more thorough understanding of this mechanism will require structural analysis of the ternary complex of PAP, ATP, and RNA.

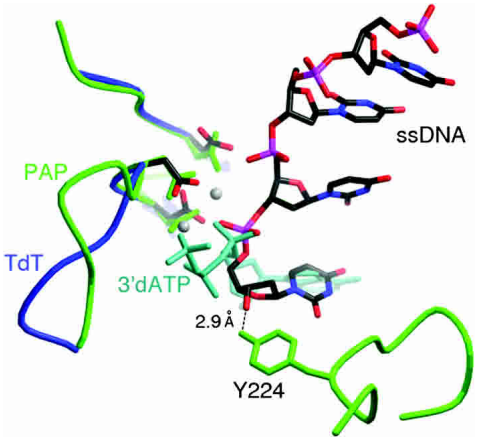

FIGURE 4. Superimposition of the binary complexes of Pap1/3′dATP and TdT/ssDNA (Delarue et al. 2002), using the alpha carbons of the three catalytic aspartates to align the two structures. Regions of Pap1 are depicted in green and TdT in purple. Also shown are manganese ions as gray balls.

MATERIALS AND METHODS

Nucleic acids and mutagenesis

Radioactive nucleotide triphosphates were from Perkin Elmer and nonradioactive ones from Promega. Oligo$(A)_{14}$ and oligo$(A)_{26}$ were from Oligo Etc. Mutagenesis was performed by PCR of a Pap1 template using a 3′ primer for K215A (TTTGGGCCCATA GCGCAATAGCTCTTA) with a point mutation adjacent to the ApaI site and a 5′ primer adjacent to the SacI site (CATATGAG CTCTCAAAAGGTTTTT), and 5′ primers for Y224F/S (TGGGCC CAAAGAAGGGCTGTTTYTGCTAATATT) (Y = C + T), N226A (TATGGGCCCAAAGAAGGGCTGTTTATGCTGCTATTTTTG), and F230Y (TGGGCCCAAAGAAGGGCTGTTTATGCTAATAT TTTTGGTTATCCTGGTGGT) with point mutations adjacent to the ApaI site and with the 3′ primer (GTTTACGTCAACAGCTG CGGTTGT) adjacent to the PvuII site, and high fidelity DeepVent polymerase (New England Biolabs). PCR products were subcloned into pJPAP plasmid (a kind gift of J. Lingner and W. Keller [Biozentrum, Univ. of Basel, Switzerland]; Lingner et al. 1991) by replacing the corresponding part of the wild-type sequence. Plasmids containing the correct mutation were determined by DNA sequencing.

Protein expression and purification

Recombinant wild-type and mutant Pap1, as well as Fip1, were expressed using the T7 expression system (Studier et al. 1990) and purified to near homogeneity as described elsewhere (Zhelkovsky et al. 1995, 1998).

Pap1 assays

Pap1 assays were performed as described previously (Zhelkovsky et al. 1998; Helmling et al. 2001) in 10 µL of solution containing 20 mM HEPES-KOH (pH 7.5), 10% glycerol, 1 mM $MnCl_2$, 50

mM KCl, 0.25 mM EDTA, 0.5 mM dithiothreitol, 0.5 mg/mL bovine serum albumin, 250 μM NTP or dNTP, 1 μCi [^{32}P]NTP or [^{32}P]dNTP, 10 μM oligoA_{14} or oligoA_{26} primer, and 20–250 ng of Pap1 or mutant Pap1 at 30°C for various periods of time. For kinetic studies, at least three independent reactions with variable concentrations of oligo$(A)_{14}$ (1–500 μM range) or ATP (25–250 μM range) and 20 ng of Pap1 (32 nM) were used, and product accumulation sampled over a 10–15-min time course, during which the reaction rate remains linear. Reaction products were quantitated by Cherenkov counting of acid-precipitable counts. For determination of the K_m for ATP, primer was used at a concentration of 50–60 μM. For processivity assays, the amounts of mutant and wild-type proteins that gave equal units of activity in polymerization of ATP were first determined experimentally using the above assay. The products formed over time in reactions using equal units of activity were then analyzed by fractionation on 18% polyacrylamide-8.3 M urea gels and visualization with a PhosphorImager (Molecular Dynamics). UV cross-linking of Pap1 was performed as described earlier (Zhelkovsky et al. 1995) using randomly labeled GAL7–9 RNA and 100 ng of protein per 10 μL reaction.

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