Mouse CAF1 Can Function As a Processive Deadenylase/3′–5′-Exonuclease in Vitro but in Yeast the Deadenylase Function of CAF1 Is Not Required for mRNA Poly(A) Removal*

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The mouse CAF1 (mCAF1) is an ortholog of the yeast (y) CAF1 protein, which is a component of the CCR4-NOT complex, the major cytoplasmic deadenylase of Saccharomyces cerevisiae. Although CAF1 protein belongs to the DEDD family of RNases, CCR4 appears to be the principle deadenylase of the CCR4-NOT complex. Here, we present evidence that mCAF1 is a processive, 3′–5′-RNase with a preference for poly(A) substrates. Like CCR4, increased length of RNA substrates converted mCAF1 into a processive enzyme. In contrast to two other DEDD family members, PAN2 and PARN, mCAF1 was not activated either by PAB1 or capped RNA substrates. The rate of deadenylation in vitro by yCCR4 and mCAF1 were both strongly influenced by secondary structures present in sequences adjacent to the poly(A) tail, suggesting that the ability of both enzymes to deadenylate might be affected by the context of the mRNA 3′-untranslated region sequences. The ability of mCAF1 to complement a ycaf1 deletion in yeast, however, did not require the RNase function of mCAF1. Importantly, yCAF1 mutations, which have been shown to block its RNase activity in vitro, did not inactivate yCAF1 in vivo, and mRNAs were deadenylated in vivo at nearly the same rate as found for wild type yCAF1. These results indicate that at least in yeast the CAF1 RNase activity is not required for its in vivo function.

The control of the rate of mRNA degradation is important to regulating the abundance and translation of mRNA in the cell. In eukaryotes a major pathway of mRNA degradation involves a poly(A)-shortening step followed by removal of the mRNA cap structure and 5′–3′ degradation of the body of the mRNA by the XRN1 exonuclease (1). Deadenylation of the poly(A) tail is also a prerequisite to 3′–5′ degradation of the message by the exosome, most notably in control of nonsense-mediated decay (2, 3). Other analysis indicates that at least in yeast changes in the rate of mRNA deadenylation contribute to the greatest changes in the rates of mRNA degradation (4). Identification of the functional deadenylases in eukaryotes and characterization of their properties is, therefore, most important to understanding the mRNA degradation process.

In the yeast Saccharomyces cerevisiae the major cytoplasmic deadenylase is the CCR4-NOT complex (5–8). CCR4 is the principle deadenylase within this complex and is a member of the exo III family of nucleases (9). PAN2/PAN3 also functions as a deadenylase in yeast and is involved particularly in trimming of the poly(A) tail (5, 10). Both the CCR4-NOT complex and PAN2/PAN3 are evolutionarily conserved (11–13) and hCCR4 and hPAN2 display deadenylase activity in vitro (7, 14). Higher eukaryotes also contain the PARN deadenylase, which is a principle component of cell-free extracts capable of efficient poly(A) shortening (15). Both PAN2 and PARN are members of the DEDD family of RNases (16, 17). PARN is specifically activated by the poly(A)-binding protein (PAB1), whereas PARN becomes activated in the presence of the mRNA cap structure (15).

A fourth deadenylase CAF1 (also known as POP2) exists within the CCR4-NOT complex. CAF1, which binds CCR4 and links it to the remainder of the CCR4-NOT complex (11, 18), is a member of the DEDD family of RNases (16). In vitro studies have demonstrated that yeast CAF1 can display deadenylase activity (8, 19), but the role of this activity in vivo is unclear. Removal of CAF1 does not impair CCR4 activity in vitro (5, 7), whereas mutations in the CCR4 putative catalytic residues abolish CCR4-NOT deadenylase function in vivo (6, 7). Although a caf1 deletion reduces the rate of in vivo poly(A) shortening, overexpression of CCR4 can complement this defect. In contrast, overexpression of CAF1 can not complement a ccr4 defect (5, 20). All of these data point toward CCR4 as comprising the major functional deadenylase activity in yeast.

Previously (7), we have been unable to detect enzymatic activity in yeast CAF1 (yCAF1).¹ Because the mouse CAF1 (mCAF1) protein displays a greater homology to the canonical RNase motifs in the DEDDh family of proteins (17), we decided to characterize the putative deadenylase function of mCAF1. mCAF1 has also been shown previously to partially complement a ycaf1 deletion in yeast (21) and to interact very strongly with yeast CCR4 and NOT1 (11),2 suggesting that it can act within a functional CCR4-NOT complex in vivo. To address the role of CAF1 in mRNA degradation, we have purified the mCAF1 to homogeneity and studied its in vitro and in vivo properties.

We find that mCAF1 is a robust 3′–5′ processive exonuclease with a preference for poly(A) substrates. mCAF1, unlike PARN, deadenylates both capped and non-capped substrates and un-

¹ The abbreviations used are: yCAF1, yeast CAF1; mCAF1, mouse CAF1; GST, glutathione S-transferase; nt, nucleotide.
² Y-C. Chiang and C. L. Denis, unpublished observations.
like PAN2 is inhibited by PAB1. mCAF1 deadenylase activity was found, however, to be 1000–10,000-fold less than that of CCR4. Both mCAF1 and CCR4 deadenylase function was inhibited by stem structures in regions upstream of the poly(A) sequence, suggesting that both proteins may recognize long stretches of RNA (22). Most importantly, mutations in either yCAF1 or mCAF1 that completely abrogate the in vitro function of each protein had no effect on the ability of either protein to functionally complement a yeast caf1 deletion. The yCAF1 deadenylase-deficient protein, moreover, allowed normal deadenylation of mRNA in yeast. These results indicate that CAF1 deadenylase activity is not required for in vivo deadenylation nor for the other important roles of CAF1 in vivo.

MATERIALS AND METHODS
Yeast Strains and Growth Conditions—The following yeast strains were used: A790, MATa aro7 his3 leu2 ura3; A792, isogenic to A790 except caf1::LEU2; EGY186-c1, MATa ura3 Leu2-ADE2 trp1 his3 caf1::URA3; and RP1620, MATa cap1::LEU2/PM his4-539 ccr4::NEO pan2::URA3 ura3-53 leu2-3,112 trp1. In vivo deadenylation of the GAL1 mRNA was monitored in strain KY803-c1 containing LexA-CAF1 proteins. Following growth on YEP (1% yeast extract, 2% bactopeptone) medium supplemented with 2% galactose, 2% raffinose for 8 min, repression of transcription was initiated with the addition of 4% glucose as described (23). Caffeine sensitivity was monitored on medium containing YEP, 9 mM caffeine, 2% agar, and either 2% glucose or 2% galactose as the carbon source.

Plasmids—The GST-mCAF1 plasmid was a kind gift from A. Sakai (21). Point mutations (D39A/E41A and Y159A/D160A) of GST-mCAF1 were generated and inserted into the pGEX-KG plasmid. All the plasmids were expressed in the BL21 Escherichia coli strain prior to purification of GST proteins.

RNA Substrates—The pT7A38 plasmid containing MFA2 was the kind gift of S. Singer (Oak Ridge National Laboratory, Oak Ridge, TN), and the MFA2 RNA was synthesized as described (24). Briefly, DNA was prepared by Qiagen mini-prep, cut with HindIII, and treated with mung bean nuclease to trim the overhangs. The linearized DNA was transcribed, and synthesis of capped and non-capped MFA2 RNA was prepared with the Maxiscript in vitro transcription kit (Ambion) using [α-32P]UTP as a radioactive probe. The transcribed RNA was eluted and quantified as described by the manufacturer of the kit. The capped transcription RNA substrate was 25N-poly(A)20A (22) and 50N + 10A containing 50 nucleotides (nt) of the 3′ untranslated regions of COX17, and AHA1 (see Fig. 4B) RNAs with 10 adenine residues at the 3′end, 50N + 10A hybrid COX17 and AHA1 chimera RNAs containing 25 nt of COX17 or AHA1 attached, respectively, to 25 nt of AHA1 and COX17 with 10 residues at their 3′end (see Fig. 4B), and the 50N + 10A COX17 stem that had the sequence as depicted in Fig. 5B.

Protein Preparations—GST-mCAF1 protein expression and purification on glutathione-agarose beads were performed as suggested by the manufacturer with slight modifications. The E. coli cells were collected and sonicated with Tris-HCl buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM MgCl2) plus a protease inhibitor mixture. The cleared supernatants were incubated with pre-equilibrated glutathione-agarose beads for 4 h at 4 °C. After extensive washes the GST-mCAF1 proteins were eluted with Tris buffer containing 50 mM glutathione or mCAF1 proteins purified following cleavage with factor-α or thrombin to remove the mCAF1 protein from the host GST fusions. The Flag-yCCR4 was prepared by anti-Flag beads purification as described previously (7).

In Vitro Deadenylase Assays—In vitro enzymatic assays with commercially synthesized RNA or in vitro transcribed RNA were conducted with CCR4 as described previously (22) and similarly with mCAF1 except the following buffer was used, 50 mM Tris-Cl, pH 7.9, 50 mM NaCl, 1 mM MgCl2, 0.1 units of RNase inhibitor, 10% glycerol. The RNA substrates were radiolabeled as indicated (7). In vitro enzymatic assays with total RNA extracted from strain RP1620 was conducted as described above, but the products were visualized by Northern analysis following the separation of the RNA in gels containing 8 M urea, 6% polyacrylamide. Radiolabeled p-g-specific probe (5) that was specific to the poly(G) tract in MFA2pG was used to probe the Northern blot. The mCAF1 kinetic assays were conducted as described for CCR4 (22).

RESULTS
mCAF1 Is a Deadenylase/3′-5′-Exonuclease—We have shown previously that mammalian CCR4 can function as a deadenylase in vivo (7). The strong homology of mammalian CAF1 to that of members of the DEDD RNases suggested that it too may function as a deadenylase. To examine mCAF1 function in vitro we purified it to homogeneity (Fig. 1A, lane 6). Incubation of in vitro synthesized MFA2 RNA containing 38 A residues at its 3′end (24) (Fig. 1B, lanes 1–4) indicated that mCAF1 processively removed the poly(A) tail and proceeded as a 3′-5′-exonuclease into the body of the mRNA before stalls at a site 99 nt into the RNA. Point mutations in the conserved putative catalytic residues in the exo I domain of mCAF1, D39A/E40A, and the exo II domain, Y159A/D160A, completely abolished the exonuclease activity (Fig. 1B, lanes 5–12). Similar results with these mutants were obtained with several other substrates and with GST-mCAF1 versions of the mCAF1 variants (data not shown), establishing that the enzymatic activity observed is because of mCAF1. It should be noted in Fig. 1B, lane 4 (identified with an asterisk) that mCAF1 formed a small amount of the MFA2-1 or 0 A product as if it were pausing at this site before proceeding into the body of the mRNA. Using increasing concentrations of mCAF1 (Fig. 1C), the MFA2-1A form becomes more apparent. The 267-nt final product was not the result of an endonucleolytic cleavage as no 99-nt fragment was observed even at increased mCAF1 concentrations (Fig. 1C). The 267-nt stall site occurs just prior to the presence of the first stable stem-loop structure in the MFA2 RNA (24). These results indicate that mCAF1 is a 3′-5′-processive RNase that displays a preference for poly(A) sequences.

Substrate Specificity of mCAF1—Optimization of the mCAF1 enzyme reaction conditions indicated that mCAF1 functioned well with Mg2+ concentrations from 0.5 to 1 mM and with Na+ concentrations of 50–100 mM. Because the PARN deadenylase, also a member of the DEDD family of proteins, is much more active with capped mRNA substrates, we examined the activity of mCAF1 with capped and uncapped substrates. In vitro synthesized capped and non-capped MFA2 RNAs were degraded by mCAF1 at the same rates (data not shown). As in vitro transcribed RNA may only be partially capped in vitro, we prepared total RNA from yeast cells and analyzed the ability of mCAF1 to degrade the capped MFA2pG RNA. Uncapped MFA2pG was prepared by hybridizing an oligonucleotide to the start codon of MFA2 followed by digesting with RNase H prior to incubation with mCAF1. This procedure cleaved ~80 nt of the 5′-end of the RNA including the cap structure. Both capped and non-capped MFA2pG RNAs were incubated with mCAF1, and the products were detected by a probe specific to MFA2pG (Fig. 2). Equal rates of deadenylation were observed for capped and uncapped MFA2pG as well as for the pG-poly(A) fragment that results from decapping and 5′–3′-RNase digestion of the RNA in vivo (23). These results indicate that mCAF1 is not a cap-dependent RNase. It should be noted, however, that MFA2pG when present in a complex mixture of total RNA was degraded in a distributive fashion by mCAF1. Degradation beyond the poly(A) tract into the body of the mRNA also did not occur within the time period analyzed, consistent with a preference of mCAF1 for poly(A) substrates. We also determined the effect of PAB1 on mCAF1 activity. PAB1 inhibited the ability of mCAF1 to deadenylate a 25N+20A substrate and did so to the same extent as it inhibited CCR4 activity (data not shown) (6).

To determine the kinetic parameters of the deadenylase activity of mCAF1, a 25N+20A RNA substrate was used (22). Typical reactions at different substrate concentrations are displayed in Fig. 3. With this short RNA molecule, mCAF1 be-
had like a distributive enzyme and paused at the 25N+2A state before proceeding more slowly to the 25N+1A and even more slowly to the 25N+0A. In contrast, CCR4 paused only at 25N+1A with this same substrate (22). We determined the $K_m$ for mCAF1 with this substrate to be 24.4 ± 2.6 μM and its $k_{cat}$ to be 0.18 ± 0.02 A/s. The $K_m$ value for mCAF1 was 7-fold higher than it was for yeast CCR4 with a similar substrate (22), whereas the $k_{cat}$ value was 1000-fold lower.

Deadenylation by mCAF1 and γCCR4 Is Affected by Secondary Structure in Upstream Non-poly(A) Sequences—We have shown previously (22) that CCR4 prefers substrates of at least 17 nt in length, not necessarily poly(A), suggesting that the non-poly RNA region was capable of being recognized by the enzyme. The importance of non-poly(A) sequences to mCAF1 activity was investigated by examining the poly(A) removal rate of mCAF1 on synthetic 50N+10A RNA fragments modeled on the 3'-untranslated region sequences of COX17 and AHA1 (Fig. 4A). The 50N+10A COX17 RNA was degraded very rapidly (processively within 5 min) to the 50N+2A form and after that to near completion apparently, as no smaller fragments were observed, and the 50N+0A fragment disappeared at 15 and 30 min time intervals. In contrast the AHA1 50N+10A RNA was deadenylated much more slowly and resulted in a 50N+2A product. The rate of formation of the COX17 50N+2A

FIG. 1. A, SDS-PAGE analysis of mCAF1 proteins. GST-mCAF1 proteins were analyzed on a 10% denaturing polyacrylamide gel and stained with Coomassie Blue. Lanes 1–4, GST and GST-mCAF1 proteins purified following glutathione-agarose chromatography. Lanes 5 and 6, GST and GST-mCAF1 cleaved with factor-a. Lanes 7 and 8, and GST-mCAF1 mutant proteins cleaved with thrombin. Lanes 1 and 5, control GST proteins; lanes 2 and 6, GST-mCAF1; lanes 3 and 7, GST-mCAF1 (D39A/E41A); lanes 4 and 8, GST-mCAF1 (Y159A/D160A). The molecular mass of the proteins (in kilodaltons) is indicated on the left. B, mCAF1 displays the 3’–5’-exonuclease activity with in vitro synthesized MFA2 RNA. Deadenylation reactions were performed as described under “Materials and Methods.” 5-μl aliquots were removed from a 30-μl reaction volume at the indicated time periods, the RNA products were separated by electrophoresis on a 6% polyacrylamide, 8 M urea gel, and the products were visualized with a PhosphorImager. The asterisk denotes the MFA2-1A or -0A product. C, mCAF1 is not an endonuclease. A uniformly labeled MFA2 RNA substrate was incubated in a 30-μl reaction volume containing purified mCAF1 (5 μg as 1X), and aliquots were removed at the indicated time. In vitro synthesized Ambion RNA markers are shown on the left.
Fig. 2. In vitro deadenylation of capped and uncapped in vivo synthesized MFA2pG transcripts. Total yeast mRNA extracted from strain RP1620 was incubated with mCAF1 for the times indicated, and the products were visualized by Northern analysis. Capped mCAF1 refers to untreated RNA and uncapped RNA refers to RNA incubated with a oligonucleotide sequence complementary to the 5’-end of MFA2 and digested with RNase H. The MFA2pG fragment that results following in vivo decapping and 5’-3’-exonuclease activity is indicated at the bottom of the figure. The dT-treatment of capped and uncapped RNA with (dT) oligomer and RNase H was conducted to indicate the size of the deadenylated RNA. The asterisks indicated two things about mCAF1 enzyme activity. First, the rate of deadenylation of RNAs can be influenced by non-poly(A) sequences. We therefore tested the relative ability of mCAF1 and CCR4 to deadenylate through a stem structure that was embedded within the poly(A) tail. Using a 50N+2A substrate fused to AHA1 was in sequences upstream of the poly(A) sequence, this conclusion could not be made because the stem structure may be less sensitive to the stem structure than mCAF1.

We also examined the ability of CCR4 to shorten poly(A) tails with these same RNA substrates. With COX17 RNA CCR4 exhibited rapid and processive deadenylation (Fig. 4C, lanes 1–4), whereas AHA1 was slowly and distributively deadenylated by CCR4 (lanes 5–8). As observed with mCAF1, the 25-nt proximal sequences of AHA1 inhibited CCR4 activity (Fig. 4C, lanes 5–12, relative rates are summarized in Table I), suggesting these proximal sequences are also affecting CCR4 deadenylation of RNAs. In contrast to mCAF1, however, CCR4 was able to deplete AHA1 to the 50N+0A form suggesting it may be less sensitive to the stem structure than mCAF1.

The previous results indicate that mCAF1 can not degrade RNA upon encountering a stem structure. For CCR4, however, this conclusion could not be made because the stem structure for AHA1 was in sequences upstream of the poly(A) sequence, and CCR4 does not readily degrade RNA through non-poly(A) sequences. We therefore tested the relative ability of mCAF1 and CCR4 to deadenylate through a stem structure that was embedded within the poly(A) tail. Using a 50N+10A substrate based on the COX17 sequences, we created a stem encompassing 5 of the As in the 10A tail (Fig. 5A). As expected mCAF1 had difficulty depleting RNA through the stem structure of COX17 stem RNA and actually paused two and three nucleotides before encountering the stem (Fig. 5B). CCR4 in contrast, although obviously slowed by the stem as compared with COX17 50N+10A, distributively deadenylated through the stem struc-

Fig. 3. Deadenylase assays for determining the kinetic parameters of mCAF1. The indicated concentrations of 5'-labeled 25N+2A substrates were incubated with 20 μg of pure mCAF1 under standard assay conditions for the times indicated. The products were separated on a 15% polyacrylamide, 8M urea gel and visualized by a PhosphorImager.

The rate of deadenylation of RNAs can be influenced by non-poly(A) sequences that lie within 25 nt of the poly(A) sequences. Second, mCAF1 can function as a strong processive enzyme and stalls within 2–4 nt downstream of a stem structure.

Fig. 4. Deadenylation of AHA1 and COX17 RNA with mCAF1. AHA1 and COX17 RNAs. As shown in Fig. 4B, COX17 displays little secondary structure, whereas AHA1 forms two stem structures one of which occurs just upstream of the 10A tail. The inability of mCAF1 to deplete beyond the 50N+2A form of AHA1 suggests that mCAF1 activity is strongly inhibited by stem structures.

To examine this further we created hybrid 50N+10A substrates that contained either the 5’-25 nt of COX17 followed by the 3’-25 nt of AHA1 or inversely the 5’-25 nt of AHA1 fused to the 3’-25 nt of COX17. The predicted structures for these two RNA fragments are shown in Fig. 5B. COX17+AHA1 50N+10A RNA was slowly degraded to the 50N+2A form, whereas AHA1+COX17 was rapidly deadenylated to the 50N+2A form and then further degraded to two smaller species marked with asterisks in Fig. 4A. These smaller species correspond to stall sites immediately downstream of the major stem-loop structure of the AHA1+COX17 RNA (Fig. 4B). These results indicate that mCAF1 enzyme activity. First, the rate of deadenylation of mCAF1 can be influenced by non-poly(A) sequences that lie within 25 nt of the poly(A) sequences. Second, mCAF1 can function as a strong processive enzyme and stalls within 2–4 nt downstream of a stem structure.

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product was estimated to be at least twice that of the rate of formation for the AHA1 50N+2A product (Table I). The difference in the behavior of these two RNAs could be attributed to sequences or secondary structures present on the 50N segment of COX17 and AHA1 RNAs. As shown in Fig. 4B, COX17 displays little secondary structure, whereas AHA1 forms two stem structures one of which occurs just upstream of the 10A tail. The inability of mCAF1 to deplete beyond the 50N+2A form of AHA1 suggests that mCAF1 activity is strongly inhibited by stem structures.

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Fig. 4. A, mCAF1 deadenylase activity is influenced by non-poly(A) sequences. 200 nmol of 5’-radiolabeled RNAs as indicated were incubated with 10 μg of mCAF1 for the times indicated. RNAs were resolved in a 12% polyacrylamide, 8 M urea gel and visualized with a PhosphorImager. The asterisks denote the intermediates that accumulated during the exonuclease activity of mCAF1. B, secondary structure model of synthetic 50N+10A RNAs. The figure was drawn with the help of the mFOLD program at the RNA server (30) available via bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi. Arrow marks indicates the stalled positions following incubation with mCAF1. C, non-poly(A) sequences influence CCR4 deadenylase activity. 200 nmol of radiolabeled 50N+10A RNA substrates as indicated were incubated with 300 pg of purified CCR4 protein using a standard CCR4 deadenylation assay (22). Products were resolved on a 12% polyacrylamide, 8 M urea gel and visualized with a PhosphorImager.
ture with no obvious pause sites (Fig. 5B, lanes 13–16). These data and data described above indicate that both mCAF1 and CCR4 deadenylation function can be influenced by upstream secondary structures not located in the poly(A) region and that mCAF1 has difficulty deadenylating through stem structures.

**The Deadenylase Function of yCAF1 and mCAF1 Are Not Required in Yeast**—Because both yCAF1 (8, 19) and mCAF1 have been shown to display deadenylase functions in vitro, we determined subsequently whether the activities of yCAF1 and mCAF1 were required in vivo. LexA-yCAF1 proteins were created containing either the S199A/E201A alteration that inactivated yCAF1 deadenylase function in vitro (19), which is comparable with the D39A/E41A mutation in mCAF1 that blocked its deadenylase activity (Fig. 1B), or the Y320A/D321A mutation that is comparable with the Y159A/D160A of mCAF1 that inhibited its activity. The expression of LexA-yCAF1 (S199A/E201A) and LexA-yCAF1 (Y320A/D321A) completely complemented the caffeine sensitivity of the ycaf1 deletion (Fig. 6 and data not shown) as did a LexA-yCAF1 fusion carrying all four mutations (data not shown).

We additionally analyzed the ability of LexA-yCAF1 (S199A/E201A) to allow deadenylation of the GAL1 mRNAs in vivo. GAL1 mRNA is polyadenylated at two locations 110 nt apart (25, 26). Following the induction of GAL1 mRNA synthesis for 8 min with galactose and repression at time zero with glucose, the rate of deadenylation of the two GAL1 mRNAs was followed in a ycaf1 strain containing LexA-yCAF1, LexA-yCAF1 (S199A/E201A), or LexA. LexA-yCAF1 allowed an efficient deadenylation of both GAL1 long and GAL1 short mRNA (Fig. 7), and CCR4 deadenylated the two mRNAs at rates of 4.7 and 3.4 As/min, respectively. In contrast, in a caf1 background expressing only LexA, deadenylation was slowed for both mRNA (rates of 1.9 and 1.3 As/min, respectively). Incomplete deadenylation was also observed at late times in the caf1 background (Fig. 7) in agreement with previous observations that caf1 causes a deadenylation end-point defect (5). In contrast, the caf1/LexA-

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**TABLE I**

Relative rates of deadenylation activity with RNA containing different non-poly (A) sequences

| 50N+10A RNA | Relative rate mCAF1 | CCR4 |
|-------------|---------------------|------|
| COX17       | 100                 | 100  |
| AHA1        | 49 ± 4.5            | 69 ± 6.5 |
| COX17 + AHA1| 45 ± 2.5            | 50 ± 10 |
| AHA1 + COX17| 112 ± 3.2           | 83 ± 12 |

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Fig. 5. A, a stem structure stalls deadenylation activity of mCAF1 and CCR4. Radiolabeled 50N+10A COX17 and 50N+10A COX17 stem RNAs were incubated with 10 μg of mCAF1 and 300 pg of CCR4 proteins as described in the legend to Fig. 4. The asterisk denotes the 50N+7A product. The concentration of mCAF1 and CCR4 was adjusted so as to give relatively similar rates of reaction with COX17 50N+10A. B, secondary structure of COX17 stem RNA. The predicted secondary structure for COX17 stem RNA was obtained as described in the legend to Fig. 4.
Deadenylase-deficient yCAF1 and mCAF1 genes complement caffeine-sensitive growth defects of the caf1Δ allele. The yCAF1/mCAF1 carried residues 1–147 of yCAF1 and the full-length version of mCAF1 (1–231) for the right panel, and in the left panel it carried residues 1–181 of yCAF1 and full-length mCAF1. The N-terminal residues 1–147 or residues 1–181 of yCAF1 are completely inactive in yeast (11). Growth was detected on rich medium containing glucose and 8 mM caffeine at 30 °C (18). Left panel, plasmids expressing LexA-yCAF1 (11) and the mutant versions as indicated were introduced into strain A792; caf1 refers to transformation with the LexA plasmid alone. Right panel, yCAF1 (strain A790), ycaf1, and yCAF1/mCAF1 derivatives were expressed from a yCAF1 promoter on a pRS316 plasmid and were transformed into strain A792.

yCAF1 (S199A/E201A) strain deadenylated both GAL1 mRNAs at comparable rates and to similar extents as that observed with LexA-yCAF1 (Fig. 7, rate of deadenylation for GAL1 long was 4.4 As/min and for GAL1 short was 2.7 As/min). These data indicate that the yCAF1 deadenylase function is not required in vivo in the deadenylation process. We also tested whether the deadenylase function of mCAF1 was required for complementing a caf1 deletion. A yCAF1/mCAF1 fusion (carrying the unique N-terminal of yCAF1 and the DEDDh domain of mCAF1) was used for this analysis, because it gives better complementation than does mCAF1 alone (21). We found that mCAF1 mutations (D39A/E41A and Y159A/D160A), which inactivated mCAF1 in vitro, like similar mutations in yCAF1 did not interfere with the ability of the yCAF1/mCAF1 hybrid protein to complement the caffeine sensitivity of a ycaf1 deletion (Fig. 6).

DISCUSSION

We demonstrate herein that mCAF1, a member of the DEDDh family of RNases, displays 3‘–5‘-exonuclease activity in vitro. Although non-poly(A) sequences were readily degraded by mCAF1, its preference was clearly for poly(A) sequences. The mCAF1 protein with long substrates was a strongly processive enzyme in contrast to yCAF1, which appeared to act distributively (19). With very short RNA substrates (25N+20A), mCAF1 could not efficiently degrade the RNA past the 25N+1A substrate (Fig. 4) and with longer substrates (COX17 50N+10A) or the 366-nt MFA2-38A mRNA, there is clear pausing also immediately following deadenylation. This ability of mCAF1 to also processively degrade non-poly(A) sequences makes mCAF1 unique among the four known eukaryotic deadenylases, CAF1, CCR4, PARN, and PAN2 (10, 14, 22, 27, 28). yCAF1 has also shown to act distributively with poly(U) and poly(C) substrates (19).

mCAF1 displays other differences in its substrate specificities when compared with the two other deadenylases in the DEDDh family, PARN and PAN2. PARN is activated specifically by the cap structure (28), whereas mCAF1 was not. mCAF1 was also inhibited by PAB1, unlike PAN2, which is activated by PAB1 (10, 14). mCAF1, therefore, more closely resembles CCR4, a member of the exo III nuclease family, in its substrate preferences. With short 45-nt substrates, mCAF1 was distributive in its mode of deadenylation, whereas with 60 nt or longer substrates mCAF1 acted primarily as a processive nuclease. CCR4 also shifted from the distributive to processive mode with substrates in which the length was at least 45 nt (22).

The effect of the length of the substrate on the processivity of mCAF1 and CCR4 enzymes may reflect the ability of these enzymes to bind significant lengths of RNA sequences. Consistent with this interpretation was the observation that non-poly(A) sequences upstream of the poly(A) tail had significant effects on the deadenylase activity of both mCAF1 and CCR4 (Fig. 5 and Table I). A stem structure in the 25 nt proximal to the poly(A) sequence, as present in the AHA1 50N+10A RNA, reduced the deadenylation activity of both mCAF1 and CCR4. Although we also showed that mCAF1 stalled in its RNase function when immediately encountering a stem structure, the effect of the upstream stem on mCAF1 and CCR4 activity occurred well before either enzyme was within a couple of
nucleotides of the stem. These observations indicate that the activities of both enzymes can be influenced by portions of the RNA sequence a significant distance upstream from the site of exonucleolytic cleavage, suggesting that the enzymes actually make physical contact with a large segment of the RNA sequence. The recent observation that the leucine-rich repeat region of CCR4 is vital for CCR4 deadenylase activity (29) suggests that a long surface of the CCR4 protein may be involved in its contact to the RNA and therefore is capable of recognizing a long stretch of RNA sequence.

The demonstration that both mCAF1 and yCAF1 display deadenylase activity in vitro raises the question of the importance of this deadenylase activity to mRNA degradation in vivo. We determined the $k_{cat}$ value for mCAF1 with the 25N+20A substrate, for which it was acting distributively, and found that this value was about 1000-fold less than that observed for CCR4 with similar substrates. Although kinetic parameters for the yCAF1 enzyme have not been reported (8, 19), an estimate based on the rate of yCAF1 deadenylation over several substrate concentrations (19) suggests that yCAF1 is similarly very inactive compared with CCR4. Although longer RNA substrates convert mCAF1 to a processive enzyme, these substrates also convert CCR4 to the processive mode, and comparisons of mCAF1- and CCR4-relative activities for a given amount of enzyme (Figs. 4 and 5) clearly indicate that mCAF1 is extremely slow in deadenylating processively as compared with CCR4. These in vitro data suggest that CAF1 may not contribute significantly to the in vivo deadenylation process.

Most importantly, mutations in yCAF1 putative catalytic residues, which are known to inhibit yCAF1 enzyme activity in vitro (S199A/E201A) (19) or are presumed to inhibit the activity based on corresponding mutations inhibiting mCAF1 (Y320A/D321A) (presented herein), did not impair the ability of yCAF1 to complement a caf1 deletion in vivo. We also showed that yCAF1 (S199A/E201A) resulted in deadenylation of the two different GAL1 mRNA rates that were comparable with what was observed for wild type CAF1. Similarly, mCAF1 (E39A/D41A or Y139A/D140A), which is enzymatically inactive in vitro, complemented a caf1 deletion in vivo as well as the wild type mCAF1 did. These data establish that the deadenylation activity of yCAF1 is not required for its in vivo functions and is not necessary for in vivo deadenylation of mRNA.

Based on the above considerations, we present the following model of CAF1 function in yeast in terms of mRNA deadenylation. CCR4 is the principal, if not sole, functional deadenylase in the CCR4-NOT complex. A caf1 deletion obviously reduces the rate of CCR4-dependent deadenylation in vivo and results in an inability to completely deadenylate mRNA, leaving an oligonucleotide (A) form of about 17 As (5, 6). Both of these phenotypes are capable of being suppressed by overexpression of CCR4 (6), suggesting that CAF1 plays a role in stabilizing CCR4 contacts with the mRNA, in allowing CCR4 access to the mRNA involved in restrictive messenger ribonucleoprotein structures, or in mediating CCR4 contacts to other proteins that control CCR4 deadenylation. Future studies with CAF1 will have to discriminate between these different models with regard to the important biological role CAF1 plays in mRNA degradation.

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Mouse CAF1 Can Function As a Processive Deadenylase/3′→5′-Exonuclease in Vitro but in Yeast the Deadenylase Function of CAF1 Is Not Required for mRNA Poly(A) Removal

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