

Identification of Multiple RNA Features That Influence CCR4 Deadenylation Activity*

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Palaniswamy Viswanathan, Junji Chen, Yueh-Chin Chiang, and Clyde L. Denist

From the Department of Biochemistry and Molecular Biology, University of New Hampshire,
Durham, New Hampshire 03824

The CCR4 family proteins are 3′–5′-deadenylases that function in the first step of the degradation of poly(A) mRNA. Here we report the purification to homogeneity of the yeast CCR4 protein and the analysis of its substrate specificities. CCR4 deadenylated a 7N+23A substrate (seven nucleotides followed by 23 A residues) in a distributive manner. Only small differences in CCR4 activity for different A length substrates were observed until only 1 A residue remained. Correspondingly, the K_m for a 25N+2A substrate was found to be at least 20-fold lower than that for a 26N+1A substrate, although their V_{max} values differed by only 2-fold. In addition, the total length of the RNA was found to contribute to CCR4 activity: up to 17 nucleotides (not necessarily poly(A)) could be recognized by CCR4. Poly(U), poly(C), and poly(G) were also found to be 12–30-fold better inhibitors of CCR4 compared with poly(A), supporting the observation that CCR4 contains a non-poly(A)-specific binding site. Surprisingly, even longer substrates (≥ 45 nucleotides) stimulated CCR4 to become a processive enzyme, suggesting that CCR4 undergoes an additional transition in the presence of such substrates. CCR4 also displayed no difference in its activity with capped or uncapped RNA substrates. These results indicate that CCR4 recognition of its RNA substrates involves several features of the RNA that could be sites *in vivo* for controlling the rate of specific mRNA deadenylation.

The regulation of eukaryotic gene expression occurs at multiple levels, including the control of mRNA stability. The poly(A) tail at the 3′-end of eukaryotic mRNA plays a major role in controlling mRNA and protein levels (1). In many cases, deadenylation of the poly(A) tail is the first step required for decapping and 5′ to 3′ degradation of the mRNA sequence (2, 3). Not only does rapid deadenylation lead to degradation of the mRNA and the shutoff of protein synthesis, but deadenylation itself reduces the efficiency of translation of mRNA (4). In yeast, mRNAs are deadenylated by first shortening the poly(A) tail to ~15 nucleotides, after which the 5′-cap is removed. Sequences within the RNA body play a role in controlling the rate of poly(A) removal as well as *trans*-acting proteins, including presumably the deadenylase itself (5, 6).

In yeast, the CCR4 protein, as part of the CCR4-NOT complex, has been shown to be responsible for cytoplasmic deadenylation (7–10). The CCR4 protein contains three major functional domains: an N-terminal activation domain, a central leucine-rich repeat (LRR)¹ domain that binds CAF1, and a C-terminal exonuclease III-like domain (9, 11, 12). The exonuclease III-like domain comprises the apparent CCR4 deadenylation function based on mutagenic studies (8, 9). We (9) and others (8) have shown that CCR4 is a 3′–5′-RNase and a single-strand DNA-specific DNase that acts in a distributive manner with strong preferences for poly(A) substrates. The CCR4 protein is enzymatically active even in the absence of the CAF1 protein and other components of the CCR4-NOT complex. In addition, point mutations of the putative catalytic residues in the exonuclease domain of CCR4 abrogate its *in vitro* and *in vivo* activities (8, 9). These observations strongly indicate that the CCR4 protein is the catalytic subunit of the mRNA deadenylase family of proteins, although CAF1 may display some deadenylase activity under certain conditions (10).

Model studies have indicated that alterations in deadenylation rates will have the greatest effects on changes in RNA degradation (13). We have therefore investigated the substrate preferences and enzymatic properties of CCR4 to understand the role CCR4 plays in selecting its mRNA substrates. We found that CCR4 prefers RNA substrates with at least 2 A residues at their 3′-ends. However, it was most efficient with substrates that were at least 17 nucleotides in length, although these sequences need not necessarily be poly(A). Inhibition studies further support a non-poly(A)-specific binding site for CCR4. Moreover, longer RNA substrates (at least 45 nucleotides) converted CCR4 to a processive enzyme. In addition, CCR4 by itself displayed no preference for capped substrates. These results suggest that the distributive-to-processive transition for CCR4 and the ability of CCR4 to contact a certain length or type of sequence may be sites by which other factors may regulate CCR4 deadenylase activity *in vivo*.

EXPERIMENTAL PROCEDURES

Plasmids and RNA Substrates—The full-length CCR4 open reading frame was cloned into a modified pYES2/CT vector (Invitrogen). The resultant CCR4 gene under the control of the *GAL1* promoter contained an N-terminal FLAG tag and a C-terminal His₆ tag. Expression of CCR4 was in strain KY803-1a-1 (*MAT α leu2-1 PET56 trp1-1 ura3-52 gal2 gcn4-1 ccr4::ura3*). Commercially synthesized RNA oligonucleotides are described in Table I. The RNA substrates were generally labeled with T4 polynucleotide kinase and [γ -³²P]ATP and further purified by gel filtration on Sephadex G-25 spin columns. The capped and uncapped 50N+5A and 50N+30A substrates (pT3-L3(A₅) and pT3-L3(A₃₀)) were synthesized *in vitro* as previously described (14). Poly(A) polymerase was used to attach radioactive 3′-AMP to the RNA sub-

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† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of New Hampshire, Rudman Hall, Rm. 387, 46 College Rd., Durham, NH 03824. Tel.: 603-862-2427; Fax: 603-862-4013; E-mail: cldenis@cisunix.unh.edu.

¹ The abbreviation used is: LRR, leucine-rich repeat.

TABLE I
RNA substrates

RNA substrate	Oligonucleotide sequence
26N+1A	5'-UAU GUG AAU UCU AUG CCA CCC CCC CCA-3'
25N+2A	5'-UAU GUG AAU UCU AUG CCA CCC CCC CAA-3'
12N+2A	5'-UGC CAC CCC CCC AA-3'
6N+2A	5'-CCC CCC AA-3'
22N+5A	5'-UAU GUG AAU UCU AUG CCA CCC CAA AAA-3'
12N+5A	5'-UGC CAC CCC CCC AAA AA-3'
6N+5A	5'-CCC CCC AAA AA-3'
7N+23A	5'-UCU AAA U + 23A-3'
25N+20A	5'-UAU GUG AAU UCU AUG CCA CCC CCC C + 20A-3'
12N+20A	5'-UGC CAC CCC CCC + 20A-3'
6N+20A	5'-CCC CCC + 20A-3'

strates as described (7), and the resultant labeled RNAs were gel-purified.

Protein Extraction and Purification—FLAG-CCR4-His₆ purification was conducted by a few modifications as described previously (9). The yeast culture containing FLAG-CCR4-His₆ fusion protein was grown in a selective medium with 4% glucose to $A = 1.0$ and then shifted to 4% galactose/raffinose for growth to $A = 1.5$. The cells were washed and lysed in Tris acetate buffer (buffer A: 50 mM Tris HOAc (pH 7.9), 150 mM KOAc, 0.1 mM MgCl₂, 10% glycerol, and 0.1% Nonidet P-40) plus a protease inhibitor mixture. After clarification of the crude lysate (15,000 × *g* at 4 °C for 10 min), the supernatants were ultracentrifuged at 100,000 × *g* for 45 min at 4 °C. The supernatants were subsequently incubated with washed Ni²⁺-agarose beads (with buffer A) for ~4 h at 4 °C. After incubation, the Ni²⁺-agarose beads were washed extensively with buffer A containing 50 mM imidazole, and then the bound proteins were eluted with same buffer containing 250 mM imidazole. The eluted proteins were dialyzed against buffer A (with 5% glycerol) for 24 h with two changes of buffer. The dialyzed proteins were incubated with anti-FLAG M2 antibody-agarose (Sigma) at 4 °C for 4 h. After extensive washes with buffer A, the FLAG beads were washed once with buffer B (buffer A without Nonidet P-40). To remove the background proteins, the beads were then incubated for 15 min at 4 °C in buffer B containing 100 μg/ml nonspecific hemagglutinin competitor peptide (YPYDVP-DYA, Research Genetics). After a pre-elution step, the bound FLAG fusion proteins were eluted twice with buffer B containing 200 μg/ml FLAG peptide (Sigma). The eluted proteins were dialyzed against buffer B (with 5% glycerol) for 24 h with two changes of buffer. The purified proteins were then quantified using the Bradford assay. The CCR4 protein was further identified by SDS-PAGE and Western analysis.

In Vitro Deadenylation Assay—The following assay conditions were used for *in vitro* deadenylation: 50 mM Tris HOAc (pH 7.9), 0.01 mM MgCl₂, 20 mM KOAc, 0.10 units of RNase inhibitor, 10% glycerol, 1–100 μM RNA substrate, and the indicated amount of purified CCR4. The RNA substrates were radiolabeled as previously indicated (9). The reaction volume was 30 μl, and the incubations were performed at 37 °C unless otherwise indicated. Five-μl aliquots of the reaction were removed, and the reaction was terminated by the addition of an equal volume of formamide/EDTA buffer. Reaction products were resolved by subsequent electrophoresis on 8 M urea and 16% polyacrylamide (19:1 (v/v) acrylamide/bisacrylamide) sequencing gels. The products were analyzed and quantified using PhosphorImager software.

Kinetic Assays for Distributive Activity of CCR4—To determine the kinetic parameters for the distributive activity of CCR4, experiments were performed with radiolabeled 26N+1A, 25N+2A, and 22N+5A RNA oligonucleotides using purified CCR4 protein. The enzyme concentration was adjusted to obtain linear reaction rates (at least up to 1 h). Five μl of sample volume were removed at appropriate times following incubation at 37 °C. The products were fractionated using a Tris borate/EDTA-polyacrylamide sequencing gel, on which the poly(A)-excised products were distinguished as discrete bands. The reaction rate was determined by measuring the quantity of substrate remaining after 5 min for the 26N+1A and 25N+2A substrates, for which the predominant products were 26N and 25N+1A, respectively. For the 22N+5A substrate, the distributive rate was determined from the total amount of deadenylation that occurred in 5 min after quantitating the abundance of the 22N+5A, 22N+4A, 22N+3A, 22N+2A, and 22N+1A products. For this analysis at low substrate concentration (see Fig. 3B, lower panel), we assumed that the relative rate of deadenylation of the 5A substrate to the 1A product was uniform based on our analysis showing uniform changes in abundance of each of these products with time (see Fig. 2 and Ref. 9; data not shown). At substrate concentrations

TABLE II
Kinetic values of the CCR4 distributive reaction

Experiments were conducted in triplicate, except 26N+1A was analyzed in duplicate. The representative value is the mean ± S.E. The amount of purified CCR4 was 0.3 ng for 22N+5A and 25N+2A and 3 ng for 26N+1A in a reaction volume of 30 μl. The K_{cat} values for 22N+5A and 25N+2A were 1.4×10^2 and 3.5×10^2 s⁻¹, respectively.

Substrate	K_m μM	V_{max} μM/min
22N+5A	3.5 ± 0.22	0.86 ± 0.3
25N+2A	5.4 ± 1.5	2.1 ± 0.6
26N+1A	102 ± 14	2.2 ± 0.6

>5 μM, the predominant reaction was 22N+5A to 22N+4A (see Fig. 3B, lower panel). To determine K_m and V_{max} values, each kinetic experiment contained up to eight different substrate concentrations. Each substrate was analyzed in duplicate (sometimes in triplicate), and the experiments were conducted at least three times. The values obtained were fitted by linear regression analysis to the Michaelis-Menten equation and used to determine, from the intercepts, V_{max} and K_m constants (Table II).

The relative rates of the distributive deadenylation reaction were determined for the 2A substrates as described above, for the 5A substrates by quantitating the total amount of deadenylation that occurred within 5 min, and for the 20A substrates by determining the total amount of deadenylation that occurred within 1 min. For the 20A substrates, within 1 min, little of the completely deadenylated substrate is found; and a substantial amount of the 20A substrate remains, with only 19A, 18A, and 17A substrates being predominantly formed. Therefore, quantitating the total amount of deadenylation within 1 min provides a reasonable estimate of the 20A reaction rate, given that very little difference in 20A, 19A, and 18A reaction rates have been observed (see Fig. 2 and Ref. 9).

RESULTS

Conditions for in Vitro Deadenylation—We optimized the conditions for CCR4 deadenylation *in vitro* using a radiolabeled 22N+5A RNA substrate. We reported previously (9) that the exonuclease activity of CCR4 is dependent upon Mg²⁺. Here we found that CCR4 displayed a very low Mg²⁺ optimum (0.01–0.1 mM) (Fig. 1). Other divalent cations such as Mn²⁺, Zn²⁺, Ni²⁺, and Co²⁺ were inactive with CCR4 (data not shown). The monovalent K⁺ (17 mM), pH (7.9), and temperature (37 °C) optima were also obtained (Fig. 1). The Na⁺ optimum was also found to be low (20 mM) (data not shown). Moreover, we did not find any difference in these optimum conditions when we compared the partially purified CCR4 preparation (single-step FLAG purification) (9) with a pure CCR4 enzyme preparation (see below) (data not shown).

Distributive Action of CCR4 on RNA Substrates—We initially analyzed the activity of the CCR4 protein on a 7N+23A RNA substrate. A time course of CCR4 deadenylation of this substrate (Fig. 2) revealed that CCR4 clearly acted in a distributive manner. A plot of the relative abundance of each of the RNA species as a function of time showed that, as CCR4 catalyzed deadenylation, the abundance of the different species

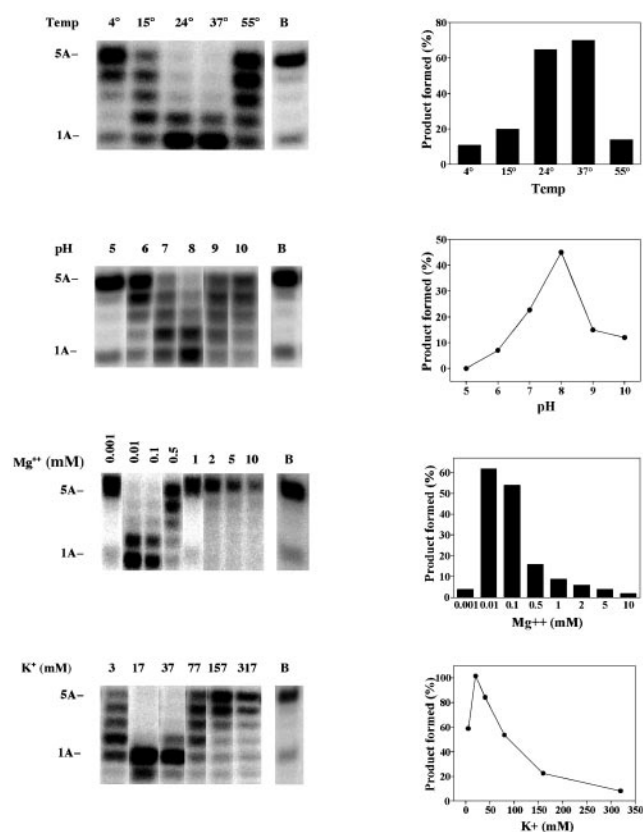


FIG. 1. **Optimum reaction conditions for CCR4 deadenylase activity.** Left panels, the deadenylase activity of CCR4 was assayed under various conditions using the 22N+5A RNA substrate. Upper panel, temperature dependence; second panel, pH dependence; third panel, Mg²⁺ concentration dependence; lower panel, K⁺ concentration dependence. Right panels, shown are graphical representations of the results, where the percent of the 22N+1A product formed in 5 min is plotted versus the relevant parameter. B lanes, no protein.

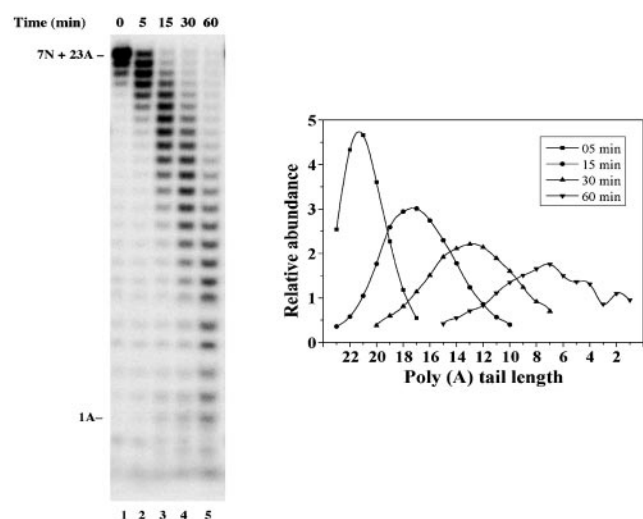


FIG. 2. **Distributive action of CCR4.** Left panel, radiolabeled 7N+23A RNA substrate (0.48 μ M) was incubated with 30 ng of partially purified CCR4 under the optimum assay conditions, and aliquots were taken at the times indicated. Samples were analyzed on a denaturing 8% urea and 14% polyacrylamide gel. Right panel, shown is a graphical representation of deadenylated products at the indicated times as quantified using PhosphorImager software.

formed a nearly symmetric bell-shaped curve. This type of profile is consistent with CCR4 displaying no large differences in its binding and/or catalysis for substrates containing poly(A) tails from 23 A residues to at least 5 A residues (Fig. 2). Our

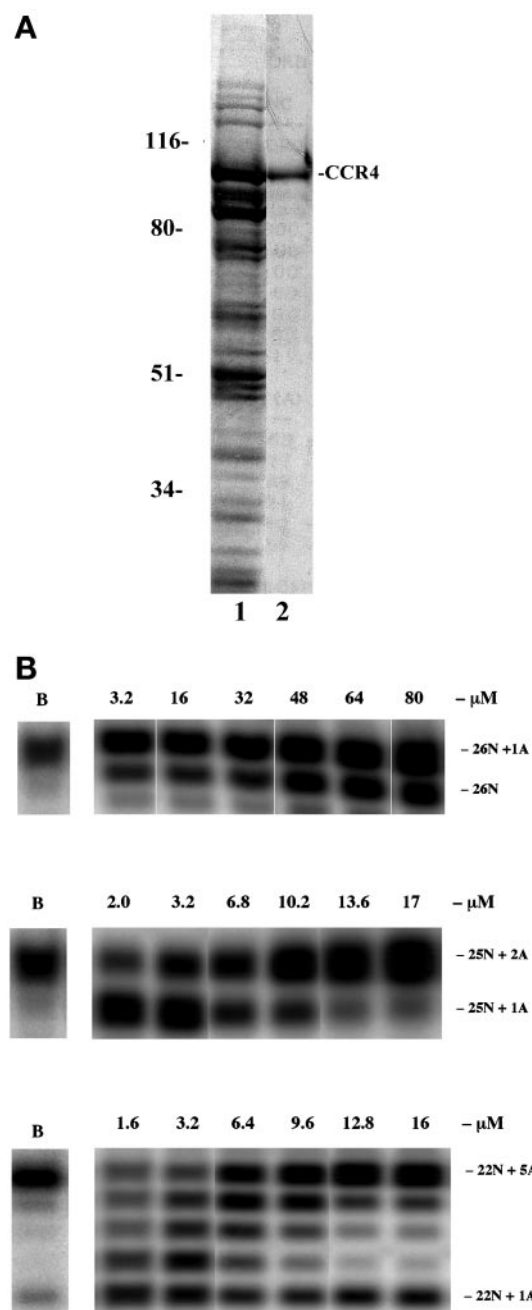


FIG. 3. A, SDS-PAGE analysis of the CCR4 protein. FLAG-CCR4-His₆ fusion protein was analyzed on a denaturing 8% polyacrylamide gel and stained with Coomassie Blue. Lane 1, CCR4 protein following Ni²⁺-agarose chromatography; lane 2, purified CCR4 following both Ni²⁺-agarose chromatography and anti-FLAG M2 antibody-agarose chromatography. The molecular mass of proteins (in kilodaltons) is indicated on the left. B, sample reactions determining the effects of poly(A) tail length on the kinetic parameters of CCR4. The indicated concentrations of radiolabeled 26N+1A, 25N+2A, and 22N+5A RNA substrates were incubated for 5 min under standard assay conditions for *in vitro* deadenylation in the presence of 300 pg of pure CCR4, except 26N+1A was incubated with 10 times the amount of enzyme. For the 22N+5A substrate, please note that for the 16, 12.8, 9.6, and 6.8 μ M concentrations, the 22N+1A band represents a contaminating band present in the original material (B lanes) (9).

previous results indicated, however, that CCR4 had major difficulty in deadenylating a substrate with only 1 terminal A residue (9). Careful analysis of the curves in Fig. 2 does indicate, however, a slight spreading of the abundance of the product toward shorter poly(A) lengths, suggestive of a slowing of the reaction rate with shorter substrate lengths (see below).

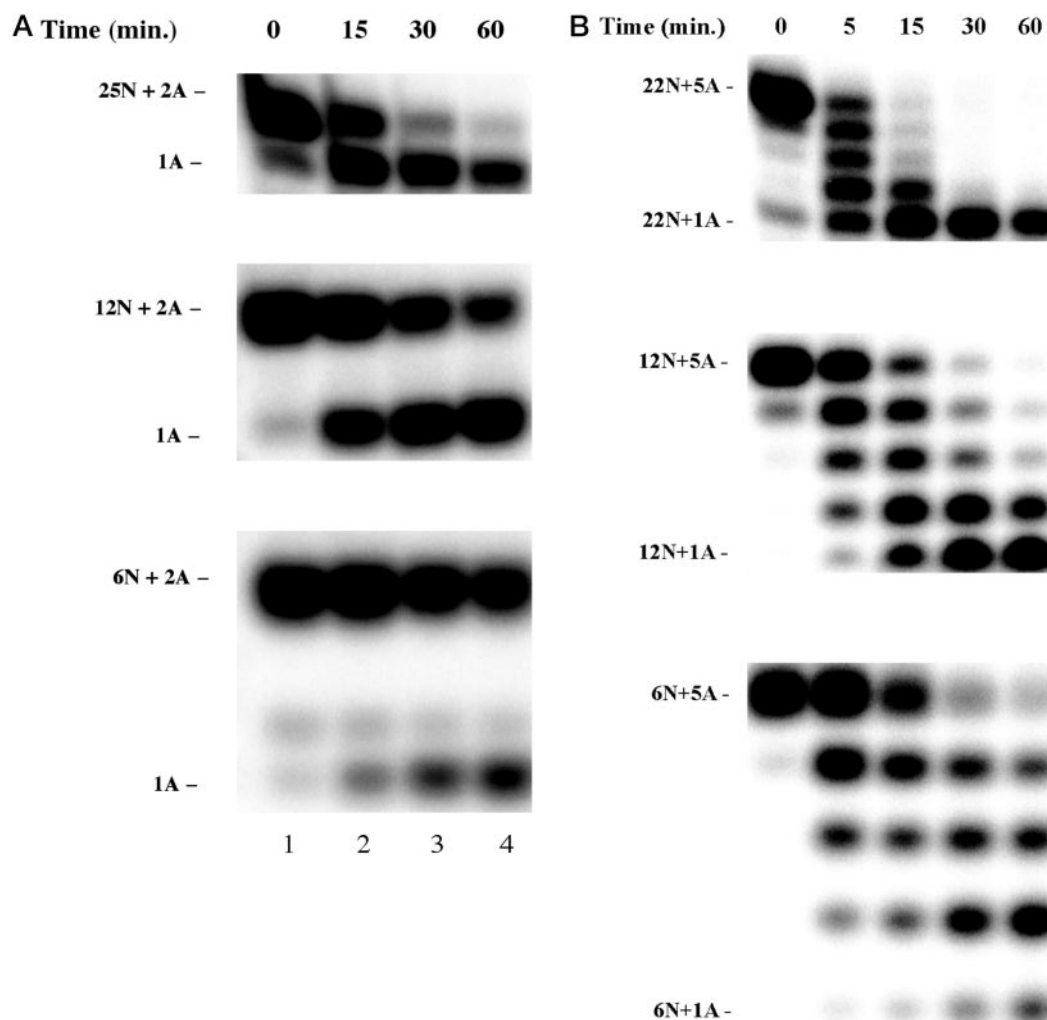


FIG. 4. Assays analyzing effects of non-poly(A) RNA length on CCR4 deadenylase activity. A, 2A RNA substrates. Radiolabeled 22N+2A, 12N+2A, and 6N+2A RNA substrates (0.31 μ M) were incubated with 300 pg of purified CCR4 protein, and aliquots were removed at the times indicated. The lengths of the RNA substrates are indicated on the left. B, 5A RNA substrates. Reactions were conducted as described for A. C, CCR4 becomes a processive enzyme with long RNA substrates. Radiolabeled 25N+20A, 12N+20A, and 6N+20A RNA substrates were incubated with 150 ng of purified CCR4. Reactions were conducted as described for A. The length of each of the RNA substrates is indicated on the left. The reaction product labeled 1A for each substrate denotes the locations of the completely deadenylated products. D, CCR4 does not act endonucleolytically. The 25N+20A substrate was radiolabeled at its 3'-end with poly(A) polymerase to which ~3–33 A residues were added. The asterisks denote the range in positions from the 22A to 52A products that would have formed if endonucleolytic cleavage had occurred at the 1A–2A phosphodiester of the original 25N+23A to 25N+53A substrates.

CCR4 Requires at Least 2 A Residues at the 3'-End to Efficiently Deadenylate the RNA—To address whether CCR4 exhibited a binding or catalytic deficiency with the 1A substrate, we purified CCR4 to homogeneity prior to analyzing its kinetic parameters. To facilitate purification of the CCR4 protein from yeast, the *CCR4* gene fused with FLAG and His₆ epitopes was overexpressed in a strain deleted for *CCR4*. The resultant fusion protein was purified to near homogeneity by a two-step affinity chromatographic procedure from yeast strain KY803-1a-1 (*ccr4*) (Fig. 3A, lane 2).

Using radiolabeled 26N+1A and 25N+2A RNA substrates, we determined the apparent K_m and V_{max} for each substrate with CCR4. Typical reactions as a function of substrate concentration are displayed in Fig. 3B. With the 25N+2A RNA substrate, CCR4 displayed a K_m of 5.4 μ M and a V_{max} of 2.1 μ M/min. Whereas the V_{max} value for the 26N+1A substrate was comparable to that for the 25N+2A substrate (2.2 μ M/min), its K_m was increased by 20-fold. The inability of CCR4 to efficiently deadenylate an RNA substrate with 1 A residue (9) can be attributed to a decreased apparent binding ability of the 1A substrate. We also determined an apparent K_m of 3.5 μ M

and a V_{max} of 0.86 μ M/min for the 22N+5A substrate, which are similar to those found for the 25N+2A substrate.

Increased Length of Non-adenylated Nucleotides of RNA Molecules Enhances the Distributive Activity of CCR4—The substrate specificity of CCR4 was further investigated using different lengths of non-adenylated nucleotides bearing constant length poly(A) tails of 2, 5, and 20 A residues. The relative activity of CCR4 was determined using a concentration of the RNA molecules of 0.31 μ M, which was significantly below the K_m for the 25N+2A and 25N+5A RNA substrates. A typical reaction is displayed in Fig. 4 (A–C) for RNA substrates containing 2, 5, and 20 A residues at their 3'-ends, respectively. Table III summarizes the relative reaction rates for the substrates analyzed. Several observations can be made from the changes in relative activity for CCR4 distributive deadenylation as a function of the length of the non-poly(A) region of the RNA substrate. First, for the 2A and 5A substrates, increasing the N length from 6 to 12 nucleotides substantially enhanced the reaction rate by 2–3-fold. In contrast, increasing the N length further from 12 to 25 nucleotides increased the relative activity by only 1.4–1.7-fold (Table III). Second, once the total

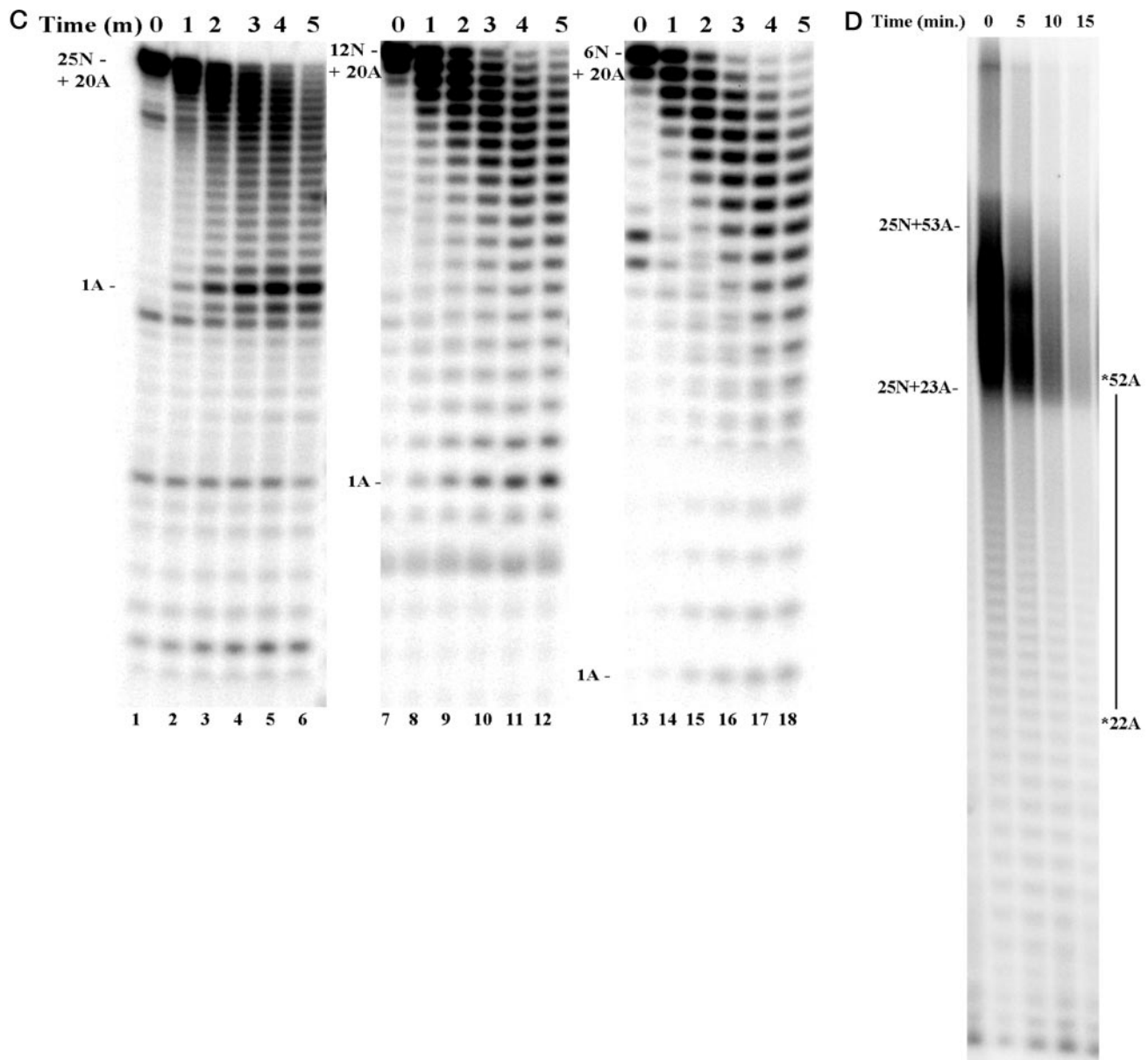


FIG. 4—continued

TABLE III
Relative rates of CCR4 distributive activity with RNA oligonucleotides of different lengths

Standard deadenylation reactions were conducted with $0.31 \mu\text{M}$ RNA substrates. The relative rates of deadenylation were standardized against 22N+5A, which was set at 100, and were determined as described under "Experimental Procedures." The values obtained represent the mean \pm S.E. of triplicate analysis, except for 6N+2A and 25N+20A (duplicate analysis).

Substrate	Relative rate
25N+2A	70 ± 7.6
12N+2A	45 ± 6.0
6N+2A	13 ± 4.2
22N+5A	100
12N+5A	69 ± 5.8
6N+5A	30 ± 6.8
25N+20A	84 ± 11
12N+20A	75 ± 9.1
6N+20A	76 ± 7.1

RNA length became ~ 17 – 26 nucleotides, no major differences in the distributive reaction rate were observed for different N lengths irrespective of the poly(A) length (Fig. 5). Third,

12N+5A was as active with CCR4 as 12N+20A, indicating that the length of the poly(A) sequences *per se* does not alter CCR4 activity. These observations suggest that CCR4 is able to recognize a certain length of RNA substrate (at least 17 nucleotides) and that the A content is not necessarily important for this recognition. These results are also in agreement with those shown in Fig. 2, where a slight spreading of the distribution of 7N+23A products indicated a slight slowing of the reaction rate at shorter substrate lengths.

CCR4 Protein Transitions to a Processive Enzyme with Substrates Greater than 44 Nucleotides—It is shown in Fig. 4C (lanes 2–6) that a significant portion of the 25N+20A substrate was converted directly to the 25N+1A product. These data suggest that CCR4 can also act in a processive manner. Because it is also possible that the 25N+1A product was created by an endonucleolytic cleavage occurring at the 1A–2A phosphodiester bond, we 3'-end-labeled the 25N+20A substrate with poly(A) polymerase and reconducted the CCR4 deadenylation reaction. As shown in Fig. 4D, at time 0, an additional 3–33 A residues were added to the 25N+20A substrate. Deadenylation of this substrate by CCR4 under conditions in which

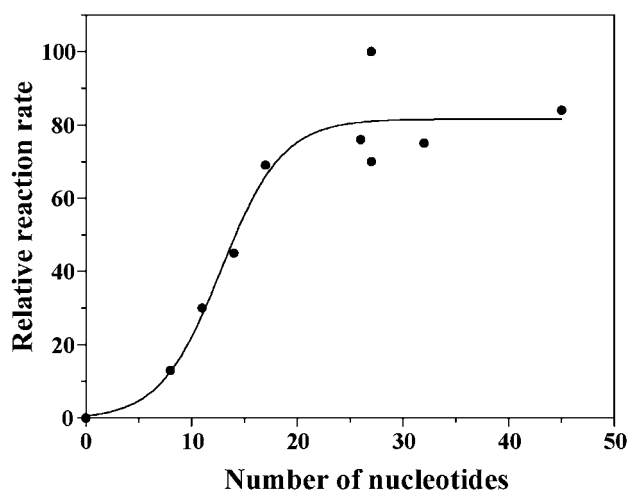


FIG. 5. **Total length of the RNA influences the distributive activity of CCR4.** The relative reaction rates for different RNA substrates with CCR4 are plotted *versus* the lengths of the RNA substrates (see Table III).

CCR4 displayed a processive reaction with the 25N+20A substrate (Fig. 4C; data not shown) or a 50N+10A substrate (data not shown) indicated that the poly(A) tail was being removed without the concomitant endonucleolytic cleavage at the 1A–2A phosphodiester that would have resulted in a range of 22–52 poly(A) products (marked with *asterisks* in Fig. 4D). Similarly, using an *in vitro* synthesized 50N+30A substrate uniformly labeled with A (14), no 29A endonucleolytic product was observed following CCR4 processive deadenylation that resulted in a 50N+1A product (data not shown). These data confirm that CCR4 can act in a processive manner. CCR4 behaved as a processive enzyme to a much greater extent with the 25N+20A substrate than with the 12N+20A substrate (Fig. 4C, lanes 7–12), indicating that the total length of the RNA substrate is important for CCR4 processivity and not just the A tail length. No processivity was observed with the 7N+23A substrate (Fig. 2), although for the 6N+20 substrate displayed in Fig. 4C (lanes 16–18), a very small amount of 6N+1A substrate was formed as a result of an apparently processive reaction.

Similarly, CCR4 reacted with the 50N+5A RNA substrate in a processive manner (Fig. 6, lane 6), indicating that it is not the length of the poly(A) sequence that dictates the distributive-to-processive transition for CCR4. In fact, CCR4 acted primarily in a processive manner with the 50N+5A, 50N+10A, and 50N+30A substrates (Fig. 6; data not shown), suggesting again that the total length of the substrate, not its poly(A) length, dictates the distributive-to-processive transition for CCR4. We conclude that CCR4 can act as a processive enzyme in the presence of RNA substrates with lengths of at least 45 nucleotides. No difference in CCR4 reaction rate or ability to act in a processive manner was observed with capped and uncapped 50N+5A RNA substrates (Fig. 6).² It should be noted that the apparent faster rate of conversion with uncapped *versus* capped substrates shown in Fig. 6 was not borne out by other experiments. Similar results were obtained with capped and uncapped 50N+30A RNA substrates (data not shown).

CCR4 Activity in the Presence of Various Inhibitors—To investigate further the CCR4 deadenylase activity specificity for its RNA substrate, we analyzed the effect of various competitive inhibitors on CCR4 poly(A) removal. The above described deadenylation assay using the 22N+5A RNA substrate was performed in the presence of increasing amounts of 5'-AMP,

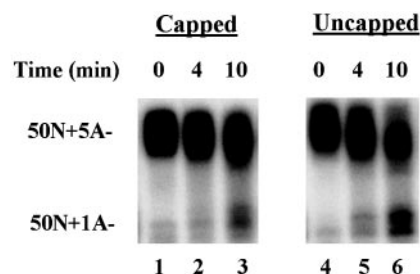


FIG. 6. **Comparison of deadenylation rates with capped and uncapped substrates.** Capped and uncapped uniformly labeled 50N+5A RNA substrates, as indicated, were incubated with 30 ng of partially purified CCR4 under standard conditions for the indicated times. The products were fractionated by electrophoresis on a denaturing 7% polyacrylamide gel. The resultant products are indicated on the left.

TABLE IV

In vitro deadenylation assay conducted in the presence of inhibitors

The rate of enzymatic activity was calculated by the rate of disappearance of the 22N+5A substrate (0.48 μ M) using the standard deadenylation assay conditions with purified CCR4. K_i is the concentration required for 50% inhibition as determined by PhosphorImager quantitation.

Inhibitor	K_i
5'-AMP	80 μ M
5'-GMP	80 μ M
5'-UMP	45 μ M
5'-CMP	80 μ M
Poly(A)	33 μ g/ml
Poly(G)	1.6 μ g/ml
Poly(C)	2.6 μ g/ml
Poly(U)	1.2 μ g/ml

5'-GMP, 5'-UMP, 5'-CMP, poly(A), poly(U), poly(G), and poly(C). Table IV shows that UMP was an \sim 2-fold better inhibitor than AMP, CMP, and GMP. We also observed that the K_i of inhibition for ATP (1.2 μ M) was much lower than that for AMP (data not shown). However, as was previously observed for the poly(A) ribonuclease (PARN) deadenylase (15), this inhibition was shown to result from the chelating effect of ATP with Mg^{2+} ions, which thereby prevented the catalytic potential of the CCR4 enzyme (data not shown). Interestingly, we observed that poly(U), poly(C), and poly(G) were much more inhibitory than poly(A), consistent with our above conclusion that the CCR4 protein may be able to recognize a certain length of the RNA substrate and that the poly(A) sequence is of little importance to this recognition.

DISCUSSION

In this study, we report on the substrate specificities of the yeast CCR4 deadenylase. Several features of its substrate recognition were identified. First, as previously indicated (9), CCR4 acted in a distributive fashion to deadenylate a 7N+23A substrate. With other substrates of comparable or shorter length, CCR4 was also a distributive enzyme; yet we observed that, with substrates 45 nucleotides or longer, CCR4 could act in both a processive and distributive manner. The ability to be a processive enzyme was also independent of the length of the poly(A) region of the substrate. One model that could account for this distributive-to-processive transition is that CCR4 binds better to longer substrates and therefore remains attached for a longer period of time. This model is unlikely, however, in that no difference was observed in the relative distributive activity with RNA substrates longer than 17 nucleotides (Figs. 4C and 5), implying that CCR4 even with longer substrates displays no differences in inherent affinity for the substrate. An alternative model is that CCR4 itself undergoes a transition in the pres-

² R. Parker, personal communication.

ence of longer substrates such that a pool of CCR4 can now act in a processive fashion. The oligomeric structures of the highly processive λ -exonuclease and poly(A) ribonuclease (PARN) deadenylase (16, 17) suggest that one such transition may be for CCR4 to become oligomeric. Perhaps longer RNA substrates provide the structure that accelerates this process by, for instance, providing significantly long enough sequences to which multiple CCR4 proteins can attach.

Second, we found that, as a distributive enzyme, CCR4 preferred substrates with lengths of at least 17 nucleotides. The decreases in reactivity with shorter substrates appeared to be a result of CCR4 decreased binding affinity, as the substrate concentrations used in these experiments were significantly below the K_m of binding. The length of the poly(A) sequence did not alter these preferences, confirming observations of others² and ours³ that CCR4 cannot specifically bind to poly(A). Whether this binding preference results from a sequence-specific recognition is not known. However, inhibition studies with poly(U) and poly(G) indicated that these compounds were 20–40-fold better inhibitors of the CCR4 distributive reaction than was poly(A). CCR4 might display sequence specificity for non-poly(A) sequences that might be enriched with U or G. Given that poly(A) sequences in yeast are up to 70–80 nucleotides in length, one model suggests that only the extreme 3'-end of the poly(A) tail and the 3'-untranslated region sequences are bound by CCR4, with most of the poly(A) sequence looped out and not in contact with CCR4.

Third, either as a distributive or processive enzyme, CCR4 was deficient in removing a single terminal A residue from the mRNA (9). This requirement for 2 A residues at the 3'-end was shown to be the result of a much higher (20-fold) K_m for the 1A substrate than for the 2A substrate. CCR4 can remove the terminal A residue and can also remove C or other nucleotides, albeit at very slow rates (9). Fourth, CCR4 itself did not display any difference with capped or uncapped substrates, suggesting that other proteins interacting with CCR4 aid its recognition of capped mRNA (8).

These substrate specificity analyses suggest the following model for CCR4 recognition of its RNA substrate. CCR4 binds to at least 2 terminal A residues, presumably in its active site. It also displays recognition for at least another 15 nucleotides; whether they are completely contiguous to these 2 A residues is not known. Finally, CCR4 may change its conformation or alter its oligomeric structure with substrates of even greater length (≥ 45 nucleotides) to become a processive enzyme. Preliminary modeling of CCR4 binding to a 17-mer RNA based on the structure of the exonuclease III family protein HAP1 (18–20) indicates that the C-terminal catalytic domain of CCR4 would not easily accommodate binding to RNA of such length. However, other regions of CCR4, especially its LRR, could be involved in RNA binding. Given that two other LRR-containing proteins require their LRR domain for contacting their cognate RNA substrates (21, 22), it is quite possible that the LRR of CCR4 directly or indirectly facilitates CCR4 recognition of its substrate.

These features of CCR4 recognition of its substrates may, of course, be targets for control of the deadenylation process. Regulating the ability of CCR4 to bind specific mRNA or to transition to its processive state would greatly influence its

enzymatic activity. The CAF1 protein and other components of the CCR4-NOT complex do not appear to be likely regulators of these features. For example, partially purified preparations of CCR4 that contain the complete CCR4-NOT complex (9) do not display significant differences in deadenylation compared with the purified protein in terms of requiring at least 17 nucleotides or in the transition to processivity. Other *trans*-acting proteins such as PUF3 and PAB1 could control these processes (7, 8, 23). The role of the other components of the CCR4-NOT complex may be to confer additional regulatory aspects to the deadenylation process. These could include binding to the mRNA cap structure or recognition of additional specific RNA sequences not present in our model RNA substrates used in this study.

Previous studies on the deadenylation of several mRNAs *in vivo* (5, 7, 8) clearly indicate that the rate of deadenylation can vary considerably for different mRNAs. Careful analysis of these *in vivo* deadenylation studies indicates that *PGK1*, which deadenylates slowly, does so in a distributive manner (5). In contrast, faster deadenylated mRNAs such as *MFA2*, *STE3*, *COX17*, and *GAL10* deadenylate with a combination of both processive and distributive processes (5, 7, 23). This is an expected result given that processivity would allow the more rapid accumulation of product compared with the comparatively slow distributive reaction. Because CCR4 can display both distributive and processive activities, future studies will need to address how regulating these processes relates to the observed differences in mRNA deadenylation. In addition, the previously observed initially slow deadenylation of all mRNAs up to loss of the first 10 A residues (5) followed by more rapid deadenylation of the bulk of the poly(A) tail suggests an additional site at which CCR4 deadenylation may be controlled.

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³ P. Viswanathan, J. Chen, Y.-C. Chiang, and C. L. Denis, unpublished data.