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The Enzyme That Adds Poly(A) to mRNAs Is a Classical Poly(A) Polymerase

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Virtually all mRNAs in eucaryotes end in a poly(A) tail. This tail is added posttranscriptionally. In this report, we demonstrate that the enzyme that catalyzes this modification is identical with an activity first identified 30 years ago, the function of which was previously unknown. This enzyme, poly(A) polymerase, lacks any intrinsic specificity for its mRNA substrate but gains specificity by interacting with distinct molecules: a poly(A) polymerase from calf thymus, when combined with specificity factor(s) from cultured human cells, specifically and efficiently polyadenylates only appropriate mRNA substrates. Our results thus demonstrate that this polymerase is responsible for the addition of poly(A) to mRNAs and that its interaction with specificity factors is conserved.

Thirty years ago, an enzymatic activity was identified that added poly(A) to RNA by polymerizing ATP (5). A decade later, the observation that mRNAs contained a 3′-terminal poly(A) segment (3, 6, 9) rekindled interest in the activity and led to the purification of poly(A) polymerases from several sources (4, 7, 21; M. Edmonds, Methods Enzymol. in press). By 1973, although the enzyme was available in nearly pure form, its role, if any, in mRNA polyadenylation remained obscure, since it would add poly(A) to any RNA, including tRNA and short oligonucleotides. In this report, we exploit recently developed assays for mRNA-specific polyadenylation to determine whether this classical polymerase is, in fact, the enzyme responsible for adding poly(A) to mRNA.

In this study, biologically meaningful mRNA polyadenylation was assayed in vitro (Fig. 1) (10, 23). In vivo, RNA cleavage, not transcription termination, generates the 3′ end to which poly(A) is then added (13, 14, 16). To eliminate the need for cleavage and thus assay polyadenylation in isolation, we prepared RNAs that end at the polyadenylation site by transcription in vitro (23). An RNA containing the last 58 nucleotides of simian virus 40 (SV40) late mRNA up to the polyadenylation site was incubated in a crude HeLa cell nuclear extract (Fig. 1). This RNA received approximately 200 nucleotides of poly(A), as judged by its increase in gel mobility (compare lanes 1 and 2) and retention on an oligo(dT)-cellulose column (data not shown). The sequence AAUAAA, located 5 to 30 nucleotides upstream of the polyadenylation site of virtually all mRNAs (15, 20), is required for this reaction: AAUCAA-containing RNA is polyadenylated less efficiently (lane 3) (23).

The requirement for AAUAAA is the criterion by which we judge whether any observed polyadenylation is biologically significant. Genetic analyses (23), competition experiments (24), and biochemical fractionation of the extract (1a, 2, 11, 17) further justify the use of this criterion.

As shown previously, the AAUAAA-specific polyadenylation activity is separated into two fractions by DEAE-Sepharose chromatography (see legend to Fig. 1) (1a, 2, 11, 17). These fractions are designated DE-100 and DE-600, according to the salt concentration at which they fail to bind to the resin. The DE-100 fraction contains a poly(A) polymerase, whereas the DE-600 fraction contains a factor(s) that provides specificity for the AAUAAA sequence (1a, 11, 17). Neither fraction was active alone (Fig. 1, lanes 4 and 5). However, when mixed together, the two fractions reconstituted efficient polyadenylation (lane 6). This reaction, like that in extract, required the AAUAAA sequence (lane 7).

To determine whether the enzyme responsible for polyadenylation of mRNAs is a classical poly(A) polymerase, we assayed a classical polymerase purified from calf thymus (19, 21), either alone or in combination with the specificity factor fraction from HeLa cells (DE-600). The polymerase alone displayed no activity (Fig. 2, lane 2), nor did the HeLa specificity factor(s) fraction (lane 3). Strikingly, the mixture of the HeLa factors with the calf polymerase restored efficient polyadenylation (lane 4). This polyadenylation requires AAUAAA, since a substrate containing AAUCAA was inert (lanes 5 to 8). We conclude that the classical poly(A) polymerase is in fact capable of specific polyadenylation of mRNAs and that it gains sequence specificity by interacting with a distinct molecule or molecules.

Since the calf polymerase was purified on the bases of its ability to add poly(A) to any RNA (21, 22), the failure of the enzyme alone to polyadenylate SV40 RNAs, as in Fig. 2, may appear surprising. However, the concentration of RNA used in the purification assay is more than 1,000 times greater than that used in our analytical experiments.

The ability of the HeLa DE-600 fraction to stimulate the calf polymerase on AAUAAA-containing RNAs was indeed due to a specificity factor(s) in that fraction (Fig. 3). The

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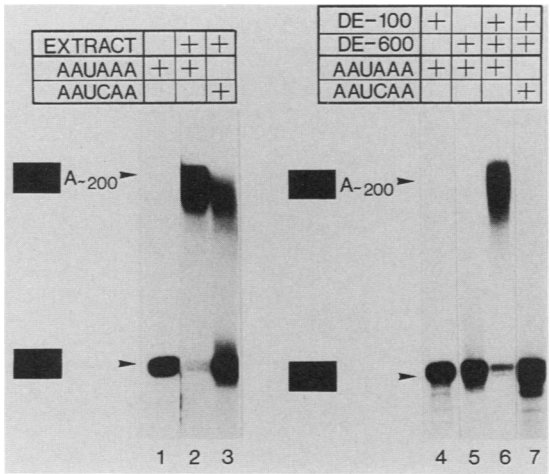

FIG. 1. Demonstration that polyadenylation requires separable components. Synthetic SV40 RNA was incubated in nuclear extract (lanes 2 and 3) or fractions derived from that extract (lanes 4 to 7) for 1 h at 30°C. RNA was analyzed by electrophoresis on an 8% polyacrylamide gel containing 7 M urea. A black box indicates the portion of the RNA before the poly(A) site. Lanes: 1, −58/+1 RNA containing AAUAAA without incubation in extract; 2, −58/+1 RNA containing AAUAAA incubated in extract; 3, −58/+1 RNA containing AAUCAA incubated in extract; 4 to 6, −58/+1 RNA containing AAUAAA incubated with either DE-100 (polymerase fraction) (lane 4), DE-600 (specificity fraction) (lane 5), or DE-100 plus DE-600 (lane 6); 7, −58/+1 RNA containing AAUCAA incubated in DE-100 plus DE-600. RNA was prepared by runoff transcription, using SP6 polymerase in the presence of [α-^{32}P]UTP (8, 12). −58/+1 RNA (1) contains nucleotides −58 to +1 of SV40 late mRNA (where +1 is the natural site of polyadenylation) preceded by 12 nucleotides of vector-derived sequence. Preparation of nuclear extract and in vitro polyadenylation assays were performed as described elsewhere (1), except that 3′-dATP was omitted and 3 to 6 μl of extract or fractions was used in 12.5 μl. Nuclear extract was fractionated by variation of a published protocol (1a). Extract was precipitated with saturated ammonium sulfate (pH 7.5). The 25 to 40% saturated fraction was suspended at 2 mg/ml in buffer D (20% [vol/vol] glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [HEPES]-KOH [pH 7.9], 0.125 mM EDTA, 100 mM KCl, 0.5 mM dithiothreitol) and dialyzed against 50 volumes of buffer D for 5 h, with one change of buffer. DEAE-Sepharose Fast Flow (Pharmacia, Inc.) was equilibrated with buffer A (10% [vol/vol] glycerol, 20 mM HEPES-KOH [pH 7.9], 1 mM $MgCl_2$, 0.125 mM EDTA, 0.5 mM dithiothreitol) containing 100 mM KCl. Ammonium sulfate-fractionated nuclear extract was applied to the column, and the flowthrough fraction (DE-100) was pooled and dialyzed against buffer D. To prepare DE-600, unfractionated nuclear extract was applied to the column in buffer A. The bound material was eluted with buffer A containing 600 mM KCl and dialyzed against buffer D as described above. All extracts and fractions were stored at −100°C after quick freezing in liquid nitrogen.

stimulatory activity was abolished by heat treatment (lane 12) and was not due to the large amount of RNA in the DE-600 fraction, since RNA alone did not stimulate (lane 11). More important, stimulatory activity could be enriched by further chromatographic steps (heparin-Sepharose and Mono-Q) (1a) that removed most of the RNA and increased the specific activity of the fraction (data not shown). Like the crude fraction (DE-600), this more highly purified preparation of specificity factor(s) (Fig. 3, Mono-Q) conferred specificity for AAUAAA on the enzyme (Fig. 3, lanes 1 to 10); the fraction was inactive on its own (lane 3) but stimulated

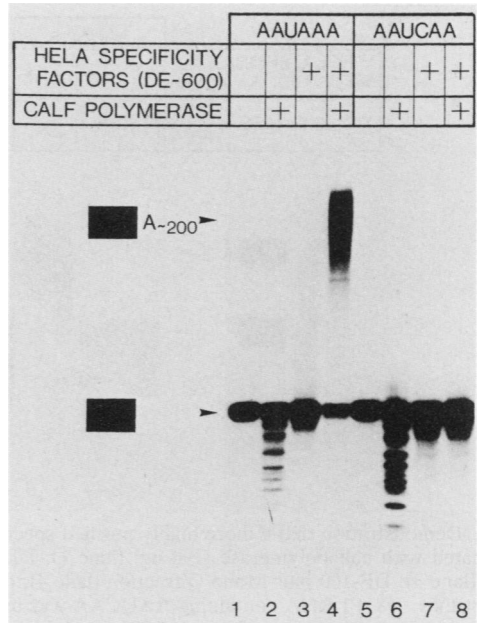

FIG. 2. Demonstration that classical poly(A) polymerase is responsible for polyadenylation of mRNA. Lanes 1 to 4, −58/+1 RNA containing AAUAAA incubated with calf poly(A) polymerase (lane 2), DE-600 (lane 3), or calf poly(A) polymerase plus DE-600 (lane 4); 5 to 8, identical to lanes 1 to 4 except that −58/+1 RNA containing AAUCAA was used. Each reaction contained 65 ng of calf poly(A) polymerase prepared as described previously (21), except that after phosphocellulase chromatography, the proteins were passed over a second DEAE-cellulose column in the absence of salt, and the flowthough was loaded onto a Blue Sepharose column and eluted with 0.45 M NaCl. The Sephadex G-200 column was omitted.

poly(A) addition when mixed with either the calf or HeLa (DE-100 fraction) polymerase (lanes 4 and 5). This stimulation was abolished if the substrate contained AAUCAA (lanes 6 to 10). Purification of DE-600 also resulted in concentration of a nuclease and addition of shorter poly(A) tails.

The calf and HeLa polymerase activities are similar in several respects. First, we and others (2, 11, 17) have further purified the HeLa cell enzyme on the basis of its AAUAAA-dependent activity when mixed with DE-600 and found that it and the calf enzyme exhibit similar chromatographic properties (1a, 2, 4, 7, 11, 21, 22; Edmonds, in press; D. Zarkower, unpublished data). Similarly, antibodies raised against the classical polymerase from rat hepatoma cells inhibit AAUAAA-specific polyadenylation in the HeLa extract, suggesting that classical and AAUAAA-specific enzymes share antigenic determinants (18). Second, both calf and HeLa activities have apparent molecular masses of approximately 60 kilodaltons (1a, 2, 4, 19; Edmonds, in press). Third, our most highly purified preparation of HeLa enzyme, like the DE-100 fraction, became AAUAAA specific in the presence of HeLa cell specificity factor(s) (DE-600) (Fig. 4, lanes 1 and 2). The efficiency and specificity of this more highly purified HeLa preparation are comparable to those observed with the calf enzyme (Fig. 4, lanes 3 and 4). Finally, like the HeLa polymerase (2, 17), the calf enzyme possesses a nonspecific activity that is stimulated by Mn^{2+} relative to Mg^{2+}: with Mn^{2+}, the calf enzyme added poly(A) to both mutant and wild-type RNAs, and in the absence of any specificity factor (Fig. 4, lanes 5 and 6).

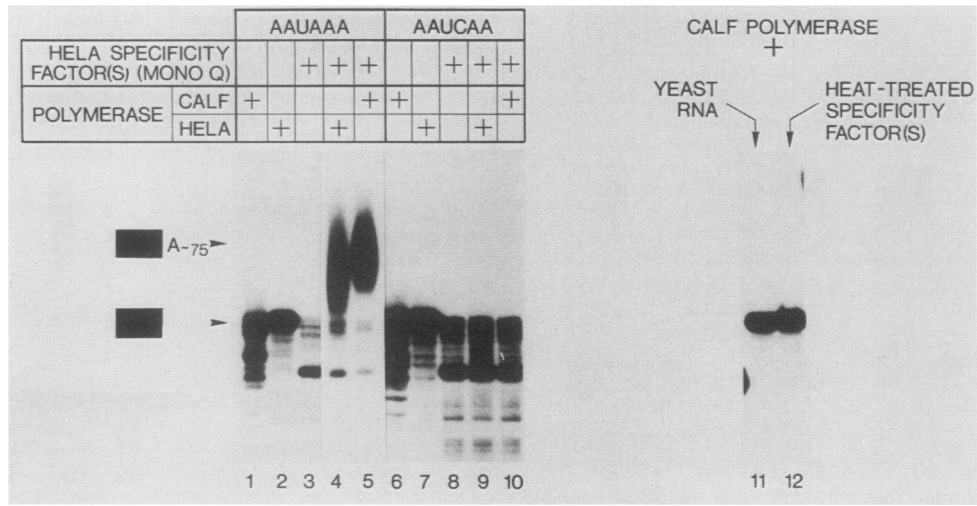

FIG. 3. Demonstration that a more highly purified specificity factor confers AAUAAA dependence. −58/+1 RNA containing AAUAAA was incubated with calf polymerase (190 ng) (lane 1), DE-100 (HeLa polymerase fraction) (lane 2), 3 μl of Mono Q fraction (specificity factor[s]) (lane 3), DE-100 plus Mono Q fraction (lane 4), or calf polymerase plus Mono Q fraction (lane 5). Lanes 6 to 10, Same as lanes 1 to 5 except that −58/+1 RNA containing AAUCAA was used; lane 11, −58/+1 RNA containing AAUAAA incubated with calf polymerase plus 0.75 μg of yeast RNA; lane 12, same RNA incubated with DE-600 treated for 45 min at 50°C. In this crude DE-100 fraction, we did not detect nonspecific activity in Mg^{2+} or Mn^{2+}. For preparation of the Mono Q fraction, heparin-Sepharose (Pharmacia) was equilibrated with buffer A containing 100 mM KCl. DE-600 (see legend to Fig. 1) was loaded onto the column, and the bound material (HS-500) was eluted with buffer A containing 500 mM KCl. HS-500 was further purified by chromatography on a Mono Q HR 10/10 column (Pharmacia) equilibrated with buffer A containing 100 mM KCl. Bound material was eluted with a 120-ml linear gradient of buffer A containing 100 to 500 mM KCl. Fractions of 2 ml were collected, dialyzed against buffer D, and assayed for stimulation of polyadenylation in the presence of DE-100. The fraction used in these experiments eluted at approximately 300 mM KCl.

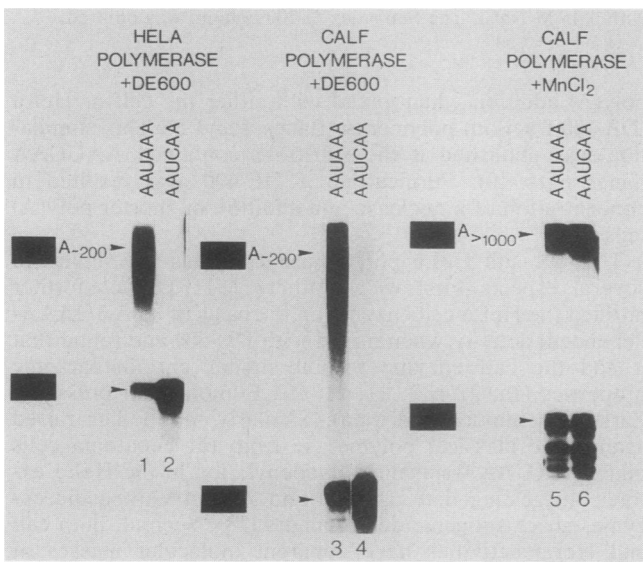

FIG. 4. Comparison of more highly purified HeLa enzyme with calf polymerase. Lanes 1 to 4, Comparison of highly purified calf and human polymerases combined with DE-600: −58/+1 RNA containing AAUAAA incubated with HeLa polymerase (35 ng) plus DE-600 (lane 1), −58/+1 RNA containing AAUCAA incubated with HeLa polymerase plus DE-600 (lane 2), and the same preparations as in lanes 1 and 2 except with calf polymerase instead of HeLa polymerase (lanes 3 and 4). Lanes 5 and 6, Manganese-dependent activity of calf thymus polymerase (378 ng) incubated with −58/+1 RNA containing AAUAAA and 0.3 mM $MnCl_2$ (lane 5) and the same preparation except with −58/+1 RNA containing AAUCAA (lane 6). The relative efficiency with which calf and human enzymes

Our data directly demonstrate that the enzyme responsible for polyadenylation of mRNAs is the same as the poly(A) polymerases first purified 15 years ago and is consistent with the observations of others on the HeLa enzyme (2, 11, 17, 18). In retrospect poly(A) polymerase was the first enzyme involved in nuclear mRNA processing to have been identified and characterized. The polymerase becomes specific for mRNAs by interaction with specificity factor(s), which as yet are not pure and may consist of a small nuclear ribonucleoprotein or protein components (1a, 11, 17). This interaction is evolutionarily conserved between cows and hu-

interact with the human specificity factors is difficult to quantitate, since neither enzyme is pure, and the ratio of Mn^{2+} activity to Mg^{2+} activity differs between the two enzymes, so that standardization to Mn^{2+} nonspecific units is not meaningful. Nevertheless, the levels of nonspecific activity exhibited by the amounts of the enzymes used here are within an order of magnitude of one another. HeLa poly(A) polymerase was purified from the DE-100 fraction, which was dialyzed against buffer A containing 20 mM KCl and applied to a Mono Q HR 10/10 column equilibrated with buffer A containing 20 mM KCl. Bound material was eluted with a 70-ml linear gradient of buffer A containing 20 to 200 mM KCl. Fractions of 1 ml were collected and dialyzed against buffer D. Activity eluted in three broad peaks, at approximately 45, 100, and 145 mM KCl. Fractions in the first peak were pooled and applied to a Biorex-70 column (Bio-Rad Laboratories) equilibrated with buffer A containing 100 mM KCl. The bound material (Bio-600) was eluted with buffer A containing 600 mM KCl. Bio-600 was dialyzed against buffer A containing 50 mM KCl and applied to a poly(A)-Sepharose column (Pharmacia) equilibrated with the same buffer. Bound material (PA-250) was eluted with buffer A containing 250 mM KCl and dialyzed against buffer D. The PA-250 fraction was used for the experiments described.

mans, as are the general biochemical properties of the polymerase.

In addition to polymerizing ATP into poly(A), the polymerase possesses several other activities, each of which merits detailed analysis. It is required for the cleavage reaction that precedes polyadenylation in vivo (1a, 2, 17), interacts with a specificity factor(s), and undergoes a transition from AAUAAA dependence to independence after it has added 10 adenosines to the mRNA (1, 16a). Dissection and analysis of these functions is likely to be facilitated by the findings reported here, since the interaction between factors, which renders the enzyme specific for mRNAs, is conserved.

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ADDENDUM IN PROOF

E. Wahle and W. Keller (personal communication) have recently carried out experiments analogous to those reported here and have reached identical conclusions.

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