Polyadenylation of mRNA: Minimal Substrates and a Requirement for the 2' Hydroxyl of the U in AAUAAA

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mRNA-specific polyadenylation can be assayed in vitro by using synthetic RNAs that end at or near the natural cleavage site. This reaction requires the highly conserved sequence AAUAAA. At least two distinct nuclear components, an AAUAAA specificity factor and poly(A) polymerase, are required to catalyze the reaction. In this study, we identified structural features of the RNA substrate that are critical for mRNA-specific polyadenylation. We found that a substrate that contained only 11 nucleotides, of which the first six were AAUAAA, underwent AAUAAA-specific polyadenylation. This is the shortest substrate we have used that supports polyadenylation: removal of a single nucleotide from either end of this RNA abolished the reaction. Although AAUAAA appeared to be the only strict sequence requirement for polyadenylation, the number of nucleotides between AAUAAA and the 3' end was critical. Substrates with seven or fewer nucleotides beyond AAUAAA received poly(A) with decreased efficiency yet still bound efficiently to specificity factor. We infer that on these shortened substrates, poly(A) polymerase cannot simultaneously contact the specificity factor bound to AAUAAA and the 3' end of the RNA. By incorporating 2'-deoxuryridine into the U of AAUAAA, we demonstrated that the 2' hydroxyl of the U in AAUAAA was required for the binding of specificity factor to the substrate and hence for poly(A) addition. This finding may indicate that at least one of the factors involved in the interaction with AAUAAA is a protein.

The mature 3' termini of most eucaryotic mRNAs are formed by cleavage of the mRNA precursor and addition of poly(A) to the newly formed end (reviewed in references 3, 10, 11, and 18). Two sequences are required for efficient cleavage: the highly conserved AAUAAA sequence, commonly located 15 to 25 nucleotides upstream of the poly(A) site, and a less conserved sequence downstream of the poly(A) site. AAUAAA is also required for polyadenylation of synthetic RNAs that end at the poly(A) site, so-called precleaved RNAs. This requirement is observed both in vitro, in a HeLa cell nuclear extract (e.g., reference 28) and in vivo, after introduction into frog oocyte nuclei (6a).

Cleavage and polyadenylation occur in a specific processing complex, as demonstrated by sedimentation (16, 23), RNase resistance (8, 9, 23), oligonucleotide and RNase H analysis (23, 29), and non-denaturing gel electrophoresis (9, 22, 30, 31). The latter assay reveals the existence of a distinct postcleavage complex, obtained if polyadenylation is blocked after cleavage (30). Synthetic RNAs that end at the poly(A) site form the postcleavage complex de novo (29, 30).

At least three separable factors are required for cleavage: a specificity factor, a cleavage factor, and a poly(A) polymerase (4, 5, 7, 12, 24, 25). Although none are yet pure, it is clear that only the specificity factor and polymerase fractions are required for AAUAAA-specific poly(A) addition to precleaved RNAs (5, 7). Candidates for the factor that recognizes AAUAAA have been identified, though none have been demonstrated directly to play a role in the reaction. These include U11 small nuclear ribonucleoprotein (snRNP) (4) and a 64- or 68-kilodalton protein that can be cross-linked by UV light to RNAs containing AAUAAA (1, 15, 26). The enzyme that adds poly(A) is a classical poly(A) polymerase (2, 5, 24). It becomes selective for AAUAAA-containing RNAs by interacting with specificity factor (2, 4, 12, 24).

The use of precleaved RNAs makes possible the analysis of polyadenylation alone, uncoupled from cleavage. The polyadenylation observed in these uncoupled reactions is very similar if not identical to that which occurs after cleavage, as judged by several criteria. First, both cleavage and polyadenylation are dependent on AAUAAA (29). Second, AAUAAA mutants with different severities affect the two reactions comparably (M. Sheets, manuscript in preparation). Third, substrates for both reactions form very similar complexes with processing factors (29, 30). Similarly, uncleaved precursors, cleaved RNAs, and precleaved RNAs all can be UV cross-linked to a 64- or 68-kilodalton protein (1, 15, 26). Fourth, poly(A) addition and cleavage apparently require common specificity factors and poly(A) polymerase. This was initially deduced from competition experiments (29) and has since been demonstrated more conclusively by copurification of AAUAAA-specific cleavage and polyadenylation activities (4, 5, 25).

In this report, we examine detailed requirements for polyadenylation in vitro, when uncoupled from cleavage. We identify a minimal substrate for polyadenylation and analyze the effect of altering the distance between AAUAAA and the 3' end of an RNA. We investigate the mechanism by which AAUAAA is recognized by determining the effect of perturbing the ribose moiety of the U in AAUAAA.

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MATERIALS AND METHODS

Transcription in vitro. RNAs were prepared by in vitro transcription of synthetic oligonucleotide templates with T7 RNA polymerase (New England BioLabs, Inc.), as described previously (14) except that our T7 promoter oligonucleotide extends from -17 to -1 rather than to +1. Also, some T7 transcriptions were performed by using the buffer for SP6 RNA polymerase (13); the RNA yield is equivalent to that obtained with the buffer of Milligan et al. (14). α-32P-labeled nucleotide triphosphates were included in transcription reactions to label RNAs. 2′-Deoxyuridine (2′-dU) was incorporated by inclusion of 100 μM 2′-dUTP (Pharmacia) in the transcription reaction, with no UTP added. All RNAs lacked a cap. Transcripts were purified by electrophoresis through polyacrylamide gels containing 7 M urea.

As described by Milligan et al. (14), the yield of transcripts synthesized by T7 polymerase varies considerably, depending on the first few nucleotides in the transcript. For example, a high yield is obtained with GG or GC as the first two nucleotides, whereas initiating with AAA or AUA results in very low yields.

RNA substrates. All RNAs correspond to the region upstream of the simian virus 40 (SV40) late mRNA poly(A) site (designated +1) or are variants of this sequence. All sequences are given in the 5′-to-3′ direction.

(i) -23/+1 and derivatives. -23/+1 is a 24-nucleotide RNA, representing the SV40 late sequence from 23 nucleotides 5′ to and including the poly(A) site at +1. The sequence of -23/+1 RNA is GCUCCAAUAACACGCUAAACA ACA. Shorter RNAs (-23/-2, -3, -4, -5, -6, -7, -8, -9, and -10) were isolated as premature termination products of a transcription reaction of the full-length -23/+1 template. -23/+1 mutant RNA contains AAUACA rather than AAUAAA.

(ii) -20/-7 and derivatives. A DNA template for -20/-7 RNA was used to generate -20/-6, -7, -8, and -9 RNAs. The sequence of -20/-7 RNA is GCAUAUCAOGUUUUAUUAA. -20/-7 single-U RNA was transcribed from a different DNA and contains an altered -20/-7 sequence, with only a single U and a single C residue. The sequence of this RNA is GGAUAUAAACAGAA.

(iii) -38/+1 single-U RNA. -38/+1 single-U RNA represents the SV40 late sequence from -36 to +1, with two additional G residues at the 5′ end and with all U residues changed to C except for the U in AAUAAA. A total of seven uridines have been changed. Its sequence is GGGCCAGCCA CCACAGCAGCCAAUAAACAGCAAACACAACA. In spite of the seven mutations, this RNA is polyadenylated as efficiently as the natural sequence in the combined Mono Q and DE-600 fractions (not shown).

(iv) Minimal substrates and derivatives. The sequences of these RNAs differ entirely from that of SV40 RNAs. The sequences of these RNAs are AAUAAACCCCA, AAGAAACCCCA, AAUAAACCCCA, AAUAAACCCCA, and AAUAAACCCCA. These RNAs are analogous to -18/-8 and -18/-9 RNAs and contain only 10 or 11 nucleotides.

Characterization of RNAs. To characterize purified transcription products, transcripts were analyzed by digestion with RNase T1. Labeled RNAs (10 fmol, mixed with 25 μg of yeast RNA) were digested with RNase T1 (5 U) at 37°C for 30 min. Digestion products were analyzed by electrophoresis on denaturing polyacrylamide gels next to RNase T1 digestion fragments of known length. To identify the 3′-terminal oligonucleotide, T1 digestion products were treated with calf intestinal phosphatase. All internal oligonucleotides decrease in electrophoretic mobility after treatment with phosphatase, since phosphatase removes the 3′ phosphate groups left by RNase T1. Only the 3′-terminal T1 oligonucleotide, which already has a 3′ hydroxyl group, is unaffected in mobility by phosphatase. We used this lack of effect to identify this oligonucleotide.

-23 RNAs were characterized by their migration on denaturing gels relative to RNase T1 digestion products of known length and by comparison with -23/-9 RNA. This RNA (CAUUAACAGGAUU) was readily identified because unlike all other RNAs in the -23 series, it ends in a G residue. RNase T1 treatment of this RNA generates CAUUAACAGGAUU; RNase T1 treatment of all longer RNAs (e.g., -23/-8) generates the same oligonucleotide carrying a 3′-terminal phosphate instead. Thus, phosphatase treatment changes the mobility of this oligonucleotide when it is derived from -23/+1 to -23/-8 but does not affect its mobility when it is derived from -23/-9 RNA. With shorter RNAs (i.e., -23/-10), the 3′-terminal oligonucleotide is increased in mobility and insensitive to phosphatase.

Minimal substrates were identified by their migration on denaturing gels relative to -23 RNAs of known sequence.

RNase T1 analysis. To analyze the nucleotide composition of RNAs, labeled RNAs (1 fmol, mixed with 12.5 μg of yeast RNA) were digested with RNase T1 (1.5 U) at 37°C for approximately 16 h. Digestion products were separated by two-dimensional thin-layer chromatography as described previously (17, 20).

Polyadenylation assays. All polyadenylation reactions were carried out in vitro, using chromatographic fractions derived from a HeLa nuclear extract. Nuclear extract was prepared as described previously (29). Two fractions were combined for the assays: a Mono Q fraction [containing the poly(A) polymerase] and a DE-600 fraction (which confers AAU AAA specificity to the reaction). These two fractions have been described previously (2) and are analogous to crude polymerase and specificity factor fractions described by Christofori and Keller (4, 5). Reaction mixtures contained 3 μl of the Mono Q fraction from buffer D (29), 3 μl of the DE-600 fraction (in modified buffer D), 0.24 mM MgCl2, 20 mM phosphocreatine, 0.1 mM ATP, and 5 to 20 fmol of RNA in a total volume of 12.5 μl. Incubations were performed at 30°C for 0 to 30 min. RNAs were purified by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation and then analyzed by electrophoresis on denaturing polyacrylamide gels.

Each of the short substrates used in this study (-23/+1, -20/-7, -38/+1 single-U, and minimal RNAs) are polyadenylated more efficiently in the combined Mono Q and DE-600 fractions than in the unfractionated nuclear extract. The biochemical basis of this difference has not yet been determined but may involve inhibitors that are present in crude extract but missing in the more purified fractions and that interfere with polyadenylation of short RNAs.

Analysis of processing complexes. AAUAAA-specific polyadenylation complexes were analyzed on nondenaturing polyacrylamide gels (30). RNA (5 to 20 fmol) was incubated with 3 to 6 μl of DE-600 in the buffer conditions described above (total volume of 12.5 μl). 3′-dATP (1 mM) was included in the reaction mixture. After incubation at 25°C for 2 min, 4 μl was removed and added to 1 μl of heparin to give a final concentration of 0.5 mg/ml. This sample was loaded directly onto a nondenaturing, 4% polyacrylamide gel as described previously (30).

Quantitation. Autoradiograms were scanned by a laser...
RESULTS

RNA substrates and polyadenylation assays. RNAs were transcribed by T7 RNA polymerase from synthetic oligonucleotide templates. All RNAs correspond to or are variants of the region upstream of the poly(A) site of SV40 virion protein mRNA except for the minimal substrates analyzed in Fig. 1. The natural polyadenylation site is designated +1. Thus, a −23/+1 RNA contains 24 nucleotides, ending at the natural polyadenylation site. AAUAAA lies between −13 and −18.

Polyadenylation assays were performed by using crude chromatography fractions derived from a HeLa nuclear extract. For AAUAAA-specific polyadenylation, two fractions were combined: a fraction containing an AAUAAA specificity factor and another containing the poly(A) polymerase (see Materials and Methods).

An 11-nucleotide RNA is a minimal substrate. To define a minimal substrate for the polyadenylation reaction, we generated short oligoribonucleotides containing the essential AAUAAA polyadenylation signal. The complete sequences of these RNAs are provided in Fig. 1A, and the results obtained by using them as substrates are presented in Fig. 1B and C. The sequence AAUAAACCCCA was selected because CCCCA does not occur in any natural mRNA immediately downstream of AAUAAA.

AAUAAACCCCA was polyadenylated in vitro (Fig. 1B, lanes 1 and 2; Fig. 1C, lanes 1 and 2). The identity of the polyadenylated material was confirmed by its retention on oligo(dT)-cellulose (Fig. 1D, lanes 1 and 2). This reaction was AAUAAA specific: AAGAAACCCCA, containing a single-point mutation in AAUAAA, was not detectably polyadenylated (Fig. 1B, lanes 3 and 4).

AAUAAACCCCA is a minimal polyadenylation substrate, since RNAs that lacked either the first nucleotide (AAUAAACCCCA; Fig. 1C, lanes 3 and 4) or the last nucleotide (AAUAAACCCCA; Fig. 1C, lanes 5 and 6) were no longer polyadenylated. The failure of AAUAAACCCCA as a substrate was not due to the terminal cytidine, since AAUAAACCCCA also was inert (Fig. 1C, lanes 7 and 8).

Polyadenylation efficiency depends on the distance between AAUAAA and the 3′ end. The 3′ end of AAUAAACCCCA lies eight nucleotides upstream of the natural polyadenylation site generated by cleavage of SV40 late pre-mRNA (+1 [20]). RNAs that end at that natural site, such as −23/+1 or −141/+1 RNAs, are polyadenylated more efficiently than AAUAAACCCCA (not shown). To determine whether the difference in efficiency was due to the difference in the number of nucleotides between AAUAAA and the 3′ end of these RNAs, we prepared substrates that differed systematically in the length of this region. The longest RNA was −23/+1. We tested nine other RNAs, from −23/+2 to −23/+10. Each RNA is shorter than the preceding one by a single nucleotide at the 3′ end (Fig. 2A), as confirmed by RNase T1 analysis (see Materials and Methods). The ability of these RNAs to support polyadenylation is presented in Fig. 2B.

RNAs with termini at −2, −3, −4, or −5 received poly(A) as efficiently as did RNA that ends at +1, the natural poly(A) site (Fig. 2B, lanes 1 to 10). Between −6 and −8 (lanes 11 to 16), polyadenylation gradually decreased until by −9 and −10 it was no longer detectable (lanes 17 to 20). Quantitation of these data is presented in Fig. 2C. For comparison, we include data obtained with −20 to −6, −7, −8, −9 RNAs and from reactions with AAUAAACCCCA and AAUAAC CCC (−18 RNAs).

The decrease observed between −5 and −9 cannot reflect a requirement for a certain overall length in the oligoribonucleotide. −20/−6 RNA was polyadenylated but −23/−9 was not, yet both of these RNAs are 15 nucleotides in length. Thus, we conclude that the distance between AAUAAA and the 3′ end affects polyadenylation efficiency and that the reaction is optimal with eight or more nucleotides 3′ to AAUAAA.

There appear to be no strict sequence requirements for the nucleotides between AAUAAA and the 3′ end. AAUAAC CCCA is not found in any natural mRNA yet is a competent substrate (Fig. 1). Similarly, the RNAs in Fig. 2 differ in their precise sequences and in the identity of the nucleotide to
which poly(A) is added yet yield a single systematic result. On the basis of these and other results (see Discussion), we suspect that AAUAAA, (11 nucleotides of RNA, in which AAUAAA are the first 6) is not an unreasonable representation of a minimal substrate for polyadenylation.

Although the only strict sequence requirement appeared to be AAUAAA, sequences other than AAUAAA could influence polyadenylation efficiency (Fig. 2C). For example, AAUAAACCCCA (−18/−8) was polyadenylated more efficiently than either GAUAAACCAAGU (−20/−8) or GCU

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FIG. 3. Analysis of complexes formed between AAUAAA specificity factors and 3'-truncated substrates. (A) Detection of complexes. Labeled RNAs were incubated in the DE-600 fraction (containing AAUAAA specificity factors) and analyzed by electrophoresis on nondenaturing polyacrylamide gels. Lanes: 1, -23/-5; 2, -23/-7; 3, -23/-9; 4, -23/+1 containing AAUACA instead of AAUAAA; 5, -18/-8 (AAUAAACCCCA); 6, -18/-9 (AAUAAA CCCC). Within each experiment, equal amounts of radioactivity were loaded on the gel for each sample. Naked RNA was run off the gel. Nonspecific complexes (i.e., complexes that form largely independently of RNA sequence) are not formed when the DE-600 fraction is used but are formed very rapidly in unfractionated nuclear extract (e.g., references 20 and 29). We infer that the components which form these complexes have been removed during fractionation. (B) Comparison of polyadenylation and complex formation. Efficiencies of polyadenylation (from Fig. 2C) and complex formation were quantitated by densitometry. Complex formation efficiency was averaged from three independent experiments for -23 RNAs and two experiments for -18 RNAs. The efficiencies are presented relative to the value for -23/-5 as 100% for the -23 RNAs and relative to the value of -18/-8 as 100% for the -18 RNAs. P, Polyadenylation; C, complex formation. -23/-9 and -18/-9 RNAs were not detectably polyadenylated.

A. Lamond, and M. Wickens, unpublished data), we first focused on the role, if any, of the 2'-hydroxyl (2'-OH) groups present in the AAUAAA sequence. We tested the effect of removing the oxygen atom from the 2'-OH group of a single nucleotide, the U in AAUAAA. To do so, we prepared short substrates containing only a single U, that is AAUAAAA. We generated substrates that differed only in the ribose moiety of this single nucleotide by transcription in vitro in the presence of either UTP or 2'dUTP. The abilities of these two types of RNA to support polyadenylation were then compared.

Figure 4A shows a time course of polyadenylation in vitro for -20/-7 RNA containing either a normal ribose-containing uridine (rU) (lanes 1 to 3) or 2'dU (lanes 4 to 6). The transcript containing 2'dU was polyadenylated less efficiently.

To test whether this result was peculiar to the particular RNA used, the same analysis was performed with a derivative of -38/+1 RNA that contains only a single U. This RNA contains the natural SV40 sequence except that the only U is the U in AAUAAA; all other U's have been changed to C. This RNA is polyadenylated as efficiently as natural, unmodified -38/+1 RNA (P. Wigley, unpublished data). Again, as
with −20/−7 RNAs, lack of the 2′-OH dramatically impaired polyadenylation (Fig. 4B).

The 2′-dUTP preparation used to generate these transcripts was contaminated with unmodified rUTP (J. Milligan and O. Uhlenbeck, personal communication; Fig. 4C). Therefore, the RNA generated in a transcription reaction using this dUTP preparation is a mixed population: some RNAs contain rU, whereas other RNAs contain 2′-dU. It was therefore essential to determine whether the polyadenylated products produced by using this mixed population contained rU or 2′-dU, or both. To quantify the relative amounts of rU- and 2′-dU-containing RNAs, −38/+1 RNA (labeled with [α-32P]ATP) were digested with RNase T2, and the products were analyzed by two-dimensional thin-layer chromatography (Fig. 4C). RNase T2 cleaves after each ribonucleotide, generating 3′-ribononophosphates. It does not cleave after 2′-deoxynucleotides. Thus, T2 digestion of RNA containing a single U will generate rUp if the RNA contains rU but will instead generate 2′-dUpAp if the RNA contains 2′-dU. As a result, the ratio of rUp to 2′-dUpAp can be used to determine the relative abundance of the two types of RNA in a mixed population.

As expected, RNase T2 digestion of RNA transcribed in the presence of rUTP yielded only unmodified rUp as well as dUp (Fig. 4C, panel 1; no Gp was detected because the RNA was labeled with [α-32P]ATP and contained no GpA dinucleotides). RNA transcribed in the presence of 2′-dUTP generated both rUp and dUpAp, as expected (Fig. 4C, panel 2). To assess unambiguously the relative ability of rU- and dU-containing RNAs to support polyadenylation, we determined the proportions of rU and dU in polyadenylated RNA produced by using this mixed population as a substrate. Almost all of the polyadenylated RNA contained rUp, not dUp (Fig. 4C, panel 3). In contrast, RNA that failed to react during the incubation (i.e., not polyadenylated) was enriched for dUp (panel 4). From the ratios of rUp to dUp in the substrate (0.5) and in the polyadenylated products (15.0), we deduce that polyadenylation exhibits a 30-fold preference for rU over 2′-dU (see legend to Fig. 4C for details). Whether 2′-OH groups elsewhere in AAUAAA are also essential has not yet been determined.

The requirement for a 2′-OH group is not observed outside of AAUAAA. To investigate the specificity of the requirement for a 2′-OH group, 2′-dC was incorporated into −20/−7 RNA, which contains a unique C residue immediately 3′ to AAUAAA. We compared the polyadenylation of this RNA (Fig. 5A, lanes 4 to 6) with that of the same RNA containing rC (lanes 1 to 3). These two RNA species were polyadenylated with the same efficiency. Moreover, RNase T2 digestion demonstrated that the 2′-dC-containing RNA contained only deoxyyrididine: dCpAp was generated, but rCp was not (Fig. 5B). Thus, we infer that removal of a 2′-OH group in the nucleotide immediately downstream of AAUAAA does not affect polyadenylation. In this sense, the requirement for a 2′-OH group is specific for the U in AAUAAA. Whether 2′-OH groups elsewhere in AAUAAA are also essential has not yet been determined.

The 2′-OH group is required for interaction with specificity factors. To determine how the lack of a 2′-OH group prevents polyadenylation, we analyzed the binding of specificity factor to 2′-dU-containing RNAs, using a gel retardation assay. Labeled −38/+1 RNA, containing either rU or 2′-dU in AAUAAA, was incubated with unfraccionated extract (Fig. 6A, lanes 1 to 6) or with fractions of the extract that contain AAUAAA specificity factor but no detectable poly(A)-polymerase (Fig. 6A, lanes 7 to 14). In both cases, the 2′-dU-containing RNA did not support the formation of polyadenylation-specific complexes as efficiently as did normal, rU-containing RNA. The specificity of the complexes formed on the rU- and 2′-dU-containing −38/+1 RNAs was demonstrated by comparison with a longer, −58/+1 RNA (Fig. 6A, lane 15 to 18): the slowly migrating complexes formed on all −38/+1 and −58/+1 RNAs comigrated (Fig. 6A, lane 15 to 17), and as demonstrated with the −58/+1 RNA, their formation required AAUAAA, since it was abolished by a mutation to AAUAAA (Fig. 6A, lane 18). The components in the extract that gave rise to nonspecific complexes were missing from the specificity factor fraction; as a result, RNA that failed to enter complexes after incubation with this fraction comigrated with free RNA (Fig. 6A, lanes 7 to 18). On the basis of these results, we conclude that the 2′-dU-containing RNA interacts inefficiently with components in the specificity factor fraction, presumably with specificity factor itself.

To confirm this conclusion and to quantitate the magnitude of the effect, we determined the ratio of rU to 2′-dU in those RNAs that formed complexes with specificity factor (Fig. 6B). [32P]ATP-labeled 2′-dU-containing RNA was incubated with the specificity factor fraction for 2 min. Specific complexes were then purified by gel electrophoresis. RNA from these complexes was analyzed by RNase T2 digestion and two-dimensional thin-layer chromatography (Fig. 6B, panel 1). As a control, we analyzed RNA that was incubated with specificity factors but failed to form a complex (Fig. 6B, panel 2).

The dU-containing substrate, before incubation with specificity factor, yielded both rUp and 2′-dUpAp in a 1:2 ratio, as already shown (e.g., Fig. 4). In contrast, RNA that entered the specific complex yielded predominantly rUp; the ratio of rUp to 2′-dUpAp spots derived from this RNA was 7.5:1 (Fig. 6B, panel 1). Thus, RNA containing 2′-dUp was
preferentially excluded from the complex. As expected, RNA that failed to enter the complex yielded the same 1:2 ratio (rUp to 2'-dUpAp) as did the substrate (Fig. 6B, panel 2). This RNA was not enriched for 2'-dUpAp because only a small fraction of the substrate entered the complex in the brief, 2-min incubation with specificity factor. Comparing the ratios of rUp to 2'-dUpAp in the substrate (0.5) and in the RNA from the specific complex (7.5), we conclude that specificity factors exhibit at least a 15-fold preference for rU-containing RNAs. Thus, this defect in the interaction with specificity factor is nearly sufficient, quantitatively, to account for the 30-fold defect in polyadenylation activity (Fig. 4).

**DISCUSSION**

Our results demonstrate that AAUAAA not only is necessary but is virtually sufficient for polyadenylation of pre-cleaved RNAs. Very short substrates (as few as 14 nucleotides) are polyadenylated efficiently as long as they contain AAUAAA. The shortest oligoribonucleotide we have used that supports polyadenylation, AAUAAACCCCA, contains only 11 nucleotides (Fig. 1). RNAs lacking a single nucleotide from one end or the other are not polyadenylated, demonstrating that operationally, this 11-nucleotide RNA is a minimal substrate in vitro. Thus, AAUAAACCCCA is sufficient to interact with the polyadenylation machinery.
which contains at least a poly(A) polymerase and a factor that recognizes AAUAAA, possibly an snRNP (2, 4, 5). The requirement for 11 nucleotides reflects the minimal length of RNA capable of interacting productively with both an AAUAAA recognition factor and poly(A) polymerase, since polymerase alone can efficiently add adenylylate residues to a tetraneucleotide substrate (24).

AAUAAACCCCA is the shortest RNA we have used that is capable of being detectably polyadenylated. However, optimal polyadenylation requires eight or more nucleotides downstream of AAUAAA (Fig. 2). As a result, polyadenylation of AAUAAACCCCA is less efficient than that of an RNA which extends three more nucleotides downstream. A computer-assisted analysis of 262 mRNA sequences reveals that the poly(A) site is within seven nucleotides of AAUAAA in fewer than 4% of those mRNAs (Wickens, in preparation). This is consistent with our results and suggests that aside from any constraints imposed by the cleavage reaction, efficient polyadenylation in vivo may also be optimal with eight or more nucleotides 3' to AAUAAA.

The sequence between AAUAAA and the 3' end appears to have little influence on polyadenylation, consistent with its lack of conservation (Wickens, in preparation). As the 3' end is brought progressively closer to AAUAAA (Fig. 2), substrates necessarily differ in sequence yet yield a systematic and progressive reduction in activity. Furthermore, the CCCCCA of AAUAAACCCCA, the minimal substrate, is not found in any natural mRNA yet supports polyadenylation (Fig. 2). No single base between AAUAAA and the polyadenylation site of SV40 late mRNA can be essential, since chemical modification of each base in this region does not reduce polyadenylation (6). On the basis of these several independent lines of evidence, we deduce that the requirement for five nucleotides beyond AAUAAA reflects a requirement for a minimum absolute distance, not a specific sequence. We infer that the polymerase must simultaneously contact specificity factor and the 3'-OH group and cannot do so on too short a tether (see below). Different sequences should vary in precisely how many nucleotides beyond AAUAAA are required, depending on their three-dimensional structure, yet each should exhibit a minimum length requirement. This is observed by using three slightly different sequences in Fig. 2. Further experiments, using more substrates in which the sequence between AAUAAA and the 3' end is varied systematically, will be needed to test this prediction conclusively.

RNAs with fewer than eight nucleotides 3' to AAUAAA interact efficiently with specificity factors yet are poor substrates for polyadenylation (Fig. 3). Thus, addition of poly(A), and not association with specificity factors, is the step affected by the distance between AAUAAA and the 3' end. Either the poly(A) polymerase interacts less stably with 3'-shortened substrates or the polymerase interacts stably but has reduced access to the 3' terminus of the RNA.

Not surprisingly, sequences outside of AAUAAA can influence the efficiency of the reaction. We anticipate that these nucleotides influence the structure of the substrate and alter either the effective distance between AAUAAA and the 3' end (see above) or the accessibility of AAUAAA. To assess the influence of sequences outside of AAUAAA, systematic structural analyses may be necessary. We therefore stress, at this point, two main conclusions: very short oligoribonucleotides support polyadenylation, and a minimal distance between AAUAAA and the 3' end is required.

Specificity factors can interact with AAUAAA in the absence of poly(A) polymerase. AAUAAA-specific complexes are formed with factors present in a specificity factor fraction that contains no poly(A) polymerase activity (Fig. 3 and 6). Furthermore, these complexes are very similar in electrophoretic mobility to those formed in unfractionated nuclear extract by using longer, more conventional substrates (not shown). Although our data demonstrate that polymerase is not required for interaction with AAUAAA, it remains possible that in unfractionated extract and in vivo, polymerase and specificity factors preassemble into a complex before interaction with the substrate.

The 2'-OH group of the U in AAUAAA is required for efficient polyadenylation (Fig. 4) and for the binding of specificity factor (Fig. 6). These effects are specific, since removal of the 2'-OH of the C immediately 3' to AAUAAA has no effect on polyadenylation (Fig. 5). The failure of 2'-dU-containing RNAs to interact with specificity factor is nearly sufficient, quantitatively, to account for their defect in polyadenylation (Fig. 4 and 6). Further analysis will be required to determine whether the 2'-OH group of each ribose in AAUAAA is required.

The 2'-OH group of the U in AAUAAA could be necessary for interaction with specificity factor either because the hydroxyl is a contact point for that factor or because the hydroxyl is required to form a structure in AAUAAA that is recognized via other contact points. Removal of the single oxygen atom at the 2' position can result in rather dramatic effects on nucleotide conformation, affecting the pucker of the ribose and the rotational position of the base relative to the sugar (19). Such changes in structure could perturb interactions distant from the hydroxyl group. Although removal of a single 2'-OH group thus can have effects at a distance, we stress that the effect we observe is specific for the 2'-OH of the U in AAUAAA; removal of the 2'-OH of the first nucleotide downstream of AAUAAA has no effect on the reaction.

AAUAAA may be recognized either by a protein or by an snRNP. If an snRNA recognizes AAUAAA by base pairing, then the 2'-OH of the U must be required for that interaction. Thus, the simplest interpretation implied by the data is that at least one of the components that interacts with AAUAAA is a protein. Further analysis of the precise involvement of the 2'-OH group will require purified specificity factor and an understanding of the structure of AAUAAA in the RNAs with which it interacts. Attaining both of these goals may be facilitated by the use of very short oligoribonucleotide substrates of the type described in this report.

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