Site-Directed Ribose Methylation Identifies 2'-OH Groups in Polyadenylation Substrates Critical for AAUAAA Recognition and Poly(A) Addition

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Summary

The importance of sugar contacts for the sequence-specific recognition that occurs during polyadenylation of mRNAs was investigated with chemically synthesized substrates containing 2'-O-CH₃ groups at selected riboses. An RNA (5'–CUGCAAUAAACAAGU–AAU–3') with 2'-O-CH₃ ribose at each nucleotide except for the AAUAAA sequence and 3'-terminal adenosine was efficiently polyadenylated in vitro. Methylation of single riboses within AAUAAA inhibited both poly(A) addition and binding of the specificity factor, but the magnitude of inhibition varied greatly at different nucleotides. Nucleotides that showed sensitivity to base substitutions did not necessarily show sensitivity to ribose methylation, and vice versa. The data indicate that the specificity factor interacts with AAUAAA through RNA–protein contacts involving essential recognition of both sugars and bases at different nucleotide positions.

Introduction

The availability of powerful methods for in vitro mutagenesis, combined with chemical modification techniques, has led to a wealth of information concerning nucleotide bases and phosphates that are critical for the sequence-specific binding of proteins to DNA. Similarly, in RNA–protein interactions mutagenesis and base modifications can identify bases that are important. However, these methods do not provide information about potentially important contacts between proteins and riboses in the sugar phosphate backbone. Yet RNA-binding proteins are known to make intimate contacts with riboses as well as with bases and phosphates. For example, the crystal structure of tobacco mosaic virus reveals that the viral coat protein binds to its RNA genome by contacting bases, phosphates, and the 2' hydroxyl of the ribose (reviewed in Holmes, 1980). In this case the RNA is predominantly bound through attachment of a nucleotide triplet to an α-helical domain of the coat protein. A major component of this binding interaction involves H-bonds formed between aspartate side chains and 2' ribose hydroxyls. These results emphasize the importance of mapping protein–ribose interactions when studying nucleic acid–binding proteins, especially in the case of factors that recognize RNA.

The method described by Beijer et al. (1990) for chemically synthesizing RNA sequences containing riboses with either 2'-OH or 2'-O-CH₃ groups at specific nucleotides offers a rapid way to study the contributions of specific ribose residues to protein recognition of RNA. Here we describe the application of this chemical approach to analyze the importance of specific ribose moieties for the sequence-specific recognition of RNA during pre-mRNA processing. The addition of poly(A) to the 3' terminus of pre-mRNA has been used as a model system for this study. A HeLa cell in vitro system has been shown to add poly(A) to exogenous pre-mRNAs accurately and efficiently (Moore and Sharp, 1985; reviewed in Wickens, 1990). This reaction critically depends on the sequence AAUAAA being present in the substrate. This highly conserved sequence typically lies 10 to 25 nucleotides upstream of the nucleotide to which poly(A) is added. The in vitro system will both cleave the pre-mRNA substrate downstream of AAUAAA and then add poly(A) to the resulting 3' terminus, as occurs in vivo. However, poly(A) addition can be uncoupled from cleavage by using a substrate already having a free 3'-OH 8 to 25 nucleotides downstream of AAUAAA. With such "precleaved" RNAs, AAUAAA-dependent 3'-poly(A) addition occurs in the absence of any cleavage reaction. This assay—addition of poly(A) to the 3' end of a precleaved substrate—was used in this study. Point mutations in any of the six positions of AAUAAA reduce the efficiency of poly(A) addition, although the magnitude of the inhibition caused by base changes varies with the position tested and the base substituted (Sheets et al., 1990). The RNA sequence AAUAAA is sufficient to direct efficient polyadenylation in vitro provided there are at least eight additional nucleotides downstream (i.e., 3') (Wigley et al., 1990). The identity of the nucleotides downstream of AAUAAA is not critical (Wigley et al., 1990).

In vitro, AAUAAA-dependent polyadenylation is catalyzed by two separate components (Takagaki et al., 1988; Christofori and Keller, 1988; Gilmartin and Nevins, 1989; reviewed in Wickens, 1990): an AAUAAA specificity factor (also called CPF; Christofori and Keller, 1988) or PF2 (Gilmartin and Nevins, 1989) and a poly(A) polymerase (Christofori and Keller, 1989; Ryner et al., 1989; Bardwell et al., 1990; Wahle, 1991). The specificity factor probably interacts directly with AAUAAA, as suggested by the analysis of short RNA substrates using nondenaturing gel mobility retardation assays (Wigley et al., 1990), and by UV cross-
linking and modification interference experiments (K. Lang, S. Bienroth, G. Christofori, and W. Keller, unpublished data). The specificity factor appears to be a complex of several polypeptides (S. Bienroth and W. Keller, unpublished data) and may include an snRNA component (Christofori and Keller, 1989).

In this report, we use a ribose methylation interference assay to systematically probe whether specific ribose residues are critical for AAUAAA recognition and polyadenylation. RNAs that contain 2'-O-CH₃ groups at specific riboses are examined for their ability to receive 3' poly(A) and to bind to purified specificity factor. These data provide strong evidence for the importance of ribose contacts during the interaction of factors with functionally important RNA sequences.

Results

RNA Substrates and Quantitation of Polyadenylation Efficiency

Oligoribonucleotides were synthesized chemically and contained either 2'-OH or 2'-O-methyl (2'-O-CH₃) ribose residues. For simplicity we will refer to all substrates as RNA even though not all are conventional ribonucleic acids. We have adopted the convention that 2'-OH-containing nucleotides are indicated with uppercase letters, and 2'-O-CH₃-containing nucleotides with lowercase. Thus AAUAAA indicates a sequence in which only the second nucleotide contains a 2'-O-CH₃ group.

Unless noted otherwise, all the substrates we have used contain the same 18 nucleotide sequence: 5'-CUGCAAUAACAAAGUUA-3', derived from the polyadenylation site of simian virus 40 late pre-mRNA. The 3' end of this sequence does not precisely correspond to the site to which poly(A) is added in vivo, but instead terminates five nucleotides upstream of the wild-type SV40 cleavage position. Nonetheless, this RNA is polyadenylated in vitro with a high efficiency, as are RNAs that have the additional six 3' nucleotides present in the wild-type sequence (Wigley et al., 1990).

The efficiency of polyadenylation of each substrate is indicated, expressed relative to a wild-type RNA that has all 2'-OH groups. We define polyadenylation efficiency as the fraction of substrate remaining after 10 min that has received poly(A). In each case, the estimate of polyadenylation efficiency shown represents an average obtained from two to four separate experiments (see Experimental Procedures), including the representative data shown in part (B) of each figure. The values are presented primarily to allow a convenient comparison of the effects of ribose methylation and base substitution at different nucleotides in the substrate and are not an attempt to represent initial rates. It is important to note, however, that these estimates are necessarily imprecise as the individual substrates contain different numbers of 2'-O-CH₃ groups and thus are differentially sensitive to nuclease degradation (Sproat et al., 1989; Beijer et al., 1990).

2'-OH Groups Are Required for Polyadenylation

To determine whether 2' hydroxyl groups are required for poly(A) addition, we analyzed a series of substrates that contained predominantly, or exclusively, either 2'-OH or 2'-O-CH₃ groups. For each substrate, 5' end-labeled RNA was incubated with crude fractions of a HeLa cell nuclear extract that contained the specificity factor and poly(A) polymerase. The extent of polyadenylation was determined by denaturing polyacrylamide gel electrophoresis. The structures of these RNAs are diagrammed in Figure 1A, as is the quantitation of the results. The data are presented in Figure 1B.

An 18 nucleotide RNA that contained exclusively 2'-OH ribose moieties (RNA 1) was efficiently polyadenylated (Figure 1B, lanes 1–3). However, a substrate RNA of identical sequence, in which every ribose, except for the 3'-terminal nucleotide, had a 2'-O-CH₃ group (RNA 2), was not polyadenylated (Figure 1B, lanes 4–6). Similarly, polyadenylation did not occur with a substrate that was entirely 2'-O-CH₃ (RNA 3), even if the RNA sequence extended upstream of AAUAAA and downstream to the natural poly(A) addition site used in vivo (Figure 1B, lanes 7–9). RNA 1, unlike RNAs 2 and 3, was sensitive to cleavage by nucleases in the extract, resulting in the appearance of a shorter RNA fragment (lanes 1–3). The 2'-O-CH₃-containing oligoribonucleotides are resistant to such nuclease attack (Sproat et al., 1989).

These results led us to three conclusions. First, chemically synthesized RNAs as short as 18 nucleotides are efficiently polyadenylated in HeLa cell nuclear extracts, confirming previous results obtained with substrates prepared by in vitro transcription (Wigley et al., 1990). Second, uniform substitution of ribose 2'-OH groups with 2'-O-CH₃ groups inhibits polyadenylation. Third, the failure of RNAs containing 2'-O-CH₃ groups at every nucleotide to receive poly(A) addition site is the quantitation of the results. The data are presented in Figure 1B.

Figure 1. 2' Hydroxyl Groups Are Required for Polyadenylation

(A) Sequences and structures of RNA substrates. Uppercase letters indicate 2'-OH nucleotides and lowercase letters indicate 2'-O-CH₃ nucleotides. Footnote (a): the value of 1.0 for RNA 1 corresponds to 87% of the molecules receiving poly(A) in 10 min (average from four independent determinations).

(B) Each 5' end-labeled RNA was incubated in crude specificity factor and poly(A) polymerase fractions for 0, 5, or 10 min. RNAs were then recovered from each incubation and analyzed by gel electrophoresis.
Poly(A) is not due solely to a requirement for a 2'-OH on the 3'-terminal ribose, to which poly(A) polymerase might be expected to be particularly sensitive.

Polyadenylation Is Sensitive to 2'-O-CH₃ Substitution in AAUAAA
To identify more precisely positions at which ribose 2'-OH groups are required, an RNA was prepared in which the only 2'-OH groups were in AAUAAA and at the 3'-terminal nucleotide; all other nucleotides were 2'-O-CH₃ (cf. RNA 4; Figure 2). To our surprise, this RNA received poly(A) quite efficiently (Figure 2B, lanes 1-3). Polyadenylation of RNA 4 was also specific, since an otherwise identical substrate with a U to G mutation in AAUAAA (i.e., AA-GAAA) was inert (RNA 4-mut; Figure 2B, lanes 7-9). Thus an 18-nucleotide RNA containing ribose 2'-OH groups only in AAUAAA and at the 3' terminus is a substrate for AAUAAA-dependent polyadenylation.

We have reproducibly observed that RNA 4 is polyadenylated less efficiently than RNA 1, which has no 2'-O-CH₃ groups. To test whether this reduced efficiency was due to the 2'-OH groups downstream or upstream of AAUAAA, RNAs were prepared that contained 2'-OH groups in AAUAAA and at all nucleotides either upstream or downstream of AAUAAA. In the latter case, having 2'-OH groups upstream of AAUAAA did not increase the polyadenylation efficiency (data not shown). In the former case, having 2'-OH groups at all positions downstream of AAUAAA, polyadenylation efficiency was restored to a similar level to RNA 1 (Figure 2, cf. RNA 1 and RNA 5). The presence of 2'-OH groups at just one to three nucleotides in the region between AAUAAA and the 3' terminus was not sufficient to restore activity fully (data not shown). We infer from these results that, in addition to recognition of AAUAAA, the polyadenylation machinery may make important contacts with backbone riboses downstream (i.e., 3'), but not upstream, of the AAUAAA sequence.

Poly(A) Can Be Added Efficiently to a Terminal 2'-O-CH₃ Nucleotide
Poly(A) polymerase must interact with the 3'-terminal nucleotide of the substrate RNA in order to add the first adenosine of the poly(A) tail. To determine whether a ribose 2'-OH at the 3'-terminal nucleotide is essential for this interaction, an RNA was synthesized containing 2'-OH groups only in AAUAAA and with 2'-O-CH₃ groups at all other positions (RNA 6; Figure 3). This RNA was polyadenylated less efficiently than an otherwise identical RNA (RNA 4) with a 2'-OH at its 3'-terminal ribose (Figure 3B, lanes 1-6). Thus, in the context of a mixed 2'-OH/2'-O-CH₃ substrate, the presence of a 3'-terminal ribose 2'-OH increases the efficiency of polyadenylation.

To test whether a 2'-OH group at the 3' terminus is similarly important in an RNA otherwise containing only 2'-OH groups, a substrate with a single 2'-O-CH₃ group at the 3'-terminal ribose was analyzed (RNA 7). In contrast to RNA 6, this RNA was polyadenylated nearly as efficiently as an all 2'-OH substrate (Figure 3B, lanes 7-12, compare both 5 and 10 min time points). These data indicate that

Figure 2. Polyadenylation Is Sensitive to 2'-O-CH₃ Substitution in AAUAAA
(A) Sequence and structures of RNA substrates. Footnote (a): the value of 1.0 for RNA 1 corresponds to 87% of the molecules receiving poly(A) in 10 min (average from four independent determinations). Footnote (b): the value of 1.0 for RNA 1 corresponds to 87% of the molecules receiving poly(A) (average from four independent determinations). (B) Each 5' end-labeled RNA was incubated in crude specificity factor and poly(A) polymerase fractions for 0, 5, or 10 min. RNAs were then recovered from each incubation and analyzed by gel electrophoresis.
poly(A) polymerase can efficiently add adenosine to a 2'-O-CH₃-containing nucleotide. However, this interpretation assumes that the 2'-O-CH₃ nucleotide at the 3' terminus of RNA 7 was not removed by nucleases in the HeLa extract prior to polyadenylation. To validate this assumption, we determined whether the 2'-O-CH₃ group was still present in RNA 7 molecules that had received poly(A). To do this, 5' end-labeled RNA 7 was used as a substrate. RNA molecules that received poly(A) were isolated, subjected to limited alkaline hydrolysis, and then analyzed by denaturing polyacrylamide gel electrophoresis (Figure 4). RNA is susceptible to alkaline cleavage at phosphodiester bonds adjacent to 2'-OH but not 2'-O-CH₃ residues. As a result, alkaline hydrolysis of 5' end-labeled RNAs generates a "ladder" of bands: each band represents an RNA cleaved at phosphodiester bonds adjacent to a 2'OH ribose group. A 2'-O-CH₃ group results in a gap in the ladder.

The polyadenylated RNA 7 molecules yielded a ladder of products, extending from mononucleotides to polyadenylated RNA, each ending with a 3' phosphate (Figure 4, lane 3). At the position corresponding to the terminal nucleotide of the RNA 7 substrate, a single band was missing. As expected, the nonpolyadenylated RNA 7 control had no missing bands (lane 2). No missing bands were observed upon alkaline hydrolysis of a polyadenylated substrate containing no methylated riboses (data not shown). This proves that poly(A) can be added to an RNA that carries a 2'-O-CH₃ group in its 3'-terminal ribose moiety.

Specific 2'-OH Groups in AAUAAA Are Sensitive to Ribose Substitution
To identify the critical 2'-OH groups within the AAUAAA sequence, a series of six substrates was prepared based on the structure and sequence shown in Figure 5(A). Each 5' end-labeled RNA was incubated in crude specificity factor and poly(A) polymerase fractions for 0, 5, or 10 min. RNAs were then recovered from each incubation and analyzed by gel electrophoresis.
on the structure of RNA 4. In each case, a different, single ribose in the AAUAAA sequence of RNA 4 was converted from 2'-OH to 2'-O-CH₃ (Figure 5A). The presence of a 2'-O-CH₃ group at either the second or fifth position in AAUAAA reduced polyadenylation of the substrate by at least 10-fold (Figures 5A and 5B). Furthermore, this inhibition is a consequence of the structure of these RNAs and is not caused by any impurity in the samples, as neither inhibits polyadenylation of RNA 4 when present in the same reaction (data not shown). Upon substitution at the fourth and sixth positions, more modest reductions of approximately 3- to 5-fold were observed. Ribose methylation at the first and third positions had even smaller (approximately 2-fold) effects.

As the inhibitory effect of ribose methylation appeared to vary so markedly at different nucleotide positions, we considered it essential to verify that the individual substrates really had the expected methylation structure. Therefore, each of the RNAs shown in Figure 5 was subjected to limited alkaline hydrolysis and analyzed by denaturing gel electrophoresis (Figure 6). Each RNA was susceptible to cleavage at all phosphodiester bonds within the AAUAAA sequence except for the single position that carried a 2'-O-CH₃ group. This confirmed that the ribose composition of each substrate was correct. The inhibitory effects of 2'-O-CH₃ substitution are therefore proven to be highly specific and to vary in magnitude between different nucleotide positions in AAUAAA, with positions 2 and 5 displaying the largest effects.

Introduction of a Single 2'-O-CH₃ Group Can Inhibit Polyadenylation

In the preceding experiment (Figure 5), function was restored to all 2'-OH RNA by introducing a minimal number of 2'-OH groups. We next performed the reciprocal experiment and sought to inactivate an RNA containing only 2'-OH groups by introducing a minimal number of 2'-O-CH₃ groups. For this analysis we focused on positions 2 and 3 of the AAUAAA sequence. RNAs were prepared that contained 2'-OH groups at every nucleotide except for position 2 or position 3 of AAUAAA (RNA 1–2 [AaUAAA] and RNA 1–3 [AAuAAA]). In these experiments purified specificity factor and poly(A) polymerase were used rather than crude fractions. This permits a more precise evaluation of the effect of ribose methylation on AAUAAA recognition and polyadenylation and circumvents complications arising from the presence of contaminating nucleases in the crude extract fractions. The specificity factor used had been enriched approximately 2000-fold (S. Bienroth, E. Wahle, and W. Keller, unpublished data); the poly(A) polymerase had been purified 6000-fold and was essentially homogeneous (Wahle, 1991). The purified specificity factor and polymerase were obtained from calf thymus, but in all respects tested thus far they are equivalent to their HeLa cell counterparts. In particular, HeLa and calf activities can functionally substitute for one another in polyadenylation assays (Bardwell et al., 1990; Wahle, 1991; S. Bienroth, E. Wahle, and W. Keller, unpublished data). The efficiencies with which each of these singly substituted RNAs received poly(A) are given in Figure 7A and the corresponding data are shown in Figure 7B.

As expected, an RNA that contained only 2'-OH groups (RNA 1) received poly(A) efficiently (Figure 7B, lanes 1–3). In contrast, an RNA with a single 2'-O-CH₃ group in the second position of AAUAAA was polyadenylated poorly (RNA 1–2; Figure 7B, lanes 4–6). Methylation at the third position of AAUAAA (RNA 1–3), or at a single position downstream of AAUAAA (RNA 8), had only a modest effect (Figure 7B, lanes 7–12).

These results corroborate the data obtained in the previous experiments with RNAs that contain predominantly 2'-O-CH₃ groups (Figure 5). In particular, with both highly purified components and crude fractions, polyadenylation is dramatically reduced by the presence of a single 2'-O-CH₃ group at the second position of AAUAAA, but is only modestly affected by a 2'-O-CH₃ moiety at the third position. We have also observed that in some experiments with purified components, the length of the poly(A) tail added to substrates was reduced by both base substitution and ribose methylation. As this effect was not observed in all experiments, its significance is unclear.

Comparison of Base Substitutions and Ribose Substitutions

To compare directly the effects of base and ribose substitutions, we again focused on positions 2 and 3 of AAUAAA. Accordingly, RNAs were prepared in which every ribose
Table A

<table>
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</tr>
<tr>
<td>RNA 8</td>
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<tr>
<td>RNA 1-mut2</td>
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<tr>
<td>RNA 1-mut3</td>
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Figure 7. Introduction of a Single 2'-O-CH3 Group Can Inhibit Polyadenylation

(A) Sequence and structures of RNA substrates. Footnote (a): the length of poly(A) added varied slightly between substrates in certain experiments, as in the example shown. This was not considered in calculating relative efficiencies. Footnote (b): the value of 1.0 for RNA 1 corresponds to 73% of the molecules receiving poly(A) in 10 min (average from two independent determinations).

(B) Each 5' end-labeled RNA was incubated with highly purified specificity factor and poly(A) polymerase for 0, 5, or 10 min. RNAs were then recovered from each incubation and analyzed by gel electrophoresis.

Figure 8. Polyadenylation and Binding of the Specificity Factor Are Similarly Affected by Ribose and Base Substitutions

The 5' end-labeled RNAs used in Figure 7 were incubated with highly purified specificity factor, then were analyzed by gel electrophoresis under nondenaturing conditions. The position of specific (i.e., AAUAAA-dependent) complexes and of protein-free RNA are indicated. Identical amounts of radioactivity were present in each incubation.

Discussion

We have presented a ribose methylation interference study of the RNA elements necessary to direct addition of poly(A) to pre-mRNAs in vitro. Methylation of single ribose 2'-OH groups inhibits both polyadenylation and the binding of the specificity factor in a nucleotide-specific manner. It is striking that at the same nucleotide position both polyadenylation and specificity factor binding can be affected differently by ribose methylation and base substitution. Base changes in the second position of AAUAAA have only modest effects, while a ribose methylation is inhibitory. At the adjacent third position, base substitutions greatly reduce activity, but a ribose methylation has little effect. Recognition of the AAUAAA sequence by the polyadenylation machinery thus appears to require important contacts with both bases and riboses at different nucleotide positions. These results also suggest an explanation for the
common observation that within consensus sequences certain positions show minimal sequence conservation, while the adjacent bases are strictly conserved. One reason for this could be that the nucleotides showing little base conservation are predominantly recognized through contacts with the ribose or phosphate backbone. From the data in this study it is clear that nucleotides that are crucial components of sequence-specific RNA–protein binding interactions need not be particularly sensitive to base substitutions.

In principle, the inhibitory effects of 2'-O-CH₃ substitution at specific nucleotides could arise either because of a direct requirement for a hydroxy group at that position, or from an induced structural distortion of the furanose ring or base. However, crystallographic and spectroscopic data indicate that structural distortion does not play a major role in the effects we have observed. Crystallographic studies of 2'-O-CH₃-containing oligoribonucleotide homopolymers show that the furanose ring adopts the standard, RNA-like 3'-endo conformation (Bobst et al., 1969; Leslie and Arnett, 1977; Zmudzka et al., 1989), although crystals of the unpolymerized nucleoside 2'-O-CH₃ cytidine do not (Hingerty et al., 1974). Similarly, in solution, 2'-O-methylation does not perturb RNA–RNA hybrids, as judged by UV and circular dichroism spectroscopic analyses using homopolymers (Bobst et al., 1969), and by Tₘ measurements using oligoribonucleotides (Inoue et al., 1987). Furthermore, 2'-O-CH₃ hybrids of mixed sequence form very stable hybrids with complementary RNAs (Barabino et al., 1989, 1990; Blencowe et al., 1989). The presence of a single 2'-O-CH₃ group in an RNA substrate would not therefore be expected to induce any major structural distortion of the polymer or prevent base pairing to a complementary sequence. We thus argue that ribose methylation at critical positions within AAUAAA inhibits specificity factor binding by disturbing essential contacts with protein.

Methylation of the ribose 2'-OH group could interfere with protein contacts in two ways. First, the bulkier methyl group could cause steric hindrance. Second, the methyl group alters the chemical properties of the 2' oxygen, changing it from an alcohol to an ether. In tobacco mosaic virus, the crystallographic data show that the predominant mode of protein contact with the 2' position of the ribose involves direct hydrogen bonding between carbohydrate side chains of amino acids and the 2'-OH. These interactions will be disrupted by 2'-O-methylation. We note, however, that although the electronegativity of the oxygen is decreased by methylation, it may still function as a donor of electrons to an electron-deficient partner. This behavior of ethers occurs in complexes such as diethyl ether boron trifluoride. In the context of protein–nucleic acid interactions, this means that functional interactions with basic amino acid side chains may be maintained after methyl substitution. For example, an interaction with the NH₂ group of an amino acid could still be possible. Thus, the absence of interference by ribose methylation does not formally exclude the possibility of an important contact at that position.

Comparison of the effects of 2'-O-CH₃ and 2'-H substitution may help to illuminate precisely what features in the RNA are recognized. For example, at the third position in AAUAAA, substitution of 2'-OH with 2'-H impairs polyadenylation (Wigley et al., 1990), while substitution with 2'-O-CH₃ does not (this study). This could mean that at the third nucleotide of AAUAAA the 2'-OH serves as an electron donor in the interaction with the protein, as discussed above. Alternatively, inhibition by 2'-H substitution could result because the structure of the ribose is altered and discriminated by the binding factor. Analysis of substrates substituted with other ribose derivatives may resolve these possibilities.

We argue here that the binding of the specificity factor requires an RNA–protein contact. In particular, the data are not consistent with recognition of AAUAAA occurring exclusively through RNA–RNA base pairing, as might be expected if the specificity factor is a snRNP (Christofori and Keller, 1988). In the simplest view, a protein component of the specificity factor binds directly to AAUAAA. This is consistent with the observation that a 155 kd protein, present in highly purified specificity factor, can be cross-linked by UV light to AAUAAA-containing substrates (Moore et al., 1988; K. Lang, S. Bienroth, G. Christofori, and W. Keller, unpublished data). The ribose methylation results do not exclude the possibility that an RNA molecule is an important component of the specificity factor, but rather emphasize the importance of a protein–RNA contact in AAUAAA recognition. As the efficiency with which the specificity factor binds to ribose-methylated or base-substituted substrates parallels the ability of that RNA to be polyadenylated, this suggests that it is an inhibition of specificity factor binding that is responsible for failure to receive poly(A). However, quantitative comparisons between binding and polyadenylation activity will be required to determine whether the poly(A) polymerase is also affected by ribose methylation within AAUAAA.

Site-directed ribose methylation is clearly a powerful companion to site-directed mutagenesis in probing RNA–protein interactions. A major advantage of the chemical synthesis approach is flexibility: 2'-O-CH₃ groups, or nucleotide substitutions, can be placed at any preselected position of an oligonucleotide substrate. By examining the effects of an array of substitutions at a specific nucleotide position, the crucial features at that position may be established. We therefore expect that this methodology will prove useful in the analysis of a wide variety of protein–RNA interactions.

Experimental Procedures

Synthesis and Preparation of Oligoribonucleotides

Oligoribonucleotides, including 2'-O-methylated derivatives, were synthesized from base-protected phosphoramidites as previously described (Beijer et al., 1990; Sproat et al., 1989). All syntheses were done using an Applied Biosystems synthesizer, model 396B-02 (Foster City, CA). Deprotection of 2'-F-PMO-blocked ribose residues was done as described by Beijer et al. (1990). For polyadenylation assays approximately 5 pmol of oligoribonucleotides was 5' end-labeled with polyadenyl kinase and 50 μCi of [γ-32P]ATP (Maniatis et al., 1982) and loaded directly onto a 7 M urea, 15% polyacrylamide gel. Full-length RNA was excised and eluted as previously described (Bardwell and Wickens, 1990). Glycogen (10 μg) was included as carrier in the ethanol precipitation.
Preparation and Fractionation of Nuclear Extract

Preparation of HeLa cell nuclear extract was performed as described previously (Bardwell et al., 1990). A DE-600 fraction and a Mono Q fraction contain crude specificity factor and poly(A) polymerase, respectively, and were prepared as described (Bardwell et al., 1990). Nearly homogeneous poly(A) polymerase was prepared from calf thymus and consisted of a prominent polypeptide of 60,000 daltons (Wahle, 1991). It possessed a specific activity of $\approx 10^5$ U/mg (1 U is defined as the amount of polymerase required to incorporate 1 pmol of AMP in 1 min), and had been purified $\approx 6000$-fold. The specificity factor previously (Bardwell et al., 1990). A DE-600 fraction and a Mono Q

Polyadenylation In Vitro

Polyadenylation assays with crude fractions contained 3 $\mu$l of the specificity factor fraction (DE-600 in Bardwell et al., 1990), 3 $\mu$l of poly(A) polymerase fraction (Mono Q in Bardwell et al., 1990), 20 mM phosphocreatine, 0.1 mM ATP, 0.3 mM MgCl$_2$, and 5,000 to 15,000 cpm of 5' end-labeled RNA in a total volume of 12.5 $\mu$l. In reactions with purified components, reactions contained 2 $\mu$l of poly(A) polymerase (diluted from concentrated stock to 20 $\mu$l), 2 $\mu$l of the specificity factor (8 U), 2.25 $\mu$l fraction buffer (10% glycerol, 50 mM Tris-HCl [pH 7.9], 3 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 100 mM KCl), 10 mM phosphocreatine, 0.8 mM ATP, 0.1 mg/ml yeast tRNA, 2.6% polyvinyl alcohol, 0.25 mg/ml bovine serum albumin, 2 mM dithiothreitol, 0.15 $\mu$l (8 U) of RNA Guard (Pharmacia), and 3000 to 6000 cpm of 5' end-labeled RNA in a total volume of 12.5 $\mu$l. Reactions were incubated at 30°C and 3 $\mu$l samples taken at 0, 5, and 10 min. RNA was prepared as described previously (Zarkower and Wickens, 1987) except that the phenol-chloroform extraction step was omitted with the purified components.

It was noted that purified fractions of poly(A) polymerase and the specificity factor were relatively less active on RNAs that contained predominantly 2'-O-CH$_3$ nucleotides than were the crude nuclear extract fractions. The explanation for this difference is not clear.

Mobility Retardation Analyses

For nondenaturing gel mobility retardation assays, 6000 cpm of 5' end-labeled RNA was incubated with 3 $\mu$l (9 U) of purified specificity factor, 20 mM phosphocreatine, 1.6 mM ATP, 0.1 mg/ml yeast tRNA, 2.6% polyvinyl alcohol, 2 mM MgCl$_2$, 2 mM dithiothreitol, and 0.06 $\mu$l (24 U) of RNA Guard in a total volume of 6 $\mu$l. Incubation was done at 30°C for 10 min, and then the sample was loaded directly onto a 4% nondenaturing gel as described previously (Humphrey et al., 1987) but without the addition of heparin.

Alkaline Hydrolysis of RNA

End-labeled RNA was mixed with 10 $\mu$g of Escherichia coli tRNA in 10 $\mu$l of 50 mM sodium bicarbonate. The solution was boiled for 4 min. An equal volume of 80% formamide was added and the solution boiled again for 30 s. RNA was analyzed by electrophoresis through a 15% polyacrylamide gel containing 7 M urea.

Electrophoresis and Autoradiography

RNAs were separated by electrophoresis through 12% polyacrylamide gels containing 7 M urea. Gels were then dried and exposed to film.

Quantitation of Polyadenylation Efficiency

For each RNA, we determined the fraction of the RNA remaining after 10 min that had received poly(A). To provide a standard for comparison, the average value for one RNA in each figure was normalized to 1.0. Absolute values for the RNA used as a standard are given in each figure legend. For all RNAs, the range of experimental variation between separate assays was no greater than (and typically less than) $\pm 0.1$ of the standard.

Quantitation was performed with a $^{32}$P imaging device (Beta-detector; Betagen), or by determining the amount of radioactivity in a gel slice by full spectrum counting in Intagel scintillation fluid (Packard).


Moore, C. L., Chen, J., and Whoriskey, J. (1988). Two proteins cross-linked to RNA containing the adenovirus L3 poly(A) site require the AAUAAA sequence for binding. EMBO J. 7, 3159–3169.


