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Assembly of a Polyadenylation-Specific 25S Ribonucleoprotein Complex In Vitro

JAMES E. STEFANO†* AND DAVID E. ADAMS‡

Department of Molecular and Cellular Biology, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206

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Extracts from HeLa cell nuclei assemble RNAs containing the adenovirus type 2 L3 polyadenylation site into a number of rapidly sedimenting heterodisperse complexes. Briefly treating reaction mixtures prior to sedimentation with heparin reveals a core 25S assembly formed with substrate RNA but not an inactive RNA containing a U→C mutation in the AAUAAA hexanucleotide sequence. The requirements for assembly of this heparin-stable core complex parallel those for cleavage and polyadenylation in vitro, including a functional hexanucleotide, ATP, and a uridylate-rich tract downstream of the cleavage site. The AAUAAA and a downstream U-rich element are resistant in the assembly to attack by RNase H. The poly(A) site between the two protected elements is accessible, but is attacked more slowly than in naked RNA, suggesting that a specific factor or secondary structure is located nearby. The presence of a factor bound to the AAUAAA in the complex is independently demonstrated by immunoprecipitation of a specific T_1 oligonucleotide containing the element from the 25S fraction. Precipitation of this fragment from reaction mixtures is blocked by the U→C mutation. However, neither ATP nor the downstream sequence element is required for binding of this factor in the nuclear extract, suggesting that recognition of the AAUAAA is an initial event in complex assembly.

The 3′ termini of most eucaryotic mRNAs are generated by the sequence-directed cleavage of the primary transcript followed by addition of a long poly(A) tail. This step is essential for expression; alterations in polyadenylation signals are included among known mutations destroying gene function (17, 33). Accumulating evidence suggests that this processing event produces effects at a number of levels. In oocytes, the translational capacity of synthetic mRNAs is increased in vivo by the poly(A) tract (6). The sequence of the region containing the polyadenylation site also affects the level of gene expression, suggesting that the efficiency of this step may be used to modulate the mRNA level (34). Finally, the selection between competing poly(A) sites in the same primary transcript may be the pivotal event in the tissue-specific choice between two alternate gene products (4, 16, 29, 45). In at least one case, polyadenylation also appears to affect termination by RNA polymerase II (42).

A highly conserved hexanucleotide sequence element, AAUAAA, is found 5 to 25 nucleotides upstream of the site of cleavage (8, 27). Mutations in this element inhibit both cleavage and poly(A) addition to the 3′ terminus, suggesting that the factor recognizing the AAUAAA is required both for marking the site of cleavage and the newly formed 3′ terminus for poly(A) addition (22, 46). However, in vivo the AAUAAA is not sufficient; a sequence downstream of the cleavage site is required for accurate or efficient cleavage (2, 3, 10, 14, 23, 26, 27, 36, 37, 40, 44). At least one of two partially conserved elements, either a U-rich or G+U-rich tract, is required (11, 25) and may be recognized by separate factors (25).

The factors which recognize the critical sequence elements have not been identified, although a variety of indirect evidence suggests the involvement of a member of the class of particles called small nuclear ribonucleoproteins (sn RNP). Polyadenylation in vitro is inhibited by antibodies directed against the U1 snRNP (30) or the m_3G cap structure present on all known snRNAs except U6 (32). After exposure of synthetic substrate RNAs to nuclear extracts capable of polyadenylation in vitro, fragments containing the AAUAAA can also be immunoprecipitated by both a monoclonal antibody directed against the Sm determinant common to snRNPs and antibody against the m_3G cap structure (15). However, although specific degradation of the RNA component of the U1, U2, and U4 + U6 snRNPs in extracts inactivates splicing, it does not block polyadenylation (1). Similarly, the inhibition produced by treating extracts with micrococcal nuclease (15) can be overcome by the addition of bulk *Escherichia coli* RNA (35), suggesting that the factors involved lack a unique and essential RNA component. A nuclease-insensitive particle, such as the protein bearing an Sm determinant which recognizes 3′ splice sites (9, 41) may be involved.

The recognition of signals required for the splicing of pre-mRNA proceeds within the context of assembly of a large ribonucleoprotein complex termed the spliceosome. Native gel electrophoresis and affinity chromatography of spliceosomes resolved by gradient centrifugation have demonstrated the presence of the U2, U5, and U4 + U6 snRNPs in the complexes from mammalian cells (12, 19). As a prerequisite to identifying some of the factors involved in polyadenylation, we have isolated and characterized the complexes formed with substrate RNAs containing the L3 polyadenylation site from adenovirus type 2. In this report we demonstrate that RNAs which are substrates for both cleavage and polyadenylation in vitro are specifically assembled into a heparin-resistant complex which sediments at 25S and which contains a factor bound to the AAUAAA.

* Corresponding author.
† Present address: Gene Trak Systems, Framingham, MA 01701.
‡ Present address: Department of Microbiology, University of California, Berkeley, CA 94720.

MATERIALS AND METHODS

Plasmid construction. The *Msp*I-*Dde*I restriction fragment isolated from spL3 (15) (a gift of C. Hashimoto) and containing the adenovirus type 2 L3 polyadenylation site was cloned into the *Hin*cII site of pEMBL8(+) after the 5′ overhanging ends had been filled with Klenow fragment. After the orientation of the cloned insert had been verified, the T→C transition was introduced into the AATAAA element by oligonucleotide-directed mutagenesis by the method of Kunkel (20), starting with a dUMP-substituted phagemid template obtained by f1 infection of a plasmid-bearing *ung dut* host. Mutants detected by the higher T_m of hybrids forming between immobilized single-stranded phagemids and the mutagenizing primer deoxyoligonucleotide (49) were verified by sequencing. The *Eco*RI-*Hin*dIII fragment containing the wild-type and mutant inserts were subsequently transferred to pSP65 to generate pSPL3MD and pSPL3MD-14, respectively.

Substrate RNAs. Plasmids bearing the wild-type or mutant sequences were digested with either *Hin*dIII or *Tha*I prior to transcription with SP6 RNA polymerase in a total volume of 20 μl as described previously (28), except that the GTP concentration was lowered to 25 μM and 0.5 mM G(5′)ppp(5′)G was included to force initiation with the cap dinucleotide (18). The *Ava*I template was prepared by first treating pSPL3MD with *Hpa*II methylase to block cleavage of an upstream *Ava*I site in the polylinker region (15). The plasmid spL3 was cleaved with *Dra*I. To obtain higher transcript yields, the 1-h transcription reactions were supplemented with 25 μM GTP every 15 min and 10 U of SP6 RNA polymerase after 30 min. When labeled UTP was used, its concentration was lowered to 25 μM and both UTP and GTP were supplemented during the reaction. The transcripts were resolved on 8.3 M urea–5% polyacrylamide gels and eluted. Isolated transcripts were stored in 30% ethanol (15). Yields were typically 15 to 20 pmol from 2 pmol of template.

Nuclear extract and polyadenylation reactions. Nuclear extracts of HeLa cells were prepared by the method of Dignam et al. (5) with modifications as described by Moore and Sharp (31). Except as noted, reaction mixtures (12.5 to 50 μl) contained 0.1 to 0.3 pmol of labeled substrate RNA, 1 mM ATP or 3′-dATP, 20 mM creatine phosphate, 1% polyvinyl alcohol, 2 μg of carrier RNA, and 44% nuclear extract containing 100 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.6) and were incubated at 30°C. When ATP was omitted, creatine phosphate was also omitted and yeast hexokinase (10 U; Sigma Chemical Co.) and 10 mM glucose were included to turn over endogenous ATP in the extract (see text). For electrophoretic analysis of RNA products, the mixtures were digested with 20 μg of proteinase K in the presence of 0.2% sodium dodecyl sulfate for 30 min at 37°C, phenol extracted, and ethanol precipitated. The RNAs were resolved on 8.3 M–5% polyacrylamide gels.

*Ava*I runoff transcripts (1 pmol) were tailed with *E. coli* poly(A) polymerase (Bethesda Research Laboratories, Inc.) for 10 min at 37°C under conditions recommended by the supplier. When ATP was substituted with UTP or CTP, the reaction time was increased to 3 h and the triphosphate concentration was increased to 1 mM.

Sedimentation analysis. After 20 min at 30°C, heparin (4 mg/ml) was added to polyadenylation reaction mixtures, and the mixtures were incubated for an additional 5 min at 30°C. The mixtures were diluted to 200 μl with cold RB (44 mM KCl, 0.7 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM dithiothreitol, 1 mM ATP, 8.8 mM HEPES [pH 7.6]) and applied to 4.8-ml linear 5 to 20% sucrose gradients prepared in RB. The gradients were centrifuged in an SW 55Ti rotor for 3 h at 50,000 rpm (368,000 × *g*). rRNA markers were centrifuged in parallel in 5 to 20% gradients prepared in 0.1 M NaCl–20 mM Tris (pH 7.4). Gradients were dripped from the bottom; 20 nine-drop fractions (250 ± 20 μl) were collected. Fractions or aliquots were counted for Cerenkov radiation.

RNase H protection assays. Reaction mixtures (40 μl) containing 34 μl of gradient fraction brought to 1% polyvinyl alcohol by addition of a 10% aqueous stock, 4 μl of DNA oligonucleotide (1 to 300 μM), and 4 U of RNase H (Bethesda Research Laboratories) were incubated at 30°C. Aliquots (10 μl) were removed and added to 0.5 μl of 10% sodium dodecyl sulfate–5 μg of proteinase K (Boehringer Mannheim Biochemicals)–5 μg of carrier RNA. After 30 min at 30°C, RNAs were ethanol precipitated, dissolved in 8.3 M urea, and resolved on 8.3 M urea–5% polyacrylamide gels.

Immunoprecipitations. Immunoprecipitation reactions were performed as described previously (15) with minor modifications. Aliquots (25 or 50 μl) of polyadenylation reaction mixtures or gradient fractions were placed on ice. RNase T_1 (30 U; Sankyo) and 10 μg of immunoglobulin G (IgG) purified by step pH elution from protein A-Sepharose (7) were added, and the mixture was kept at 0°C. After 20 min, the mixture was transferred to 10 mg of protein A-Sepharose (Pharmacia, Inc.) equilibrated with NET-2 (0.05% Nonidet P-40, 150 mM NaCl, 50 mM Tris [pH 7.4]). The beads were kept in suspension by brief but repeated stirring with a pipette tip over 30 min, diluted to 1 ml with ice-cold NET-2, and pelleted. The supernatant was discarded, and the pellet was washed four to six times with 1 ml of cold NET-2. The washed pellet was digested with proteinase K (0.5 mg/ml) in the presence of 0.2% sodium dodecyl sulfate, 100 μl of NET-2 was added, and the mixture was cleared of protein and Sepharose by phenol extraction. RNA fragments in the aqueous phase were ethanol precipitated with 20 μg of carrier RNA. Recovery of counts per minute starting from the washed protein-A Sepharose pellet was typically 85%. RNA fragments were resolved on 8.3 M urea–15% polyacrylamide sequencing gels which were autoradiographed (without drying) with an intensifying screen at −70°C. The Sm monoclonal antibody (Y12) (21) was harvested from culture supernatants. A mixture of two myeloma IgG2a (Miles Laboratories, Inc.) was used as control.

RESULTS

To produce RNAs for this analysis, a *Msp*I-*Dde*I fragment of adenovirus type 2 containing the L3 polyadenylation site was cloned into pSP65 to generate the template vector pSPL3MD (Fig. 1A). The AAUAAA hexanucleotide was altered to AACAAA by oligonucleotide-directed mutagenesis of the same fragment cloned in a phagemid vector (pEMBL), which was subsequently transferred to pSP65 to generate pSPL3MD-14. This U→C mutation was shown to block polyadenylation of the simian virus 40 late transcript both in vivo and in vitro (43). Both clones were transcribed with SP6 RNA polymerase after truncation with *Tha*I to generate labeled 211-nucleotide (nt) RNAs in which the L3 polyadenylation site is centrally disposed. These RNAs were assayed for cleavage and poly(A) addition in a nuclear extract from HeLa cells in the presence of ATP (31). After a 2-h incubation, the RNA containing the AAUAAA sequence produced a mixture of poly(A)$^+$ product RNAs which mi-

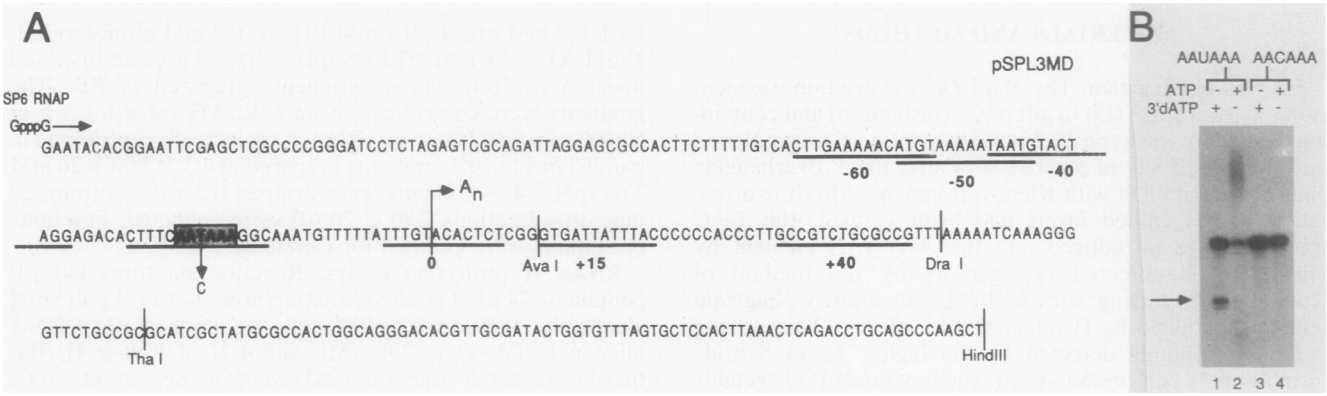

FIG. 1. A Template sequence. The sequence of the sense strand of pSPL3MD, including nucleotides 22719 to 22953 of adenovirus type 2 and the L3 polyadenylation site, is shown. The vertical bars are sites for restriction enzyme cleavage in the template strand. The underlined regions indicate sequences complementary to oligonucleotide probes used in RNase H protection experiments (see Fig. 5). The arrow indicates the site of cleavage and poly(A) addition. Sequences are numbered according to their position relative to the polyadenylation site (0). (B) U→C Transition blocks site utilization. Purified transcripts (100 fmol each) of *Tha*I-cleaved pSPL3MD and the mutant clone (pSPL3MD-14) labeled with [α-^{32}P]GTP were incubated in a HeLa nuclear extract in the presence of ATP or 3′-dATP as indicated. RNA products were isolated and resolved on an acrylamide gel containing 8.3 M urea. The dark bands in the center are the remaining precursor RNA; the slowly migrating material in lane 2 is poly(A)$^+$ product. The arrow shows cleaved, 3′-dAMP-adduct product produced with 3′-dATP.

grated more slowly on polyacrylamide gels (Fig. 1B, lane 2). In contrast, the RNA bearing the U→C change in the AAUAAA was not active as a substrate for polyadenylation (lane 3). In a second set of reactions, 3′-dATP (cordecypin triphosphate) was substituted for ATP. Addition of a terminal cordecypin residue to the 5′ cleavage product blocks the addition of subsequent adenylate groups (38). In the presence of this analog, the wild-type AAUAAA RNA generated a ca. 140-nt RNA product (Fig. 1B, arrow) corresponding to the 5′ cleavage product, verifying that the mutation renders the RNA incapable of cleavage at the correct site.

We decided to use the effect of this mutation to identify polyadenylation-specific complexes. Each of the RNAs was incubated for 20 min in the nuclear extract, and the mixtures were diluted and sedimented through sucrose gradients (Fig. 2, left panel). After exposure to the extract, most of each RNA sedimented significantly faster than its naked form (fraction 16). Below 30S, both RNAs displayed similar profiles. The wild-type RNA was found in greater abundance specifically in a region at ca. 30S (fraction 3) and to a lesser degree further down the gradient, suggesting that the active RNA is specifically assembled into higher S structures. However, sedimentation at lower speeds or shorter times did not reveal any repeaking in the range of 30S to 80S (data not shown).

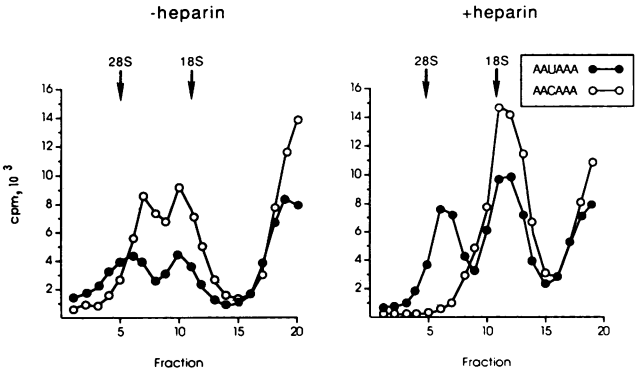

FIG. 2. Sedimentation analysis of complex formation. Transcripts of *Tha*I-truncated pSPL3MD were incubated for 20 min at 30°C in the extract as described in Materials and Methods. The mixtures were divided in half, and heparin (4 mg/ml) was added to one portion. After 5 min, the reaction mixtures were diluted and applied to 5 to 20% sucrose gradients. The direction of sedimentation is from right to left. (Left) Reactions not receiving heparin treatment. (Right) Reactions treated with heparin.

The other halves of the reaction mixtures were briefly treated with the polyanion heparin (4 mg/ml for 5 min at 30°C) prior to sedimentation. After this treatment, a complex sedimenting at ca. 25S appeared with the wild-type RNA which was not formed with the mutant RNA (Fig. 2, right panel). Thus, the single nucleotide change in the RNA which blocks processing also blocks formation of this complex. In other experiments, the 25S complex preceded the appearance of product RNA in the reaction (see Fig. 3 below) and increased with time of exposure to the extract, typically reaching a maximum in 20 min (not shown).

A complex of ca. 15S also formed with both the wild-type and mutant RNAs. This non-AAUAAA-dependent assembly was observed at the earliest times in the reaction and varied between 12S and 20S with different extract preparations; stability experiments suggested that this complex contains a number of weakly bound components (data not shown). By several other criteria, this complex appears to be nonspecific (see below).

Sequence and cofactor requirements for complex assembly. We decided to extend our observations to determine whether other portions of the substrate RNA are necessary for formation of the heparin-resistant 25S complex. The pSPL3MD template was truncated at each of the restriction sites shown in Fig. 1 to generate a series of RNAs containing various portions of the sequences downstream of the polyadenylation site. In addition, another clone of the L3 site (spL3 [15]), containing an additional 145 base pairs of upstream sequence, was truncated at the *Dra*I site and transcribed to produce a 329-nt RNA with the L3 site near

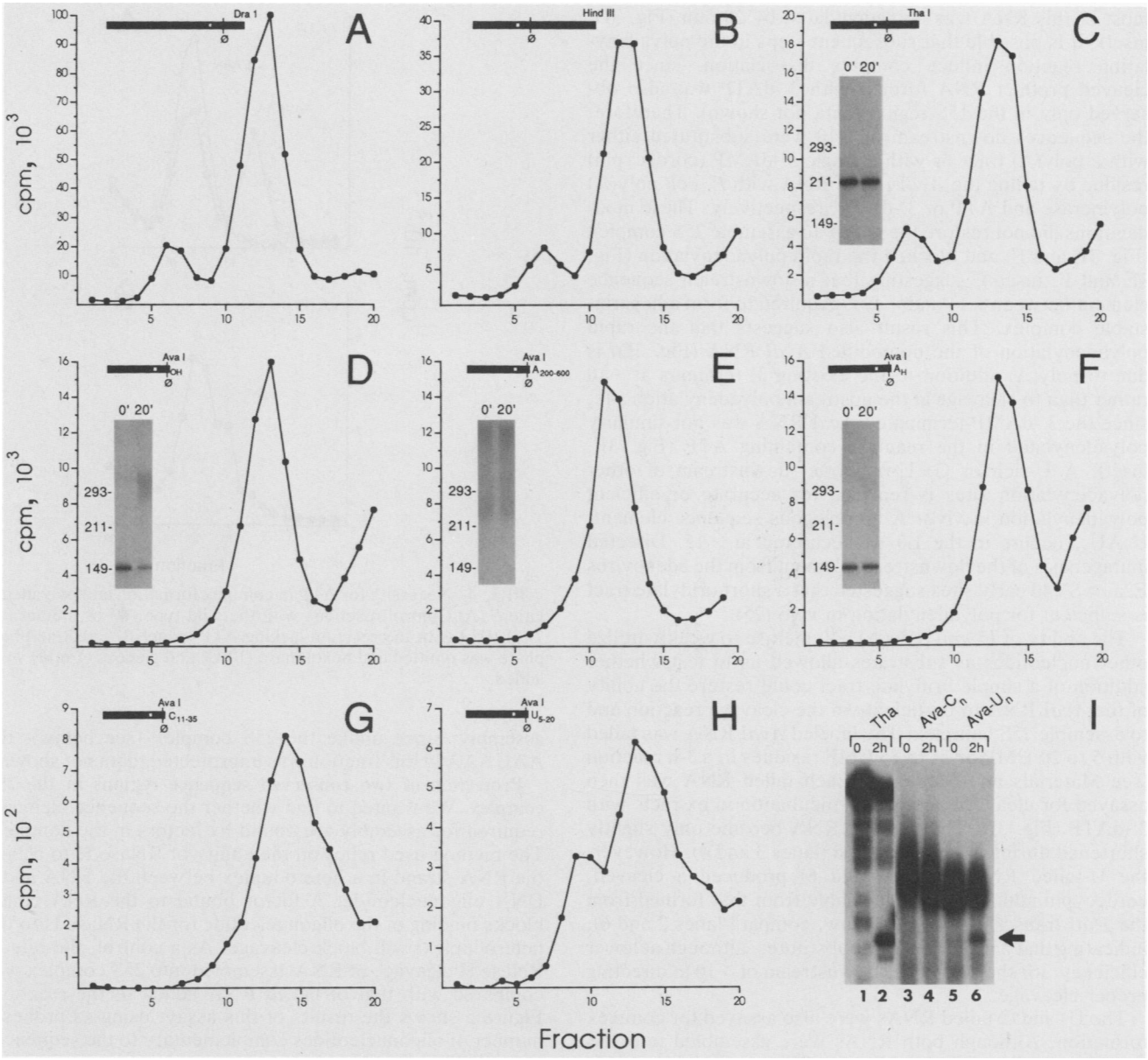

FIG. 3. Sequences required for complex formation. Transcripts truncated at sites indicated in Fig. 1 were tested for formation of complexes which survive heparin treatment as described in Fig. 2. (A) A 329-nt transcript of spL3, which possesses an additional 145 nt of adenovirus type 2 sequence upstream of those in pSPL3MD, truncated at the *Dra*I site was assayed. (B to D) Transcripts of pSPL3MD truncated with *Hin*dIII (B), *Tha*I (C), or *Ava*I (D). The lengths of each transcript were as follows: *Hin*dIII, 294 nt; *Tha*I, 211 nt; *Ava*I, 149 nt. A diagrammatic structure of each RNA to scale is included above the plot. The white box and vertical line indicate the position of the AAUAAA and the cleavage site, respectively. (Insets to C to F) Labeled RNAs before and after a 20-min incubation in the nuclear extract. (E to H) A uridylate tract can substitute for sequences downstream of +10 in complex formation. The *Ava*I transcript was tailed with *E. coli* poly(A) polymerase and either ATP (E), cordecypin triphosphate (3′-dATP) (F), CTP (G), or UTP (H) before being incubated with the extract. (I) A uridylate tract can substitute for sequences downstream of +10 in directing cleavage. The *Tha*I transcript or the *Ava*I transcript previously tailed with 11 to 35 CMP or 5 to 20 UMP residues was tested for product formation. Each RNA was incubated with the nuclear extract in the presence of 3′-dATP. RNAs isolated from the reaction were electrophoresed on a 5% denaturing gel. The arrow indicates the correctly cleaved cordecypin adduct. The minor amount of shorter product, which probably results from cleavage at an upstream site, was not characterized.

the 3′ terminus. Each of these RNAs was assayed for complex formation as in Fig. 2.

RNAs containing the AAUAAA and sequences upstream of the *Dra*I site (+49) were all assembled into ca. 25S heparin-stable complexes (Fig. 3A to C). Therefore, the sedimentation rate of the complex is not strongly affected by the size of the RNA or the position of the polyadenylation site in the RNA. Denaturing gel electrophoresis of RNA from the reaction mixture containing the *Tha*I transcript indicated that poly(A)$^+$ product had not yet appeared in the reaction (Fig. 3C, inset).

In contrast, RNA terminated at the *Ava*I site at +10 was not assembled into a heparin-stable 25S complex, but was found exclusively in the 15S region (Fig. 3D). However,

most of this RNA was polyadenylated by 20 min (Fig. 3D, inset). It is possible that subsequent steps in the polyadenylation reaction induce complex dissociation, since the cleaved product RNA formed with 3'-dATP was also observed only in the 15S region (data not shown). Therefore, the sequences downstream of +10 were substituted either with a poly(A) tract or with a single 3'-dAMP (cordecypin) residue by tailing the *Ava*I runoff RNA with *E. coli* poly(A) polymerase and ATP or 3'-dATP, respectively. These modifications did not restore the ability to assemble 25S complex (Fig. 3E and F) and blocked the rapid polyadenylation (Fig. 3E and F, insets), suggesting that a downstream sequence element between +11 and +49 is required to form a heparin-stable complex. This result also suggests that the rapid polyadenylation of the unmodified *Ava*I RNA (Fig. 3D) is due to poly(A) addition to the existing 3' terminus at +10 rather than to cleavage at the upstream polyadenylation site, since the 3'-dAMP-terminated *Ava*I RNA was not similarly polyadenylated in the reaction containing ATP (Fig. 3F, inset). A U-rich or G+U-rich tract downstream of other polyadenylation sites is required for accurate or efficient polyadenylation in vivo. A homologous sequence element, U_2AU_3, occurs in the L3 site centered at +15. Directed mutagenesis of the downstream element from the adenovirus E2a or SV40 early sites suggested that a short uridylate tract is sufficient for polyadenylation in vivo (25).

The ability of *E. coli* poly(A) polymerase to weakly utilize other nucleotides as substrates allowed us to test whether addition of a simple uridylate tract could restore the ability of the *Ava*I RNA to participate in the cleavage reaction and to assemble 25S complex. The labeled *Ava*I RNA was tailed with 5 to 20 UMP or 11 to 35 CMP residues in a 3-h reaction (see Materials and Methods). Each tailed RNA was then assayed for cleavage activity by incubation in extracts with 3'-dATP (Fig. 3I). The C-tailed RNA became only slightly shortened during a 2-h incubation (lanes 3 and 4). However, the U-tailed RNA (lanes 5 and 6) produced a cleaved, cordecypin adduct indistinguishable from that formed from the *Tha*I transcript (Fig. 3I, arrow, compare lanes 2 and 6), indicating that a uridylate tract substitutes, although at lower efficiency, for the sequences downstream of +10 in directing proper cleavage.

The U- and C-tailed RNAs were also assayed for complex formation. Although both RNAs were assembled into 15S complexes (Fig. 3G and H), the RNA containing the 3' terminal uridylate tract also formed a complex sedimenting at ca. 20S after heparin treatment (Fig. 3H), suggesting that in addition to sequences upstream of +10, a simple uridylate tract in this position is sufficient to direct assembly of the heparin-stable complex. The lower sedimentation rate may reflect a conformation resulting from the altered position of the U-rich tract or the missing downstream portion of the RNA. As shown below, the AAUAAA in the 25S complex is protected against RNase H attack. By the same method, the AAUAAA was protected in the 20S complex containing the U-tailed *Ava*I RNA, indicating that it is analogous to the complex assembled with other L3 RNAs (data not shown).

Moore and Sharp (31) have extensively characterized the requirements for the polyadenylation reaction at the L3 site in vitro. ATP is required for cleavage as well as the addition of poly(A) to the new 3' terminus. We therefore tested the requirement for ATP in complex assembly. Less than 10% of the amount of 25S complex was observed in reactions lacking ATP (Fig. 4B). The minor shoulder appearing on the heavy side of the 15S peak appears to be a nonspecific

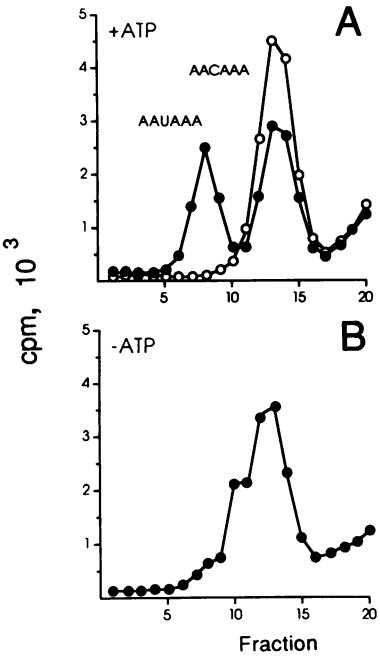

FIG. 4. Necessity for ATP in complex formation and polyadenylation. (A) Control reactions with the wild-type (●) or mutant (○) *Tha*I RNAs. In the reaction lacking ATP (panel B), creatine phosphate was omitted and hexokinase (10 U) and glucose (1 mM) were added.

assembly, since unlike the 25S complex (see below), the AAUAAA in this fraction was unprotected (data not shown).

Protection of two conserved sequence regions in the 25S complex. We wanted to find whether the sequence elements required for assembly are bound by factors in the complex. The method used relies on the ability of RNase H to cleave the RNA strand in a heteroduplex between the RNA and a DNA oligonucleotide. A factor bound to the RNA which blocks binding of the oligonucleotide (or the RNase H to the heteroduplex) will block cleavage. As a control, the rate of RNase H cleavage of RNA assembled into 25S complex was compared with that of naked RNA added to the reaction. Figure 5 shows the results of this assay, using as probes a number of oligonucleotides complementary to the sequences underlined in Fig. 1.

Although several sites in the complex were cleaved at nearly identical rates in the control RNA (gels marked −40 and +40), other sites were resistant to cleavage in the complex. Over 95% of the control RNA was cleaved at the AAUAAA or the +15 sequence, whereas the complex-associated RNA was unaffected during the 15-min digestion. The polyadenylation site between these two protected sites was partially susceptible to cleavage: both RNAs were cleaved, but the complex-bound RNA was cleaved more slowly. Neither degree of resistance was observed at any site in the 15S complex (panel 15S).

Surprisingly, one site (gel marked −50) was efficiently cleaved in the complex, but was resistant to cleavage in the control RNA. The naked RNA was also cleaved slowly at this site when assayed independently, indicating that the RNA contains a structure which inhibits cleavage at this site. Secondary structures may inhibit the RNase H reaction by blocking hybridization of the DNA oligonucleotide. A stable structure can be drawn in which this region is paired with a U-rich tract upstream of the polyadenylation site. Consistent

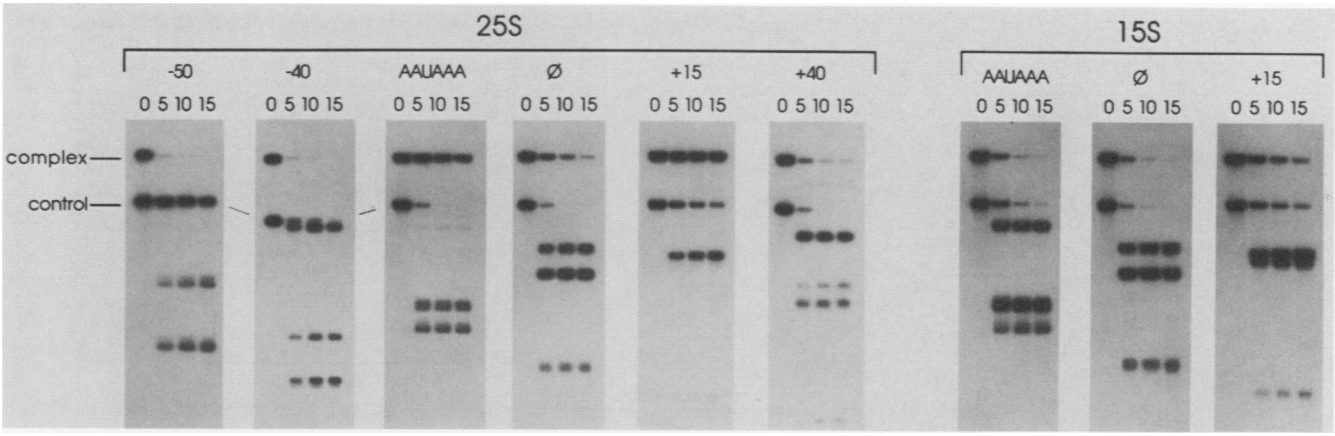

FIG. 5. Protection of specific sequence elements in the 25S complex. Complexes formed with the 294-nt *Hin*dIII runoff transcript were isolated on gradients as shown in Fig. 2. Identical molar amounts of the smaller *Tha*I runoff transcript were added as a control (see text). The mixtures were then incubated with RNase H and oligodeoxynucleotides complementary to the sequences underlined in Fig. 1. Cleavage products were resolved by denaturing gel electrophoresis. The numbers above each lane indicate minutes of RNase H digestion. The gels labeled 15S show the slower-sedimenting complex observed after heparin treatment (Fig. 2); the gels labeled Ø show the reaction containing the oligonucleotide overlapping the polyadenylation site (Fig. 1). Note that the *Hin*dIII transcript cleaved with the anti-−40 oligonucleotide produces a 3′ cleavage product which migrates close the intact control RNA. The lower member of the doublet is the cleavage product.

with this possibility, the naked RNA was cleaved rapidly at −50 if the complementary −5 sequence was first removed by truncating the RNA at −40 (data not shown). This observation raises the possibility that protection of the AAUAAA and the U-rich element in the complex may reflect the presence solely of secondary structures involving these sequences in the complex. As shown below, however, an RNA oligonucleotide containing the AAUAAA can also be immunoprecipitated from RNase T_1 digests of the 25S fraction, indicating that a factor is bound to this element in the complex. The protection of the U-rich sequence element is discussed below.

Mapping of primary sequence contacts. The experiments above demonstrate that two specific sequence elements are protected in the complex. The major regions of the RNA contacted in the complex was assayed by another approach. The isolated complex RNA was first cleaved with RNase H at accessible sites, and the digests were then diluted and sedimented through a second gradient. After cleavage, a fraction of the radioactivity continued to sediment at about 25S (Fig. 6), another being found at the top of the gradient in the position of free RNA (fraction 16). RNA recovered from each region was analyzed by denaturing gel electrophoresis (panels next to each profile). In every instance, including cleavage at the polyadenylation site, the RNA fragment containing the AAUAAA hexanucleotide was found in the 25S region; the other portion of the RNA was found exclusively at the top of the gradient, suggesting that the major contacts lie between −40 and the polyadenylation site. None of the cleavages produced complexes sedimenting between the 25S complex and naked RNA, suggesting that the components of the complex other than the precursor RNA are tightly associated.

Since minor contacts to the downstream +15 region may not be apparent in the above experiment, we also examined the effect of removing this region on the stability of the RNA-complex interaction. If contacts exist to this region, an RNA containing only the upstream region would be less stably bound. The complex was first treated with RNase H in the presence of the anti-Ø oligonucleotide to cleave the RNA which would allow release of the downstream fragment (Fig. 6). The RNase H was then inhibited by the addition of excess EDTA, the digest was mixed with untreated complex, and the mixture was incubated at 30°C. Aliquots of the incubation mixture taken after various periods were diluted in cold buffer and subjected to a second round of sedimentation. About half of the complexes dissociated during the 50-min incubation (Fig. 7). Similar first-order kinetics were observed for the disappearance of AAUAAA protection when a complex containing the full-length RNA was used (data not shown). The RNAs from the 25S region were extracted, and the relative abundance of the full-length and cleaved RNAs was determined electrophoretically (Fig. 7B). The ratio of the two RNAs remaining bound in the 25S complex did not vary, indicating that the downstream fragment does not contribute to the interaction with the bound RNA. We infer that the downstream sequence region is not contacted by other complex components (see Discussion).

Immunoprecipitation of a fragment containing the AAUAAA from the 25S complex. Fragments of the RNA containing the AAUAAA sequence can be immunoprecipitated by antibodies directed against the Sm determinant common to snRNPs, as well as to the m_3G cap structure common to all the known snRNAs except U6, suggesting that the AAUAAA is recognized by a member of this class of particles (15). We decided to find whether this interaction is sequence specific and, if so, whether the 25S complex also contains this factor. We first performed immunoprecipitation experiments in extracts by using the wild-type and mutant L3 RNAs. After a 20-min incubation of each of the *Tha*I RNAs, the reaction mixtures were chilled and digested with RNase T_1, and the fragments were immunoprecipitated with a monoclonal antibody against the Sm determinant (Y12) essentially as described previously (15), except that the IgG was affinity purified (7). A mixture of two myeloma IgG of the same subclass (IgG2a) was used as a control.

As observed previously (15), the 15-nt T_1 oligonucleotide containing the AAUAAA was precipitated with the Sm monoclonal antibody (Fig. 8, lane 1). This interaction is sequence dependent, since the homologous oligonucleotide from the mutant RNA containing the AACAAA sequence was not precipitated (lane 3). In contrast to the assembly of the 25S complex, binding of the AAUAAA does not require ATP, since the same amount of AAUAAA oligonucleotide

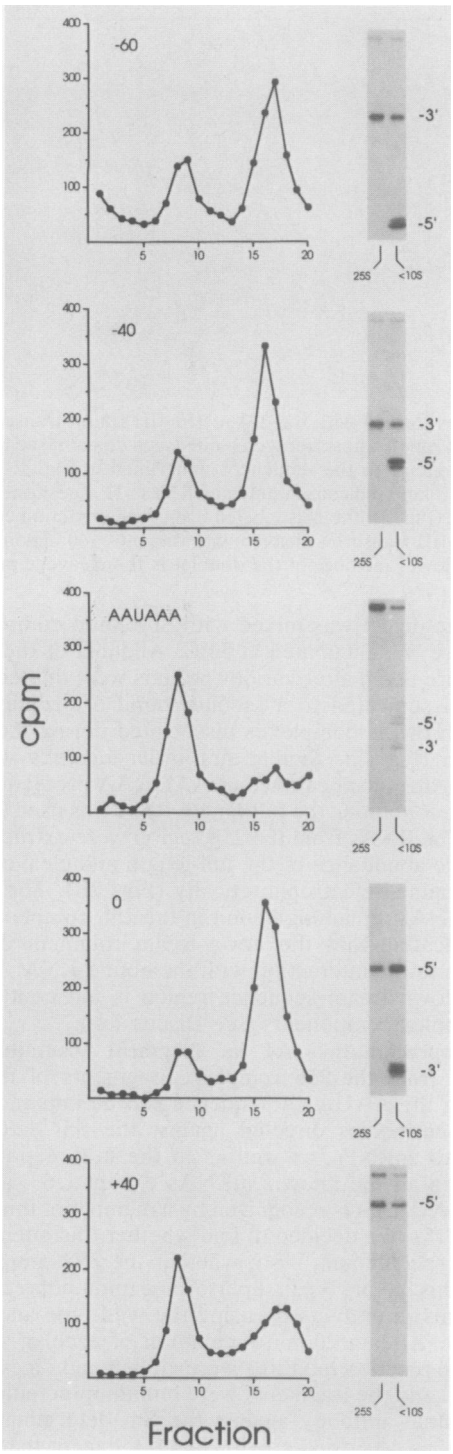

FIG. 6. Cleavage-resedimentation analysis. Isolated complexes containing the *Tha*I runoff RNA were treated with RNase H and oligonucleotides and subjected to a second round of sedimentation. The free *Tha*I RNA sedimented in fraction 16 (not shown). RNA recovered from each peak was resolved by denaturing gel electrophoresis (right panels). The top band in each lane is the residual uncleaved RNA. Note that in each case, the cleavage product containing the AAUAAA hexanucleotide sequence sediments with the complex (3′ fragment for −60- or −40-cleaved RNAs, 5′ fragment of Ø-cleaved RNA).

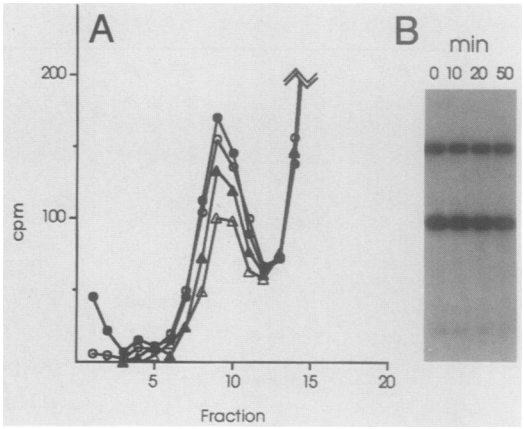

FIG. 7. Relative stability of complexes containing full-length and ϕ-cleaved RNAs. (A) A mixture of untreated complex and complex digested with RNase H and the anti-ϕ oligonucleotide was incubated at 30°C for 0 min (●), 10 min (○), 20 min (▲), or 50 min (△) prior to a second round of sedimentation. (B) RNAs retained in the complex were isolated and resolved on a 5% polyacrylamide urea gel. Identical counts per minute in total RNA from the 25S region were loaded in each lane.

was precipitated if ATP and creatine phosphate were omitted from the reaction (lane 4) or if hexokinase was added (data not shown), conditions which suppress complex formation (Fig. 4). This interaction also does not require a downstream sequence element, since the oligonucleotide was also efficiently precipitated from a reaction containing the truncated *Ava*I RNA (lane 10).

In addition to the AAUAAA, a 21-nucleotide oligomer containing the downstream U-rich sequence (Fig. 8, +15 oligo) was specifically precipitated with the Sm monoclonal antibody (lane 1), indicating that an Sm-containing moiety in the extract interacts with this fragment. Consistent with its nucleotide composition, this fragment was much more strongly labeled if an [α-^{32}P]UTP-labeled RNA was used (lanes 9 to 16). Identical amounts of this oligonucleotide were precipitated from reactions with the AACAAA mutant RNA (lane 3), showing that this interaction does not require a perfect AAUAAA sequence element. The identities of the 15-mer and the 21-mer as containing the AAUAAA and the +15 U-rich element, respectively, were verified by the loss of these elements after cleavage of the transcript with specific DNA oligonucleotides and RNase H (lanes 12 to 16); the precipitated fragments were resistant to further T_1 treatment (data not shown). Other protected fragments were also seen in the immunoprecipitates. Most of these were also seen in a precipitate obtained from a *Tha*I RNA first truncated by cleavage at −40 to remove the AAUAAA hexanucleotide and sequences further downstream (lane 11), suggesting that these fragments arise from the 5′ region of the RNA and reflect interactions which are probably unrelated to polyadenylation.

In contrast to the complex pattern seen in the extract, only the AAUAAA oligonucleotide (and, in a few cases, minor amounts of other fragments) was immunoprecipitated from the gradient-purified 25S complex (lane 5) and not from the 15S complex (lane 7). Immunoprecipitation of the downstream +15 sequence element oligonucleotide was not observed.

We also tested which complexes observed without heparin treatment (Fig. 2) also contain a factor interacting with the hexanucleotide. Partial protection of the AAUAAA from

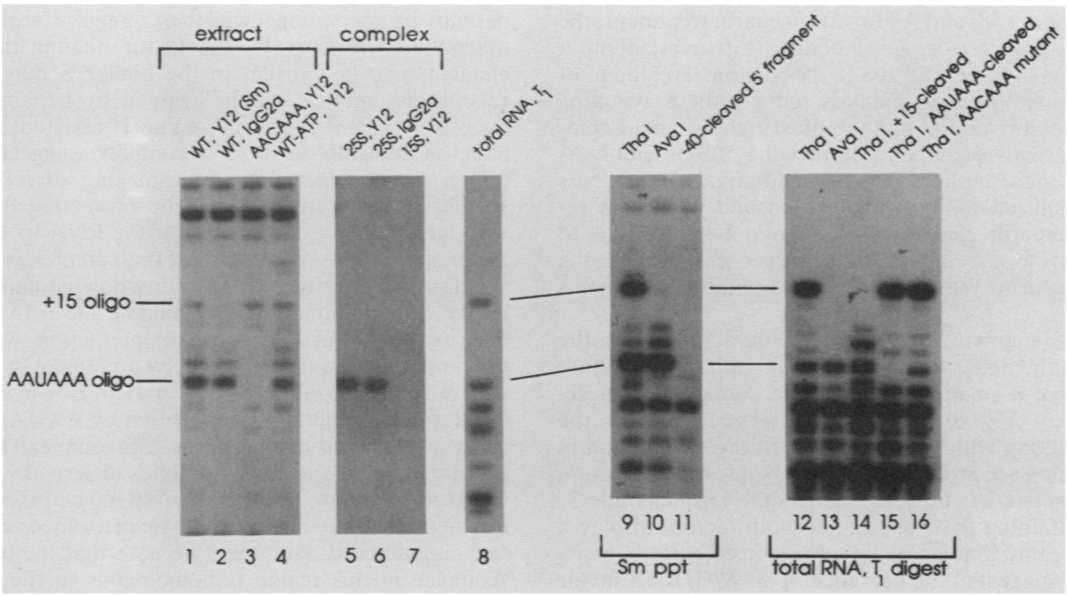

FIG. 8. Immunoprecipitation analysis. Processing reactions (lanes 1 to 4 and 9 to 11) or the isolated complex (lanes 5 to 7) were digested with RNase T_1 and immunoprecipitated with an Sm monoclonal antibody (Y12 [21]) or a mixture of two irrelevant monoclonal antibodies of the same subclass (IgG2a; Miles). Immunoprecipitated T_1 fragments were resolved on 15% sequencing gels. Extract reaction mixtures contained either the wild-type *Tha*I transcript (lanes 1, 2, 4, and 9), the AACAAA mutant *Tha*I transcript (lane 3), the wild-type *Ava*I transcript (lane 10), or the 5′ fragment of *Tha*I RNA cleaved with the anti-−40 oligonucleotide and RNase H (lane 11). Gradient-isolated complexes all contained *Tha*I RNA. The RNAs in lanes 1 to 8 were labeled with [α-^{32}P]GTP; those in lanes 9 to 16 were labeled with [α-^{32}P]UTP. Lane 8: T_1 digest of the *Tha*I transcript used in the experiments in lanes 1, 2, and 4 to 7. Lanes 12 to 16: Identification of T_1 oligonucleotides containing the AAUAAA and U-rich downstream element. The *Tha*I transcript ([α-^{32}P]UTP labeled) was cleaved with either no (lane 12), the anti-+15 (lane 14), or the anti-AAUAAA (lane 15) oligonucleotides. The fragments were purified, pooled, and digested with RNase T_1 prior to electrophoresis. The largest oligonucleotide containing the downstream U-rich element (+15 oligo) is not present in the *Ava*I transcript (lane 13). Note that the U→C transition causes a slight increase in the mobility and reduction in the intensity of the 15-mer (lane 16).

RNase H (maximally 25 to 50%) and immunoprecipitation of the oligonucleotide were observed only at ca. 30S (e.g., Fig. 2, left panel, fractions 3 to 6). The extent of protection reflected the yield of 15-mer in the immunoprecipitate from each fraction (data not shown).

Unexpectedly, similar amounts of 15-mer containing the AAUAAA were precipitated with the Sm monoclonal antibody or control IgGs either from the extract or the 25S complex (Fig. 8, compare lane 1 with lane 2 and lane 5 with lane 6). In contrast, the oligonucleotide containing the downstream U-rich tract was not precipitated with control antibody (lane 2), indicating that the precipitation of this fragment was due to interaction with an Sm component. A series of immunoprecipitations was performed by using the intact 25S complex and various amounts of each IgG. With 10 μg of IgG, 59% of the 25S complex was precipitated with the Sm monoclonal antibody, and about half that was precipitated with control IgG. A 100-fold reduction in IgG reduced the amount of complex precipitated with the Sm monoclonal antibody about 40%, while lowering the amount precipitated with the control IgG about fivefold. A similar low specificity was observed with serum samples from patients (data not shown).

DISCUSSION

In an effort to identify factors involved in polyadenylation, we have characterized a specific heparin-resistant 25S particle assembled with precursor RNA in nuclear extracts from HeLa cells. The AAUAAA hexanucleotide, ATP, and a uridylate-rich sequence element downstream of the polyadenylation site are necessary both for the assembly of this complex and for cleavage and polyadenylation in vitro. These results demonstrate that the requirements for assembly of this complex match the requirements for cleavage and polyadenylation, suggesting that this complex is an intermediate in the process. A number of other observations support this interpretation. First, the assembly of the complex precedes the appearance of product RNA (Fig. 3). RNAs containing the simian virus 40 late and IgM (secreted form) polyadenylation sites are also assembled into complexes sedimenting at 25S after heparin treatment. The simian virus 40 complex is also inhibited by a mutation in the AAUAAA, suggesting that this complex is assembled in response to all polyadenylation signals (J. Stefano, unpublished observations). The sequence elements in the adenovirus substrate essential for polyadenylation are also protected from RNase H in the complex, suggesting that factors or structures involved in marking the polyadenylation site for cleavage are present.

While this paper was in preparation, several reports identifying polyadenylation-specific complexes appeared (32, 39, 48). Zhang and Cole (48) observed complexes forming with both the herpes simplex virus TK and L3 sites by gel mobility shift following heparin treatment. Deletion of the entire signal region at the TK site blocked complex formation, and RNAs lacking the AAUAAA or the downstream G+U-rich element failed to compete for factors directing complex formation. Skolnick-David et al. (39) and Moore et al. (32) observed a 50S complex with an L3 substrate RNA which required the AAUAAA, sequences

between +5 and +48, and ATP. After heparin treatment, the complex was detected by gel mobility shift overlapping a nonspecific assembly in the 20S to 25S region. Treatment of spliceosomes with heparin similarly reduces the S, resulting in a core assembly containing the most tightly bound components (19). Thus, it is possible that the 50S assembly as identified by these authors contains additional components which are required for polyadenylation and which are removed by heparin. However, we have been unable to observe discrete assemblies larger than ca. 30S in the absence of heparin. We do not understand the basis for this discrepancy.

We have demonstrated a factor interacting with the AAUAAA in the heparin-resistant 25S complex by immunoprecipitation of a small oligonucleotide containing that sequence element (Fig. 8). Although not formally proven, the factors interacting with the AAUAAA in the extract and in the 25S complex are probably the same, since the interaction in each case appears to be delineated by the 15-nucleotide T_1 fragment containing the element and both factors display a strong nonspecific binding to IgG. Previous RNase H resistance assays suggested the presence of an AAUAAA factor in a product-containing complex formed with 3'-dATP (47; our unpublished results). Our finding of a complex with precursor RNA which both requires the AAUAAA and contains a factor bound to this element indicates that recognition of the AAUAAA occurs within the context of assembly of the 25S complex which precedes cleavage. Interaction with the AAUAAA itself is distinguishable from the assembly of the 25S complex, however, since the latter also requires the downstream sequence element and ATP. We infer that binding of the AAUAAA factor is a single and separable component of the interactions involved in complex assembly.

The assay for complex formation used here differs from the immunoprecipitation method in that the former requires the use of heparin to observe a discrete assembly. Thus, the requirements for complex assembly may also reflect a requirement(s) for producing a heparin-stable state. For example, ATP may be required for a conformational change in the complex which converts an initial, unstable interaction to a heparin-stable form. This is consistent with the finding that the AAUAAA fragment is not immunoprecipitated from any of the complexes obtained after heparin treatment of reaction mixtures lacking ATP, suggesting that the complex formed with the AAUAAA factor alone is not stable to heparin (J. Stefano, unpublished results). The lack of the downstream sequence element may also render the assembly susceptible to heparin attack, consistent with our finding both the tailed *Ava*I RNA (Fig. 3) and correctly cleaved and poly(A)$^+$ product RNA (not shown) only in the 15S region. This is supported by the finding of precursor, precleaved, and product RNAs in a larger 50S polyadenylation-specific assembly in the absence of heparin (32). Nevertheless, for the L3 site, the requirements for the heparin-stable 25S assembly best correlate with the requirements for cleavage, as shown by the inability of the 3'-dAMP-terminated *Ava*I RNA which lacks the downstream U-rich element either to be cleaved or to assemble 25S complex (Fig. 3). A similar conclusion was drawn for the herpes simplex virus TK site (48).

Removal of the downstream portion of the RNA had no detectable effect on the stability with which the RNA is bound to the 25S complex, suggesting that the downstream sequence element is not contacted in the complex. This sequence may be recognized by a *trans*-acting factor or perhaps by a component of the complex transiently during assembly. Alternatively, the factor binding this sequence element may be present in the higher S polyadenylation-specific assemblies, but is stripped by heparin treatment. Surprisingly, this sequence region is resistant to RNase H attack specifically in the 25S complex, suggesting that this region is sequestered without making direct contact. A secondary structure may also be protecting this sequence without stabilizing the binding of the RNA to the complex. Secondary structure analysis of the complex is in progress.

One candidate for a *trans*-acting downstream factor may be the Sm-containing moiety binding the +15 oligonucleotide in the extract. This weak interaction was observed previously and was enhanced by treatment of the extract with micrococcal nuclease (Fig. 6) (15). However, binding of this extract component is unaffected by AAUAAA mutation and was observed neither in the 25S complex (Fig. 8) nor in polyadenylation-specific assemblies observed without heparin treatment (data not shown). We have also observed this fragment to bind micrococcal nuclease-degraded snRNPs (our unpublished data), and we note that the uridylate-rich sequence in this region is homologous to the Sm protein-binding site in snRNAs (24). Whether this interaction is important in polyadenylation is unknown.

The possibility that the factor recognizing the AAUAAA is an snRNP was previously suggested by immunoprecipitation with the Sm-specific monoclonal antibody used in this study. We found that the AAUAAA oligonucleotide was also precipitated by an irrelevant monoclonal antibody and nonimmune human IgG, suggesting that the AAUAAA factor or an associated component strongly but nonspecifically binds immunoglobulins. It is conceivable that nonspecific binding of an snRNP or snRNP components to the complex, as for the IgG, is responsible for its weakly Sm-specific reaction. However, these results do not exclude the presence of an Sm component in the particle. In contrast to our observation of the nonspecific binding to IgG, Hashimoto and Steitz (15) found that sera specific for determinants on the U1 or U2 snRNP did not immunoprecipitate the component interacting with the AAUAAA. It is possible that those autoimmune sera have a low nonspecific affinity for the factor or that the longer incubations we used to obtain 25S complex assembly allow the binding of an extract component which potentiates nonspecific binding to IgG. Resolution of this issue will probably require a approach compatible with more stringent conditions such as Western immunoblot analysis.

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