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Classes of polyadenylation sites revealed by native gel electrophoresis of *in vitro* assembled complexes and sensitivity to U RNA cleavage

Rose, Scott Daniel, Ph.D.

Rice University, 1988
RICE UNIVERSITY

CLASSES OF POLYADENYLATION SITES
REVEALED BY NATIVE GEL ELECTROPHORESIS
OF IN VITRO ASSEMBLED COMPLEXES AND
SENSITIVITY TO U RNA CLEAVAGE

by

SCOTT D. ROSE

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE:

Dr. Susan M. Berget, Director
Associate Professor of Biochemistry

Dr. George N. Bennett
Associate Professor of Biochemistry

Dr. George J. Schroepfer
Professor of Biochemistry

Dr. Frederick B. Rudolph
Professor of Biochemistry

Dr. Ronald J. Parry
Professor of Chemistry

Houston, Texas
August, 1988
Classes of Polyadenylation Sites Revealed by Native Gel Electrophoresis of In Vitro Assembled Complexes and Sensitivity to U RNA Cleavage

by

Scott D. Rose

Abstract

The sequences that comprise a polyadenylation signal are varied. With the exception of the conserved hexanucleotide AAUAAA, other required sequence elements vary from site to site. This variability in sequence content may be indicative of different types or classes of polyadenylation signals. We have used an in vitro polyadenylation system to investigate the possibility that classes of poly(A) sites exist. Precursor RNAs from the SV40 late and adenovirus 2 L3 polyadenylation sites were examined for differences in: assembly into RNA-protein complexes; sequence requirements for complex assembly; and interactions with small nuclear ribonucleoproteins (snRNPs). Both SV40 late and L3 precursor RNA required an intact hexanucleotide and downstream sequence elements for complex formation. The stability of the complexes assembled using the two precursor RNAs was different. The L3 RNA complex was unstable in the presence of the anion poly(ACU); whereas the SV40 late complex or a chimeric L3/SV40 late complex were not.

SV40 late and L3 precursor RNAs associated with the Sm protein determinant (common to U1, U2, U4/U6, U5 and U7 snRNPs)
and a U1 snRNP-specific protein determinant early in the polyadenylation reaction. This association was reduced as the polyadenylation reaction progressed. Formation of the polyadenylation specific complexes was shown to require the small nuclear RNAs (snRNAs) U1, U2 and U4. The two polyadenylation precursor RNAs showed a different sensitivity to oligonucleotide directed RNase H cleavage of U RNAs. The SV40 late site was sensitive to cleavages of U1, U2 and U4 RNA. The L3 site showed sensitivity to only U4 cleavage. When the two polyadenylation signals were preceded by a functional intron with 5' and 3' splice sites, sensitivity to U RNA cleavages was altered. However even as chimeric polyadenylation splicing templates, the two sites exhibited different sensitivities to U4 cleavage in the region of U4 in which it hybridizes to U6 RNA. The observed differences in complex stability, and sensitivity to U RNA cleavage suggest that different classes of polyadenylation sites exist.
Acknowledgements

I would like to thank Dr Susan Berget for providing me an excellent graduate research background, and for allowing me to mature into a research scientist at my own pace. I would like to extend my thanks to Barbara Robberson for both her technical help and friendship. I would also like to thank the members of the Berget lab for their helpful insights. A special thank you goes to my good friends and fellow researchers Dr. Martin Zillmann, Dr. Gil Cote, Ira Schulman, and Sandra Pennington for showing me how valuable good friends are and for keeping me sane during those times when even the simple experiments just would not work.

I would also like to acknowledge the support of my family during my graduate career, especially my grandfather Milford Rose for his unwavering faith in my abilities. And finally, I must thank my wife Renee Rose for her support and love during both the good and bad times of my graduate career.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bis</td>
<td>N, N'-methylenebisacrylamide</td>
</tr>
<tr>
<td>C</td>
<td>cytidine</td>
</tr>
<tr>
<td>CAP</td>
<td>diguanosine triphosphate G(5')ppp(5')G</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene related peptide</td>
</tr>
<tr>
<td>cleav.</td>
<td>cleavage</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>3'-dATP</td>
<td>3' deoxyadenonsine triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>G</td>
<td>guanosine</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine</td>
</tr>
<tr>
<td></td>
<td>N'-2-ethanesulfonic acid</td>
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<tr>
<td>hnRNA</td>
<td>heterogenous nuclear ribonucleic acid</td>
</tr>
<tr>
<td>hnRNP</td>
<td>heterogenous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin mu</td>
</tr>
<tr>
<td>Kd</td>
<td>kilodalton</td>
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<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>ml</td>
<td>milliliter</td>
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MOPS 3-(N-morpholino)propanesulfonic acid
mRNA messenger ribonucleic acid
PEG polyethylene glycol
poly(A) polyadenylic acid
Pre. precursor
PVA polyvinyl alcohol
RNA ribonucleic acid
RNase ribonuclease
RNasin ribonuclease inhibitor
RNP ribonucleoprotein
SDS sodium dodecyl sulfate
T thymidine
TEMED N, N, N’, N’-tetramethylethylediamine
Tris tris(hydroxymethyl)aminomethane
tRNA transfer ribonucleic acid
U uridine
ug microgram
ul microliter
U RNA uridine rich ribonucleic acid
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Introduction

One of the early nuclear processing events that primary transcripts must undergo is the formation of 3' termini. For RNA polymerase II transcripts this process is known as polyadenylation. The sequence requirements for 3'-end formation in a number of individual polyadenylation sites have been studied extensively. The sequence requirements for different polyadenylation sites vary. Apart from the hexanucleotide, AAUAAA, sequences within polyadenylation sites vary considerably. The interactions between those sequences and the factors necessary for polyadenylation in the nucleus are poorly understood. In vitro RNA processing systems developed from HeLa cell nuclear extracts allow questions to be asked about the interactions between simple RNA transcripts containing polyadenylation or combination splicing and polyadenylation signals and the factors required for their processing. The goals of these studies are to examine the in vitro assembly of RNAs containing a single polyadenylation signal into active polyadenylation specific complexes and determine if different classes of polyadenylation sites exist based on sequence requirements for complex formation and their interactions with nuclear factors.
The Polyadenylation Reaction

The 3' terminus of the RNA polymerase II primary transcript extends far beyond the 3' end of the mature messenger RNA. It has been documented for the adenovirus major late transcription unit, mouse B globin gene, mouse dihydrofolate reductase gene, mouse alpha amylase gene, and chicken ovalbumin gene that transcription termination occurs downstream of the mature 3' termini (Nevin and Darnell, 1978; Fraser et al., 1975; Hagenbuckle et al., 1984; Frayne et al., 1984; Lemur et al., 1984; Hoefer and Darnell, 1981; Hoefer et al., 1982; Salditt-Georgieff et al., 1984). In several cases, transcription termination occurs kilobases away from the 3' end of the mature message. The process of generating the mature 3' terminus for most RNA polymerase II transcripts is known as polyadenylation. Polyadenylation is a two step process (fig. 1), the first step involves endonucleolytic cleavage at a defined site by an unidentified endoribonuclease, resulting in a capped pre-mRNA with a free 3' hydroxyl, and an uncapped RNA consisting of sequences downstream of the cleavage site, ending at the transcription termination site. The second step is the addition of several hundred adenylate residues to the newly generated 3' end of the capped pre-mRNA by an AAUAAA hexanucleotide-dependent poly(A) polymerase. The other half of the primary transcript has never been detected in vivo and is presumably degraded.

In this thesis, polyadenylation is used to refer to the
Figure 1: Mechanism of the Polyadenylation Reaction

Polyadenylation is a two step nuclear processing event; the first step is an endonucleolytic cleavage of the capped primary transcript at a defined site 10 to 30 nucleotides downstream of the hexanucleotide AAUAAA, usually at an A residue. The second step is the polymerization of approximately 250 adenylate residues to the new 3' hydroxyl. The uncapped half of the primary transcript is presumably degraded.
overall process of cleavage and adenylate addition; poly(A)-addition to refer to the polymerization of adenylate residues onto an RNA cleaved at the site of (A)-addition. The term polyadenylation site will refer to an RNA containing all the signals necessary for both cleavage and (A)-addition. Polyadenylation signal will refer to the primary sequence elements that direct polyadenylation.

Significance of Polyadenylation

The role of polyadenylation is not yet understood. The fact that almost all mRNAs are polyadenylated argues that the process must have some significance to the cell. Early theories postulated a role for the poly(A) tail in stabilizing the message to ribonucleases in the cytoplasm. Rabbit beta globin with a very short A tail (less than 30 adenylate residues) is unstable when injected into frog oocytes or HeLa cells (Huez et al., 1971, Nudel et al., 1974). The length of the poly(A) tail decreases with age of the message in the cytoplasm; however, the correlation is not perfect because of the existence of cytoplasmic poly(A) polymerases that add on small stretches of adenylate residues to existing 3’ termini of messages (Brawerman, 1976; Brawerman, 1981).

A second role postulated for poly(A) was as a signal recognized during nuclear processing and transport of the message. Experimental evidence argues against this second role. RNA that has been correctly spliced can be detected in
the presence of ATP analogues, including 3' deoxyadenosine triphosphate (cordycepin triphosphate), that inhibit polyadenylic acid synthesis. In the presence of 3'dATP, correctly spliced but non adenylated adenovirus 2 RNA was detected in the cytoplasm and in polyribosomes; however the RNA exhibited a shorter half-life than did poly(A)+ adenovirus 2 RNA (Zeevi et al., 1982).

Several recent reports now link polyadenylation to transcription termination as a third function. A thalassemic alpha globin gene with a point mutation in the polyadenylation signal does not undergo polyadenylation at the normal site, but downstream of a cryptic poly(A) signal. Transcription termination also fails to occur at the normal site in the mutant (Higgs et al., 1983; Whitelaw and Proudfoot, 1986), suggesting a link between transcription termination and 3' end processing. Furthermore, the sequence element necessary for transcription termination in the mouse beta major globin gene (Hofer et al., 1982, Salditt-Georgieff and Darnell, 1983) was introduced into the adenovirus E1A transcription unit and only functioned in the presence of a polyadenylation signal (Falck-Pedersen et al., 1985). Mutations in the polyadenylation signal caused a reduction in the level of correct transcription termination (Logan et al., 1987).

Gene expression is regulated through choices of polyadenylation sites. The calcitonin/calcitonin gene related peptide (CGRP) and the IgM transcription units are examples of
genes in which tissue specific expression is regulated through differential use of alternative polyadenylation and splicing sites. The calcitonin/CGRP transcription unit is composed of 6 exons. The first four are utilized to generate calcitonin mRNA, predominantly manufactured in the thyroid C cells, with the fourth exon containing the polyadenylation signal (Amara et al., 1982). The CGRP mRNA (produced in brain, spinal cord, and cranial nerve ganglia) is generated by splicing the first three exons to the fifth and sixth exon and utilizes a polyadenylation signal within the sixth exon (Amara et al., 1984; Leff et al., 1987; Crenshaw et al., 1987).

Differentiation of B lymphocytes is marked by the change in expression of membrane bound IgM to secreted IgM. The difference in the two mRNAs exists at the 3' end, where the membrane bound form contains an additional 1.8 Kb of sequence not found within the secreted form. The secreted form utilizes an internal polyadenylation signal, whereas the membrane form utilizes a polyadenylation signal further downstream (Rogers et al., 1980, Early et al., 1980). The regulation of production of the two RNAs appears to be a result of poly(A) site usage instead of splice site choice (Galli et al., 1988).

Role of Consensus Sequence AAUAAA in Polyadenylation

Examination of the primary sequences of over 30 poly(A) sites indicated one conserved sequence, AAUAAA, found 10 to 30 residues upstream of the (A)-addition site (Proudfoot and
Brownlee, 1974). The hexanucleotide AAUAAA was subsequently shown to be part of the polyadenylation recognition sequence through a series of deletion experiments carried out on SV40 late mRNA in vivo (Fitzgerald and Shenk, 1981). Three classes of deletions were constructed. The first class of mutants lacked sequences between the hexanucleotide AAUAAA and the normal poly(A) cleavage site. The mutants from this class polyadenylated at new cleavage sites downstream of the normal in vivo site. The second class of mutants had deletions upstream of the hexanucleotide AAUAAA. A slight altering of the site of poly(A)-addition was observed; however, the steady state levels of polyadenylated RNA were not affected. The third class of mutants contained deletions that encompassed the AAUAAA. In this case, no mRNA production was detected. From this work it was concluded that the spacing between the hexanucleotide and the poly(A) cleavage site was crucial, and the hexanucleotide itself was probably part of the recognition sequence.

Direct proof of the role of the AAUAAA in polyadenylation was provided by the introduction of a single U to G transversion in the AAUAAA consensus sequence of the adenovirus early region 1A gene (Montell et al., 1983). By this time it was clear that in the case of adenovirus 2 and several other cellular mRNAs, the site of transcription termination was downstream of the polyadenylation site. Therefore, polyadenylation must be a two step process involving a cleavage of the pre-mRNA followed by addition of the A tail. The point mutant reduced the efficiency
of RNA cleavage by 95%. The remaining 5% that was cleaved was also polyadenylated, although microheterogeneity of the cleavage site was observed. From this observation it was concluded that the hexanucleotide AAUAAA was essential for cleavage, but at least partially dispensable for A addition. A similar observation concerning the importance of the AAUAAA sequence was noted in an alpha thalassemic patient found in Saudi Arabia (Higgs et al., 1983). The alpha 2 gene differed from the wild type gene in an A to G transition in the hexanucleotide sequence AAUAAA (AAUAAA to AAUAG). In this case high levels of message were produced; however, the message was abnormally processed. Instead of the normal in vivo poly(A) cleavage site being used, the transcript was polyadenylated downstream at a cryptic AAUAAA. Further substantiating evidence for the role of AAUAAA in polyadenylation was obtained from a beta thalassemic patient, in whom an U to C transition inactivated the normal beta globin gene polyadenylation signal (Orkin et al., 1985). Once again, polyadenylation occurred downstream of the normal site at a cryptic AAUAAA. To date, all polyadenylated mRNAs contain an AAUAAA sequence. Extensive examination of 3' end sequences indicate, however, that the sequence is not invariant. Naturally occurring and functional minor variations have been observed including AAUUAA, AAUACA, AAUAAU, AGUAAA (Wickens and Stefenson, 1984; Hagenbuchle et al., 1980; Nunberg et al., 1980; Tosi et al., 1981; De Greve et al., 1982).
Identification and Function of Downstream Sequence Elements

The hexanucleotide cannot be the only signal necessary for polyadenylation. The sequence AAUAAA occurs in the coding region of certain mRNAs, and clearly does not constitute a polyadenylation signal in this context. Extensive sequence analysis fails to show significant homology other than the AAUAAA element near the poly(A) cleavage site. Deletion analysis does indicate that sequences downstream of the cleavage site do play a vital role in polyadenylation (fig. 2). Simian virus 40 (SV40) late mRNA requires sequences from +6 (6 nucleotides downstream of the cleavage site) to +30 for efficient cleavage and polyadenylation (Conway and Wickens, 1985). Additional sequences downstream of +30 have also been shown to contribute to the efficiency of polyadenylation for SV40 late mRNA (Sadofsky and Alwine, 1984; Sadofsky et al., 1985). Similar to the alpha globin thalassemia point mutation in the AAUAAA element, deletions in downstream sequences result in longer than normal transcripts that utilize cryptic downstream polyadenylation signals (Sadofsky and Alwine, 1984).

The bovine growth hormone gene also contains downstream sequences required for accurate polyadenylation (Woychik et al., 1984). Analysis of the SV40 early and adenovirus E2A polyadenylation sites also indicate a role for downstream sequences for efficient polyadenylation. A region of alternating TGT residues was shown to be necessary in both sites by introducing point mutations in those regions (McDevitt et
SV40 late

CUGCAAUAACCAAGUUAACA*ACAACAUUGCAUUCAUUUAUGUUUCAGGGUUCAGGGGAGG
UGUGGGAGGUGUUU

L3

UUUCAAUAAAAGGCAAAUGUUUUUUUUUGUA*CACUCUCGGGUGAUUAUUUACCCCCACCCCUU
GCCGUCUGCGCGGUU

Figure 2: SV40 late and L3 Polyadenylation Signals
The sequences comprising the polyadenylation sites SV40 late and L3 that are functional in vitro. The conserved hexanucleotide AAUAAA and sequences downstream of the cleavage site known to be important for polyadenylation by deletional or point mutational analysis are underlined. The polyadenylation cleavage site is indicated by an asterisk.
al., 1984; McDevitt et al., 1986). Examination of sequences just 3' to the cleavage site for several genes does show a tendency for runs of T's with interdispersed G's. This loose region of homology is now referred to as the "GT" cluster (Birnstiel et al., 1985). An additional region consisting of a run of T's has been identified as a downstream sequence element in the case of the rabbit beta globin gene (Gil and Proudfoot, 1984, Gil and Proudfoot, 1987). Each of the elements by themselves is not sufficient for wild type efficiency of 3' end processing, and the spacing between the elements is critical.

An additional recognition sequence element, CAYUG, was postulated to play a role in polyadenylation (Berget, 1984). A representative of the CAYUG was found upstream or downstream of the site of poly (A) addition in 61 different RNAs examined. Point mutations in the CACUG sequence of the SV40 Early polyadenylation signal inhibit efficient RNA processing in vitro (Sperry and Berget, personal communication, 1988).

**In Vitro RNA Processing Systems**

Recently a large amount of information about sequence requirements for polyadenylation has been obtained by using in vitro processing systems. Two types of cell extracts have been used, the HeLa whole cell extract and the HeLa cell nuclear extract. The whole cell extract was originally developed as a transcription extract and shown to be competent to transcribe genes from the adenovirus late transcription unit (Manley et
al., 1980, Manley, 1983). This transcription extract was able to carry out coupled transcription and polyadenylation of SV40 early RNA (Manley et al., 1985). The in vitro polyadenylation reaction was very specific, only RNAs with intact hexanucleotides were competent to be polyadenylated.

The second type of extract, the HeLa cell nuclear extract, was also originally made for in vitro transcription (Dignam et al., 1983). It was quickly shown to be a superior extract for RNA processing in vitro. The nuclear extracts are competent for most of the major RNA processing steps, including removal of intervening sequences (splicing), polyadenylation, and histone 3' end maturation (Grabowski et al., 1984; Ruskin et al., 1984; Zillmann et al., 1986; Moore and Sharp, 1984; Moore and Sharp 1985; Hart et al., 1985; Sheets et al., 1987; Zarkower et al., 1986; Sperry and Berget, 1986; Gick et al., 1986; Mowry and Steitz; 1987 Mowry and Steitz, 1987). Using a HeLa cell nuclear extract, the initial cleavage event in polyadenylation was demonstrated to be an endonucleolytic cleavage by isolating the intact downstream cleavage fragment (Moore and Sharp, 1985). The cleavage event was also shown to be independent of the synthesis of a poly(A) tract by uncoupling the two events using ATP analogues (Moore and Sharp, 1985; Zarkower et al., 1986).

In vitro analysis also indicated that the AAUAAA hexanucleotide sequence was required for both cleavage and A addition (Zarkower et al., 1986). The need for downstream sequence elements for efficient processing was also confirmed in vitro (Hart et al.,
Interaction of Nuclear Polyadenylation Factors and Consensus Sequence Elements

Primary gene transcripts in the nucleus exist complexed to a large number of polypeptides known as hnRNP proteins. The hnRNP proteins are very abundant at a similar level to histones. At least 24 hnRNP polypeptides associate with hnRNA, and can be detected in HeLa cells (Pinol-Roma et al., 1985). At least some of these probably associate with the 3' ends of pre-mRNA. Limited nuclease digestion of hnRNP releases a 15S ribonucleoprotein particle containing polyadenylated RNA (Baer and Kornberg, 1980; Tomcsany and Tügyi, 1983). A 55 kd poly(A) binding protein (PABP) has been detected in nuclei of rat liver and yeast. A 75 kd yeast cytoplasmic poly(A) binding protein appears to come from the same gene as the nuclear PABP (Sachs and Kornberg, 1985; Sachs et al., 1986). At present very little is known concerning the roles of the hnRNP polypeptides, PABPs, or other factors in polyadenylation. Through the use of in vitro nuclear extracts the factors associated with and responsible for polyadenylation are slowly being identified.

The hnRNP polypeptides are likely candidates for possible factors in pre-mRNA processing. Monoclonal antibodies against the hnRNP polypeptide C inhibit in vitro splicing (Choi et al.,
1986). Protein-RNA cross-linking studies using polyadenylation precursor RNAs and HeLa cell nuclear extracts show close contacts between RNA and the C polypeptide. A previously unidentified 64 kd nuclear protein was recently shown to UV cross-link in vitro to RNAs containing the AAUAAA sequence (Wilusz and Shenk, 1988). The protein was specifically cross-linked to three different polyadenylation precursor RNAs in the presence of a 4000 fold molar excess of tRNA, and failed to cross-link to a RNA containing bacterial sequences. Association of the 64 kd protein was only detected at reaction temperatures of 30°C. The binding of the 64 kd protein was not ATP dependent, was unaffected by EDTA, and was inhibited at Mg++ concentrations greater than 4 mM. The 64 kd polypeptide failed to bind to a point mutant in the AAUAAA sequence, demonstrating the specificity of binding. The presence of the downstream sequence elements of SV40 late precursor RNA were not essential for binding of the 64 kd polypeptide but did enhance binding. The identity and function of the 64 kd protein is unclear but it is not a poly(A) polymerase.

Several other peptides associating with polyadenylation precursor RNAs were also detected by UV crosslinking. Two peptides of 155 (p155) and 68 (p68) kd are crosslinked to the adenovirus 2 L3 precursor RNA (Moore et al., 1988). A single point mutation introduced into the AAUAAA sequence (AAUAAA to AAGAAA) abolished the ability to detect crosslinking of these two proteins. Both polypeptides were not detected on product
RNAs that have been cleaved and polyadenylated. p155 associated with L3 RNA in the absence of downstream sequences and is, therefore, probably dependent on only the AAUAAA sequence for binding. The p155 polypeptide is recognized by the Sm class of antibodies but is not associated with snRNPs containing a RNA bearing a trimethylated guanosine cap structure, indicating that p155 is not part of a major known snRNP.

In Vitro Detection of RNA-Protein Complexes

By utilizing the in vitro polyadenylation extracts, it has been possible to detect the specific interaction between nuclear factors and precursor RNAs containing polyadenylation signals. Hashimoto and Steitz reported detecting a small nuclear ribonucleoprotein that associates with the AAUAAA polyadenylation signal (Hashimoto and Steitz, 1986). Their approach was to incubate radiolabeled RNA containing various polyadenylation signals with nuclear extract, add a anti-snRNP antibody and ribonuclease T1, immunoprecipitate the antibody-antigen complexes, and examine the RNase T1-protected RNA fragments remaining in the antigen-antibody complex. In four different substrates one of the RNA fragments protected contained the AAUAAA sequence. The results were identical using either a monoclonal antibody that recognizes the Sm protein antigen (common to all snRNPs) or an antibody that recognizes the unique trimethyl guanosine cap structure found on almost all U RNAs. Antibodies unique for U1 or U2 failed to detect
protected sequences containing the hexanucleotide.

A second method for detecting complexes relies on the accessibility of small oligonucleotides to base pair with exposed region of RNA and the subsequent cleavage of the heteroduplex with RNase H (fig. 3). This technique has been used extensively to examine the interaction of splicing consensus sequences with splicing factors (Kramer et al., 1984; Ruskin and Green, 1986; Rymond and Rosbash, 1986). A complex protecting the AAUAAA sequence of SV40 late against digestion has been detected (Zarkower and Wickens, 1987). The complex forms with precursor RNA and is transient. A stable complex can be detected on the RNA when a single cordycepin residue has been incorporated on the newly cleaved RNA. Complex association can be inhibited by the addition of RNAs containing the poly(A) signal, AAUAAA. Complex association is not detected when an RNA containing a single point mutation in the AAUAAA sequence (AAUAAA to AAUACA) is used. Finally, complex association cannot be detected on an RNA that already has undergone processing.

Several investigators have recently reported detection of large 30-60S RNA-protein complexes generated in vitro, on splicing precursor RNAs, by sucrose gradient analysis or native gel electrophoresis (Frendewey and Keller, 1985; Grabowski et al., 1985; Kaltwasser et al., 1986, Brody and Abelson 1985; Konarska and Sharp, 1986; Zillmann et al., 1987; Zillmann et al., 1988). These complexes contain both precursor and product RNAs. Using similar systems, formation of polyadenylation
specific complexes has been examined. A 25S ribonucleoprotein complex was detected in vitro on sucrose gradients using precursor RNAs containing the adenovirus type 2 L3 polyadenylation signal (Stefano and Adams, 1988). The 25S complex formation was dependent upon having a functional AAUAAA. An inactive RNA with a U to C mutation in the AAUAAA failed to assemble into the 25S complex. Complex formation was also dependent upon ATP, and a uridylate rich tract downstream of the cleavage site. The AAUAAA sequence and the U rich downstream sequence in the 25S complex were protected from oligonucleotide directed RNase H cleavage, indicating the presence of a factor bound to the RNA at those sequences. A T1 protected RNA fragment containing the AAUAAA was immunoprecipitated with Sm or anti trimethyl guanosine cap antibodies, however, no fragments corresponding to the downstream U rich regions were immunoprecipitated. Similar results were obtained in other laboratories using the L3 polyadenylation signal (Moore et al., 1988).

Gel retardation assays using in vitro generated complexes have yielded similar results. Detection of polyadenylation specific complexes on nondenaturing gels has been recently reported for L3, SV40 late, and herpes TK polyadenylation precursor RNAs (Zarkower and Wickens, 1987; Humphrey et al., 1987; Zhang and Cole, 1987). Each precursor RNA assembles into a slowly migrating complex that is dependent upon an intact AAUAAA, ATP, and downstream sequences for formation. Point
Figure 3: Oligonucleotide Directed RNase H Cleavage

The technique of oligonucleotide directed RNase H cleavage can be used to detect the presence of a factor bound to the RNA. A complimentary oligonucleotide will hybridize to the RNA only in the absence of any bound factors. The RNA-DNA duplex is recognized by RNase H and the RNA is cleaved. Cleaved RNA indicates that region is free of bound factors.
mutations in the hexanucleotide that inhibit in vitro polyadenylation also prevent formation of the specific complexes. RNAs that are unable to undergo polyadenylation in vitro also fail to assemble into a specific complex. Specific complexes form prior to the detection of any polyadenylation activity and, therefore, may be required for the reaction. Cleaved and polyadenylated RNA is detected in a different complex.

Experimental Objectives

The goal of these studies was to: investigate the in vitro formation of polyadenylation specific complexes with precursor RNAs containing the SV40 late and adenovirus 2 L3 polyadenylation sites; determine the sequences necessary for complex assembly and determine if assembly differences in the two sites could be detected; and examine for the possible involvement of small nuclear RNAs in polyadenylation. The detection and resolution of polyadenylation specific complexes was by native gel electrophoresis. Sequence requirements were determined by deletion and point mutation analysis. Involvement of snRNPs in polyadenylation was probed using oligonucleotide directed RNase H cleavage of small nuclear RNAs and native gel electrophoresis.
MATERIALS AND METHODS

Plasmid Construction

The plasmid containing the SV40 late polyadenylation signal pSVL was a gift from M. Wickens. pSVL (fig. 4) is a BamHI/HindIII fragment of SV40 late (nucleotides 2533 to 2745, with a HindIII linker added at 2745) inserted into the plasmid vector pSP65 (Promega Biotech). This template DNA when digested with DraI (Boehringer Mannheim Biochemicals, BMB) will give a SP6 RNA polymerase (Bethesda Research Laboratories, BRL) transcript of 224 nucleotides. The plasmid pBSSVL KS+ was constructed by subcloning the BamHI/HindIII fragment of pSVL into the BamHI/HindIII sites of pBlueScript KS+ (Stratagene). Digesting pBSSVL with BamHI (BMB) will give a 266 nucleotide T3 RNA polymerase (BRL) transcript that is antisense to the SV40 late precursor RNA. The plasmid pSVL₅', (fig. 4) was constructed by subcloning the BamHI/HpaI fragment (nucleotides 2533 to 2666) into the BamHI/EcoRV sites of the plasmid vector pBlueScript KS+. The RNA SVL₅', can be generated either by digesting pSVL with HpaI (BMB) to give a SP6 RNA polymerase transcript of 163 nucleotides or by digesting pSVL₅' with AccI (BMB) to give a T7 RNA polymerase (BRL) transcript of 193 nucleotides. The plasmid pAAUACA (fig. 4) was a kind gift from M. Wickens and consist of an A to C transversion at nucleotide 2661. pAAUACA₅', was constructed by subcloning the BamHI/HpaI fragment of pAAUACA into the BamHI/EcoRV sites of pBlueScript
Figure 4: Polyadenylation Precursor RNAs

Maps of the SV40 late (SVL), adenovirus 2 L3, E2A, and chimeric L3-SV40 late (L3/SVL) polyadenylation sites showing overall length and position of cleavage sites. The thin lines represent bacterial vector sequences and the open boxes represent polyadenylation site specific sequences. The deletion mutants of the SV40 late (SVL\textsubscript{5'}) and L3 (L3\textsubscript{5'}) sites were constructed by appropriate restriction endonuclease digestion of the parent plasmids as detailed in the material and methods section. The SV40 late point mutant AAUACA was a kind gift of M. Wickens. The short L3 substrate CML3 and point mutant AAGAAA substrate were kind gifts from C. Moore. The combination splicing/polyadenylation templates MXSVL and MXL3 are shown with exon sequences represented by hatched boxes and polyadenylation site specific sequences represented by open boxes.
KS+. Plasmid pBSAAUACA was constructed by subcloning the BamHI/HindIII fragment of pAAUACA into the BamHI/HindIII sites of pBlueScript KS+. A T3 transcript of pBSAAUACA digested with BamHI (BMB) will give an antisense RNA of 266 nucleotides.

The plasmid containing the adenovirus 2 L3 polyadenylation signal, pSPL3 (fig. 4), was a kind gift from C. Moore. The plasmid pSPL3 was constructed by inserting a HindIII linker (Pharmacia, 5'-pd[CAAGCTTG]-3') at adenovirus 2 nucleotide 22237 and a SalI linker (Pharmacia, 5'-pd[GGTGACCC]-3') at nucleotide 22866. The HindIII/SalI fragment was subcloned into the HindIII/SalI sites of pSP64 (Promega). DraI digested pSPL3 will give a SP6 RNA polymerase transcript of 267 nucleotides. The plasmid pSPL3SAL was constructed by filling in the HindIII site and adding a SalI/TaqI linker (Pharmacia) (5'-pd[GGTGACCC]-3'). The plasmid pL35+ (fig. 4) was generated by deleting the sequences from the AvaI site at nucleotide 22448 to the AvaI site in the polylinker of the vector. pL35+ gives a SP6 transcript of 226 nucleotides when digested with AvaI. The plasmid AAGAAA was also a kind gift from C. Moore, and contained an U to G transversion in the AAUAAA sequence. This plasmid will give a T3 RNA polymerase transcript of 101 nucleotides when digested with DraI. The plasmid pBSL3 was constructed by subcloning the HindIII/SalI fragment of pSPL3 into the HindIII/SalI sites of pBlueScript KS+. This plasmid will give a T3 RNA polymerase transcript of antisense orientation when digested with HindIII.
The adenovirus 2 E2A polyadenylation signal was subcloned to give the plasmid pG4E2A (fig. 4). The plasmid pG4E2A was generated by subcloning the AhaIII/HindIII fragment of pSPL3 into the HindIII/SmaI sites of the plasmid vector pGem4 (Promega Biotech). This plasmid will give a SP6 RNA polymerase transcript of 158 nucleotides when digested with HaeII. Digesting the plasmid with EcoRI will give a 287 nucleotide T7 RNA polymerase transcript of antisense orientation relative to the E2A RNA.

A chimeric polyadenylation signal (L3/SVL) (fig. 4) was created by replacing the downstream sequences of pSPL3 with downstream sequences of SV40 late. The plasmid pL3/SVL was generated by subcloning the HpaI/PvuII fragment from pSVL into the SmaI/PvuII sites of pL3\(_5\)\(_S\). This plasmid, when cut with DraI, will give a SP6 RNA polymerase transcript of 284 nucleotides. The plasmid pBSL3/SVL was created by subcloning the HindIII fragment from pL3/SVL into pBlueScript KS+ at the HindIII site. The orientation of this clone was determined by dideoxy sequencing. Digestion of this plasmid with DraI gives a T3 RNA polymerase transcript of 328 nucleotides.

The splicing/polyadenylation template pMXSVL (fig. 4) was constructed by inserting the BamHI/XbaI fragment from the plasmid pSVL into the BamHI/XbaI sites of pMINX (Zillmann et al., 1988) a plasmid containing a duplicated second exon from the adenovirus 2 leader sequence. Digestion with DraI gives a SP6 RNA polymerase transcript of 416 nucleotides. A second
splicing/polyadenylation template, pMXL3 (fig. 4), was made by inserting a SalI fragment from pSPL3SAL into the SalI site of pMINX. Digestion with DraI gives a SP6 RNA polymerase transcript of 501 nucleotides.

Transformation of DH1 or AG1 Competent Cells

E. coli strains DH1 or AG1 (BRL) cells were made competent through the CaCl₂/RbCl procedure (Kushner 1978). Cells were grown at 37°C in approximately 50 ml of Luria Broth [1% Bacto-Tryptone (Difco), 1% Bacto-Yeast Extract (Difco), 0.17M NaCl (Sigma), pH 7.5] to a cell density of 5x10⁷ cells/ml. Cells were centrifuged at 5,000 rpm in a Sorvall SS-34 rotor for 10 minutes. The cells were resuspended in 25 ml of 10 mM MOPS (Sigma) pH 7.0, 10 mM RbCl (Sigma) and centrifuged for 10 minutes at 5,000 rpm. The cell pellet was resuspended in 25 ml of 100 mM MOPS pH 6.5, 10 mM RbCl, 50 mM CaCl₂ (Baker) and incubated on ice for 30 minutes. The cells were centrifuged at 6,000 rpm for 10 minutes. The cell pellet was resuspended in 2.5 ml of 100 mM MOPS pH 6.5, 10 mM RbCl, 50 mM CaCl₂ and 0.038 ml of DMSO (Sigma, ACS reagent grade) was added. The competent cells were aliquoted into 200 microliter lots and either quick frozen in a dry ice/ethanol bath or were used immediately. DNA was added to the cells in a volume no greater than 10% of the competent cell volume. The cells were incubated on ice for 30 minutes, heat shocked at 42 °C for 45 seconds,
and 1 ml of Luria Broth (LB) added. The cells were then incubated at 37\(^0\) C for 1-2 hours without shaking. The cells were centrifuged at 3,000 rpm for 10 minutes and the cell pellet was resuspended in 200 microliters (ul) of LB and plated out on LB plates [Luria Broth, 1.5 % Bacto-Agar (Difco)] containing either ampicillin (Sigma) at 100 micrograms/ml or tetracycline (Sigma) at 15 micrograms/ml. The plates were incubated overnight at 37\(^0\) C.

**Small Scale Plasmid DNA Preparation**

Colonies grown on drug containing plates were hand picked using sterile sticks and 5 ml of LB were inoculated and grown overnight at 37\(^0\) C with shaking. The saturated overnight culture was centrifuged at 3,000 rpm for 10 minutes. The pellet was resuspended in 0.5 ml of 50 mM Tris (Sigma) pH 8.0, and 50 ul of 250 mM Tris pH 8.0/10 ug/ml lysozyme (Sigma) was added. The mixture was incubated 20 minutes at room temperature. An equal volume of phenol (Baker) pH 8.0 was added and the tube was inverted gently 5 times. The tube was centrifuged for 5 minutes in the eppendorf microcentrifuge. The aqueous phase was removed, 300 ul of phenol/CHCl\(_3\) (Baker) pH 8.0 added, and the tube was vortexed for 10 seconds. The tube was centrifuged for 5 minutes in the microfuge. The aqueous phase was removed, 300 ul of CHCl\(_3\)/Isoamyl alcohol (Fisher) added, and the tube vortexed and centrifuged for 3 minutes. The aqueous phase was
removed, 50 ul of 2.5 M NaOAc (Sigma) added, and 1.0 ml of ethanol were added. The tube was incubated at -70 C for 15 minutes and centrifuged for 5 minutes. The DNA pellet was resuspended in 100 ul of 10 mM Tris pH 7.5/1 mM EDTA (Sigma), 3 ul of pancreatic RNase (Sigma) 10 mg/ml was added and the sample incubated at 37° C for 30 minutes. One fifth of the sample volume was then subjected to restriction endonuclease digestion.

**Large Scale Plasmid DNA Isolation**

Approximately 5 ml of a saturated overnight culture were added to a 1 L flask of LB containing the appropriate antibiotic. The flask was incubated in an incubator shaker (Labline) at 37° C until the cells reached an OD₆₀₀ of 0.6-0.8. Chloramphenicol (Sigma) was added to a final concentration of 170 ug/ml, and the cells were incubated for an additional 10-12 hours at 37 C. The cells were harvested by centrifugation at 6,000 rpm for 10 minutes in a Sorvall GSA rotor. The cell pellet was resuspended in 10 ml of solution 1 [25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose (Sigma)] containing 10 mg of lysozyme (Sigma) and incubated on ice for 30 minutes. 10 ml of solution 2 [0.2 M NaOH (Matheson, Coleman, and Bell), 1% sodium dodecyl sulfate (Bio-Rad)] were added and the mixture was incubated for 10 minutes on ice. 15 ml of solution 3 [3 M KOAc (Sigma), 1.8 M formic acid (Fisher)] were added and the mixture was left on ice for 30 minutes, followed
by centrifugation at 10,000 rpm for 15 minutes. At this point the supernatant was filtered through two thicknesses of wet cheesecloth and divided equally between 4 Oak Ridge centrifuge tubes. 15 ml of cold 95% ethanol were added to each tube and the tubes incubated at -20°C for 20 minutes. The tubes were centrifuged at 10,000 rpm for 10 minutes, the supernatant was removed and the pellet was dried. The pellet was resuspended in 2 ml TE (10 mM Tris, 1 mM EDTA), 1 ml of solution 5 [7.5 M NH₄Ac (Sigma)] was added and the tubes were left on ice for 20 minutes. The tubes were then centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed, 6 ml of ethanol added, and the tubes were incubated at -70°C for 15 minutes. The tubes were centrifuged for 10 minutes at 10,000 rpm. The supernatant was removed and the pellet dried. The pellet was resuspended in 5 ml of urea buffer [7 M urea (BMB), 10 mM Tris pH 7.5, 10 mM EDTA, 350 mM NaCl, 1% SDS], extracted twice with phenol/chloroform 1:1, and once with chloroform/isoamyl alcohol 24:1. The aqueous phase was added to 10 ml of ethanol and incubated at -70°C for 15 minutes. The tubes were centrifuged at 10,000 rpm for 10 minutes, the supernatant removed, and the pellet dried. The pellet was resuspended in 2 ml of TE, 10 microliters (μl) of RNase A (Sigma)(10 mg/ml) added, and incubated for 30 minutes at 37°C. One ml urea buffer was added to the tube and the tube was extracted twice with phenol/chloroform and once with chloroform/isoamyl alcohol. Six ml of ethanol was added and the tube was incubated
at -70° C for 15 minutes. The tube was centrifuged for 10 minutes at 10,000 rpm, the supernatant was removed and the pellet air dried. The final DNA pellet was resuspended in 1 ml of TE and its concentration determined by absorbance measurements at 260 and 280 nm.

**In Vitro Transcription**

Uniformly labeled and capped RNA transcripts were generated using a modified protocol of Melton (Melton, 1984). The standard transcription reaction contained: 17 ul H2O, 10 ul 5x transcription buffer (200 mM Tris pH 7.5, 30 mM MgCl2 (Sigma), 10 mM spermidine (Sigma), 50 mM NaCl), 0.5 ul 1 M dithiothreitol (BMB), 2 ul RNasin, (60 u/ul)(Promega), 2.5 ul ribonucleoside triphosphates (BMB) (10 mM ATP, 10 mM CTP, 4 mM UTP, 2 mM GTP), 2.5 ul diguanosine triphosphate (CAP, Pharmacia), 10 ul of linearized template DNA (2 ug), 5 ul of 32P-UTP (DuPont) (50 uCi), and 1 ul of polymerase (10 units). The transcription mixture was incubated for 90 minutes at 42° C, 2 ul of RQ DNase (Promega) added and incubation was continued for 10 minutes. 200 ul of urea buffer and 200 ul of TE were added to the mixture. The reaction was extracted with 200 ul of phenol/chloroform and 1 ml of ethanol was added. The tube was incubated at -70° C for 15 minutes then centrifuged in a microcentrifuge for 5 minutes. The RNA pellet was washed twice with 70% ethanol and dried. The pellet was resuspended in 5 ul
of TE and purified by gel electrophoresis.

**Gel Purification of RNA Transcripts**

An equal volume of denaturing dye [95% formamide (Aldritch), 1% xylene cyanol (Sigma), 1% phenol red (Sigma)] was added to the RNA sample and the sample was loaded onto a 5% acrylamide [30:1 acrylamide (Bio-Rad) to bis-acrylamide (Bio-Rad)], 7 M urea gel. The gel was run until the phenol red marker had moved off the bottom of the gel. The gel was covered with plastic Reynolds film and exposed to a piece of X-ray film to visualize the radiolabeled transcripts. The region of the gel containing the radiolabeled transcript was excised with a sterile razor blade and soaked in 500 ul of RNA elution buffer (0.5 M NH₄Ac, 0.5% SDS, 0.005 M EDTA pH 7.0) for 60 minutes at room temperature, with gentle agitation. The gel slice was removed and the eluent was extracted with 0.3 ml of phenol/CHCl₃. The tube was centrifuged for 5 minutes in the microcentrifuge. The aqueous phase was removed and 2 volumes of ethanol were added. After a 20 minute incubation at -70°C the RNA was pelleted in the microcentrifuge and dried. The pellet was resuspended in TE (10 mm Tris ph 8.0, 1mm EDTA) to a final concentration of 50,000 cpm/ul.
Silver Staining Acrylamide Gels

The acrylamide gel was soaked in 200 ml of H_2O for 5 minutes to remove the urea. The gel was then soaked in 200 ml of fix solution [50% methanol (Baker), 12% acetic acid (Baker)] for 5 minutes. The fix solution was removed and 200 ml of wash solution added (10% methanol, 5% acetic acid) for 30 minutes. The wash was removed and the gel was soaked in 200 ml of 0.0034 M K_2Cr_2O_7 (Baker), 0.0032 M HNO_3 (Fisher) for 5 minutes. The gel was then rinsed in H_2O several times, 200 ml of 0.0141 M AgNO_3 (Fisher) added, and allowed to soak for 20 minutes. The gel was then rinsed several times in H_2O and developed by the addition of 0.27 M Na_2CO_3 (Sigma), 0.2% formaldehyde. The development was stopped by the addition of 1% acetic acid. The silver stained gel was then dried down either by heating under vacuum or by sandwiching the gel between two pieces of dialysis membrane (Bio-Rad) on a special plexiglass frame.

Polyadenylation Reactions

Polyadenylation reactions contained 44% HeLa cell nuclear extract, 20 mM creatine phosphate (Sigma), 1 mM ATP (BMB), 1.5 mM MgCl_2, 3% polyethylene glycol 5000 (Sigma), 1 ug poly(ACU) (Pharmacia), 44 mM KCl (Sigma), 0.22 mM dithiothreitol, and 5-10 ng radiolabeled precursor RNA in a final volume of 0.025 ml. In ATP analogue experiments, cordycepin triphosphate (BMB)(3'-dATP)
at 1 mM was used in place of ATP. Total RNA from reactions was analyzed by electrophoresis on denaturing acrylamide gels containing 7 M urea.

**HeLa Cell Nuclear Extract Preparation**

The HeLa cell nuclear extract was prepared following the procedure initially described for making a transcription extract (Dignam et al., 1983). Approximately 8 L of log growth phase HeLa cells were harvested by centrifugation at 1200 rpm for 20 minutes in a Sorvall HG-4L rotor. The cell pellet was washed in 30 ml of sterile PBS [0.137 M NaCl, 2.7 mM KCl, 10.6 mM Na₂HPO₄ (Baker), 1.5 mM KH₂PO₄ (Fisher) pH 7.4] and centrifuged at 1000 rpm for 5 minutes in a Sorvall HL-8 rotor. The volume of the wet cell pellet was noted and resuspended in 30 ml of buffer A (10 mM HEPES (Sigma) pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol). The cell suspension was incubated on ice for 10 minutes followed by centrifugation at 1500 rpm for 10 minutes. The cell pellet was resuspended in 10 ml of buffer A and homogenized ten times in a Kontes all glass homogenizer. The homogenized cells were spun at 2000 rpm for 15 minutes. The supernatant was removed and the nuclei pellet was resuspended in buffer C (20 mM HEPES pH 8.0, 25% glycerol (Fisher), 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol) at 0.83 volumes of the initial wet cell volume. The nuclei were homogenized an additional 3 times to complete
resuspension. The nuclei suspension was placed in a small glass beaker and stirred slowly on ice for 30 minutes. The suspension was centrifuged at 13,000 rpm for 30 minutes in a Sorvall SS-34 rotor at 4°C. The supernatant was removed and dialyzed against 250 ml of buffer D (20 mM Tris pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1.5 mM MgCl₂) in Spectrapor membrane tubing (6,000-8,000 m.w. cutoff) at 4°C. The buffer was changed after 2 hours and dialyzed for an additional 2 hours. The dialysate was aliquoted into 0.5 ml microfuge tubes pre-chilled to -20°C and quick frozen in liquid nitrogen.

RNA Analysis of Polyadenylation Intermediates and Products

Aliquots from various time points during a standard polyadenylation reaction were added to tubes containing 200 ul of TE, 200 ul of urea buffer, and 200 ul of phenol/chloroform 1:1. The tubes were vortexed briefly and spun in the microcentrifuge for 3 minutes. The aqueous phases were removed and 1 ul of a carrier glycogen (Sigma) solution (10 mg/ml) was added. The RNA was precipitated by addition of 1 ml of ethanol and incubation at -70°C for 15 minutes. The RNA pellets were resuspended in 5 ul of TE and 7 ul of denaturing dye (98% formamide, 1% xylene cyanol, 1% bromphenol blue), followed by heating the samples at 100°C for 5 minutes with subsequent rapid chilling on ice. The samples were subjected to 7M urea 8%
denaturing polyacrylamide gel electrophoresis. The gel was autoradiographed to visualize the location of the radiolabeled RNA precursor and products. A dark smear running above the precursor band corresponds to the poly(A) RNA. A band approximately 50 nucleotides shorter than the precursor RNA corresponds to the cleaved and singly adenylated RNA that is seen in reactions containing 3' dATP.

**RNase T2 Mapping of Polyadenylated RNA**

The precursor RNA can be polyadenylated in two ways, the A residues can be added to the 3' terminus of the precursor RNA (end adenylation) or the precursor can be endonucleolytically cleaved then adenylated (in vivo polyadenylation). In order to distinguish the level of cleavage in the presence of ATP, a hybridization RNA protection experiment was done. The RNA was hybridized to either single stranded DNA or RNA that is complimentary to the entire precursor RNA. RNase T2 (Sigma) was added to the hybridization mixture to digest any regions of single stranded RNA. The digestion mixture was then analyzed on a denaturing acrylamide gel and visualized by autoradiography. If the RNA was end adenylated, then a band the size of precursor RNA is seen. If the RNA was cleaved then adenylated, a band with a length corresponding to the site of cleavage will be seen. This band size depends on the distance between the 5' end of the precursor RNA and the cleavage site.
The RNA from the reaction was purified as stated in the previous section. The RNA was resuspended in 10 μl of 20 mM Tris pH 7.5, 3 μl of 5x hybridization buffer (5 M NaCl, 250 mM Tris pH 7.5, 5 mM EDTA), 2 μg of single stranded DNA. The hybridization mixture was incubated at 65°C for 5 minutes, then incubated for 60 minutes at 50°C. 200 μl of NTE (0.2 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA) and 4 units of RNase T2 was added and the mixture was incubated at 30°C for 60 minutes. An equal volume of urea buffer was added and the mixture extracted with one-half volume of phenol/chloroform. The RNA was precipitated with ethanol. The digestion products were separated by electrophoresis on denaturing acrylamide gels and autoradiographed.

Native Composite Agarose Acrylamide RNP Gels

RNA-protein complexes were resolved on native composite agarose-acrylamide gels. Heparin (Sigma) was added to a final concentration of 5 mg/ml to polyadenylation reactions and the samples were incubated on ice for 5 minutes or quick frozen in liquid nitrogen and stored at -70°C. The samples were warmed to room temperature and loaded onto RNP gels. The gels consisted of 4% acrylamide (80:1 acrylamide to bis-acrylamide), 0.5% agarose (Sigma), 50 mM Tris pH 8.8, 50 mM glycine (Glycine), cast to 0.75 mm thickness. The gels were run at 11 volts/cm for 4 hours. The gels were soaked in 50% methanol/12%
acetic acid for 3 minutes and subjected to autoradiography overnight to visualize the RNA-protein complexes.

**Extraction of RNA from RNP Gels**

The regions of the gel containing the RNP complexes were excised with a razor blade and soaked in 0.5 ml of RNA elution buffer (0.5 M NH₄Ac pH 7.5, 0.5% SDS, 5 mM EDTA) for 30 minutes with vigorous shaking. The eluent was separated from the gel slice and extracted with 0.3 ml of phenol/chloroform. The aqueous phase was removed, 10 ug of glycogen and 1 ml of ethanol were added. The solution was incubated at -70°C for 15 minutes to precipitate the RNA.

**Oligonucleotide Directed RNase H Cleavage of snRNAs**

Specific regions of the small nuclear RNAs U1, U2, and U4 were destroyed by using the technique of oligonucleotide directed RNase H cleavage (fig. 5). Six oligonucleotides were synthesized (Baylor College of Medicine, Department of Biochemistry) that were complementary to various regions of the snRNAs U1, U2, U4 (fig. 6) or as a control nonspecific oligonucleotide, to a region of the murine dihydrofolate reductase gene. The oligonucleotide denoted as U1 5' was complimentary to nucleotides 1-16 of U1 RNA. Oligonucleotides
U2 5' and U2 loop were complimentary to nucleotides 1-16 and 28-42 of U2 RNA respectively. Oligonucleotides U4 5' and U4 loop were complimentary to nucleotides 2-15 and 66-85 of U4 RNA respectively. The control oligonucleotide consisted of the sequence 5' GCTATCGTAAAGAAGTCAG 3'. The oligonucleotide cleavage reactions were carried out under conditions optimal (Sperry and Berget, 1986; Berget and Robberson, 1986) for polyadenylation. To each polyadenylation reaction containing 11 ul of nuclear extract, approximately 0.02 OD260 of oligonucleotide was added and the cleavage reaction was carried out at 30°C for 30 minutes. The radiolabeled substrate RNA was added to the reaction mixture and the mixture was incubated at 30°C for 90 minutes. The RNA was extracted from the mixture by treatment with urea buffer and phenol/chloroform followed by ethanol precipitation. The RNA was run out on a 10% denaturing polyacrylamide gel and the level of cleavage of the U RNAs was monitored by silver staining the gel.
Figure 5: Oligonucleotide Directed RNase H Cleavage of U RNAs

Complimentary oligonucleotides were hybridized to unprotected single stranded regions of the U RNA. RNase H recognizes the duplex and cleaves the RNA. This technique allows for specific removal of portions of the U RNA without perturbing the rest of the snRNP. The extent of U RNA cleavage was monitored by electrophoresis of isolated RNA followed by silver staining to visualize the U RNAs.
**Figure 6: Regions of U RNAs Targeted for Oligonucleotide-mediated Cleavage**

U RNA sequences to which oligonucleotides were hybridized to are underlined. * indicates pseudouridines. A control oligonucleotide complimentary to mouse dihydrofolate reductase cDNA (5'-GCTATCGTTAGAACTCAG-3') was used to insure inhibition was not due to the nonspecific addition of an oligonucleotide.
U15' 1-16
AmUmACU*U*ACCUGGCAGGGGAGAUACCAUGAU

U25' 1-16
AmUmCGCU*U*CUCGmGmCCU*UUUGmGCUAAGmAU

U2loop 28-42
AGmAUCAAmGmUGU*AGU*AU*CmU*GU*U*CUUmAU

U45' 2-15
AmGCU*UUGC GCAGUGGCAGUAUCGUAGCCAAUG

U4loop 66-85
AAUUGAmAAACUUU*UCCCAAU*ACCCCGCCGUGACG
Detection of Polyadenylation Specific Complexes with SV40 Late Precursor RNA by Native Gel Electrophoresis

Time Course of Assembly of SV40 Late Complexes

In vitro generated assemblies of RNA-protein complexes specific for RNAs containing splicing signals have been resolved by native gel electrophoresis (Konarska and Sharp, 1986; Konarska and Sharp, 1987; Lamond et al., 1987; Pikielny and Rosbash, 1986; Zillmann et al., 1987, Zillmann et al., 1988). Using a similar system, we attempted to resolve polyadenylation specific complexes generated in vitro with a HeLa cell nuclear extract and exogenously transcribed precursor RNA. Radiolabeled precursor RNA containing the SV40 late polyadenylation signal was incubated with ATP in a HeLa cell nuclear extract under conditions previously determined to be optimal for polyadenylation (Sperry and Berget, 1986). Aliquots were removed from the reaction at various time points, heparin was added to the samples to quench nonspecific protein binding, and samples were directly analyzed by TG (Tris-glycine) native gel (RNP) electrophoresis (fig. 7A). The radiolabeled RNA rapidly assembled into a broadly migrating smear, denoted the I or initial complex. The I complex formed on ice and was the first
Figure 7: Assembly of the SV40 late Polyadenylation Complexes

Radiolabeled precursor RNA (SVL) was incubated in the nuclear extract under optimal polyadenylation conditions. Reactions were terminated at the indicated times (minutes) by the addition of heparin to a final concentration of 5 mg/ml and polyadenylation complexes were resolved by native RNP gel electrophoresis.  A) Native RNP gel of assembly of SV40 late precursor RNA in the presence of 1 mM ATP, of SV40 late precursor RNA in the presence of 1 mM 3′-dATP (cordycepin triphosphate).  B) Denaturing RNA gel of the RNA products from the samples analyzed in panel A.
detectable complex. The I complex mobility decreased by 2 minutes of incubation, and by 5 minutes was replaced by a discrete complex labeled A, and a second, slower-migrating complex labeled B. At late times in the reaction, 30 and 90 minutes, another complex appeared between B and A. This complex was labeled C. A weak band having mobility similar to that of complex C was occasionally observed at 0 minutes. This band was not observed under reduced MgCl₂ concentrations and was, therefore, thought to be artifactual. During the first 30 minutes of reaction, the amount of complex B increased as the level of complex A decreased. However, by the end of the reaction, the level of complex B declined, and the level of complex C increased. This order of complex appearance suggested an ordered pathway of assembly of I to A to B to C.

Analysis of RNA in the reactions analyzed for assembly in figure 7B showed the appearance of polyadenylated RNA by 10 minutes, with a strong signal 30 minutes into the reaction. The kinetics of appearance of Poly(A)+ product RNAs was equivalent to the kinetics of appearance of complex C, suggesting that it might contain Poly(A)+ RNA. When the polyadenylation reaction was carried out in the presence of the ATP analog, 3’ dATP (cordycepin triphosphate), a similar pattern of complex formation was observed with one major exception - the absence of complex C. Cordycepin triphosphate is a chain terminator; it is incorporated as the first adenylate residue added to the newly cleaved 3’ terminus. Further addition is impossible due to the
lack of a 3' OH necessary for the formation of the phosphodiester bond with the next adenylate residue. The loss of complex C when 3' dATP was used further suggested that complex C might contain polyadenylated product RNA. The lack of formation of an additional complex in the presence of cordycepin also suggested that the intermediate in the polyadenylation reaction, the cleaved and singly adenylated RNA, must reside in a complex with similar mobility to either A or B, yet that complex would have to be distinct from the A or B complex formed in ATP-containing reactions.

The requirement for ATP in complex formation was explored by depleting the extract of ATP before the addition of substrate RNA and analysis of assembly patterns (fig. 8). In the presence of ATP, complexes A, B, and C are visible by 20 minutes. In the absence of ATP, neither complex A or C was formed; instead only A and a novel, slightly slower migrating form of A were observed. This indicates that complexes B and C require ATP for formation; and suggests that complexes B and C are functional complexes. In contrast, complex A had properties more suggestive of its being a non-specific complex.

**Effect of Heparin on Resolution of Polyadenylation Complex**

Polyadenylation reactions were stopped by addition of heparin to 5 mg/ml and incubation on ice for 10 minutes. The heparin stops the reaction and quenches nonspecific protein
Figure 8: ATP is Required for Complex B Formation

Radiolabeled precursor RNA (SVL) was incubated in nuclear extract that had been depleted of ATP (lanes 1-5) or mock treated (lanes 6-10). Complex formation was analyzed by RNP gel electrophoresis at the indicated times (minutes).
Figure 9: Effect of Heparin on Resolution of Polyadenylation

Complexes

Polyadenylation reactions containing ATP and SV40 late precursor RNA site were terminated at the indicated times (minutes) by increasing concentrations of heparin (0.0-5.0 mg/ml). The reactions were analyzed by RNP gel electrophoresis. Complexes A, B, and C are labeled
binding, possibly by virtue of its negative charge. The effect of heparin on the detection and resolution of polyadenylation complexes was explored. A heparin titration was performed using a concentration range of heparin from 0 to 5 mg/ml (fig. 9). In the absence of heparin, little material entered the gel, as judged by the level of signal observed in the wells. Almost no resolution of complexes was observed in the absence of heparin. Between 0.5 mg/ml to 5 mg/ml, the addition of heparin had little effect on complex resolution, although the amount of material not entering the gels deceased with increasing heparin. The other effect observed was a reduction in mobility of complex B as the heparin concentration increased. From these results, it appeared that added heparin was essential for good resolution of polyadenylation complexes, although high heparin may remove some components from the polyadenylation assemblies.

Two Dimensional Analysis of SV40 Late Polyadenylation Complexes

To determine the structure of the RNAs present in each of the gel-resolved complexes, two dimensional gel analysis was performed. Polyadenylation reactions containing either ATP or cordycepin triphosphate were analyzed on RNP gels. The gel lanes were cut into 0.5 cm slices and the RNA in each slice was eluted. The eluted RNA was then analyzed on a denaturing polyacrylamide gel. In the polyadenylation reaction containing ATP (fig. 10), the bulk of the precursor RNA was observed in
Figure 10: Two Dimensional Analysis of SV40 late Polyadenylation Complexes

Polyadenylation reactions containing ATP or 3'-dATP were subjected to RNP gel electrophoresis after incubation for 120 minutes. RNA was eluted from 0.5 cm slices of the RNP gel lanes as indicated and analyzed on denaturing RNA gels. Polyadenylation complexes A, B, and C are identified for the RNP gel. Unreacted precursor RNA, poly(A)+ RNA, and cleaved and singly adenylated RNA (cleav.) are identified for the RNA gel.
lanes 3-5 and 9-11, corresponding to the gel-slices containing complexes B and A, respectively. The polyadenylated RNA was seen in lanes 5-8, corresponding to the position of complex C. From this analysis, we concluded that complexes A and B are complexes of precursor RNA and complex C is the complex of polyadenylated product RNA.

In the presence of cordycepin triphosphate, complexes A and B were observed (fig. 10). The bulk of the precursor RNA was seen in lanes 2-5 and lanes 7-9, corresponding to the positions of complexes B and A, respectively. The majority of cleaved and singly-adenylated RNA was found in lanes 7-10 at the position of complex A. This indicates the existence of a fourth complex, observable only when the reaction is halted following cleavage but before (A)-addition, and having mobility similar to complex A.

Based on the gel mobility of naked RNA, it is clear that all precursor and product RNAs have factors associated with them throughout the entire reaction. Similar results are seen in the analysis of splicing complexes. The intermediates and end products of the reaction are always complexed to factors (Zillmann et al., 1988; Konarska and Sharp, 1987; Pikielny et al., 1986). The migration differences between complexes A and B suggested that the two complexes differed by the presence or absence of factors specific for polyadenylation. Formation of complex B was competed only with RNAs containing polyadenylation signals (data not shown). This indicates complex B is probably
the active pre-complex.

**Assembly of SV40 Late RNAs Containing Deletion and AAUAAA Point Mutations**

Extensive deletion studies indicate the presence of at least 2 sequence elements, the hexanucleotide AAUAAA and the downstream sequence element, absolutely required for polyadenylation both in vivo and in vitro. To determine which sequences were required for complex formation, a deletion mutant was constructed. The mutant SVL 5' lacks sequences from 6 nucleotides prior to the cleavage site to 55 nucleotides past the cleavage site. This RNA still contains an intact AAUAAA and can end adenylate at levels equal to or greater than the wild type RNA. The deletion mutant assembled into the I complex at zero time, and into complex A by 5 minutes (fig. 11A). Complex C was also observed; formation of C correlated with appearance of polyadenylated RNA (fig. 11B). The striking difference between the assembly pattern of the deletion mutant and wild type RNA was the lack of formation or complex B with the mutant. To insure that the absence of complex B was due to the removal of specific downstream RNA sequences, nonspecific bacterial sequences were added to the end of the truncated SVL' RNA. The addition of nonspecific RNA failed to restore formation of complex B. This data suggests both that an intact hexanucleotide is not sufficient for complex B formation, and that specific downstream sequence information is absolutely
Figure 11: Consensus Sequence Requirements for Assembly of the SV40 late Polyadenylation Complex

Polyadenylation reactions using SVL, SVL₅⁰, and SVLAAUACA precursor RNAs were analyzed on RNP gels (A) and RNA gels (B) at the reaction times (minutes) indicated. Polyadenylation complexes I, A, B, and C are indicated.
essential

The role of the hexanucleotide in polyadenylation has been well documented. Point mutations in the hexanucleotide lower the efficiency of polyadenylation in vivo and in vitro. However, naturally occurring minor variants of AAUAAA do exist (Birnstiel et al., 1985). A factor has recently been shown to directly interact with the hexanucleotide of SV40 late precursor RNA (Zarkower and Wickens, 1987). Point mutations that severely inhibit polyadenylation also fail to associate with the unidentified factor. The point mutant, SVLAAUACA, was tested for its ability to assemble into polyadenylation complexes (fig. 11A). This point mutant polyadenylates at 5 to 10 percent the efficiency of wild type levels. The mutant assembled into complexes A and B efficiently, but complex C was not observed. Analysis of the RNA showed a low level of end-adenylation 30 minutes into the reaction. The formation of complex B with the point mutation may be due to the ability of the point mutant to undergo low levels of polyadenylation. Other SV40 late hexanucleotide point mutations have been shown to inhibit formation of complex B (Zarkower and Wickens, 1987).

The results from the downstream deletion RNA and the work of Zarkower and Wickens suggest that formation of complex B is dependent on both an intact hexanucleotide and downstream sequence information. Formation of complex C is independent of downstream sequence information and may suggest the loss of association of certain factors once the RNA has been cleaved.
The formation of complex A is independent of downstream sequence information and may represent the random binding of hnRNP polypeptides.
Detection of Polyadenylation Specific Complexes with L3 Precursor RNA by Native RNP Gel Electrophoresis

Time Course of Assembly of L3 Complexes

Sequences comprising polyadenylation sites, with the exception of the AAUAAA, are diverse. The diversity in sequence content has hindered the delineation of sequence elements necessary for polyadenylation. Each poly(A) site must be examined extensively to determine which regions are important. This diversity in sequence content led us to wonder if there are different types of polyadenylation signals, and if so, are there different factors that recognize different sites. One approach we took was to see if the pattern of assembly was identical between RNAs containing the SV40 late and adenovirus L3 poly(A) signals. Radiolabeled precursor RNA containing the L3 polyadenylation signal was incubated in a HeLa cell nuclear extract under conditions identical to those used in the assembly of SV40 late precursor RNA. Aliquots of the reaction were analyzed by native gel electrophoresis. A time course of assembly is shown in figure 12A. The RNA assembled into a rapidly migrating diffuse complex very similar to the I complex seen with SV40 late RNA. As the reaction progressed, the I complex slowly reduced in mobility and became a discrete complex, labeled A. By 90 minutes two complexes were observed. The slowest migrating complex was labeled C and was similar in
Figure 12: Assembly and 2 Dimensional Analysis of the adenovirus 2 L3 Polyadenylation Complex

Aliquots of a polyadenylation reaction containing adenovirus L3 RNA and 1 mM ATP (lanes 1-6) or 1 mM 3'-dATP (lanes 7-12) were analyzed on RNP gels (A). RNP complexes A and C are defined in the text. 0.5 cm slices of the gel lanes containing the ATP (B) and 3'-dATP (C) 120 minute time points were analyzed for RNA content. Lane numbers on the RNA gels correspond to indicated slices on the RNP gel. Unreacted precursor RNA (L3), poly(A)+ RNA, and cleaved and singly-adenylated RNA (cleav.) are identified for the RNA gels.
appearance and migration to the complex C formed with SV40 late precursor RNA. In the presence of 3' dATP only one complex, complex A, was observed at the end of the reaction; no complex C was observed. The dependence of complex C on the presence of ATP, suggested that complex C contained polyadenylated product RNA. Even at very late times in the reaction, no complex resembling SV40 late complex B was detected.

Two Dimensional Analysis of L3 Polyadenylation Complexes

To verify the RNA content of each L3 complex, two dimensional gel analysis of the complexes generated at the 120 minute time point was performed as previously outlined. In the presence of ATP, the bulk of the polyadenylated RNA was seen in lanes 4-6, corresponding to the region of gel containing complex C (fig 12B and 12C). The bulk of the precursor RNA was observed in lanes 6-8, corresponding to the region of the gel containing complex A. In reactions containing 3' dATP, precursor RNA was observed in lanes 6-8, and the cleaved and singly adenylated RNA was seen in lanes 7-9. Both precursor and product RNAs migrate in the same region of the gel as complex A. The migration of the complexes C and A containing polyadenylated product and cleaved and singly-adenylated RNA, respectively was consistent between SV40 late and L3. Only SV40 late RNA assembled into complex B. The complex formation pattern observed with wild type L3 RNA was similar to the pattern seen with a truncated
SV40 late RNA (SVL₅₅) containing no downstream information. To further illustrate the differences between the assembly patterns of L3 and SV40 late precursor RNAs, assembly patterns of precursor RNAs containing the two sites were analyzed on the same RNP gel together with a control RNA containing bacterial sequences (pBR322) (fig. 13). The RNA containing bacterial sequences never assembled beyond the I region, and was rapidly degraded. The position in the gel of uncomplexed RNA is indicated. SV40 late RNA clearly assembled into three complexes by 120 minutes. In contrast, the L3 RNA only assembled into two complexes, C and A.

Assembly of a L3 Deletion RNA

A deletion mutation was constructed in which downstream sequences were removed starting 5 nucleotides beyond the cleavage site of L3. This clone was designated L3 5' and was used to investigate the possible role of downstream sequences in assembly of L3 complexes. The L3 5' RNA is an excellent substrate for end-adenylation in the extract. This RNA end adenylates with an efficiency equal to or greater than that of the wild type RNA. When incubated in the extract (fig. 14A), L3 5' RNA assembled into two complexes, A and C. The appearance of complex C was coincidental to the appearance of end adenylated product RNA. The efficiency at which this substrate end adenylated is seen by the almost complete conversion of
Figure 13: Comparative RNP Gel Analysis of the SV40 late and adenovirus 2 L3 Polyadenylation Complexes

Polyadenylation reactions using SV40 late (SVL), adenovirus 2 L3 (L3), and control plasmid (pBR) RNA containing no consensus sequences were sampled for RNP gel electrophoresis at the indicated times (minutes).
Figure 14: Assembly of the L3 Complex Does Not Depend On Downstream Sequences

Polyadenylation reactions containing L3 or L3₅, RNA were analyzed by RNP (A) or RNA (B) gel electrophoresis at the indicated times (minutes). An assembly reaction using L3 RNA and containing 3'-dATP is shown for comparison. The ATP containing reactions using wild type L3 were also mapped for cleavage activity (insert in B).
precursor to product by 90 minutes (fig. 14B). This efficiency was also reflected in the rapid disappearance of complex A and the appearance of complex C.

Assembly of a Chimeric Polyadenylation Substrate

The observed differences in the assembly patterns of the two poly(A) sites suggested that SV40 late precursor RNA associated with some factor that was either absent or transiently associated with complexes containing L3 RNA. Experimental data from immunoprecipitation experiments suggested that a factor with an Sm protein determinant associated with sequences downstream of the SV40 late polyadenylation cleavage site but not the L3 site (Hashimoto and Stietz, 1987). To determine if the differences we observed could be accounted for by downstream sequences, a chimeric polyadenylation substrate was constructed that contained the hexanucleotide and cleavage site of L3 fused to the cleavage site and downstream sequences of SV40 late. This RNA, L3/SVL, was incubated in the extract under standard polyadenylation conditions and compared to SV40 late for complex assembly (fig. 15A). The SV40 late RNA exhibited the standard complex assembly profile. The chimeric RNA produced an assembly pattern very similar to that of SV40 late RNA. Complex B was observable at five minutes, and complex C was seen by 30 to 90 minutes. Less complex B was formed, however, with the chimera; more RNA was observed in complex A, suggesting that the chimeric
Figure 15: Assembly of a Chimeric Polyadenylation Substrate

Polyadenylation reactions containing the SVL or L3/SVL precursor RNAs were terminated with heparin at the indicated times and were analyzed on RNP gels (A). The complexes A, B, and C are indicated for both substrates. (B) The RNA from each time point was RNase T2 mapped to determine the level of cleavage and analyzed on a denaturing acrylamide gel.
RNA was not as efficient in assembly as was SV40 late RNA. When the RNAs were examined for the level of cleavage and (A)-addition (fig. 15B), both showed reasonable levels of activity by 30 minutes. Approximately 50% of the SV40 late RNA was cleaved and adenylated; whereas only 20%-30% of the chimeric RNA had been correctly processed. The chimeric RNA contains two possible cleavage sites; however, due to the small spacing between them, the actual site utilized could not be determined by RNA mapping. It is clear, however, that polyadenylation efficiency of the chimeric RNA was lower than that of wild type SV40 late RNA.

**Effects of Poly(ACU) on Complex Stability**

Several explanations could account for the inability to detect a complex similar to B with the L3 precursor RNA. The L3 polyadenylation signal may not associate with some factor(s) that the SV40 late polyadenylation signal does. Or if precursor RNA did assemble into a B-like complex but was rapidly converted into product, then the formation of complex B would be short lived and perhaps nondetectable by the native gel system. Alternatively, the L3 B-like complex may not be very stable and may fall apart during electrophoresis. The last explanation seemed to be easily testable. One of the components of the polyadenylation reaction added to repress binding of nonspecific proteins was a random polymer of adenosine, cytosine and
Figure 16: Poly(ACU) Destabilizes Complex B of L3

Precursor RNAs SVL, L3, and L3/SVL were incubated in the nuclear extract in the presence of increasing amounts of poly(ACU). Complex assembly was analyzed by RNP gel electrophoresis. The complexes A and B are indicated.
poly (ACU)

1 mg/ml  3 mg/ml  5 mg/ml

SVL  L3  L3/SVL  SVL  L3  L3/SVL  SVL  L3  L3/SVL

B

A
uracil. It seems possible that the poly(ACU) was destabilizing the B complex of L3. A comparison of complex assembly between SV40 late, L3, and L3/SVL RNAs in the presence of increasing amounts of poly (ACU) was carried out (fig. 16). At low concentrations of poly(ACU), all three RNAs produced complex B. When the concentration was increased, complex B was no longer seen with the L3 RNA; however, both SV40 late and L3/SVL RNAs continued to produce observable complex B. From this experiment we concluded that L3 RNA does assemble into a B-like complex, but that the stability of the L3 complex B is greatly reduced in comparison to the B complex generated by SV40 late RNA or the chimeric RNA.

All of the previous experiments done with precursor L3 RNA were repeated under low ACU conditions to facilitate the detection of complex B. A time course of assembly under ATP or 3' dATP conditions is shown in figure 17. In the absence of poly(ACU), L3 RNA formed complex B by 10 minutes. The observed complex B persisted throughout the reaction. The results of complex formation using L35', RNA indicated that formation of complex B was dependent upon the presence of L3 downstream sequences as was the case for the SV40 late RNA (data not shown). A point mutation in the hexanucleotide, a U to C transition, was used to examine the requirements of complex B for an intact AAUAAA. This mutant RNA failed to show any formation for complex B, as compared to wild type L3 (fig. 18A). This mutant does not undergo any detectable level of
Figure 17: Assembly of L3 Polyadenylation Complexes in the Absence of Poly(ACU)

Polyadenylation reactions devoid of poly(ACU), containing ATP or 3'-dATP, and the precursor RNA L3 were terminated at the indicated times and analyzed by RNP gel electrophoresis. The position of complexes A, B, and C are indicated.
Figure 18: Complex B Formation Requires the Hexanucleotide AAUAAA

Polyadenylation reactions containing ATP, using precursor RNAs CML3 or L3AAGAAA, were terminated at the indicated times (minutes) and analyzed by RNP gel electrophoresis (A). The positions of complexes A, B, and C are indicated. (B) RNA from the reactions was purified and analyzed by denaturing polyacrylamide gel electrophoresis. The position of the precursor RNAs and the polyadenylated product are indicated.
polyadenylation in vitro (fig. 18B).

**SnRNP Association with Polyadenylation Substrate RNAs**

**Immunoprecipitation of snRNPs Associated with SV40 Late and L3 RNAs**

It has been demonstrated that cleavage and polyadenylation are inhibited in vitro by anti-Sm antibodies directed against the family of snRNPs involved in the splicing of pre-mRNAs (Moore and Sharp, 1984; Moore and Sharp, 1985). Polyadenylation is also inhibited by antisera against U1 snRNPs. The required polyadenylation signal AAUAAA associates with a factor that can be immunoprecipitated by snRNP-specific antibodies (Hashimoto and Steitz, 1986). It is possible that snRNPs may associate with precursor RNAs to make up part of the polyadenylation complex. The involvement of snRNPs in premessenger RNA splicing has been well documented (Kramer et al., 1984; Padgett et al., 1984; Black et al., 1985; Krainer and Maniatis, 1985; Berget and Robberson, 1986; Black and Steitz, 1986). To examine the association of snRNPs with polyadenylation precursor RNAs, immunoprecipitation experiments were performed. Radiolabeled RNA was incubated in the extract under conditions optimal for polyadenylation. Aliquots from the reaction were removed at 0, 5, and 90 minutes. Antibodies recognizing the whole class of U
snRNPs (Sm antibody), U1 snRNPs (RNP antibody), or a control IgG fraction were added to the reaction mixture. The antibody-antigen complexes were precipitated by the addition of pansorbin. The radiolabeled RNA associated with the complexes was isolated and analyzed on a denaturing acrylamide gel. The immunoprecipitation reactions were first done under standard polyadenylation conditions including 3% polyethylene glycol (PEG). The RNP antibody that is specific for U1 snRNPs coimmunoprecipitated other snRNPs when PEG was present in the reaction (data not shown). To avoid this problem, PEG was omitted from subsequent immunoprecipitation experiments. Both SV40 late and L3 precursor RNAs rapidly associated with both the Sm protein determinant and the U1 snRNP-specific polypeptide (fig. 19A and 19B). The association was maximal very early in the polyadenylation reaction, and decreased later, with only trace amounts of RNA immunoprecipitating by 90 minutes. The loss of immunoprecipitability with incubation was concomitant with the appearance of polyadenylated RNA, suggesting that snRNP interaction with RNA ceased after the RNA was processed. No polyadenylated RNA appeared to be associated with snRNPs in the in vitro reaction. The specificity of the antibodies was illustrated by gel electrophoretic display of the small nuclear RNAs contained in the immunoprecipitates (fig. 19C). The RNP antibody brought down U1 and U1* RNA (U1 star is a truncated version of U1 with no currently known function). The Sm antibody brought down U1, U2, U4, U6 snRNAs. The tRNA present
Figure 19: SnRNPs Associate With Polyadenylation Precursor RNAs

Early in the Polyadenylation Reaction

Polyadenylation reactions containing ATP were incubated with the (A) SV40 late or (B) L3 polyadenylation precursor RNAs. Aliquots were removed at 0, 5, and 90 minutes and either a control IgG fraction (C), SM (S), or RNP (R) antibodies were added. The antibody-antigen complexes were precipitated by addition of pansorbin. The RNAs were eluted from the complexes and analyzed by denaturing polyacrylamide gel electrophoresis. The gels were stained with silver to visualize the small nuclear RNAs. The silver stained gel (C) was identical for both poly(A) sites used. U RNAs are identified in panel C.
in the Sm and RNP lanes was also seen in the control sera lanes and is the level of background observed for these experiments.

**Sensitivity of Polyadenylation to U RNA Cleavage**

Previous experiments indicated that in vitro polyadenylation was not inhibited by oligonucleotide-directed cleavage of U1, U2, U4, or U6 RNAs under conditions that effectively inhibit in vitro splicing (Berget and Robberson, 1986; Black and Steitz, 1986). However, previous experiments were performed in the presence of polyethylene glycol or polyvinyl alcohol, reagents that enhance in vitro polyadenylation activity. Certain poly(A) sites are active enough to be assayed in vitro in the absence of such reagents. The U RNA cleavages were repeated under conditions lacking PEG to investigate further the requirements of U snRNPs during polyadenylation. The technique of oligonucleotide directed RNase H cleavage allows one to selectively remove a portion of a specific U RNA without perturbing the extract in any other fashion. The utilized oligonucleotides and the U RNA sequences they target are diagrammed in figure 6. U RNA cleavage was efficient to greater than 95% as judged by the silver staining of the U RNAs in the extract (fig. 20). After U RNA cleavage, polyadenylation was initiated by the addition of radiolabeled polyadenylation precursor RNAs containing the SV40 late, adenovirus L3, or adenovirus E2A poly (A) site. Polyadenylation occurs by a two
Figure 20: U RNA Oligonucleotide-mediated Cleavage

Acrylamide gel analysis of U RNA cleavages. The gel is stained with silver. The oligonucleotide used are indicated, with C being a control oligonucleotide. Intact U RNAs are indicated.
step mechanism in which cleavage at the site of (A)-addition precedes and is rapidly followed by the addition of adenylate residues to the new 3' terminus (Moore and Sharp, 1984; Moore and Sharp, 1985; Zarkower et al., 1986; Sheets et al., 1987). The two steps responded differently to cleavage of individual U RNAs with some U RNA cleavages inhibiting both steps whereas others only inhibited cleavage. All cleavages of U1, U2, or U4 inhibited polyadenylation cleavage of SV40 late precursor RNA as judged by the relative amounts of cleaved versus uncleaved substrate RNA (fig. 21A). Cleavage of U1 RNA was more inhibitory than cleavage of U2 or U4 RNA. None of the U RNA cleavages substantially affected the level of radiolabeled RNA observed at the end of the reaction. Poly(A)-addition was inhibited by internal cleavage of U2 RNA and 5' proximal cleavage of U4 RNA (fig. 21B). The other cleavages of U2 or U4 had no effect on ((A))-addition; neither did cleavage of U1 RNA. The level of poly(A) addition to SVL5' (a precleaved SV40 late precursor RNA truncated near the normal cleavage site which undergoes end-poly(A)-addition with high efficiency) was reduced following internal U2 cleavage or 5' U4 cleavage (fig. 21D), the same U RNA requirement as that for poly(A)-addition of uncleaved precursor RNA (fig. 21B). Therefore, both in vitro cleavage and (A)-addition of SV40 late RNA were sensitive to cleavage of U RNAs, although the two steps exhibited different requirements for individual U RNAs.

To determine the reason for the difference between the
**Figure 21: Effect of U RNA Cleavage on In Vitro Polyadenylation of SV40 late Precursor RNA**

In vitro synthesized and labeled SV40 late precursor RNAs of the structures diagrammed were incubated in in vitro polyadenylation reaction that had been subjected to the indicated U RNA cleavages prior to addition of substrate. (A) Effect of U RNA cleavage on polyadenylation cleavage of full length SVL RNA, (B) effect of U RNA cleavage on poly(A)-addition of truncated SVL₅, RNA, (C) effect of U RNA cleavage on polyadenylation cleavage of full length SVL RNA in the presence of 3% PEG, and (D) effect of U RNA cleavage on poly(A)-addition of full length SVL RNA. Precursor [Pre], poly(A)+ [A+]. and mapped cleaved RNA [Cleav.] are indicated. Reaction RNAs were isolated and displayed directly on acrylamide gels to examine total RNA (B and D) or subjected to hybridization to complementary DNA and subsequent ribonuclease T2 digestion (Moore and Sharp, 1984; Moore and Sharp, 1985) prior to gel electrophoresis to examine cleavage activity (A and C). In panel C, PEG to a final concentration of 3% was added to the reaction after U RNA cleavage but before substrate addition.
results in figure 21A and those reported earlier for other poly(A) sites, cleavages were repeated under previously used assay conditions. Following U RNA cleavage and prior to the addition of radiolabeled precursor RNA, PEG was added to 3%. Under these conditions, none of the U RNA cleavages significantly inhibited cleavage of SV40 late precursor RNA (fig. 21C). The factor concentration affected by the addition of PEG must bypass the sensitivity to individual U RNA cleavage during in vitro polyadenylation. We interpret this result to indicate that the residual snRNP structure remaining after U RNA cleavage, functions in the absence of the removed RNA sequences. In this context, the removed sequences would be considered as stimulatory, but not absolutely necessary, for processing activity.

Cleavage of U RNAs also affected polyadenylation of adenovirus L3 precursor RNA, although the cleavage response pattern was different than that of SV40 late RNA. Cleavage of U1 RNA had no effect on either cleavage (fig. 22Aa) or poly(A)-addition (fig. 22B) of L3. Therefore, L3 and SV40 late precursor RNAs exhibited different requirements for U1 RNA during in vitro polyadenylation. Neither 5'proximal or internal cleavage of U2 RNA was markedly inhibitory to polyadenylation cleavage although substrate RNA was more sensitive to degradation following cleavage (fig. 22A and 22B). In contrast, both internal and 5' cleavage of U4 RNA had an effect on polyadenylation of L3 RNA. Removal of the 5' 15 nucleotides of
Figure 22: Effect of U RNA Cleavage on In Vitro Polyadenylation of adenovirus 2 L3 RNA

Adenovirus 2 L3 RNAs of the diagrammed structures were assayed for various activities following U RNA cleavage as described in figure 21. (A) Effect of U RNA cleavage on polyadenylation cleavage of full-length L3 RNA, (B) effect of U RNA cleavage on (A)-addition of full-length L3 RNA, and (C) effect of U RNA cleavage on (A)-addition of truncated L35, RNA. Precursor, poly(A)+, and mapped cleaved RNA are indicated. Equal amounts of L3 RNA were used in each reaction; varying signal strength reflects differential stability as discussed in the text. The additional band in panels A and B in the U4L lanes resulted from cleavage of substrate due to partial complimentarity between the U4L oligonucleotide and L3 RNA.
U4 RNA strongly inhibited polyadenylation cleavage as judged by the ratio of cleaved to uncleaved RNA. In addition, L3 substrate survival severely decreased following 5' U4 cleavage. We interpret this susceptibility to suggest that L3 RNA is not assembled into stable polyadenylation complexes following U4 5' terminal cleavage, resulting in substrate degradation. Internal cleavage of U4 RNA did not inhibit L3 cleavage or poly(A)-addition. This cleavage stimulated accumulation of poly(A)+ RNA. This, internal cleavage of U4 RNA, in contrast to 5' cleavage, stimulated the polyadenylation process for L3 RNA.

The effect of U RNA cleavage on poly(A)-addition of L3 RNA was also examined using an L3 precursor RNA truncated near the cleavage site (L35'). L35'RNA undergoes efficient end-adenylation in vitro. Internal U2 or 5' proximal U4 cleavage reduced the level of surviving polyadenylated RNA (fig. 22C). Internal U4 cleavage stimulated the level of observed poly(A)+ RNA. Therefore, poly(A)-addition of L3 5' responded to U RNA cleavage similarly to full length L3 and SV40 late precursor RNAs. Thus, all poly(A)-addition reactions were affected by the same U RNA cleavages. In contrast, polyadenylation cleavage responded to U RNA cleavage in a site specific fashion. In this context, it should be noted that most of the sequence elements that distinguish one poly(A) site from another lie downstream of the cleavage site and are absent during the poly(A)-addition step of polyadenylation.

The requirement of U RNAs for polyadenylation of adenovirus
E2A precursor RNA was also examined. Cleavage, poly(A)-addition, and substrate stability were dependent upon intact U RNAs. The cleavage response pattern and substrate stability were identical to that of adenovirus L3 RNA (data not shown).

**Effect of U RNA Cleavage on In Vitro Assembly of Polyadenylation Specific Complex**

In vitro assembly of splicing complexes is dependent upon U RNAs in the nuclear extract (Zillmann 1987). We therefore wanted to determine if the in vitro formation of polyadenylation specific complexes was dependent on intact U RNAs. The nuclear extract was depleted of U RNAs by oligonucleotide-directed RNase H cleavage. Radiolabeled SV40 late RNA was incubated in the U RNA-depleted extract and assembly was analyzed on a 4% non-denaturing acrylamide gel (fig. 23). Addition of a control oligonucleotide failed to have any effect on assembly of complex B. Cleaving U1 at the 5' terminus inhibited the formation of complex B. Cleavage of U2 at the 5' terminus had very little effect on assembly of complexes migrating in the region of the gel containing B. The mobility of the observed complex B after U2 cleavage, however, appeared greater than the mobility of the complex B in lanes 1 and 2. Internal cleavage of U2 as well as removal of the 5' terminal nucleotides of U4 had a very dramatic effect on assembly of complex B. Very little complex B was
Figure 23: Effect of U RNA Cleavage on In Vitro Assembly of Polyadenylation Specific Complexes

Oligonucleotides complimentary to targeted U RNAs were added to the nuclear extract in the presence or absence of Mg++. SV40 late and L3 RNAs were assayed for complex formation 60 minutes following U RNA cleavage. Equivalent amounts of RNA were used in each reaction. Each reaction was analyzed for complex formation by RNP gel electrophoresis.
formed following these cleavages; the majority of the RNA resided in the region of the gel containing complex A. Internal cleavage of U4 RNA had an effect similar to the 5' cleavage of U2 RNA. Complex B formed, but had increased mobility.

A second set of control reactions were carried out to insure that the oligonucleotides themselves don’t interfere with assembly. A mock reaction was performed by incubating the extract with oligonucleotides in the presence of 0.5 mM EDTA to inhibit RNase H activity (fig. 23). RNase H activity is inhibited in the absence of Mg++, but polyadenylation cleavage and complex assembly are not (Zarkower and Wickens, 1987). The degree of complex B formation was constant in the presence of all oligonucleotides, indicating that oligonucleotide-mediated inhibition of assembly required U RNA cleavage. The mobility of complex B appeared to be slightly increased when the U4 internal oligonucleotide had been added. Analysis of the U RNAs at the end of the reaction indicated that a significant portion of the U4 RNA has been cleaved in the presence of the U4 internal oligonucleotide despite the presence of EDTA (data not shown).

Assembly reactions were carried out with RNA containing the L3 polyadenylation signal to examine L3 requirements for U RNAs (fig. 23). The effects on assembly were similar to those of the SV40 late RNA. Cleavage of U1 and U2 RNA at their 5' termini decreased formation of complex B, although the inhibition was slight. Internal U2 cleavage or 5'-proximal cleavage of U4 RNA depressed the amount of observed complex B. Interestingly, the
amount of radiolabeled complex present at the end of the reaction was greatly reduced following internal cleavage of U2 RNA or 5' proximal cleavage of U4 RNA similar to the case when the level of cleaved and adenylated L3 was measured in previous experiments. Once again, the level of L3 complex surviving was enhanced following internal cleavage of U4 internal cleavage. When EDTA was included in the reaction mixture, to inhibit RNase H, loss of L3 complex was no longer observed, indicating that the oligonucleotides themselves were not responsible for the destabilization effects. The level of complex B was fairly constant regardless of the identity of the added oligonucleotide.

Analysis of Splicing/Polyadenylation Chimeric RNAs

In higher eucaryotes, most polyadenylated RNAs are also spliced. Most precursor RNAs, therefore, contain multiple processing signals including 5' and 3' splice sites. In order to more closely mimic the in vivo situation, chimeric splicing/polyadenylation substrates were constructed and tested for their ability to function in vitro.

The chimeric substrates MXSVL and MXL3 were incubated in the nuclear extract in the presence of ATP or 3'-dATP, aliquots were removed from the reaction at 0, 10, and 45 minutes, and the RNA products were analyzed on a denaturing gel (fig. 24). Three species of processed RNA were detected in the presence of
Figure 24: Analysis of Splicing/Polyadenylation Chimeric RNAs

Precursor RNAs, containing both splicing and polyadenylation signals, MXSVL and MXL3 were incubated in the nuclear extract in the presence of ATP or 3'-dATP and aliquots of the reactions were taken at indicated time points. The partial and fully processed RNAs were analyzed by denaturing gel electrophoresis. The various RNA products and their lengths are diagrammed schematically. The filled boxes represent exon sequences, the thin line intron sequences and open boxes represent polyadenylation sequences.
3'-dATP; unspliced-cleaved and adenylated RNA (S-A+)
spliced-noncleaved RNA (S+A-) and spliced-cleaved and adenylated
(S+A+). The designation of RNA species is based on migration
compared to DNA size markers and the disappearance of the
polyadenylation cleavage bands in the presence of ATP. Any RNA
containing an AAUAAA polyadenylation signal near the 3' terminus
is competent for end-adenylation. Therefore, all three product
species, A-S+, A+S-, A+S+, should appear as poly(A)+ RNAs in the
presence of ATP. The unspliced-cleaved and adenylated form
(S-A+) was first observed at 10 minutes. Appearance of the
spliced-noncleaved (S+A-), and the spliced-cleaved and
adenylated (S+A+) RNAs was not observed until later times.
These kinetics suggest that polyadenylation can precede splicing
in a chimeric substrate RNA in vitro, which is in agreement with
in vivo experimental results (Nevins and Darnell, 1978).

Interestingly, chimeric substrates containing the SV40 late
or adenovirus L3 poly(A) sites exhibited different efficiencies
of splicing and polyadenylation and sensitivities to nucleases.
The substrate MXSVL accumulated a large amount of the
spliced-noncleaved (S+A-) RNA species, whereas the MXL3
substrate was more efficient at converting precursor RNA into
fully processed (S+A+) RNA. Conversely, the MXL3 substrate RNA
was more susceptible to nucleases in the extract. Greater than
50% of the input RNA was degraded using MXL3; little to no
degradation was observed using MXSVL. This difference in
nuclease sensitivity was similar to the situation observed using
precursor RNAs containing isolated SV40 late and L3 polyadenylation sites. L3 RNA was highly sensitive to nuclease degradation and the SV40 late RNA was not.

Optimization of Splicing/Polyadenylation In Vitro

The in vitro splicing and polyadenylation magnesium requirements have been previously determined for RNAs containing isolated poly(A) sites. To determine if the optimal reaction conditions were different for the chimeric RNAs, the substrates MXSVL, SVL, MXL3, and L3 were analyzed under varying MgCl₂ concentrations (fig 25). The reactions were carried out in the presence of 3'dATP in order to visualize the cleaved and singly-adenylated polyadenylation products. The previously reported optimal MgCl₂ concentration for splicing was 2.5 mM (Hardy et al., 1984; Zillmann, Ph.D. thesis 1987) with higher levels being strongly inhibitory. The poly(A) cleavage reaction for RNAs containing the L3 or SV40 late polyadenylation signals were reported to be efficient in the absence of MgCl₂, although concentrations up to 0.7 mM were not inhibitory (Moore and Sharp, 1985; Zarkower et al., 1986). The substrate MXSVL exhibited a broad MgCl₂ optima for splicing, ranging from 0.6 mM to 2.7 mM (fig. 25). The poly(A) cleavage activity also exhibited a broad optima, with the peak of activity seen in the range of 0.9 to 1.5 mM MgCl₂. RNA containing the SV40 late polyadenylation signal showed an identical MgCl₂ optima,
Figure 25: Optimization of Splicing/Polyadenylation Reaction In Vitro

Polyadenylation reactions were performed in the presence of 3'-dATP in order to visualize the cleaved and singly adenylated polyadenylation products. Increasing amounts of MgCl₂ (0.0 mM to 2.7 mM) were added to the polyadenylation reaction containing either the chimeric splicing/polyadenylation substrates MXSVL and MXL3 or the polyadenylation substrates SVL and L3. The reaction intermediates and products were analyzed by denaturing polyacrylamide gel electrophoresis. The intermediates, products and their corresponding lengths in nucleotides are depicted schematically.
between 0.9 to 1.5 mM (fig. 25). The substrate MXSVL was more efficient at polyadenylation cleavage and A-addition than was the substrate containing the SV40 late site by itself.

The MgCl₂ optimum for the substrate MXL3 was similar to that of MXSVL. Spliced-uncleaved (S+A-) and spliced-cleaved-adenylated (S+A+) RNAs were detected at MgCl₂ concentrations between 0.3 mM and 2.7 mM (fig. 25). The unspliced-cleaved-adenylated (S-A+) RNA was first detected in the absence of MgCl₂. Fully processed RNA was observed at concentrations from 0.3 to 2.7 mM MgCl₂. L3 and MXL3 RNAs were processed at equal efficiency over the entire magnesium range tested (fig. 25). Both MXL3 and L3 were susceptible to nuclease degradation at high MgCl₂ concentrations, although the substrate MXL3 was less sensitive than the substrate L3.

Effect of U RNA Cleavage On Polyadenylation Using Chimeric Splicing/Polyadenylation Substrates

Cleavage of individual U RNAs has well documented effects on splicing activity. The effect of U RNA cleavage on combination splicing/polyadenylation substrates has never been examined in vitro. It was conceivable that creating a second exon that contained both splicing and polyadenylation signals might alter the response of the chimeric substrate to U RNA cleavage. To test this possibility, chimeric substrates were examined for their sensitivity to U2 and U4 RNA cleavage (fig.
Figure 26: Effect of U RNA Cleavage on In Vitro Polyadenylation Using Chimeric Splicing/Polyadenylation Substrates

Radiolabeled chimeric splicing/polyadenylation substrates MXL3 and MXSFL were incubated under optimal processing conditions (1.2 mM MgCl₂, 1 mM 3'-dATP) in the nuclear extract, that had been subjected to the indicated U RNA cleavages. Partially and fully processed RNAs were analyzed on denaturing polyacrylamide gels. The various forms of the RNAs and their length in nucleotides are indicated schematically.
26). To examine both splicing and poly(A) cleavage (but not poly(A) addition), 3'd-ATP was used in place of ATP. Both 5' proximal and internal cleavages of U2 and U4 were inhibitory to splicing as well as to poly(A) cleavage with the substrate RNA MXSVL. The levels of inhibition varied, but the degree of inhibition was similar for both splicing and poly(A) cleavage. This pattern was identical to that observed using precursor RNA containing the isolated SV40 late site.

U RNA cleavage had an interesting effect on the second chimeric substrate, MXL3 (fig. 26). Polyadenylation of MXL3 was only marginally sensitive to internal cleavage of U2 and U4 RNA under conditions that completely inhibited splicing. 5' proximal cleavages of U2 and U4 RNA were more inhibitory. This is a different pattern than previously observed with the isolated L3 poly(A) site (fig. 22A). Cleavage of U4 RNA within its 5' end inhibited polyadenylation cleavage at the L3 site regardless of whether this site was alone or downstream of an intron. In contrast, cleavage of U2 RNA did not inhibit poly(A) cleavage of an isolated polyadenylation precursor RNA but did inhibit polyadenylation cleavage of the chimeric RNA, suggesting that the presence of an upstream intron necessitated U2 involvement in processing.

The instability of isolated L3 poly(A) precursor RNA observed following U RNA cleavage was not observed with the chimeric substrate. Therefore, the presence of an upstream intron permitted the formation of stable assemblies of the
chimeric RNAs even in the absence of functional U RNAs. In general, regardless of the structure of precursor RNA, substrate RNAs containing the SV40 late poly(A) site exhibited a greater sensitivity to U RNA cleavage than did RNA containing the L3 poly(A) site, suggesting site-specific interactions with U snRNPs and the nuclear factors associated with them.
DISCUSSION

Certain sequence features are common to all polyadenylation sites, primarily the hexanucleotide AAUAAA; however, differences also exist. Mutational analysis has defined additional elements in addition to AAUAAA necessary for polyadenylation. In different polyadenylation sites, these elements vary in sequence content and number. These differences may indicate classes of polyadenylation sites. Two experimental approaches were used to address the question of the existence of classes of polyadenylation sites. Both approaches yielded results that suggest classes of poly(A) sites exist.

The first approach utilized a nondenaturing polyacrylamide gel electrophoresis system to examine differences in the RNA-protein complexes formed by different precursor RNAs. Polyadenylation-specific complexes were generated in vitro with two different precursor RNAs containing either the SV40 late or adenovirus 2 L3 polyadenylation signals. The sequences required for assembly were determined and the stability of the respective complexes was examined. Precursor RNA containing the SV40 late poly(A) site assembled into a complex that was stable to the anion poly(ACU), whereas precursor RNA containing the L3 poly(A) site did not.

The second approach was to scrutinize the requirements for U RNAs in polyadenylation for each of the poly(A) sites. The
requirement for U RNAs was demonstrated by selectively removing portions of the U RNAs by the technique of oligonucleotide-directed RNase H cleavage. Intact U RNAs were required for both assembly of polyadenylation specific complexes and efficient polyadenylation activity; however, the requirements for U RNAs of the two polyadenylation sites differed.

Detection of Polyadenylation Specific Complexes

Using nondenaturing polyacrylamide-agarose composite gel electrophoresis, polyadenylation specific complexes for RNAs containing the SV40 late and L3 poly(A) sites were resolved. The SV40 late RNA assembled into several complexes, denoted B, A, and C. B complex formation required the hexanucleotide AAUAAA and downstream sequence information. These results are in close agreement to those of Zarkower and Wickens (Zarkower and Wickens, 1987). Using a similar gel system and RNA containing the SV40 late poly(A) site, they also detected a specific complex that required the conserved sequence AAUAAA and sequences downstream of the polyadenylation cleavage site. Several lines of evidence support the idea that complex B is the pre-cleavage complex formed between the precursor RNA and the nuclear factors required for polyadenylation cleavage. Complex B formation is detected prior to polyadenylation cleavage activity and contains precursor RNA only. Complex B formation
is not detected with a precursor RNA containing bacterial sequences. The formation of complex B is dependent on the hexanucleotide AAUAAA and requires specific sequences downstream of the polyadenylation cleavage site. Replacement of downstream sequences with nonspecific bacterial sequences fails to restore complex B formation, demonstrating the need for downstream sequence elements.

Complex C is clearly the product complex. C contains polyadenylated RNA. The formation of complex C would be predicted to only be dependent on the AAUAAA sequence and not on the presence of downstream sequences. All RNAs lose their downstream sequences once they have been cleaved. Experimental results indicate that C is not dependent on sequences downstream of the cleavage site, as precursor RNAs truncated just prior to the cleavage site still undergo end (A)-addition and assembly into complex C.

If the sequences specifying classes of poly(A) sites were downstream of the cleavage site, then detectable differences between poly(A) sites would be expected to be observed in the B complex. Under conditions identical to those used for assembly of SV40 late precursor RNA, precursor RNA containing the L3 poly(A) site did not form a stable B complex. Initial experiments only detected the non-specific complex A and the product complex C. These results were in contrast to those of Humphrey et al. (Humphrey et al., 1987). Humphrey reported the presence of a complex similar to B when a precursor RNA
containing the L3 polyadenylation site was assembled. Their polyadenylation reactions did not contain poly(ACU). Repeating our earlier experiments in the absence of poly(ACU), complex B formation could be detected with the L3 precursor RNA. Comparing complex B formation, in the presence of increasing amounts of poly(ACU); with SV40 late, L3, and a chimeric poly(A) site (consisting of the hexanucleotide and cleavage site of L3 fused to the downstream sequences of SV40 late), clearly showed an instability of complex B when it was formed with the L3 precursor RNA. The stability of complex B was enhanced when the downstream sequences of SV40 late site replaced those from the L3 site. We conclude from these observations that the interactions between nuclear factors and L3 downstream sequences are weaker than those of SV40 late. This may reflect different proteins or small nuclear RNAs recognizing differential signals unique to one class of poly(A) sites. In this case, the SV40 late site has additional signals that are lacking in the L3 polyadenylation site.

Association of Small Nuclear Ribonucleoproteins with Polyadenylation Precursor RNAs

Small nuclear ribonucleoproteins have been shown to be essential for several RNA processing events including splicing and histone 3' end maturation (Kramer et al., 1984; Chabot et al., 1985; Mount et al., 1983; Berget and Robberson, 1986; Mowry
and Steitz, 1987; Ruskin and Green, 1985). However, their involvement in polyadenylation is unclear. Several laboratories have documented inhibition of in vitro polyadenylation by anti-Sm (antibody directed against the whole class of snRNPs), and anti-RNP antibodies (U1 snRNP specific), (Moore and Sharp, 1984; Moore and Sharp, 1985; Sperry and Berget, 1986). We were able to immunoprecipitate antibody-antigen complexes that were associated with polyadenylation signal containing precursor RNAs. Antibodies recognizing the whole class of snRNPs (Sm antibody) or U1 snRNPs (RNP antibody) gave identical results. The snRNP association was maximal early in the reaction, with decreased association at later times. These results are consistent with those of Hashimoto and Steitz who reported a small nuclear ribonucleoprotein associated with the AAUAAA polyadenylation signal in vitro (Hashimoto and Steitz, 1986). They reported a strong initial association of a snRNP with polyadenylation precursor RNAs, with a rapid decrease in association as the polyadenylation reaction progressed.

We interpret these data to suggest that snRNPs are associated with precursor RNA during the early substrate recognition events preceding polyadenylation. In this light, in vitro antibody inhibition data may reflect an interference of the association of snRNPs with the precursor RNA, with this early association being important for efficient RNA processing. This interpretation is further strengthened by the results from our oligonucleotide directed RNase H cleavage experiments.
Cleavage of U RNAs U1, U2, and U4 inhibited the formation of complex B with both SV40 late and L3 RNAs. If snRNPs were required after the formation of the pre-cleavage complex, then complex B formation should not be affected by U RNA cleavage, yet it clearly is. This further suggest that the U RNAs are an integral part of the polyadenylation pre-cleavage complex.

**Differential Requirements for snRNAs in In Vitro Polyadenylation**

The results from the U RNA cleavage studies directly contradicts previous studies that indicated U1, U2, and U4 were not required for in vitro polyadenylation (Berget and Robberson, 1986; Black et al., 1986). In the previous studies, one of the reagents used to enhance the level of polyadenylation was polyethylene glycol 5000 (PEG). We noticed that inclusion of PEG contributed to artifactual results in certain experiments. Because of this, our U RNA cleavage studies were performed in the absence of PEG. Table 1 summarizes the effects of U RNA cleavage on polyadenylation. The inhibition of polyadenylation cleavage seen was reversed by addition of PEG to the oligonucleotide directed RNase H cleavage reactions. The two poly(A) sites, SV40 late and L3, exhibited different sensitivities to U RNA cleavage. The polyadenylation cleavage step for SV40 late was inhibited by all U RNA cleavages. The poly(A)-addition step was inhibited only by internal cleavages of U2 and U4. The L3 poly(A) site showed a different pattern of
Table 1: Summary of the Effect of U RNA Cleavage On Polyadenylation

The effects of U RNA cleavage on polyadenylation is summarized. A plus indicates inhibition of that activity by U RNA cleavage, a minus indicates no inhibition.
Table 1. Effect of U RNA Cleavage on Polyadenylation

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<thead>
<tr>
<th>Property</th>
<th>U15⁺</th>
<th>U25⁺</th>
<th>U2L</th>
<th>U45⁻</th>
<th>U4L</th>
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<td>Cleavage Activity(^a)</td>
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<td>Adeno. E2A</td>
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<td>Poly(A)+ RNA(^b)</td>
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<td>SV40 late</td>
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<td>Adeno. E2A</td>
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<td>RNA Survival(^d)</td>
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<td>SV40 late</td>
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+ equivalent to that observed using a control oligonucleotide; - less than control; ++ greater than control.

\(^a\)based on the relative amount of cleaved versus uncleaved RNA remaining at 90 min

\(^b\)based on the absolute amount of poly(A)+ RNA remaining after 90 min

\(^c\)considerable substrate instability under these conditions

\(^d\)based on the total amount of radiolabeled RNA remaining after 90 min
sensitivity to U RNA cleavage. The L3 poly(A) site was only
sensitive to cleavage of U4 RNA at the 5' proximal end.
Cleavage of U4 internally stimulated polyadenylation activity,
an oligonucleotide mediated response that was unique for L3.
The L3 RNA also showed a startling sensitivity to nucleases when
U2 RNA was cleaved or when U4 RNA was cleaved at the 5' proximal
region. This nuclease sensitivity is not observed for the SV40
late site under identical conditions. The nuclease sensitivity
for L3 partially reflects the inability of U RNA cleaved extract
to complex the RNA to nuclear factors. Since the SV40 late site
does not appear to be as sensitive to nucleases even in the
presence of U RNA cleaved extract, there must be inherent
differences in the two RNAs that contribute to their respective
stabilities.

The differences in sensitivities to U RNA cleavages were
altered, but not abolished when chimeric
splicing/polyadenylation substrates were utilized. MXL3 was
inhibited by 5' proximal U RNA cleavages of U2 and U4. MXL3 was
not inhibited by internal cleavage of U2 and U4. MXSVL was
inhibited by all cleavages of U2 and U4, although the degree of
inhibition varied. One of the prominent differences in U RNA
cleavage sensitivities between MXL3 and MXSVL was the total
inhibition of MXSVL by the internal U4 cleavage; whereas MXL3
was not inhibited by internal U4 cleavage.

We interpret these results as indicating the snRNPs do play
a role in in vitro polyadenylation most likely at the packaging
or complex assembly level. Experimental results from nuclear extract fractionation studies suggest that the major snRNPs are not the catalytic components of polyadenylation (Takagaki et al., 1988). SnRNPs most likely play ancillary roles.

The experimental results based on RNase T1 protection (Hashimoto and Steitz, 1986), complex assembly, and U RNA cleavage data, all suggest that polyadenylation sites are not identical and different types or classes of polyadenylation sites exist.
References


Appendix: Publications

Papers


Abstracts

