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ANALYSIS OF HELA CELL PREMESSENGER RNA SPICING COMPLEXES CONTAINING THE SNRNP U1 BY NATIVE GEL ELECTROPHORESIS

by

MARTIN ZILLMANN

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

DOCTOR OF PHILOSOPHY

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August, 1987
ANALYSIS OF HELEA CELL PREMESSENGER RNA SPlicing COMPLEXES
CONTAINING THE SNRNP U1 BY NATIVE GEL ELECTROPHORESIS

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Martin Zillmann

ABSTRACT

The typical eukaryotic RNA polymerase II primary transcript is divided into regions that encode information expressed at the protein level (exons) and those which do not (introns). The latter must be removed from the transcript rapidly and with proper joining of the coding sequences during the maturation of the transcript in the nucleus. This process is termed splicing and is accompanied by the sequential addition of factors to the primary transcript resulting in the formation of a series of large ribonucleoprotein particles. The splicing reaction can be studied in vitro in HeLa cell nuclear extracts by the addition of a capped, in vitro transcribed splicing precursor RNA. A native gel electrophoresis system was developed which allowed resolution of various ribonucleoprotein complexes and the study of the splicing complex and intermediates in its formation.

The HeLa cell nuclear extracts were found to immediately assemble exogenously added precursor RNAs into rapidly migrating complexes. With time, complexes migrating more slowly were observed. The complex migrating the most slowly appeared concurrently with the products of 5' junction cleavage. This complex was identified as the "active" splicing complex by the presence of reaction intermediates and the requirement for both ATP and splicing consensus sequences for its formation. Later in the reaction, when large amounts of ligated RNA had been generated, rapidly migrating complexes reappeared. All of these complexes contained U snRNPs as defined by immunoprecipitation of gel-fractionated complexes. In
particular, eluted active complex contained U1 snRNPs as defined by the ability of anti-U1 antiserum to immunoprecipitate this particle in the presence of competing free, unlabelled precursor RNA. Previously reported gel systems appear to resolve active complexes devoid of U1 snRNPs, a snRNP known to be required for splicing both \textit{in vivo} and \textit{in vitro}. Furthermore, the 5' splice junction, the region to which U1 snRNPs bind, was protected from oligonucleotide directed RNase H cleavage in eluted complexes, indicating that a 5' factor remained bound during electrophoresis. Apparently, complexes eluted from the native system retain most of the properties, other than activity, found for these complexes in whole extracts, suggesting that this gel system is an ideal tool for the study of the ribonucleoprotein complexes involved in splicing.
ACKNOWLEDGEMENTS

I wish to thank Dr. Berget for providing me with what I believe to be an excellent general background in the techniques of molecular biology and for bearing with me through the somewhat tough early years of my graduate career. I thank her also for teaching me the difference between doing experiments and conducting research. I would also like to acknowledge the friendship and help provided to me by my fellow lab-mates. Scott Rose taught me everything I know about cloning; Ann Sperry, Maria Zapp, and Barbara Robberson helped me both directly and through excellent suggestions.

I acknowledge the aid of my committee members, Dr. George Bennet, Dr. Kathleen Matthews, Dr. Steve Olson, and Dr. Joe Martin. I thank each of you for your suggestions and for bearing with me through several project changes.

Lastly, I want to thank my wife, Kathy, for providing moral support through the years and seeing me through to the final culmination of my work, especially since the end of my graduate career has been an ill-defined terminus.
ABBREVIATIONS

A: ampicillin
APS: ammonium persulfate
ATP: adenosine triphosphate
BIS: N, N'-methylenebisacrylamide
bp: base pairs (DNA)
C: cytidine
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
EGTA: ethyleneglycol bis(β-aminoethyl ether) N, N', N'-tetraacetic acid
exon: sequence in the precursor which is found in the mature message
G: guanosine
HEPES: N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
hnRNA: heterogeneous nuclear RNA
hnRNP: heterogeneous nuclear ribonucleoprotein
intron: intervening sequence removed upon precursor maturation
Kd: kilodalton
ul: microliter
LB: Luria broth
MOPS: 3-(N-morpholino)propanesulfonic acid
mRNA: messenger RNA
PEG: polyethylene glycol
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<td>RNA:</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase:</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNaSin:</td>
<td>commercial (Promega) RNase inhibitor</td>
</tr>
<tr>
<td>RNP:</td>
<td>ribonucleoprotein</td>
</tr>
<tr>
<td>SDS:</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>snRNA:</td>
<td>small nuclear RNA</td>
</tr>
<tr>
<td>snRNP:</td>
<td>small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>TEMED:</td>
<td>N, N, N', N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TRIS:</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>tRNA:</td>
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<td>U:</td>
<td>uridine</td>
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INTRODUCTION

The discovery that many eukaryotic genes are not continuous but are interrupted by sequences which do not appear in the mature messenger RNA opened the doors to the field of RNA splicing. The term splicing has been coined to describe the rapid and accurate removal of extra sequences from the nascent transcript as it matures in the nucleus.

The preparation of in vitro extracts that could conduct this process (Hernandez and Keller, 1983; Krainer et al., 1984; Lin et al., 1985) and the ability to make large amounts of precursor RNAs in vitro (Contreras et al., 1982; Konarska et al., 1984) has led to the accumulation of an amazing wealth of information in the last six years. It has been discovered that for some RNA molecules splicing can occur in the absence of any macromolecular catalysts. These RNAs have been subdivided into groups based upon the differences in the intermediates of the reaction. Nascent transcripts produced by RNA polymerase II, however, require the presence of macromolecular catalysts for the removal of their intervening sequences. For example, it has been found that the ribonucleoprotein structure which conducts this reaction in vitro is structurally complex, consisting of five or more ribonucleoprotein subunits and additional protein factors. This complex has been termed the spliceosome. The spliceosome easily rivals the ribosome in complexity and may be very large in vivo. A single transcript has been found in an RNP structure sedimenting at 200S.

It is the purpose of these studies to examine both the in vitro assembly of a simple precursor RNA into active spliceosomes and the subsequent disassembly of these spliceosomes upon completion of catalysis.
LITERATURE REVIEW

The Splicing Reaction at the RNA Level

The coding sequences (exons) in many higher eukaryotic genes are disrupted by noncoding regions of variable size (introns). In order for a functional mRNA to be produced from such genes the noncoding portions must be removed. This process is termed splicing. Transcripts that contain introns fall into several classes depending on the details of the splicing reaction. Mitochondrial introns are divided between two groups that differ in whether a circular or branched intermediate is produced, but are alike in that intron removal is catalyzed by the RNA itself. Similarly the intron of the nuclear 26S ribosomal RNA of *Tetrahymena thermophila* is removed autocatalytically via a circular intermediate and has been studied extensively by Cech and coworkers (Cech *et al.*, 1981). Introns in tRNA are removed by a splicing endonuclease that has been highly purified from yeast (Peebles *et al.*, 1985). Of interest in the work reported here is the class of intron-containing transcripts that require trans-acting factors for intron removal. All intron-containing RNA polymerase II transcripts are in this class and all further discussions of splicing will be restricted to this class.

The development of *in vitro* splicing extracts (Dignam *et al.*, 1983; Hernandez and Keller, 1983; Krainer *et al.*, 1984) and *in vitro* transcription systems (Contreras *et al.*, 1982; Konarska *et al.*, 1984) has greatly advanced our understanding of the splicing reaction with respect to the machinery that catalyzes the process and with respect to the intermediates of the reaction itself. When a simple (two exon, one intron) *in vitro* synthesized precursor RNA is added to a splicing extract, it undergoes a two-step reaction as diagrammed in Figure 1 (Grabowski *et al.*, 1984; Ruskin *et al.*, 1984). In the first reaction, cleavage occurs at the 5' intron-exon boundary, resulting in a linear species.
containing exon 1 and a partially circular species (lariat) containing a novel 2' to 5' linkage of the intron's 5' terminal nucleotide to a site internal to the intron (branch point). In the second step, cleavage occurs at the 3' intron-exon boundary with ligation of the two exons to yield the product RNA. The intron is released as a lariat, but an activity exists in the cell that is capable of resolving this species into its linear form (Ruskin and Green, 1985a). It is apparent from this reaction scheme that the intermediates of the first reaction must not be able to freely diffuse if the second step of the reaction is to occur or if it is to occur without exon interchange between different RNAs undergoing simultaneous processing in the same location. Trans-splicing of RNAs has been demonstrated in vitro by two laboratories (Konarska et al., 1985; Solnick, 1985) and has just recently been demonstrated in vivo. A 22 nucleotide leader has been found spliced onto the 5' end of several C. elegans RNAs (Krause and Hirsh, 1987) in a fashion similar to that of the 35 nucleotide leader found on all Trypanosome RNAs (Murphy et al., 1986; Sutton and Boothroyd, 1986). The mRNA coding for the ribosomal protein rps12 in tobacco chloroplasts contains three exons, two of which are coded for by contiguous DNA sequences. The first exon is separated from the others by more than 100 kb of DNA and is joined via trans splicing (Koller et al., 1987). The mechanism by which trans-splicing occurs is poorly understood at present.

Nature has shown us that exon exchange is not prevented by spliceosome formation and splicing can occur for some RNAs in its absence. Exactly why has this very complicated system evolved to deal with intron removal from polII transcripts? Perhaps this system allows greater flexibility in the regulation of splicing for multi-intron RNAs. Numerous examples exist of RNAs that demonstrate tissue specific splicing. One of the more complicated alternately spliced RNAs is the transcript for troponin T, containing up to 17 exons (Breitbart and Nadal-Ginard, 1987). It has been postulated that spliceosomes prevent "exon-skipping" through their formation on the nascent transcript, thereby.
Figure 1: Mechanism of the Splicing Reaction at the RNA Level. The splicing of single intron mRNA precursors proceeds in two distinct steps. The RNA first undergoes cleavage at the 5' splice junction with simultaneous ligation of the free 5' phosphate to a hydroxyl group on an internal adenosine residue to give a 5' to 2' linkage. This type of structure has been termed a lariat. In a second step, the RNA is cleaved at the 3' junction and the exons are ligated with the release of the intron as a lariat. The first step of the reaction can be uncoupled from the second either by mutations in the RNA or fractionation of the splicing extracts. Cofactor requirements are also indicated.
committing neighboring junctions for cleavage and ligation. This has been termed the "first come, first serve" hypothesis (Aebi and Weissmann, 1987).
The Role of Macromolecular Factors in Splicing

As the in vitro splicing system was studied in greater detail, it became apparent that as the reaction proceeded, the exogenously added, radiolabelled RNA associated with factors that gave it a high sedimentation velocity upon gradient analysis. Furthermore, these factors gave rise to particles of discrete sizes. The term "spliceosome" was coined to describe the 40S (yeast) to 50S (mammalian) complex that formed with 400 to 500 nucleotide, single-intron RNAs that contained the reaction intermediates (Brody and Abelson, 1985; Grabowski et al., 1985).

It had long been known that pre-mRNA was associated with small nuclear ribonucleoproteins in vivo. U1 snRNA could be found base-paired to hnRNP in vivo (Calvet and Pederson, 1981) and other U RNAs could be found associated with it (Guimont-Ducamp et al., 1977). Antibodies produced against U1 snRNPs in patients with systemic lupus erythematosus (SLE) inhibit splicing when injected into frog oocytes (Bozzone et al., 1984; Fradin et al., 1984). These same antibodies also inhibit splicing in nuclear extracts (DiMaria et al., 1985; Kramer et al., 1985; Padgett et al., 1983; Yang et al., 1981). Cleavage of the RNA moiety of the U snRNPs also resulted in an inhibition of splicing for all of the U snRNPs cleaved successfully to date (Kramer et al., 1984; Krainer and Maniatis, 1985; Black et al., 1985; Berget and Robberson, 1986; Black and Steitz, 1986).

The association of snRNPs with precursor RNAs in vitro has been recently demonstrated directly in both mammalian and yeast extracts. By incorporating biotinylated nucleotides into a precursor RNA to permit recovery of assembled complexes through affinity chromatography on streptavidin, it was possible to affinity purify a reconstituted splicing complex containing
U2, U4/U6, and U5 snRNPs (Grabowski and Sharp, 1986). A recent study made use of substrate-dependent mobility shifts of extract snRNPs upon analysis in RNP gels to determine the snRNP composition of the spliceosome as well as to characterize snRNP/snRNP associations (Konarska and Sharp, 1987). Interestingly, the U1 snRNP was not found in the spliceosome by either approach and has not been found participating in any higher order assemblies by this group. A similar approach had been taken earlier to identify several yeast snRNAs associated with the yeast spliceosome (Pikielny and Rosbash, 1986). How these snRNAs are related to mammalian snRNAs is only known in some instances (Parker et al., 1987).

Another approach to assess snRNP involvement in splicing that has been used successfully in vitro has taken advantage of antibodies directed against snRNP polypeptides. U1 and U2 can be immunoprecipitated separately from the other snRNPs because they contain unique polypeptides (see below). Another commonly used antibody recognizes an antigen present on all snRNPs. It was found by several researchers that the intermediates of the reaction were immunoprecipitable with antibodies directed against U1 snRNPs and against all snRNPs both out of whole extract (Binereif and Green, 1986; Zillmann et al., 1987) and from gradient fractions containing the splicing complex (Grabowski et al., 1985). Antibodies directed against U2 also immunoprecipitate the intermediates of the reaction (Black et al., 1985). These studies indicate that at least the snRNPs U1 and U2 are integral components of the active splicing complex and are associated in a manner that survives immunoprecipitation.

There are yet other abundant nuclear factors that are known to be associated with RNA in vivo. Newly synthesized RNA does not exist as free RNA but is assembled into hnRNP particles that form immediately after transcription (Knowler, 1983). When isolated, these structures were found to consist of RNA and about nine major, highly interrelated and evolutionarily conserved proteins (mw = 32Kd-42Kd), now termed hnRNP core proteins
by analogy to nucleosomes and histones (Wilk et al., 1985; Lesser et al., 1984; Kruz-Alvarez et al., 1985). Antibodies to these polypeptides inhibit splicing in vitro (Sierakowska et al., 1986; Choi et al., 1986), suggesting that the hnRNP structure of the splicing substrate is also essential for activity.

In addition, there are protein factors required for splicing, especially 3' cleavage that are defined only through fractionation studies (Krainer and Maniatis, 1985; Chabot et al., 1985; Gerke and Steitz, 1986 Tazi et al., 1986). There are also genetically defined factors in yeast that are essential for splicing in this organism. Mutations in the genes coding for these factors have been classified into complementation groups (Lustig et al., 1986).
The Ordered Assembly of Splicing Complexes

The data detailed above led researchers to postulate the existence of large RNP structures that conducted RNA processing. Nuclear RNP particles containing the transcript from an amplified CAD gene (carbamoyl-phosphate synthetase, aspartate transcarbamylase, and dihydroorotase) were shown to be as large as 200S. The snRNPs U1, U2, and U6 were colocalized with the CAD transcript in sucrose gradients (Sperling et al., 1986). Free snRNP particles sediment at 10 to 15S. Other snRNPs not probed for were most likely also present.

The formation of RNP particles containing exogenously added splicing precursors has been studied extensively in vitro using gradient and electrophoretic analysis. Both techniques yield data in good agreement. Exogenously added single-intron RNA 400 to 500 nucleotides in length rapidly assembles into complexes in mammalian extracts that sediment at 15 to 20S. With time, factors are added that increase the sedimentation rate to 35S. In the presence of ATP, additional factors are added that increase the sedimentation rate to about 50 to 60S (Frendewey and Keller, 1985; Grabowski et al., 1985; Kaltwasser et al., 1986; Bindereif and Green, 1986), about the value for a large ribosomal subunit. This complex appears to represent at least a portion of the active complex as it exists in vivo, because the intermediates of the splicing reaction can be found in the same region of the gradient, and because the appearance of this complex is coincident with the detection of activity. The analogous complex in yeast extracts sediments at 40S (Brody and Abelson, 1985).

The formation of splicing complexes in extracts has also been studied by electrophoretic analysis. Again, rapidly migrating complexes are observed early in the reaction. Factors are added that retard migration to give a complex that matures to the
slowest migrating complex only in the presence of ATP. The complex of slowest mobility contains splicing intermediates; the 35S "spliceosome" peak from heparin-containing gradients yielded a comigrating complex upon electrophoretic analysis (Konarska and Sharp, 1986). In the presence of heparin, the sedimentation rate of the active complex was reduced from 50S to 35S, indicating a conformational change or loss of factors. A similar correlation was made using a heparin-free native gel system (Konarska and Sharp, 1987).
Interactions Between Factors and Precursor Consensus Elements

Both mammalian and yeast introns contain highly conserved elements necessary for splicing. The mammalian consensus sequences are shown in Figure 2. It is interesting to note that the consensus elements in plant RNAs are sufficiently similar to those of mammals to allow their correct splicing in HeLa cell extracts. This is also true for yeast RNAs (Ruskin et al., 1986). The yeast elements are essentially identical to mammalian elements with the exception that the sequence UACUAAC is found invariably at the branch point in yeast RNAs. The GU dinucleotide at the 5' splice junction is highly conserved and absolutely required. The actual 5' consensus element appears to extend into the intron for 6-8 additional bases. Alterations several nucleotides from the 5' splice junction depress splicing, but splicing can be restored by compensatory mutations in U1 snRNP (Zhuang and Weiner, 1986). Although the branch point sequence in mammals is not well conserved, the presence of an A residue 20 to 40 nucleotides from the 3' splice junction appears to be critical (Green, 1986). Mutations in this region in mammalian RNA result in activation of cryptic branch points. All four nucleotides will support branch point formation, although A and C appear to function better than U or G (Hornig et al., 1986). In yeast, mutations in this region reduce splicing severely (Brody and Abelson, 1985), but compensatory mutations can be made in the yeast U2 snRNA analog that restore activity (Parker et al., 1987). The polypyrimidine track appears to be an essential element in mammals. Its deletion inhibits the formation of the 50S spliceosome structure in vitro, but partial deletion does not appreciably affect splicing activity (Frendewey and Keller, 1985). Surprisingly, the highly conserved AG dinucleotide at the 3' splice junction does not appear to be involved in spliceosome assembly and cleavage at the 5' splice junction either in mammals (Frendewey and Keller, 1985; Ruskin and Green, 1985b) or in yeast
Mammalian Consensus Sequences

Figure 2: Mammalian Splicing Consensus Sequences. Higher eukaryotic splice junctions are highly conserved at the sequence level. The common features include a GU dinucleotide at the 5' junction, an AG dinucleotide at the 3' junction, and a polypyrrimidine track preceding the 3' junction. The sequence around the branchpoint is not highly conserved, but branchpoint formation is almost exclusively to an A residue. Selection of this A residue appears to depend on spatial constraints as well as neighboring sequence elements. There are also constraints on the minimum length of the intron. Precursors with an intron length below about 70 nucleotides do not undergo efficient splicing in the cases examined unless the intron is elongated above this length.
(Rymond and Rosbash, 1985).

Two powerful approaches have been used to study the interaction of factors with precursor RNA consensus elements. One approach makes use of antibodies raised against snRNP polypeptides to immunoprecipitate RNA fragments bound to these proteins and hence protected from RNase T1 digestion. In this fashion, the site of factor binding and the identity of the binding factor can be established simultaneously. This approach has been used to show that U1 snRNPs bind to the 5' splice junction and U2 snRNPs to the branch point as early events in the splicing reaction (Black et al., 1985). Protection of the 5' junction can be demonstrated much earlier in the reaction than the protection of the branch point, suggesting that U1 binding may precede U2 binding. Furthermore, the same sequences protected on the precursor RNA are protected from digestion in the intermediates, suggesting that the association of factors persists through the reaction. In particular, U2 snRNP appears to remain bound to the branch point sequence even after the formation of the branch (Chabot and Steitz, 1987a). Another interesting result found in these experiments was the discovery that U1 and U2 snRNPs appear to be in close proximity on the precursor when activity is detected (see below). This technique is unfortunately limited to mammalian systems due to the inability of the antibodies used to efficiently bind yeast snRNPs. Another approach was developed that took advantage of the ability of RNase H to specifically cleave RNA in RNA/DNA heteroduplexes (Kramer et al., 1984). This technique was employed to examine the order of sequence protection from RNase H cleavage along the precursor. The rationale behind oligonucleotide-directed RNase H cleavage is shown in Figure 3. The same sequences already known to be associated with factors by T1 protection and immunoprecipitation were also identified by this analysis in the mammalian system (Ruskin and Green, 1986) and the yeast system (Rymond and Rosbash, 1986). However, in the mammalian system, protection of the branch point from digestion was observed much
Figure 3: Oligonucleotide-Directed RNase H Cleavage. The technique of oligonucleotide-directed RNase H cleavage has proven to be an extremely powerful tool in the study of splicing. This technique has been used to both "knock out" extract RNAs as well as to probe for factor association with sequences complementary to the oligonucleotide used. The technique is based upon the fact that RNase H cleaves only RNA hybridized to DNA. The RNA in the hybrid is cleaved by an endonucleolytic activity, but the DNA remains intact.
earlier than protection of the 5' junction, in conflict with T₁ analysis.

Another approach that has been taken is to immunoprecipitate RNAs that have consensus sequences deleted. As discussed above, immunoprecipitations done with wild-type precursor have shown that at least U1 and U2 snRNPs are associated with the native spliceosome as found in the extract and within the spliceosomes purified by gradient centrifugation. Similar experiments performed with deleted precursors have led to some interesting conclusions. Most surprisingly, U1 snRNPs, known to interact with the 5' splice site via RNA/RNA hybridization, are detected associated with RNAs having intact 3' consensus elements but no first exon or 5' splice junction (Zillmann et al., 1987). RNAs lacking consensus elements are not immunoprecipitable by any antibody directed against U snRNPs.

The association of a U snRNP with the 3' splice site has been demonstrated by two groups using totally different approaches. In one case, a Western blot was probed with a variety of RNA probes and it was discovered that RNA lacking the 3' splice site was not bound specifically to any of the proteins on the filter, whereas RNA containing the 3' splice site was bound to a protein of a molecular weight ranging between 70 and 100 kd. This activity co-migrated upon fractionation with a common snRNP antigen (Tazi et al., 1986; Gerke and Steitz, 1986). There is some evidence that this protein might be associated with U5 snRNP. Both 3' splice site protection by T₁ analysis and the RNA moiety of U5 snRNP are highly resistant to micrococcal nuclease digestion. No other U snRNP shows such high resistance to degradation by this nuclease (Chabot et al., 1985).
snRNP/snRNP Interactions and Spliceosome Formation

Both gradient and native gel analysis of splicing reactions have shown that the spliceosome is assembled in discrete steps. Recent studies have given much insight into what these steps might be.

Affinity purification has shown that early complexes appear to be devoid of snRNPs (Grabowski and Sharp, 1986) and probably reflect association with hnRNP polypeptides. A complex of intermediate size was found to contain only U2 snRNP, whereas the largest complex was found to contain all of the major snRNPs with the exception of U1.

Other researchers have found that U1 snRNPs interact with precursor RNA very early in the reaction, U2 snRNP associating only later in the reaction (Black et al., 1985). As mentioned above, evidence also exists supporting the reverse order (Ruskin and Green, 1986). Several observations have been made which may help to resolve this conflict. In experiments where the intron size has been reduced below about 60 nucleotides, 5' cryptic sites are activated and not 3' cryptic sites (Wierenga et al., 1984; Ruskin et al., 1985). This suggests that the association of factors at the 5' splice site might be weaker than the association of factors with the 3' site and might not survive some types of analysis. It has been demonstrated that factors bound to the 3' splice site affect the binding of factors at the 5' splice site and vice versa. For instance, binding of U1 snRNP to β-globin cryptic sites could be detected only after incubation in the extract for a short time, in sharp contrast to U1 snRNP binding to wild-type 5' junctions, where binding could be detected immediately (Chabot and Steitz, 1987b). This experiment suggests that other extract factors stabilize the weaker interaction of U1 snRNP with cryptic sites. It has also been noted that in the absence of the polypyrimidine tract and 3' splice site, binding of factors to the 5' splice site (presumably U1 snRNP) is greatly diminished as assayed by RNase H protection (Ruskin and Green, 1985b). The reverse has been found to also be
true. In the absence of a 5' splice site the immunoprecipitability of the RNA via its association with common snRNP antigen was greatly reduced. Removal of U1 snRNP from the extract by immunoprecipitation prior to the addition of wild-type RNA produced the same effect, suggesting that binding of U1 snRNP to the 5' splice site stabilized the association of 3' factors (Zillmann et al., 1987). Perhaps the best piece of evidence that U1 and U2 snRNPs stabilize each other's association with RNA substrates via interaction comes from the fact that later in the reaction an antibody directed against U2 snRNP will immunoprecipitate 5' splice site protection fragments (Chabot and Steitz, 1987a). It is interesting to note in this regard that the relative order of the 3' and 5' elements is also essential as demonstrated by the fact that an RNA containing a 5' splice junction downstream of the 3' elements behaves just like an RNA lacking the 5' junction upon gradient analysis (Frendewey and Keller, 1985).
Supplement: snRNP Structure and Composition

The sequence of all the abundant U snRNAs is known (Reddy, 1986), and secondary structures have also been proposed based upon nuclease sensitivity studies (Mount and Steitz, 1981; Keller and Noon, 1985; Rinke et al., 1985; Branlant et al., 1983). Sequence comparison among species demonstrates that the U RNAs have been highly conserved throughout evolutionary time. These RNAs are found in vivo as highly abundant RNP's, existing at $10^5$ or more copies per cell and localized primarily in the nucleus (Zieve, 1981).

Each U snRNP is associated with at least ten proteins, some of them common to all U snRNPs (Hinterberger et al., 1983; Kinlaw et al., 1983). The individual U snRNPS U1, U2, U4/U6 and U5 have been purified through the use of combined affinity and column chromatography so that the polypeptides associated with each snRNP could be identified (Fig. 4). These particles in their free form sediment at between 10 and 15S and are assembled via an RNA free core particle containing polypeptides D, E, F, and G which has a sedimentation coefficient of 6S. The C polypeptide associated with U1 undergoes a post-assembly modification in vivo which results in an apparent increase of about 1 Kd by electrophoretic analysis (Fisher et al., 1985).
**Figure 4: Known snRNP Associated Polypeptides.** Twelve snRNP associated polypeptides have been identified by combined column and affinity chromatography approaches. Plus symbols indicate the presence of the particular protein on a particular snRNP. The polypeptides in the shaded boxes are those which are common to all of the abundant snRNP. A subset of these, the D, E, F, and G polypeptides have been identified in an RNA-free core particle which is presumed to be a precursor in the assembly of snRNPs.
EXPERIMENTAL OBJECTIVES

The splicing reaction has been fairly well characterized at the RNA level, but very little is known about how the reaction actually occurs and what factors are involved in this process. Several independent lines of evidence suggested that small nuclear ribonucleoproteins (snRNPs) probably play a critical role, but the exact nature of their involvement remains unclear.

It is the purpose of these studies to characterize the splicing reaction with respect to cofactor requirements and sensitivity to various perturbations. The relationship between extract factors and simple mRNA precursors is to be explored using a native gel electrophoretic assay. Using this assay, the formation of the splicing complex is to be analyzed in detail with respect to intermediate complexes and their composition. The sequences within the precursor necessary for splicing and splicing complex formation are to be determined by deletion analysis, and it is to be demonstrated that the deleted RNAs display similar assembly behavior as already documented by gradient analysis.

Most importantly, the complexes resolved by native gel analysis must be shown to have a composition and properties similar to, or identical to, complexes found in unfractionated extracts. This criterion must be satisfied to establish the validity of native gel electrophoresis as a tool for the study of splicing complexes.
MATERIALS AND METHODS

Preparation of Hela Cell Nuclear Extracts: Preparation of the nuclear extract was essentially as described for a HeLa cell soluble transcription extract (Dignam et al., 1983). The pH of all solutions used in this protocol was measured at the temperature at which they were to be used. Logarithmically growing cells were harvested from 8 liters of RPMI (Gibco) supplemented with 10% horse serum (Gibco) by centrifugation at 1.2 krpm in a Sorvall HG-4L rotor for 20 minutes at 4°C. The cell pellet (about 14 ml of wet cells) was resuspended in 3 volumes of PBS (137 mM NaCl, 2.7 mM KCl, 10.6 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) in a Corning 50 ml tube and centrifuged at 1 krpm for 5 minutes as before, except in a Sorvall HL-8 rotor. The pellet was resuspended in 5 packed cell pellet volumes of ice-cold Solution A (10 mM HEPES, pH 8.0, 1.5mM MgCl₂, 10 mM KCl, 0.5 mM DTT) and held on ice for 10 minutes. The cell pellet was collected by centrifugation at 1.5 krpm for 10 minutes and resuspended in two packed cell pellet volumes of ice-cold Solution A. This suspension was dounced (ten strokes) in a Kontes all glass Dounce homogenizer (B type pestle) and nuclei were then pelleted by centrifugation at 2 krpm in a Sorvall HL-8 rotor for 10 minutes at 4°C. The cytoplasmic fraction was removed from the nuclear pellet by careful decantation. The nuclear pellet was resuspended in 8 ml of Solution C (20 mM HEPES, pH 8.0, 25% glycerol, 420 mM NaCl, 0.75 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT). The nuclei were resuspended by douncing (6 strokes). The resulting suspension was gently stirred with a magnetic stirrer for 30 minutes on ice in the cold room and then cleared by centrifugation at 13 krpm for 30 minutes in a Sorvall type SS-34 rotor at 4°C. The supernatant was dialyzed for 4 hours against two 1 liter changes of Solution D (20 mM HEPES, pH 8.0, 20% glycerol, 100 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.5 mM DTT) at 4°C and quick frozen in 100 or 200 microliter aliquots in liquid
nitrogen. The final protein concentration of the extract was usually between 6 to 8 mg/ml. For some experiments, such as RNase H cleavage of U RNAs and precursor dependent mobility shifts on RNP gels, the extract was centrifuged in a microfuge for 5 to 10 minutes. This method was found to remove snRNPs bound to hnRNP which apparently were not available to participate in the processing of added precursor. For some experiments, $^{32}$P labelled extract was used. This was made by incubating the HeLa cells with 25 mCi of $^{32}$P orthophosphate for one hour prior to harvesting and extract preparation. These extracts had lower protein concentrations and were usually supplemented with active, unlabelled extracts prior to use.

**SP6 Polymerase Transcriptions:** Uniformly labelled, *in vitro* capped transcripts were generated from plasmids containing the SP6 promoter (Kassavetis *et al.*, 1982) in the following way. The standard transcription reaction was conducted in a volume of 50 ul and contained the following, added in the order given: 17 ul H$_2$O, 10 ul 5X transcription buffer (200 mM TRIS, pH 7.5, 30 mM MgCl$_2$, 10 mM spermidine, 100 mM NaCl), 0.5 ul 1 M DTT, 2 ul RNasin (Promega), 2.5 ul 20X ribonucleoside triphosphates (10 mM ATP and CTP, 4 mM UTP, 2 mM GTP), 2.5 ul 20 mM diguanosine triphosphate (Pharmacia), 10 ul of linear DNA (2 ug), 5 ul of $^{32}$P-UTP (50 uCi), and 1 Ô of SP6 polymerase (Promega, 10 U/ul). After 2 hours of incubation at 42º, 2 ul of RQ DNase (Promega) were added, and incubation was continued for another 15 minutes. Transcripts not requiring gel purification were then purified by the addition of 150 ul of urea buffer, extraction with an equal volume of buffered phenol/chloroform, extraction with an equal volume of chloroform/isoamyl alcohol, and precipitation by the addition of one volume of isopropanol. This mixture was placed at -80º for 10 minutes and the RNA was collected by centrifugation in a microfuge. The RNA pellet was resuspended in 200 ul of TE and 2 microliters of this were subjected to electrophoretic analysis (see below) to determine
the intactness of the transcript.

Transcripts requiring gel purification were precipitated in the reaction mix by the addition of 400 ul of ethanol. The reaction was placed at -80° for 10 minutes and the RNA was collected as before and dried under vacuum. The RNA pellet was resuspended in 10 ul of denaturing dye and loaded onto a small, sequencing thickness, 5% polyacrylamide denaturing gel. Electrophoresis was conducted at a constant amperage of 25 mA until the phenol red dye had reached the bottom. The plates were pried apart, leaving the gel attached to one of the plates. The gel was covered with plastic wrap and carried to the darkroom where autoradiography was conducted (1 minute) by placing a piece of film on top of the gel. Register marks were made by piercing the film and gel simultaneously with a needle. This left both black marks on the autoradiogram and holes in the plastic wrap which could be aligned. Bands were excised with a razor blade and placed into 300 ul of buffer X. Elution was allowed to proceed at room temperature for 30 minutes. The mixture was shaken thoroughly throughout. After 30 minutes the buffer was removed and replaced with 300 ul of fresh buffer. After another 30 minutes, the buffer was removed and added to that removed earlier. The mixture was extracted with an equal volume of phenol/chloroform and nucleic acids were precipitated with isopropanol after the addition of 10 ug of glycogen carrier. Recoveries were dependent upon RNA length, but were between 50% and 80% of the counts present in the original band.

**Standard Splicing Assay:** A standard splicing reaction contained 1 ul of 60% PEG, 1 ul of 0.5 M phosphocreatine, 1.6 ul of 25 mM ATP, 2.5 ul of 25 mM MgCl₂, 5 ul of Roeder Solution D, 10 ul of nuclear extract, and 1-4 ul of RNA precursor in a 25 ul reaction. This reaction was incubated at 30° for 60 minutes and worked up for either RNA or RNP analysis as described below.
Reaction In Extract Containing Cleaved RNAs: Reactions were assembled as above with the exception that no precursor was added and that 5 μg of an oligonucleotide complementary to the RNA to be cleaved and 1 μl of 800 U/ml RNase H (Boehringer-Mannheim) were added in addition. This mixture was incubated at 30° for 20 minutes, precursor was added and the reaction was allowed to proceed another 30 minutes at 30°. The extent of cleavage was assayed by denaturing gel electrophoresis. Reactions were also performed in extracts in which endogenous RNAs had been cleaved by micrococcal nuclease (MCN) digestion. The cleavage reaction contained 50 μl of cleared extract, 10 μl of 50 mM CaCl$_2$, 5 μl of 0.5 M phosphocreatine, 8 μl of 25 mM ATP, 12.5 μl of MgCl$_2$, 25 μl of Roeder buffer D, and 10 μl of 4500 U/ml MCN (Sigma). Cleavage was allowed to proceed for 45 min. at 30°, followed by the addition of 10 μl of 0.1 M EGTA (Sigma) and 10 min. of further incubation at 37°. The treated extract could be divided into aliquots and stored at -80°. At the time of use, 1 μl of 0.5 M phosphocreatine and 1.6 μl of 25 mM ATP were added per 25 μl of treated extract along with 1 μl of substrate. Reaction was allowed to proceed at 30° for the desired length of time.

RNA Analysis of Splicing Products: 175 μl of urea buffer were added to the 25 μl splicing reaction, followed by 200 μl of buffered phenol/chloroform solution. The mixture was vortexed vigorously and the phases were then separated by centrifugation in a microfuge for 5 minutes at room temperature. The supernatant was removed and extracted with 200 μl of chloroform/isoamyl alcohol solution. The phases were separated by a 1 minute centrifugation in the microfuge. The supernatant was retained and the RNA was precipitated from it by the addition of an equal volume of isopropanol. Precipitation was carried out at -80° for 15 minutes. The RNA was collected by centrifugation in the cold in a microfuge for 5 minutes. The isopropanol solution was aspirated off and the pellet was washed once with 200 μl of 70% ethanol. The RNA pellet
was dried and resuspended in 10 μl of denaturing sample buffer (90% deionized formamide, 10 mM EDTA, 0.2% each of xylene cyanole and phenol red). Samples were heated in a boiling water bath for 5 minutes, during which time they were vortexed briefly several times. Samples were then quick-chilled in a slush bath prior to loading onto a denaturing gel.

**Denaturing RNA Gels:** Three different types of RNA gels were used in these experiments, the particular type used depending upon the number of samples and the degree of resolution required. All gels were cast to a thickness of 0.8 mm and were 10% in polyacrylamide. The resolving gel consisted of 5 ml 10X TBE (see Standard Solutions), 24 gm urea, 10 ml of 50% deionized polyacrylamide:bisacrylamide (30:1), and water to 50 ml. Polymerization was induced by the addition of 20 ul of TEMED and 400 ul of 10% APS. Gels 20 cm wide by 30 cm high (tall) required 50 ml of gel solution and were electrophoresed at 800-1000 V (about 25 mA). Gels 30 cm wide by 20 cm high (wide) required 50 ml of gel solution and were electrophoresed at 600 V (about 50 mA). Gels 20 cm wide by 16 cm high (small) were electrophoresed at 600 V (about 25 mA). Electrophoresis was conducted in all cases so that the gel was hot to the touch and was of sufficient length to allow the xylene cyanole dye to reach the bottom of the gel.

**Silver Staining of RNA Gels:** After electrophoresis, the gel was soaked in several changes of water until all urea had been removed (approximately 15 minutes for 0.8 mm thick gel). The gel was then covered with fixative (50% methanol, 12% acetic acid) for 3 minutes. The fixative was removed by aspiration and replaced with enough washing solution (10% methanol, 5% acetic acid) to cover it. Washing was allowed to proceed for 30 minutes. The washing solution was then replaced with enough oxidizing solution (3.4 mM K₂Cr₂O₇, 3.2 mM HNO₃) to cover it and oxidation was allowed to proceed for 10
minutes. The oxidant was then removed and replaced with a 12 mM solution of AgNO₃, which was left in place for 20 minutes. After removal of the silver solution, the gel was rinsed well with water to remove any residual silver. The gel was developed in a solution of 0.28 M Na₂CO₃ containing 0.2% formaldehyde. This solution was changed when it turned brown. The reaction was allowed to proceed until the background began to turn dark and then was stopped by replacing the developing solution with a solution of 1% acetic acid. When CO₂ evolution had ceased, the gel was dried down between two sheets of dialysis membrane (Bio-Rad) held taut in a specially designed plexiglass stretcher.

RNP Gel Analysis of Splicing Reactions: The native gel systems employed in this study were all based upon work on the electrophoretic analysis of bacterial polyribosomes (Dahlberg et al., 1969). The TGE system (see below) is a modification of a recently published heparin-free, native system (Konarska and Sharp, 1987). Splicing reactions to be analyzed in the TAE system (see below) were stopped by the addition of 6 ul of RNP gel dye (62 ul of 50 mg/ml heparin (Sigma, 162 USP U/mg), 77 ul of 200 mM EDTA, 8.0, 154 ul glycerol, 0.2% each of xylene cyanole, phenol red, and bromphenol blue) and frozen in liquid nitrogen until needed. Reactions to be analyzed in the TGE system (see below) were stopped by the addition of 5 ul of stop solution (15 mg/ml heparin, 62.5 mM EDTA, 8.0) and frozen in liquid nitrogen. Prior to loading, the samples were incubated at 30° for 5 minutes and 5 ul of tri-dye glycerol (0.2% each bromphenol blue, xylene cyanol, and phenol red in glycerol) were added.

The gel matrix was prepared from a 10X RNP buffer stock (TAE: 250 mM TRIS-acetate, pH 8.0, 100 mM EDTA. TGE: 500 mM TRIS-HCl, pH 8.8, 500 mM glycine, 100 mM EDTA), a 50% polyacrylamide:bisacylamide (80:1) stock and a 1% agarose stock. For 50 ml of gel, 2.5 ml of polyacrylamide stock, 5 ml of 10X RNP buffer, and 10 ml of glycerol
were brought up to a volume of 25 ml with deionized water. To this, 25 ml of boiling hot agarose stock were added. Polymerization was initiated by the addition of 40 ul of TEMED and 800 ul of freshly made 10% APS. The gel was cast immediately as for denaturing RNA gel (0.8 mm thickness) except that the bottom spacer was replaced by an appropriate thickness of wetted filter paper and care was taken to keep all clamps on the spacers. The bottom was left unclamped. The comb was inserted immediately to a depth of 0.5 cm and the gel was allowed to polymerize for 30 minutes or more at room temperature in an upright position. The gel was then moved to a 4° cold room and allowed to cool to 4°. Care was exercised in the removal of the comb from the still clamped gel. The gel was transferred to the electrophoresis apparatus keeping at least two clamps on it at all times. At this time the gel sometimes either rose or fell within the plates. Rising could be corrected by moving the clamps farther toward the edges of the plates and falling by moving them away from the edges of the plates. The buffer reservoirs were filled with 1X RNP buffer at this time. A thin membrane usually remained in the wells and this was removed by careful flushing. After sample was loaded to a depth of 1 mm (about 50° for standard wells), electrophoresis was conducted at about 10 V/cm for 3 to 4 hours. Upon completion of electrophoresis, the gel was briefly fixed (50% methanol, 12% acetic acid) and transferred to filter paper. This paper was wrapped in plastic wrap and subjected to autoradiography.

**Extraction of RNA from RNP Gel:** Upon completion of electrophoresis, the RNP gel was either subjected to autoradiography at 4° for 30 minutes in an unclamped folder with bands cut from the gel using the autoradiogram as a guide, or the gel was cut into ordered segments directly. In either case, the gel slices were crushed in three to five volumes of TBE containing 10% glycerol by repeated passage through a 22½ gauge needle. This suspension was incubated on ice for 60 minutes, whereupon the gel matrix
was pelleted by 5 minutes of centrifugation in a microfuge. The supernatant was carefully removed from the clear pellet and extracted with phenol/chloroform, extracted with chloroform/isoamyl alcohol, and nucleic acids were precipitated at -80° for at least 15 minutes with isopropanol after the addition of sodium acetate to 0.1 M. The RNA was pelleted as described above. The pellet usually contained some agarose and was dissolved in 20 μl of denaturing sample buffer by heating and vortexing. Samples were loaded hot to prevent the setting of the agarose. RNA could also be readily be purified from particles in the RNP gel by blotting to a membrane and then eluting RNA from the membrane with a high salt buffer. The RNP gel was capillary blotted to Z-Probe (Bio-Rad) membrane in 1X TEA (10 mM TRIS, pH 7.8, 5 mM sodium acetate, 0.5 mM EDTA) for 2 hours. This blot was subjected to autoradiography with sections cut using the autoradiogram as a guide, or the blot was cut into ordered sections directly. The membrane slices had an area of less than 1 cm² and were placed in a tube with 900 μl of urea buffer, never allowing the membrane to dry out. The buffer was heated to 60° for 10 minutes and removed from the membrane. RNA was recovered by the addition of 600 μl of isopropanol, chilling to -80° for 15 minutes, and pelleting in a microfuge as before. Both methods of RNA recovery from RNP gels gave comparable yields (about 50%) and resulted in comparable amounts of degradation. The RNA composition of particles resolved in the RNP gel was also examined by two-dimensional electrophoresis. One lane was removed from the RNP gel and carefully placed onto a piece of Parafilm. This slab was covered with a solution of 0.1% SDS, 0.5 mg/ml protease K (Boehringer-Mannheim) in TBE buffer and placed at 37° for 10 minutes. This solution was removed and replaced with loading buffer, which was left in place for 5 minutes. The slab was then loaded onto a pre-warmed denaturing RNA gel and electrophoresis was conducted as usual (see above).
Recovery of RNP Particles from RNP Gels: The location of the desired particles was discerned by autoradiography as described above. The cut pieces of gel were crushed in 3 to 5 volumes of Roeder Solution D (see Standard Solutions) as described above. The gel matrix was cleared by centrifugation and the supernatant retained for further analysis. About 50% of the original counts were recovered at this stage. A typical experiment consisted of division of the supernatant into 3 aliquots, usually about 100 ul in volume. RNA was prepared directly from one as described, and the other two were subjected to immunoprecipitation as detailed below, one with control serum and the other with the antiserum of choice. It was noted that the crushed gel could be frozen at -80° overnight without any undesirable effects.

Immunoprecipitation of Eluted Complexes: Roughly 50 ng of unlabelled competitor substrate was added per 100 ul of eluted complex and this mixture was incubated on ice for 10 minutes prior to the addition of antibody. Complexes were immunoprecipitated by the addition of 2 to 5 ul of antibody. The antibodies used were a patient preimmune control serum, a patient αRNP antibody, and a monoclonal αSm antibody (Y12 monoclonal). Specificities were determined by use of these antibodies as probes for Western analysis of HeLa cell nuclear proteins. After a 30 minute incubation on ice, the immune complexes were collected on prewashed (see below) Pansorbin (Calbiochem). Prewashed Pansorbin (10 ul) were added to the mixture and incubation was continued for an additional 15 minutes. The Pansorbin was collected by 30 seconds of centrifugation in a microfuge in the cold. The pellet was washed three times with 1 ml of ice cold NET buffer (50 mM TRIS, pH 7.4, 150 mM NaCl, 0.05% NP40, 0.5 mM DTT). The antigen was then dissociated from the Pansorbin by the
addition of 250 ul of urea buffer and heating to 70° for 5 minutes. The Pansorbin was removed from the urea buffer by 5 minutes of centrifugation in a microfuge at room temperature. The RNA was precipitated by the addition of 10 ug of E. coli tRNA (Sigma) or glycogen (Sigma) carrier and 250 ul of isopropanol. Subsequent steps were the same as described above for RNA samples. tRNA carrier was used in experiments where a silver-stained RNA profile was not needed.

Pansorbin was prewashed by washing two times in each of the Pansorbin washing buffers: Buffer A (0.15 M NaCl, 5 mM EDTA, 50 mM TRIS, pH 7.4, 0.05% NP40, 0.5% NaN3), Buffer B (0.30 M NaCl, 5 mM MgCl2, 50 mM TRIS, pH 7.4, 0.5% NP40, 0.5% SDS), and Buffer C (0.3 M NaCl, 5 mM MgCl2, 50 mM TRIS, pH 7.4, 2 mM EDTA). Washing was accomplished by adding 10 volumes of ice-cold buffer to the Pansorbin, resuspending the Pansorbin by vortexing, and spinning down the Pansorbin in a microfuge. The length of time required to collect the Pansorbin varied with the buffer used. The final Pansorbin pellet was resuspended in an equal volume of NET buffer. Pansorbin which had been stored in this buffer for more than one week was washed again once with each of the washing buffers before use.

RNase H Protection for Eluted Complexes: Rnase H cleavages were conducted in 100 ul aliquots. Four volumes of Roeder D had been added to the gel slice, making the final EDTA concentration roughly 2 mM, which is inhibitory to RNase H (Zarkower and Wickens, 1987). To overcome this effect, 1 ul of 250 mM MgCl2 was added, bringing the final concentration to 2.5 mM, which was found to restore activity. Regions of the radiolabelled RNA which were capable of forming DNA/RNA hybrids with added oligonucleotide were then cleaved with
RNase H (Fig. 3). RNase H has the property that it only cleaves regions of RNA in DNA/RNA hybrids. Cleavage was initiated by the addition of 0.8 U of RNase H (Pharmacia) and 500 ng of the appropriate oligonucleotide. Oligonucleotides were synthesized at the Baylor College of Medicine on an Applied Biosystems 380A 3-column DNA synthesizer using β-cyanoethyl phosphoramidite chemistry. Some of these oligonucleotides were used to prime cDNA synthesis from their complementary U RNA. Incubation was at room temperature for twenty minutes. The reactions were then extracted with phenol/chloroform and isopropanol precipitated. The pellet was resuspended in 15 to 20 μl of formamide loading dye (see above), placed in a 95° water bath for 5 minutes and loaded straight from the bath onto the gel due to the presence of agarose in the samples.

Generation of Splicing Precursors and Deletion Mutants: All splicing precursor constructs used in this work were derivatives of pRSP1 (Konarska et al., 1985). The initial constructions were done by Scott Rose and consisted of transfer of the insert from pSP62 into pSP64 and finally into pSP65. plVTQ was generated by cloning the TaqI fragment carrying the insert into the Accl site of pSP64 in reverse orientation. The RNA made from this clone was anti-sense. This clone also contained the BamHI-BclI fragment containing the SV40 early and late polyadenylation sites cloned into the BamHI site of the poly linker. pIVPX (see Fig. 5) was constructed from plVTQ by taking the PstI-Xbal fragment and recloning it into the identical sites of pSP65. A 442 nucleotide splicing precursor RNA could be made from this plasmid by linearization with PstI. pMZ1 was constructed by Scott Rose by inserting a BglII linker (Boehringer-Mannheim) into the vector PvuII site of plVPX. A 632 nucleotide splicing precursor could be made from this plasmid by linearization with BglII. pΔΔ5'
Figure 5: Construction of Plasmids Deleted for Consensus Elements. Plasmids containing deleted precursor sequences were derivatives of pRSP1, a plasmid containing the first two exons of the adenovirus tripartite leader and a deleted first intron. pMZ1 is shown twice for clarity. In one case a segment was subcloned (pΔΔ5') and in another a segment was deleted (pΔE2L). All inserts were cloned into pSP65, a plasmid containing the SP6 promoter element. Transcription of plasmid cut with the enzymes indicated gave transcripts of the length indicated below the schematic. The boxes represent exons. The filled box represents the first exon and the hatched box the second.
was constructed from pIVPX by taking the Rsal fragment encompassing the intron and half of the second exon and cloning it into the Smal site of pSP65. Splicing precursors of 289 and 506 nucleotides lacking the 5' splice junction could be made from this plasmid by linearization with BamHI and PvuII, respectively. pΔE2L was constructed from pMZ1 by removal of the HindIII fragment between a HindIII site in the intron and the HindIII site in the polylinker. Precursors lacking the 3' junction of 255 and 440 nucleotides could be made from this plasmid by linearization with HindIII and PvuII, respectively. pIVXX was constructed from pMZ1 by filling in the vector HindIII site using dNTPs and Klenow enzyme (Boehringer-Mannheim) and adding a XhoI linker (Pharmacia). pEIEIO (exon-intron-exon-intron-etc.) was constructed from pIVXX by inserting the HindIII fragment containing half of the intron and all of the second exon from pIVPX into the HindIII site of pIVXX in the sense orientation (Fig. 6).

A two intron, three exon precursor of 651 nucleotides could be made from this plasmid by linearization with XhoI. pMINX (mini-exons) was constructed from pEIEIO by cloning the Rsal fragment containing half of the second exon sequence, the second intron sequence, and the third exon sequence into the Smal site of pSP65. A miniature splicing precursor containing only second exon sequences and having an artificial 120 nucleotide intron could be generated from this plasmid by linearization with BamHI.

**Transformation of Competent DH1 Cells:** *E. coli* strain DH1 was made competent using the RbCl/CaCl₂ protocol (Goddard et al., 1983). Cells were grown in a 50 ml flask to 35 Klett units. Cells were then harvested by 10 minutes of centrifugation at 8 krpm in a Sorvall SS-34 rotor at 4°C. The cells were resuspended in half the culture volume of 10 mM MOPS, pH 7.0/10 mM
**Construction of Novel Splicing Precursors**

![Diagram of construction of novel splicing precursors]

**Figure 6: Construction of Novel Splicing Precursors.** A novel precursor construction was made to study factors affecting the order of splice site usage. pEIEIO was constructed by inserting the HindIII-HindIII fragment of pIVPX back into the HindIII site in the intron sequence of pIVPX. pMINX was constructed to examine the rate and efficiency of the removal of the second intron in the absence of the first intron and surrounding sequences. All inserts were cloned into pSP65, a vector containing the SP6 promoter. Transcription of plasmid cut with the indicated enzymes gave transcripts of the length indicated below the schematic. The boxes represent exons. The filled box represents the first exon and the hatched box the second.
RbCl and harvested as above. The cell pellet was resuspended in half the culture volume of 100 mM MOPS, pH 7.0/10 mM RbCl/50 mM CaCl$_2$ and held on ice for 30 min. Cells were harvested as before and resuspended in one tenth to one twentieth of the original culture volume of the buffer just used and DMSO was added to a final concentration of 3%. This mixture was divided into 200 aliquots which were kept frozen at -80° until needed. When needed, competent cells were thawed on ice and up to 50 ul of DNA solution were added. The cells and DNA were incubated together for 30 minutes on ice. The cells were then heat-shocked by a three minute incubation at 42° and 1 ml of LB was added to the tube. The cells were then allowed to recover at 37° for 2 hours before they were plated out onto LB/Amp (100 ug/ml) plates. To plate the cells, the tube was centrifuged for 30 seconds in a microfuge, the supernatant was poured off, and the cells were resuspended in the small amount of LB remaining. This mixture was transferred to a plate and spread. The plate was incubated at 37° overnight and colonies were picked for minipreps.

**Screening of Recombinant Bacterial Colonies by Miniprep:** Ampicillin resistant colonies were picked and used to inoculate 6 ml LB culture tubes. The cultures were grown overnight at 37° with shaking, transferred to 15 ml Corning tubes, and harvested by centrifugation at 5 krpm in a Beckman TJ6 rotor for 15 minutes. The small amount of fluid left in the original culture tube was used after screening to inoculate the large culture used for obtaining milligram quantities of the desired plasmids. The supernatant was discarded and the cell pellet was resuspended in 0.5 ml of 50 mM TRIS-HCl, pH 8.0 by vortexing. To each tube 50 ul of 10 mg/ml lysozyme (Sigma) prepared fresh in 250 mM TRIS-HCl, pH 8.0 were added. Digestion was allowed to proceed for 30
minutes at room temperature and the contents of the large tubes were transferred to 1.5 ml microtubes. To each tube 0.5 ml of buffered phenol (no chloroform) were added and the contents were gently mixed by several inversions. The tubes were centrifuged in a microfuge for 5 minutes at room temperature. The clear supernatant was recovered and placed into a fresh 1.5 ml microtube along with 150 ul of 9M ammonium acetate and 0.5 ml of buffered phenol/chloroform. Centrifugation was repeated and the supernatant was transferred to a fresh 1.5 ml microtube. The plasmid DNA was precipitated by filling the tube with isopropanol and chilling to -80° for 15 minutes. The tubes were centrifuged in a microfuge for 5 minutes in the cold, the supernatant was discarded, and the pellet was washed with 70% ethanol at room temperature. After drying, the DNA was dissolved in 100 ul of TE containing 3 ul of 10 mg/ml boiled RNase A (Sigma). Digestion was allowed to proceed for 30 minutes at 37°. Approximately 15 ul of this mixture were digested with the restriction endonuclease of choice by addition of 2 ul of the appropriate 10X restriction buffer (Boehringer-Mannheim), 3 ul of water, and 2-3 units of the desired enzyme. Digestion at 37° was usually allowed to proceed overnight. The digestion products were analyzed by native gel electrophoresis on agarose or polyacrylamide gels as was appropriate. Miniprep DNA linearized by restriction endonuclease cleavage could be used for transcription reactions after further purification. One third volume of 8.7 M ammonium acetate was added to the digested miniprep DNA (whole miniprep digested in 100 ul of restriction buffer) and the DNA was extracted with phenol/chloroform and chloroform/isoamyl alcohol as above. The DNA was precipitated with isopropanol and washed with 70% ethanol as described. The DNA after resuspension in 250 ul of TE was ready for use in transcription reactions. Transcripts made in this fashion were often
not stable to prolonged storage unless gel purified.

**Large Scale Plasmid DNA Isolation:** About 6 ml of LB were added to the small amount of cells left in the culture tube from the miniprep and this culture was incubated overnight at 37° with shaking. This culture was used to inoculate a 1 liter flask of LB/Amp (100 ug/ml) at 37° and the cells were allowed to grow with shaking to Klett 80. At this point, chloramphenicol (Sigma) was added to 100 ug/ml and incubation was continued overnight. Cells were harvested by centrifugation at 6 krpm for 10 minutes in a Sorvall GSA rotor at 4°. The cell pellets were resuspended in a total volume of 50 ml of TE, which was split among two Oakridge tubes. The cells were collected by centrifugation as before, but in a Sorvall SS-34 rotor. The cell pellet was resuspended by vortexing in 2 ml of Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM TRIS-HCl, pH 8.0). Another 8 ml of Solution 1 were added and the suspension was divided between two Oakridge tubes. To each tube 5 mg of lysozyme (Sigma) were added and digestion was allowed to proceed on ice for 30 minutes. After 30 minutes, 10 ml of Solution 2 (0.2 M NaOH, 1% SDS) were added to each tube, the contents were mixed, and the mixture was left on ice for 10 minutes. After 10 minutes, 7.5 ml of Solution 3 (3 M potassium acetate, 4.5% formic acid) were added to each tube and the contents were mixed by inversion. The tubes were left on ice for 30 minutes, whereupon the precipitate was removed by a 10 minute centrifugation at 8.5 krpm at 4° in a Sorvall SS-34 rotor. The supernatant was filtered through cheesecloth and divided equally between three Oakridge tubes. The plasmid DNA was precipitated by the addition of 2 volumes of ethanol to each tube and placement at -20° for 20 minutes. The DNA was collected by centrifugation as in the preceding step. The pellets were dissolved in a total of 5 ml of Solution
4 (0.1 M sodium acetate, 50 mM MOPS, pH 8.0) and combined in one Oakridge tube. The DNA was reprecipitated by the addition of 10 ml of ethanol and placement of the tube at -80° for 10 minutes. The DNA was collected by centrifugation at 10 krpm for 10 minutes at 4° in a Sorvall SS-34 rotor after the contents of the tube had been warmed to room temperature. The pellet was redissolved in 2 ml of TE and 1 ml of Solution 5 (7.5 M ammonium acetate) was added. The mixture was left on ice for 20 minutes and centrifuged as in the previous step. The supernatant was removed and 6 ml of ethanol were added. The DNA was precipitated and collected as before. The pellet was dissolved in 2.5 ml of Solution 4 and extracted three times with buffered phenol/chloroform. The DNA was collected as before by the addition of two volumes of ethanol. The pellet was redissolved in 2 ml of TE containing 20 ug/ml of boiled RNase A (Sigma). Digestion was allowed to proceed for 30 minutes at 37°. Ammonium acetate was added to a final concentration of 2.5 M and the mixture was extracted three times with an equal volume of buffered phenol/chloroform and two times with chloroform/isoamyl alcohol (24:1). The DNA was precipitated as before by the addition of two volumes of ethanol. The pellet was rinsed with ethanol and dried. The plasmid DNA was redissolved in 0.5 ml of TE and the OD_{260} was measured to determine its concentration. To prepare templates for transcription, 50 ug of plasmid were digested overnight with 50 units of the appropriate restriction enzyme in a total volume of 1 ml. This was extracted once with 0.5 ml of phenol/chloroform and once with 0.5 ml of chloroform/isoamyl alcohol and precipitated with 0.5 ml of isopropanol. When the restriction enzyme used required low salt buffer (no NaCl), sodium acetate was added to a final concentration of 100 mM prior to precipitation. The DNA pellet was resuspended in 250 ul of TE so that 10 ul of this solution would contain 2 ug of linearized
plasmid. Templates prepared in this manner and stored at 4° produced excellent transcripts for 3 or more months.
STANDARD SOLUTIONS

Buffered Phenol/Chloroform: Buffering solution consisted of 100 mM TRIS-HCl, pH 7.4, 300 mM NaCl, 1 mM EDTA, 0.1% i-mercaptoethanol, and 0.1% 8-hydroxyquinoline. Redistilled phenol, 500 gm (Fisher), was dissolved in 467 ml chloroform. Two liters of buffer were added and the mixture was equilibrated at 4° overnight. The pH was readjusted as necessary with NaOH. Phenol prepared in this fashion and stored at 4° in a capped brown glass bottle was stable for at least one year. Transition in color from yellow to orange or brown indicated oxidation.

Buffer X: 0.5 M ammonium acetate, pH 7.5, 0.5% SDS, and 5 mM EDTA.

Denaturing Dye: To make ten milliliters of loading dye 9.0 ml of deionized formamide, 0.5 ml of 200 mM EDTA, pH 8.0, 0.5 ml H₂O, 0.02 gm xylene cyanole, and 0.02 gm of phenol red were mixed. The migration of phenol red in a denaturing gel is the same as that of bromphenol blue.

Roeder Buffer D: For 500 ml: 20 ml of 0.5 M TRIS-HCl, pH 8.0, 200 ml of 50% glycerol, 12.5 ml of 4 M KCl, 0.2 ml of 0.5 M EDTA, and 250 ul of 1 M DTT.

TAE: 25 mM TRIS and 10 mM EDTA, adjusted to pH 8.0 with glacial acetic acid.

TBE: For 1 liter of 10X stock: 121.1 gm TRIS base, 51.4 gm boric acid, and 3.7 gm EDTA.

TE: 10 mM TRIS, pH 7.4 and 1 mM EDTA.

TEA: 10 mM TRIS-acetate, pH 7.8, and 0.5 mM EDTA.

TGE: 50 mM TRIS-HCl, pH 8.8, 50 mM glycine, and 10 mM EDTA.

Urea Buffer: 2X buffer was 0.35 M NaCl, 10 mM TRIS, 7.4, 10 mM EDTA, and 1% SDS. To use, 4.2 gm of urea and 5 ml of 2X buffer were brought to a final volume of 10 ml. Final urea concentration is 7 M.
RESULTS

Splicing Extract and Reaction

Optimization of the Splicing Reaction. The splicing reaction has been shown to have discrete optima for the concentration of Mg$^{++}$ and K$^+$ ions at about 1 mM and 30 mM, respectively (Hardy et al., 1984). It was desirable to redetermine these values and those of several other parameters for extracts prepared in our laboratory. This determination is shown in Figure 7. The splicing reaction is accompanied by the creation of various intermediate and product RNA species. The linear species were identified strictly by their mobility with respect to denatured marker DNA fragments of known length. The lariat species were identified by their aberrant mobilities in gels of different acrylamide percentage with respect to denatured DNA fragment markers. In some

It was found that splicing activity was optimal at pH=8.0, [Mg$^{++}$]=2 mM, [K$^+$]=40 mM to 60 mM, and a polyethylene glycol (PEG) concentration of 2% to 4% in the final reaction. These values are in good agreement with reported values and were used throughout these studies. It was also noted that much of the ribonuclease activity found in some nuclear extracts could be removed by a 5 minute centrifugation step in the microfuge. This procedure removed some snRNPs and much of the hnRNP, but resulted in no apparent loss of splicing activity (data not shown). The amount of ribonuclease activity observed varied between extracts and depended upon the RNA added. RNAs lacking consensus
Figure 7: The Splicing Reaction has Discrete pH, K⁺, and Mg²⁺ Optima. One hour splicing reaction were conducted with IVPX precursor in the presence of the indicated conditions and the RNA was displayed by denaturing polyacrylamide (10%) gel electrophoresis. The cofactors not being optimized remained at standard concentrations (2.5 mM MgCl₂ and 60 mM KCl). The standard pH was 8.0. Note that the addition of about 3% PEG to the reaction greatly stimulated conversion of precursor to intermediates. It was found in some experiments that extracts which appeared inactive in the absence of PEG showed activity in its presence during a one hour reaction, indicating that the concentration of some factor was below a critical level. All of the cofactors were found to have narrow concentration optima in good agreement with published values. The reaction intermediates and products are shown schematically. The open box denotes the first exon, the filled box the second exon, and the line in between, the intron. The intron is removed as a lariat—an RNA having a 2' to 5' linkage of the 5' terminal nucleotide to an internal nucleotide.
RNA Composition of the Extract and Requirement for Splicing. The nuclear extract was found to contain all of the most abundant small nuclear RNAs of the U class as well as tRNA, pre-tRNA, and ribosomal 5.8S and 5.0S RNAs as is shown in Figure 8. This figure also shows RNAs extracted from other sources. Compare the whole cell RNA profile shown with the extract RNA lane in Figure

RNA species in the extract can be specifically degraded by micrococcal nuclease, a calcium requiring enzyme. The enzyme can be inactivated at the end of the reaction by the addition of EGTA, a calcium specific chelator. This prevents the degradation of RNA species added subsequently, such as precursor RNA. When most of the RNA in the extract was degraded in this fashion, splicing activity was abolished (Fig. 9), as has been shown previously (Furneaux et al., 1985; Krainer and Maniatis, 1985). Addition of fresh extract allowed splicing to occur, demonstrating that activity could be restored and that the MCN treatment did not add an inhibitory factor. It was of interest to determine whether activity could be restored by RNA alone, because if this were possible, it would allow the study of the interaction of each U snRNP with the splicing precursor simply by providing the RNA of interest in radiolabelled form to the micrococcal nuclease digested extract. However, RNA alone did not restore splicing activity to detectable levels after 30 minutes of preincubation with treated extract at 30°. The addition of tRNA to the treated extract served as a control in the event that the

Although catalysis (i.e. cutting and joining of RNA molecules) is usually thought of as being conducted by proteins, there is evidence for RNA catalysis (Cech and Bass, 1986) and catalysis by ribonucleoproteins (Gold and Altman, 1986). In the case of the splicing reaction, it is quite clear that the reaction does not proceed in the absence of extract RNA. It has been speculated that snRNPs, through their interaction with precursor, serve as a scaffold which
Figure 8: HeLa Cell Small Nuclear RNA Profile. RNA was extracted from several types of cells including F9, a mouse cell line; HeLa, a human cell line; *Tetrahymena thermophila*, a ciliated protozoan; and *Xenopus laevis* oocytes. RNA was extracted from whole cells by detergent lysis and displayed by denaturing polyacrylamide (10%) gel electrophoresis. RNAs were visualized by silver stain. The RNA profile from HeLa cell nuclear extract is identical to the HeLa whole cell RNA profile with the following exceptions. The amount of hnRNP in nuclear extract cleared by centrifugation is less compared with U1 RNA, the amount of 5.8S ribosomal RNA is less in the cleared nuclear extract compared with the levels of U1 RNA, and U3 RNA is absent from the cleared nuclear extract. Note that the amount of U snRNA in the lower organisms is dramatically decreased with respect to ribosomal RNAs. It has been estimated that the number of snRNPs in a *Tetrahymena* cell is 50 to 100 fold lower than in a mammalian cell.
Figure 9: Micrococcal Nuclease Digestion Inactivates the Extract.
Whether splicing could occur in the absence of intact extract RNAs was tested through the micrococcal nuclease (MCN) digestion of extract RNAs and inhibition of the nuclease with EGTA prior to the addition of extract. Panel A is a silver stain of the gel in panel B. The silver stain shows that the bulk of the U RNA, tRNA, and hnRNA was destroyed by this treatment. A 30 min. reaction with MINX precursor gave all of the expected products in both the control reaction and in a MCN digested extract supplemented with untreated extract when reaction RNAs were analyzed by denaturing polyacrylamide (13%) electrophoresis in Panel B. This indicated that MCN treatment did not contribute factors to the untreated extract which would inhibit its activity. The amount of activity seen in the supplemented MCN extract is about half of that seen in the untreated extract because equal amount of treated and untreated extract was used in the reaction, activity being derived only from the untreated extract. Supplementation of the MCN treated extract with purified RNA from two volumes of untreated extract and 30 min. of preincubation under reaction conditions prior to the addition of substrate failed to produce any detectable activity. The control for this reaction was the addition of E. coli tRNA to the MCN treated extract which was not expected to produce activity.
Reed, 1987). In this regard, it is well documented that U1 binds the 5' splice junction and that U2 binds the branch point, two spatially separated domains. Yet, during catalysis, these two snRNPs are in intimate contact (Chabot and Steitz, 1987a). This requires the juxtaposition of the 5' and 3' elements.

Effect of Denaturants on Splicing. Denaturants were added to splicing reactions to address the question of whether errors in splicing would be made as a result of the incorrect interaction of factors and that this would give some clue as to the mechanism of the reaction. SDS was chosen as a reagent which would primarily perturb protein/protein and protein/RNA interactions and urea as one which would perturb RNA/RNA interactions in addition. It was also hoped that this approach could be used in combination with native electrophoresis to demonstrate differential stability in the interaction of various RNAs with splicing complexes. As shown in Fig. 10, the addition of consecutively higher levels of either denaturant abolished splicing activity without the appearance of anomalous intermediates. As activity, by definition, correlates with the presence of the active splicing complex, it can be assumed that the active complex exists in extracts which have been treated with levels of denaturant which do not inhibit splicing. This observation could be applied in fractionation techniques in which nonspecific binding of factors to chromatographic supports is undesirable, the

Comparison of the Splicing Efficiency of MINX and IVBX. pMINX was constructed primarily to facilitate the analysis of the RNA products of the splicing reaction. RNA made from this construction was about half of the length of RNA made from pIVBX and had about the same length as RNAs made from plasmids containing consensus sequence deletions (Fig. 5). This was important
Figure 10: Effect of Denaturants on Splicing. One hour splicing reactions were conducted with IVPX precursor at 37° in the presence of denaturant at the final concentration shown and the RNA from the reactions was displayed by denaturing polyacrylamide (10%) gel electrophoresis. Splicing was inhibited by urea concentrations greater than 0.5 M and SDS concentrations greater than 0.012%. The appearance of the products of 5' cleavage in the absence of 3' cleavage was never detected. The lariat reaction intermediates are indicated schematically.
for assembly studies, as length effects on complex migration have been observed by others (Konarska and Sharp, 1986) and were to be avoided. Lengthening the deletion RNAs to the same length as IIVX RNA resulted in the activation of cryptic sites (see Appendix 1). pMINX can best be thought of as an exchange of the 5' junction sequence present in the first exon of pIVBX for the 5' junction sequence present in the second exon sequence of this plasmid. Both RNAs splice with approximately the same efficiency (percent of input RNA converted to product). Addition of pBR322 sequence to the 3' terminus of MINX RNA which made it the same length as IIVX RNA increased the efficiency slightly for unknown reasons (Fig. 11). The short RNA appeared to behave identically to previously characterized precursors and was used throughout this work.
Figure 11: MINX Precursor RNA Splices Both Correctly and Efficiently. The short length of the MINX (MX) precursor RNA make it ideally suited for studying splicing. It is technically easier to make large amounts of intact short RNA than intact long RNA using the SP6 system. In addition, the small sizes of linear products also measurement of lengths to the nucleotide possible. The splicing of this RNA precursor was compared to the splicing of IVPX RNA (PX) and MINX Long RNA (MXL). Aliquots were taken after incubation at 30° in the presence of 3% PEG for the length of time indicated and analyzed by denaturing electrophoresis on a 10% gel. The latter transcripts have the same length within several nucleotides. The precursor RNAs are shown schematically at the bottom of the figure. MINX and MINX Long should both give identical first exon and released lariat RNAs. The reaction products are shown schematically on the right of the figure. It was observed that both MINX and MINX Long yielded intermediates of expected mobility and spliced as efficiently as IVPX RNA.
Native Electrophoresis of Splicing Complexes

Time Course of Splicing Complex Assembly. Radiolabelled RNA was added to nuclear extract under splicing conditions and aliquots were analyzed at various times by either TGE (Fig. 12A) or TAE (Fig. 13B) native electrophoresis. Labelled RNA was found to assemble immediately into a rapidly migrating, heterogeneous complex, denoted I (initial). This complex formed on ice, did not require ATP for its formation, and formed in the presence of 10 mM EDTA and 2.5 mg/ml heparin. As reaction proceeded, further factors associated with the RNA, reducing its mobility further. Within 5 minutes, complexes in the I region were replaced by a new complex, A. As reaction proceeded further, complexes A' and B appeared, the latter appearing coincident with activity. Very late in the reaction, when the products of the reaction had accumulated to significant levels, the rapidly migrating complexes C and D appeared. Although the migration of these complexes was similar to that of the initial complexes, these species may be distinct. Figs. 12B and 13A show the RNA intermediates present in the reactions analyzed by native electrophoresis. Note the appearance and disappearance of complex B with the products of cleavage at the 5' junction, LE₂ and E₁ in either gel system. The products of 3' cleavage and ligation, L and E₁E₂, appeared at the same time that 5' cleavage was first detected. This taken together with the observation that the products of 5' cleavage did not accumulate, but remained at low levels after activity was detected, suggests that the formation of complex B is the rate limiting step in the splicing reaction. The two gel systems differed mainly in that the TGE system reproducibly resolved complexes
Figure 12: Time Course of Splicing Complex Formation (TGE System).
Splicing reactions containing 3% PEG and MINX precursor RNA were incubated at 30° for the times indicated (min) and aliquots were analyzed by either TGE native gel electrophoresis (Panel A) or denaturing polyacrylamide (10%) electrophoresis (Panel B). Complexes in the I region were detected early in the reaction. Complex A was seen at later times prior to and during splicing. Complexes A' and B appeared coincident with the intermediates of 5' cleavage. Although 5' cleavage is known to precede 3' cleavage and ligation, this order is not apparent in this experiment. Cleavage at the 3' splice junction and ligation appear to occur immediately after 5' cleavage and lariat formation. This may be peculiar to MINX precursor RNA. Abbreviation are: LE2 (lariat-exon 2), Pre (precursor), L (lariat), E1E2 (product), and E1 (exon 1).
Figure 13: Time Course of Splicing Complex Formation (TAE System). Splicing reactions containing 3% PEG and MINX precursor RNA were incubated at 30° for the lengths of time indicated (min). Aliquots were taken and analyzed either by denaturing electrophoresis on a 10% gel (Panel A) or TAE native electrophoresis (Panel B). Panel A shows that at 155 min, the primary species in the extract is ligated exon RNA. Approximately 40% of the input RNA was converted to product. The high efficiency of this particular reaction allowed the visualization of two diffuse complexes, C and D, at late times when analyzed by TAE native electrophoresis. The trend of rapidly migrating complexes early in the reaction to slowly migrating complexes later in the reaction was identical to that observed in the TGE system. The difference between the systems resided mainly in the more reproducible separation of the A and B regions in the TGE system. Early complexes behaved identically in the two systems.
A and B, whereas the TAE system did not. The α complex (see below) was detected and migrated identically with respect to other complexes in both systems. It is interesting to note that a large fraction of the lariat product (L) is converted to its linear form by a debranching activity, in contrast to findings that this region is protected from debranching for β-globin precursors (Ruskin and Green, 1985). The linear intron was identified by gel purification of lariat followed by debranching by enzyme present in the nuclear extract. The extent of this debranching, however, varied between different lots of nuclear extract.

Identification of the Intermediates Present in Complexes. Previous studies have shown the assembly of exogenously added RNA precursors into RNP structures is an orderly process in that factors appear to be added sequentially until the active splicing complex had been obtained (Grabowski et al., 1985; Frendewey and Keller, 1985; Brody and Abelson, 1985; Konarska and Sharp, 1986; Konarska and Sharp, 1987; Pikielny et al., 1986). Although comparison of the time course of complex appearance by electrophoretic and gradient analysis gave some clue as to the relationship between the complexes observed by the two techniques, identification of the splicing intermediates present in the complexes was necessary to help in establishing their relationship to previously identified complexes. Figure 14 shows the two-dimensional analysis of splicing complexes resolved in the TGE system and Figure 15 in the TAE system. The first dimension was a native gel and the second a denaturing RNA gel. Most of the region containing the initial complexes was omitted in this analysis of the TGE system but may be seen in Figure 15. Precursor was found to be present in all three of the slow-migrating complexes. Most of the products of 5' cleavage were found to be localized in the region of the B complex in both systems,
Figure 14: Two-Dimensional Analysis of Splicing Intermediates In Complexes (TGE System). The reaction intermediates contained in each of the complexes visualized by TGE native gel electrophoresis were analyzed by two-dimensional electrophoresis. A splicing reaction incubated for 30 min. at 30° was loaded onto a TGE native gel and electrophoresis was conducted. The RNP first dimension was treated with protease K and denaturing dye prior to electrophoresis in a denaturing RNA gel (13%) second dimension. This analysis demonstrated that splicing intermediates migrated in complex B and that the products of the reaction were in complexes which migrated more rapidly in the first dimension. The comigration of LE₂ and E₁ in complex B is quite evident in this figure.
Figure 15: Two-Dimensional Analysis of Splicing Intermediates In Complexes (TAE System). The distribution of intermediates among the complexes observed by TAE native electrophoresis was analyzed by two-dimensional electrophoresis. A splicing reaction containing 3% PEG was incubated at 30° for 75 min. and loaded onto a TAE native gel. After electrophoresis, the RNP gel was treated as detailed in Figure 14. Reaction intermediates are indicated schematically on the left of a marker lane of unfractionated splicing reaction. The denaturing gel was 12% polyacrylamide and the lariat RNA is not well resolved from the precursor RNA. Again, the intermediates of splicing comigrated with complex B which has already been demonstrated to appear coincident with activity (Fig. 13). The complexes migrating rapidly in the first dimension that contain ligated exons and released lariat are readily evident. The migration in the RNP dimension of the C and I complexes is distinct, the I complex migrating slightly slower.
identifying this species as the "active spliceosome".

It is important to note, however, that this complex as resolved in the gel may have lost components with respect to the actual complex as present in the extract. In this regard, it has been demonstrated that omission of heparin from the loading mixture resulted in the slower mobility of complexes, suggesting that the heparin may displace factors naturally associated with the complexes (Konarska and Sharp, 1987). It has also been demonstrated that in yeast extracts, the addition of excess non-specific RNA to the loading mixture causes more rapid migration of the complexes analyzed by native electrophoresis, in parallel to the effect of heparin addition (Pikielny, et al., 1986). The excess non-specific RNA is added under conditions that allow only the formation of the initial complexes and the effect of RNA addition may simply be to bind up all the non-specific factors such as hnRNP polypeptides, which could otherwise bind weakly to the

A complex migrating in the region of the early complex A in both systems was found to contain both of the products of 3' cleavage and ligation. The precursor remaining in this region of the first dimension probably is in a complex which has not assembled all of the factors required for activity, and the products are probably in a complex which has lost factors as the spliceosome disassembles into simpler RNP structures. The late complex A appears to be rather unstable as the released lariat and the ligated exons were both observed in the very rapidly migrating complexes, C and D. These complexes are probably analogous to the rapidly migrating complexes observed by other researchers when splicing reactions were analyzed by velocity centrifugation through gradients containing high concentrations of salt (Grabowski et al., 1985; Bindereif and Green, 1986). A small amount of the ligated exon species also migrated as a free RNA in the first dimension.
The relationship of complex A' to complexes A and B remains unclear. Both complexes A' and B appeared simultaneously in time courses, making the assignment of an assembly order difficult. This complex is the terminal assembly product for extracts in which U4 snRNA had been cleaved by oligonucleotide-directed RNase H cleavage (see below), indicating that it probably is a discrete species.

Assembly of Deleted Precursors. Studies of the consensus sequence requirements of splicing and of splicing complex formation have revealed that both 3' and 5' sequences are essential for the assembly of the active splicing complex and hence for splicing activity. Gradient analysis of the complexes formed with RNAs containing consensus sequence deletions or point mutations within consensus sequences have demonstrated the absolute requirement of 3' sequences for any specific assembly (Bindereif and Green, 1986; Frendewey and Keller, 1985; Grabowski et al., 1985). In an effort to further correlate the complexes observed by gradient analysis with those resolved by native electrophoresis, several RNAs deleted for consensus sequences were made. The inset of Figure 16 depicts the structure of these RNAs schematically. The RNAs were of roughly the same length, minimizing length effects on complex migration. When these RNAs were added to extract under splicing conditions, each RNA gave a characteristic terminal complex when the reactions were analyzed by native electrophoresis (Fig. 16). A 0 minute time point for a reaction containing MINX RNA and an ATP depleted reaction containing MINX RNA were included as references. As seen earlier (Fig. 12), MINX RNA produced complexes migrating in the I region early in the reaction. All of the RNAs containing consensus element deletions also produce these complexes early in the
Figure 16: Assembly of Complex B Requires ATP and Consensus Elements (TGE System). Splicing reactions containing either the deleted RNAs ΔΔ5' or Δ3' or MINX precursor RNA were analyzed by TGE native gel electrophoresis after incubation for the time indicated and under the conditions indicated. These RNAs are shown schematically in the inset. Thin lines denote transcribed vector sequence, thick lines intron sequence, open circles the site of branch formation, and the hatched boxes exon sequences. ATP was depleted from the extract by preincubation of the extract under reaction conditions prior to the addition of precursor RNA. The RNAs used in this experiment had the structures shown schematically below. At 0 minutes MINX precursor produced only complexes migrating in the I region. These can be divided into \textit{I}_H and \textit{I}_D,  

\[ \text{MINX(220)} \]

\[ \Delta \Delta 5'(289) \]

\[ \Delta E 2(255) \]

denoting the heterogenous and discrete complexes migrating in this region. A diffuse complex with the same mobility as the \textit{I}_D complex was produced when MINX precursor RNA was incubated in ATP depleted extract. ΔΔ5' RNA produced a complex, α, that migrated slightly but reproducibly faster than complex A produced in a wild-type reaction. Δ3' (=ΔE2) produced only complex \textit{I}_H as did RNAs devoid of consensus elements. Much of the Δ3' RNA migrated as free RNA in the native gel suggesting that it either associated with factors inefficiently or its association with factors was not stable to electrophoretic analysis.
reaction (data not shown), suggesting that the factors which give rise to these complexes bind non-specifically. RNA containing the branch point and 3' elements produces a complex, α, later in the reaction that migrated slightly more rapidly than complex A. The same RNA containing an additional 219 nucleotides of pBR322 sequence at its 3' end failed to produce this complex, and produced mainly complexes migrating in the I region (data not shown). It appears that 3' elements present in this RNA are no longer protected from T1 digestion as is the case for the shorter RNA (Rick Padgett, personal communication). Why the addition of 3' sequences should have this effect remains unclear. The addition of this same sequence to the 3' end of either IVPX RNA (producing MZ1 RNA) or MINX RNA (producing MINX Long RNA) did not diminish splicing activity or the ability to form complex B.

Both RNAs containing no consensus elements and RNAs containing the 5' consensus element but no branch point or 3' elements produce only complexes which migrate in the I region. In addition, much of this RNA either fails to assemble or assembles in a fashion that does not survive electrophoresis as is indicated by the large amount of label migrating at the position of free RNA. If gel mobility is a rough measure of particle size, as indicated by several other studies (Konarska and Sharp, 1986; Konarska and Sharp, 1987), RNAs containing the 3' consensus elements are able to tightly bind more factors than RNAs containing the 5' consensus element. The latter appears only to be capable of binding non-specific factors. MINX precursor RNA produces heterogeneous complexes migrating in the upper portion of the I region in the absence of ATP. In theory, these complexes could contain U1 snRNPs because U1 snRNPs are known to bind to precursor RNAs in the absence of ATP (Hinterberger et al., 1983). Analysis of reactions containing deleted RNAs by TAE native gel electrophoresis gave
identical results (data not shown). For all intents and purposes, TGE and TAE native gels produced identical results with the exception that the latter system did not resolve complex A'.

**Assembly In Extracts Containing Cleaved U RNAs.** Cleavage of the RNA moiety of U snRNPs in nuclear extracts inactivates splicing (Kramer et al., 1984; Krainer and Maniatis, 1985; Black et al., 1985; Berget and Robberson, 1986; Black and Steitz, 1986). Potentially, cleavage of each U RNA in turn could assay the step in the pathway to assembly of the active spliceosome requiring a particular. In practice, however, U5 is resistant to RNase H cleavage, and, therefore, its role in spliceosome assembly has not been determined by this technique. Splicing reactions done with MINX precursor RNA in extracts containing cleaved U RNAs did not form complex B when aliquots were analyzed by (TGE) native electrophoresis (Fig. 17). Cleavage of the 5' terminus of either U1 or U2 resulted in the formation of very little complex A. Cleavage U2 RNA at sequences further removed from the 5' terminus allowed the formation of complex A but no slower migrating complexes. Cleavage of the 5' terminus of U4 arrested complex formation at complex A'. From these data, a simple model for spliceosome assembly can be proposed. U1 and U2 snRNPs appear to be required very early in the assembly of the spliceosome because not even complex A is formed in their absence. Complex A is formed, however if internal nucleotides of U2 snRNA are cleaved. Perhaps complex A contains both U1 and U2 snRNPS bound to precursor RNA. It is possible that cleavage of the internal nucleotides of U2 snRNPs allows them to bind precursor RNA in a fashion that stabilizes the interaction of U1, but destroys a recognition site for factors which bind subsequently. U4 snRNP might be such a factor, since cleavage of the RNA in this snRNP allows assembly to the A' complex.
Figure 17: Assembly of Complex B Requires Intact snRNPs (TGE System).

snRNAs in the nuclear extract were cleaved or mock-cleaved using a variety of complementary DNA oligonucleotides and RNase H as described in Methods. MINX precursor RNA was added and incubation was continued for an additional 30 min. The reactions were analyzed by TGE native electrophoresis. A reaction containing RNA lacking 5' consensus elements was included as a marker. U1\(_{O}\) cleaves bases 2 to 11 of U1 RNA. U2\(_L\) and U2\(_S\) cleave bases 30 to 45 and 2 to 15 of U2 RNA, respectively. U4\(_O\) cleaves bases 1 to 15 of U4 RNA. U5\(_O\) is complementary to bases 86 to 102 of U5 RNA but does not cleave U5 RNA presumably because the site of hybridization is inaccessible. DHFR is a sequencing primer and has no homology to any known U snRNA. Control reactions were untreated. Cleavage of extract RNA with U1\(_O\) and U2\(_S\) resulted in the accumulation of complexes migrating in the I region. Treatment with U2\(_L\) resulted in the accumulation of complex A and cleavage with U4\(_O\) resulted in the formation of complexes A and A', but not B. Cleavage with all of these oligonucleotides other than that complementary to U5 RNA, resulted in the complete inhibition of activity (data not shown).

None of the control oligonucleotides had any effect on splicing or complex formation, with the exception that more complex I remained than in untreated extract. These data suggest that the binding of U1 and U2 snRNPs to precursor RNA are early steps in spliceosome formation, that the loop portion of U2 snRNA may play some role in the U1/U2 interaction which allows the addition of other snRNPs, and that the addition of U4 occurs subsequent to the addition of U1 and U2.
Analysis of the Migration of Extract Components. Extract snRNPs, which are subunits of the spliceosome, have sedimentation coefficients between 10 and 15S. Binding of these snRNPs to RNA and to other factors raises their sedimentation coefficient. The same should be true for migration in native gels. In theory, it should be possible to add unlabelled RNA to the extract and monitor the shift in migration of endogenous components as they bind this RNA and bind other factors either by stain or radioactive label. This technique could then be used to identify which factors participate in the formation of spliceosomes simply by looking for factors which show substrate-dependent mobility shifts in reactions analyzed by native gel electrophoresis. In practice, this type of analysis is only possible in lower eukaryotes such as yeast which do not have many intron-containing RNAs. This allows the addition of enough exogenous precursor that it comprises the bulk of the RNA undergoing processing. Extract as prepared from HeLa cells contains large amounts of RNA associated with splicing factors without the addition of any exogenous substrate. Centrifugation of the extract which results in the removal of large hnRNP particles aids this type of analysis somewhat.

Splicing reactions containing $^{32}$P labelled, cleared extract and unlabelled precursor or pBR322 RNAs (50 ng) were analyzed by two-dimensional electrophoresis (Fig. 18). Reactions were first electrophoresed on a TGE native gel and the on a 10% polyacrylamide denaturing gel. The migration of each of the abundant small RNAs in the native dimension is clearly evident from this analysis. Examination of the panel containing pBR322 RNA (Control) shows that of the abundant U snRNPs, U3 snRNPs (RNA=217 bases) migrate most slowly and in the region also occupied by 5.8S ribosomal RNA. U1 (RNA=164 bases) and U5 snRNPs (RNA=115 bases) migrate further into the first dimension. U4 (RNA=145 bases) and U6 snRNPs (RNA=108 bases) comigrate perfectly and have been shown to be in the same particle by a variety of techniques including psoralen cross-linking (Rinke et al., 1985). U2 snRNPs (RNA=188 bases) migrate the
Figure 18: Migration of Endogenous Particles and Substrate-Dependent Mobility Shifts. Substrate-dependent mobility shifts for endogenous snRNPs which place them in the region of the active spliceosome as identified with radiolabelled substrate have been used to identify snRNPs which may participate in spliceosome formation. Splicing reactions containing 12 ul of $^{32}$P labelled extract (see Methods) and 3 ul of unlabelled extract (and no Roeder D) cleared of particulates by 5 minutes of centrifugation and roughly 50 ng of either unlabelled IVPX (SUBSTRATE) or pBR322 (CONTROL) RNAs were incubated at 30° for 30 minutes and analyzed by TGE native electrophoresis. The RNP dimension was then electrophoresed in a denaturing RNA gel (10%) second dimension and RNAs were visualized by autoradiography. U2, U4, and U6 snRNPs were seen to undergo substrate dependent mobility shifts, the movement of U2 snRNP being the most pronounced. U1 and U5 snRNPs did not show a substrate-dependent mobility shift and remained dispersed through a large portion of the RNP first dimension. It is also evident from this analysis that some portion of pre-tRNA migrated as an RNP in the first dimension, possibly indicating the presence of factors which process this RNA. tRNA migrated solely as free RNA in the first dimension, indicating that it lacks whatever sequence or conformation is necessary for the formation of RNP structures. The RNP dimension marker was a reaction incubated just long enough to give complex A. The RNA dimension markers were RNAs from the $^{32}$P labelled extract. A small amount of extract was placed into formamide loading dye and electrophoresed.
most rapidly in the first dimension. It is obvious from this analysis that RNA size does not
determine mobility in the native gel. Of special interest is the migration of pre-tRNA, a
portion of which migrated as free RNA and a portion of which migrated as a complex of
unknown nature. The analysis of the same reactions by TAE native gel electrophoresis
gave identical results.

Comparison of the analysis of a reaction which contained unlabelled MINX precursor
RNA (Substrate) with that containing pBR322 RNA (Control) showed that the snRNPs U2,
U4, and U6 underwent substrate-dependent mobility shifts. U1 and U5 snRNPs did not,
but remained dispersed throughout a large region of the first dimension. This analysis
demonstrates that U2, U4, and U6 snRNPS associate with substrate RNA in some manner
which produces a change in their mobility. Why U1 and U5 snRNPs do not undergo a
mobility shift in the presence of substrate is not known. U5 was demonstrated to undergo
a substrate-dependent mobility shift in the heparin-free TG system described by
Konarska and Sharp, but U1 snRNPs failed to show a shift (Konarska and Sharp, 1987). It
is important to point out that the controls in each experiment were different. Konarska
and Sharp used the addition of no RNA with and without ATP as their controls, whereas
the addition of pBR322 RNA in the presence of ATP was used in the experiment in
Figure 18.
Characterization of Complexes Eluted from Native Gels

Immunoprecipitations from Whole Extract: The experiments presented up to this point have shown that the assembly of the splicing complex is a complicated but orderly process. Factors bind to precursor RNAs added to splicing extracts in a stepwise fashion until a 50S particle is formed that catalyzes splicing and then dissociates into simpler structures. But the question of what factors are in each complex resolved by native electrophoresis has not yet been addressed. Two approaches were used to answer this question. Precursor RNA in the gel should still be associated with the factors which retarded its migration. These complexes should be immunoprecipitable with antibodies directed against such factors. The antibodies which were available were antibodies directed against a common snRNP antigen and against p68 of U1 snRNPs. Of primary concern was to demonstrate that U1 snRNPs were still associated with precursor RNA and intermediates in the native gel, since in the other gel systems available this association was apparently not maintained. The other approach which can be used is to demonstrate protection from degradation for the RNA residing in gel isolated complexes. Either the immunoprecipitation of T1 nuclease resistant fragments or RNase H protection could be used to monitor the association of factors with RNA in complexes. Data obtained by T1 analysis were somewhat preliminary, but indicated that the pattern of protection is nearly identical for gel isolated complexes and whole extract. Only the results of RNase H protection experiments will be given here.

The association of factors with precursor RNA consensus elements was initially probed in whole extract using deleted precursor RNAs. Figure 19 shows the results for such an experiment. Panel A shows the immunoprecipitation of complexes containing added RNA from whole extract and Panel B from extract in which U1 snRNPs have been cleaved using oligonucleotide-directed RNase H cleavage. Note that cleaved U1 RNA no
Figure 19: U1 snRNP's Recognize RNA by Mechanisms In Addition to Hybridization. Reactions in normal extract (Panel A) or in extract in which U1 snRNA had been cleaved (Panel B) and containing various precursor RNAs deleted for consensus elements were assembled on ice and immunoprecipitated with control serum (C), anti-U1 sera (R1,R2), and anti-U snRNP monoclonal (S). The RNAs used were IVS (wild-type), IVSΔ3' (deleted for branch point and 3' elements), IVSΔ5' (deleted for active 5' splice junction), IVSΔΔ5' (deleted for both 5' splice sites, contaminated with IVSΔ5', and pBR322 (containing no consensus sequences). Panel A shows that in whole extract, U1 snRNPs were able to interact with all RNAs containing at least some consensus elements, although less well than with IVS precursor RNA. This was somewhat surprising because U1 snRNPs were thought to interact with RNAs solely by RNA/RNA hybridization at the 5' splice junction. IVSΔ5' and IVSΔΔ5' both lack this sequence, and hence should not be immunoprecipitable if this were the sole mechanism of interaction. Removal of the 5' end of extract U1 snRNPs, the sequence which has been shown to hybridize to the 5' splice junction, did not alter the relative amount of each RNA immunoprecipitable with each antibody, but the net amount of each RNA immunoprecipitable dropped dramatically with respect to immunoprecipitations done in the presence of intact U1 snRNPs. A much longer exposure was used to generate the autoradiogram in panel B than in panel A. The extraneous RNA in the IVSΔΔ5' lanes that comigrates with IVSΔ5' is RNA arising from incomplete digestion of the template DNA from which the RNA was made and has the same sequences present as IVSΔ5'.
longer interact with the 5' consensus element via RNA-RNA hybridization. Equal cpm amounts of each RNA were used in both experiments.

IVSΔ3' still retains 5' consensus sequence and could potentially have hybridized with intact U1 snRNPs. However, this hybridization appears only to occur in RNA which retains the 3' elements as well (IVS). The immunoprecipitability for 5' deleted RNAs (IVSΔ5', IVSΔΔ5') with anti-snRNP antibody (S) has also decreased with respect to the control (IVS,S), indicating that the binding of factors to 5' elements stabilizes the interaction of factors with 3' elements. Note that the removal of the 3' terminal 5' splice junction from IVSΔ5' RNA, creating IVSΔΔ5' RNA, had no detectable effect on immunoprecipitability with any antibody. This observation suggests that this splice junction is not recognized by any factors. pBR322 RNA was not immunoprecipitable with any antibody. Note that although relative band intensities are approximately identical in both Panels A and B, a much longer exposure was required to visualize radiolabelled RNA in Panel B, indicating that the removal of sequences from U1 RNA destabilizes interaction of this particle with RNA, even interactions at the 3' terminus of the intron. It is not clear why the removal of U1 sequences which have been demonstrated to interact with 5' intron sequences should affect the interaction of this particle with 3' intron sequences. Perhaps there is a change in the conformation of the U1 snRNP particle which reduces its ability to bind 3' intron sequences or factors bound there. Alternatively, cleavage of the 5' terminus of U1 RNA could result in the loss of as yet undescribed protein factors essential for the observed binding to the 3' end of the intron.

Prior to attempts to immunoprecipitate complexes eluted from native gels, it was necessary to demonstrate that the EDTA/heparin treatment did not dissociate factors from RNA in the native gel. This was done by subjecting whole splicing reactions to treatments designed to simulate gel conditions (Fig. 20). The splicing reactions were placed into loading dye and incubated at 30° just as they would have been prior to
Figure 20: EDTA Stabilizes the Association of U1 Antigen with the Splicing Complex Under Gel Conditions. Splicing reactions containing radiolabelled MINX precursor RNA were incubated at 30° for 30 min. Reactions were either immunoprecipitated without further treatment (-) or after dilution to 200 ul (dil) or after dilution and addition of EDTA to 10 mM (dil+EDTA) or glycerol to 10% (dil+gly) or both (dil+gly+EDTA). EDTA was also added to 10 mM for one reaction without dilution (EDTA). All reactions containing EDTA had been brought to 2.5 mg/ml heparin and heated to 30° prior to dilution. Immunoprecipitation were conducted with either control serum (C), anti-U1 serum (R), or anti-U snRNP monoclonal (S), RNA was prepared and analyzed by electrophoresis on a 13% denaturing gel and visualized by autoradiography. The amount of splicing intermediates recovered by virtue of their association with antigen was always greater for the anti-UsnRNP antibody that for the anti-U1 antibody. However, the best recoveries for diluted samples were obtained either after the addition of glycerol or EDTA. Abbreviations are: LE2=lariat-exon 2, L=released lariat, E1E2=product.
electrophoresis. Entry into the gel was simulated by dilution into gel buffer (TAE). Of special interest was immunoprecipitability with anti-U1 antibody (R). It was found that both glycerol and EDTA stabilized association of the U1 antigen with radiolabelled intermediates.

**Immunoprecipitation of Gel Isolated Particles:** As a means to address the question of what factors are associated with RNA in the complexes resolved by native electrophoresis, complexes were eluted from the gel and immunoprecipitated with a variety of antibodies. Early experiments involving the elution of complexes containing IVPX precursor RNA (442 bases) from TAE native gels containing 10% glycerol demonstrated the feasibility of this technique and that the hnRNP C polypeptide was associated with RNA in this system. A trend of decreased immunoprecipitability with decreasing complex mobility was observed. This has also been seen for whole extract in that as the reaction proceeds, complexes containing the input RNA and intermediates become less immunoprecipitable (Gideon Dreyfuss, personal communication). Whether this reflects loss of the C polypeptide as other factors are added to the RNA or burial of its epitope is not known at present. Immunoprecipitations were also done with anti-U1 and anti-snRNP antibodies in which intermediates were immunoprecipitated.

Similar experiments were done for complexes eluted from TGE native gels after location by brief autoradiography. The complexes B, A, and I_D were eluted into Roeder buffer D and immunoprecipitated using either control serum or anti-U1 (RNP) and anti-snRNP (Sm) antibodies after the addition of approximately 50 ng of unlabelled MINX RNA (Fig. 21). The RNA recovered was analyzed by electrophoresis on a 13% denaturing polyacrylamide gel. The amount of unlabelled RNA added was in gross excess to any extract RNAs as could be demonstrated by silver stain. Analysis of aliquots of eluted complexes showed that the distribution of intermediates among the complexes was
Figure 21: Complex B Contains U1 Antigen as Assayed by Immunoprecipitation (TGE System). Splicing reactions containing radiolabelled MINX precursor RNA were electrophoresed on a TGE native gel. The gel was subjected to brief autoradiography at 4° to locate complexes. The areas containing complexes were excised and complexes were eluted as described in Methods. Approximately 50 ng of unlabelled MINX RNA were added to bind extract factors which comigrated in the gel with the labelled complexes and were also eluted. These factors could have potentially bound to labelled MINX RNA released from complexes during elution. The unlabelled RNA was incubated on ice with the eluted complexes for 10 minutes prior to the addition of antibody. After incubation with antibody, antibody-antigen complexes were recovered with Pansorbin and RNA was prepared and displayed on a 13% denaturing gel. Splicing intermediates are indicated schematically on the left. The eluted complexes B, A, and I (see Fig. 12) were subjected to immunoprecipitation with control antibody (Control), anti-U1 antibody (RNP), and anti-snRNP antibody (Sm). The starting material is also shown, either by silver stain (lanes 1-3) or autoradiography (lanes 4-6). Lanes 1-3 show that the amount of unlabelled precursor RNA was in gross excess to any U snRNPs eluted from the gel for any of the complexes analyzed. Lanes 4-6 show the same distribution of intermediates for each of the complexes as was seen before (Figs. 14,15) and represents the starting material for immunoprecipitation. Lanes 7-9 represent material which either bound non-specifically to antibody or Pansorbin or else was particulate in nature. Lanes 10-11 were immunoprecipitated with an anti-U1 antibody reacting specifically with p68 (Fig. 4). It is apparent that for the B complex (lane 10), the immunoprecipitated distribution of intermediates was not the same as that for the starting material (lane 4). The complex containing the L2 intermediate appears to contain U1 snRNPs and to be highly antigenic. A corresponding amount of E1 was also immunoprecipitated. The lariat species, L, does not appear to be associated in a stable fashion with U1 snRNPs. The amount of each intermediate immunoprecipitable with anti-snRNP antibody was much higher than that immunoprecipitable with the anti-U1 antibody. This may reflect in part the different titres of the antibodies used. The lariat product was highly precipitable with anti-snRNP antibody, indicating that it may still be associated with some snRNP other than U1 (lanes 13,14). Although large quantities of product, E1E2, were present in complex I (lane 6), this species was not immunoprecipitable with either antibody (lanes 12,15).
identical to that seen by two-dimensional analysis (Fig. 14). Complexes B and A were highly immunoprecipitable with either anti-U1 or anti-snRNP antibody compared with complex $I_D$. As is the case in whole extract, the LE$_2$ and E$_1$ intermediates remained co-immunoprecipitable. For this particular substrate RNA, the product RNA, E$_1$E$_2$, does not appear to be immunoprecipitable with either anti-U1 or anti-snRNP antibody.

A similar experiment to that described above was conducted for complexes formed with the 5' deleted RNA, ΔΔ5'. Complexes $\alpha$ and $I_D$ were eluted and subjected to immunoprecipitation with the same antibodies used in the previous experiment (Fig. 22). Both complexes were found to be slightly immunoprecipitable with anti-U1 antibody, suggesting weak interaction of U1 snRNPs with the RNA in these complexes. Complex $\alpha$ was highly immunoprecipitable with anti-snRNP antibody indicating the presence of a snRNP in addition to U1.

**Oligonucleotide-Directed RNase H Cleavage Studies:** Eluted complexes were also analyzed by oligonucleotide-directed RNase H cleavage. Several oligonucleotides complementary to consensus elements within MINX precursor RNA were synthesized (Fig. 23). Prior to experiments on eluted complexes, cleavage was optimized using free RNA under the conditions required for assay of eluted complexes. It was found that MgCl$_2$ must be added to the eluted complexes to overcome the inhibitory effect of EDTA present in the native gel buffer (Figure 24).

As controls for the assay of eluted complexes, whole reactions were analyzed using all four oligonucleotides with or without the addition of EDTA/heparin (Fig. 25). The amount of protection for each sequence element was found to be virtually identical in both cases, with the exception of the polypyrrimidine track, which appeared to be more protected in the sample containing EDTA/heparin. Analysis of eluted complexes demonstrated slight protection of 3' elements in the $I_D$ complex and protection of all elements in the A
Figure 22: Complex α Contains a snRNP In Addition to U1 as Assayed by Immunoprecipitation (TGE System). Splicing reactions containing ΔΔ5' RNA were electrophoresed on a TGE native gel. The gel was subjected to brief autoradiography at 4° to locate complexes. The areas containing complexes I₀ and α were excised and complexes were eluted as described in Methods. Approximately 50 ng of unlabelled MINX RNA were added to bind extract factors which comigrated in the gel with the labelled complexes and were also eluted. Immunoprecipitation and RNA analysis were conducted as described in Figure 21. Although more radiolabelled complex was eluted from the I₀ region (lane 17) than the α region (lane 16), the signal from the α region was higher than that from the I₀ region when immunoprecipitation was conducted with control antibody. This probably reflects the larger size of the α complex in that it is more likely to be found in the pellet in the absence of antibody binding. Although approximately equal amounts of RNA from the I₀ were recovered with either anti-U1 or anti-snRNP antibodies, complex α was much more immunoprecipitable with anti-snRNP anti-bodies than with anti-U1 antibodies, indicating the possible association of a snRNP in addition to U1.
Figure 23: Oligonucleotides Complementary to MINX Consensus Elements. Several DNA oligonucleotides were synthesized which are complementary to the recognized consensus elements in MINX RNA. The 5' oligonucleotide encompasses the 5' splice junction, the Bp oligonucleotide the branch point, the Py oligonucleotide the polypyrimidine track, and the 3' oligonucleotide the 3' splice junction. The boxed sequences are exons and the bold letter A indicates the site of branch formation. Key nucleotides are numbered. All of these oligonucleotides are also complementary to the same elements in IVPX RNA, with the exception that the 5' oligonucleotide now is complementary to an unused 5' splice site rather than the active one located closer to the cap.
Figure 24: Optimization of RNase H Activity Under Assay Conditions. MINX precursor RNA in 100 µl of Roeder buffer D containing 2 mM EDTA and 0.5 µg/ml yeast RNA was subjected to cleavage in the presence of 50 µg/ml of Bp oligonucleotide (Fig. 23). MgCl₂ was added to a final concentration of 0 mM, 2.5 mM, or 5 mM to overcome the inhibition of RNase H by EDTA. Cleavage was initiated by the addition of 1 µl (0.8 U), 0.1 µl (0.08 U), or 0.01 µl (0.08 U) of RNase H. Cleavage was for 20 minutes at room temperature. The RNA was precipitated by the addition of 200 µl of ethanol and analyzed by denaturing gel (15%) electrophoresis. The condition chosen for the analysis of gel isolated material was 2.5 mM MgCl₂ and 1 µl (8 U/ml) of RNase H. These concentrations gave almost complete cleavage of the input RNA in an environment suitable for eluted complexes. The radiolabelled markers were made by labelling HpaI cut pBR322 with α-³²P dCTP and Klenow enzyme. The DNA fragments have the sizes in base pairs: 622, 527, 404, 309, 242/238, 217, 201, 190, 180, 160, 147, 122, 110, 90, 76, 67, 34, 26, 15, and 9.
Figure 25: 5' Splice Junction and Other Consensus Elements are Protected from RNase H Digestion in Gel Isolated Complexes. The conditions given in Figure 24 were used to analyze complexes from whole extract (Rx), reaction prepared for gel analysis by the addition of EDTA and heparin (Load) and complexes I_H, I_D, and A eluted from a TGE native gel after electrophoresis of a 30 minute reaction containing MINX precursor. I_H and I_D refer to the heterogenous and discrete bands in the I region (see Fig. 12). RNA from each sample was analyzed on a 10% polyacrylamide denaturing gel prior to cleavage (T), or after cleavage with 5' oligonucleotide (5'), branch point oligonucleotide (B), polypyrimidine track oligonucleotide (P), or 3' oligonucleotide (3') in the presence of RNase H. Although cleavage of the RNA was much more complete than desired, several things are evident. The 5' splice junction was found to be most susceptible to cleavage in both whole extract or heparin and EDTA treated extract. The amount of cleavage observed with each oligonucleotide was approximately equivalent in both cases, with the exception that the polypyrimidine track was less susceptible to cleavage in the latter. Very little protection was seen for the eluted I complexes for any of the oligonucleotides tested with the exception that some protection of the 3' elements was seen for the I_D complex. The pattern of protection seen for the eluted A complex was very similar to the pattern seen for whole extract. The major difference was that the 3' splice junction was much less protected than for the whole extract with or without EDTA and heparin treatment. This suggests that the association of factors which protect the RNA from oligonucleotide directed RNase H cleavage is very similar in eluted complexes and whole extract, the primary differences being at the 3' splice site and not the 5' splice site. The ▼ denotes the RNase H cleavage fragments. The markers were Hpa II digested pBR322 and had the lengths given in Fig. 24.
complex. The relative amount of protection seen with each oligonucleotide was similar to that seen in treated or untreated whole extract, indicating that the complexes resolved in this native system have not lost essential factors.
DISCUSSION

Data have been presented which show, by native gel analysis, the orderly, step-wise assembly of the splicing complex onto RNAs which contain all of the consensus elements shown to be essential for splicing by others. Lack of 3' or 5' elements leads to the formation of abortive complexes as does the cleavage of the RNA moiety of various snRNPs. The requirement of extract RNAs and ATP has also been demonstrated.

The native gel system used in these experiments seems to maintain native structure well enough to allow protection experiments and immunoprecipitation experiments for complexes eluted from the gel. These techniques have been used to probe the identity of factors associated with RNA in the gel which retard its migration with respect to free RNA. This has allowed the identification of at least U1 snRNPs in several of the complexes resolved by native gel analysis. Other snRNPs can be postulated to be present in some complexes based upon complex formation in extracts containing cleaved snRNPs, RNase protection of known binding sites, and ATP requirements for assembly. Based on these observations, a model correlating the complexes resolved by native gel electrophoresis with snRNP composition can be proposed (Fig. 26). This model is reasonably consistent with all of the data presented as well as that of others, but postulates the existence of several complexes for which there is not much data. This model is, however, quite testable. This model also allows the construction of a hypothetical assembly scheme for an RNA having all consensus elements (Fig. 27). The complex having both U1 and U2 snRNPs bound to the 3' consensus element has the same composition and structure as has been postulated for the α complex (Fig. 26) and has been seen in reactions containing MINX precursor which have undergone many freeze-thaw cycles prior to electrophoretic analysis. Again, the predictions made by this
Figure 26: Hypothetical Correlation of Complexes Resolved by Native Gel Electrophoresis with snRNP Composition of These Complexes. The model presented above is reasonably consistent with the data presented. The complexes visualized by native gel electrophoresis are shown in their relative positions with respect to one another and are labelled as discussed in the text. Precursor RNA is shown schematically, boxes denoting exon sequence and lines intron sequence. The small circles represent U snRNPs as indicated. The 1H complexes appear not to contain any specific factors based upon their immunoprecipitability and formation with RNAs having no consensus sequences. The consensus elements of RNA in these complexes are not protected as demonstrated by RNase H analysis. The 1D complexes appear to contain U1 snRNPs based upon that they are immunoprecipitable from ATP depleted reactions and also when isolated from gels. Furthermore, RNase H analysis demonstrated weak protection of 3' elements in this complex. Complex α appears to contain weakly bound U1 snRNPs, as well as U2 snRNPs based on the immunoprecipitation of gel isolated material. Complex A probably has a similar snRNP composition, but the 3' and 5' elements may now have been brought into juxtaposition, giving a circular RNA with aberrant migration properties. Both 3' and 5' elements are protected from RNase H cleavage in this complex. Complex A' may represent the association of U4/U6 snRNPs with the snRNPs already present in complex A. It is likely that this complex occurs as an artifact of U5 snRNP dissociation during electrophoretic analysis. The only evidence that this complex contains U4 snRNPS comes from experiments conducted in extracts containing cleaved U4 snRNPs. In these extracts, assembly is aborted at the level of complex A'. Complex B contains all of the common U snRNPs as has been demonstrated by a number of techniques. With respect to the difference between complexes A' and B, it is equally likely that complex A' contains U5 snRNPs but not U4/U6 snRNPs, because it cannot be ascertained what effect cleavage of U4 snRNPs has on assembly. The U4/U6 particle may be rendered unable to bind a U1/U2/U5 particle (complex A' for this assumption), or it may block the addition of further factors such as U5 snRNPs, as depicted above.
Gel Complex

- B
- A'
- A
- α
- I_D
- I_H

snRNP Composition

- U1
- U2
- U4
- U6
- U5
- U8
- U11

- A
- A'
- α
- I_D
- I_H
Figure 27: Hypothetical Scheme for Spliceosome Assembly. A hypothetical assembly scheme for the splicing complex based upon data presented herein as well as experiments of others. Again, boxes represent exon sequences and the lines in between, intron sequences. The small spheres represent the hnRNP C polypeptide and the large spheres the snRNPs, as indicated. Reaction intermediates and products are shown schematically as before. The first step in assembly is the association of hnRNP polypeptides in a weak and non-specific fashion with the precursor which would give rise to the heterogeneously migrating complexes seen early in the splicing reaction and seen with RNAs lacking consensus sequences. The next stage involves the association of U1 snRNPs by an unstable protein/RNA interaction. After the addition of U2 snRNPs, U1 snRNPs interact with the 5' splice site. In the event that the association of one snRNP is not possible, aberrant complexes which are less stable than the correct complex are formed. After the U1/U2 complex forms, other snRNPs are added. There is some evidence for the existence of a U4/U5/U6 preparticle which may bind at this stage to give a complex containing all the snRNPs known to be in the spliceosome. During the first step of splicing, the 5' consensus element with U1 bound is brought very close to the branch point which has U2 bound to it. The presence of two 10S particles in such close proximity may destabilize the association of one, probably the U1 snRNP, since U2 snRNPs are known to remain associated with the branch point after its formation. U1 snRNP may dissociate completely or bind to sequences in the first exon to prevent its diffusion. There is very little evidence concerning this point, but if this occurred, it might not be unreasonable to expect to find U1 associated with ligated product. After 3' cleavage and ligation, the U4/U5/U6 complex probably dissociates, awaiting another round of catalysis.
U2_i metastable
stable
Addition of other snRNPs and factors
Catalysis and Resolution
model could be tested using the techniques described in this work.

Further analysis of the splicing complex will probably involve fractionation experiments. The native gel system provides a rapid tool for studying the complexes made by various fractions and is already being used in this fashion.
APPENDIX I

Splicing of 3’ Deletion RNA to Cryptic Sites. An RNA deleted for 3’ consensus elements was made which had about the same length as IVPX RNA through the addition of pBR322 sequence to its 3’ terminus. This RNA was found to undergo splicing when placed in nuclear extract under splicing conditions (Fig. 28). The released first exon had the size expected for proper use of the 5’ splice junction still present in this RNA. Two product RNAs were observed which differed in length by 10 to 20 nucleotides. The length of the product RNAs defined the 3’ splice junction and all other 3’ elements, including the polypyrimidine track, in pBR322 sequences. No attempts were made to map the exact location of the 3’ splice site, although it is quite feasible to do this.

Splicing of a Three-Exon, Two-Intron RNA. A more complicated RNA was constructed to examine factors influencing the order of intron removal from a multi-intron RNA. Figure 29 shows the possible fates of such an RNA. Two pathways lead to correctly spliced product and can be differentiated by the intermediate RNAs. One pathway leads to removal of the second exon from the product RNA. This RNA was synthesized and gel purified and placed in extract under splicing conditions. It was found that both of the pathways leading to correctly spliced product were used (Fig. 30), but more RNA was processed by removal of the first intron prior to the second. The intermediates diagnostic for exon-skipping were not observed, even upon lengthy autoradiography. This RNA could potentially be used as a model for alternate splicing through the addition of sequences which specifically bind a protein which can be supplied externally. The Ψ sequence of MMLV might provide such a sequence since it is recognized specifically by coat proteins. If this sequence were placed in proximity to a splice junction, the addition of purified MMLV coat protein might lead to skipping of this junction.
Figure 28: Detection of Cryptic 3' Splice Junction in pBR322 Sequences. In an effort to make deletion RNAs equivalent in length to IVPX RNA (MINX had not been constructed yet), ΔE2L (see Fig. 5) was constructed. It was initially very distressing to find that ΔE2L RNA formed both complexes A and B upon RNP analysis of reactions containing it. Only complexes migrating in the I region had been observed previously with shorter RNAs lacking the branch point and 3' junction. When RNA from a splicing reaction containing ΔE2L was analyzed on a 10% denaturing gel, the reason for formation of complexes A and B became evident. This RNA was indeed being spliced. The length of exon 1 was the size expected for proper use of the 5' splice junction. The migration of the lariat species was about the same as for IVPX RNA (see Fig. 11), indicating that the 3' splice junction was approximately in the same location as the 3' junction in IVPX RNA. This is roughly 231 nucleotides 3' of the 5' splice junction, or 79 nucleotides into pBR322 sequences brought in by the deletion of a HindIII fragment during cloning. A better estimate can be made from the sizes of the product RNAs, roughly 230 and 240 nucleotides. This would make the 3' exon roughly 124 or 134 nucleotides in length.
Figure 29: Alternate fates of a Three-Exon, Two-Intron RNA. A two intron, three exon RNA species can undergo splicing by one of the several pathways indicated. Exons are indicated by boxes and introns by lines. Each exon is shaded differently for clarity. The intermediates of the pathways on the left are different, but both result in properly spliced product. The splicing of exon 1 to exon 3 results in incorrectly spliced product, the result of "exon-skipping." These reactions are all distinguishable because they result in characteristic intermediates. The pathway shown on the right, RNA circularization, occurs readily with uncapped RNA and is dependent on the lot of extract used. Whether this circular RNA is a substrate for splicing or not is unknown.
Figure 30: Splicing of a Three-Exon, Two-Intron RNA. As discussed in the previous figure, a two intron RNA can be spliced by a number of pathways. Although exon skipping is rarely observed *in vivo* for RNAs which do not exhibit alternate splicing, it is often observed *in vitro*. EIEIO RNA was incubated in extract under splicing conditions (+ 3% PEG), for the times indicated and the reaction RNA was analyzed by denaturing polyacrylamide (5%) gel electrophoresis. Reactions containing MINX and MZ1 precursor RNAs were also analyzed and their reaction intermediates provide useful markers. Neither the super-lariat species nor the short product RNA were detected in this assay, indicating that the RNA undergoes exon skipping at undetectable levels. What was observed, however, was the more rapid removal of the first intron than the second. The lariat-exon2-intron2-exon3 species indicating cleavage at the 5' junction of intron1 was detectable before the lariat-exon3 species which indicates cleavage at the 5' junction of intron2 (before or after the removal of the first intron). But, for some molecules of precursor the second intron was removed prior to the first, as indicated by the presence of the exon1-intron1-exon2-exon3 species. For this substrate, the order of intron removal appears random, but the first exon appears to be removed with more rapid kinetics. In contrast, each half of EIEIO (IVPX and MINX) is spliced with roughly the same kinetics (see Fig. 11). The intermediates of the reaction were identified by comigration with linear DNA markers for the linear intermediates and comigration with lariat intermediates of identical configuration for the lariat intermediates. The latter were provided by the reaction of MINX or MZ1 RNAs.
Assembly of Exogenously Added U RNAs Into RNPs. In an effort to identify the snRNPs in complexes resolved in native gels directly, $^{32}$P labelled snRNAs were incubated in HeLa cell nuclear extracts. It was hoped that the free RNAs might interchange with unlabelled RNAs in the corresponding snRNPs at some low level, thereby "tagging" the snRNP. In the experiment shown in Figure 31, the labelled RNAs were only incubated in the extract for 30 minutes. Much longer incubation times are probably feasible since the U RNAs are highly stable in the extract. Surprisingly, association of factors with the added RNA was observed upon native gel analysis. The addition of factors appears to be quite rapid for U1 RNA and occurs more slowly for the other U RNAs. The composition of these complexes could be assayed by immunoprecipitation of eluted complexes, or in the cases where only one complex is visible, by immunoprecipitation of the whole reaction. Pre-tRNA was found to form a complex having the same mobility as the complex seen in unfractionated extracts. This might represent the pre-tRNA processing endonuclease which has been shown to have a separate binding and cleaving activity (Baldi et al., 1986). Note that less radioactivity was present in the slowly migrating complex after incubation than prior to it, and more radioactivity was present at the position of tRNA after incubation. This suggests conversion of the pre-tRNA in the complex to material comigrating with tRNA. The assembly of snRNAs into snRNPs is currently under investigation.
Figure 31: Assembly of Exogenously Added U RNAs Into RNPs. $^{32P}$ labelled RNAs from in vivo labelled extract were purified from a denaturing polyacrylamide (10%) gel as described in the text. These RNAs were then added to nuclear extract under splicing conditions and incubated at 30° for 0 or 30 min. The RNP particles formed were then analyzed by TAE native electrophoresis. All of the snRNAs which could be detected by autoradiography assembled into RNP structures of unknown composition, some extremely rapidly. The nature of these complexes is currently under investigation. In addition, 5S ribosomal RNA and pre-tRNA both gave complexes which migrated in roughly the same location as the complexes containing these RNAs from $^{32P}$ labelled whole extract (see Fig. 18). Note that the amount of pre-tRNA in complex form at 0 minutes is greater than the amount of pre-tRNA in complex form at 30 minutes, with a corresponding increase in the amount of tRNA. This conversion of pre-tRNA to tRNA was also observed upon RNA analysis by denaturing electrophoresis (data not shown), indicating that the HeLa cell nuclear RNA is also capable of processing pre-tRNA to tRNA. The complex observed with pre-tRNA in this extract may or may not be responsible for this conversion.
APPENDIX 2: PUBLICATIONS

Papers


Manuscripts Submitted


Manuscripts in Preparation

Zillmann, M., M. Zapp, and S.M. Berget. Analysis of splicing complexes containing U1 snRNPs by electrophoresis in a native gel system containing high concentrations of EDTA. Manuscript in preparation.

Abstracts


LITERATURE CITED


