Yeast mRNA Splicing in Vitro*

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Synthetic actin and CYH2 pre-mRNAs containing a single intron are accurately spliced in a soluble whole cell extract of yeast. Splicing in vitro requires ATP. The excised intron is released as a lariat in which an RNA branch connects the 5' end of the molecule to the last A in the "intron conserved sequence" UACUAAC. Two other discrete RNA species produced during splicing in vitro may represent reaction intermediates: free, linear exon 1 and a form of the intron lariat extending beyond the 3' splice site to include exon 2. Both lariat forms correspond to molecules previously shown to be produced during yeast pre-mRNA splicing in vivo.

Many eukaryotic genes contain intervening sequences which must be precisely removed from the primary transcript by RNA splicing before the genetic information can be expressed. Recently, the development of mammalian cell-free systems capable of splicing messenger RNA precursors has provided the opportunity to study this process in vitro (Hernandez and Keller, 1983; Padgett et al., 1983a; Krüner et al., 1984). Analysis of the RNA species generated during mammalian pre-mRNA splicing in cell-free systems has led to the formulation of a two-step for intron removal (Ruskin et al., 1984; Padgett et al., 1984). The first discernible event is a cut at the 5' splice site, accompanied by the formation of an unusual RNA branch, which links the 5' end of the intron to a specific site a short distance upstream of the intron-exon 2 junction. Hence, the nucleotide at the base of the branch participates simultaneously in both 2'-5' and 3'-5' phosphodiester bonds, and most of the intron forms a closed loop. Such structures have been christened lariats (Ruskin et al., 1984; Grabowski et al., 1984; Padgett et al., 1984). The second step involves cleavage at the 3' splice site to release the intact, excised intron, still in the form of a lariat, and ligation of the two exons to produce the messenger RNA. Lariat RNAs and RNA branches have also been detected in vivo in animal cells (Wallace and Edmonds, 1983; Zeitlin and Efratadias, 1984).

Studies of yeast pre-mRNA splicing in vivo suggest that a broadly similar mechanism could operate in yeast (Domdey et al., 1984; Rodriguez et al., 1984). Splicing of yeast actin pre-mRNA in vivo leads to the formation of intron lariat, containing an RNA branch, and another lariat molecule analogous to the intron-exon 2 species described for the mammalian system. Nevertheless, the introns in yeast messenger RNA precursors are distinguished in certain respects from their mammalian counterparts. Firstly, all Saccharomyces cerevisiae introns sequenced to date contain a sequence TACTAAC near their 3' end, and this sequence is essential for splicing (Langford and Gallwitz, 1983; Pikiely et al., 1983). The third A in this conserved sequence is the site of the branch in the intron lariat (Domdey et al., 1984; Rodriguez et al., 1984). Secondly, the 5' end of yeast introns is characterized by the almost invariant sequence GTATGG. The strict sequence conservation of these splicing signals in yeast is in contrast to the variability of their counterparts in mammalian introns (Mount, 1982; Keller and Noon, 1984). Accordingly, introns of higher eukaryotes are generally refractory to splicing in yeast (Langford et al., 1983; Watts et al., 1983).

There are compelling technical advantages for studying pre-mRNA splicing in yeast. Not least is the opportunity to use genetic approaches to identify and analyze the components of the splicing machinery. The genetic approach, however, demands the development of an in vitro system, capable of splicing messenger RNA precursors, and the biochemical fractionation of the system. Here we describe the development of an in vitro system for yeast mRNA splicing. We present a detailed analysis of the products and presumed intermediates in the splicing of synthetic actin pre-mRNA in vitro.

MATERIALS AND METHODS

Yeast Strains and Yeast Transformation—Yeast strains used in this study are J401 (a, ura3-52, his3, his7, ade3, rna2-1) and EJ101 (a, trpl, pro1-126, prb1-112, pep4-3, prcl-126). Yeast transformation was carried out according to the lithium acetate procedure (Ito et al., 1983).

Plasmids and Plasmid Construction—Modification of the exon 2 sequence of the yeast CYH2 gene (Knäfer et al., 1983) entailed the following manipulations. A 592-bp Xhol-BglII fragment including parts of the intron and exon 2 was removed from YEpl CYH2 (a 2-μM13-actin-CYH2 clone was used as the template in a mutagenesis reaction primed with the yeast transformation was carried out according to the lithium acetate procedure (Ito et al., 1983)).

Xhol-BglII fragment including parts of the intron and exon 2 was removed from YEpl CYH2 (a 2-μM13-actin-CYH2 clone was used as the template in a mutagenesis reaction primed with the yeast transformation was carried out according to the lithium acetate procedure (Ito et al., 1983)).

1 The abbreviations used are: bp, base pair; kb, kilobase pair; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HEPES, N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid; PEG, polyethylene glycol; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid).

2 A. J. Newman, R.-J. Lin, S.-C. Cheng, and J. Abelson (1985) Cell 42, 335-344.

3 Synthetic actin and CYH2 pre-mRNAs containing a single intron are accurately spliced in a soluble whole cell extract of yeast. Splicing in vitro requires ATP. The excised intron is released as a lariat in which an RNA branch connects the 5' end of the molecule to the last A in the "intron conserved sequence" UACUAAC. Two other discrete RNA species produced during splicing in vitro may represent reaction intermediates: free, linear exon 1 and a form of the intron lariat extending beyond the 3' splice site to include exon 2. Both lariat forms correspond to molecules previously shown to be produced during yeast pre-mRNA splicing in vivo.

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intact CYH2 gene with a modified exon 2 sequence (designated CYH2m).

The SP6-CYH2m transcription plasmid was constructed using M13mp11 as an intermediate vector (Fig. 4). YEp CYH2m was digested with HindIII, and the HindIII ends were filled in with the Klenow fragment of DNA polymerase I. The digest was then recut with XhoI to yield a 241-bp fragment extending from the middle of exon 1 into the intron (Kauf er et al., 1983); this fragment was gel purified. A 608-bp fragment extending from the XhoI site to the unique EcoRI site was inserted into M13mp11 cut with SmaI and in exon 2 sequence (designated AluI fragment of the yeast actin gene, including the intron and fragments were inserted together into M13mp11 cut with SmaI and EcoRI. Subsequently the CYH2m insert was transferred to the SP6 vector pSP64 using the HindIII and EcoRI sites.

The SP6-actin transcription plasmid was made by inserting a 544-bp AluI fragment of the yeast actin gene, including the flanking portions of exon 1 and exon 2 (Gallwitz and Sures, 1980; Ng and Abelson, 1980), into the SmaI site of the SP6 vector (Fig. 4).

Preparation of Synthetic CYH2m and Actin Pre-mRNA—CYH2m transcripts for in vitro splicing and subsequent oligonucleotide/ST1 assay were synthesized in 50-μL SP6 transcription reactions containing the following: 20 mM NaCl, 40 mM Tris-Cl, pH 7.5, 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 0.5 mM each of CTP, UTP, and ATP, 50 μM GTP, 0.5 mM [α-32P]GTP (P-L Biochemicals), 100 ng/μl [32P]UTP, 1000 units/ml RNAsin (Promega Biotec), 50 μg/ml linearized template, and 400 units/ml SP6 RNA polymerase (Promega Biotech or Boehringer-Mannheim). Transcription was at 37 °C for 2 h and full-length transcripts were purified by polyacrylamide gel electrophoresis. Using this ratio of [32P]GTP:GTP, the majority of transcripts initiate with [32P]GTP. This was shown by two-dimensional thin layer chromatography analysis (Sanesyahi et al., 1972) of T2 digestion products from a 58-base long model transcript, labeled with [32P]ATP, and synthesized using BarnHI-linearized pSP64 DNA as template (data not shown). Actin transcripts for in vitro splicing reactions were synthesized and purified as above, but the UTP concentration was reduced to 25 μM, and [α-32P]UTP was present at 5 μCi/μl. To prepare [α-32P]CTP-labeled actin transcripts, the CTP concentration was reduced to 25 μM, and [α-32P]CTP was present at 100 μCi/μl. Unlabeled NTP concentration was kept at 500 μM in all cases. In actin transcripts, the cap dimer was not included in the SP6 transcription reaction.

Preparation of Whole Cell Extracts of Yeast—Yeast strain EJ101 was grown, shaking at 30 °C in YPD medium (1 liter) to A600 = 2–4. We use this strain because it is deficient in protease, but we have made active extracts from other strains of S. cerevisiae. The cells were harvested by centrifugation at 3000 rpm for 5 min (Sorval GS3 rotor) and resuspended in 15 ml of 1 M sorbitol, 50 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 30 mM DTT. After 15-min incubation at 25 °C without shaking, the cells were collected by centrifugation at 3000 rpm for 5 min and resuspended in 15 ml of 1 M sorbitol, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 3 mM DTT (called Buffer S). 90 μl of 0.0 mg/ml zymolyase 60,000 (Seikagoku Kogyo Co., Ltd.) were added and the cell suspension was shaken very gently at 30 °C for 20 min. All subcellular layers were carried out at 30 °C. The spheroplasts were collected by centrifugation at 3000 rpm for 5 min, washed by gentle resuspension in 15 ml of Buffer S, collected by centrifugation at 3000 rpm for 5 min, and resuspended gently in 10 ml of 0.5 M HEPES-K+ (pH 7.0 at 4 °C), 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM DTT (Buffer A). The spheroplasts were lysed in this hypotonic buffer by five strokes with a tight-fitting pestle in a glass Dounce homogenizer. Loose-fitting pestles have consistently given inactive extracts and we therefore judge the tightness of the pestle to be an important factor in this step. 2.0 M KCl was added to give a final KCl concentration of 0.4 M. The lysate was gently mixed on ice for 30 min and DTT was removed by centrifugation at 17,000 rpm for 30 min (Sorval SS34 rotor). The supernatant was then centrifuged at 37,000 rpm for 60 min (Beckman Ti60 rotor) and dialyzed for 3 h against 1 liter of 20 mM HEPES-K+ (pH 7.0, 0.2 mM EDTA, 0.5 mM DTT, 50 mM KCl, 20% (v/v) glycerol. Finally, the extract was centrifuged at 17,000 rpm for 60 min (Sorval SS34 rotor) to remove small amounts of insoluble material. The supernatant was frozen at −70 °C in small aliquots. Extracts made by this procedure had protein concentrations of about 25–30 mg/ml and were stable for at least 6 months at −70 °C.

In Vitro Splicing Reactions—Standard splicing reactions were carried out at 25 °C for 10 min in a volume of 10 μl. They contain 4 μl of whole cell extract, 1 μl each of 20 mM ATP, 25 mM MgCl2, 30% (w/v) PEG 8000, 0.6 M potassium phosphate, pH 7.0, and 1 nM of synthetic pre-mRNA. The whole cell extract was in 20 mM HEPES-K+, pH 7.0, 20% (v/v) glycerol, 50 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT. Hence, the final concentrations of the various components present were as follows: 2 mM ATP, 2.5 mM MgCl2, 3% (w/v) PEG 8000, 60 mM potassium phosphate, 20 mM KCl, 8 mM HEPES, 8% (v/v) glycerol, 80 μM EDTA, 0.2 mM DTT, and 1 nM actin or CYH2m pre-mRNA. Yeast proteins were present at about 10–12 mg/ml. The reaction was stopped by the addition of 2 μl of stop solution (1 mg/ml proteinase K, 50 mM EDTA, 1% SDS) and the mixture was incubated at 37 °C for 15 min. 200 μl of a mixture containing 50 mM sodium acetate, pH 5.3, 1 mM DTT, 0.1% SDS, 25 μg/ml Escherichia coli RNA were then added and proteins were removed by extraction with an equal volume of phenol-chloroform-isooamyl alcohol (50:50:1). The nucleic acids were precipitated with 2.5 volumes of ethanol, rinsed with 70% ethanol, and dried in vacuo. The RNA was then either analyzed directly by fractionation on thin polyacrylamide-urea sequencing gels, or was subjected to oligonucleotide/S1 analysis.

Oligonucleotide/S1 Assays—The oligonucleotide probe 5' TTCACCCTTTACCGGCTGAGACGTGTGGAGA was 5' -end labeled using T4 polynucleotide kinase and [γ-32P]ATP, to a specific activity of 1–5 × 106 Cerenkov cpm/pmol and freed of unincorporated ATP by chromatography on DEAE-cellulose (Whatman DE82). RNA samples were hybridized to probe (2 × 106 Cerenkov cpm, 0.1 pmol) in 20 μl of a solution containing 150 mM NaCl, 15 mM PIPES, pH 6.4, 0.5 mM EDTA, 100 μg/ml E. coli RNA, and 0.1% SDS by cooling from 37 °C to 25 °C. 100 μl of a mixture containing 0.1 M NaCl, 30 mM sodium acetate, pH 4.6, 3 mM ZnCl2, 25 μg/ml E. coli RNA, and 0.1% SDS were added, followed by 1000 units of nuclelease S1. After 30 min at 37 °C, the S1 digestion was stopped by addition of 2.5 volumes of ethanol. The S1-resistant products were fractionated by electrophoresis on 16% polyacrylamide-8 M urea gels and visualized by autoradiography.

RNA Structure Analysis—RNase digestions were performed according to Domdey et al. (1984). Fingerprinting procedures and secondary analyses were carried out according to Volckaert et al. (1976) and Volckaert and Fiers (1977), as applied by Gegenheimer and Volckaert (1980). The first dimension of fingerprinting was cellulose acetate membrane electrophoresis, pH 3.5, and the second dimension was homochromatography on polyethylenimine (PEI)-cellulose thin-layer plates. Secondary analysis was performed by elution of the oligonucleotides with 30% triethylamine bicarbonate, pH 10, appropriate RNase digestions, and two-dimensional thin-layer chromatography (PEI for T1 and A) or cellulose plates (for T2) (Domdey et al., 1984).

RESULTS

Design of the pre-mRNA Splicing Assay—In order to detect pre-mRNA splicing activity in a crude yeast extract, we needed a sensitive assay which specifically and unequivocally detected the rearranged RNA sequence resulting from correct splicing of an input pre-mRNA substrate. We developed a technique based on the S1 nuclease procedure (Berk and Sharp, 1977), which uses a 5' -end labeled oligonucleotide probe, specific for the exon 1-exon 2 splice junction in CYH2 mRNA (Kauf er et al., 1983), to detect conversion of pre-mRNA to mRNA in vitro (Tabak et al., 1981). The basic principle of the assay is illustrated in Fig. 1.

The oligonucleotide probe is allowed to hybridize with CYH2 sequences in the RNA sample under analysis, and the hybrids are digested with nuclease S1. The S1-resistant products are then fractionated by polyacrylamide gel electrophoresis and visualized by autoradiography. CYH2 mRNA and pre-mRNA protect the end-labeled probe to different extents, yielding two characteristic sets of protected products. The probe is provided with a 6-base extension at the 3' end, which cannot pair with mRNA or pre-mRNA and so is always removed by nuclease S1. This feature allows a distinction to be made between any residual undigested full-length probe as opposed to probe protected by virtue of hybridization to CYH2 RNA sequences.

* E. Jones, personal communication.
pre-mRNA Exon 1 Intron Exon 2 mRNA

pre-mRNA mRNA

mRNA

Pre-splice Junction

Probe

mRNA specific products

pre-mRNA specific product

16% polyacrylamide gel urea

FIG. 1. Diagram illustrating the basic features of the oligonucleotide/nuclease S1 assay. The 5' end-labeled oligonucleotide probe is complementary to the region flanking the splice junction in the mRNA. Hybridization to mRNA and pre-mRNA is driven by the excess probe, and the hybrids plus unhybridized probe are then digested with nuclease S1. Pre-mRNA and mRNA each yield a characteristic set of labeled S1-resistant products, which are visualized after electrophoresis and autoradiography. The 3'-terminal six nucleotides of the probe do not pair with either mRNA or pre-mRNA, and so are always removed by S1. This feature allows nonspecific and specific protection of the probe to be distinguished.

FIG. 2. Identity of the three conservative, single base changes made in exon 2 of the yeast CYH2 gene. Also shown are the hybrids formed between the oligonucleotide probe (a 5' end-labeled 30-mer specific for modified CYH2 mRNAs) and its mRNA and pre-mRNA partners.

Early attempts to detect splicing of CYH2 pre-mRNA in vitro using this assay revealed a serious difficulty: many yeast extracts contained a small, but nevertheless readily detectable amount of CYH2 mRNA. This gave rise to a false positive, even in zero time on "extract alone" reactions. Any low level of real splicing of added CYH2 pre-mRNA was then obscured by this background signal.

Alteration of CYH2 Exon 2 Nucleotide Sequence—To solve this problem we took advantage of the ability of nuclease S1 to cut efficiently at single base mismatches in an otherwise perfect duplex (Shenk et al., 1975). We synthesized an oligonucleotide designed to introduce three single-base changes into the exon 2 sequence (Fig. 2). These changes do not alter the identity of the amino acids encoded by this region of the gene; they merely replace one frequently used codon with another. We used the mutagenic oligonucleotide to introduce these changes into exon 2 and then reintroduced the modified CYH2 gene into yeast by transformation, using a multicopy plasmid vector. We used a strain (J401) carrying the recessive conditional mutation rna2-1, a temperature-sensitive lesion in a gene involved in pre-mRNA splicing (Teem and Rosbash, 1983; Lee et al., 1984; Last et al., 1984). Poly(A)+ RNA was prepared from cultures of strain J401 harboring the CYH2 plasmid, after growth at 23 or 36°C. Similar RNA samples were isolated from the same strain harboring 2-μ plasmid carrying the unmodified CYH2 gene.

For the S1 analysis shown in Fig. 3, we used an oligonucleotide probe designed to pair with CYH2 mRNA sequences. Three conclusions can be drawn from Fig. 3. First, this probe is indeed specific for CYH2 mRNA sequences, since no S1-resistant products survive digestion if only unmodified CYH2 sequences are present. Apparently S1 is able to cut efficiently at one or more of the mismatched bases in such hybrids. Second, the assay readily distinguishes between precursor and mRNA allowing assay of conversion of one to the other. Third, not only is the altered CYH2 pre-mRNA spliced normally at 23°C in vitro, this splicing also exhibits the normal sensitivity to the rna2-1 lesion, so that at 36°C the steady state level of spliced CYH2 mRNA is dramatically reduced. This behavior faithfully mimics that of normal CYH2 transcription products, and that of other intron-containing polymerase II transcripts in rna2-1 strains of yeast (Teem and Rosbash, 1983; Teem et al., 1983).

Artificial CYH2 Pre-mRNA—We have used the bacteriophage SP6 RNA polymerase transcription system (Green et al., 1983; Melton et al., 1984) to produce synthetic pre-mRNA from the modified CYH2 gene. An internal fragment of the CYH2 gene was inserted downstream of the SP6 promoter in the vector pSP64. This fragment included 80 base pairs of DNA linearized at a unique EcoRI site at the 3' end of the CYH2 insert (Fig. 4). In some experiments, transcripts were capped by including m'GpppG as a priming dinucleotide at 10-fold excess over GTP.

Before using this synthetic precursor, we carried out an experiment in vivo, to demonstrate that the CYH2 mRNA sequences represented in the SP6 transcript included all of the information required for accurate splicing. The same CYH2 fragment was fused to the promoter from the yeast alcohol dehydrogenase gene ADC1, on a plasmid carrying the 2-μ replication control region (Ammerer, 1983). This construction was introduced into yeast by transformation, and poly(A)+ RNA was purified from the transformed strain. The RNA was analyzed by primer extension with reverse transcriptase, using an oligonucleotide primer for CYH2 exon 2. The results of this analysis established that the chimeric ADC1-CYH2 pre-mRNA was efficiently spliced in vivo (see Newman et al., 1985).

Splicing of Synthetic CYH2 Pre-mRNA in Vitro—Multiple attempts to detect splicing of CYH2 pre-mRNA in vitro using extracts of crude yeast nuclei produced uniformly negative results. This was despite extensive manipulation of the method of preparation of nuclei, of the method of solubiliza-
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The addition of an ATP regenerating system (phosphocreatine and creatine phosphokinase from rabbit muscle) to the splicing reactions did not alter this result, although it did stabilize the ATP against otherwise rapid hydrolysis in most nuclear extracts (data not shown).

We surmised these nuclear extracts might lack one or more components involved in pre-mRNA splicing, and so we turned our attention to whole cell extracts made from yeast spheroplasts. Yeast spheroplasts were prepared by zymolyase treatment and lysed in a Dounce homogenizer under hypotonic conditions. Components were solubilized from the crude lysate by extraction with 0.2 M KCl. Cellular debris was removed by centrifugation, and the supernatant was dialyzed and frozen in aliquots at −70 °C (see “Materials and Methods” for details).

In an early experiment, a yeast whole cell extract made in this way was incubated with or without synthetic unlabeled CYH2 pre-mRNA (Fig. 5A). Samples were withdrawn at intervals, deproteinized, and analyzed by the oligonucleotide/S1 method, using the CYH2-specific oligonucleotide (cf. Fig. 2). There is no protection of the oligonucleotide against S1 digestion in the absence of the synthetic CYH2 pre-mRNA. At the beginning of the reaction containing synthetic CYH2 pre-mRNA, only pre-mRNA-specific S1 products are visible, whereas, after a few minutes of incubation, a second set of S1 products appears. This set is identical to that produced after S1 analysis of poly(A) RNA containing authentic CYH2m
A

![Image](image1.png)

B

![Image](image2.png)

**FIG. 5.** Time course of synthetic CYH2 mRNA pre-mRNA splicing in vitro. A, first detection of pre-mRNA splicing in vitro. The splicing reaction was carried out at 30 °C in 40 μl of mixture containing 40% (v/v) yeast whole cell extract (in 20 mM HEPES, pH 7.8, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 20% (v/v) glycerol), 1 mM each of all four ribonucleoside triphosphates, 3 mM MgCl₂, 5 mM DTT, 1 mM spermidine, 50 mM creatine phosphate, 1 mg/ml creatine kinase, 3% (w/v) PEG 8000, with or without the presence of synthetic CYH2 pre-mRNA. Samples were withdrawn from the reaction at intervals (time of incubation is given in minutes), deproteinized, and analyzed using the oligonucleotide/nuclease S1 assay. The sets of S1 products characteristic of authentic CYH2 pre-mRNA and mRNA are shown in the lane at the center (poly(A)⁺ RNA from an rna2-1 strain harboring YEplac67 at 23 °C). B, CYH2 pre-mRNA was incubated in a yeast whole cell extract under optimal conditions. The preparation of the yeast whole cell extract and the standard splicing reactions are described under "Materials and Methods.”

**Optimization of Pre-mRNA Splicing Reaction Conditions**—We have systematically varied the reaction conditions, one parameter at a time, in order to establish optimum conditions for splicing and to investigate the requirements for exogenous cofactors. Fig. 6 shows the results of several such experiments. The important features to emerge are summarized below. CYH2 pre-mRNA splicing in the whole cell extract requires ATP (≥500 μM). UTP, GTP, and CTP can substitute poorly for ATP, perhaps by inefficient phosphate transfer to endogenous ADP. We note that the addition of an ATP regeneration system will support splicing in the absence of added ATP or ADP (data not shown). Nonhydrolyzable ATP analogs (β-γ methylene ATP (AMPPCP) and α-β methylene ATP (AMPCCP), either by itself or in combination) cannot substitute for ATP, implying that splicing has a requirement for ATP hydrolysis.

Splicing was abolished by the addition of 5 mM EDTA, indicating a divalent cation requirement, and displayed a broad optimum for Mg²⁺ ion concentration centered at about 2–3 mM. A monovalent cation is also required: K⁺, Na⁺, and NH₄⁺ all support splicing over a broad concentration range, with an optimum of 80–100 mM. The addition of PEG 8000 to 3% (v/v) enhanced the yield of spliced product, presumably by an excluded volume effect. Optimum temperature for CYH2 pre-mRNA splicing was 25 °C, and optimum pH was 7.0 (data not shown). The placental ribonuclease inhibitor RNAsin (from Promega Biotec) at 1 unit/μl does not inhibit splicing in vitro. On the other hand, another ribonuclease inhibitor, vanadyl ribonucleoside complex (from Bethesda Research Laboratories) completely inhibits splicing in vitro at a concentration of 10 mM (data not shown).

**Caps and Cap Analogs**—The splicing reactions shown in Fig. 5 used synthetic CYH2 pre-mRNAs carrying 7mGpppG caps, as a result of the inclusion of 7mGpppG as initiating dinucleotide in the SP6 polymerase transcription reactions. Nevertheless, uncapped transcripts, initiated with pppG, were spliced with apparently equal efficiency (data not shown). We have not ruled out the possibility, however, that such transcripts might be capped in the whole cell extract during the reaction.

We also show in Fig. 6 that the addition of the cap analog dinucleotide 7mGpppG to the splicing reaction did not affect the yield of CYH2 mRNA, even at concentrations as high
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A.

![Fig. 6. Composite of S1 assay data from several experiments in which individual reaction parameters were varied one by one in order to optimize conditions for CYH2 pre-mRNA splicing. Also shown are the effects of the ATP analog AMPPCP and the cap analog m'GpppG on splicing in vitro.](image)

as 250 μM. This is in contrast to the splicing of the adenovirus major late leader RNA in whole cell extracts from HeLa cells (Konarska et al., 1984) where even 10 μM cap analog dramatically inhibited splicing.

Analysis of RNA Splicing Produced during Pre-mRNA Splicing in Vitro—The experiments outlined above allowed us to establish optimum conditions for splicing synthetic CYH2 pre-mRNA in vitro. We have subsequently used 32P-labeled synthetic pre-mRNA as substrates in order to isolate and characterize the products of splicing in vitro. Here we present the analysis of the RNA species generated in the whole cell extract from a synthetic actin pre-mRNA. This substrate was synthesized by SP6 transcription from linearized plasmid DNA carrying an internal fragment of the yeast actin gene (Gallwitz and Sures, 1980; Ng and Abelson, 1980) inserted downstream of a bacteriophage SP6 promoter (Fig. 4). The yeast DNA fragment included the 309-bp intron, 73 base pairs of exon 1, and 162 base pairs of exon 2.

32P-labeled run-off transcripts were produced from the SP6-actin clone after linearization of the template with EcoRI or HpaII which cut within exon 2 of the actin gene. Synthetic actin pre-mRNAs of two different sizes were then synthesized, differing only in the size of the exon 2 portion of the transcript. These pre-mRNAs were then separately incubated with the whole cell extract under conditions optimized for splicing and samples were withdrawn at intervals. The RNA was deproteinized and fractionated by electrophoresis on an 8% polyacrylamide-8 M urea gel. Fig. 7 shows the result.

![Fig. 7](image)

Four novel RNA species arise during the course of each reaction. The smallest of these, produced from both substrates, has the mobility expected for free, linear exon 1. HpaII substrate (short exon 2) also generates a product of mobility 135–140 nucleotides, exactly that predicted for the correctly spliced product from this pre-mRNA. Moreover, EcoRI substrate (long exon 2) gives rise to a product of mobility 250–260 nucleotides, again corresponding to that expected for the correctly spliced product. Both of these putative spliced RNA species display some size heterogeneity, with a tendency for the more slowly migrating forms to predominate as the reaction proceeds. This presumably reflects some covalent modification, probably polyadenylation, apparently peculiar to the putative spliced products.

Two other discrete RNA species also appear in each splicing reaction. These molecules share the striking characteristic that they migrate more slowly than the synthetic actin pre-mRNA from which they arise. Furthermore, the apparent mobility of these RNA species varies, relative to DNA markers, according to the concentration of the acrylamide gel on which the samples are fractionated: their electrophoretic behavior becomes more anomalous as the gel matrix concentration is increased (data not shown). This sort of behavior is characteristic of molecules which contain an element of circularity (Bruce and Uhlenbeck, 1978; Sanger et al., 1979) and in the present context suggests that these species might be RNA lariats (Ruskin et al., 1984; Grabowski et al., 1984).

For the RNA analysis experiments described below, pSP6
FIG. 7. Splicing of synthetic actin pre-mRNA in vitro. Radioactive actin pre-mRNAs synthesized as run-off transcripts from templates linearized with either HpaII or EcoRI (see Fig. 4) were incubated in the yeast whole cell extract under appropriate conditions. Samples were withdrawn at intervals (time of incubation is numbered in minutes), deproteinized, and analyzed by electrophoresis on an 8% polyacrylamide-8 M urea sequencing gel, followed by autoradiography. End-labeled DNA fragments (pBR322 cut with HpaII (M1) or EcoRI (M2)) were run alongside as size markers. El and E2 refer to the exons, and lariat forms are denoted by *.

R and H refer to RNA molecules produced from EcoRI and HpaII transcripts, respectively.

actin plasmid DNA, which was linearized at the HpaII site (Fig. 4) was used as template to produce run-off transcripts in vitro, labeled to high specific activity with one of the four [α-32P]NTPs. Preparative scale splicing reactions were performed, and the RNA species of interest were purified by gel electrophoresis. Unspliced transcripts from the splicing reactions, termed precursor RNA, were also recovered from the gel to serve as control RNA in the subsequent RNA analyses.

Exon 1 and Exon 2 Are Accurately Spliced—The [α-32P]UTP-labeled spliced exon 1-2 RNA was digested with RNase A, and the products were fractionated by two-dimensional homochromatography (Volckaert et al., 1976). The [α-32P]UTP-labeled precursor RNA was fingerprinted for comparison. The sequence of the various spots was determined by comparing relative mobilities and nucleotide compositions with the known sequence (cf. Fig. 8). A pentanucleotide, GAGGUp, which spans the splice junction, was detected in the fingerprint of the spliced RNA (Fig. 9A). RNase A digestion of the precursor RNA does not produce an oligonucleotide of this sequence (Fig. 9B). Furthermore, this pentanucleotide will be produced only if splicing is accurate (cf. Fig. 8). An equivalent set of two-dimensional fingerprints was done with the spliced exon 1-2 and precursor RNA labeled with [α-32P]ATP. The same pentanucleotide, GAGGUp, was detected in the fingerprint of the spliced RNA but not the precursor RNA (Fig. 9, C and D). This result indicates that the phosphate at the splice junction of the mRNA comes from the 3' splice site. This pentanucleotide would not have been labeled if the phosphate was derived from the 5' splice site or from exogenous ATP.

This [32P]ATP-labeled pentanucleotide was eluted from the chromatograph and digested with RNase T1, and the products were fractionated by PEI thin-layer chromatography (Volckaert and Fiers, 1977). A labeled guanosine 3' monophosphate was the only product detected as expected (data not shown). We conclude that the intron was accurately excised and exon 1 and exon 2 were precisely joined together.

The Excised Exon 1 Has a 3' OH Terminus—One of the intermediates which appeared early in the splicing reaction was tentatively identified as exon 1 RNA. It presumably results from a cleavage at the exon 1-IVS junction. The putative exon 1 RNA was digested with RNase T1 and fingerprinted as shown in Fig. 10A. An *in vitro* transcript of the
indicated by nucleotides correspond to the numbered spots in the T1-fingerprints. A digestion of both T1 oligonucleotides produced 32P-labeled consistent with T17' derived from the exon 1 RNA having a 3' hydroxyl. Second, a unique oligonucleotide, T27', containing the TACTAAC sequence of the IVS, moves faster in the first dimension and slower in the second dimension when compared to the related T27 from the control RNA (Fig. 11). Both T27 and T27' were eluted from the chromatographs and digested with RNase A, and the products were fractionated by PEI thin-layer chromatography (Fig. 12). The RNase A digestion products of the T27 from the control RNA are ACP and AU5, as expected from the sequence (Fig. 12B; cf. Fig. 8). Digestion of the T27' derived from the IVS* RNA produced the same two dinucleotides, as well as a unique oligonucleotide that barely moved from the origin (Fig. 12A). This unique RNase A product of T27' must contain the branch point observed previously in the in vivo study (Domdey et al., 1984) and by other groups in the mammalian system (Ruskin et al., 1984; Padgett et al., 1984). Indeed, this unique oligonucleotide contains an RNase T2-resistant component as determined by RNase T2 digestion and cellulose thin-layer chromatography (Saneyoshi et al., 1972) (Fig. 13A). The same RNase T2-resistant species, which migrated slowly in the second dimension, was present in the RNase T1 oligonucleotide T27' (Fig. 13B). We have previously suggested that one of the last two, A's in the UACUAAC sequence is the site of a branch point in the IVS (Domdey et al., 1984). In other words, the unique RNase A oligonucleotide in the T27' has a sequence AACp linked 2'-5' to a G, presumably the G at the 5' end of the IVS. The fact that the RNase T2-resistant oligonucleotide can be labeled with [α-32P]UTP is consistent with this assumption, because the sequence of the 5' end of the IVS starts with GU. The location of the branch in T27' was unambiguously determined by analysis of the IVS* RNA labeled with each of the four [α-32P]NTPs. The 32P-labeled IVS* RNA was digested with RNase T1 and RNase A, and the products were fractionated by PEI thin-layer chromatography. The tetranucleotide, AA5, was further analyzed by RNase T2 digestion. The results are summarized in Table I. RNase T2 digestion of the [α-32P]ATP-labeled tetranucleotide, AA5, produced labeled Ap as well as the RNase T2-resistant component (Fig. 13D). This result indicates that the G is linked to the second A in the AACp. If the G is linked to the first A in the AACp, RNase T2 digestion would have produced labeled Cp. An equivalent set of experiments was done with the putative splicing intermediate, IVS*exon 2 RNA (the IVS-exon 2 in a lariat form), and similar results were obtained (data not shown). We conclude that both IVS* and IVS'exon 2 RNA are lariat structures with the G at the 5' end of the IVS linked to the last A in the TACTAAC sequence via a 2'-5' phosphodiester bond.

**DISCUSSION**

We have shown that a soluble whole cell extract of yeast will accurately splice synthetic yeast pre-mRNAs containing a single intron. Splicing in vitro generates the correctly spliced mRNA, and three other discrete RNA species: free exon 1 bearing a 3' OH terminus; a molecule consisting of the intron and exon 2; and the intact, excised intron. The last two of these contain an intramolecular branch at a specific site near
**Fig. 9.** RNase A fingerprint analysis of *in vitro* spliced mRNA. The SP6 *in vitro* transcript of proteus 6-actin linearized at the *HpaII* site was used as pre-mRNA in the splicing reaction. The spliced mRNA as well as pre-mRNA were recovered from polyacrylamide-urea gel, digested with RNase A, and fingerprinted as described under "Materials and Methods." The directions of the first dimension, high-voltage paper electrophoresis at pH 3.5, and second-dimension homochromatography on PEI plates, are indicated by arrows. Nucleotide composition of each oligonucleotide is indicated. RNA sequences surrounding the splice sites in pre-mRNA and the splice junction in mRNA are given below the fingerprints. Exon-intron boundaries are indicated by two colons. Two oligonucleotides of interest, AGAGGU from pre-mRNA and GAGGU from mRNA, are encased in boxes. Upper half, [*32P*]UTP-labeled mRNA (A) and pre-mRNA (B). Lower half, [*32P*]ATP-labeled mRNA (C) and pre-mRNA (D).
Fig. 10. RNase T1 fingerprint analysis of exon 1 RNA. A, the free exon 1 RNA was digested with RNase T1 and fingerprinted (see Fig. 9 legend for details). B, an SP6 in vitro transcript of proteus 6-actin, linearized at the XhoI site, was also fingerprinted for comparison. Both RNA molecules are [32P]UTP-labeled. The spots on the fingerprints are given numbers, corresponding to the numbered T1 oligonucleotides in Fig. 8. The T17', derived from the 3' end of exon 1 RNA, migrated slower in the first dimension and faster in the second dimension than the related T17. Some spots of low intensity are present on the fingerprint of exon 1 due to contamination by degraded pre-mRNA in the splicing reaction.

Fig. 11. RNase T1 fingerprint analysis of IVS* RNA. The IVS* RNA (panel A) and pre-mRNA (panel B), both [32P]UTP-labeled, were digested with RNase T1 and fingerprinted (see Fig. 9 legend for details). The spots on the fingerprints are given numbers, corresponding to the numbered T1 oligonucleotides in Fig. 8. The T14', derived from the IVS* RNA, migrated slower in the first dimension and faster in the second dimension than the related T14. On the other hand, the T27', derived from the IVS* RNA, migrated faster in the first dimension and slower in the second dimension than the related T27. A predicted A-rich 17-mer from the sequence in Fig. 8, A9CUG, which streaks in the first dimension, and a large 55-mer, which is hardly transferred from the first dimension cellulose acetate membrane onto the second dimension PEI plate, may not be observed on the fingerprints.
were eluted from the fingerprint chromatographs, digested with RNase A, and the products were analyzed by two-dimensional thin layer chromatography on PEI plates as described under "Materials and Methods." A, T27' derived from IVS* RNA; RNase A, and the products were analyzed by two-dimensional thin-layer chromatography on cellulose plates as described under "Materials and Methods." A, T27' derived from IVS* RNA; B, T27' derived from pre-mRNA, both were [32P]UTP-labeled. Arrows indicate the directions of chromatography. The origin of chromatography is marked with an X. The nucleotide sequence of each 32P-labeled spot is indicated. A unique oligonucleotide derived from T27', migrating very slowly in both dimensions, contains the branched nucleotide. Several RNase A digestion products, visualized by ultraviolet light illumination, were circled for alignment. The proposed nucleotide sequence of T27' is shown below the chromatograms. Nearest neighbor nucleotides are given in parenthesis.

The 3' end of the intron, corresponding to the final A of the essential "intron conserved sequence" UACUAAC (Langford and Gallwitz, 1983; Pikielny et al., 1983). This A residue is linked by a 3'-5' phosphodiester bond to the adjacent C, and by a 2'-5' phosphodiester bond to a G, the first nucleotide of the intron.

Hence, the intron and intron-exon 2 molecules consist of a 5' circular component and a 3' linear component downstream of the intramolecular branch. These RNA species correspond precisely to the lariats produced during splicing of globin and adenovirus pre-mRNAs in mammalian cell extracts (Ruskine et al., 1984; Padgett et al., 1984).

We have found that ATP is required for yeast pre-mRNA splicing in vitro. What could be the molecular basis for this requirement? ATP analogs such as AMPCCP and AMPCP will not substitute for ATP itself, implying a need for ATP hydrolysis. When these analogs replace ATP, no specific cleavage or rearrangement of the pre-mRNA occurs (data not shown). One possibility is that the component responsible for making the 2'-5' phosphodiester linkage at the UACUAAC sequence needs ATP-dependent activation. There is a precedent for this in yeast: the tRNA splicing ligase is activated by ATP-dependent adenylylation (Greer et al., 1983). If cleavage at the 5' splice site and formation of the branch are in some way coupled, neither would occur in the absence of ATP.

Alternatively, ATP might be required to organize the precursor and the components of the splicing machinery into a precisely folded structure preparatory to splicing. Perhaps energy is needed to scan for the specific RNA sequences which define the splice sites and intron branch point to be used or to impose a specific three-dimensional organization on such sequences. Biochemical characterization of the splicing machinery may enable us to address these questions.

There are evidently extensive similarities in the way introns are removed from pre-mRNAs in yeast and higher eukaryotes, at least in respect to the intermediates observed and the fate of the excised intron RNA. Whereas in yeast introns, the 5' splice site and intramolecular branch point are rigorously defined by highly conserved sequences, in mammalian introns, and those of higher eukaryotes in general, the corresponding sequences exhibit considerable variability. Indeed, deletion analysis of the rabbit P-globin large intron failed to show a requirement for any specific internal sequence corresponding to the UACUAAC branch point motif from yeast introns (Wierenga et al., 1984). Nevertheless, the sequences surrounding those mammalian intron branch points which have been examined show clear similarities, not only among themselves but also with the yeast TACTAAC sequence (Ruskin et al., 1984; Keller and Noon, 1984). Computer analysis of metazoan intron sequences revealed that similar potential branch-point sequences were invariably present near the 3' splice site (Keller and Noon, 1984). Presumably, then, a specific but more flexible branch-point sequence is also re-
TABLE I

Nucleotide composition of the RNase T2-resistant oligonucleotide

The SP6-actin pre-mRNAs were synthesized in vitro using each of the four [32P]NTPs in separate reactions and incubated in the in vitro splicing reactions, and the IVS* RNA was purified and digested with RNase T1 + A. The digestion products were fractionated by two-dimensional TLC on PEI plates. The unique oligonucleotide indicated by + or −. A schematic diagram of the oligonucleotide containing the RNase T2-resistant component is shown below the table. The RNase T1, A (pancreatic RNase (Panc.), and T2 cleavage sites are indicated. The 2'−5' and 3'−5' phosphodiester linkages of the branched nucleotide are also indicated.

| RNAase | T1 and Panc. | T1, Panc., and T2 |
|--------|--------------|--------------------|
|        | ApA | ApA | ApA |
| label | PpG | PpG | PpG |

T1 ↓

CpUp

2'

UpApA

Panc. T2

5' CpAp

Panc.

required in metazoan introns. When the normal branch point is removed by deletion, the splicing machinery is able to recruit an alternative branch-point sequence elsewhere in the intron (unpublished data cited in Raskin et al., 1984). Are the mechanistic similarities between yeast and mammalian splicing likely to be related in similarities in the components of the splicing machinery? In mammalian cells, there is molecular evidence that U1 snRNAs are involved in pre-mRNA splicing (Padgett et al., 1983b; Kramer et al., 1984). Furthermore, there is a specific interaction between U1 snRNP and the 5′ splice site in mouse β-globin pre-mRNA in vitro (Mount et al., 1983). Yeast also does contain small nuclear RNAs resembling the U class of mammalian snRNAs in respect to their size, 5′ cap structure, and the presence of modified bases (Wise et al., 1983). These yeast snRNAs are present in low abundance, however, and their involvement in yeast pre-mRNA splicing is open to question. Fractionation and characterization of the components of the splicing apparatus should shed some light on this point.

Are the RNA species produced during yeast pre-mRNA splicing in vitro and the likely sequence of events inferred from their structure a fair reflection of splicing as it occurs in the cell? Lariat forms of the actin and rp51A introns are indeed produced by splicing in vivo (Domdey et al., 1984; Rodriguez et al., 1984). Moreover, the intramolecular branch occurs at precisely the same position within the UACUAAC sequence in intron lariats generated in vitro and in vivo. In a separate study, we have examined the effects of specific mutations in the intron sequences which define the 5′ splice site and branch point on splicing of the yeast CYH2 pre-mRNA in vitro and in vivo. The results are again consistent with the belief that the RNA metabolism we observe in vitro is an accurate representation of these events as they occur in vivo.

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REFERENCES

Ammerer, G. (1983) Methods Enzymol. 101, 192–201
Berk, A. J., and Sharp, P. A. (1977) Cell 12, 721–722
Bruce, A. G., and Uilenbeck, O. C. (1978) Nucleic Acids Res. 5, 3665–3677
Domdey, H., Apostol, B., Lin, R.-J., Newman, A., Brody, E., and Abelson, J. (1984) Cell 39, 611–621
Gegenheimer, P., and Apyrion, D. (1980) J. Mol. Biol. 143, 237–257
Gallwitz, D., and Sures, I. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2546–2550
Grabowski, P. J., Padgett, R. A., and Sharp, P. A. (1984) Cell 37, 415–427
Green, M. R., Maniatis, T., and Melton, D. A. (1983) Cell 32, 681–694
Greer, C. L., Peebles, C. L., Cgenheimer, P., and Abelson, J. (1983) Cell 32, 537–546
Hardy, S. F., Grabowski, P. J., Padgett, R. A., and Sharp, R. A. (1984) Nature 308, 375–377
Hernandez, N., and Keller, W. (1983) Cell 35, 89–99
Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
Kaufert, N. F., Fried, H. M., Schwindinger, W. F., Jasins, M., and Warner, J. R. (1983) Nucleic Acids Res. 11, 3123–3135
Keller, E. B., and Noon, W. A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7417–7420
Kosarska, M. M., Padgett, R. A., and Sharp, P. A. (1984) Cell 38, 737–756
Kraimer, A. R., Maniatis, T., Raskin, B. and Green, M. R. (1984) Cell 36, 993–1005
Kramer, A., Keller, W., Apel, B., and Löhmann, R. (1984) Cell 38, 293–307
Langford, C. J., and Gallwitz, D. (1983) Cell 33, 519–527
Langford, C. J., Nellen, W., Niessing, J., and Gallwitz, D. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1496–1500
Last, R. L., Stavenhagen, J. B., and Woolf, J. L., Jr. (1984) Mol. Cell. Biol. 4, 2396–2405
Lee, M. G., Young, R. A., and Beggs, J. D. (1984) EMBO J. 3, 2825–2830
Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) Nucleic Acids Res. 12, 703–706
Mount, S. M. (1982) Nucleic Acids Res. 10, 459–472
Mount, S. M., Petterson, L., Hinterberger, M., Karmas, A., and Steitz, J. A. (1983) Cell 33, 509–518
Nol, R., and Abelson, J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3912–3916
Padgett, R. A., Hardy, S. F., and Sharp, P. A. (1983a) Proc. Natl. Acad. Sci. U. S. A. 80, 5230–5234
Padgett, R. A., Mount, S. M., Steitz, J. A., and Sharp, P. A. (1983b) Cell 35, 101–107
Padgett, R. A., Kosarska, M. M., Grabowski, P. J., Hardy, S. F., and Sharp, P. A. (1984) Science 225, 898–903
Pikielny, C. W., Teem, J. L., and Rosbash, M. (1983) Cell 34, 395–403
Rodriguez, J. R., Pikielny, C. W., and Rosbash, M. (1984) Cell 39, 605–619
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Ruskin, B., Krainer, A. R., Maniatis, T., and Green, M. R. (1984) Cell 38, 317–331
Saneyoshi, M., Ohashi, Z., Harada, F., and Nishimura, S. (1972) Biochem. Biophy. Acta 262, 1–10
Sänger, H. L., Ramm, K., Domdey, H., Gross, H. J., Herce, K., and Riesner, D. (1979) FEBS Lett. 99, 117–122
Shenk, T. E., Rhodes, C., Rigby, P. W. J., and Berg, P. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 989–993
Volckaert, G., Min Jou, W., and Fiers, W. (1976) Anal. Biochem. 72, 433–446
Volckaert, G., and Fiers, W. (1977) Anal. Biochem. 83, 228–239
Watts, F., Castle, C., and Beggs, J. (1983) EMBO J. 2, 2085–2091
Wieringa, B., Hofer, E., and Weissmann, C. (1984) Cell 37, 915–925
Wise, J. A., Tollervey, D., Maloney, D., Swerdlow, H., Dunn, E. J., and Guthrie, C. (1983) Cell 35, 743–751
Zeitlin, S., and Efstratiadis, A. (1984) Cell 39, 589–602