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**A combination of RNase H and S1 nuclease circumvents an artefact inherent to conventional S1 analysis of RNA splicing**

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**ABSTRACT**

S1 nuclease mapping is commonly used to analyze transcription and processing of unlabelled RNAs. However, the S1 protocol that appears best suited to demonstrate splicing of a particular RNA (using an intronless probe that is 5' end-labelled in the downstream exon) is not diagnostic as expected. Rather, both intron-containing RNA and intronless RNA confer protection of probe across the splice juncture. To unambiguously demonstrate correctly spliced RNAs that begin at a specific initiation site, we present a procedure in which unspliced RNA molecules are first cleaved by RNase H following annealing to an intronic DNA fragment and the remaining RNA is then subjected to S1 analysis using an intronless probe present in vast excess. Only spliced, correctly initiated transcripts can protect the probe across the splice junction and up to residue +1. This RNase H/S1 method provides a broadly applicable technique with which to demonstrate splicing and initiation of a variety of transcripts, especially ones from transfected genes that can arise both from the normal and from activated cryptic initiation sites.

**INTRODUCTION**

The S1 mapping protocol of Berk and Sharp (1) has become a standard analytical technique that is widely utilized in molecular biology. Its value derives from its ability to map precisely the boundaries of even rare RNAs. In the standard application, an RNA preparation containing the transcript of interest is hybridized to excess of a denatured, end-labelled probe, and the unhybridized, single-stranded nucleic acid regions are specifically hydrolyzed by treatment with S1 nuclease. The S1 resistant probe fragments are then electrophoretically resolved under denaturing conditions and are visualized by autoradiography. Their sizes serve to map the location of the termini or other discontinuities of the RNA and their signal intensity allows quantitation of the transcript under study.

Notwithstanding its great usefulness, the S1 technique has several limitations. First, S1 nuclease does not readily attack small mismatches in RNA-DNA heteroduplexes (1). Second, rU:dA is an especially unstable duplex (2) and is readily cleaved by S1 nuclease, making oligo-U regions of RNA

artefactually appear like ends in S1 analysis (3). Third, contrary to the initial expectation that branching discontinuities in duplex structures would be sites for S1 attack on both strands (1), we have recently found that a single DNA molecule can be stably protected from S1 digestion by its simultaneous hybridization to two different RNA molecules (4). Such trimolecular hybrids can arise whenever an RNA sample contains both the transcript of interest and an abundant RNA species that hybridizes to an adjacent region of each probe molecule. At the junction of the two RNAs, the probe is refractory to S1 cleavage, making it appear as if the probe were protected by a single RNA species.

In this article, we report a complication that can arise in S1 nuclease analysis of RNA splicing. An obvious and frequently used method to demonstrate that a transcript has been spliced involves S1 mapping, using a probe prepared from the analogous cDNA construct. While one might expect that protection of probe across the splice junction would be diagnostic of spliced RNA and that unspliced RNA would only protect the probe up to the position of the intron (1), we show that intron-containing transcripts can also protect a cDNA probe from cleavage at the splice junction. Modifications of this analysis using cRNA probes and digestion with S1 or RNases do result in a higher percentage of probe molecules being cleaved opposite the looped out intron, but the efficiency never approaches 100%. To circumvent this limitation in conventional S1 analysis, we have developed a two-step analysis in which the RNA sample is first hybridized to an intronic DNA fragment and the heteroduplexed RNA region is degraded with RNase H. The resultant RNA is then analyzed by S1 nuclease mapping using an intronless probe that extends across the splice junction and upstream of the initiation site. Since the S1 probe is present in excess and unspliced RNAs are cleaved by RNase H at the site of the intron, protection of probe up to residue +1 is diagnostic of correctly initiated and spliced RNA molecules.

## **MATERIALS AND METHODS**

### **DNA templates and their transcription**

Plasmids containing the recombinant chicken  $\beta 3$  tubulin gene (p $\beta$ G3; 5) and the *X. borealis* 5S maxigene (6) were previously described. Single-strand recombinant M13mp18 phage containing chicken  $\beta 3$  tubulin cDNA was isolated as described (7). The construction of the 5S/ $\beta$ -tubulin chimeric plasmids from these DNAs is shown in Fig. 2.

Plasmid DNAs were transcribed in vitro using mouse S-100 extracts (3) in

reactions containing 90 mM KCl, 5 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM Hepes (pH 7.9), 1 mM DTT, 0.1 mM EDTA, 10 µg/ml of plasmid template, and 500 mM of each rNTP. After a 90 minute synthesis period, the reactions were stopped, treated with RNase-free DNase (Promega Biotec), phenol extracted, and ethanol precipitated following addition of yeast tRNA carrier.

#### **DNA probe preparation and S1 nuclease analysis**

Double-stranded S1 probes, prepared from the intron-containing and intronless plasmids p5Sβ3 and p5Scβ3, were 5' end-labelled with <sup>32</sup>P at the Ava I site in the downstream β3 exon, 69 nucleotides beyond the 3' splice junction. (When using the intron-containing β3 probe to map intronless RNA, a 72 base protected fragment can be formed because the trinucleotide CAG occurs immediately upstream of the 5' donor splice as well as immediately upstream of the acceptor splice site.) An excess of probe (0.01 pmole) was mixed with in vitro transcribed RNA (from 1/500 of a 25 µl reaction), lyophilized to dryness, and suspended in 20 µl of a solution containing 80% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4), and 1 mM EDTA. Following heating to 90°C for 5 minutes, the hybridization reactions were incubated for 12-16 hours at 58.5°C, diluted 15-fold by addition to ice-cold S1 nuclease buffer [0.2 M NaCl, 30 mM sodium acetate (pH 4.5), 5 mM ZnCl<sub>2</sub>, 50 µg/ml denatured salmon sperm DNA], and incubated in the presence of varying amounts of S1 nuclease (BRL) at 23°C for 60 minutes. S1-resistant probe fragments were resolved on 9 M urea/6% polyacrylamide gels and visualized by autoradiography.

For primer extension analysis (8), a 60 nucleotide Rsa I/Ava I fragment from the downstream β3 exon was isolated. The 5' terminus of this primer is at the same position as the 5' terminus of the S1 probes.

#### **RNA probe preparation and utilization**

The Sp6-derived antisense 5Scβ3 construct (pSPcβ3(R); see Fig. 6A) was linearized with Hind III beyond the β tubulin and 5S sequences and was transcribed using Sp6 polymerase (Promega Biotec) in the presence of α-<sup>32</sup>P-CTP according to the manufacturer's specifications. Following incubation for 60 minutes at 40°C, reactions were treated with RNase-free DNase (Promega), extracted with phenol/chloroform and ethanol precipitated in the presence of yeast tRNA carrier. Hybridizations (at 53°C) using this RNA probe (0.01 pmole) and S1 nuclease treatment were conducted as described above. For analysis with RNase, hybridization reactions were diluted 15-fold with a buffer containing 10 mM Tris (pH 7.5), 5 mM EDTA, 0.3 M NaCl, 40 µg/ml RNase A (P/L Biochemicals), and 2 µg/ml RNase T1 (P/L Biochemicals). Reactions were incubated for 60 minutes at the indicated temperatures, terminated by addition

of 0.15 mg/ml Proteinase K (Sigma) in the presence of 0.5% SDS, and further incubated for 15 minutes at 37°C. Following phenol extraction and ethanol precipitation, resultant protected fragments were fractionated and visualized as described above.

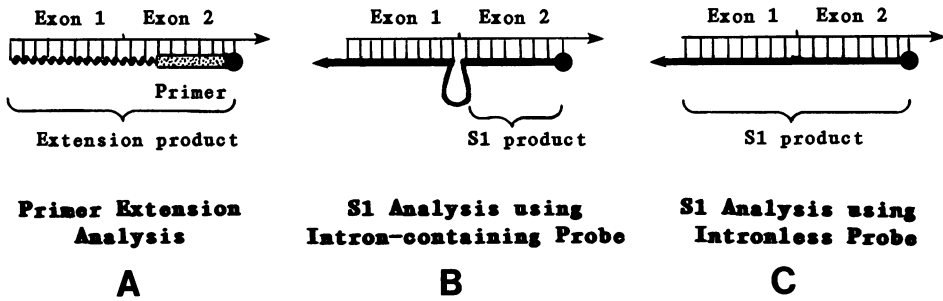
#### **RNase H/S1 assay**

For analysis of RNA by the combined RNase H/S1 assay, RNA was first hybridized to a DNA fragment from the intron region of the template. Approximately 0.1 pmole of the appropriate DNA fragment [gel isolated and purified by NACS (BRL) chromatography] was mixed with the RNA, lyophilized to dryness, suspended in 20 µl of a solution containing 80% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4), and 8 mM EDTA, and then heated to 90°C for five minutes. The sample was transferred to a 60°C water bath which was allowed to cool to 46°C and remain at this temperature for eight hours. The reaction was then diluted with 10 volumes of a solution containing 10 mM Tris (pH 7.5), 1 mM EDTA, and 20 mM NaCl, and was ethanol precipitated and suspended in 10 µl of 5 mM Tris (pH 7.5). Ten µl of 2X RNase H buffer [20 mM MgCl<sub>2</sub>, 40 mM Tris (pH 7.5), 100 mM NaCl, 2 mM DDT, 60 µg/ml BSA (Boehringer Mannheim), and 0.1 U/µl RNase H (9 ; P-L Biochemicals)] was added and the reaction was incubated at 30°C for 60 minutes. Following phenol extraction and ethanol precipitation, the samples were subjected to S1 nuclease analysis using the intronless probe as described above.

#### **RESULTS**

In the last several years a large number of studies have focused on RNA splicing. Those conducted with cell-free extracts generally employ a radiolabelled, in vitro synthesized RNA substrate, the splicing of which is determined directly from its altered electrophoretic mobility (reviewed in ref. 10). For splicing occurring in intact cells, on the other hand, the RNA substrates are generally unlabelled and determination of their fate requires indirect analyses, three methods of which are commonly in use:

(i) First is primer extension analysis (Fig. 1A), in which a primer is hybridized to sequences downstream of the splice site and extended, using reverse transcriptase, up to the 5' end of the RNA. The size of the full length extension product defines whether RNA that begins at the normal initiation site contains or lacks the intron. Although potentially definitive, this analysis is complicated by artefactual bands resulting from the propensity of reverse transcriptase to terminate prematurely, especially when transcribing relatively large (>100 nucleotide) distances, and by limited



**Figure 1: Methods commonly employed to demonstrate splicing of unlabelled RNAs.** In each panel the upper line depicts RNA, which has been spliced to remove the intron between exon 1 and exon 2.

**A. Primer extension analysis.** A primer (-----) homologous to a portion of the downstream exon is end-labelled (●), hybridized to the RNA, and extended to the 5' end of the RNA by treatment with reverse transcriptase (~~~~).

**B. S1 analysis using an intron-containing probe.** Intron-containing S1 probe, 5' end-labelled (●) in the downstream exon, is hybridized to the RNA, and treated with S1 nuclease, resulting in protection of the indicated downstream exon region of the labelled probe.

**C. S1 analysis using an intronless probe.** Intronless probe, 5' end-labelled (○) in the downstream exon, is hybridized to RNA. S1 digestion results in a protected fragment that extends across the splice junction.

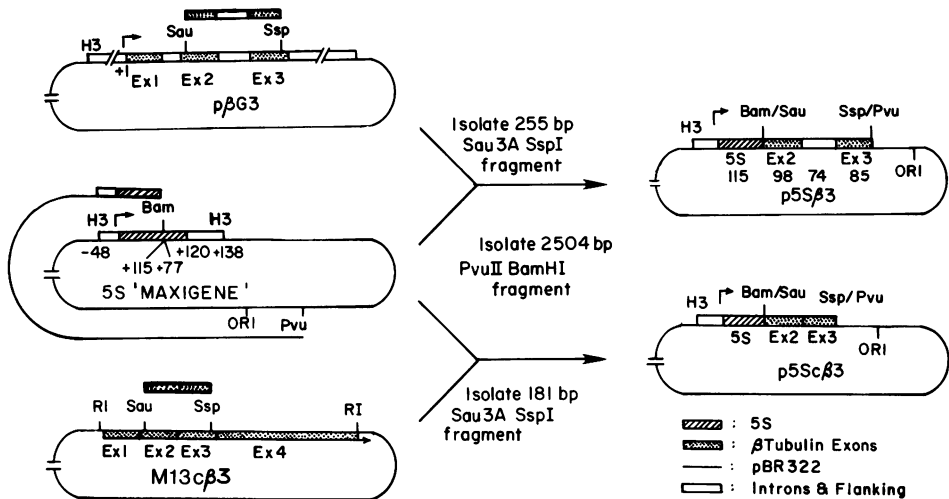
sensitivity due to difficulty in obtaining extension products from each input RNA molecule.

(ii) A second method of analyzing RNA splicing involves S1 mapping using a hybridization probe prepared from the intron-containing template DNA, 5' end-labelled in the downstream exon (Fig. 1B). Protection of a probe fragment that extends up to the proximal intron/exon boundary is diagnostic of spliced RNA. However, this method does not allow determination of whether the spliced RNAs began at the correct or at an aberrant initiation site.

(iii) The third method frequently employed to analyze splicing also involves S1 mapping, but uses a hybridization probe prepared from a cDNA clone (Fig. 1C). In this instance, a correctly spliced transcript affords protection of a labelled probe fragment that extends across the splice junction. When the intronless probe DNA is engineered to also contain the appropriate gene initiation region, the protected region will extend up to residue +1. It is this S1 methodology (which should allow the simultaneous assessment of splicing and initiation site utilization) that the present study directly addresses.

#### **S1 analysis of intron-containing and intronless transcripts**

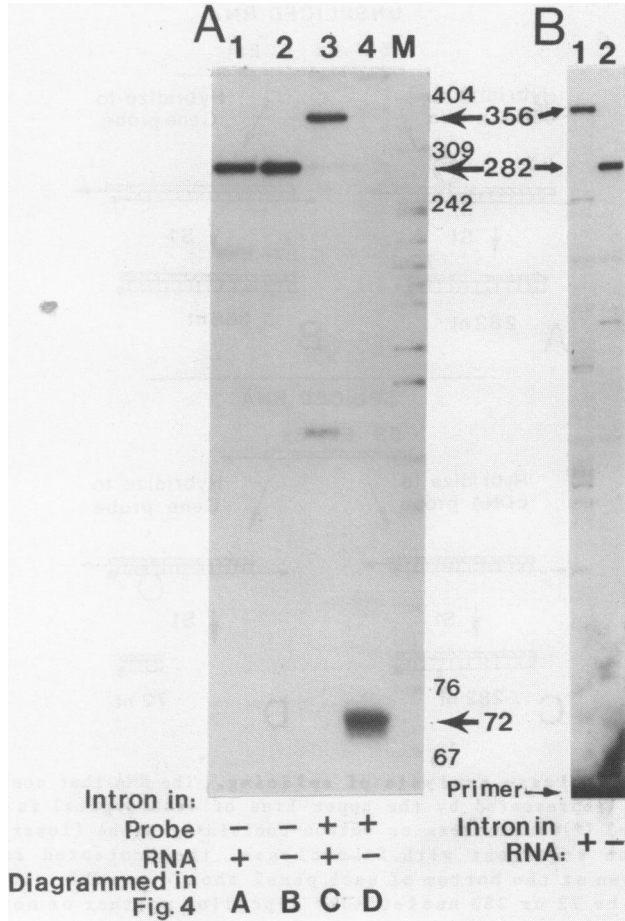
To obtain efficient production of an intron-containing RNA both in vitro



**Figure 2: Construction of p5Sβ3 and p5Scβ3.** A plasmid bearing the 5S maxigene (6) cloned into the Hind III site of pBR322 was digested with Pvu II and Bam HI to yield the overlined 2504 bp fragment containing the 5S promoter and the replication origin. This was ligated to the overlined 255 bp Sau 3A-Ssp I segment of the chicken β3 tubulin gene from plasmid pβG3, producing p5Sβ3 (top line). An analogous 5S driven plasmid lacking the intron sequences was constructed using the corresponding overlined 181 bp Sau 3A-Ssp I fragment from the cDNA clone, M13cβ3. The chicken β-tubulin gene region is devoid of T clusters and thus should not cause premature termination by RNA polymerase III.

and *in vivo*, the chimeric 5S/β3 plasmids shown in Fig. 2 were prepared. These constructs contain the promoter region of the *Xenopus borealis* somatic 5S gene (which is active both in mammalian cells and cell extracts; 11) linked to a segment of a chicken β-tubulin gene. In plasmid p5Sβ3, this segment consists of the second intron of the genomic β3 tubulin gene and ~180 bp of flanking 5' and 3' exon sequences (Fig. 2, upper construct). Plasmid p5Scβ3 is a completely analogous intronless construct that contains the correctly spliced exon sequences (Fig. 2, lower construct).

RNA was transcribed from the intron-containing and intron-less templates *in vitro* and was subjected to S1 nuclease analyses using intron-containing and intronless probes, 5' end-labelled in the downstream exon (Fig. 3A; diagrammed Fig. 4). With the intronless probe, intronless RNA protected the predicted 282 base segment (Fig. 3A, lane 2; Fig. 4C). Paradoxically, however, an identical 282 base fragment of the intronless probe was also protected by intron-containing p5Sβ3 RNAs (Fig. 3A, lane 1). If S1 nuclease had out the

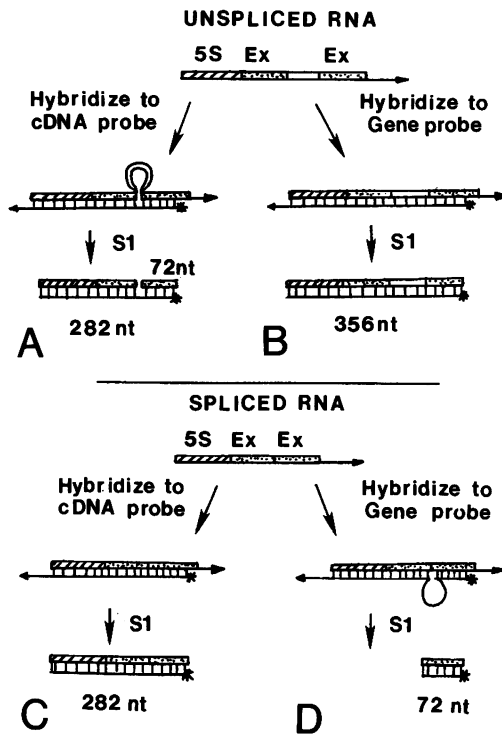


**Figure 3: Analysis of in vitro transcribed RNA.**

**A. S1 analysis.** RNA transcribed in vitro from templates containing (lanes 1, 3) or lacking (lanes 2, 4) the intron was subjected to S1 nuclease analysis using intronless (lanes 1 and 2) or intron-containing (lanes 3 and 4) probe.

**B. Primer extension analysis.** Using a primer from the downstream exon, intron-containing (lane 1) and intronless (lane 2) RNAs were assessed by primer extension analysis.

cDNA probe opposite the site of the looped out intron RNA, as is generally assumed to occur, a 72 nucleotide protected fragment would have been obtained (Fig. 4A). Yet little, if any, of the expected 72 base fragment is detected (Fig. 3A, lane 1). This indicates that either (i) the intron had been removed from the p5Sβ3 RNA in the in vitro transcription reaction or (ii) S1 nuclease failed to cleave the cDNA probe opposite the unhybridized intron loop.



**Figure 4: S1 nuclease analysis of splicing.** The RNA that contains or lacks the  $\beta 3$  intron (represented by the upper line of each duplex) is hybridized to 5' end-labelled (\*) intronless or intron-containing probe (lower line of each duplex). Upon treatment with S1 nuclease, the protected radiolabelled fragments shown at the bottom of each panel should result. In Part A, this product will be 72 or 282 nucleotides depending whether or not S1 nuclease clips the intronless probe opposite the looped out intron.

To confirm that the *in vitro* catalyzed transcripts of the p5S $\beta 3$  and p5Sc $\beta 3$  templates were indeed the anticipated intron-containing and intronless RNAs, respectively, two further analyses were performed. First, the RNAs were assessed by S1 nuclease mapping, using an intron-containing probe (diagrammed in Fig. 4B and D). As expected, the transcripts containing and lacking the intron protected 356 and 72 nucleotide regions of the intron-containing probe, respectively (Fig. 3A, lanes 3 and 4). Second, these RNAs were subjected to primer extension analysis using an end-labelled single-stranded primer complementary to a 60 nucleotide segment of the downstream exon and 5' end-labelled at the same *Ava* I site as was the S1 probe. The resultant reverse transcripts are the 356 and 282 bp lengths predicted to arise from intron-

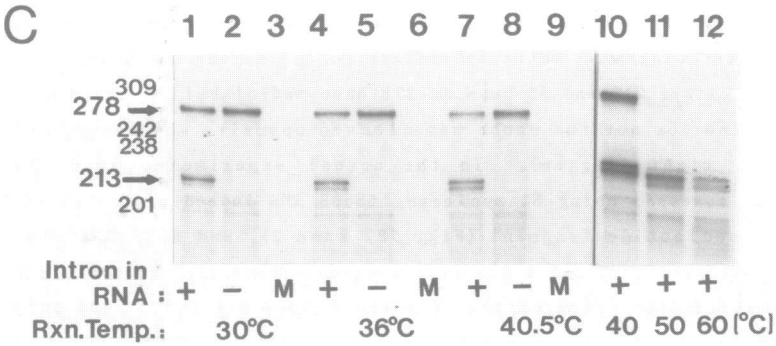
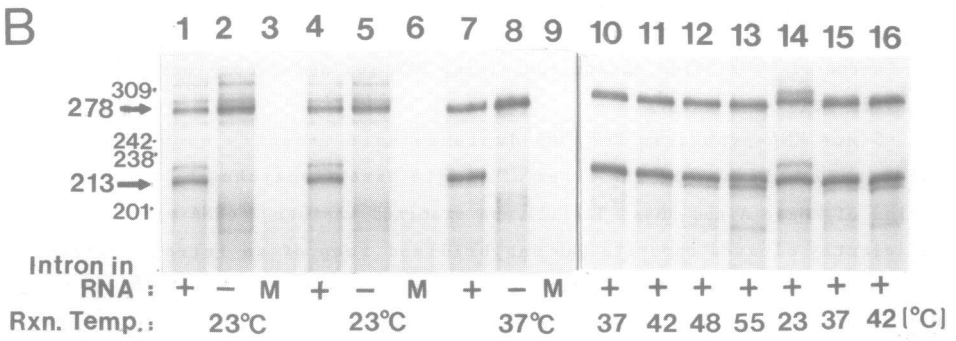
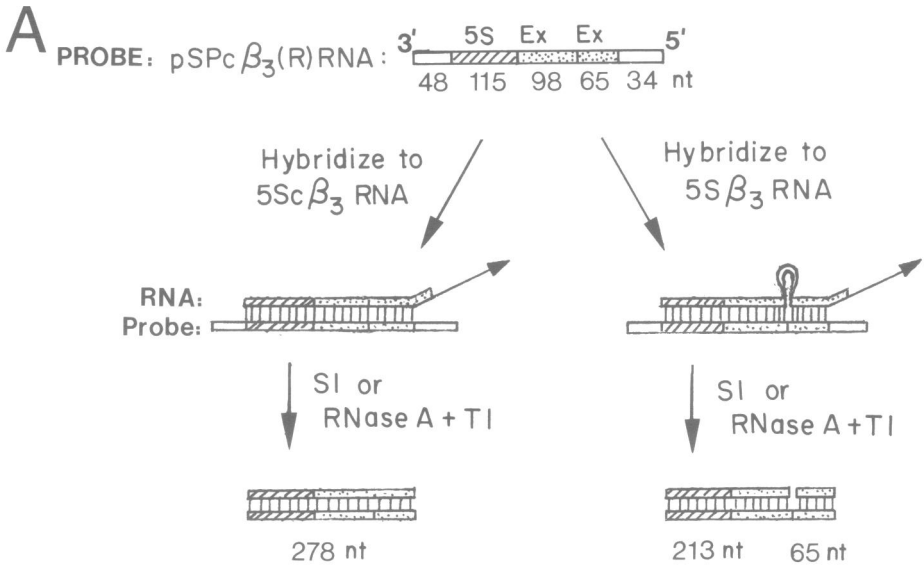


containing and intronless RNAs, respectively (Fig. 3B). These results independently confirm that the RNA transcribed *in vitro* from p5S $\beta$ 3 had not been spliced. Thus, in the experiment of Fig. 3A, lane 1, protection of the 282 base fragment must be due to a failure of S1 to cleave the intronless probe, opposite the looped out intron of a hybridized, intron-containing RNA.

In an effort to favor cleavage of the cDNA probe opposite the looped out intron, S1 nuclease analysis (as in Fig. 3A, lane 1) was repeated at varying enzyme concentrations and at increased temperature (data not shown). The 282 nucleotide fragment of intronless probe artefactually protected by intron-containing RNA is resistant to as much as an order of magnitude higher concentration of S1 than is used in our normal digestion conditions. Although at  $\geq 30$  times the normal amount of S1 some of the probe molecules are cleaved at the site of the intron to yield a limited amount of the 72 base protected fragment, the cleavage is far from quantitative and the resultant bands are markedly broadened and diminished in overall intensity (indicative of S1 nibbling at the ends of the duplex region). Similar results were obtained when the S1 digestions were conducted at 50°C. In these and a number of other experiments (data not shown), digestion of the cDNA probe opposite the looped out intron of hybridized intron-containing RNA was never complete.

#### Analysis using RNA probes

Since RNA probes in RNA/DNA duplexes have recently been shown to be efficiently cleaved at the sites of single base mismatches by the combined action of RNase A and RNase T1 (12), we examined whether a cRNA probe could be efficiently cleaved opposite the unhybridized loop of an intron-containing RNA, either by S1 nuclease or by RNase A plus RNase T1. The cRNA probe we used was a uniformly labelled SP6-derived transcript that was otherwise homologous to the coding strand of the p5S $\beta$ 3 DNA. Hybridization to intronless p5S $\beta$ 3 RNA should protect a 278 base fragment of this intronless probe (Fig. 5A, left diagram), while hybridization to intron-containing 5S $\beta$ 3 RNA would protect either 213 and 65 base or 278 base radiolabelled fragments, depending on whether or not the probe was cleaved opposite the looped out intron (Fig. 5A, right diagram). In the actual experiment, when the hybridization was assessed using S1 nuclease, 5S $\beta$ 3 RNA indeed protected the anticipated 278 nucleotide fragment (Fig. 5B, lane 2), and 5S $\beta$ 3 RNA also yielded some of the predicted 213 nucleotide fragment (lane 1). However, the efficiency of cutting the probe across from the looped out intron was only ~50%, as shown both by the ~50% diminution in the 278 base protected fragment and by the approximately equal molar ratio of the 213 and 278 nucleotide



fragments (compare lanes 1 and 2). Decreasing the salt concentration of the S1 reaction 4-fold did not appreciably increase the efficiency of this cleavage (Fig. 5B, lanes 4-6), nor did increasing the temperature of the reaction (Fig. 5B, lanes 7-13) or the amount of S1 nuclease (lanes 14-16).

When the hybrids involving the cRNA probe were assessed using a combination of RNase A and RNase T1 digestion (Fig. 5C), the results were quite similar to those obtained using S1 nuclease. Increasing the reaction temperature did increase the relative yield of the 213 base species diagnostic of probe cleavage opposite the looped out intron of unspliced RNA, but this was only at the expense of significant overdigestion, as manifested by the heterogeneous size of the resultant protected fragments (lanes 10-12). Thus, cRNA probes do not adequately overcome the artifactual appearance of splicing by unspliced RNA.

#### **RNase H allows definitive analysis of splicing**

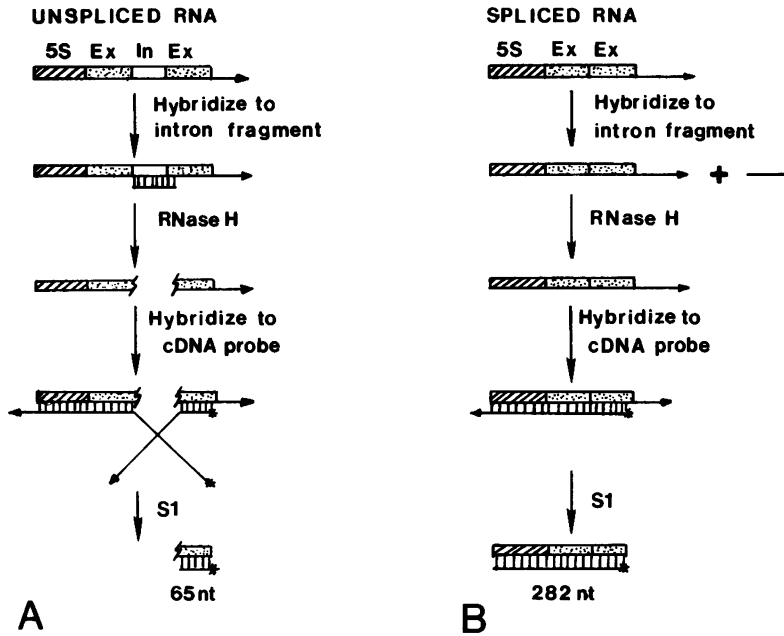
The reason that the upstream and downstream exons of the intron-containing RNA artifactually protect the intronless probe from digestion at the splice site is that the two exon transcripts are held in close proximity by virtue of their being tethered onto the same RNA molecule. Thus, even though the hybridization is performed in vast probe excess, both exon

#### **Figure 5: Splicing analysis using a cRNA probe.**

**A. Diagram of the predicted resultant fragments.** The intronless gene was cloned into pSP65 and used as template for an in vitro transcription reaction involving Sp6 polymerase and radiolabelled rXTPs. In the top line, the resultant anti-sense strand RNA is shown, extending from the Sp6 initiation site (far right) through the 34 nucleotide polylinker, the  $\beta$ -tubulin and the 5S sequences, to a Hind III site 48 nucleotides upstream of the 5S initiation site. This internally labelled, intronless RNA probe was hybridized to intronless or intron-containing RNA (the upper line in the hybrids). Upon nuclease treatment, the radiolabelled fragments depicted at the bottom of the panel should result. In the right panel, depending on whether or not the probe is cleaved opposite the looped out intron of the hybridized transcript, fragments of 213 and 65 nucleotides or 278 nucleotides would be obtained.

**B. Analysis using S1 nuclease.** RNA transcribed in vitro from intron-containing (+) or intronless (-) templates or in mock reactions containing no added template (M) was annealed with the cRNA probe, and the hybrids were assessed using S1 nuclease at 2U/ $\mu$ l (lanes 1-13) or 5U/ $\mu$ l (lanes 14-16) at the indicated temperature in normal S1 buffer (lanes 1-3) or 1/4 this buffer concentration (lanes 4-16).

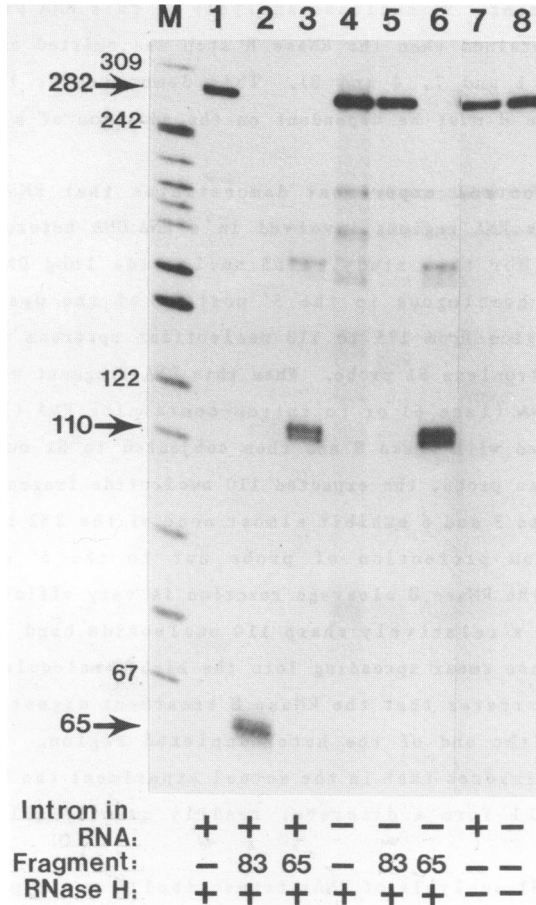
**C. Analysis using RNase A + RNase T1.** RNA transcribed in vitro from intron-containing (+) intronless (-) templates or in mock reactions containing no added template (M) was annealed with the cRNA probe, and the hybrids were assessed using a combination of RNase A (40  $\mu$ g/mg) and RNase T1 (2  $\mu$ g/ml) at the indicated temperatures.



**Figure 6: Schematic diagram of the RNase H/S1 method for analyzing transcription and splicing of RNA. Intron-containing and intronless RNAs (diagrammed in the upper line) are hybridized to an intron fragment (second line) and treated with RNase H (third line). This RNA is subsequently hybridized to an intronless probe in probe excess (fourth line) and treated with S1 nuclease (bottom line) to yield fragments which definitively reveal whether or not the original RNA contained the intron.**

transcripts hybridize to the same probe molecule (Fig. 4A). If the upstream and downstream exons were not part of the same RNA molecule, they would hybridize to different probe molecules, and S1 nuclease treatment would then accurately reveal the splicing status of the RNA transcript.

To overcome this tethering of upstream and downstream exons of unspliced RNA and allow analysis of splicing using an intronless S1 probe, we have developed a combined RNase H/S1 nuclease assay (diagrammed in Fig. 6). The RNA is first hybridized with a DNA fragment that is complementary to the intron region, and the reaction is treated with RNase H to cleave unspliced RNA in the RNA:DNA duplex region (Fig. 6A). Intronless RNA is not affected by this treatment (Fig. 6B). The RNA is then hybridized to an intronless probe and subjected to S1 nuclease analysis. The two separated exons deriving from unspliced RNAs will now hybridize to different probe molecules; S1 nuclease treatment of this material should yield only probe fragments that are



**Figure 7: RNase H/S1 analysis of intron-containing and intronless RNA.** RNA that contains (lanes 1-3, 7) or lacks (lanes 4-6, 8) the intron was hybridized to the 83 bp intron fragment (lanes 2 and 5), to the 65 bp upstream exon fragment (lanes 3 and 6), or to no fragment (lanes 1, 4, 7, 8). The reactions of lanes 1-6 were subsequently treated with RNase H. All resultant RNAs were then analyzed by S1 mapping using the intronless probe. The 110 nucleotide fragment obtained from intron-containing RNA using the 65 bp exon fragment in the RNase H step (lane 3) results from a hybrid in which the intron region of the transcript is looped out, forming a S1-resistant junction.

protected up to the site of the intron and no artefactual longer fragments (Fig. 6A). Thus, protection of probe out to the transcription initiation site should be diagnostic of spliced, correctly initiated RNA (Fig. 6B).

To verify the RNase H/S1 protocol, two control experiments were first performed. In one, no DNA fragment was added to hybridize to the RNA prior to

the RNase H treatment. S1 nuclease analysis of this RNA yielded the same results as were obtained when the RNase H step was omitted entirely (in Fig. 7, compare lanes 1 and 7, 4 and 8). This demonstrates that any effect obtained with RNase H must be dependent on the addition of a hybridizing DNA fragment.

The second control experiment demonstrates that RNase H treatment efficiently digests RNA regions involved in a RNA:DNA heteroduplex (Fig. 7, lanes 3 and 6). For this study, a 65 nucleotide long DNA fragment was prepared that is homologous to the 5' portion of the upstream exon; it encompasses the region from 175 to 110 nucleotides upstream of the labelled terminus of the intronless S1 probe. When this DNA fragment was hybridized to the intronless RNA (lane 6) or to intron-containing RNA (lane 3) and the reaction was treated with RNase H and then subjected to S1 nuclease analysis using the intronless probe, the expected 110 nucleotide fragment was obtained. The fact that lanes 3 and 6 exhibit almost none of the 282 nucleotide band which results from protection of probe out to the 5' end of the RNA demonstrates that the RNase H cleavage reaction is very efficient. Moreover, the appearance of a relatively sharp 110 nucleotide band in these lanes, rather than a diffuse smear spreading into the higher molecular weight region of the gel, demonstrates that the RNase H treatment digests the RNA quite precisely out to the end of the heteroduplexed region. This result is important, for it insures that in the actual experiment the RNase H cleaved unspliced RNA will form a discrete, readily quantifiable band upon S1 analysis.

The RNase H/S1 analysis of RNAs transcribed from the p5S $\beta$ 3 and p5Sc $\beta$ 3 templates is shown in Fig. 7, lanes 2 and 5. For this analysis, an 83 nucleotide fragment which contains virtually the entire intron and ends 65 nucleotides upstream from the labelled terminus of the S1 probe was prepared. When intron-containing RNA was hybridized to this DNA fragment, the intron region of the transcript was efficiently degraded by treatment with RNase H (Fig. 7, lane 2). This digestion is detected in the subsequent S1 nuclease analysis as efficient cleavage of the probe to yield the expected 65 nucleotide protected fragment (see Fig. 6A). Virtually none of the artefactually protected 282 nucleotide fragment was obtained from this S1 analysis of RNase H-treated intron-containing RNA (Fig. 7, lane 2). In contrast, intronless RNA is not digested by RNase H when annealed with the 83 nucleotide intron fragment, and the expected 282 nucleotide fragment of the

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cDNA probe is protected without yielding any of the 65 nucleotide fragment (lane 5). Thus, the RNase H pretreatment enables accurate S1 nuclease assessment of splicing using an intronless probe.

## **DISCUSSION**

In the present manuscript, we have identified a source of ambiguity, and thus potential misinterpretation, in conventional S1 nuclease analysis of splicing using cDNA probes. With this normal S1 protocol, unspliced RNA artefactually appears to be spliced because S1 nuclease cannot efficiently cleave the intronless probe opposite the looped out intron of a hybridized, unspliced RNA molecule (illustrated in Fig. 4A). Although reaction conditions can be manipulated to increase the amount of cleavage at the site of the intron, it is only at the expense of significant overdigestion, resulting in low signal intensities and reduced lengths of cleavage products. Furthermore, while a promising alternate analysis which utilizes a cRNA hybridization probe and digestion by RNases A and T1 does result in a higher efficiency of probe cleavage opposite the intron, the digestion is still far from quantitative.

These results mandate that caution be exercised in interpreting a number of experiments in the literature which have exploited cDNA probes to analyze transfected and/or in vitro derived RNA products. (For examples of the common, general usage of this kind of S1 analysis see refs. 13-22.) In most of these cases, total cellular RNA was analyzed, but because hnRNA constitutes an appreciable fraction of certain RNA species, these unspliced molecules could give rise to artefactual protection of the cDNA probe. Furthermore, while one initially might think that S1 analysis of cytoplasmic RNA would be less likely to be affected by artefactual protection due to unspliced molecules, the production of predominantly unspliced cytoplasmic transcripts of transfected tk/ $\beta$ -globin chimeric genes (23) indicates that unspliced cytoplasmic RNAs could also give rise to artefactual protection of cDNA probes.

Both of the major alternative techniques used to demonstrate splicing of unlabelled RNAs have certain drawbacks (Fig. 1). The first, primer extension analysis using a primer complementary to downstream exon sequences is potentially extremely powerful, but technical problems such as premature terminations and limited extension efficiencies make it very challenging to use this technique on long or low abundance transcripts. A second alternative is S1 nuclease analysis using an intron-containing probe. Although this is

the method of choice to demonstrate that a particular RNA is not spliced, it is less suitable for assessing spliced RNA because it can only directly measure RNA discontinuity at the 3' splice border, and not accurate splicing per se. In addition, this method is unable to distinguish whether the detected RNAs had initiated correctly or aberrantly. This latter point is especially significant when assessing transcripts of templates that have been transfected into mammalian cells, since the prokaryotic vector sequences frequently provide fortuitous transcription initiation sites and thus a significant fraction of the RNA from certain transfected plasmids can derive from fortuitous initiations (by any one of the three eukaryotic RNA polymerases). Moreover, such large and/or heterogeneously initiated RNAs can readily escape detection when the analysis utilizes either primer extension or S1 nuclease mapping with probes that do not detect such divergent RNAs in a discrete, quantifiable band.

To overcome the inherent limitations in the procedures described above, we have developed an analytic method that makes use of RNase H and S1 nuclease. First, the RNA sample of interest is hybridized to a DNA fragment (or oligonucleotide) complementary to intronic sequences, and the RNA of the duplex region is degraded by treatment with RNase H. The resultant RNA is then subjected to S1 mapping using an intronless hybridization probe that is constructed to be homologous to the gene and its promoter region. Protection of a segment of probe that extends from the labelled terminus across the splice juncture and up to residue +1 is diagnostic of an accurately transcribed and spliced RNA species. We suggest that this RNase H/S1 analysis may provide the most rigorous and convenient method by which even a low abundance transcript can be definitively assessed for its accurate initiation and splicing.

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