Cell, Vol. 32, 681–694, March 1983, Copyright © 1983 by MIT

Human β-Globin Pre-mRNA Synthesized In Vitro Is Accurately Spliced in Xenopus Oocyte Nuclei

Michael R. Green, Tom Maniatis and D. A. Melton
Department of Biochemistry and Molecular Biology
Harvard University
7 Divinity Avenue
Cambridge, Massachusetts 02138

Summary

To study the mechanisms of RNA splicing we have synthesized β-globin mRNA precursors by in vitro transcription using a plasmid in which a human β-globin gene is fused to an efficient bacteriophage promoter. The structural requirements for accurate splicing of the in vitro synthesized pre-mRNAs were investigated by injection into Xenopus oocyte nuclei. We detect splicing only if the pre-mRNA is capped in vitro prior to injection; uncapped RNA is rapidly degraded. In addition, we find that in vitro synthesized pre-mRNAs that are not polyadenylated or lack a normal 3′ end are spliced following injection into oocytes. The observation that a purified pre-mRNA can be spliced in oocytes indicates that transcription and splicing are not obligatorily coupled. When an in vitro synthesized β-globin pre-mRNA containing a splice junction mutation is microinjected, the affected junction is not spliced, indicating that sequences necessary for accurate splicing in human cells are also necessary for splicing in oocytes.

Introduction

Many eukaryotic genes are interrupted by intervening sequences which are present in the primary transcripts of these genes but are subsequently removed by splicing. Although several models for splicing have been proposed, they remain largely unsubstantiated (see Lewin, 1980; Sharp, 1981; Flint, 1983, for reviews). In addition, the proposed models do not address many crucial features of mRNA splicing such as: the features of a pre-mRNA that allow recognition by the appropriate splicing enzymes; the potential role of ribonucleoprotein structure(s) in RNA processing; the identification and localization of pre-mRNA splicing enzyme(s); the chemical mechanism of intron removal; the factors that govern the selection of donor and acceptor sequences; and what, if any, regulation of gene expression is imposed at the level of pre-mRNA splicing.

An important clue regarding the RNA signals required for splicing has emerged from the nucleotide sequence analysis of numerous splice junctions. Intervening sequences ordinarily begin with the dinucleotide GT and end with AG. In addition, it is possible to formulate consensus 5′ and 3′ splice junction sequences which extend in both directions beyond the conserved dinucleotides (Breathnach et al., 1978; Mount, 1982). The requirement for the GT dinucleotide in mRNA processing has been directly demonstrated by analysis of transcripts derived from mutant genes containing single base changes in the G (Treisman et al., 1982; Wieringa et al., 1982) or T (Montell et al., 1982).

The detailed mechanisms and enzymology of RNA processing could be most effectively addressed by using an in vitro system for splicing which would allow the identification and purification of the requisite components. A major difficulty in developing such systems is obtaining precursor RNA substrates for splicing. Primary transcripts for a given gene represent only a small fraction of the total cellular RNA even for those of highly abundant cytoplasmic mRNAs (see, for example, Hofer and Darnell, 1981). Moreover, primary transcripts are rapidly processed and are, therefore, present at low steady state levels (for review see Darnell, 1979). In addition, an efficient cell-free system for RNA processing has not been developed. Although several reports show that splicing occurs in isolated nuclei (Blanchard et al., 1978; Manley, Sharp, and Gefter, 1979; Yang and Flint, 1979; Hamada, Igarashi, and Muramatsu, 1980), the efficiency of splicing is very poor (Manley, Sharp, and Gefter, 1982) and splicing has not been separated from transcription. Three groups have reported splicing in cell-free systems (Weingartner and Keller, 1981; Goldenberg and Raskas, 1981; Kole and Weissman, 1982). However, because of difficulties in reproducibility (see, for example, Handa et al., 1981; Cepko et al., 1981) and relatively inefficient splicing, significant new insights into the mechanisms of splicing have not yet emerged.

In an alternative approach to the study of splicing we injected unspliced transcripts of the human β-globin gene into Xenopus oocyte nuclei. These RNAs were synthesized in vitro by using a plasmid in which a highly efficient SP6 bacteriophage promoter (Butler and Chamberlin, 1982) is fused to a human β-globin gene (E. Butler and P. Little, unpublished). In vitro transcription of this plasmid with SP6 RNA polymerase yields substantial quantities of a single RNA species that resembles a genuine primary transcript. For convenience, we refer to in vitro synthesized β-globin RNAs as 'pre-mRNAs', noting that they are not identical to authentic β-globin primary transcripts. Xenopus oocytes were chosen as a test system because DNA and RNA can be readily introduced into oocyte nuclei by microinjection (reviewed by Gurdon and Melton, 1981). Moreover, mature proteins are produced following microinjection of DNA containing intervening sequences, indicating that injected oocytes are able to splice accurately pre-mRNAs (reviewed by Gurdon and Melton, 1981). In this report we describe experiments that demonstrate accurate splicing of in vitro synthesized β-globin pre-mRNA in oocyte nuclei.

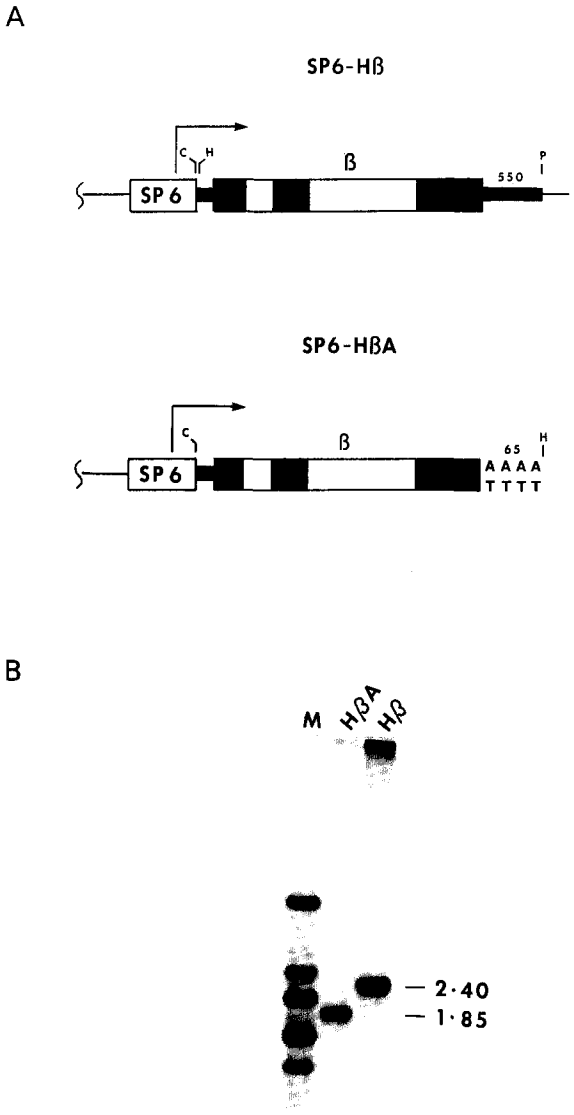


Figure 1. In Vitro Transcription of SP6-Hβ and SP6-HβA

A. The two plasmids are represented in the linear form normally used for in vitro transcription. The approximate location of the SP6 initiation site and direction of transcription are indicated by the arrow. For SP6-Hβ, the distance from the polyadenylation site to the Pst I site used to linearize the plasmid is 550 bp. For SP6-HβA the region from the Eco RI site in the third exon to the AT homopolymer tract is derived from the β-globin cDNA clone JW102 (Wilson et al., 1978). (Solid blocks) β-globin gene exons. (Open blocks) intervening sequences. (Thin lines) pBR322 sequences. The 65 base pair AT tract in SP6-HβA and SP6 DNA sequences in both plasmids are indicated. H: Hind III sites. P: Pst site. C: Cla I site. B. In vitro transcription reactions were conducted as described in Experimental Procedures using as templates Pst-digested SP6-Hβ and Hind III–digested SP6-HβA DNAs. A small portion of the purified ^{32}P-RNAs were denatured with glyoxal (McMaster and Carmichael, 1977) and subjected to electrophoresis in a 1% agarose gel in 20 mM MOPS (morpholinopropanesulfuric acid), 5 mM sodium acetate, 1 mM EDTA. Following electrophoresis the gel was dried and autoradiographed. 3′-end labeled ^{32}P-DNA markers (M) were derived from pBR322 DNA and include fragments of 4.36, 2.67, 2.04, and 1.69 kb.

Results

Strategy for Preparing Large Amounts of Human β-Globin Pre-mRNA

The human β-globin gene transcription unit extends from the mRNA capping site to the polyadenylation site and includes intervening sequences (IVS) of 130 and 850 nucleotides in length (Figure 1, Lawn et al., 1980). Normal β-globin pre-mRNAs are capped and polyadenylated (for review see Curtis et al., 1977; Lingrel et al., 1979; Ross et al., 1981), their 5′ and 3′ ends are identical to mature β-globin mRNA (Weaver and Weissman, 1979; Grosveld, Koster and Flavell, 1981) and they contain both intervening sequences (Tilghman et al., 1978). Although β-globin pre-mRNA transcripts can be detected in nucleated erythroblasts (Bastos and Aviv, 1977; Curtis, Mantei and Weissmann, 1977; Lingrel et al., 1979; Ross et al., 1981) they cannot be obtained in amounts necessary for detailed biochemical study.

To obtain large amounts of β-globin pre-mRNA we used a recombinant plasmid containing a 400 base pair promoter fragment from the bacteriophage SP6 (R7Δ7, E. Butler and P. Little, unpublished). SP6 is a Salmonella phage that encodes an RNA polymerase specific for SP6 promoters (Butler and Chamberlin, 1982). One advantage of this in vitro transcription system is that SP6 RNA polymerase initiates transcription exclusively at the SP6 promoter, thus avoiding transcripts that initiate elsewhere on the plasmid DNA (Butler and Chamberlin, 1982). In addition it is possible to obtain long transcripts that extend through cloned DNA sequences (E. Butler, unpublished results and our unpublished data). Finally, SP6 RNA polymerase is relatively easy to purify in large amounts and is remarkably stable (Butler and Chamberlin, 1982).

Derivatives of the plasmid R7Δ7 containing human β-globin genes fused to the SP6 promoter are shown in Figure 1A. The plasmid SP6-Hβ contains the entire human β-globin gene transcription unit, 46 bp of 5′ flanking sequence and greater than 1 kb of 3′ flanking sequence (E. Butler and P. Little, unpublished). When this plasmid is linearized by Pst I and transcribed with SP6 RNA polymerase, a discrete 2.4 kb RNA is produced (Figure 1B). S1 nuclease mapping experiments indicate that initiation of transcription occurs about 200 base pairs from the right end of the SP6 promoter fragment as it is shown in Figure 1A (data not shown). Thus, this nonpolyadenylated in vitro transcript contains 246 additional nucleotides at the 5′ end and 550 nucleotides 3′ to the polyadenylation site of the human β-globin gene (Lawn et al., 1980).

In order to synthesize unspliced β-globin transcripts with polyadenylated 3′ ends, we constructed a β-

globin gene derivative of SP6-Hβ in which the third exon of the gene is followed by a poly dT sequence in the coding strand (SP6-HβA, Figure 1A; see experimental procedures for details of the construction). DNA sequence analysis of SP6-HβA confirmed the presence of a 65 nucleotide dT tract in the coding strand adjacent to the Hind III site, but revealed that 45 nucleotides of the 3′ untranslated region, including the AATAAA putative polyadenylation signal (Proudfoot and Brownlee, 1974; Fitzgerald and Shenk, 1981) was deleted. (See experimental procedures for details). When SP6-HβA DNA is digested with Hind III and transcribed with SP6 RNA polymerase, a 1.85 kb polyadenylated β-globin pre-mRNA is obtained (Figure 1B). This transcript binds quantitatively to oligodT cellulose; digestion of the RNA with sequence-specific ribonucleases produces a resistant ^{32}P-polyA fragment of 65 nucleotides, indicating that the SP6 polymerase can efficiently transcribe the 65 base pair AT tract (data not shown).

Stability of β-Globin Pre-mRNA In Vitro Transcripts and β-Globin mRNAs in Xenopus Oocytes

To determine whether the in vitro synthesized β-globin pre-mRNA can serve as a substrate for splicing, ^{32}P-labeled RNA was microinjected into Xenopus oocytes. When either SP6-Hβ or SP6-HβA-derived RNA is injected into oocyte nuclei or cytoplasm, no discrete ^{32}P-RNA product is observed after short incubations. The analysis of injected SP6-HβA transcripts is shown in Figure 2A, identical results were obtained with SP6-Hβ RNA (data not shown).

To determine whether any of the injected SP6-HβA RNA is accurately spliced, total RNA from injected oocytes was analyzed by using an S1 nuclease assay (Weaver and Weissmann, 1979). A 5′ ^{32}P-labeled Bam HI DNA fragment, which extends from exon 2 to the 5′ end of the β-globin sequence, was annealed to oocyte RNA, digested with S1 nuclease and the products fractionated by polyacrylamide gel electrophoresis (Figure 2B). The unspliced in vitro transcript protects 526 nucleotides of the probe corresponding to the distance between the Bam HI site and position −46 of the β-globin gene. Authentic β-globin mRNA generates a 209 nucleotide S1 nuclease-resistant DNA fragment which measures the distance from the Bam HI site in the second exon to the IVS-1 3′ splice site (Figure 2B). We detect a 526 nucleotide fragment immediately after injection; this fragment decreases in amount as a function of time after injection. A 209 nucleotide band representing an accurate endonucleolytic cleavage at the IVS-1 3′ splice site is not detectable at any time. This failure to observe splicing could be a consequence of the rapid turnover of the injected RNA or the structure of the SP6-HβA transcript may be incompatible with normal splicing. Alternatively, transcription and splicing may be obligatorily coupled. These possibilities are considered below.

The rapid turnover of β-globin pre-mRNA is consistent with previous studies that demonstrate degradation of a variety of RNAs, including eukaryotic polyA(+) RNAs, following injections into the cytoplasm of oocytes (Allende, Allende and Firtel, 1974). In contrast, some mRNAs, including globin mRNAs, are stable following cytoplasmic injections and are actively translated for up to 2 weeks (Gurdon, Lingrel and Marbaix, 1973). To our knowledge, the stability of globin mRNAs or pre-mRNAs in Xenopus oocyte nuclei has not been reported. We therefore injected human β-globin mRNA into oocyte nuclei and cytoplasm and assayed for stability by an S1 nuclease analysis. As shown in Figure 3, the mRNA remains intact in oocytes for up to 24 hr following either nuclear or cytoplasmic injection. Identical results were obtained when RNA stability was analyzed by the RNA blotting procedure (data not shown). Thus, β-globin mRNA is stable following nuclear injections as has been previously shown for cytoplasmic injections.

We next investigated the basis for the significant difference in stability of SP6-generated in vitro transcripts and mature β-globin mRNA. One obvious difference is that the mRNA contains a 5′ terminal cap structure (reviewed by Banerjee, 1980), while the in vitro transcript contains a 5′ triphosphate terminus. Several studies have suggested that the cap plays a role in mRNA translation (Lockard and Lane, 1978; Paterson and Rosenberg, 1979) and cytoplasmic stability (Furuichi, LaFiandra and Shatkin, 1977), but the effect of a 5′ cap on RNA stability in the nucleus has not been reported. To determine whether the cap is important for nuclear stability we removed the cap from the β-globin mRNA with the enzyme tobacco acid pyrophosphatase to produce 'decapped' mRNA (Efstratiadis et al., 1977). The enzyme preparation we used was shown to be free of contaminating ribonucleases by incubation with several in vitro–synthesized ^{32}P-RNAs (data not shown). The results of injecting decapped β-globin mRNA into oocyte nuclei are shown in Figure 3B. In this case, a 5′ ^{32}P-labeled Mst II–Hae III probe specific for the 5′ end of β-globin mRNA (Figure 3B) was employed in an S1 nuclease assay. Immediately after injection, the 5′ end of the decapped mRNA is intact and protects a 70 nucleotide fragment identical to the untreated mRNA. Enzymatic decapping results in only a very small amount of RNA degradation, as evidenced by minor protected bands shorter than 70 nucleotides. The integrity of the decapped β-globin mRNA was also demonstrated with the Acc I probe (Figure 3A) producing the expected 211 nucleotide S1 nuclease–resistant ^{32}P-DNA fragment (data not shown). After 5 hr of incubation in injected oocytes, the decapped mRNA is almost entirely degraded which is in marked contrast to the results with mature β-globin mRNA (Figure 3A). The small amount of RNA that remains intact in the oocyte is most likely due to mRNA that was originally resistant to enzymatic decapping (Efstratiadis et al., 1979). We

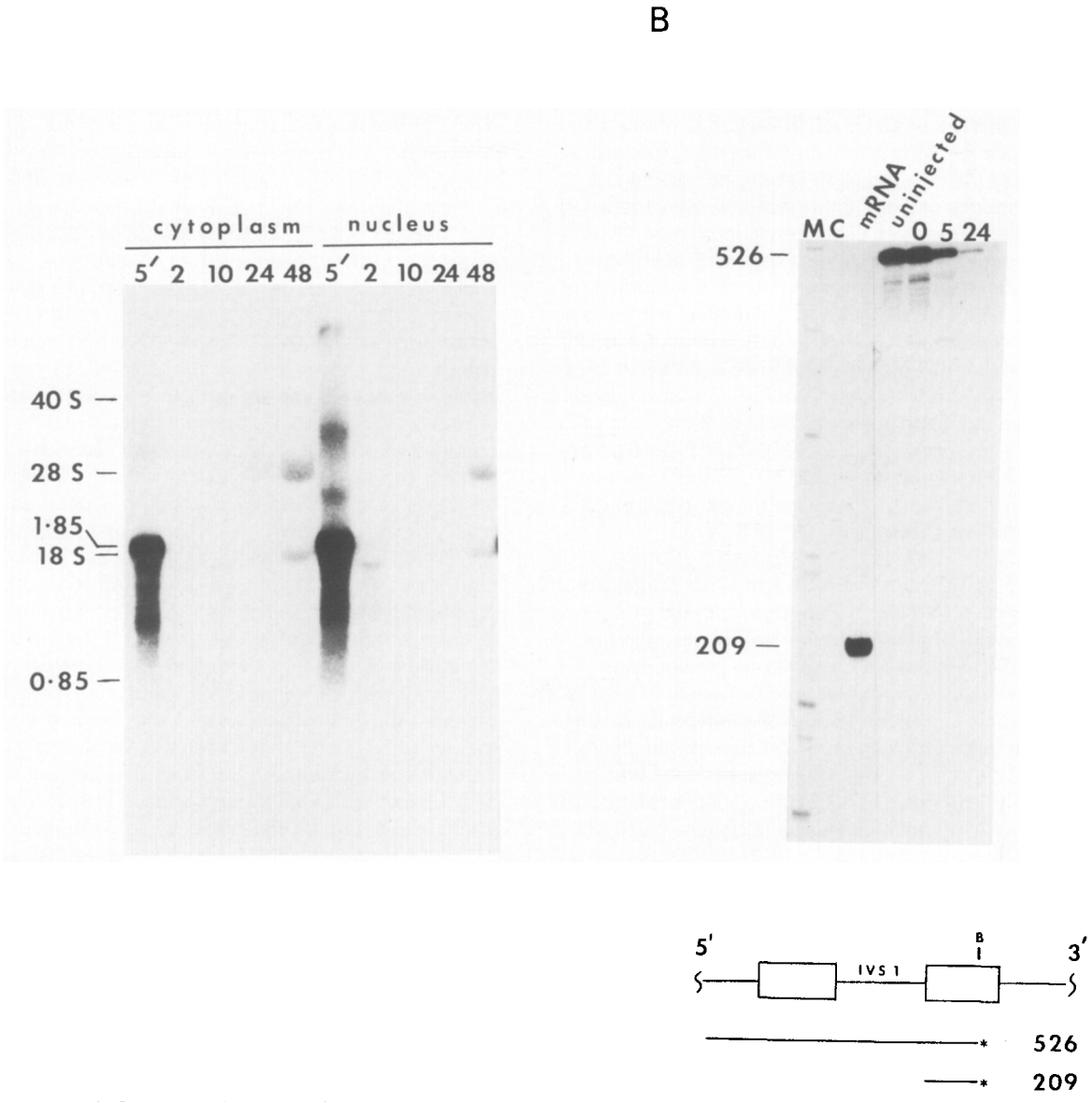


Figure 2. SP6-HβA RNA Is Rapidly Degraded in Oocytes

A. ^{32}P-RNA was transcribed from Hind III digested SP6-HβA DNA, purified, and injected into the nucleus and cytoplasm of oocytes as described in Experimental Procedures. At the indicated times (in hr except for the 5 min point) total RNA was extracted from oocytes and a portion denatured with glyoxal and analyzed on a 1% agarose gel. Size standards include denatured ^{32}P-DNA markers run in parallel and endogenous oocyte ribosomal RNAs. Sizes are indicated on the left and include the expected position of the fully spliced RNA (0.85 kb, see Figure 6). The bands that appear at late times are due to reincorporation of ^{32}P into oocyte 40S, 28S, and 18S ribosomal RNAs. B. RNA was transcribed from Hind III–digested SP6-HβA DNA, purified, and injected into oocyte nuclei. At the indicated times (hr) total RNA was extracted from the oocytes and a portion subjected to S1 nuclease analysis. The probe was a double-stranded 1.8 kb Bam HI fragment, 5′ ^{32}P-labeled in the second exon at nucleotide 481. The expected S1 nuclease–resistant DNA products are shown in the diagram below the figure; ^{32}P-labeled ends are shown as stars and the Bam HI site as B. The control reactions include: C, total RNA from uninjected oocytes; mRNA, uninjected oocyte RNA plus human 9S globin RNA; uninjected, uninjected oocyte RNA plus the original in vitro β-globin transcript. ^{32}P-DNA markers (M) are from a Hpa II digest of pBR322 DNA. The products were fractionated on a 5% denaturing polyacrylamide gel.

conclude that the stability of mature β-globin mRNA in oocyte nuclei is critically dependent upon a 5′ cap structure.

In Vitro Capping of SP6-Derived β-Globin Pre-mRNAs

The observation that a 5′ terminal cap structure is necessary for stabilizing β-globin mRNA in the oocyte nucleus suggested that the failure to observe specific processing of uncapped in vitro transcripts may be due to rapid nonspecific degradation. To test this possibility, we capped the SP6-generated transcripts by using vaccinia virus guanylyltransferase, which is active on RNAs with 5′ triphosphate termini (Monroy, Spencer and Hurwitz, 1978; Venkatesan, Gershowitz and Moss, 1980).

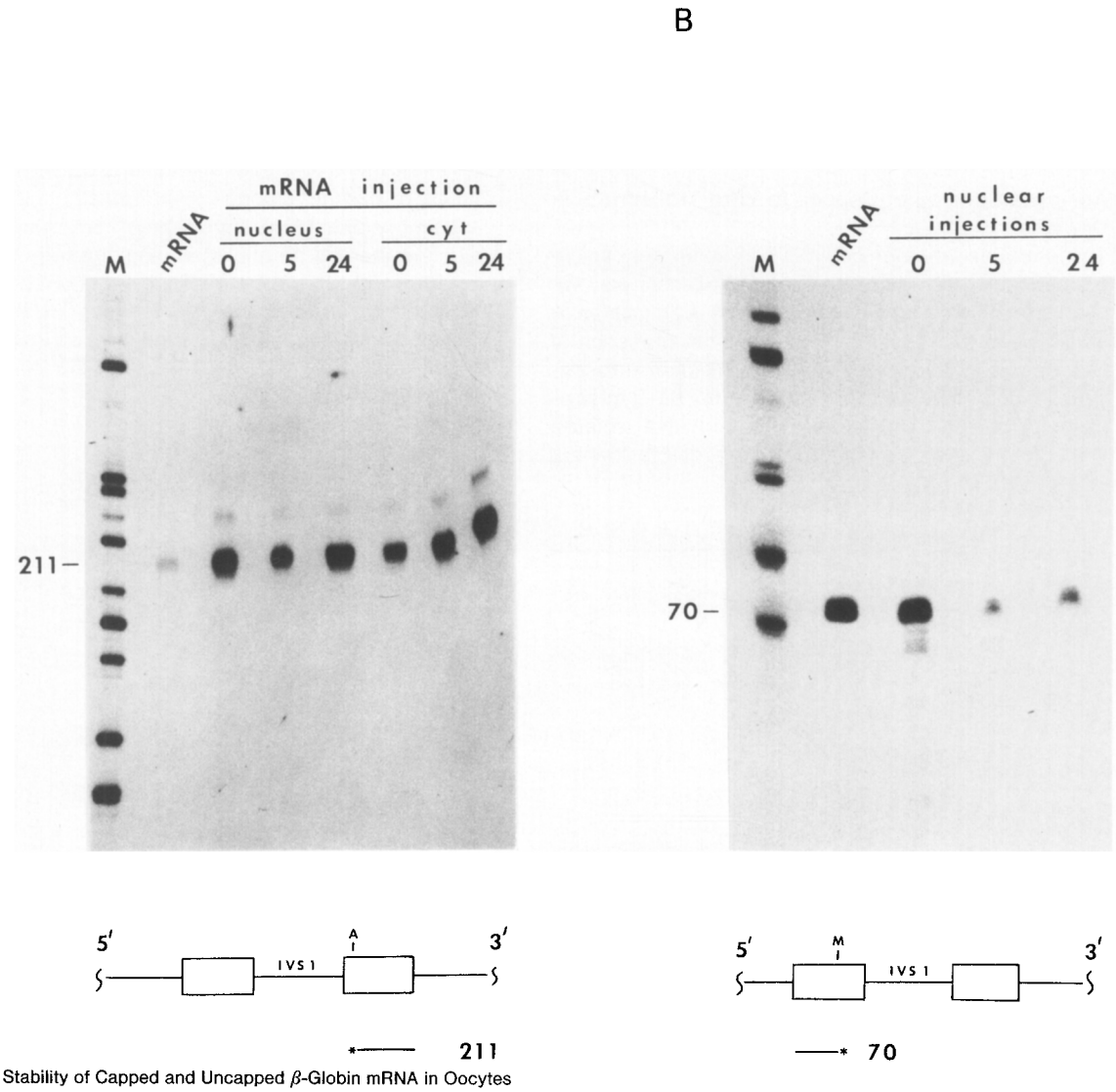


Figure 3. Stability of Capped and Uncapped β-Globin mRNA in Oocytes

A. Approximately 6 ng of human 9S globin RNA was injected into the nucleus or cytoplasm of each oocyte. At the indicated times (hr) total RNA was extracted from the oocytes and a portion subjected to S1 nuclease analysis. The probe was a double-stranded 1.7 kb Acc I fragment 3′ end-labeled within the second exon at position 282. The expected S1 nuclease-resistant DNA product is shown in the diagram below the figure; ^{32}P-labeled DNA ends are shown as stars and Acc I site as A. The control reaction designated mRNA contains uninjected oocyte RNA plus human 9S globin RNA. ^{32}P-DNA markers (M) are from a Hpa II digest of pBR322 DNA. The products were fractionated on a 5% denaturing polyacrylamide gel. B. Human 9S globin RNA was enzymatically decapped as described in Experimental Procedures. Approximately 6 ng of decapped RNA was injected into the nucleus of each oocyte. Although this experiment was not carried out with the same batch of oocytes used for the experiment of Figure 3A, the integrity of the oocytes was documented by their ability to transcribe efficiently a tRNA gene and by the oocytes' morphology 24 hr after injection. At the indicated times (hr) total RNA was extracted from the oocytes and a portion subjected to S1 nuclease analysis. The probe was a single-stranded 5′ ^{32}P-labeled Mst II–Hae III fragment (−76–+170). The expected S1 nuclease-resistant DNA product is shown in the diagram below the figure; ^{32}P-labeled DNA ends are shown as stars and Mst II site as M. The control reaction designated mRNA contains oocyte RNA from uninjected oocytes plus human 9S globin RNA. ^{32}P-DNA markers (M) are from a Hpa II digest of pBR322 DNA. The products were fractionated on a 8% denaturing polyacrylamide gel.

The efficiency of capping SP6-generated transcripts in vitro was tested by using ^{32}P end-labeled RNA as a substrate. Transcription from the SP6 promoter in R7Δ7 plasmids is initiated with a guanosine residue (M. Chamberlin, personal communication). Thus, by conducting transcription reactions in the presence of ^{32}P-β-GTP it is possible to obtain β-globin pre-mRNA in which only the terminal nucleotide G is labeled. Following the capping reaction, the RNA was digested with nuclease P1 and the radioactive products analyzed by thin layer chromatography (Shuman and Hurwitz, 1981) along with fluorescent markers for 5′ terminal structures (Figure 4). P1 nuclease digestion of uncapped end-labeled RNA produces a single product, ^{32}P-GTP, as expected. When reacted with guanylyltransferase, the RNA 5′ termini are quantitatively converted to the 5′ structure GpppG. When the methyl donor S-adenosyl-methionine is included in the

capping reaction, the capped structure is ^{7}mGpppG (Figure 4). When the capped ^{32}P-RNAs are analyzed by denaturing gel electrophoresis there is no evidence of RNA degradation during the enzymatic reaction (data not shown).

Accurate Splicing of Capped In Vitro Transcripts in Oocytes

To determine whether oocytes will accurately splice capped and polyadenylated in vitro transcripts, we used the Hind III linearized SP6-HβA as a template and capped the resulting ^{32}P-RNA in the presence of S-adenosyl-methionine. Following injection into oocyte nuclei, RNA was analyzed by an S1 nuclease assay (Figure 5). Uninjected RNA and RNA isolated immediately after nuclear injection protects the expected 526 nucleotide fragment from S1 nuclease digestion. Several shorter fragments also appear; these may result from the trace amounts of nicked RNA in the original sample or artifactual cleavage of the DNA–RNA hybrid by S1 nuclease. The amount of full length 526 nucleotide fragment decreases over the 24 hr period, although some remains even at the last time point. Most importantly, immediately after injection there is no nuclease-resistant fragment that migrates with the 209 nucleotide fragment produced by authentic β-globin mRNA. However, within 2 hr following injection an RNA is produced which protects

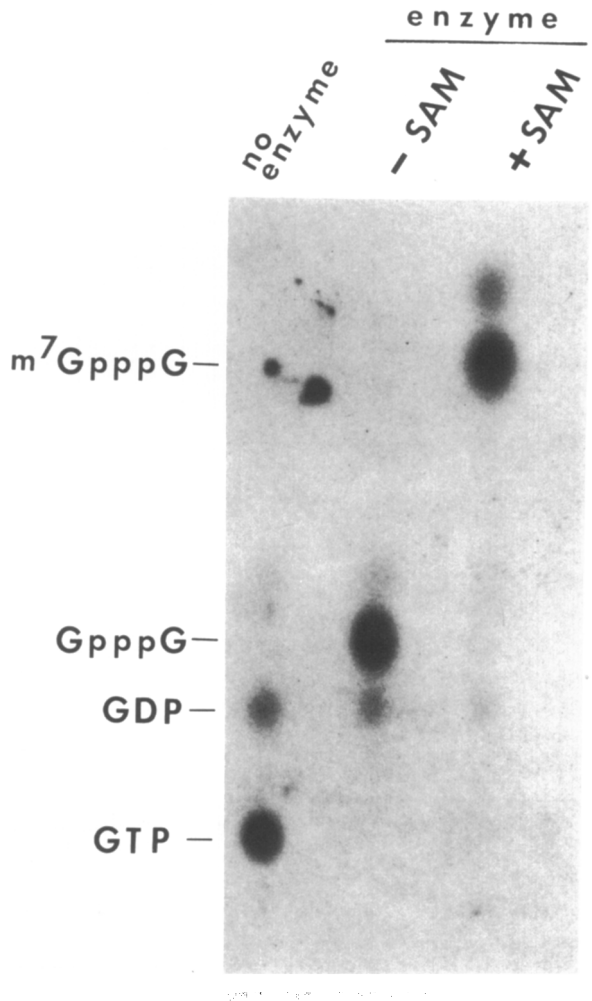


Figure 4. Efficient In Vitro Capping of SP6-HβA RNA

Transcription of Hind III–cleaved SP6-HβA was conducted as described in Experimental Procedures in the presence of ^{32}P-β-GTP. Purified ^{32}P-RNA was added to the standard capping reaction; or the guanylyltransferase (no enzyme) or S-adenosyl-methionine (-SAM) were omitted. The 5′ termini were subsequently analyzed by P1 nuclease digestion and thin layer chromatography. The positions of fluorescent markers for 5′ terminal structures are shown on the left.

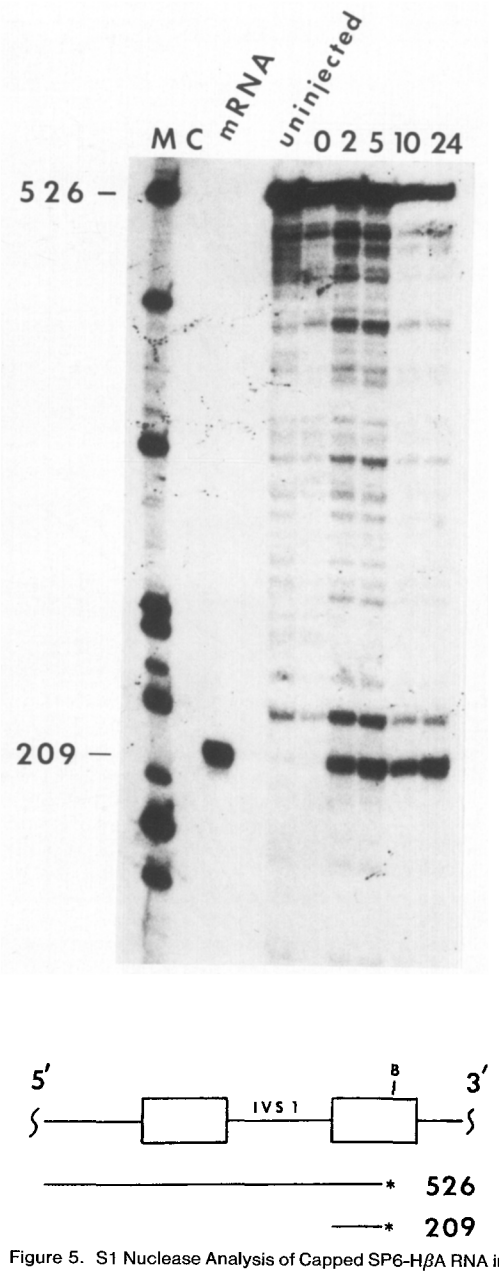


Figure 5. S1 Nuclease Analysis of Capped SP6-HβA RNA in Oocytes

The experiment was conducted exactly as described in the legend to Figure 2B except that the RNA was capped in vitro prior to injection. S1 nuclease analysis was as described in the legend to Figure 2B.

a 209 nucleotide fragment of the 5′ labeled DNA probe. The amount of this RNA is relatively constant through at least 24 hr of incubation in oocytes. We estimate that this 209 nucleotide S1 nuclease–resistant fragment represents one to several percent of the input β-globin RNA. These results differ in two respects from the previous experiment in which uncapped polyadenylated RNA was analyzed (Figure 2B). First, these data show that capped and polyadenylated β-globin pre-mRNAs can be accurately cleaved at a splice junction in oocytes. Second, the capped pre-mRNA is more stable in injected oocytes than RNA containing a 5′ terminal triphosphate.

We also directly assayed for RNA processing by denaturing agarose gel electrophoresis. The results in Figure 6A demonstrate that the predominant stable ^{32}P-RNA produced in oocytes is approximately 850 nucleotides, a size expected if both intervening sequences are excised from the original 1.85 kb in vitro transcript. No similar RNA was observed when uncapped RNAs were tested (Figure 2A). Although the vast majority of full-length input RNA is degraded in

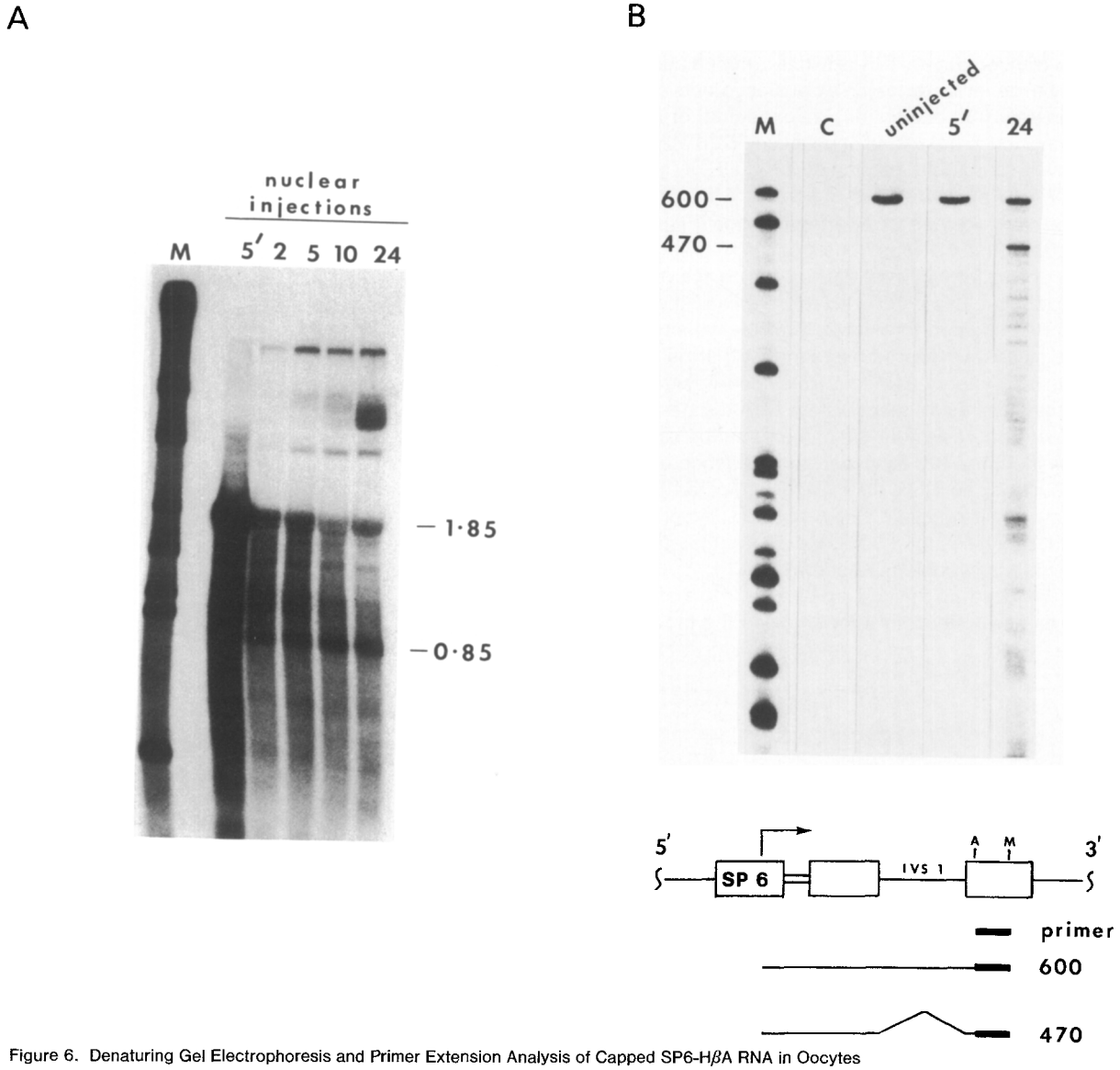


Figure 6. Denaturing Gel Electrophoresis and Primer Extension Analysis of Capped SP6-Hβ A RNA in Oocytes

A. The experiment was conducted as described in the legend to Figure 2A except that the RNA was capped in vitro prior to injection. RNAs were denatured with glyoxal and electrophoresed in a 1.4% agarose gel. 3′ end-labeled ^{32}P-DNA markers (M) were derived from pBR322 DNA and include fragments of 4.36, 2.97, 1.86, 1.40, 1.1, 0.93 and 0.38 kb. B. RNA from uninjected oocytes (C), SP6-Hβ A RNA plus uninjected oocyte RNA (uninjected), and RNA from oocytes purified immediately (5′) and 24 hr (24) after injection were subjected to primer extension analysis using a 5′ ^{32}P-labeled Mst II–Acc I probe (359–287) from the second exon of the β-globin gene. Following reverse transcription, RNA was degraded by alkaline hydrolysis and the ^{32}P-cDNAs were electrophoresed on a 5% denaturing polyacrylamide gel along with ^{32}P-DNA markers (M) from a Hpa II digest of pBR322 DNA. The expected ^{32}P-cDNAs are shown in the diagram below the figure. (Solid rectangle) ^{32}P-labeled DNA primer. A: Acc I site. M: Mst II site.

oocytes, some RNA still remains after 24 hr, again in contrast to the rapid turnover of uncapped RNA (Figure 2A).

These results suggest that a portion of the capped and polyadenylated pre-mRNA is cleaved at splice junctions and the resulting exons ligated. To confirm that both cleavage and ligation of the injected RNA occur, we analyzed the RNA products by using a primer extension assay shown in Figure 6B. A single-stranded 5′ ^{32}P-labeled DNA primer from the second exon was hybridized to the RNAs from the experiment of Figure 6A. The hybridized primer was then extended with unlabeled deoxyribonucleoside triphosphates and reverse transcriptase. The original uninjected transcript and RNA extracted immediately after injection generate a major extension product of approximately 600 nucleotides as expected for reverse transcription to the 5′ end of unspliced SP6-HβA RNA. However, an additional major product of approximately 470 nucleotides is observed in the RNA extracted from oocytes 24 hr after injection (Figure 6B). The size of this cDNA is consistent with the presence of an accurately spliced RNA in which the 130 nucleotide IVS-1 has been precisely removed, i.e., exon 1 has been ligated to exon 2. The relative intensities of the major cDNA products suggest that half of the remaining β-globin RNA that remains undegraded is spliced. This result is consistent with the denaturing gel electrophoresis analysis of the same RNA samples shown in Figure 6A. However, the presence of heterogenously sized RNA after 24 hr indicates that the fraction of the remaining RNA that is spliced may be considerably less than 50%. Moreover, the efficiency of splicing is variable (see below).

To demonstrate the ligation of exon 2 to exon 3, we used an assay described for studying the processing of mouse globin pre-mRNA (Kinniburgh, Mertz and Ross, 1978) and the kinetics of splicing of the adenovirus 2 major late tripartite leader sequence (Keohavong et al., 1982). ^{32}P-RNA is hybridized to an unlabeled cDNA clone under conditions of DNA excess. Following digestion with ribonuclease the protected ^{32}P-RNA is analyzed by denaturing gel electrophoresis. In this assay the introns of unspliced ^{32}P-RNA are unpaired and are degraded by ribonuclease. Thus, protected RNA fragments result from hybridization to the individual exons. In contrast, spliced RNA is entirely homologous to the cDNA and therefore produces a longer protected fragment equal in length to the sum of the exon lengths (Figure 7A).

As shown in Figure 7B, 24 hr after injection, an expected 460 nucleotide fragment is produced in addition to ^{32}P-RNA products of 223 and 215 nucleotides. In contrast, no discrete ^{32}P-RNA products are observed if the cDNA is omitted from the hybridization reaction. RNA isolated immediately after oocyte injection yields a 223 nucleotide ^{32}P-RNA fragment resulting from protection by the second exon and 215 nucleotide ^{32}P-RNA fragment corresponding to the shortened third exon in SP6-HβA RNA. The human β-globin cDNA plasmid lacks most of exon 1 (Wilson et al., 1978) so the corresponding protected ^{32}P-RNA fragment would not be visualized on this gel.

Accurate Splicing of Capped, polyA$^-$ pre-mRNA

To determine whether splicing of SP6-derived transcripts requires polyadenylation, we analyzed capped RNA transcripts from the SP6-Hβ plasmid which produces RNA devoid of polyA sequences (Figure 1) and extends 550 nucleotides beyond the normal polyadenylation site. Although polyadenylation usually precedes splicing (Nevins and Darnell, 1978) drug

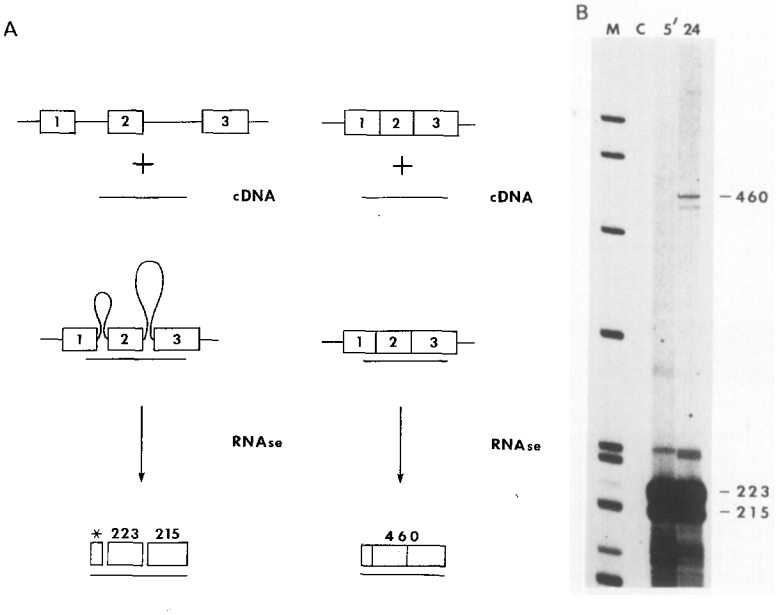
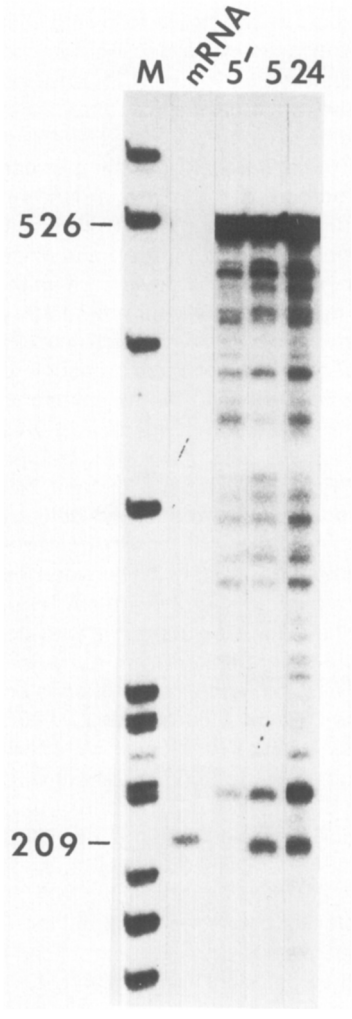


Figure 7. Ligation of Exon 2 to Exon 3 of Capped SP6-HβA RNA in Oocytes

A. Expected RNAse digestion products following β-globin cDNA protection of SP6-HβA ^{32}P-RNAs in oocytes. (Thin line) β-globin cDNA. (Open boxes) exons of the ^{32}P-RNAs; (thin lines) intervening sequences. The expected sizes of RNAse digestion products for unspliced and spliced SP6-HβA ^{32}P-RNAs are shown. (Star) expected truncated first exon ^{32}P-RNA product using JW102 cDNA which lacks the majority of the first exon (Wilson et al., 1978). B. Purified capped ^{32}P-RNA from SP6-HβA was injected into oocyte nuclei and total RNA extracted 5 min (5′) or 24 hr (24) after injection. RNA samples were hybridized to 0.5 μg of unlabeled Hind III linearized JW102 DNA (Wilson et al., 1978), followed by ribonuclease digestion. In a control reaction (C) DNA was omitted from the hybridization reaction with RNA extracted immediately following injection. The resultant ^{32}P-RNA products were electrophoresed on a 5% denaturing polyacrylamide gel along with ^{32}P-DNA markers (M) from a Hpa II digest of pBR322 DNA.

inhibitor studies suggest that splicing does not require polyadenylation (Zeevi et al., 1981). SP6-Hβ-derived RNA was microinjected into oocyte nuclei, incubated for 5 and 24 hr, and polyA$^-$ RNA isolated by oligodT cellulose chromatography. An S1 nuclease analysis of the polyA$^-$ RNA revealed a fragment of 209 nucleotides, indicative of accurate cleavage at the 3′ end of IVS-1 (Figure 8). However, using a 3′ probe that spans the normal β-globin polyadenylation site, we detect a small amount of correct 3′ ends, suggesting that in vitro transcripts which extend beyond the polyadenylation site can be accurately but inefficiently cleaved at the polyadenylation site in oocyte nuclei (data not shown). It is therefore possible that some or all of the spliced transcripts contain correct 3′ termini. Thus, we do not know whether correct cleavage at the 3′ end is necessary for splicing. However, the fact that the spliced RNA does not bind oligodT cellulose suggests that polyadenylation is not required for splicing (see Discussion).



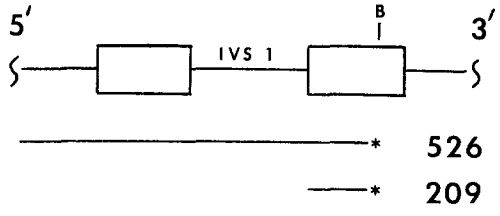


Figure 8. S1 Nuclease Analysis of Capped SP6-Hβ RNA in Oocytes

The experiment was conducted as described in the legend to Figure 5 except that RNA was transcribed from Pst-digested SP6-Hβ DNA. Total oocyte RNA was extracted at the indicated times following injection. The polyA$^-$ RNA selected by oligodT cellulose chromatography was subjected to S1 nuclease analysis.

Processing of a Synthetic β°-Thalassemia Pre-mRNA in Oocytes

To determine whether splicing of RNAs injected into oocytes faithfully reflects the expression of β-globin genes in human cells, the normal β-globin gene was replaced in SP6-HβA with a β° allele (SP6-Hβ°FA). The mutation in this β° gene occurs at position 1 of the IVS-2 sequence and changes the dinucleotide GT to AT at the 5′ splice site (Treisman et al., 1982). When this gene is introduced into human cells in culture via an appropriate expression vector, the IVS-2 splice site is completely inactive and a previously silent splice site located within IVS-2 is used (Treisman et al., 1982).

Transcripts were synthesized in vitro from SP6-HβA and SP6-Hβ°FA DNA, capped and injected into oocyte nuclei. The resulting RNAs were analyzed for the activity of the IVS-2 5′ splice site by an S1 nuclease assay by using a 3′ ^{32}P-labeled Acc I–Rsa I probe (Figure 9). Oocyte RNA isolated immediately following injection of both the wild-type and mutant β-globin pre-mRNA produces the full-length 290 nucleotide product as well as minor shorter bands. RNAs transcribed from the wild-type gene are cleaved in oocytes at the IVS-2 5′ splice site, producing the 211 nucleotide nuclease-resistant fragment. In contrast, the S1 nuclease assay reveals no detectable 211 nucleotide product when the in vitro transcript derived from the β° gene is injected into oocytes. The aberrant splice that is detected when this gene is expressed in human cells (Treisman et al., 1982) is not observed in injected oocytes. However, we cannot rule out that this cryptic splice occurs at an efficiency below the sensitivity of the assay. As expected, the IVS-1 3′ splice site of the mutant gene is active in oocytes as indicated by an S1 nuclease assay in which the 5′ ^{32}P-labeled Bam HI probe is used (not shown). We conclude that the β° mutation that prevents splicing in human cells also prevents splicing in oocytes.

Discussion

We have demonstrated accurate splicing of an in vitro–synthesized human β-globin pre-mRNA transcript injected into Xenopus oocyte nuclei. This work may provide a useful approach for studying pre-mRNA

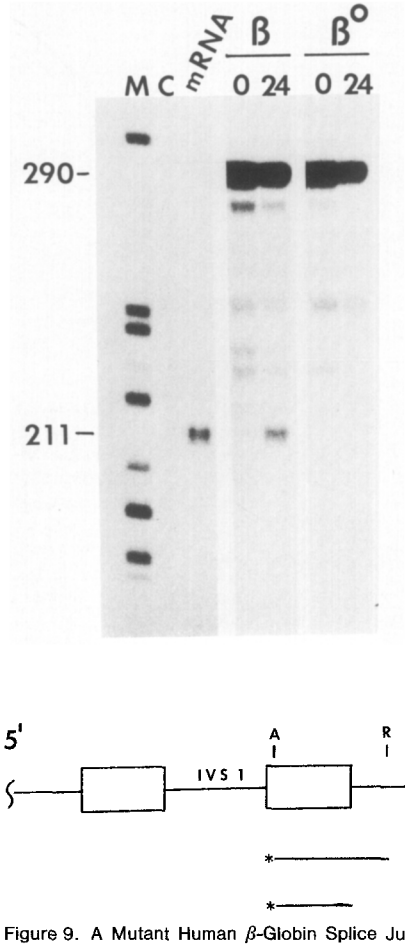


Figure 9. A Mutant Human β-Globin Splice Junction Is Inactive in Oocytes

Capped RNAs from SP6-Hβ A and SP6-Hβ°FA were injected into oocyte nuclei and total RNA extracted immediately (0) or 24 hr (24) after injection. RNAs were subjected to S1 nuclease analysis using the double-stranded 3'-^{32}P-labeled Acc I–Rsa I probe (282–581). The expected S1 nuclease–resistant DNA products are shown in the diagram below the figure. (Stars) ^{32}P-labeled ends. A: Acc I site. R: Rsa site. The control reactions include: C, total RNA from uninjected oocytes; mRNA, uninjected oocyte RNA plus human 9S globin RNA. ^{32}P-DNA markers (M) are from a Hpa II digest of pBR322 DNA. The products are displayed on a 5% denaturing polyacrylamide gel.

processing as well as a means for obtaining substrates for the further development of in vitro splicing systems. In addition, these studies have yielded new information regarding the requirements for RNA stability and processing in oocytes. In particular, we have shown that transcription and splicing can be uncoupled. This conclusion has both practical and mechanistic significance. From a practical point of view, this observation affords the opportunity to study the structural and biochemical requirements for splicing independent of those required for transcription. Mechanistically, the ability to uncouple transcription and splicing of pre-mRNA indicates that completed primary transcripts contain the information necessary to form a structure that can be accurately spliced. We do not know whether this pre-mRNA folds into a unique configuration or associates with proteins to form a ribonucleoprotein complex (RNP) which may be necessary for splicing (Munroe and Pederson, 1981). In any case, the ability to produce large amounts of highly labeled substrate provides the opportunity to evaluate critically the possible relationship between RNP structure and mRNA processing. Moreover, it should be possible to analyze the rates of pre-mRNA splicing.

Although we have not examined the kinetics of splicing during the first two hours following injection, we note that the amount of spliced RNA is constant from 2 to 24 hours (see Figure 5). In addition, unspliced pre-mRNA is present even after 24 hours. These observations could be explained by a relatively high stability of the spliced RNA or by a continuous slow rate of processing of the pre-mRNA with the concomitant turnover of the spliced RNA. Alternatively, it is possible that both spliced and unspliced RNAs are transported to the cytoplasm within the first two hours after injection. Thus, the pre-mRNA would slowly turn over in the cytoplasm where it could not be spliced. The possibility of rapid transport of unspliced β-globin pre-mRNA to the cytoplasm is consistent with the observation that unspliced SV40 RNA is detected in the cytoplasm of oocytes injected with SV40 DNA (M. Wickins, personal communication).

Our results also address the question of the role of polyadenylation and the 5′ cap on RNA stability and splicing. The difference in stability between capped and uncapped authentic β-globin mRNA in oocyte nuclei shows that one function of the cap is to stabilize RNA, presumably by protection from nuclease degradation. Thus, the rapid appearance of caps on nascent hnRNA in vivo (Salditt-Georgieff et al., 1980) and in vitro (Weil et al., 1979; Proudfoot et al., 1980) may reflect the requirement to protect potential mRNA sequences from degradation. The cap may also play a role in translation (reviewed by Banerjee, 1980) and in cytoplasmic mRNA stability (Furuichi et al., 1977). It is likely that our ability to detect splicing with capped in vitro transcripts is a consequence of the increased stability of capped precursors and their spliced products. However, the possibility that the cap plays some direct role in splicing cannot be excluded.

Several studies have indicated that poly(A) is an important determinant of RNA stability (e.g., Nudel et al., 1976). However, we show that poly(A) is clearly not sufficient for RNA stability in oocytes since both polyadenylated uncapped in vitro transcripts and polyadenylated decapped β-globin mRNA are highly unstable. Our data also suggest that polyadenylation is not required for splicing in oocytes. Both the polyadenylated SP6-Hβ A transcripts and the unpolyadenylated SP6-Hβ transcripts are spliced. Although we detect spliced RNA in the polyA$^-$ fraction from an oligodT cellulose column, we cannot exclude the possibility that a small number of adenylate residues are added to SP6-Hβ RNA in the oocyte. Drug inhibitor

studies also suggest that polyadenylation is not required for splicing (Zeevi et al., 1981), but again the presence of a small number of 3′ adenine residues could not be excluded (Zeevi et al., 1981).

Most of the injected β-globin pre-mRNA is not spliced. Moreover, there is a significant experimental variation in the amount of injected RNA that is spliced (compare Figures 5 and 6 with 7 and 9). There are several possible explanations for the inefficiency of splicing in this system. First, it is possible that the splicing capacity of the oocyte has been exceeded. However, preliminary titration experiments suggest that this is not the case (data not shown). Second, the inefficiency of splicing may relate to the fact that the SP6 β-globin transcripts differ in several potentially important ways from authentic β-globin pre-mRNA. As noted above, the 5′ and 3′ ends of the in vitro transcripts differ from authentic β-globin pre-mRNA. Moreover, the synthetic precursor may be lacking some posttranscriptional modification important for efficient splicing. For example, a recent study suggests that internal RNA methylation may be necessary for splicing (Stoltzfus and Dane, 1982). Furthermore, the 5′ terminal structures we have synthesized in vitro lack the 2′-0 methylations normally present at the 5′ ends of mRNA in higher eukaryotes (Banerjee, 1980). Third, the inefficient splicing in oocytes may result from some feature of the human β-globin primary sequence. For example, it is possible a pre-mRNA transcribed from a Xenopus gene, particularly one expressed during oogenesis, may be processed more efficiently. Fourth, only a minority of the naked RNA injected into oocytes may be able to assume a correct secondary structure or RNP configuration required for efficient splicing.

An alternative possibility is that inefficient processing is an inherent characteristic of oocyte RNA metabolism. Unfortunately, kinetic data pertaining to the synthesis of spliced RNAs ordinarily expressed in oocytes is not available. What is known is that 5% of rapidly labeled endogenous nuclear RNA of stage 6 oocytes enters the cytoplasm as stable RNA (Anderson and Smith, 1977). It is also relevant to note that greater than 90% of the viral RNA synthesized in oocytes injected with SV40 DNA is rapidly degraded in the nucleus. Only a small proportion of the remaining 10% eventually gives rise to a polyadenylated cytoplasmic RNA species similar to mature SV40 19S mRNA (Miller, Stephens, and Mertz, 1982). Thus, the inefficient splicing of synthetic β-globin pre-mRNA in oocytes could be the natural consequence of the turnover of RNA in the oocyte nucleus or the result of differences between the synthetic and natural pre-mRNAs or both. In this regard, our most recent studies with a modified SP6-HβA transcript that contains less SP6 5′ flanking sequence indicate that a significantly higher proportion of the injected RNA is spliced in oocytes (unpublished data). Thus, the splicing efficiency of injected pre-mRNAs can be increased by constructing pre-mRNAs which more closely resemble their natural counterparts. In any case, the efficiency of splicing that we observe is clearly adequate for obtaining information relevant to the mechanism of mRNA splicing. Moreover, the availability of large amounts of a well-defined biochemically active substrate for RNA splicing should be useful in the further development of cell-free systems for studying RNA processing.

Experimental Procedures

Materials

Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. S1 nuclease and guanylate kinase was from Sigma. Tobacco acid pyrophosphatase (lot #1285), guanylyltransferase (lot #2311), and vanadyl-ribonucleosides were purchased from Bethesda Research Laboratories. T4 polynucleotide kinase, terminal transferase, mung bean nuclease, and fluorescent cap markers were from P-L Biochemicals. P1 nuclease was from Boehringer-Mannheim. Pyruvate kinase was from Calbiochem. E. coli DNA polymerase Klenow fragment was from New England Biolabs. RNAsin was from Biotec. Calf intestine alkaline phosphatase was prepared by M. Alonso. SP6 RNA polymerase was prepared by P. Little and M. Alonso according to the published procedure with minor modifications (Butler and Chamberlin, 1982). RNAse-free DNAse (Worthington) was repurified as described (Maxwell et al., 1977). Oligo(dT) cellulose was from Collaborative. γ-^{32}P-ATP was prepared according to (Walseth and Johnson, 1979). α-^{32}P-deoxynucleoside triphosphates (>3000 Ci/mmole) and α-^{32}P-ribonucleoside triphosphates (>400 Ci/mmole) were purchased from New England Nuclear. Reverse transcriptase was a gift from T. Papas and globin mRNA was a gift from S. Orkin.

Plasmid Constructions

SP6-Hβ (Figure 1A) was constructed by E. Butler and P. Little starting with a pBR322 derivative containing the SP6 promoter, R7Δ7 (E. Butler and P. Little, unpublished). SP6-HβA (Figure 1A) was prepared from SP6-Hβ by destroying the Hind III site and inserting a Hind III site 3′ to the β-globin gene. The resulting plasmid was cut at the Hind III site, filled in with DNA polymerase I (Klenow) in the presence of deoxyribonucleoside triphosphates, T tailed with terminal transferase (Deng and Wu, 1981), and cut with Eco RI. The human β-globin cDNA insert was purified by a modification of a procedure described by Hofstetter et al. (1976). JW102 DNA (Wilson et al., 1978) was incubated in 30 mM sodium acetate (pH 4.5), 250 mM NaCl, 5 mM $ZnSO_4$, 50% formamide with 2500 U/ml mung bean nuclease at 48° for 30 min followed by electrophoresis on a 7% nondenaturing polyacrylamide gel. Under these conditions, it is likely that mung bean nuclease can cut in the highly AT rich 3′ untranslated region of cDNA, resulting in the 45 nucleotide deletion in SP6-HβA. The gel-purified cDNA insert was A tailed with terminal transferase, cut with Eco RI, and ligated to the SP6-Hβ vector described above. Following bacterial transformation individual colonies were screened (Birnboim and Doly, 1979) for a Hind III site and the distance between the Hind III and Eco RI site. Colonies that were likely to contain a sufficiently long AT tract by restriction digest were analyzed for the length and integrity of the AT tract by DNA sequencing (Maxam and Gilbert, 1980). SP6-Hβ°FA was made by replacing the β-globin Eco RI–Bam HI fragment of SP6-HβA with the Eco RI–Bam HI fragment from πSVHPβ° (Treisman et al., 1982).

In Vitro Transcription

Standard transcription reactions (100 μl) contained 3.5 μg of linearized DNA template, 40 mM Tris–HCl (7.5), 6 mM $MgCl_2$, 10 mM DTT, 4 mM spermidine, 100 units of RNAsin, 500 μM of each ribonucleoside triphosphate, 100 μCi of one α-^{32}P-ribonucleoside triphosphate, and 18 units of SP6 RNA polymerase. Reactions were incubated at

37° for 1 hr followed by the addition of vanadyl-ribonucleosides to 10 mM and RNAse-free DNAse to 20 μg/ml and subsequent incubation for 10 min at 37°. Following extraction with phenol–chloroform the ^{32}P-RNA was purified from unincorporated nucleotides by sephadex G-100 chromatography. Typically, 1–3 micrograms of ^{32}P-RNA was obtained.

In Vitro Capping and Enzymatic Decapping of RNAs

β-globin mRNA was decapped with tobacco acid pyrophosphatase as described (Efstratiadis et al., 1977). The preparation of enzyme used was free of any nonspecific ribonuclease activity as determined by incubation with ^{32}P-labeled in vitro transcripts followed by denaturing gel electrophoresis (data not shown).

RNAs were capped in a 30 μl reaction volume containing 50 mM Tris–HCl (7.9), 1.25 mM $MgCl_2$, 2.5 mM DTT, 50 μM S-adenosylmethionine, 1000 units/ml of RNAsin, and 3.6 units of guanylyltransferase at 37° for 45 min. Following phenol–chloroform extraction and ethanol precipitation the RNA was resuspended in water. For assaying capping in vitro β-^{32}P-GTP was synthesized from γ-^{32}P-ATP as described (Furuichi and Shatkin, 1977), purified by sephadex G-100 chromatography, and concentrated by lyophilization. RNAs were end-labeled by the addition of 200 μCi of β-^{32}P-GTP in a standard transcription reaction. ^{32}P-RNAs were digested in 30 mM sodium acetate (pH 5.3) with 2 μg of P1 nuclease at 37° for 1 hr. Subsequently the sample was applied to a PEI cellulose plate along with fluorescent markers, developed with 1.2 M LiCl, and autoradiographed.

Oocyte Injections and RNA Extraction

Stage V and stage VI oocytes (Dumont, 1972) were removed from anesthetized X. laevis females and incubated at 20°C in a modified Barth solution (Gurdon, 1976). A 20–40 nl solution of RNA (approximate concentration: 100 μg/ml) was injected into the oocyte nucleus. Typically, a set of twenty oocytes was injected for each sample, incubated for a specified time in Barth solution, frozen on solid CO_2 and then transferred to −20°C until analyzed further. As a control, all experiments included extraction of RNA shortly after injection which generally required between 0 and 5 min.

RNA was extracted from frozen oocytes by digestion in proteinase K, phenol–chloroform extraction and ethanol precipitation as described elsewhere (Melton and Cortese, 1979).

S1 Nuclease Mapping and Primer Extension

S1 nuclease mapping was conducted as described by Treisman et al. (1982) using the higher nuclease concentration (1000 μ/ml) for mapping intervening sequence boundaries. Typically, the RNA from the equivalent of ½–2 oocytes was used for each assay. Following S1 nuclease digestion, any remaining ^{32}P-RNA was destroyed by treatment with pancreatic ribonuclease or alkaline hydrolysis. Hybridization of ^{32}P-RNA to unlabeled cDNA was in 80% formamide buffer at 51° for 3 hr. Following hybridization, ribonuclease digestion was performed as described (Keohavong et al., 1982).

Primer extensions were done using the 73 nucleotide 5′ ^{32}P-labeled Mst II–Acc I probe. Hybridizations were in 50 μl of 40 mM PIPES (6.7), 0.4 M NaCl, 1 mM EDTA, 0.2% SDS at 62° for 4 hr. Hybrids recovered by ethanol precipitation were extended with reverse transcriptase as described by Treisman et al. (1982) at 41° for 3 hr. Following alkaline hydrolysis and phenol extraction the cDNAs were analyzed by gel electrophoresis.

Acknowledgments

We would like to acknowledge Eugene T. Butler, III and Peter Little for their contributions to the initial stages of this work, and Richard Treisman for helpful discussions. We are especially grateful to B. Moss for initially providing us with guanylyltransferase. We thank Sophia Stern for help in preparing the manuscript. Michael Green is a Helen Hay Whitney Fellow. T. M. acknowledges grant support from the National Institutes of Health and D. M. acknowledges grant support from the National Science Foundation and the Dreyfus Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 1, 1982; revised January 6, 1983

References

Allende, C. C., Allende, J. E. and Firtel, R. A. (1974). The degradation of ribonucleic cells injected into Xenopus laevis oocytes. Cell *2*, 189–196.

Anderson, D. M. and Smith, L. D. (1977). Synthesis of heterogeneous nuclear RNA in full-grown oocytes of Xenopus laevis (Daudin). Cell *11*, 663–671.

Banerjee, A. K. (1980). 5′-Terminal cap structure in eukaryotic messenger ribonucleic acids. Microbiol. Rev. *44*, 175–205.

Bastos, R. N. and Aviv, H. (1977). Globin RNA precursor molecules: biosynthesis and processing in erythroid cells. Cell *11*, 641–650.

Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. *7*, 1513–1523.

Blanchard, J. M., Weber, J., Jelinek, W. and Darnell, J. E. (1978). *In vitro* RNA–RNA splicing in adenovirus mRNA formation. Proc. Natl. Acad. Sci. USA *75*, 5344–5348.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. and Chambon, P. (1978). Ovalbumin gene: evidence for a leader sequence in mRNA and DNA sequences at the exon–intron boundaries. Proc. Nat. Acad. Sci. USA *75*, 4853–4857.

Butler, E. T. and Chamberlin, M. J. (1982). Bacteriophage SP6-specific RNA polymerase: isolation and characterization of the enzyme. J. Biol. Chem. *257*, 5772–5778.

Cepko, C. L., Hansen, U., Handa, H. and Sharp, P. A. (1981). Sequential transcription translation of SV40 by using mammalian cell extracts. Mol. Cell Biol. *1*, 919–931.

Curtis, P. J., Mantei, N. and Weissmann, C. (1977). Characterization and kinetics of synthesis of 15S β-globin RNA, a putative precursor of β-globin mRNA. Cold Spring Harbor Symp. Quant. Biol. *42*, 971–984.

Darnell, J. E. (1979). Transcription units for mRNA production in eukaryotic cells and their DNA viruses. Prog. Nucl. Acids Res. Mol. Biol. *22*, 327–353.

Deng, G. and Wu, R. (1981). An improved procedure for utilizing terminal transferase to add homopolymers to the 3′ termini of DNA. Nucl. Acids Res. *9*, 4173–4188.

Dumont, J. N. (1972). Oogenesis in Xenopus laevis (Daudin) stages of oocyte development in laboratory-maintained animals. J. Morphol. *136*, 153–180.

Efstratiadis, A., Vournakis, J., Donis-Keller, H. Chaconas, G., Dougall, D. K. and Kafatos, F. C. (1977). End labeling of enzymatically decapped mRNA. Nucl. Acids Res. *4*, 4165–4174.

Fitzgerald, M. and Shenk, T. (1981). The sequence 5′-AAUAAA-3′ forms part of the recognition site for polyadenylation of late SV40 mRNAs. Cell *24*, 251–260.

Flint, J. (1983). RNA splicing *in vitro*. In Enzymes of Nucleic Acid Synthesis and Modification, 2, S. T. Jacob, ed. (Boca Raton, Florida: CRC Press), in press.

Furuichi, Y., LaFiandra, A. and Shatkin, A. J. (1977). 5′-terminal structure and mRNA stability. Nature *266*, 235–239.

Furuichi, Y. and Shatkin, A. J. (1977). A simple method for the preparation of β-^{32}P purine nucleoside triphosphates. Nucl. Acids Res. *4*, 3341–3355.

Goldenberg, C. J. and Raskas, H. J. (1981). *In vitro* splicing of purified precursor RNAs specified by early region 2 of the adenovirus 2 genome. Proc. Nat. Acad. Sci. USA *78*, 5430–5434.

Grosveld, G. C., Koster, J. and Flavell, R. A. (1981). A transcription map for the rabbit β-globin gene. Cell *23*, 573–584.

Gurdon, J. B., Lingrel, J. B. and Marbaix, G. (1973). Message stability in injected frog oocytes: long life of mammalian α and β globin messages. J. Mol. Biol. *80,* 539–551.

Gurdon, J. B. (1976). Injected nuclei in frog oocytes: fate, enlargement, and chromatin dispersal. J. Embryol. Exp. Morphol. *36,* 523–540.

Gurdon, J. B. and Melton, D. A. (1981). Gene transfer in amphibian eggs and oocytes. Ann. Rev. Genet. *15,* 189–218.

Hamada, H., Igarashi, T. and Muramatsu, M. (1980). *In vitro* splicing of SV40 late mRNA in isolated nuclei from CV-1 cells. Nucl. Acids Res. *8,* 587–599.

Handa, H., Kaufman, R. J., Manley, J., Gefter, M. and Sharp, P. A. (1981). Transcription of simian virus 40 DNA in a HeLa whole cell extract. J. Biol. Chem. *256,* 478–482.

Hofer, E. and Darnell, J. E., Jr. (1981). The primary transcription unit of the mouse β-major globin gene. Cell *23,* 585–593.

Hofstetter, H., Schambock, A., VanderBerg, J. and Weissmann, C. (1976). Specific excision of the inserted DNA segment from hybrid plasmids constructed by the poly (dA):poly (dT) method. Biochem. Biophys. Acts *254,* 587–591.

Kinniburgh, A. J., Mertz, J. E. and Ross, J. (1978). The precursor of mouse β-globin messenger RNA contains two intervening RNA sequences. Cell *14,* 681–693.

Kole, R. and Weissman, S. M. (1982). Accurate *in vitro* splicing of human β-globin RNA. Nucl. Acids Res. *10,* 5429–5445.

Keohavong, P., Gattoni, R., LeMoullec, J. M., Jacob, M. and Stevenin, J. (1982). The orderly splicing of the first three leaders of the adenovirus-2 major late transcript. Nucl. Acids Res. *10,* 1215–1229.

Lawn, R. M., Efstratiadis, A., O'Connell, C. and Maniatis, T. (1980). The nucleotide sequence of the human β-globin gene. Cell *21,* 647–651.

Lewin, B. (1980). Alternatives for splicing: recognizing the ends of introns. Cell *22,* 324–326.

Lingrel, J. B., Lowenhaupt, B. A., Haynes, J. R., Rosteck, B. S., Kalb, V. F. and Smith, K. (1979). Processing and turnover of globin mRNA during erythroid maturation. In Cellular and Molecular Regulation of Hemoglobin Switching, G. Stamatoyannopoulos and A. W. Nienhuis, eds. (New York: Grove and Stratton), pp. 675–692.

Lockard, R. E. and Lane, C. (1978). Requirement for 7-methylguanosine in translation of globin mRNA *in vivo.* Nucl. Acids Res. *5,* 3237–3247.

Manley, J. L., Sharp, P. A. and Gefter, M. L. (1979). RNA synthesis in isolated nuclei: identification and comparison of adenovirus 2 transcripts synthesized *in vitro* and *in vivo.* J. Mol. Biol. *135,* 171–197.

Manley, J. L., Sharp, P. A. and Gefter, M. L. (1982). RNA synthesis in isolated nuclei: processing of adenovirus serotype 2 late messenger RNA precursors. J. Mol. Biol. *159,* 581–599.

Maxam, A. M. and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. Meth. Enzymol. *65,* 499–560.

Maxwell, I., Maxwell, F. and Hahn, W. E. (1977). Removal of RNAse activity from DNAse by affinity chromatography on agarose-coupled aminophenylphosphoryl-uridine-2′(3′) phosphate. Nucl. Acids Res. *4,* 241–246.

McMaster, G. K. and Carmichael, G. G. (1977). Analysis of single and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Nat. Acad. Sci. USA *74,* 4835–4838.

Melton, D. A. and Cortese, R. (1979). Transcription of cloned tRNA genes and the nuclear partitioning of a tRNA precursor. Cell *18,* 1165–1172.

Miller, T. J., Stephens, D. L. and Mertz, J. E. (1982). Kinetics of accumulation and processing of SV40 RNA in *Xenopus laevis* oocytes injected with SV40. Mol. Cell. Biol. *2,* 1581–1594.

Monroy, G., Spencer, E. and Hurwitz, J. (1978). Purification of mRNA guanylyltransferase from vaccinia virions. J. Biol. Chem. *253,* 4481–4489.

Montell, C., Fisher, E. F., Caruthers, M. H. and Berk, A. J. (1982). Resolving the functions of overlapping viral genes by site-specific mutagenesis at a mRNA splice site. Nature *295,* 380–384.

Mount, S. M. (1982). A catalogue of splice junction sequences. Nucl. Acids Res. *10,* 459–472.

Munroe, S. H. and Pederson, T. (1981). Messenger RNA sequences in nuclear ribonucleoprotein particles are complexed with protein as shown by nuclease protection. J. Mol. Biol. *142,* 437–449.

Nevins, J. R. and Darnell, J. E., Jr. (1978). Steps in the processing of Ad2 mRNA: poly(A)$^+$ nuclear sequences are conserved and poly(A) addition precedes splicing. Cell *15,* 1477–1493.

Nudel, U., Sureq, H. Littauer, U. Z., Marbaix, G. Huez, G., Leclercq, M., Hubert, E. and Chantrenne, H. (1976). Globin mRNA species containing poly (A) segments of different lengths: their functional stability in Xenopus oocytes. Eur. J. Biochem. *64,* 115–121.

Paterson, B. M. and Rosenberg, M. (1979). Efficient translation of prokaryotic mRNAs in a eukaryotic cell-free system requires addition of a cap structure. Nature *279,* 692–696.

Proudfoot, N. J. and Brownlee, G. G. (1974). Sequences at the 3′ end of globin mRNA shows homology with immunoglobin light chain mRNA. Nature *253,* 359–362.

Proudfoot, N. J., Shander, M. H. M., Manley, J., Gefter, M. and Maniatis, T. (1980). The structure and *in vitro* transcription of human globin genes. Science *209,* 1329–1336.

Ross, J., Maquot, L. E., Kinniburgh, A. J., Beach, L. R., Honig, G. R., Lazerson, J. and Ershier, W. B. (1981). Processing of the β-globin mRNA precursors in mouse and human cells. In Organization and Expression of Globin Genes, G. Stamatoyannopoulos and A. W. Nienhuis, eds. (New York: Alan P. Liss Inc.), pp. 205–217.

Salditt-Georgieff, M., Harpold, M., Chen-Kiang, S. and Darnell, J. E., Jr. (1980). The addition of 5′ cap structures occurs early in hnRNA synthesis and prematurely terminated molecules are capped. Cell *19,* 69–78.

Sharp, P. A. (1981). Speculations on RNA splicing. Cell *23,* 643–646.

Shuman, S. and Hurwitz, J. (1981). Mechanism of mRNA capping by vaccinia virus guanylyltransferase: characterization of an enzyme-guanylate intermediate. Proc. Nat. Acad. Sci. USA *78,* 187–191.

Stoltzfus, C. M. and Dane, R. W. (1982). Accumulation of spliced avian retrovirus mRNA is inhibited in S-adenosyl-methionine-depleted chicken embryo fibroblasts. J. Virol. *42,* 918–931.

Tilghman, S. M., Curtis, P. J., Tiemeier, D. C., Leder, P. and Weissmann, C. (1978). The intervening sequence of a mouse β-globin gene is transcribed within the 15S β-globin mRNA precursor. Proc. Nat. Acad. Sci. USA *75,* 1309–1313.

Treisman, R., Proudfoot, N. J., Shander, M. and Maniatis, T. (1982). A single-base change at a splice site in a β^0-thalassemic gene causes abnormal RNA splicing. Cell *29,* 903–911.

Venkatesan, S., Gershowitz, A. and Moss, B. (1980). Modification of the 5′ end of mRNA: association of RNA triphosphatase with the RNA guanylyltransferase-RNA (Guanine-7-) Methyltransferase complex from vaccinia virus. J. Biol. Chem. *255,* 903–908.

Walseth, T. F. and Johnson, R. A. (1979). The enzymatic preparation of [^{32}P] nucleoside triphosphate, cyclic [α^{32}P] AMP, and cyclic [^{32}P] GMP. Biochem. Biophys. Acta. *526,* 11–31.

Weaver, R. F. and Weissmann, C. (1979). Mapping of RNA by a modification of the Berk–Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucl. Acids Res. *6,* 1175–1193.

Weil, P. A., Luse, D. S., Segall, J. and Roeder, R. G. (1979). Selective and accurate initiation of transcription of the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell *18,* 469–484.

Weingartner, B. and Keller, W. (1981). Transcription and processing of adenoviral RNA by extracts from HeLa cells. Proc. Nat. Acad. Sci. USA *78,* 4092–4096.

Wieringa, B., Meyer, F., Reiser, J. and Weissmann, C. (1982). In Cetus-UCLA Symposium on Gene Regulation, in press.

Wilson, J. T., Wilson, L. B., DeRiel, J. K., Villa-Komaroff, L., Efstratiadis, A., Forget, B. G. and Weissman, S. M. (1978). Insertion of synthetic copies of human globin genes into bacterial plasmids. Nucl. Acids Res. *5*, 563–581.

Yang, V. W. and Flint, S. J. (1979). Synthesis and processing of adenoviral RNA in isolated nuclei. J. Virol. *32*, 394–403.

Zeevi, M., Nevins, J. R. and Darnell, J. E., Jr. (1981). Nuclear RNA is spliced in the absence of poly(A) addition. Cell *26*, 39–46.