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Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter

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ABSTRACT

A simple and efficient method for synthesizing pure single stranded RNAs of virtually any structure is described. This in vitro transcription system is based on the unusually specific RNA synthesis by bacteriophage SP6 RNA polymerase which initiates transcription exclusively at an SP6 promoter. We have constructed convenient cloning vectors that contain an SP6 promoter immediately upstream from a polylinker sequence. Using these SP6 vectors, optimal conditions have been established for in vitro RNA synthesis. The advantages and uses of SP6 derived RNAs as probes for nucleic acid blot and solution hybridizations are demonstrated. We show that single stranded RNA probes of a high specific activity are easy to prepare and can significantly increase the sensitivity of nucleic acid hybridization methods. Furthermore, the SP6 transcription system can be used to prepare RNA substrates for studies on RNA processing (1,5,9) and translation (see accompanying paper).

INTRODUCTION

Single stranded RNA copies of well characterized cloned DNA molecules can be used as substrates for studies on RNA processing, RNA structure and mRNA translation. Synthesis of the desired RNA molecule in vitro circumvents problems associated with isolating rare RNAs in amounts sufficient for detailed biochemical analysis. For example, in vitro synthesis of unprocessed RNAs has greatly facilitated studies on RNA splicing (1-6), tRNA maturation (7,8), and 3' end formation (9-11). Single stranded RNA molecules synthesized in vitro can also be labeled to very high specific activities for use as sensitive hybridization probes. These RNA probes are in some cases preferable to conventional nick translated DNA probes because they are easy to prepare and can increase the sensitivity of the detection method (12-14, and see below). Single stranded RNA is far more effective than double stranded RNA or nick translated DNA as a probe for in situ hybridization (15). Furthermore, single stranded RNA probes have made it possible to increase the detection sensitivities of RNA and DNA blot hybridization procedures (16,17).

Several methods have been reported for synthesizing RNAs *in vitro*. These include the use of *E. coli* RNA polymerase to transcribe cloned DNAs containing a procaryotic promoter (18-22) and several methods of preparing eucaryotic whole cell and nuclear extracts. These extracts either contain or can be supplemented with eucaryotic RNA polymerase and direct the synthesis of 'run off' transcripts from added DNA templates containing eucaryotic promoters. (e.g. 23,24). None of these methods are ideally suited for the synthesis of large amounts of a specific RNA molecule either because of inefficient RNA synthesis, transcription of both DNA strands, incorrect initiation, premature termination, or a combination of these problems. In order to synthesize large amounts of any specific RNA and to generate RNAs of a high specific activity, it is necessary to have a transcription system which will initiate and terminate RNA synthesis efficiently at precise positions on the DNA template. It is also desirable to have a transcription system which works efficiently in a simple buffer, eliminating the need to prepare and characterize cell extracts.

Several investigators have exploited the specificity of phage RNA polymerases and replicases to produce defined RNAs *in vitro*. For example, T7 RNA polymerase will efficiently initiate RNA synthesis at cloned T7 promoters (25). Similarly, Qβ replicase can be used to produce large amounts of RNA *in vitro* by autocatalytic replication of recombinant RNA (26). The elements of an especially attractive *in vitro* transcription system have been described by Chamberlin and his colleagues. They studied an unusually specific promoter-RNA polymerase combination that is found in the *Salmonella typhimurium* phage SP6 (27,28). The phage encoded RNA polymerase efficiently initiates transcription only at SP6 phage promoters and will transcribe any DNA sequence cloned downstream from the promoter. Transcripts resulting from initiation at other procaryotic or eucaryotic promoters, end to end transcription of DNA restriction fragments or transcription of the wrong (coding) DNA strand are rarely, if ever, observed. Moreover, the transcription reaction consists of a simple salt buffer, DNA template, ribonucleoside triphosphates and SP6 RNA polymerase and results in the synthesis of large amounts of RNA.

We have previously described the use of an SP6 transcription system for the synthesis of pre-mRNA substrates and as hybridization probes (ref above). SP6 probes for *in situ* hybridizations have been used effectively by others, notably the Angerers and coworkers (15,29). Here we describe a new set of cloning vectors that contain an SP6 promoter upstream from a po-

lylinker sequence that makes make it possible to conveniently clone any DNA sequence downstream from the SP6 promoter. The results of experiments which determine the optimal conditions for SP6 in vitro transcription are presented. We also describe the use and sensitivity of SP6 RNA probes in filter and solution hybridizations and compare these single stranded RNA probes to nick translated DNA probes.

MATERIALS AND METHODS

Materials

SP6 RNA polymerase was purified from SP6 infected Salmonella typhimurium according to the method of Butler and Chamberlin (27), with minor modifications (15). Following chromatography through a blue dextran-Sepharose column the enzyme (Fraction V) was dialyzed against 0.2 M KCl, 10 mM KPO_4, pH 7.9, 10 mM β-mercaptoethanol, 0.1 mM EDTA, 50% glycerol. A polymerase stock at 7 units/µl was stored at -20°C. SP6 RNA polymerase is now available from Promega Biotec (Riboprobe™), New England BioLabs and New England Nuclear. Restriction enzymes and nuclease Bal 31 were purchased from New England Biolabs. RNAsin (ribonuclease inhibitor) was purchased from Promega Biotec and rNTPs from PL Biochemicals. RNAse A, type IIIA, and RNAse T1, grade IV, were purchased from Sigma. ^{32}P-α-GTP and ^{32}P-α-UTP were purchased from Amersham and New England Nuclear. Recent experiments have shown that ^{32}P-α-NTP which is more than 10 days old can substantially inhibit SP6 transcription in vitro.

Plasmid constructions

SP6 cloning vectors were constructed by Bal 31 nuclease deletions at unique restriction sites. 5 µg of linear plasmid DNA was digested in 600 mM NaCl, 100 mM Tris, pH 8.0, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 0.4 mM EDTA, 15 units of Bal 31 (New England Biolabs) for 0.5-10 min as described elsewhere (30). The ends of the DNA were repaired with DNA Pol I. Hind III or EcoR1 linkers were ligated onto the blunt ended DNA and the circles were closed with DNA ligase. The DNA sequences of parts of the SP6 vectors were determined by chemical degradation (31).

The plasmid R7Δ7 was constructed by cloning a 500 bp Bgl II - Cla I fragment from bacteriophage SP6 DNA into BamH I - Cla I digested pBR322 (E. Butler and P. Little, unpublished data). The SP6 promoter is located near the center of the cloned fragment and a series of Bal 31 deletions were performed to remove excess sequences downstream from the point where transcription initiates. Hind III linkers were added to the ends of the Bal 31

treated DNA. One of the resulting plasmids, pSP62, has 42 bases of SP6 DNA between the transcription initiation site and the Hind III linker. Another plasmid, pSP63 has 12 bases of intervening DNA. Further Bal 31 deletions produced shorter fragments which were retested for SP6 promoter activity. The shortest Bal 31 product that retained promoter activity was transferred from pBR322 to the pUC high copy number plasmid, pUC12 (32). Since the BamH I site of R7Δ7 was destroyed in the original cloning, the promoter region was removed by cutting with Sal I and Hind III. To form pSP64, pUC12 was cut at the unique Nde I site, blunt ended with Klenow polymerase and then digested with Hind III. The Sal I end of the SP6 promoter fragment was filled in with Klenow polymerase and joined to the Nde I site of the pUC12 vector. From previously published sequences of pUC12 and pBR322 DNA, we conclude that pSP64 contains 2520 bases of pUC12 DNA, 278 bases of pBR322 DNA and 251 bases of SP6 DNA.

pSP65 has the polylinker cloned in the opposite orientation relative to pSP64. To construct pSP65, pSP64 was cut with Hind III and treated with mung bean nuclease (33) to remove the single-stranded regions. An Eco R1 linker was ligated to the ends and the SP6 promoter was excised and cloned into pUC13 (32).

In vitro transcription with SP6 RNA polymerase

Linear DNA templates (100 μg/ml) are transcribed in 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, RNAsin (1 unit/μl), 100 μg/ml BSA, and 500 μM of each rNTP. Typically, 1 unit of SP6 RNA polymerase is added per μg of DNA template for a 1 hour synthesis at 40°. These conditions differ slightly from those previously described by Butler and Chamberlin (27). A 5X transcription buffer of 200 mM Tris, pH 7.5, 30 mM $MgCl_2$, 10mM spermidine is autoclaved and stored at -20°C. Dithiothreitol and rNTPs stocks are prepared in water which had been previously treated with diethylpyrocarbonate and autoclaved. The components of the transcription reaction are mixed at room temperature, not on ice, because the spermidine can cause the DNA to precipitate at 0°C.

Following RNA synthesis the DNA template is removed by the addition of RNAsin and RNAse-free DNAse to final concentrations of 1 unit/μl and 20 μg/ml, respectively. After a 10 min incubation at 37° the reaction is extracted with phenol:chloroform and then precipitated directly by the addition of ammonium acetate to 0.7M and 2.5 vol of ethanol. Alternatively the RNA is purified from unincorporated NTPs by Sephadex G100 chromatography. Most commercial preparations of 'RNAse-free' DNAse are not RNAse-free and

must be further purified. RNAse-free DNAse was prepared by chromatography on UDP-agarose (Miles-Yeda) as described elsewhere (34). We have recently obtained satisfactory results with Worthington DPRF DNAse without UDP-agarose chromatography.

Gel analyses

RNA transcripts are stored in ethanol and precipitated immediately before use. The precipitates are dissolved in diethylpyrocarbonate (DEPC) treated H_2O and mixed with 3 volumes of loading buffer (67% formamide, 20% formaldehyde, 13% 10X MOPS buffer). Following incubation at 60°C for 5 min the sample is electrophoresed in agarose (0.6-2.5%) containing 7.5% formaldehyde (v:v) in 1X MOPS buffer (35,36). Electrophoresis is carried out in 1X MOPS without formaldehyde. 10X MOPS buffer is 0.2M 3-N-morpholinopropanesulfonic acid, 0.05M sodium acetate, and 0.01M EDTA.

Blot hybridizations

RNAs are electrophoresed in formaldehyde-agarose gels and transferred to nitrocellulose as described elsewhere (37). Following a 2 hr incubation at 80°C *in vacuo* the filters are pre-hybridized for 1-4 hrs at 55-60° in 50% formamide, 50 mM $NaPO_4$, pH 6.5, 5X SSC, 0.1% SDS, 1 mM EDTA, 0.05% BSA, 0.05% Ficoll, 0.05% PVP, and 200 µg/ml denatured salmon sperm DNA. We do not know which if any of these various components is necessary. Following the addition of probe, the filters are hybridized in the same buffer at 55-60°C depending on the length and sequence homology of the probe (38). Filters are washed 3-5 times in 0.1X SSC, 0.1% SDS, pre-heated to 65°C, for 20 min each wash. Autoradiographic exposures with pre-flashed Fuji X-ray film are performed at -80°C.

Solution hybridization and RNAse mapping

RNAse mapping, described previously in ref 16, is performed by dissolving 5-40 µg of test RNA in 80% formamide, 40 mM Pipes, pH 6.7, 0.4 M NaCl and 1 mM EDTA. u^{32}P-RNA SP6 probes are dissolved in the same buffer and added to the test RNA to give a final volume of about 30 µl. Following denaturation at 85°C for 5 min the mix is incubated overnight (>8hr) at 45°C or another more appropriate temperature depending on the GC content and length of the RNA-RNA hybrids. Following hybridization 300 µl of 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, RNAse A (40 µg/ml), and RNAse T1 (2µg/ml) is added and incubated for 1 hr. The time and temperature of the RNAse digestion should be determined empirically as it can significantly influence the signal to noise ratio. In many cases a 30°C digestion produces satisfactory results. The RNAse digestion is terminated by the addition of

20 µl of 10% SDS and 50 µg of Proteinase K and an additional incubation at 37°C for 15 min. The reaction is extracted with phenol:chloroform and the ^{32}P-RNA is precipitated with carrier tRNA and ethanol. The precipitate is washed with 70% ethanol, dissolved in 80% formamide and dyes, and analyzed by denaturing acrylamide-8M urea gel electrophoresis.

A high background signal can result from the presence of residual DNA template in the RNA probe as a result of incomplete digestion of the DNA template following the transcription reaction. Excessive amounts of probe can also give a high background and it is helpful to determine the optimal amount of probe needed for a given RNA sample by titration.

RESULTS

Cloning vectors containing an SP6 promoter

An SP6 promoter was originally cloned into pBR322 as a vector for SP6-driven *in vitro* transcription (1, E. Butler and P. Little, unpublished data). This vector, R7Δ7, has been used to produce *in situ* hybridization probes (15,29) and pre-mRNAs (1,9), but is not ideal because transcription initiates about 250 bases upstream from the restriction site used for cloning. We have used Bal 31 nuclease digestion to obtain SP6 transcription vectors which have a minimum number of bases between the site at which transcription is initiated and the cloning site. In addition, we have inserted polylinkers so that virtually any DNA restriction fragment can be cloned in the desired orientation relative to the promoter.

Figure 1 shows restriction maps of pSP64 and pSP65 and the DNA sequences near the SP6 promoter. Note that pSP64 has only 6 bases between the transcription start site and the Hind III site of the polylinker. pSP65 has 9 bases between the transcription start site and the EcoR1 site of the inverted polylinker. The insertion of the SP6 promoters into the pUC vectors resulted in the removal of most of the lac Z gene. Consequently, identifying recombinants by X-gal color screening is not possible with pSP64 or pSP65.

The nucleotide at which SP6 RNA polymerase initiates transcription was identified by labelling RNA transcripts with ^{32}P-β-GTP and ^{32}P-β-ATP. Only the ^{32}P-β-GTP was incorporated into RNA and RNA sequence analyses (data not shown) revealing that the G residue marked in Figure 1 is the start site for SP6 transcription.

Bal 31 digestion at the Hind III site of pSP64 was used in an attempt to make the nucleotide at which transcription initiates coincident with the

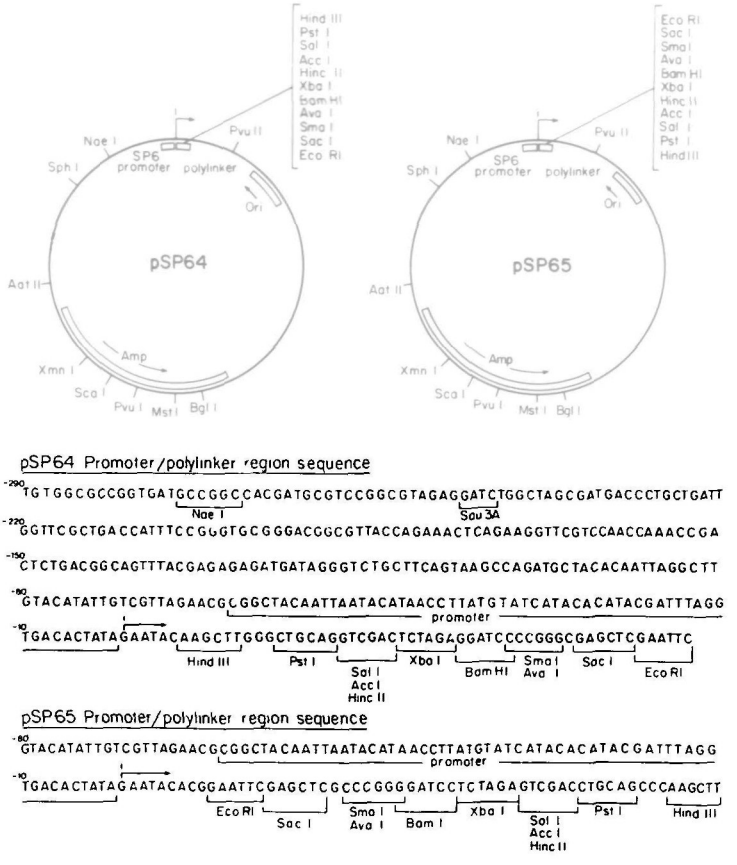

Figure 1. Restriction maps and sequences for SP6 cloning vectors pSP64 and pSP65. Restriction enzymes that cut the plasmids only once are shown in the correct positions on the circular maps. Ori, origin of replication. Amp, ampicillin resistance gene with the arrow noting the direction of transcription. Position 1 marks the nucleotide at which SP6 RNA polymerase initiates transcription as denoted by the arrow. The Sau 3A site present at about -250 marks the junction of the SP6 promoter DNA and the pBR322 sequences. The 5' end of the underlined promoter region is a FnuD II site. See Materials and Methods for further details on plasmid constructions.

G residue in the Hind III site. This would eliminate any extra sequences at the 5' end of transcripts synthesized from DNA sequences cloned into the Hind III site of pSP64. These Bal 31 deletions produced clones with small deletions upstream of the starting G residue. Unfortunately, all these clones are transcriptionally inactive. One of these deletions is noteworthy because its sequence in the promoter region is very similar to the wild type

promoter and yet this clone is transcriptionally inactive. The sequences near the wild type promoter (top) and this deletion clone (bottom) are shown below; the differences are underlined. The G* residue is the site at which transcription initiates in the wild type promoter.

5' AAGGTGACACTATAG*AATACA 3'

AAGGTGACACTCAAG*CTTGGG

Note that the G* is in the same position relative to the 5' promoter elements in both clones. Because this deletion clone is transcriptionally inactive we conclude that the bases at position -2 and -3 are important elements of the SP6 promoter. It is also possible that sequences downstream from the initiation site are important promoter elements. The results of transcription studies with this and other deletion clones suggest that a transcriptionally active promoter containing a restriction enzyme cleavage site right at the initiating G residue cannot be made.

The 5' end of the SP6 promoter has not been carefully demarcated by deletion studies. However, we have shown that the SP6 promoter is not inactivated by digestion with FnuD II. Therefore the minimum size for an excisable promoter must be less than 60 bases, the distance between the FnuD II and Hind III sites in pSP64.

Factors affecting RNA synthesis by SP6 RNA polymerase

Butler and Chamberlin examined the optimal transcription reaction conditions for SP6 RNA polymerase using total SP6 phage DNA, which contains several promoters, as a transcription template (27). We have performed a similar series of experiments using linearized SP6 plasmid DNAs as templates to determine whether there are any significant differences in the reaction conditions when a cloned SP6 promoter is used. Moreover, we have determined the minimum rNTP concentration necessary to produce full length transcripts rather than measuring rNTP incorporation. These data establish reaction conditions for synthesizing large amounts of RNAs and for synthesizing RNAs at a high specific activity from DNAs cloned into the SP6 vectors.

Nucleotide concentration. To determine the lowest rNTP concentration at which full length transcripts can by synthesized, the concentration of three NTPs was kept constant at 500μM and one was varied from 0-500μM. The DNA template was pSP62-HβA linearized with Hind III which directs the synthesis of a 1850 base RNA transcribed from the human globin gene (Figure 4 and ref 1). Measurements of the amount of RNA synthesized at varying rNTP concentrations shows that all four rNTPs are saturating at 250 μM (Figure 2a).

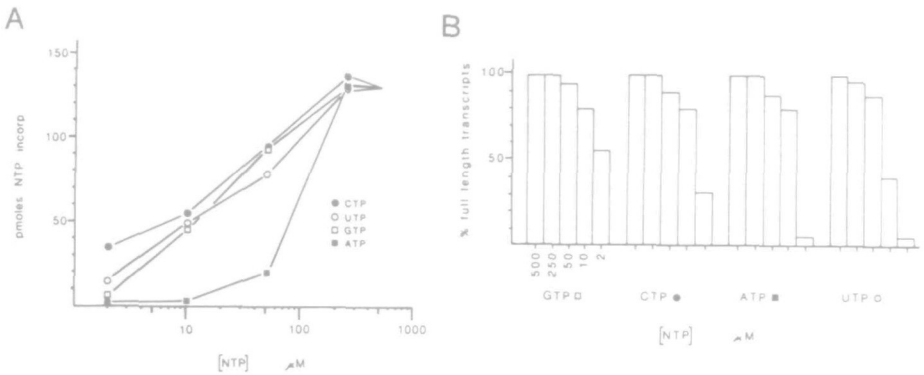

Figure 2. Effect of rNTP concentration on SP6 in vitro RNA synthesis. In vitro transcription was performed for 30 min under standard conditions (Materials and Methods) except that the concentration of one rNTP was varied from 0-500 μM as indicated. The template is Hind III digested pSP62-HβA DNA (see Figure 4) which directs the synthesis of an RNA 1850 nucleotides long. A. Total RNA synthesis as measured by incorporation of ^{32}P-α-rNTP (TCA precipitable cpm). The radiolabeled rNTP was in each case different from the limiting rNTP. B. ^{32}P-RNAs from each transcription reaction were fractionated on denaturing agarose gels and the autoradiograms were scanned with a densitometer. The percent of total RNA synthesized which is full length (1850 nts) is shown for each rNTP concentration.

Neglecting possible effects from competitive inhibition among the triphosphates in these reactions, SP6 RNA polymerase has the lowest apparent K_m for CTP and the highest for ATP. These data do not however take into account the length of the RNA synthesized and this is often an important consideration. Gel analyses of the transcriptional products shows that at low (10 μM) rNTP concentrations shorter RNAs are produced (data not shown). The percent of total RNA synthesized which is full length, measured at various rNTP concentrations, is presented in Figure 2b. These data show that the problem of premature stops is minimized if GTP is the limiting rNTP.

Requirement for BSA and spermidine. Butler and Chamberlin have reported that spermidine stimulates SP6 transcription in vitro and recommend the use of 4 mM spermidine in transcription reactions (27). We and others (Angerers, personal communication) find that the DNA template is sometimes precipitated at 4 mM spermidine at 0°C and avoid this by the use of spermidine at 2 mM. Furthermore, we find that a combination of 2 mM spermidine and 100 μg/ml BSA can stimulate transcription by a factor of 2.3 as compared to a reaction performed without these components.

Enzyme and DNA concentrations. The data in Figure 3a shows that RNA syn-

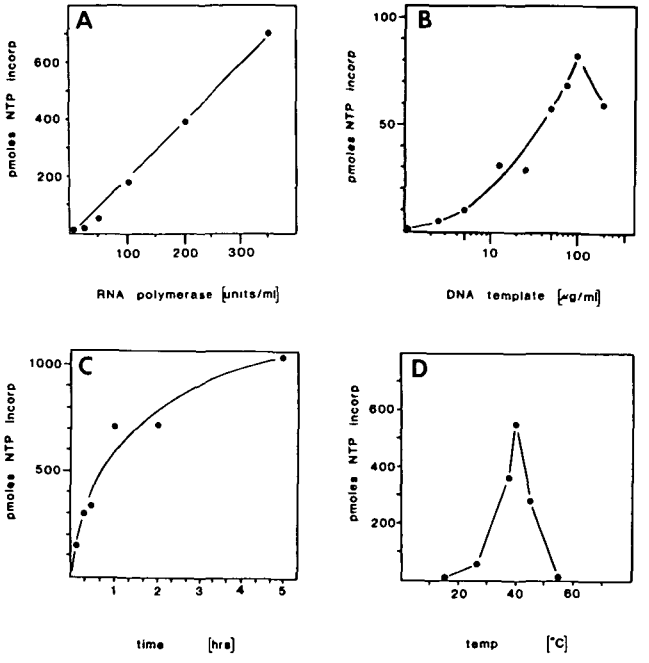

Figure 3. Effects of SP6 RNA polymerase concentration, DNA template concentration, time and temperature on SP6 RNA synthesis in vitro. A,B. Transcription reactions were performed for 30 min under standard conditions using Hind III digested pSP62-HβA DNA at 50µg/ml (see Figure 4) and measuring RNA synthesis by the incorporation of ^{32}P-α-rUTP. C,D. Transcription reactions performed as in A and B except that the incubation period (C) or temperature (D) were varied.

thesis increases linearly with the amount of enzyme added for a given amount of DNA template consistent with the results obtained by Butler and Chamberlin (27). The data in Figure 3b suggest that an optimal DNA (SP6 promoter) concentration is about 30 nM of plasmid DNA or 100 µg/ml for pSP62-HβA.

Time course and temperature optimum. Figure 3c shows that the rate of RNA synthesis is constant during the first hour of incubation and drops off slowly thereafter. A temperature curve for RNA synthesis (Figure 3d) shows a sharp temperature optimum at 40°C, the temperature at which the SP6 phage grows effectively in the Salmonella host. In vitro RNA synthesis at 40°C increases the amount of RNA made by 30% compared to synthesis at 37°C.

Amounts of RNA synthesized under optimal conditions

The data presented above suggest that optimal conditions for in vitro transcription of DNAs cloned in pSP64 or pSP65 are: 40 mM Tris-HCl, pH 7.5,

6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 100 µg/ml BSA, 1 unit/µl RNAsin, 500 µM each of CTP, UTP, GTP, and ATP, >100 units/ml SP6 RNA polymerase, and 100 µg/ml linear DNA template in a reaction incubated at 40°C. The DNA template (pSP62-HβA) used to determine these conditions has a molecular weight of about 3.3 X 10^6 so that the concentration of the SP6 promoter in the reaction is about 30 nM.

One parameter which will vary, depending on the intended use of the RNA, is the rNTP concentration. For example, when using pSP62-HβA as a template to synthesize human β-globin pre-mRNAs in large amounts the rNTP concentration is kept at 500µM for all NTPs. Under these conditions a 1 hour transcription reaction results in the synthesis, on average, of 8 moles of RNA transcript/mole of DNA template. If more enzyme is added after the first hour of incubation we typically observe the synthesis of 10-20 moles of transcript/mole of template such that 5-10 µg of pre-mRNA is made in a standard (see above) 50 µl reaction.

In other cases, such as the preparation of RNA at a high specific activity for use as a hybridization probe, the rNTP concentration is limiting. We typically use ^{32}P-α-UTP at 12 µM, 400 Ci/mmol, in a 20 µl reaction and achieve 80-90% incorporation of the label in a 1 hr reaction. This results in the synthesis of about 250 nanograms of ^{32}P-RNA at a specific activity of 6.6 X 10^8 dpm/µg. RNAs of an even higher specific activity can be synthesized by using rNTPs of a higher specific activity and by using more than one labelled rNTP.

Length of in vitro transcripts

To test whether SP6 RNA polymerase can synthesize long RNA transcripts the human β-globin gene (pSP62-HβA) was linearized at various positions, each restriction fragment serving as a different transcription template. The data in Figure 4 show that in every case full length transcripts are synthesized giving rise to RNAs from 380 to 5700 bases in length. Transcripts shorter than full length RNAs that result from premature termination are rare (faint bands in Figure 4).

We have also tested whether SP6 RNA polymerase can transcribe continuous stretches of the same nucleotide. For example, a stretch of 65 T residues is efficiently transcribed to produce a poly A tail on β-globin RNA (see Fig.4 and ref 1). Similarly, transcription by SP6 RNA polymerase proceeds through poly (C), poly (G), and poly (A) stretches of about 30 bases each (data not shown).

In vitro transcription reactions using numerous DNA templates indi-

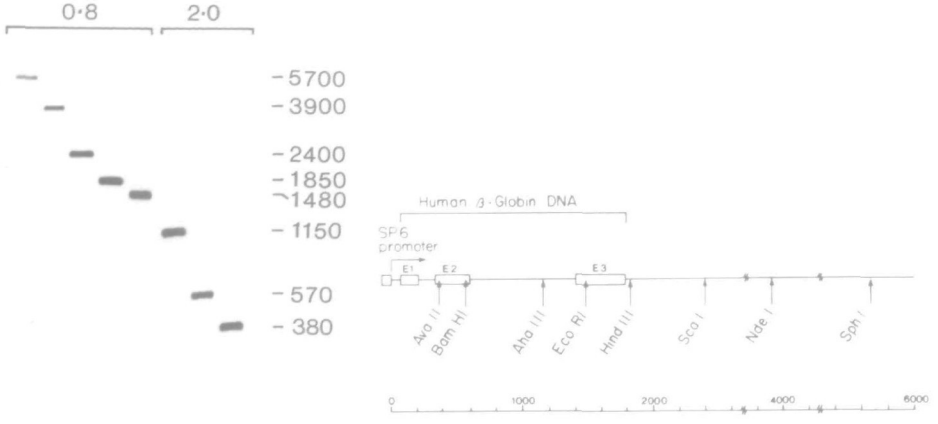

Figure 4. Full length transcription of linear DNA templates by SP6 RNA polymerase. Plasmid pSP62-HβA was digested with one of the restriction enzymes indicated at the bottom and the linear DNAs were used as templates in eight separate transcription reactions. The RNA transcripts were labeled by incorporation of ^{32}P-α-rUTP and fractionated by electrophoresis in 0.8% or 2.0% denaturing agarose gels as indicated. An autoradiogram of the dried gels is shown with the sizes of the transcripts in nucleotides. The scale at the bottom is given in nucleotides.

cates that SP6 RNA polymerase does not readily terminate transcription. A wide variety of DNAs serve as transcription templates and we have not identified any strong terminators. It would be useful to identify a sequence which will efficiently cause the polymerase to stop transcription because this would eliminate the necessity to use linear DNA templates. Presumably, such a terminator sequence can be isolated from SP6 phage DNA. In this regard it should be noted that some sequences can cause the polymerase to fall off or terminate transcription albeit rather infrequently. An as yet uncharacterized sequence found about 400 bp downstream of a chicken H2B histone gene is able to cause some premature termination of transcription and this results in the synthesis of about 80% full length molecules and 20% shorter transcripts (data not shown).

Strand and template specificity of SP6 in vitro transcription

Several experiments were performed to test the template specificity of SP6 RNA polymerase. First, to test strand specificity, β-globin transcripts were synthesized by transcription of Hinf I digested pSP64-Xβm DNA. pSP64-Xβm (details in the accompanying paper) contains a Xenopus β-globin cDNA clone and transcripts from this DNA closely resemble authentic Xenopus β-globin mRNA. These ^{32}P-labeled transcripts were hybridized to filter

Table 1. Strand specificity of SP6 transcription.

DNA bound to filter	^{32}P-RNA probe	cpm hybridized
β-globin non-coding strand	SP6-globin RNA	45,659
β-globin coding strand	SP6-globin RNA	185
single stranded vector	SP6-globin RNA	156
none	SP6-globin RNA	85

SP6 transcription of Hinf I digested pSP64-Xβm DNA was used to produce a ^{32}P-RNA resembling β-globin mRNA (see text and accompanying paper for details). 3 x 10^5 cpm of this RNA, which is complementary to the non-coding strand of the globin cDNA clone, was hybridized to filter bound single stranded DNAs as indicated. The filters were washed and the cpm hybridized were measured in a scintillation counter. The non-coding strand of the Xenopus β-globin cDNA clone is present in DNA isolated from the M13 recombinant phage, MXβ13. The coding strand of the β-globin cDNA, which has the same sequence as the mRNA, is present in DNA isolated from the recombinant phage, MXβ27. The control filter contains single stranded DNA from the cloning vector, M13mp2.

bound single-stranded DNA that contained either the coding or the non-coding strand of the globin gene. By convention, the mRNA is complementary to the non-coding strand of the DNA. The results presented in Table 1 show that only 0.2% of the labeled RNA transcripts can hybridize to the coding DNA strand (the strand with the same sequence as the mRNA). In effect, it is difficult to detect transcripts from the wrong (coding) strand. We therefore conclude that 99.8% of the SP6 RNA is derived by transcription of the non-coding strand of the cDNA clone to produce globin mRNA. A similar conclusion was reached by Cox et al (15) from a slightly different type of experiment with SP6 transcripts.

Secondly, we tested whether nicks in the DNA template can serve as transcription initiation sites. pSP62-HβA DNA and pBR322 DNA (which does not contain an SP6 promoter) were linearized with Hind III and treated with DNAse I. Examination of these nicked DNAs by denaturing gel electrophoresis showed that the average size of the fragments was about 500 bases, i.e. there was a nick in one of the two DNA strands about every 250 base pairs. Equal amounts of both DNAs were used as templates for in vitro transcription. The linear nicked pBR322 is unable to promote SP6 transcription in

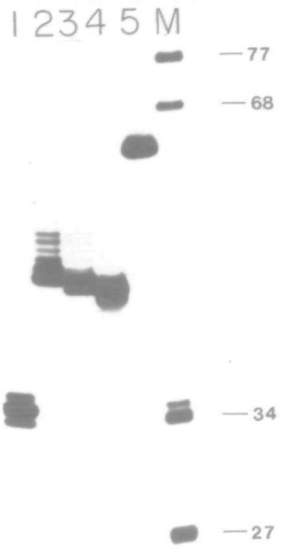

Figure 5. Transcription of DNAs with staggered or blunt 3' termini. pSP65 DNA was digested with BamH I, Sal I, Acc I, Hinc II, or Hind III, 1-5, respectively, all of which cut the DNA in the polylinker (Figure 1). Standard transcription reactions were performed and the labeled transcripts were fractionated on a 15% denaturing acrylamide gel. An autoradiogram of the gel is shown. M, DNA markers with sizes in nucleotides shown.

vitro. In contrast, linear nicked pSP62-HβA does serve as an efficient transcription template. Interestingly, the nicked pSP62-HβA template does not direct the synthesis of shorter transcripts (data not shown). We therefore conclude that nicks in the DNA do not serve as adventitious sites for transcription initiation by SP6 RNA polymerase. Moreover, SP6 RNA polymerase does not terminate at nicks in the template. Note that these and other results (e.g. Fig 4) show that SP6 RNA polymerase does not readily initiate transcription at the ends of a linear duplex.

These data confirm observation that SP6 RNA polymerase is extraordinarily specific for transcription initiation at an SP6 promoter (27,28). It is also of interest to determine where transcription actually stops with respect to the terminus of a DNA template digested with a restriction enzyme. Figure 5 shows the results of an experiment designed to determine where transcription stops on DNAs that contain either a 5' overhang or flush ends. pPS65 contains unique Sal I, Acc I and Hinc II sites in the polylinker. All three enzymes recognize the same sequence, 5' GTCGAC 3', but cleavage of the DNA occurs at different positions such that Sal I generates a 4 base 5' overhang on the template strand, Acc I a 2 base 5' overhang, and Hinc II leaves blunt-ended DNA. The various 3' ends are shown below with the template strand (the strand which is transcribed) on the bottom.

Sal I	Acc I	Hinc II
5' --G	--GT	--GTC
3' --CAGCT	--CAGC	--CAG

Transcription of the three different templates (Fig 5) shows that the DNA with the longest template strand (Sal I digested DNA) directs the synthesis of longest transcripts. Note that in each case there are two predominant bands which presumably represent termination at the last two nucleotides of the template strand. In addition, there are several minor transcription products which are longer than the template and may be the result of template independent additions at the 3' terminus. In conclusion, the results suggest that the major RNA transcripts are derived from transcription of the template strand to the last and penultimate nucleotide. Given that the RNAs have the same 5' ends, the results also show that there is some heterogeneity at the 3' ends of the SP6 transcripts.

SP6 transcripts as solution and blot hybridization probes

As mentioned above, the advantages of SP6 RNA probes over nick translated DNA probes for in situ hybridization have been carefully documented (15,29). We show here that single stranded SP6 RNA probes have several advantages, including ease of preparation and increased detection sensitivities, for solution and blot hybridization.

Northern blots. To accurately measure the limits of detection in RNA blot hybridizations, ^{3}H-globin RNA was synthesized in vitro by SP6 transcription of a Xenopus β-globin cDNA. In this case the non-coding strand of the cDNA clone is transcribed producing an RNA that resembles authentic globin mRNA. Measurement of ^{3}H incorporation allowed for the addition of known amounts of globin RNA to total Xenopus oocyte RNA. Two identical denaturing agarose gels were run, each containing lanes with decreasing amounts of ^{3}H- globin RNA and 2 μg of total oocyte RNA. After blotting to nitrocellulose, the filters were prepared for hybridization and probed with either SP6 ^{32}P-globin RNA or nick translated ^{32}P-globin DNA. The SP6 single stranded RNA probe was synthesized using 100 μCi of ^{32}P-α-GTP and 0.25 pmol of DNA template. In this case the coding strand of the cDNA clone was transcribed producing an RNA that is complementary to the mRNA. The nick translated DNA probe was prepared using 100 μCi of ^{32}P-α-dGTP and 0.25 pmol of a globin DNA restriction fragment. This restriction fragment was the same DNA that was transcribed to produce the SP6 RNA probe. While both reactions started with the same amount of ^{32}P-nucleotide, the reactions produced a single-stranded SP6 RNA probe with a specific activity 6.6 X 10^{8} dpm/μg and a double

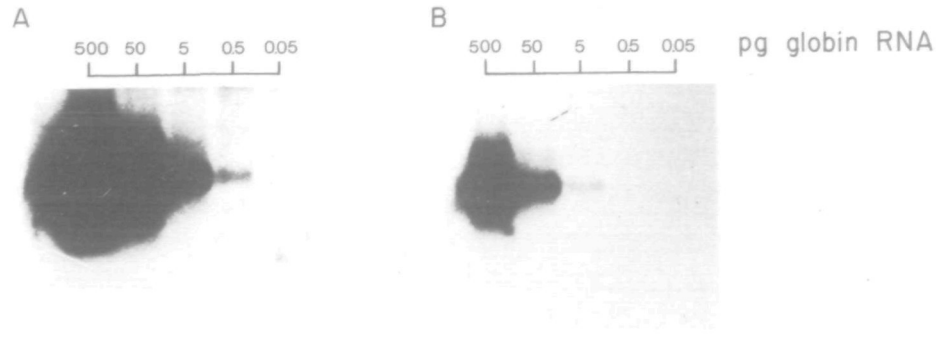

Figure 6. Northern blot comparing an SP6 single stranded RNA probe to a nick translated DNA probe. Each lane contains 2 μg of oocyte RNA supplemented with a known amount of a synthetic globin mRNA as indicated at the top. Following electrophoresis and transfer to nitrocellulose, one filter was hybridized with ^{32}P SP6 RNA (6.6 X 10^8 dpm/μg) which was prepared by transcribing the coding strand of a Xenopus β-globin cDNA. The other identical filter was hybridized with ^{32}P-globin DNA (2.1 X 10^8 dpm/μg) which was prepared by nick translating a Xenopus β-globin cDNA restriction fragment. See text for further details. A 3 day exposure of the autoradiogram is shown.

stranded DNA probe with a specific activity of 2.1 X 10^8 cpm/μg.

The autoradiogram in Figure 6 shows that the SP6 RNA probe can detect as little as 0.5 picograms of globin RNA whereas the detection limit for the nick translated DNA is ten times more RNA or 5 picograms (3 day exposure). Even after accounting for differences in the specific activities, the single stranded RNA probe is superior. While the RNA probes have the obvious attraction of increasing the sensitivity of detection there is the disadvantage, in some instances, of a higher incidence of non-specific binding to ribosomal RNA. This problem can be eliminated without a concomitant loss of signal by increasing the stringency of the wash.

Southern blots. SP6 probes can also be used effectively for DNA (Southern) blots. SP6 probes allow for the detection of femtograms of DNA in a 10 day exposure of a Southern blot when using the special hybridization conditions developed for genomic sequencing (see ref. 17). We have not carefully measured the detection sensitivities in DNA blots, though our preliminary studies indicate that it is helpful to treat the blot with RNAse after hybridization to reduce the background.

RNAse mapping

SP6 single stranded ^{32}P-RNA probes can also be used in solution hybridizations to detect and quantitate specific RNAs and to determine struc-

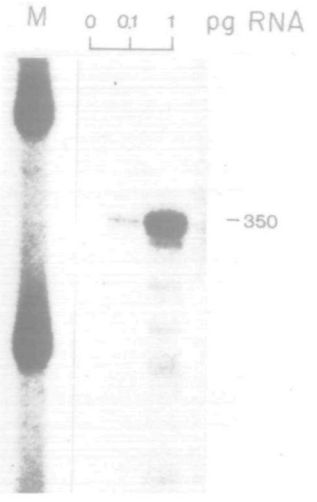

Figure 7. Solution hybridization with single stranded SP6 RNA probes. ^{3}H-globin RNA was synthesized by SP6 transcription of a restriction fragment of a globin cDNA clone. Known amounts of this ^{3}H-RNA were added to 20 µg of HeLa cell RNA and the mixtures were hybridized with a ^{32}P-labeled SP6 RNA probe. The ^{3}H-RNA and ^{32}P-RNA are homologous over a 350 base region. The ^{32}P-RNA probe was prepared by inverting the globin cDNA restriction fragment and transcribing the DNA with SP6 RNA polymerase. Following hybridization and subsequent digestion with RNAses A and T1, the products were fractionated on a 5% denaturing acrylamide gel. The amount of ^{3}H-RNA mixed in for each sample is indicated at the top of the lane. A 3 day exposure of the autoradiogram is shown. M, DNA markers.

tural features of RNA molecules. The RNAse mapping procedure described below and in ref. 16 is analogous to the well known S1 nuclease mapping procedure in which labelled DNA probes are employed (39-41). An excess of single stranded ^{32}P-RNA complementary to the test RNA is synthesized with SP6 RNA polymerase and hybridized in solution to the RNA sample in order to form a ^{32}P-RNA-RNA hybrid. Ribonuclease treatment digests the unhybridized single stranded ^{32}P-RNA probe, but double stranded ^{32}P-RNA-RNA hybrids are ribonuclease resistant and can be detected and quantitated by gel electrophoresis.

A reconstruction experiment using this RNAse mapping procedure is shown in Figure 7. Measured amounts of ^{3}H-globin RNA were added to 20 µg of HeLa cellular RNA. These RNA mixtures were hybridized with a high specific activity ^{32}P-RNA probe complementary to the ^{3}H-RNA. The ^{32}P-RNA probe was prepared by SP6 transcription of the coding strand of the globin DNA. Fol-

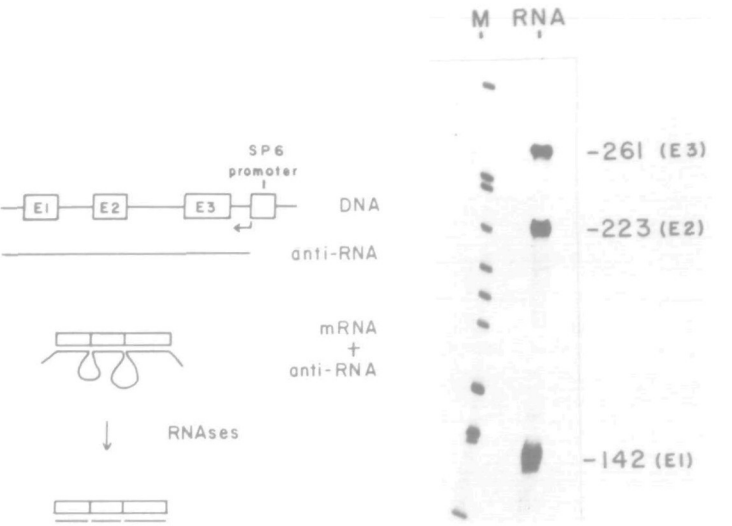

Figure 8. Exon mapping with single stranded SP6 RNA probes. A full length SP6 transcript of the coding strand of the human β-globin gene was synthesized as shown on the left. This ^{32}P-RNA, an anti-RNA, is complementary to globin mRNA. The uniformly labeled anti-RNA was hybridized with mature globin mRNA which is represented by three spliced exons (boxes) in the diagram. The globin exon lengths are 142, 223, and 261 nucleotides. The mRNA:anti-RNA hybrids were digested with RNAses A and T1 and the nuclease resistant fragments were fractioned on a 5% denaturing acrylamide gel. An autoradiogram of the gel is shown with DNA markers, M.

lowing hybridization, the samples were digested with RNAase A (C and U specific) and RNAase T1 (G specific). The data presented in Figure 7 show that as little as 0.1 pg of specific RNA can be detected with an autoradiographic exposure of 3 days. Further experiments show that the intensity of the signal is relatively insensitive to variations in RNAase concentration over at least a 10-fold range (data not shown). This is in contrast to what is often observed in S1 nuclease mapping experiments using end-labelled DNA probes (41). The relatively minor effect of ribonuclease concentration in these experiments is probably due to the absence of 'end-nibbling' and the relatively greater stability of RNA-RNA hybrids. We have observed that the signal to noise ratio is significantly affected by both the temperature and time of ribonuclease digestion. It is therefore advisable to empirically determine optimal conditions for a particular probe. We have also found that other RNAases can be used in this procedure, e.g. RNAse T2 which is not sequence specific.

This RNAse mapping procedure with SP6 RNA probes can also be used to define structural features of RNA molecules. For example, the number and sizes of exons and introns within a DNA molecule can be mapped. Figure 8 shows an example of this exon-mapping procedure using the human β-globin gene. In this case genomic human β-globin genomic DNA was transcribed to produce a ^{32}P-RNA complementary to β-globin mRNA. This ^{32}P-RNA probe, called anti-RNA in Figure 8, was hybridized to authentic human β-globin mRNA. The hybrids were digested with RNAses A and T1 and the nuclease resistant ^{32}P-RNA-RNA hybrids were analyzed by denaturing gel electrophoresis. Three ^{32}P-RNA products are observed with sizes corresponding to the three known β-globin exons which are 142, 233, and 261 nucleotides long. Thus, it is possible to map the number and sizes of the exons (and introns by subtraction) within the gene.

DISCUSSION

In this paper we describe the construction and characterization of cloning vectors that contain an SP6 promoter immediately upstream from a polylinker sequence. By removing sequences between the transcription initiation site of the SP6 promoter and cloning sites in the vector we have positioned the SP6 promoter to within 6 and 9 nucleotides of the polylinker. Thus, it is now possible to insert DNA fragments generated by digestion with a variety of different restriction enzymes into the polylinker and generate RNA transcripts containing a minimum of extraneous vector sequence.

The data presented here show that SP6 derived RNAs have several advantages as hybridization probes. SP6 RNAs can be synthesized at very high specific activities and single stranded probes can be obtained without gel isolation. These single stranded probes are often more effective in northern blot hybridizations (Fig 6) and in situ tissue section hybridizations (15,28) than nick translated DNA probes. We have not tested the relative efficiency of single stranded RNA and single stranded DNA probes like those made with M13 phage (14). Single stranded RNA probes are potentially more effective than single stranded DNA probes because RNA-RNA duplexes are more stable than DNA-DNA or DNA-RNA duplexes (38). In theory, this extra stability allows for the formation and washing of hybrids under more stringent conditions which may increase the signal to noise ratio. In addition, it is possible to use RNAse to remove non-specifically bound RNA probe while leaving the RNA-DNA hybrids untouched. These theoretical considerations are considered in more detail elsewhere (14,38).

The technique of RNAse mapping or detection with single stranded RNA probes (Fig 7 and ref. 16) is a very sensitive method for quantifying the amount of rare RNAs. As little as 0.1 picograms of RNA can be detected in a 72 hr exposure. In our experience this is about 25-50 times more sensitive than detection with end-labeled DNA probes in an S_1 protection assay. Presumably, this difference is a consequence of being able to synthesize SP6 probes at a much higher specific activity and the increased stability of RNA-RNA hybrids. The disadvantages of this method are that often necessary to titrate the amount of RNA probe needed for each RNA test sample in order to give low backgrounds and maximize the sensitivity of the assay. Moreover we note reports that SP6 RNA polymerase appears to contain an activity that can produce RNA complementary to the desired probe and this results in unacceptably high levels of background in hybridization assays (D. Ward, personal communication). This problem is most severe when relatively short DNA templates (less than 150 nts) are used and when the templates are prepared by digestion with restriction enzymes that leave a 3' overhang. We have not detected significant amounts of complementary RNAs in SP6 transcription reactions, but most of the templates we use are longer than 150 nts. In any case, it has been determined that removal of the 3' overhang minimizes the synthesis of the complementary RNA (B. Mierendorf and D. Ward, personal communications).

The technique of RNAse mapping can also be used as a rapid method to map the exons and introns within a genomic DNA clone. In this method the entire genomic copy of the gene is cloned in an SP6 vector such that SP6 transcripts will be complementary to the mRNA. Transcription of the cloned genomic DNA, after linearization at a position 5' to the gene, will produce full-length, anti-RNA probe. When the anti-RNA probe is hybridized to cellular mRNA and the hybrids digested with RNAase and gel fractionated, protected fragments corresponding to the length of each individual exon are observed. This mapping of the number and sizes of the exons is illustrated for the human β-globin gene in Figure 8. This same procedure can be extended to map the relative positions of the exons as follows. Following linearization of DNA templates at successive positions along the gene and transcription of these templates the RNA transcripts can be used for RNAse mapping as described above. Using the globin gene as an example, RNAase mapping with an RNA probe transcribed from DNA restricted at a site (X) within exon 3 will produce a single protected band which maps the position of the 3' end of exon 3. Use of a probe transcribed from DNA restricted within intron 2 will

give a band corresponding to the entire length of exon 3. Together these data define the size of exon 3 and the positions of the 5' and 3' ends of exon 3 relative to the restriction site (X) in exon 3. Similarly, restriction of the DNA template within exons 1 and 2, intron 1, and a site upstream of the 5' end of the gene will provide RNA probes which can be used map the positions and sizes of exons 1 and 2.

In vitro synthesized SP6 RNAs, produced by the methods detailed in this paper, are biologically active. We have previously shown that SP6 transcripts can function as pre-mRNAs and undergo two types of RNA processing reactions. SP6 transcripts of β-globin genes containing 2 intervening sequences are correctly spliced when injected into frog oocyte nuclei (1) or when added to HeLa cell nuclear extracts (5). In addition, histone pre-mRNAs that contain RNA sequences extending beyond the terminus of mature histone mRNA are correctly processed in injected oocytes to generate RNAs with mature 3' ends (9). In this regard, it is an attractive option to create pre-mRNA mutants for RNA processing studies by taking advantage of the numerous methods available for mutagenizing DNA templates. SP6 transcription of the altered DNAs yields mutant pre-mRNAs whose functions and activity can then be tested. The data presented in the accompanying paper show that this approach to analyzing function of RNA sequences can be extended to the study of messenger RNAs and the proteins they encode.

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