Enzymatic synthesis of RNA oligonucleotides

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ABSTRACT

Using SP6 RNA polymerase and various synthetic DNA oligomers as templates (12-65 b), we have obtained efficient synthesis of RNA transcripts without the need for either primer or promoter. Gel analysis of transcripts made from templates of known size demonstrated a predominant single species, together with some minor bands. For a given template the major band had the same 5'-nucleotide as predicted from the 3'-nucleotide of the template. This synthesis procedure makes it possible to efficiently and conveniently make labeled or unlabeled RNA from synthetic DNA oligonucleotides.

INTRODUCTION

Examination of the structural and functional roles implied for certain DNA and RNA sequences can sometimes be aided by the availability of short DNA and RNA species of defined sequence. The requirement for DNA sequences up to 100 b in length can be met by current automatic DNA synthesizing machines. However, for RNA sequences, nothing of comparable efficiency and simplicity is currently available. Current automated chemical methods yield RNA products only up to 15 b in length (1-3). Alternatively, enzymatic synthesis of a particular RNA can be carried out by the introduction of the corresponding double-stranded DNA into a suitable vector adjacent to and in the appropriate orientation relative to an RNA polymerase promoter (4). And recently this method has been simplified for the purpose of oligoribonucleotide synthesis, by chemically synthesizing DNA oligonucleotides with an adjacent T7 RNA polymerase promoter (5). We now report an even simpler method. We have shown that SP6 RNA polymerase will efficiently transcribe single-stranded DNA oligonucleotides without either primer or promoter. Our reasons for attempting this synthesis were twofold: (a) When RNA polymerases are presented with a mixture of single- and double-stranded DNA, the enzymes preferentially bind the single-stranded DNA (6,7). (b) A complication of transcription of double-stranded DNA by SP6 polymerase is that the enzyme prefers exposed single-stranded 3'-ends even relative to an internal

promoter sequence. Such ends can act as efficient initiation sites for transcription that is promoter-independent and apparently from end-to-end (8).

MATERIALS AND METHODS

Materials

SP6 RNA polymerase, DNase I and RNasin were products of Promega Biotech, Madison, WI; T₄ kinase was from Bethesda Research Laboratories, MD, and snake venom phosphodiesterase from Sigma, St. Louis, MO. Calf intestinal phosphatase, [α -³²P] UTP (833 Ci/mMol) and [γ -³²P] ATP (3000 Ci/mMol) were obtained from New England Nuclear, MA. Prepared columns of NACS-52 were from Bethesda Research Laboratories, MD. Precoated PEI-cellulose sheets for thin layer chromatography were from Brinkmann Instruments, NY.

Methods

After chemical synthesis, using an Applied Biosystems model 380A DNA synthesizer, the DNA oligonucleotides were further purified using electrophoresis into a DNA sequencing gel (9). The appropriate bands were located by UV-shadowing and eluted into a buffer of 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA and 0.1% SDS by shaking overnight at 37°C and finally precipitated with ethanol. Transcription of 50-250 ng of purified oligonucleotide into RNA by 10 U of SP6 polymerase was performed in a final volume of 10 μ l containing 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 10 mM dithiothreitol, 4 mM NaCl, 2 mM spermidine, 0.2 mM each of ATP, CTP, UTP, and GTP, and 20 U of RNasin. In order to obtain radioactive RNA, 5 μ Ci of [α -³²P] UTP was included in the reaction mixture. All the reactions were assembled at room temperature and incubated at 37°C for 1 hr, unless otherwise stated. Products were treated with DNase (1 mg/ml) for 10 min at 37°C and then sequentially extracted with phenol, ether and finally precipitated with ethanol.

For 5'-end labeling, cold RNA transcripts were made as described above, and then treated with calf intestinal phosphatase and labeled using T₄ kinase according to a published procedure (10). For polyacrylamide gel analysis samples were mixed with an equal volume of 5M urea-dye mix, and heated at 100°C for 3 min, prior to electrophoresis into 10% gels containing 7 M urea in TBE buffer (10). After electrophoresis, the gel was subjected to autoradiography and quantitation using an E-C densitometer coupled to a Hewlett-Packard model 3380 integrator.

The 5'-end labeled products were used, gel purified and then digested

Table 1. Chemically synthesized DNA oligomer templates.

Length	Sequence (5'-3')
12	TCCCCCTCCC TA
15	TGGTGAGCCC CGGGG
40	AATTCATAAG ACTACATTTC CCCCTCCCTA TGCAACTGCA
50	GTATTGCATA AGACTACATT TCCCCCTCCC TATGCAAAAG CGTATTTCGC
65	AATTCCTGGG GATCCCTGAA GGAATTCTTC CTTCCTGCTC CTATCTTTT TCTTGATGCC TGCAG

with snake venom phosphodiesterase (9) for 30 min at 37°C to release the 5'-nucleotide. An aliquot (2 µl) of the digest was applied to a PEI-cellulose sheet and chromatography was carried out in 1 M acetic acid for the first 4 cm, followed by 0.3 M LiCl for 1 hr (11). The plate was then dried and developed by autoradiography and quantitated as described above. As chromatography standards we used four unlabeled 5'-ribonucleotides (Sigma) which were located with UV light.

RESULTS

Initially we examined the lengths of RNA products transcribed from a selection of DNA oligonucleotide templates. The sizes of the templates used were 12, 15, 40, 50 and 65 bases and the sequences are presented in Table 1. The labeled RNA products were examined by gel electrophoresis with results as shown in Fig. 1a. The RNA transcripts in each case (lanes 1-4) showed a single major band. As approximate size standards, we kinased the DNA oligonucleotides and subjected them to the same electrophoresis, as shown in lane M. We noted that for each oligonucleotide, the major RNA transcript migrated slower than the corresponding kinased DNA template. Fig. 1b shows semi-logarithmic plots of the distances migrated by the oligonucleotide markers and also the RNA transcripts. In general the RNA transcripts moved 5% slower than their corresponding oligodeoxynucleotides. Thus the difference in mobility may simply be due to the average difference of molecular weights for ribonucleotides (323 daltons) versus deoxyribonucleotides (308 daltons).

We also considered the effect of the time of synthesis on the yield and quality of the RNA product. Data for the 40 b DNA template are shown in Fig. 1c. Apparently full length RNA was made within 10 min. From densitometer analysis we deduced that after 10 min of incubation 65% of the product was full length and by 60 min it was 85%.

Table 2 shows an analysis of the reaction yield as a function of the

Figure 1. Gel electrophoresis of RNA transcripts.

a) The 15-, 40-, 50- and 65-mer DNAs of Table 1 were transcribed in an SP6 polymerase reaction containing [α - 32 P] UTP for 1 hr. The labeled products were subjected to gel analysis and autoradiography with results as shown in lanes 1-4 respectively. Lane M shows the electrophoresis of 12-, 26-, 40-, 50- and 65-mer DNAs after 5'-labeling with polynucleotide kinase. b) A semi-logarithmic plot of electrophoretic mobilities of DNA templates (open circles) and RNA products (closed circles). c) The 40-mer DNA (250 ng) was transcribed, as in panel (a), with aliquots being removed for gel analysis and autoradiography at 0, 10, 30, 60 and 120 min, as shown in lanes 2-6 respectively. Lane 1 shows the mobility of the 40-mer DNA template after 5'-end labeling. In the third panel the nucleic acids were submitted to higher resolution electrophoresis so as to demonstrate the homogeneity of the RNA products. The origin region of the gel is not shown.

amount of template used. The yield was calculated from the specific activity of ribonucleotide precursor and the activity incorporated into acid insoluble material. Table 2 shows also the yield of apparently full length transcripts. Note that with the lowest amount of template, the enzyme synthesized more than 35 apparently full length RNA transcripts for each molecule of DNA template.

As part of an attempt to test whether the RNA transcripts were in fact full-length copies of the DNA template, the following experiment was undertaken to determine the 5'-nucleotide of the RNA products. Unlabeled RNA products were made from the 12-, 40- and 50-mer (Table 1). They were 5'-end labeled, hydrolysed and the released 5'-nucleotide identified with results as shown in Table 3. For the 12-mer, 94% of the label was found in pU. Not only is pU the 5'-end expected for a full length transcript, but this is the only location of U in the predicted product. For the 40-mer, 97% of the

Table 2. Efficiency of RNA transcription from 40-mer DNA template.

Mass of DNA template (ng)	Mass of total RNA product (ng)	Fraction of full length product	Yield of full length RNA product per unit of DNA template
250	540	0.85	2.5
50	360	0.90	6.5
5	180	0.98	35.3

The mass of RNA product was deduced from the fraction of labeled precursor incorporated into acid-insoluble material. The fraction of full length product was deduced from densitometer analysis of the autoradiogram.

label was again in the expected nucleotide pU. Similarly, for the 50-mer, 97% of the label was in the expected nucleotide, pG. In summary, for these three RNA transcripts, the identity of the 5'-nucleotide was consistent with the interpretation that the majority of the synthesis began as directed by the 3'-terminal nucleotide of the DNA template. We have also examined the termination of transcription. The RNA transcript of a 65-mer (Table 1) was labeled at the 3'-end using RNA ligase and radioactive pCp (12). The product was gel purified and submitted to sequencing with partial RNase digestions (13). The first five nucleotides were as expected for RNA transcription terminating at the 5'-end of the DNA template (data not shown).

DISCUSSION

We have described a successful method of transcribing single-stranded DNA oligonucleotides into RNA. Others have reported the transcription by bacterial RNA polymerase of single-stranded phage DNA. However, they obtained yields of less than one molecule of RNA per molecule of template

Table 3. Identification of the 5'-terminal nucleotides of the RNA transcripts.

DNA oligomer template	Observed 5'-terminal nucleotide				Predicted 5'-terminal nucleotide
	pC	pA	pG	pU	
12	<1	<1	6	94	U
40	<1	<1	3	97	U
50	<1	<1	97	3	G

The 5'-nucleotides of the three RNA products were identified as described under Methods. The table shows for each product, the percentage of label in each of the four possible terminal nucleotides. The predicted terminal nucleotides were obtained as described in the text.

(14,15). Their interpretation was that synthesis led to the formation of DNA-RNA hybrids which limited further transcription. Such a limitation apparently does not apply to our transcription of oligonucleotides, since we could obtain as many as 35 transcripts per molecule of template (Table 2).

In a typical one hour reaction at least 85% of the mass of product was full length. The remaining 15% was in smaller species most of which may be due to transcriptional stops caused by secondary structure in the nascent product. This interpretation was preferred because we were able to reduce this fraction from 15% to 2% by replacing rGTP with rITP, during synthesis (data not shown).

It is envisaged that the procedure we have described will have a number of applications. Short RNA sequences having potentially important functions can now be readily synthesized and used, for example, in the inhibition of translation by antisense RNA (16), and as substrates in self-cleavage (17) and in self-ligation reactions (18). We have already used the method to synthesize labeled RNA oligonucleotide probes which not only have a high specific activity but also produce more stable hybrids than the corresponding DNA probes (data not shown). Also we have used the method to generate 50 b RNAs and reconstruct the RNase H-dependent priming of retrovirus plus strand DNA synthesis (19,20).

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