Accurate *in vitro* cleavage by RNase III of phosphorothioate-substituted RNA processing signals in bacteriophage T7 early mRNA

Allen W. Nicholson, Kenneth R. Niebling^{1*}, Paul L. McOsker¹⁺ and Hugh D. Robertson¹

Department of Biological Sciences, Wayne State University, Detroit, MI 48202 and ¹The Rockefeller University, New York, NY 10021, USA

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ABSTRACT

To test the ability of an RNA processing enzyme to cleave chemically-modified RNA substrates, RNA transcripts containing RNase III cleavage sites were enzymatically synthesized in vitro to contain specific phosphorothioate diester internucleotide linkages. One transcript (R1.1 RNA) was generated using phage T7 RNA polymerase and a cloned segment of phage T7 DNA containing the R1.1 RNase III processing site. The second transcript was the phage T7 polycistronic early mRNA precursor, which was synthesized using E. coli RNA polymerase and T7 genomic DNA. The RNA transcripts contained phosphorothioate diester groups at positions including the scissile bonds. The modified RNAs were stable to incubation in Mg²⁺-containing buffer, and were specifically cleaved by RNase III. RNA oligonucleotide sequence analysis showed that the modified R1.1 RNA processing site was the same as the canonical site and contained a phosphorothioate bond. Furthermore, RNase III cleaved the phosphorothioate internucleotide bond with 5' polarity. RNase III cleavage of phosphorothioate substituted T7 polycistronic early mRNA precursor produced the same gel electrophoretic pattern as that obtained with the control transcript. Thus, RNase III cleavage specificity is not altered by phosphorothioate internucleotide linkages.

INTRODUCTION

RNA processing reactions and their role in gene expression and regulation are currently under intense investigation [1-4]. Such studies have revealed that RNA molecules can undergo unusual structural transformations [5], and that they can function directly as biochemical catalysts [6]. However, the mechanistic details of the reactions in which RNA molecules participate are still only partly understood. Since RNA processing reactions (excluding base modifications) involve cleavage or formation of phosphodiester bonds, a detailed description of the pathway by which covalent bonds are formed or broken at phosphorus can provide insight into the mechanism of catalysis by RNA processing enzymes. However, there is to date no description of the stereochemistry of phosphodiester bond synthesis or hydrolysis in an RNA processing reaction.

The introduction of a chiral phosphate into an RNA molecule can permit

the determination of the stereochemical course of reaction at phosphorous [7]. Specifically, the use of nucleoside phosphorothioates in determining the stereochemistry of substitution at phosphorus in enzyme-catalyzed phosphoryl and nucleotidyl transfer reactions is now well-established [8-10]. Replacement of a non-bridging oxygen atom in a phosphodiester bond by a sulfur atom confers chirality on the internucleotide moiety, which can provide evidence whether phosphodiester bond hydrolysis proceeds through a covalent enzyme-substrate intermediate, or through direct nucleophilic attack by water at phosphorus [7-10].

We are interested in determining the mechanism of action of RNase III, and the sequence and structural elements in its RNA substrates that specify accurate binding and hydrolysis. RNase III from *Escherichia coli* [E.C.3.1.24] consists of a dimeric homopolyptide of subunit molecular weight 25,000, and requires a divalent metal ion for activity [11,12]. RNase III processes the host ribosomal RNA precursor as well as specific host mRNAs, and it cleaves the bacteriophage T7 polycistronic early mRNA precursor at five primary sites to create mature mRNAs. RNase III can hydrolyze RNA-RNA duplexes, yielding limit digests of approximately 15 base pairs in length which contain 5' phosphate and 3' hydroxyl termini. RNA-RNA duplex structural elements are present in the host ribosomal processing signals [13], but RNA sequence elements involved in more complicated structures can also direct RNase III processing [12-14]. The introduction of specific phosphorothioate internucleotide groups within an RNase III processing signal, including the scissile bond, could permit determination of the stereochemistry of RNase III cleavage and assess the involvement of the phosphodiester backbone in specifying enzyme binding and catalysis. As an initial step towards these goals, we report herein the synthesis and purification of phage T7-specified, phosphorothioate-substituted RNA substrates, and their accurate processing *in vitro* by RNase III.

MATERIALS AND METHODS

Materials

The ribonucleoside 5'-O-(1-thiotriphosphates) GTP[α S], Sp isomer and ATP[α S], Sp isomer were kindly provided by F. Eckstein (Göttingen). The Sp isomer has the opposite configuration from the Rp isomer at the α -phosphorus; the Sp isomers are substrates for RNA polymerases, yielding RNA chains with phosphorothioate diester linkages in the Rp configuration [8,9]. Ribonucleoside triphosphates were obtained from Pharmacia-PL. [α - ^{32} P]ATP and

[α - ^{32} P]UTP (410 Ci/mmol, 10 mCi/ml) were purchased from Amersham. T7 RNA polymerase was obtained from Boehringer-Mannheim. *E. coli* RNA polymerase was kindly provided by P. Model (Rockefeller U.). DNase (DPFF) was obtained from Worthington. RNase III was isolated as described previously [15], but a phosphocellulose column was substituted for the poly(I)-poly(C) column. The source of RNase III was either from *E. coli* strain BL15 [16], or from the RNase III⁻ (rnc105) strain BL107 [16], which expressed RNase III from a multicopy plasmid containing the RNase III structural gene [17]. Restriction enzyme NciI was obtained from New England Biolabs.

In vitro synthesis of T7 R1.1 RNA

The *in vitro* transcription reactions contained plasmid pAR1450 as a DNA transcription template (kindly provided by Dr. J. J. Dunn, Brookhaven). The template consisted of a 79 base pair T7 DNA HinfI restriction fragment containing the T7 R1.1 RNase III processing signal, ligated to linkers and cloned downstream from the T7 ϕ 10 class III promoter in a pBR322 derivative [18]. The cloned T7 DNA sequence containing the R1.1 processing signal included base pairs 5837-5916 of the T7 genome [18]. Cleavage of the recombinant plasmid by restriction endonuclease NciI yielded a linear template which was transcribed by T7 RNA polymerase *in vitro* to produce a 131-nucleotide RNA (R1.1 RNA). R1.1 RNA contains 79 nucleotides of the T7 sequence, and 52 nucleotides of flanking vector and linker sequences.

Transcription reactions were carried out as described previously [19], with the following modifications: purified template DNA (2.4 μ g) was pre-incubated at 37°C for 5 minutes with T7 RNA polymerase (35 units) in 10 μ l of buffer (40 mM Tris-HCl [pH 7.9], 8 mM MgCl₂, 5 mM DTT, 4 mM spermidine, 5% Glycerol). To the preincubation mix was added 10 μ l of a nucleotide mix which consisted of ATP, CTP and UTP at 0.6 mM each, and 2 mM GTP or GTP[α S], Sp isomer. [α - ^{32} P]ATP (100 μ Ci) was included as the source of radiolabel. The reaction mix was then incubated at 37°C for 45 minutes. tRNA (1 μ g/ μ l final concentration) and DNase (50 μ g/ml final concentration) were added, and incubation continued at 37°C for 5 minutes. Samples were phenol extracted, the RNA products purified by CF11 cellulose chromatography [20] and recovered by ethanol precipitation.

In vitro synthesis of T7 polycistronic early mRNA precursor

Transcription reactions were carried out according to a previous protocol [21], with several modifications. Purified T7 genomic DNA (1.8 μ g) was pre-incubated at 37°C for 10 minutes with *E. coli* RNA polymerase in 70 μ l of buffer (final concentration: 20 mM Tris-HCl [pH 7.4], 50 mM KCl, 10 mM MgCl₂,

1 mM DTT, 0.2 mM EDTA). Thereafter, 10 μ L of a heparin solution was added (0.125 mg/ml final concentration), followed by further incubation at 37°C for 2 minutes. Nucleotide mix was then added, which consisted of 0.225 mM (final concentration) each of ATP, CTP, UTP and GTP; in specific reactions, GTP was replaced by 2.3 mM GTP[α S], Sp, isomer or ATP was replaced by 2.3 mM ATP[α S], Sp isomer. The nucleotide mix also contained 50-100 μ Ci [α -³²P]UTP as the label. The reactions (100 μ L final volume) were incubated at 37°C for 15 minutes. tRNA (0.2 μ g/ μ L final concentration) and DNase (20 μ g/ml final concentration) were added, followed by incubation for 5 minutes at 37°C. Samples were phenol extracted, the RNA products purified by CF11 cellulose chromatography and recovered by ethanol precipitation.

Gel Electrophoretic Analysis of RNA

Phosphorothioate-substituted and control RNAs were analyzed using three different gel electrophoretic systems. Vertical slab gels, composed of 2% polyacrylamide and 0.5% agarose [22], or 3% polyacrylamide and 6 M urea [23], or 10% polyacrylamide containing 7M urea [24], were prepared as described. Prior to electrophoresis on 3% and 10% polyacrylamide gels, an equal volume was added to the samples of loading buffer, consisting of 7 M urea, 10% sucrose and 0.004% each of xylene cyanol and bromophenol blue. The samples were electrophoresed at 10 V/cm (3% gels) or 15 V/cm (10% gels). The 3% gels were dried directly and exposed at -70°C to Kodak XAR-5 or Fuji RX Film, using Dupont Cronex intensifying screens; 10% gels were exposed to film without prior drying.

Prior to electrophoresis on 2% gels, one-fifth volume was added to the samples of loading buffer, consisting of 125 mM Tris-Acetate (pH 6.8), 5 mM EDTA, 2.5% SDS, 2.5% 2-mercaptoethanol, 0.15% bromophenol blue and 0.25 mg/ml tRNA. Samples were heated at 65°C for 1 minute, then loaded and electrophoresed at 7 V/cm. The gels were fixed in 10% acetic acid containing 1% glycerol, then dried and autoradiographed.

RESULTS

Synthesis and Reactivity in vitro of Phosphorothioate R1.1 RNA

We utilized R1.1 RNA, containing the T7 R1.1 primary RNase III processing signal, as a substrate with which to examine the effects of phosphorothioate substitution on RNase III action. As would be predicted, the in vitro cleavage of this RNA transcript by RNase III has been shown to occur at the same site as that utilized both in vivo and in vitro in the R1.1 processing signal of the T7 polycistronic early mRNA precursor [25-27]. The RNA sequence

Figure 1. Structural Formulae. A. Guanosine 5'-O-(1-thiotriphosphate), Sp isomer. B. Up_sG dinucleoside phosphorothioate, Rp configuration. C. Secondary structure model of a portion of the 131 nucleotide R1.1 RNA containing the T7 R1.1 RNase III primary processing signal (residues 51 to 131). The arrow indicates the RNase III cleavage site. The bold-face sequence containing the cleavage site denotes the RNase T1-resistant oligonucleotide which was isolated and subjected to secondary enzymatic analysis (see text, and Table 1).

surrounding the R1.1 cleavage site is shown in Figure 1C.

We (and others [28]), have found that T7 RNA polymerase can accept ribonucleoside 5'-O-(1-thiotriphosphates) (Sp configuration) as substrates. The RNA products contain phosphorothioate internucleotide linkages in the Rp configuration [28]. R1.1 RNA synthesized in the presence of GTP[α S], Sp isomer would contain phosphorothioate internucleotide linkages 5' to each internal guanosyl residue, including the one which is cleaved by RNase III (see Figure 1C). In the transcription reactions carried out as described in Materials and Methods, the level of synthesis of phosphorothioate-substituted R1.1 RNA was found to be comparable to that of the control R1.1 RNA. Furthermore, the synthesis of phosphorothioate-substituted R1.1 RNA was absolutely dependent on the presence of GTP[α S], Sp isomer (data not shown).

The R1.1 RNA transcripts were purified and analyzed on 10 percent polyacrylamide gels containing 7 M urea (Figure 2). The phosphorothioate-

Figure 2. RNase III Cleavage of Phosphorothioate-Substituted R1.1 RNA. R1.1 RNA was synthesized as described in Materials and Methods, using GTP[α S] where appropriate and [α - ^{32} P]ATP as the radiolabel. Aliquots (1.5×10^6 dpm) were incubated in a 50 μ l volume at 37°C for 90 minutes in a buffer consisting of 30 mM Tris-HCl (pH 8), 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT, and tRNA (0.4 mg/ml). RNase III (10 μ l) was included in the incubation as indicated. Following reaction, aliquots (10^5 dpm) were analyzed on a 10% polyacrylamide gel. The order of experiments on the gel is as follows: Lane (a), R1.1 RNA (control); Lanes (b) and (c), R1.1 RNA (control) incubated in buffer without or with RNase III, respectively; Lane (d), R1.1 RNA (phosphorothioate-substituted); Lanes (e) and (f), R1.1 RNA (phosphorothioate-substituted) incubated in buffer without or with RNase III, respectively. The arrows adjacent to lane (f) indicate the 102 and 29 nucleotide RNA cleavage products.

substituted R1.1 RNA has the same gel electrophoretic mobility as the unmodified R1.1 RNA (compare lanes a and d, Figure 2). Incubation of the phosphorothioate-substituted R1.1 RNA with RNase III yields two major species which comigrate with the 102 nucleotide and 29 nucleotide species produced by RNase III cleavage of control R1.1 RNA (compare lanes c and f, Figure 2). These data indicate that RNase III can catalyze the hydrolysis of phosphorothioate-substituted R1.1 RNA at, or very close to the canonical RNase III site. In addition, incubation of the phosphorothioate-substituted R1.1 RNA in buffer alone did not produce any noticeable breakdown products (compare lanes d and e, Figure 2). Thus, phosphorothioate-substituted R1.1 RNA has a hydrolytic stability comparable to that of unmodified R1.1 RNA in Mg²⁺-containing buffers.

Precise Site and Polarity of RNase III Cleavage of Phosphorothioate R1.1 RNA

Since RNase III can cleave phosphorothioate-substituted R1.1 RNA at or near the normal R1.1 processing site, we determined the precise site and polarity of RNase III cleavage of the phosphorothioate-substituted transcript. Our approach took advantage of the direct analysis of certain key RNase T1-resistant oligonucleotides in the immediate vicinity of the R1.1 RNase III cleavage site. Since RNase III cleaves a phosphodiester bond within a segment of R1.1 RNA defined by the 8 base RNase T1-resistant oligonucleotide, 5'CCUUUAUGp3' [#](see Figure 1C), we followed the fate of this oligonucleotide when phosphorothioate-substituted R1.1 RNA was cleaved with RNase III. R1.1 RNA was synthesized using GTP[α S], Sp, isomer and using [α -³²P]ATP as the radiolabel. This provided ³²P-label in RNase T1-resistant oligonucleotides, including the 8 nucleotide RNA spanning the RNase III cleavage site which would contain ³²P-radioactivity both from the internal A residue, and the A residue which is the 3' nearest neighbor (Figure 1C). The radiolabeled transcript was treated, in parallel with control R1.1 RNA, with purified RNase III. Aliquots of the reactions were analyzed on a 10 percent polyacrylamide gel to confirm complete reaction. The reactions were terminated by phenol extraction, and portions of the purified products and starting materials were digested with RNase T1. The RNase T1-resistant oligonucleotides were fractionated in two dimensions using gel electrophoresis followed by ascending homochromatography on DEAE-cellulose [29].

In the fingerprint of the unmodified R1.1 RNA, the 8-base oligonucleotide CCUUUAUG (species 1; Table 1) and its RNase III cleavage products CCUUUAU_{OH} (species 2) and pGp (species 3) were identified by elution and secondary analysis using pancreatic RNase, and RNase U2 (Table 1). To establish the identity of the corresponding RNase T1-resistant phosphorothioate-containing oligonucleotides, candidate spots were subjected, in parallel with the control R1.1 RNA oligonucleotides, to secondary enzymatic analyses (Table 1). The phosphorothioate-substituted R1.1 RNA starting material exhibited the RNase T1-resistant oligonucleotide CCUUUAUG (species 1) while the products of RNase III reaction lacked this oligonucleotide, and instead displayed the new species CCUUUAU_{OH} (species 2) and p_sGp (species 3).

Additional results from the secondary analyses can be summarized as follows. First, the corresponding pairs of the RNase T1-resistant RNA species 1, 2 and 3 comigrated in three different electrophoretic systems (Table 1). Second, digestion of the corresponding pairs of the RNase T1-resistant species with ribonucleases T2, U2, pancreatic ribonuclease, or nuclease P1 yielded

TABLE 1

Secondary Analysis of RNase T1-Resistant Oligonucleotides of Phosphorothioate-Substituted R1.1 RNA

Species	Sequence ^a	R1.1 RNA ^b	Presence of Species ^c		Comigration of Species ^d			Phosphatase Reactivity ^g	32P-Labeled Products of Nuclease Digestion ^h			
			-RNase III	+RNase III	3MM(3.5)	DEAE(3.5)	DEAE(1.9)		T2	P1	Panc.	U2 ⁱ
1	CCUUUAU	Unmodified	+	-				+	Up, Cp	pA, P _i	Up, Cp	CCUUAp, UGp
		Phosphorothioate	+	-	+	(+) ^e	+	+	Up, Cp	pA, P _i	Up, Cp	CCUUUAUp, Cp
2	CCUUUAU _{on}	Unmodified	-	+				-	Up	pA	Up	CCUUUAUp
		Phosphorothioate	-	+	+	(+) ^e	+	-	Up	pA	Up	CCUUUAUp
3	pGp	Unmodified	-	+				+	ND	P _i	ND	ND
		Phosphorothioate	-	+	(+) ^f	+	+	+	ND	P _i	ND	ND

a. See Figure 1C for location of RNA species within the R1.1 RNA sequence.

Radiolabeled phosphates, or residues 5' to labeled phosphates are underlined.

b. Phosphorothioate-substituted R1.1 and control RNA's were synthesized as described in Materials and Methods. GTP[α S] was used as the phosphorothioate precursor, and [α -³²P]ATP was the radiolabel.

c. Determined by the two-dimensional fractionation and autoradiography of RNase T1-resistant oligonucleotides [29], which were derived from control or phosphorothioate-substituted R1.1 RNA incubated in the presence or absence of RNase III (see legend to Figure 2).

d. The chromatographic systems used were: 3MM(3.5), Whatman 3MM paper, pH 3.5 buffer; DEAE(3.5), Whatman DE81 paper, pH 3.5 buffer; DEAE(1.9), Whatman DE81 paper, pH 1.9 buffer [31,32].

e. Both the control and phosphorothioate-substituted RNA's remained at the origin of electrophoresis.

f. p_sGp exhibits a slightly slower mobility than pGp under these conditions.

g. Calf intestine alkaline phosphatase (Boehringer) was used, and the digestions were performed in 5 μ l of 10mM Tris-HCl (pH 7.5), 1 mM EDTA for 30 minutes at 30°C followed by 30 minutes at 37°C. Analysis of the reaction products was carried out using the chromatographic systems described in (d).

h. RNase T2 digestions were carried out and the products fractionated by high voltage electrophoresis on Whatman DE81 paper in pH 3.5 buffer. Nuclease P1 digestions were performed and the products subjected to high voltage electrophoresis on Whatman 3MM paper in pH 3.5 buffer [32]. Pancreatic ribonuclease digestions products were fractionated on Whatman 3MM paper in pH 3.5 buffer [31]. RNase U2 reactions were carried out and the products separated by high voltage electrophoresis on DE81 paper, using pH 1.9 or pH 3.5 buffer, or using Whatman 3MM paper and pH 3.5 buffer [31]. In all the analyses, the radiolabeled RNA products were located by autoradiography and identified by their characteristic mobilities [31, 32]. ND=not determined; P_i=inorganic phosphate; p_s indicates a phosphorothioate internucleotide linkage.

i. UpG and Up_sG have different mobilities on DEAE paper in pH 1.9 buffer. The alteration of chromatographic properties of RNA dinucleoside phosphates upon phosphorothioate substitution has been noted elsewhere [43].

identical products. This indicated that the phosphorothioate-substituted R1.1 RNA-derived species are identical in sequence with their unmodified counterparts. Third, parallel treatment of the phosphorothioate-substituted and

control RNase T1-resistant RNA species with calf alkaline phosphatase produced similar shifts in mobilities of RNA species 1 and 3, but caused no change in the mobility of species 2. This result demonstrates that the RNase T1-resistant species 1 and 3 contain a 3'-phosphate group, and that species 2 contains a 3'-hydroxyl group (see also below). From these results we conclude that RNase III can catalyze the hydrolysis of a phosphorothioate internucleotide bond at the R1.1 processing site. Furthermore, RNase III cleaves the phosphorothioate bond with 5' polarity, yielding RNA products with 5' phosphorothiomonoester and 3' hydroxyl termini.

A variety of phosphorothiomonoesters are resistant to phosphatase action [30]. This behavior is also exhibited by phosphorothiomonoesters at the 3' end of RNase T1-resistant oligonucleotides, since under conditions of full removal of 3' phosphomonoesters with calf alkaline phosphatase, there is little, if any, hydrolysis of 3'-phosphorothiomonoesters (unpublished experiments). Hence, it is conceivable that RNase III may be cleaving the phosphorothioate internucleotide bond with 3' polarity, and that the resistance of RNA species 2 to alkaline phosphatase action may reflect the presence of a 3' phosphorothiomonoester group, rather than a 3' hydroxyl group. This possibility can be ruled out, since 1) p₅Gp is a product of RNase III cleavage followed by RNase T1 digestion of phosphorothioate-substituted R1.1 RNA; and 2) RNA species 2 from both phosphorothioate-substituted R1.1 RNA and control R1.1 RNA comigrate in three different electrophoretic systems (Table I). This is in contrast to the different electrophoretic behavior exhibited by oligonucleotides which differ only by the presence or absence of a 3' phosphate group ([31], [32], unpublished experiments).

RNase III Cleavage of Phosphorothioate-Substituted T7 Polycistronic Early mRNA Precursor

The bacteriophage T7 polycistronic early mRNA precursor contains five primary RNase III processing signals, including the R1.1 site. The RNase III cleavage sites and surrounding sequences have all been directly characterized at the RNA level [25-27,33-37]. T7 polycistronic early mRNA precursor can be synthesized *in vitro* using purified T7 DNA and *E. coli* RNA polymerase [21]. The polymerase can utilize ribonucleoside 5'-O-(1-thiotriphosphates) (Sp configuration), producing RNA chains which correctly initiate at one of the three major rightward promoters, and also contain phosphorothioate internucleotide bonds in the Rp configuration [38-40]. Direct comparison of the RNase III cleavage products of the phosphorothioate-substituted transcript with the control RNA cleavage products would indicate whether RNase III can accurately

Figure 3. Gel electrophoretic analysis of phosphorothioate-substituted T7 polycistronic early mRNA precursor and its RNase III cleavage products. The T7 mRNA precursor was synthesized *in vitro* as described in Materials and Methods, using [α - ^{32} P]UTP as the radiolabel. **A:** Synthesis of T7 RNA. Aliquots (4×10^3 dpm) of the transcription reactions were analyzed on a 2% polyacrylamide gel. Lanes (b) and (c) display control T7 RNA synthesized for 15 minutes (lane b) or 45 minutes (lane c). The prominent band in lane (b) is the 7090 nucleotide-long polycistronic early mRNA precursor. Lanes (d) and (e) display T7 RNA synthesized for 15 minutes (lane d) or 45 minutes (lane e) in the presence of ATP[α S] (0.6 mM). Lanes (f) and (g) display T7 RNA synthesized for 15 minutes (lane f) or 45 minutes (lane g) in the presence of GTP[α S] (0.6 mM). Lane (a) contains RNase III-cleaved control T7 RNA; the sizes of the RNA species are (from top to bottom): 2749, 1670, 1140, 578/561/532, and 392 (faint) nucleotides.

B. Cleavage of T7 RNA by RNase III. Control T7 RNA [lanes (a) and (d)] and T7 RNA synthesized in the presence of 2.4 mM ATP[α S] [lanes (b) and (e)] or in the presence of 2.4 mM GTP[α S] [lanes (c) and (f)] were analyzed in a 3% polyacrylamide gel. RNase III digestions [lanes (d), (e) and (f)] were carried out in 10 μ l volumes for 30 minutes at 37°C, as described in the legend to Figure 2. The arrowhead marks the position of the bromophenol blue dye.

C. A schematic of the primary structure of the T7 polycistronic early mRNA precursor [15,18]. I₁, I₂ and I₃ denote the three RNA leader sequences;

0.3, 0.7, 1.0, 1.1 and 1.3 refer to the individual mRNAs. Not all genes are denoted; missing are the 0.4, 0.5, 0.6, 0.65 and 1.2 cistrons. See Figure 3B for the order of migration of these species in a 3% polyacrylamide gel. The arrows below the lines mark the RNase III primary cleavage signals (R0.3, R0.5, R1.0, R1.3). The length scale refers to the size of the longest of the three transcripts, which initiates at the A1 promoter [18].

process the primary signals in the phosphorothioate-substituted polycistronic mRNA.

T7 genomic DNA was transcribed *in vitro* using *E. coli* RNA polymerase as described in Material and Methods, and the RNA transcription products were purified and analyzed by gel electrophoresis. Figure 3A displays the RNA products resolved in a 2% polyacrylamide gel. The control T7 polycistronic early mRNA precursor is synthesized as a discrete product under these conditions (Figure 3A, lane b). RNA products somewhat smaller in length are synthesized when ATP[α S],Sp isomer (Figure 3A, lane d) or GTP[α S],Sp isomer (Figure 3A, lane f) are substituted for ATP and GTP, respectively. The phosphorothioate-substituted RNAs are heterogeneous in size, and have an average chain length of approximately 4000 nucleotides, as indicated by their gel electrophoretic mobilities. Increasing the transcription reaction time from 15 to 45 minutes produced an increase in the average size of T7 RNA synthesized in the presence of the ribonucleoside 5'-O-(1-thiotriphosphates) (Figure 3A, compare lanes d and e, and f and g). The increased transcription time, however, did not significantly alter the size of the control T7 RNA (compare lanes b and c, Figure 3A). The two-dimensional fractionation of RNase T1-resistant oligonucleotides derived from the phosphorothioate-substituted T7 RNAs yields patterns essentially the same as that of control T7 RNA (data not shown). We conclude that the normal path of transcription of the early region of bacteriophage T7 DNA by *E. coli* RNA polymerase is not altered in the presence of ribonucleoside 5'-O-(1-thiotriphosphates), except for the production of a shorter average transcript length.

T7 RNA synthesized using ATP[α S] or GTP[α S] was incubated with RNase III, and the reaction products were analyzed by gel electrophoresis. The phosphorothioate-substituted RNAs are substrates for RNase III (compare lanes b and e, and lanes c and f, Figure 3B). The RNase III cleavage products have electrophoretic mobilities similar to the products of RNase III digestion of control T7 polycistronic early mRNA precursor (compare lanes e and f with lane d, Figure 3B). The sizes and identities of the RNase III cleavage products of

control T7 RNA which correspond in gel electrophoretic mobility to the products of phosphorothioate-substituted T7 RNA are (from the bottom to the top of the gel in Figure 3B, lane 4): 140, 264 and 392 nucleotides (the three leader RNAs I₃, I₂, I₁, cleaved from the primary transcript at the R0.3 site), 578 nucleotides ("0.3 mRNA," produced by RNase III cleavage at the R0.3 and R0.5 sites), and 1670 nucleotides ("0.7 mRNA", produced by cleavage at the R0.5 and R1.0 sites). The phosphorothioate-substituted T7 RNA products of RNase III reaction contain significantly lower levels of the RNA species corresponding to the 532 nucleotide RNA ("1.1 mRNA", resulting from cleavage at the R1.1 site and double cleavage [15,33] at the R1.3 site), the 1140 nucleotide RNA ("1.3 mRNA," resulting from cleavage at the R1.3 site), and the 2749 nucleotide RNA ("1.0 mRNA," resulting from cleavage at the R1.0 and R1.1 sites).

The presence of phosphorothioate-substituted RNA species corresponding in size to the three leader RNAs, the 0.3 mRNA and the 07 mRNA indicates that RNase III is correctly recognizing and cleaving within the R0.3, R0.5 and R1.0 processing signals. The low amounts of phosphorothioate-substituted RNA species corresponding to the 1.0 mRNA, 1.1 mRNA and 1.3 mRNA are in accord with the result (see above) that the phosphorothioate-substituted T7 primary transcript terminates on average between the R1.0 and R1.1 sites.

DISCUSSION

This report has described the in vitro synthesis and purification of phage T7-specific RNA transcripts containing phosphorothioate internucleotide linkages. T7 RNA polymerase can accept GTP[α S], Sp isomer as a substrate for RNA synthesis in vitro, producing levels of synthesis of R1.1 RNA comparable to that obtained with GTP. Using T7 DNA as a template, E. coli RNA polymerase synthesizes phosphorothioate-substituted RNA chains that are significantly shorter than the regular polycistronic mRNA precursor. The average size (approximately 4000 nucleotides) of the phosphorothioate-substituted RNA indicates that the RNA chains terminate between the R1.0 and R1.1 RNase III processing signals. This result probably reflects the less favorable substrate properties of the ribonucleoside 5'-O-(1-thiotriphosphates) [38-40], which could induce premature pausing and/or fall-off of the E. coli RNA polymerase from the T7 DNA template.

There is evidence of a specific early termination event during the in vitro transcription of T7 genomic DNA in the presence of ribonucleoside 5'-O-(1-thiotriphosphates) (unpublished experiments). The early termination site

appears to be at, or very close to, the R0.3 RNase III site, since the three RNA species produced by this event comigrate with the three leader RNAs generated by RNase III cleavage of the polycistronic mRNA at the R0.3 site. In light of this result, it is notable that the secondary structure models of several T7 primary RNase III processing signals are similar to the proposed secondary structure of a transcription terminator [41], and that in bacteriophage lambda, RNase III processing and transcription termination are signalled by the same RNA element [42].

This report represents the first direct demonstration of the accurate cell-free processing of specific phosphorothioate-substituted RNA substrates by a RNA processing enzyme. We have shown that RNase III cleavage specificity and accuracy are unperturbed by phosphorothioate internucleotide bonds either at or near a primary processing site. In addition to catalyzing the accurate hydrolysis of the $\text{Up}_\text{s}\text{G}$ scissile bond within the R1.1 processing signal, RNase III can also cleave the $\text{Up}_\text{s}\text{G}$ bond in the T7 R0.3 and R0.5 signals, as well as the $\text{Up}_\text{s}\text{A}$ bond in the R1.0 signal. The effect of phosphorothioate groups on the rate of RNase III cleavage is under current investigation. Preliminary results suggest that phosphorothiodiester linkages in a T7 processing signal cause a distinct reduction in RNase III cleavage rate.

Little is currently known about the influence of phosphorothioate diester linkages on the chemical and structural properties of RNA. This study has shown that phosphorothioate-substituted RNA molecules are stable in Mg^{2+} -containing aqueous buffers at near neutral pH. Phosphorothioate-substituted RNA is thus a good prospect for the study of RNA structure and reactivity under biologically relevant conditions. The substitution of sulfur at a non-bridging oxygen does not alter the total negative charge (-1) of a phosphodiester bond, and causes only a moderate increase in the van der Waal's radius and bond length within this nucleic acid moiety [30,43]. It is likely that these similarities in molecular properties are the reason that phosphorothioate substitution has been successfully used in probing the stereochemistry of nuclease-catalyzed reactions. Nonetheless, the differing molecular structures of the phosphodiester and phosphorothiodiester linkages can also reveal subtle mechanistic features of reactions in which RNA molecules participate.

This investigation has also demonstrated the feasibility of determining the stereochemical course of hydrolysis catalyzed by RNase III. One experimental approach would involve the large-scale enzymatic synthesis [44,45] of a phosphorothioate-containing minimal RNase III substrate, followed by RNase

III cleavage in H_2^{18}O and analysis of products by NMR spectroscopy [10]. These studies and others on phosphorothioate-containing RNA will provide essential information on the molecular mechanisms of the biochemical reactions in which RNA molecules participate.

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*Present address: Department of Cell Biology, Stanford University, Palo Alto, CA 94305, USA

+Present address: Pharmacia Inc., 800 Centennial Ave., Piscataway, NJ 08854, USA

#All RNA sequences are written 5' to 3', and contain a 3' phosphate unless otherwise indicated.

REFERENCES

1. Abelson, J. (1979) *Ann. Rev. Biochem.* **48**, 1035-1069.
2. Altman, S., Guerrier-Takada, C., Frankfort, H.M. and Robertson, H.D. (1982) in "Nucleases" (S.J. Linn and R. J. Roberts, eds) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. pp 243-274.
3. Cech, T.R. and Bass, B.L. (1986) *Ann. Rev. Biochem.* **55**, 599-629.
4. King, T.C., Sirdeskumkh, R. and Schlessinger, D. (1986) *Microbiol. Rev.* **50**, 428-451.
5. Sharp, P.A. (1987) *Science (USA)* **235**, 766-771.
6. Cech, T.R. (1987) *Science (USA)* **236**, 1532-1539.
7. Knowles, J.R. (1980) *Ann. Rev. Biochem.* **49**, 877-919.
8. Eckstein, F. (1983) *Angew. Chem. Intl. Ed.* **22**, 423-439.
9. Eckstein, F. (1985) *Ann. Rev. Biochem.* **54**, 367-402.
10. Connolly, B.A., Eckstein, F. and Pingoud, A. (1984) *J. Biol. Chem.* **259**, 10760-10763.
11. Dunn, J.J. (1982) in "The Enzymes", vol. 15 (P. Boyer, ed) Academic Press, New York pp. 485-499.
12. Robertson, H.D. (1982) *Cell* **30**, 669-672.
13. Robertson, H.D. and Barany, F. (1978) in *Proceedings of the 12th FEBS Congress*, Pergamon Press, pp. 285-295.
14. Robertson, H.D. (1977) in "Nucleic Acid-Protein Recognition" (H. Vogel, ed) Academic Press, New York pp. 549-568.
15. Dunn, J.J. (1976) *J. Biol. Chem.* **251**, 3807-3814.
16. Dunn, J.J. and Studier, F.W. (1975) *J. Mol. Biol.* **99**, 487-499.
17. Watson, N. and Apirion, D. (1985) *Proc. Nat. Acad. Sci. USA* **82**, 849-853.
18. Dunn, J.J. and Studier, F.W. (1983) *J. Mol. Biol.* **166**, 477-535.
19. Carter, A.D., Morris, C.E. and McAllister, W.T. (1981) *J. Virol.* **37**, 636-642.
20. Franklin, R.M. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 1504-1511.

21. Dunn, J.J. and Studier, F.W. (1973) *Proc. Nat. Acad. Sci. USA* 70, 1559-1563.
22. Studier, F.W. (1973) *J. Mol. Biol.* 79, 227-236.
23. Palese, P. and Schulman, J.L. (1976) *J. Virol.* 17, 876-884.
24. Sanger, F. and Coulson, A.R. (1978) *FEBS Lett.* 87, 107-110.
25. Oakley, J.L. and Coleman, J.E. (1977) *Proc. Nat. Acad. Sci. (USA)* 4266-4270.
26. Kramer, R.A., Rosenberg, M. and Steitz, J.A. (1977) *J. Mol. Biol.* 89, 767-776.
27. Rosenberg, M., Kramer, R.A. and Steitz, J.A. (1977) *J. Mol. Biol.* 89, 777-782.
28. Griffiths, A.D., Potter, B.V.L. and Eperon, I.C. (1987) *Nucleic Acids Res.* 15, 4145-4162.
29. Nicholson, A.W., Frankfort, H.M., Davis, N.G., Ferrari, S., Lamb, R.A. and Robertson, H.D. (1986) *Biochim. Biophys. Acta* 868, 153-163.
30. Eckstein, F. (1975) *Angew. Chem. Intl. Ed.* 14, 160-166.
31. Brownlee, G.G. (1972) "Determination of Sequences in RNA" (Work, T.S. and Work, E., eds) North Holland/American Elsevier Publishers, New York.
32. Barrell, B.G. (1971) in *Procedures in Nucleic Acids Research* (Cantoni, G.L. and Davies, D.R., eds) Vol. 2. Harper and Row, New York, pp. 751-771.
33. Robertson, H.D., Dickson, E. and Dunn, J.J. (1977) *Proc. Nat. Acad. Sci. (USA)* 74, 822-826.
34. Rosenberg, A.H., Simon, M.N., Studier, F.W. and Roberts, R.J. (1979) *J. Mol. Biol.* 135, 907-915.
35. Rosenberg, M. and Kramer, R.A. (1977) *Proc. Nat. Acad. Sci. USA* 74, 984-988.
36. McConnell, D.J. (1979) *Nucleic Acids Res.* 6, 3491-3503.
37. Studier, F.W., Dunn, J.J. and Buzash-Pollert, E. (1979) in "From Gene to Protein: Information Transfer in Normal and Abnormal Cells." (T.R. Russell, K. Brew, H. Faber and T. Schultz, eds) Academic Press, New York pp. 261-269.
38. Eckstein, F., Armstrong, V.W. and Sternbach, H. (1976) *Proc. Nat. Acad. Sci. USA* 73, 2987-2990.
39. Burgers, P.M.J. and Eckstein, F. (1979) *Proc. Nat. Acad. Sci. USA* 75, 4798-4800.
40. Yee, D., Armstrong, V.W. and Eckstein, F. (1979) *Biochem.* 18, 4116-4120.
41. Platt, T. (1986) *Ann. Rev. Biochem.* 55, 339-372.
42. Montanez, C., Bueno, J., Schmeissner, U., Court, D.L. and Guarneros, G. (1986) *J. Mol. Biol.* 191, 29-37.
43. Frey, P.A. and Sammons, R.D. (1985) *Science (USA)* 228, 541-545.
44. Lowary, P., Sampson, J., Milligan, J., Groebe, D. and Uhlenbeck, O.C. (1986) in "Structure and Dynamics of RNA" (P.H. van Knippenberg and C.W. Hilbers, eds) Plenum Press, New York, pp. 69-76.
45. Milligan, J.F., Groebe, D.R., Witherell, G.W. and Uhlenbeck, O.C. (1987) *Nucleic Acids Res.* 15, 8783-8798.