
Stereospecificity of nucleases towards phosphorothioate-substituted RNA: stereochemistry of transcription by T7 RNA polymerase

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ABSTRACT

Transcription by T7 RNA polymerase has been studied using a chiral ATP analogue. The Sp diastereoisomer of adenosine 5'-O-(1-thiotriphosphate) (ATP_αS) was incorporated into RNA with an apparent K_M of approximately 15 μM, similar to that for ATP; the Rp diastereoisomer was neither a substrate nor a competitive inhibitor. The configuration of the phosphodiester link in the RNA produced was analyzed with stereospecific nucleases. The rate of nuclease digestion was compared with the rate of digestion of phosphorothioate-substituted RNA of known stereochemistry synthesized by *E.coli* RNA polymerase. Surprisingly, the nucleases exhibited reduced discrimination compared with their activity on dinucleotides. The results show that phosphorothioate-substituted RNA transcribed by T7 RNA polymerase has the same configuration as that transcribed by *E.coli* RNA polymerase, ie. Rp. Thus, the reaction proceeds with inversion of configuration at phosphorus.

INTRODUCTION

Chiral analogues of substrate molecules are valuable tools in studying enzyme mechanisms. If the chiral centre is the site of nucleophilic substitution, then the stereochemical course of the reaction can be determined. Reactions involving phosphoryl or nucleotidyl transfer can be analysed using chiral phosphates (1-4). Chiral phosphodiester and anhydrides can be produced by replacing one of the non-bridging oxygen atoms with either an isotopic oxygen atom or sulphur; in the latter case, a phosphorothioate is formed. These compounds have been used widely to determine the number of elementary steps in enzyme-catalyzed reactions: inversion of configuration results from a single nucleophilic substitution, and retention from a double-displacement reaction, usually indicating the existence of a covalent enzyme intermediate (3,4). Although other reaction courses could, in principle, give rise to either outcome, there is no supporting evidence for these in biological systems. Furthermore, the reaction mechanisms determined for phosphorothioate analogues have been

found to agree with those of phosphates in those cases where analogues containing both types of chiral linkage have been tested (reviewed in refs. 3 and 4). Chiral nucleoside phosphorothioates have the further advantage that, whereas one diastereoisomer can be substituted for substrate, the other is frequently found to act as a competitive inhibitor. Thus, stable enzyme-substrate analogue complexes can be formed, or intermediates revealed.

For these reasons, deoxynucleoside α -thiotriphosphates have been used to determine the stereochemical course of reaction of DNA polymerases (5-9) and internucleotidic phosphorothioates have been used to analyse the mechanisms of reaction of nucleases and a site-specific restriction endonuclease (10-13). In contrast, very little is known of the mechanisms of sequence-specific reactions involving RNA. In order to use phosphorothioate-substituted DNA or RNA the stereochemical course of incorporation of dNMP α S or NMP α S must be known. So far all DNA polymerases studied (5-9) and *E.coli* RNA polymerase (14) are known to use Sp diastereoisomers of the substrate analogue and proceed with inversion of configuration. However, most RNAs transcribed *in vitro* are produced using the RNA polymerases of bacteriophages T7 or SP6 (15). Thus, we have sought to determine the stereochemical course of transcription by T7 RNA polymerase, both to see if it follows the general pattern of polymerases and to characterize the RNA produced as a substrate for RNA processing enzymes. In this study, we have used the two diastereoisomers of the substrate ATP α S and nucleases of known stereospecificity (previously characterized with respect to small molecules) to characterize both the process of transcription and the stereochemical discrimination of the nucleases.

MATERIALS AND METHODS

Synthesis of ATP α S and Purification of Diastereoisomers

The synthesis was performed according to published procedures (16). Purification by HPLC was performed on a Shimadzu LC-4A system with an Apex 5 μ ODS RP column using as eluant 50 mM triethylammonium bicarbonate pH 6.45 containing 2% acetonitrile. ^{31}P NMR spectroscopy was performed with broad band proton decoupling on a Bruker AM300 spectrometer operating at 121.5 MHz.

Transcription

Preparative transcriptions by T7 RNA polymerase (Boehringer) were

performed in 5 μ l volumes comprising 34 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1.7 mM spermidine, 8.8 mM dithiothreitol (DTT), 8 units RNasin (P & S Biochemicals Ltd.), 0.44 mM unlabelled NTPs or NTP α S, for 1 hour at 37°C with 20 units T7 RNA polymerase (Boehringer) and approximately 0.5 μ g template DNA. To prepare ³²P-labelled RNA, the mix was supplemented with 20 μ Ci [α -³²P] CTP at 800 Ci/mmol (Amersham); to prepare ³⁵S-labelled RNA, unlabelled UTP was replaced with 10 μ Ci [α -³⁵S] UTP at 400 Ci/mmol (Amersham). In all cases, the template DNA was a HindIII-cut recombinant of mICE10 (17) containing an insert of eukaryotic DNA, giving rise to a transcript of 470 nucleotides. After transcription, the reaction mixtures were extracted with phenol and ether, and spun through a column of Sephadex G50 to remove nucleoside triphosphates. After precipitation of the RNA with ethanol and solution in water, incorporation of radiolabelled nucleotides was measured by scintillation counting. For kinetic measurements of transcription, 2 μ l reactions of the same composition were used, with [α -³²P]CTP in all cases. The template DNA was mICE11 (17) with an insert of eukaryotic DNA which, after cleavage with EcoRI, gave rise to a transcript of 830 nucleotides. The concentration of one nucleotide was varied as shown in the results. The reaction was initiated by addition of unlabelled nucleotides to a mix of all the other components, 2 μ l volumes were dispensed into tubes, and these were placed at 37°C for the times noted. Reactions were quenched in dry ice and followed by TCA precipitation and scintillation counting. Assuming the specific activity of the enzyme supplied was 600,000 units per mg (18), these assays used about 0.1 pmole enzyme and 0.04 pmole template.

Preparative transcriptions by E.coli RNA polymerase (Pharmacia) were performed in 50 μ l 32 mM Tris-HCl pH 7.5, 120 mM KCl, 8 mM MgCl₂, 0.08 mM EDTA, 0.08 mM DTT, 0.2 mM unlabelled NTPs with 8 pmole E.coli RNA polymerase (Pharmacia) and 0.5 μ g uncut replicative form DNA comprising an insert of 808 base-pairs of eukaryotic DNA in mICE 11. 80 μ Ci [α -³²P] CTP or 40 μ Ci [α -³⁵S] UTP were used as supplements or replacements respectively, as noted above. Incorporation was assessed as described for T7 RNA polymerase. CTP α S (an epimeric mixture), GTP α S and UTP α S (both enriched in Sp isomer) were gifts from Prof. F. Eckstein, Göttingen.

DNA synthesis

Labelled DNA was prepared by primed synthesis with E.coli DNA polymerase I, large subfragment, from universal primer annealed to the single-stranded DNA form of the mICE11 recombinant described above.

^{32}P -labelled DNA was synthesized in the presence of all four dNTPs at $30\ \mu\text{M}$ with $10\ \mu\text{Ci}$ [α - ^{32}P] dATP ($3000\ \text{Ci}/\text{mmole}$) in $32\ \mu\text{l}$; ^{35}S -labelled DNA was synthesized in the presence of dCTP αS , dGTP αS and dTTP αS (all at $30\ \mu\text{M}$) and $40\ \mu\text{Ci}$ [α - ^{35}S] dATP ($1000\ \text{Ci}/\text{mmole}$), in $32\ \mu\text{l}$. Reaction products were purified as described for transcriptions.

Nuclease Digestions

Serial dilutions of each enzyme were performed in reaction buffer : snake venom phosphodiesterase (SVPD) from Crotalus durissus terrificus (Boehringer) in $100\ \text{mM}$ Tris-HCl pH 8.75, $2\ \text{mM}$ MgCl_2 , $2\ \text{mM}$ DTT; nuclease S1 (Pharmacia) in $0.28\ \text{M}$ NaCl, $50\ \text{mM}$ sodium acetate pH 4.8, $4.5\ \text{mM}$ ZnSO_4 ; nuclease P1 (Pharmacia) in $30\ \text{mM}$ sodium acetate pH 4.8, $0.1\ \text{mM}$ ZnSO_4 . The volumes of the products of transcription by E.coli and T7 RNA polymerases were adjusted to give equal counts per unit volume when digestions were performed to compare the two. $1\ \mu\text{l}$ of the purified preparative transcription products were added to $5\ \mu\text{l}$ volumes of each concentration of nuclease; when ^{32}P and ^{35}S -labelled materials were digested together, total reaction volumes were $10\ \mu\text{l}$. When digestion of ^{35}S -labelled DNA was performed in parallel with that of mixed ^{32}P and ^{35}S -labelled DNA (Figure 6), this too was performed in $10\ \mu\text{l}$. Digestions were incubated for 30 minutes at 37°C and quenched in dry ice. The results were analysed by TCA precipitation or thin-layer chromatography on polyethyleneimine (P.E.I.) plates (19). In the latter case, $1\ \mu\text{l}$ of each digest was mixed with $2\ \mu\text{g}$ of E.coli tRNA^{Phe} that had been digested to completion with nuclease P1.

RESULTS

Synthesis of Diastereoisomers of ATP αS

The epimeric mixture of ATP αS was examined by ^{31}P nmr spectroscopy. The α -thiophosphate signal showed three peaks, representing overlap of two doublets: each diastereoisomer gives rise to a doublet. One of the diastereoisomers was seen to be approximately twice as abundant as the other; on the basis of the diastereoisomer ratio from a reported synthesis (14) and chemical shifts (20), this was assigned to the Rp form. After separation of the two diastereoisomers using reverse phase HPLC (Figure 1), the products were checked by a repeated HPLC separation. No cross-contamination of the two diastereoisomers was observed, indicating that less than 5% of the Rp-ATP αS isolate might represent Sp-ATP αS and that less than 10% of the Sp-ATP αS fraction might comprise Rp-ATP αS .

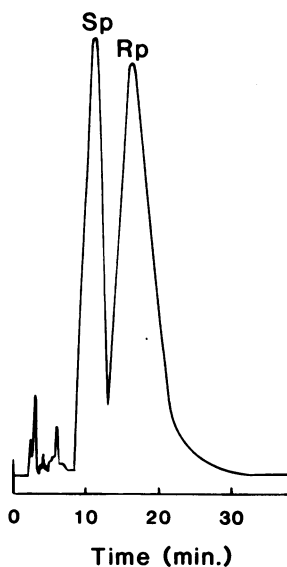


Figure 1. Separation of the diastereoisomers of ATP α S using reverse phase HPLC.

Substrate Preference and Transcription Kinetics

Each diastereoisomer was tested as a substrate for T7 RNA polymerase in a preliminary reaction. We observed that Sp-ATP α S appears to act as a substrate whereas Rp-ATP α S at a much higher concentration does not seem to increase the rate of transcription above the background level seen in the absence of ATP (data not shown).

A series of determinations of the apparent K_M (K_{app}) was undertaken, each determination relying on single values for each of the time points used for estimation of the initial rate at each substrate concentration (see Discussion). Sample results are shown in Figure 2 and all are summarised in Table 1. The apparent value for the K_M determined with Sp-ATP α S is approximately 15 μ M, which is quite similar to the value with ATP. Although the significance of the kinetic constant may be doubtful (see Discussion), it represents a practical guide in assessing the requirements for optimal transcription efficiency.

It was not possible to determine reproducible values for the maximum velocity. However, duplicate rates were measured with ATP or ATP α S at identical concentrations (about six times K_{app}) in the course of a single

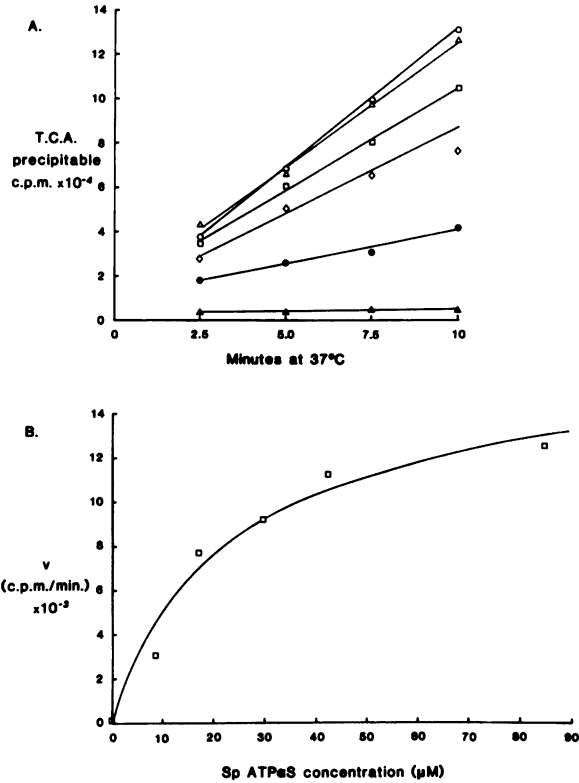


Figure 2. Determination of K_{app} for T7 RNA polymerase with Sp-ATP α S. One of the experiments summarised in Table 1 is shown. Initial rates were calculated from the upper graph at the following ATP α S concentrations: Δ 0 μ M; \circ , 8.5 μ M; \square , 17 μ M; \bullet , 30 μ M; \triangle , 42 μ M; \circ , 85 μ M. In all cases the incorporation of ^{32}P from [α - ^{32}P]CTP was followed. The slopes were calculated by linear regression. The lower graph shows the initial rate plotted against Sp-ATP α S concentration, with a rectangular hyperbola fitted by an iterative least squares procedure (27).

Table 1. Results of experiments to determine K_{app} .

Substrate	K_{app} values (μ M)	Mean (μ M)
ATP	16.5 , 29	23
Sp-ATP α S	23 , 12 , 10 , 16	15

Table 2. Comparisons of initial rates of transcription with ATP or ATP α S at 85 μ M.

<u>Experiment</u>	<u>Substrate</u>	<u>Initial Rates</u> (c.p.m./min)	<u>Means</u> (c.p.m./min)
1	ATP	931 , 1092 , 999	1007
1	Sp-ATP α S	959 , 1431	1195
2	ATP	2127 , 2153 , 1727	2002
2	Sp-ATP α S	2041 , 1378	1710

experiment. Table 2 shows that on the two occasions when the measurements were performed there did not appear to be a substantial difference between the two substrates in the rate of reaction.

The poor transcription observed with Rp-ATP α S may be attributed to weak binding or to interference with the chemical reaction, ie. a destabilised transition state. In order to test these possibilities, we measured initial rates with Sp-ATP α S at a concentration approximately corresponding to the K_{app} value in the presence or absence of the highest practicable concentration of Rp-ATP α S (Table 3). As with the previous comparisons of rates, the use of single substrate concentrations allowed duplicate initial rates to be determined for each condition. No inhibition was detected, suggesting that Rp-ATP α S was not binding at a significant level.

Configurational Analysis of Transcripts

Three nucleases have been shown to discriminate strongly between the two diastereoisomers of phosphorothioate linkages in dinucleotides and between these linkages and normal phosphodiester. Snake venom phospho-

Table 3. Initial rates of transcription with 16.9 μ M Sp-ATP α S in the presence or absence of 597 μ M Rp-ATP α S.

<u>Experiment</u>	<u>Initial Rates</u> (c.p.m./min)	<u>Means</u> (c.p.m./min)
+ Rp-ATP α S	977 , 1277 , 1105	1120
- Rp-ATP α S	1082 , 1048	1065

diesterase (SVPD) has been shown to prefer Rp- to Sp-thiophosphodiester linkages and to act most rapidly on phosphodiester linkages (10,11); nuclease S1 cleaves Sp linkages at a rate similar to phosphodiester linkages (12) and, as with nuclease P1 (21), it has not been possible to measure cleavage of Rp-linkages.

In order to use these nucleases for configurational assignments in RNA, it would be desirable to characterize the rates of digestion of RNA containing only Sp- or Rp-thiophosphodiester internucleotidic linkages (Sp- or Rp- [S]RNA). However, only Rp-[S]RNA can be made with RNA polymerases characterized to date. Nonetheless, stereospecificity could be inferred if there were consistent differences in rates of digestion between RNA with and without thiophosphodiester linkages: Rp-[S]RNA would be expected to be a comparatively poor substrate for nuclease S1 and to be digested by SVPD, whereas Sp-[S]RNA would be expected to be an extremely poor substrate for SVPD but to be almost comparable with normal RNA as a substrate for nuclease S1.

The two substrates used were synthesized by T7 RNA polymerase on the same template, giving rise to transcripts with major products of the same length and similar distributions of size for the minor products, estimated by polyacrylamide gel electrophoresis. One substrate contained only normal phosphodiester linkages and [³²P]C whereas the other was expected to contain only thiophosphodiester linkages and [³⁵S]U. The two classes of transcript were mixed in a molar ratio of approximately 25:1 and aliquots digested for a fixed time with a range of concentrations of each enzyme. Our assumption was that if no discrimination took place then, regardless of the ratio of mixed substrates, a single curve should be produced; alternatively, a high degree of discrimination would result in rapid digestion of the [³²P]RNA, and the digestion profiles of each substrate would be almost unaltered by the presence of the other substrate.

Figure 3 shows that there is comparatively little difference between the SVPD digestion profiles of the two substrates, the [³⁵S]-labelled RNA being digested slightly more rapidly than the [³²P]-labelled RNA, whereas nucleases P1 and S1 act much more slowly on thiosubstituted RNA. The digestion by nuclease P1 of these thiophosphodiester linkages is so slow that the presence of thiosubstituted RNA does not affect the activity on normal RNA, and *vice-versa*; in separate experiments, the curves from digestion of similar initial levels of the two RNAs, digested individually, were found to be almost perfectly superimposable on those shown in figure

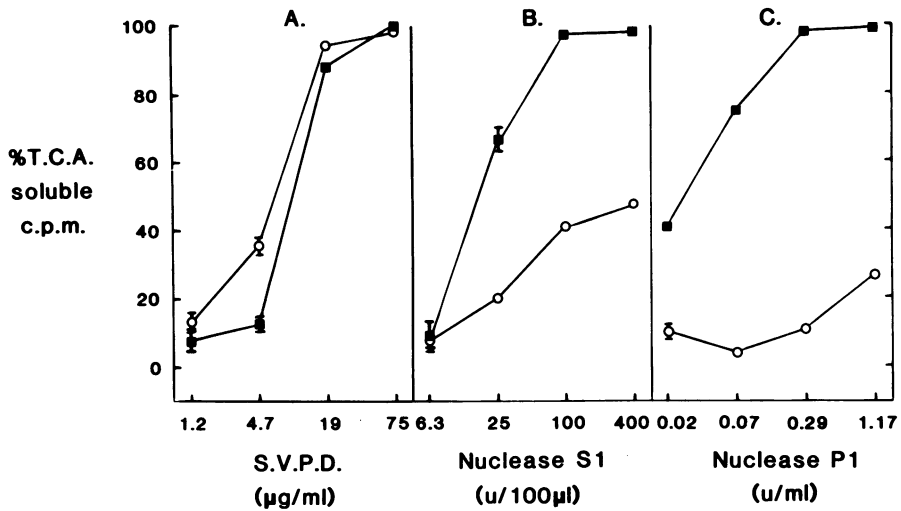


Figure 3. Experiments to determine whether nucleases discriminate between RNA and [S]RNA. Transcripts of 830 nucleotides were labelled with ^{32}P (■) or, if all internucleotidic linkages were phosphorothioate-substituted, with ^{35}S (○). Mixed transcripts were digested at the enzyme concentrations shown. The proportion of labelled material rendered soluble in T.C.A. at each enzyme concentration was determined in triplicate; for each value the mean is plotted together with the standard error of the mean (where error bars are not shown, the dimensions of the point exceed those of the error bar). Enzyme concentrations were plotted on a logarithmic scale.

3c. Thus, it is very probable that the nucleases retain stereospecific preferences with long RNA molecules and that the T7 [S]RNA has an Rp configuration.

This conclusion was reinforced by comparing digestion of T7 [S]RNA with [S]RNA known to be of Rp configuration, ie. synthesized by E.coli RNA polymerase. Transcripts were synthesized by T7 and E.coli RNA polymerases in the presence of ATP α S, CTP α S, GTP α S and [α - ^{35}S]UTP. Approximately equal molar yields, in terms of nucleotides incorporated, were incubated under standard reaction conditions with a series of dilutions of each enzyme. Undigested material was measured by TCA precipitation. The results shown in figure 4 demonstrate that both RNAs are almost equally susceptible to digestion by SVPD and nuclease S1, even though there is more double-stranded template DNA present in the samples transcribed by E.coli RNA polymerase. However, nuclease P1 digests E.coli RNA polymerase transcripts more readily than those produced by T7 RNA polymerase. It

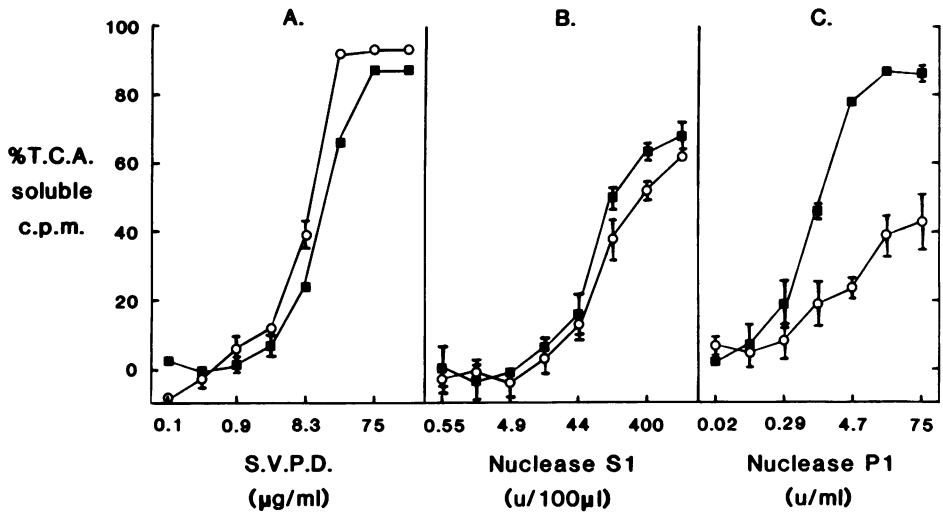


Figure 4. Comparisons of nuclease susceptibility of [S]RNA transcribed by T7 RNA polymerase (○) with that of Rp-[S]RNA transcribed by *E. coli* RNA polymerase (■).

Both transcripts were labelled with ³⁵S, and were digested in separate reactions. The means of determinations in triplicate are shown together with their standard error.

should be noted that, on the basis of studies with dinucleotides (21), the *E. coli* Rp-[S]RNA should be resistant to nuclease P1; if the T7 transcripts contained linkages of the Sp configuration they should be digested more readily. Therefore, the difference in rates of digestion cannot be attributed to the effects of thiophosphodiester configuration; instead, it was anticipated that the enzyme might be sensitive to differences in length or secondary structure of the transcripts. Both these aspects will differ substantially: the products of transcription of a cloned eukaryotic gene by T7 RNA polymerase have a sharply defined length, whereas those of M13 DNA by *E. coli* RNA polymerase are heterogeneous and shorter (data not shown). However, the control experiment in figure 5a shows that the difference in the rates of digestion by nuclease P1 is confined to thio-substituted RNA.

The analysis of TCA-precipitable material does not define the extent of digestion very precisely. Oligonucleotides as well as mononucleotides will escape precipitation. This allows the possibility that NTPs, if they were present as a contaminant in the stocks of NTPαS, will have been incorporated during transcription and will be digested preferentially by the

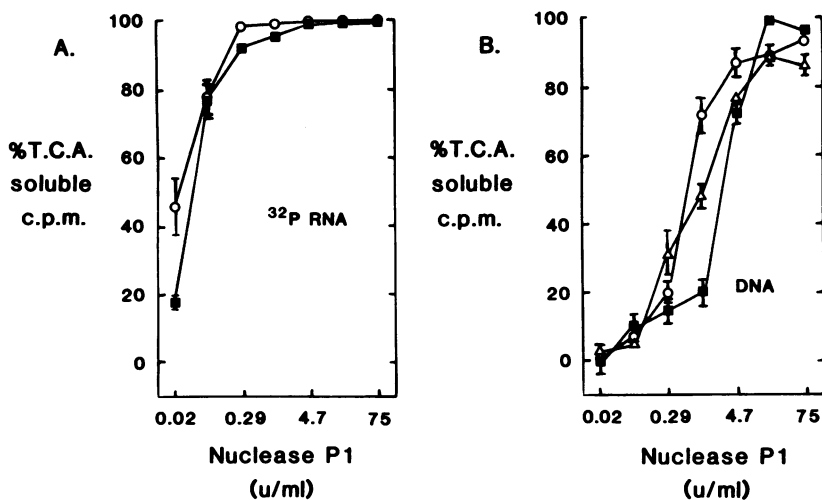


Figure 5. Investigations of nuclease P1 activity.

A. Comparison of nuclease P1 activities on RNA transcribed by T7 and *E. coli* RNA polymerases. ^{32}P -labelled RNA, lacking internucleotide phosphorothioates, was transcribed by T7 RNA polymerase (O) and *E. coli* RNA polymerase (■); after digestion, the results were plotted as described in the legend to Figure 4.

B. Experiments to determine whether nuclease P1 discriminates between DNA and [S]DNA. DNA synthesis products were labelled with ^{32}P (■) or, when all internucleotide linkages were phosphorothioate-substituted, with ^{35}S (O, Δ). Digestions were performed with a mixture of the two substrates (■, O) or with one (Δ).

stereospecific nucleases. With a sufficiently high level of contamination this would undermine our interpretation of the results with SVPD and nuclease S1 in Figures 3 and 4. In order to prove that thiophosphodiester linkages are being cleaved, we carried out the experiment shown in Figure 6. Transcripts were prepared using [α - ^{35}S]UTP as the only source of thiophosphodiester linkages. These were digested by the same three nucleases, and the products were distinguished by thin-layer chromatography. Only the results from nuclease P1 are shown; at high concentrations of nuclease S1 and SVPD the lanes became very smeary. The appearance of [^{35}S]UMP confirmed that the thiophosphodiester linkages were being hydrolyzed in the case of all three enzymes.

The rate of appearance of [^{35}S]UMP from both transcripts was identical with nuclease P1. Since we did not observe the discrepancy seen with TCA precipitation, we carried out the same experiment with transcripts which contained only thiophosphodiester linkages. The results (Figure 6)

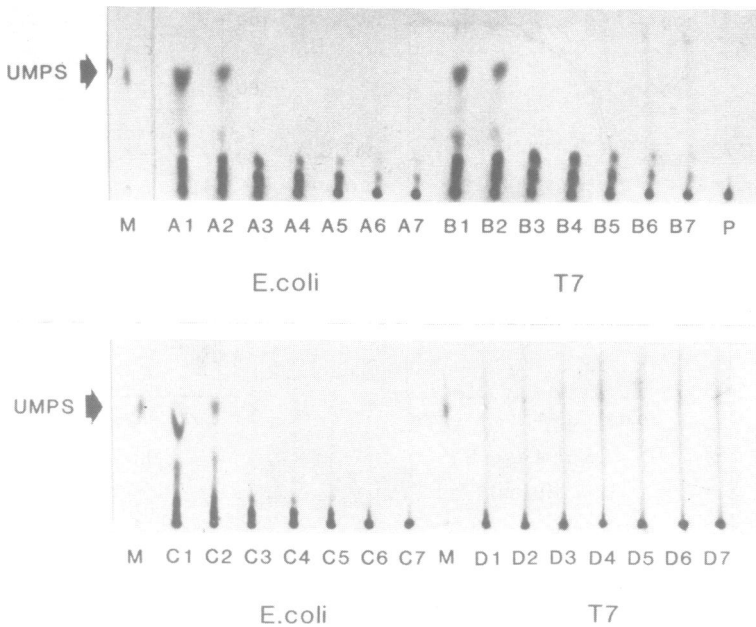


Figure 6. Chromatography on P.E.I.-cellulose to show cleavage at internucleotidic phosphorothioate linkages by nuclease P1. Samples marked A and B were products of transcription by *E.coli* and T7 RNA polymerase respectively in the presence of ATP, CTP, GTP and [α - 35 S]UTP; those marked C and D were transcribed in the presence of ATP α S, CTP α S, GTP α S and [α - 35 S]UTP. Nuclease P1 digestions were performed with successive ten-fold dilutions of enzyme from sample 1 (0.83 u/ μ l) to sample 6; sample 7 in each series was incubated without enzyme. Sample P represents digestions of [α - 35 S]UTP with nuclease P1 at 0.83 u/ μ l (showing that contamination of the transcript would not generate [35 S]UMP); M is [35 S]UMP generated by SVPD digestion of [α - 35 S]UTP.

now matched those obtained with analysis by TCA precipitation: the T7 transcripts were more refractory to nuclease P1 than those synthesized by *E.coli* RNA polymerase.

Nuclease P1 is regarded as showing no activity at all towards Rp-thiophosphodiester linkages in dinucleotides (21). Three possible reasons for digestion of Rp-[S]RNA are that contaminating phosphodiester linkages are being cleaved (shown above to be an insufficient explanation), that digestion of dinucleotides is more stringent than of polynucleotides, or that RNA and DNA are digested with different stringencies. To test the last explanation, parallel syntheses were performed to produce DNA and Rp-[S]DNA (5) by primed synthesis with DNA polymerase 1. Mixed products, as well as Rp-[S]DNA alone (in this case, in the presence of less than half as much unlabelled template DNA), were digested by nuclease P1. Figure 5b

shows that nuclease P1 does not discriminate against these Rp-thiophosphodiester linkages; it is clear that nuclease P1 stringency depends on the difference between dinucleotides and polynucleotides.

DISCUSSION

The work described above sought to establish whether nucleases could be used to assign the configuration of the diastereoisomeric inter-nucleotidic phosphorothioates in thio-substituted RNA and thus, knowing which diastereoisomer of ATP α S was a substrate for T7 RNA polymerase, whether incorporation proceeded with inversion of configuration.

After synthesis of ATP α S the ratio of Sp:Rp diastereoisomers was approximately 1:2, shown by ^{31}P NMR measurements based on known assignments (14,20). The diastereoisomers were separated by reverse phase HPLC and the peaks were assigned by their intensities. The Sp diastereoisomer was found to elute before the Rp. Separation had previously been performed using ion-exchange HPLC (14).

The initial kinetic analysis of transcription showed that Sp-ATP α S was incorporated efficiently, but there appeared to be slower incorporation in the presence of Rp-ATP α S. Much, if not all, of the latter can be attributed to contamination of the other NTPs, rather than to desulphurization of ATP α S or incorporation of Rp-ATP α S. Our later experiments suggested that Rp-ATP α S did not bind significantly to the enzyme.

In order to establish whether Sp-ATP α S could be regarded as a good or a poor substrate, the values of K_{app} for this substrate and for ATP were determined. Precise determinations of kinetic constants proved to be difficult. It was observed that incubation on ice during preparation of the reactions gave extremely unreliable results. Thus, precise incubation times could be measured for only a limited number of samples in parallel reactions with different concentrations of substrate. In practice, in one experiment it was possible to measure about 24 reactions, i.e. four time points for each of six concentrations of substrate (0 and five appropriate concentrations). It was not possible to generate duplicate values for each point, since the actual rates varied from day to day. However, the K_{app} values obtained did not vary very widely, and the mean values for Sp-ATP α S and ATP were quite similar.

The variability of rates may have arisen from the known instability of the enzyme (18) and from the decay of [α - ^{32}P]CTP substrate, for, despite attempts to correct for the decrease in activity with time of the labelled

substrate, the observed rate declined disproportionately (data not shown). Thus, any quantitative estimate of maximal velocity was impossible; furthermore, it would have reflected the substantial period of each round of transcription involved in re-initiation (22). However, it was possible to demonstrate that, in so far as the elongation rate affects the observed reaction rate, the Sp-ATP α S does not contribute to a reduction in rate. It should be noted that there are difficulties in interpreting the apparent K_M . Reactions with T7 RNA polymerase show a lag of 10-15 seconds before the steady-state is reached (22). With a maximum rate of elongation of 200 nucleotides per second (23), only about 4 seconds will be required for elongation of each transcript. Longer transcripts will require longer elongation times, and the contribution of initiation will be correspondingly reduced; thus it would be predicted that longer transcripts will give rise to higher values of K_{app} , which approach the K_M for elongation. It is of interest, and consistent with the above, to note that our value for the K_{app} of ATP is 16-30 μ M, whereas with transcription of T7 DNA a value of 47 μ M has been reported (22). This uncertainty about the significance of the value of K_{app} has been circumvented in studies with T3 RNA polymerase or E.coli RNA polymerase where preinitiated complexes have been studied during elongation (24,25). This approach was not feasible with the short transcripts used in this study.

The assignment of configuration by nuclease sensitivity yielded unexpected results. An early example of the application of "stereospecific" nucleases involved the assignment of the configuration of DNA containing thiophosphodiester linkages synthesized by E.coli DNA polymerase (5) where, as in this paper, it was not possible to synthesize a polymer with the opposite configuration. It was shown that the relative rates of digestion with SVPD of [S]DNA and DNA were similar to those of Rp-[S]RNA compared with RNA; results from dinucleotides were also more consistent with an Rp than an Sp assignment. This was confirmed by an independent method (26). These experiments did not establish whether SVPD was sensitive to configuration in polynucleotides, but the assumption had been reasonable.

Based on this precedent, we sought to show whether [S]RNA synthesized by T7 RNA polymerase behaved like [S]RNA of known configuration with respect to nucleases with opposite stereochemical preference. The comparison of the two RNAs demonstrated that, with respect to two nucleases reported to have opposite preferences, the two RNAs behaved as if they were

identical. The use of thin-layer chromatography confirmed that the rates of cleavage of thiophosphodiester linkages were the same. In all these experiments a constant time of incubation was used and the enzyme concentration was varied in order to keep the background rate of chemical hydrolysis constant.

To demonstrate that the nucleases retained any stereochemical preferences on long [S]RNA, or on RNA rather than the DNA dinucleotides tested previously, we compared the rates of digestion of [S]RNA and normal RNA. The two transcripts were mixed in an arbitrary ratio (1:25), and digestion was followed by TCA precipitation. The concentration of phosphodiester linkages of each transcript was less than one-thousandth of the K_m reported for dinucleotides with nucleases P1 and S1 (21,11). A plot of low values of percentage of reactant solubilised in a given time is equivalent to a plot of V/S against enzyme concentration. If simple Michaelis-Menten kinetics are followed, this plot should be linear, or, with a semi-log plot as in Figures 3-5, exponential.

The exponential curves for two substrates should be superimposable by a displacement along the X-axis. The divergence of the lines for the two substrates seen in Figure 3 for nucleases S1 and P1 cannot be explained by the artefacts of an assay based on TCA precipitation. It is possible that thiosubstitution of RNA results in a shift of the relative rates of endonucleolytic and exonucleolytic activity, which would give rise to altered sensitivities to TCA precipitation; alternatively, if the stereospecificity of the nucleases is greater with smaller substrates, discrimination may increase with the extent of endonucleolytic digestion. This divergence means that measurements and comparisons of rates at any given nuclease concentration cannot be generalised. However, a comparison of rates can be made by determining the enzyme concentrations required to achieve a fixed but arbitrary extent of digestion, when the distribution of sizes of material in the TCA-soluble and TCA-precipitable fractions will be similar for any two substrates that are subject to the same enzymatic activities. In order to render 25% of the substrate soluble in TCA (when initial steady-state rates should still apply) the ratios of the required enzyme concentrations with [S]RNA and normal RNA are: SVPD, 0.4; nuclease S1, 4; nuclease P1 ≥ 100 .

When characterized with dinucleotides, SVPD has been shown to digest normal linkages, $Rp\alpha[S]$ linkages and $Sp-[S]$ linkages with approximate relative values for V_{max} as follows: 185,000 : 1700 : 1 (11). Even given

that SVPD digests Rp-[S]RNA and T7 [S]RNA equally well, i.e. that T7 [S]RNA has an Rp configuration, our results suggest that SVPD may not discriminate sufficiently well (if at all) to be used alone for configurational assignments of [S]RNA. In previous experiments, a much reduced level of discrimination had been found with Rp-[S] poly(A), which was digested 10-fold less rapidly than poly(A) (5). V_{max}/K_M values have been determined (12) for nuclease S1 digestion (at 4 units per 100 μ l) of normal Sp-[S] and Rp-[S] linkages in dinucleotides, producing relative values: 10 : 1 : (0). Thus, although nuclease S1 discriminates against T7 [S]RNA (Figure 3), an assignment of Rp configuration could only be justified by the identical behaviour of known Rp-[S]RNA (Figure 4). Rates of digestion have not been published for nuclease P1, but Rp-[S] dinucleotides have been shown to resist digestion (21); from our results, nuclease P1 seems to discriminate sufficiently against Rp-[S]RNA to suggest that this nuclease will be able to distinguish Sp from Rp configurations in RNA.

It is very striking that nucleases S1 and P1 digested thiophosphodiester linkages which, in the case of E.coli RNA, were certainly in the Rp configuration, whereas this had not been observed for either enzyme with studies on dinucleotides (12,21). The thin-layer chromatography data are important, excluding the possibility that the observed digestion was due to cleavage of normal phosphodiester bonds. The high level of digestion seen in figure 4 suggests that the presence of occasional Sp-linkages in the transcripts (produced by a low rate of incorporation of contaminating Rp-NTPs during transcription) can be excluded as an alternative explanation of this phenomenon.

In conclusion, it seems that discrimination between normal and thio-phosphodiester linkages is retained with RNA, but that the thiophosphodiester linkages are digested more readily in RNA than in dinucleotides. Thus, even the unfavourable configuration is digested, but at a lower rate than normal phosphodiester linkages. Since nuclease P1 does not show even this level of discrimination with the products of DNA Polymerase 1 incorporation, it is possible that the length of the substrate determines the reduced discrimination. This phenomenon might be related to the binding of substrates, if subsidiary binding sites adjacent to the catalytic centre are able to bind extra nucleotides in longer molecules and thereby partly compensate for possible weak binding of substrates containing unfavourable thiophosphodiester linkages. Although the evidence

is circumstantial, we infer that some discrimination between thiophosphodiester diastereoisomers in [S]RNA is retained and that polymerization by T7 RNA polymerase proceeds with inversion of configuration.

The reduced sensitivity of [S]RNA to nuclease P1 when the RNA was synthesized by T7 rather than E.coli polymerase, in the presence of all four NTP α S substrates, was remarkable. In contrast, identical sensitivities were seen with the digestion of unsubstituted RNA, assayed by TCA precipitation, and with RNA wherein only one in four internucleotidic links was thio-substituted, with an assay based on thin-layer chromatography. Because the stereochemistry of the internucleotidic link in RNA synthesized by the E.coli RNA is of the Rp configuration it should be fully resistant to nuclease P1 (see above); the assays with normal or only partially thio-substituted RNAs showed that neither length nor RNA structure affected the digestion patterns. One possible explanation is that endonucleolytic activity was affected more than exonuclease activity by complete thio-substitution, and that therefore differences in transcript lengths became important. This cannot, of course, be estimated in studies with a dinucleoside phosphorothioate substrate. However, it can be seen that SVPD, an obligate exonuclease, did not show marked preferences for RNAs according to the polymerase used for transcription.

The application of these techniques to studies of RNA processing reactions will not be straightforward. In order to assign the configuration of internucleotidic RNA phosphorothioate it will be necessary to rely on stereospecific digestion by nucleases such as those used in this study. It is important to investigate the reason for the reduced stereospecificity in the digestion seen here: will full stereospecificity be seen with RNA dinucleotides present at very low concentrations? If not, then kinetic studies of digestion will be required on the scarce products of RNA processing reactions from which small oligonucleotides containing the thiophosphodiester linkage of interest have been isolated. Such studies will also require the synthesis of pure diastereoisomers of the small oligonucleotide, in order to demonstrate that the stereospecificity of the analytical enzymes used is sufficient. Characterization of the activity of a number of sequence or structure-specific nucleases on thio-substituted RNAs must be undertaken too, in order to optimize methods for probing the folding of such RNAs or for the isolation of small subfragments containing the site of a phosphoryl transfer event.

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