2′-Fluoro- and 2′-Amino-2′-deoxynucleoside 5′-Triphosphates as Substrates for T7 RNA Polymerase†

Helle Aurup, David M. Williams,‡ and Fritz Eckstein*

Max-Planck-Institut für experimentelle Medizin, Hermann-Rein Strasse 3, W-3400 Göttingen, FRG

Received May 5, 1992; Revised Manuscript Received July 14, 1992

ABSTRACT: 2′-Fluoro- and 2′-amino-2′-deoxynucleoside 5′-triphosphates have been investigated as substrates for T7 RNA polymerase. Michaelis–Menten kinetic parameters are reported for the incorporation of 2′-fluoro-2′-deoxyuridine, 2′-fluoro-2′-deoxycytidine, and 2′-amino-2′-deoxyuridine into runoff transcripts. The 2′-amino derivative of uridine is a better substrate than the 2′-fluoro derivative. Gel electrophoretic analysis shows that full-length transcripts with a length of 2500 nucleotides can be obtained with the analogues, although a considerable amount of shorter fragments accompanies the full-length product. In keeping with the kinetic analysis, the 2′-aminouridine triphosphate gives a cleaner product than the 2′-fluoro analogue. Transcription of two tRNA genes shows that such shorter templates can be transcribed to full-length products essentially without premature termination with any of the analogues.

Recently 2′-fluoro- and 2′-amino-2′-deoxynucleotides have been incorporated into oligoribonucleotides by automated chemical synthesis. These 2′-modifications of the pyrimidine nucleosides were tolerated in a hammerhead ribozyme without any significant loss of catalytic efficiency (Pieken et al., 1991). In addition, ribozymes containing these 2′-modifications were stabilized against degradation in rabbit serum by a factor of at least 10^3 compared to the unmodified ribozyme (Pieken et al., 1991; Heidenreich & Eckstein, 1992). A comparison of the catalytic activity of ribozymes where adenosines or guanosines were replaced at certain positions by the 2′-amino-, 2′-fluoro-, or 2′-deoxynucleoside analogues has provided important information about the possible functional role of 2′-hydroxyl groups in the parent molecule (Olsen et al., 1991; Williams et al., 1992). These observations suggest that transcripts containing 2′-modified nucleotides should be interesting for several reasons. First, 2′-modified RNA should show increased stability against degradation by nucleases, and second, these modifications should facilitate the identification of positions where the 2′-hydroxyl group is essential for function. In addition, judicious choice of the 2′-group might even help to specify the role played by the hydroxyl group at a given position.

At present the chemical synthesis of oligoribonucleotides is limited to relatively short pieces of approximately 50 nucleotides in length (Gait, 1991). The synthesis of longer RNA fragments has to rely on enzymatic transcription of the corresponding DNA sequence (Melton et al., 1984; Milligan & Uhlenbeck, 1989). It follows that if the preparation of transcripts containing modifications is desired, the corresponding nucleoside 5′-triphosphates have to be reasonable substrates for the polymerase used in the transcription. This will in most cases be either the T7 or the SP6 RNA polymerase.

We report here that the 5′-triphosphate of 2′-amino-2′-deoxyuridine and the 5′-triphosphates of several 2′-fluoro-2′-deoxynucleosides are substrates for T7 RNA polymerase. Kinetic parameters are presented and it is shown that these nucleotides can be incorporated into full-length transcripts of 2500 nucleotides in length.

MATERIALS AND METHODS

Nucleoside triphosphates and 2′-deoxycytidine triphosphate (special quality for molecular biology, 100 mM) were purchased from Boehringer Mannheim. [^{14}C]ATP (specific activity 50 mCi/mmol), [α-^{32}P]GTP (specific activity ~3000 Ci/mmol) and [γ-^{32}P]ATP (specific activity >5000 Ci/mmol) were from Amersham Buchler, Braunschweig, FRG. Liquid scintillant Szintillator 303 was obtained from Riedel-deHaen, Seelze, FRG. The restriction endonuclease *Bst*NI (10 units/μL) was from New England Biolabs, T4 polynucleotide kinase (30 units/μL) was purchased from U.S. Biochemicals, and ultrapure calf intestinal alkaline phosphatase (1 unit/μL) was from Boehringer Mannheim. Plasmid pSPT19 was obtained from Pharmacia. Plasmid AspUC19 containing the gene for *Escherichia coli* tRNA^{Asp} behind the T7 promoter was a generous gift from D. Schatz (Göttingen, FRG). Plasmid pTFMa containing the gene for yeast tRNA^{Asp} (Perret et al., 1990) was a kind gift from R. Giegé (Strasbourg, France). The plasmids were isolated according to the Qiagen plasmid maxi preparation method provided by Diagen Düsseldorf, FRG. The concentration of plasmid was determined by using 1 A_{260} unit equal to 50 μg of double-stranded DNA and the concentration of tRNA by using 1 A_{260} unit equal to 42 μg of tRNA (Sambrook et al., 1989). Urea stop mix contained 8 M urea, 0.09 M Tris–borate at pH 8.3, 2.5 mM Na_2EDTA, 0.1% xylene cyanol FF, and 0.1% bromophenol blue. KODAK X-Omat films were employed for autoradiography. For quantitative evaluation, autoradiographs were scanned with a Pharmacia LKB Ultrascan XL laser densitometer equipped with Gelscan XL software.

2′-Fluoro-2′-deoxyuridine (Doerr & Fox, 1967) was either prepared analogously to 2′-fluoro-2′-deoxythymidine (Williams et al., 1991) or obtained from U.S. Biochemical Corp. (Bad Homburg, FRG). 2′-Fluoro-2′-deoxycytidine (Codington et al., 1963) was prepared from 2′-fluoro-2′-deoxyuridine by the method of Li et al. (1987). 2′-Fluoro-2′-deoxyadenosine was prepared according to Olsen et al. (1991). 2′-Azido-2′-deoxyuridine was prepared as described (Ver-

† Financial support by the Deutsche Forschungsgemeinschaft (Ec28-14/1) and the Fonds der Chemischen Industrie is gratefully acknowledged.

‡ Present address: Cruachem, Todd Campus, West of Scotland Science Park, Acre Rd., Glasgow G20 0UA, Scotland.

0006-2960/92/0431-9636$03.00/0 © 1992 American Chemical Society

heyden et al., 1971). The 2'-modified nucleoside 5'-triphosphates were prepared according to Ludwig and Eckstein (1988) except the 5'-triphosphate of 2'-fluoro-2'-deoxyadenosine, which was prepared according to Ludwig (1987). 2'-Azido-2'-deoxyuridine 5'-triphosphate was reduced to the 2'-amino derivative either by hydrogenation or by reaction with triphenyl phosphine (Mungall, 1975). All triphosphates were analyzed by HPLC and ^{31}P NMR spectroscopy as described (Ludwig & Eckstein, 1988).

T7 RNA polymerase was isolated from an overproducing *E. coli* strain, BL21, containing the plasmid pAR219 (kindly provided by W. Studier, Brookhaven National Laboratories, Upton, NY), which possessed the T7 polymerase gene under control of the *lacUV5* promoter (Davanloo et al., 1984). The enzyme was purified as described previously (King et al., 1986). The concentration was 6.25 mg/mL as determined by the Bradford assay (Bradford, 1976). The specific activity of the enzyme was determined to be 16 000 units/mg using *Bst*NI-cut plasmid pSPT19 as template. One unit of RNA polymerase activity is defined as 1 nmol of AMP incorporated/h at 37 °C (Chamberlin & Ring, 1973). All templates used for transcription were prepared by linearizing the plasmids with *Bst*NI: the reaction volume (200 μL) contained 10 mM Tris/HCl, pH 7.9 (25 °C), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 120 units of *Bst*NI, and 150 μg of plasmid. After incubation overnight at 37 °C, the reaction mixture was phenol–chloroform-extracted as described by Sambrook et al. (1989). Finally the DNA was precipated with ethanol, and the pellet was washed with 70% (v/v) aqueous ethanol, dried, and dissolved in water.

Determination of Steady-State Kinetic Parameters. The template for transcription in the pSPT19 plasmid DNA, after restriction with *Bst*NI, is 3000 nucleotides long, corresponding to a molecular weight of 2×10^6 Da. The resulting transcript has a length of 2500 nucleotides. Kinetic assays of transcription were carried out at 37 °C in 500-μL Eppendorf tubes. Samples (total volume 45 μL) of the standard reaction contained 40 mM Tris/HCl, pH 8.1 (37 °C), 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 60 nM pSPT19 linearized-DNA as template, and 800 μM each GTP, UTP, CTP, and and [^{14}C]ATP (specific activity of 30 000 cpm/nmol). CTP was replaced by 2'-F-CTP[1] and UTP by its 2'-derivatives for the determination of the kinetic constants of the analogues. The concentration of the nucleotide whose kinetic parameters were to be determined was added in five different concentrations, between 30 and 1000 μM for the unmodified and the 2'-amino nucleotides and between 800 and 6300 μM for the 2'-fluoro nucleotides. The reaction mixtures were incubated for 4 min at 37 °C, and then 8 units of enzyme was added to start the reaction. To avoid evaporation of the sample during incubation, 25 μL of paraffin oil was carefully added on top of each sample.

Aliquots of 8 μL were withdrawn at five different time points between 1 and 8 min for the unmodified nucleotides, between 10 and 60 min for the 2'-amino nucleotides, and between 1 and 4 h for the 2'-fluoro nucleotides. The samples were spotted onto a strip of filter paper (2 × 13.5 cm) (Whatman no. 3MM) pretreated with 16 μL of 200 mM EDTA. The filter paper strips were developed in 1 M ammonium acetate/ethanol (1:1 vol/vol) by descending chromatography for 2 h. They were dried under an IR lamp, and the origin (2 × 2 cm) was cut out and counted in liquid scintillant (Armstrong et al., 1979). The time intervals were chosen so as to ensure a maximum incorporation of approximately 10% of the nucleotides. All steady-state kinetic parameters were determined by Eadie–Hofstee plots. The assay used in these experiments gave reproducible values for K_m and V_{max}, varying at most by a factor of 2.

[1] Abbreviations: 2'-F-NTP, 2'-fluoro-2'-deoxynucleoside 5'-triphosphate; 2'-NH_2-NTP, 2'-amino-2'-deoxynucleoside 5'-triphosphate; PAGE, polyacrylamide gel electrophoresis.

Table I: Kinetic Parameters for the Transcription of Nucleoside 5'-Triphosphates and Their 2'-Modified Analogues[a]

	K_m (μM)	V_{max} (nmol min^{-1} μg^{-1})
CTP	133	2.7
UTP	114	2.4
2'-F-CTP	2857	0.11
2'-F-UTP	2246	0.22
2'-NH_2-UTP	272	0.28

[a] Determined as described in Materials and Methods.

Transcription of Full-Length Product. Transcription samples (total volume 30 μL) contained the same buffer as used in the kinetic assays and 60 nM template, either pSPT19, pTFMa, or AspUC19 DNA, all digested with *Bst*NI. Unmodified NTPs were used at 1 mM concentration together with [α-^{32}P]GTP (final specific activity 1.7 mCi/nmol). For transcription reactions using analogues, UTP was substituted by either 3.5 mM 2'-F-UTP or 1 mM 2'-NH_2-UTP, CTP by 3.5 mM 2'-F-CTP, and ATP by 3.5 mM 2'-F-ATP. To each sample was added 300 units of T7 RNA polymerase. The samples were incubated at 37 °C for 3 h. The reactions were stopped by addition of 2 μL of 500 mM Na_2EDTA. Urea stop mix was added and the mixture was subjected to PAGE on either a 4% or a 12% denaturating gel, depending on the length of the transcript. The products were finally visualized by autoradiography.

Partial Alkaline Hydrolysis of E. coli tRNA^{Asp} Transcripts. Transcripts of the E. coli tRNA^{Asp} gene containing 2'-fluoro- or 2'-aminouridine were prepared as described above for the production of full-length products in a total volume of 80 μL except that no radioactively-labeled nucleotides were used. After incubation for 3 h at 37 °C, the reactions were stopped by addition of 6 μL of 500 mM Na_2EDTA. Urea stop mix was then added and the samples were purified by PAGE on a 12% denaturing gel. The tRNA band was visualized by UV light, the appropriate band was cut out, and the product was extracted from the gel pieces as described (Schatz et al., 1991). The transcripts were then enzymatically dephosphorylated and 5' end-labeled with [γ-^{32}P]ATP (Sambrook et al., 1989). The crude products were again purified by PAGE on a 12% denaturing gel. The labeled transcripts were visualized by autoradiography, and the full-length tRNA transcript was cut out and then eluted from the gel piece as described above. The material was then dissolved in 10 μL of 50 mM $NaHCO_3$, 10 μM of bulk carrier tRNA was added, and the solution was incubated for 10 min at 100 °C and put on ice (Beijer et al., 1990). Urea stop mix was added and the alkaline hydrolysis products were separated by PAGE on a 20% denaturing gel and visualized by autoradiography.

RESULTS

The vector pSPT19 cut with *Bst*NI, used for the kinetic studies, directs the synthesis of a transcript 2500 nucleotides in length. Both the 2'-fluoro- and 2'-amino-2'-deoxyuridine triphosphate analogues and also 2'-fluoro-2'-deoxycytidine triphosphate (Table I) served as substrates for T7 RNA polymerase. All three of them exhibited V_{max} values approximately 10-fold lower than the parent compounds. However, they differed considerably in their K_m values. The 2'-fluoro

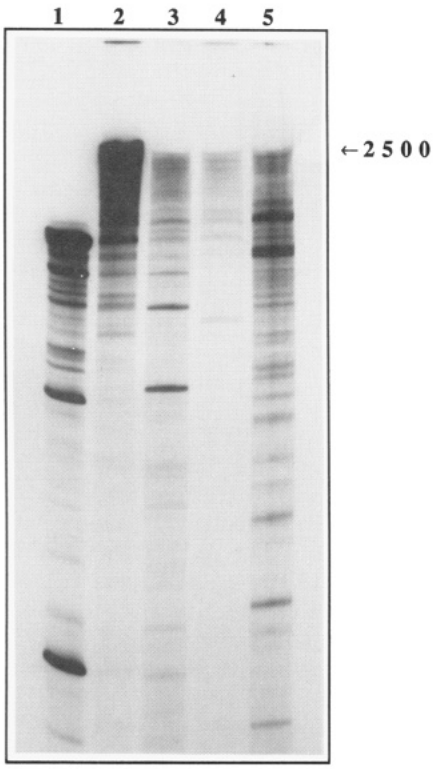

FIGURE 1: Autoradiography of PAGE of [^{32}P]GMP-labeled transcripts using pSPT19 as template. 2′-NH_2-UMP-modified transcript, lane 1; unmodified transcript, lane 2; 2′-F-UMP-modified transcript, lane 3; 2′-F-AMP-modified transcript, lane 4; 2′-F-CMP-modified transcript, lane 5. Details are as described in Materials and Methods.

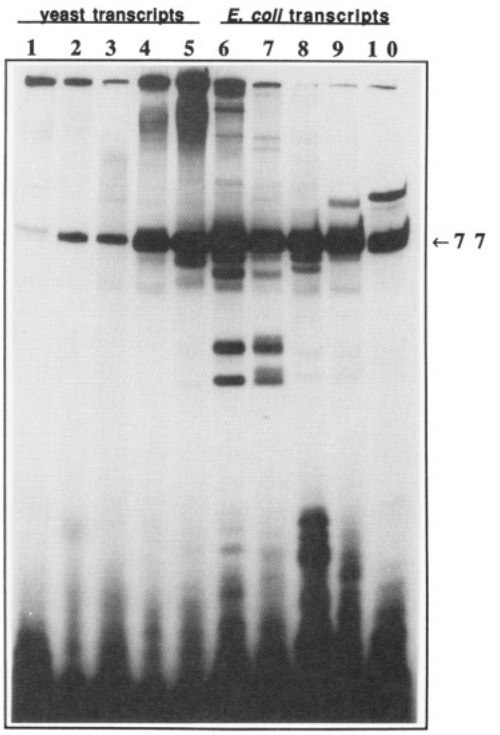

FIGURE 2: Autoradiograph of PAGE of [^{32}P]GMP-labeled transcripts of the *E. coli* and yeast tRNA^{Asp} genes. Yeast tRNA^{Asp}, lanes 1–5; *E. coli* tRNA^{Asp}, lanes 6–10; 2′-F-CMP-modified transcripts, lanes 1 and 10; 2′-F-UMP-modified transcripts, lanes 2 and 9; 2′-NH_2-UMP-modified transcripts, lanes 3 and 8; 2′-F-AMP-modified transcripts, lanes 4 and 7; unmodified transcripts, lanes 5 and 6. The full-length yeast tRNA transcript is 77 nucleotides in length. Details are as described in Materials and Methods.

derivatives showed an increase of this value by approximately 20-fold, whereas the K_m of the 2′-amino derivative was only 2-fold higher than that of the parent triphosphate. Thus, the affinity of the analogues for this enzyme seems to be much less affected by the 2′-amino compared to the 2′-fluoro substituent. Attempts to determine the kinetic constants for incorporation of 2′-fluoro-2′-deoxyadenosine or 2′-deoxycytidine were not successful under the reaction conditions described.

In order to check whether full-length transcripts could be obtained in the presence of the analogues, the reaction products were subjected to denaturing gel electrophoretic analysis. Full-length transcripts of 2500 nucleotides could be obtained with all of the 2′-fluoro analogues used in these experiments (Figure 1). Although these analogues gave full-length transcripts, this was not the main product as it was accompanied by a large number of prematurely terminated fragments. In the presence of the 2′-aminouridine triphosphate no full-length transcript could be detected. The main product which migrates faster than the full-length product seen in the normal transcription reaction derives in all probability from premature termination of transcription. This band can also be seen as a minor component when the normal triphosphates and the 2′-fluorocytidine triphosphate are used as substrates (lanes 2 and 5).

We also examined the products when shorter templates such as tRNA genes were transcribed. All of the 2′-fluoro- and the 2′-amino-modified analogues, including 2′-F-ATP, gave the full-length product when either the *E. coli* (75 nucleotides in length) or yeast tRNA^{Asp} (77 nucleotides in length) genes were transcribed (Figure 2). No product could be seen when either dCTP or 2′-azido-2′-deoxycytidine 5′-triphosphate was tested under the same conditions with the yeast tRNA^{Asp} gene. It is striking that the amount of product

Table II: Relative Yield of Full-Length tRNA Transcripts[a]

transcribed gene	unmodified transcript	transcript containing			
		2′-F-AMP	2′-F-UMP	2′-NH_2-UMP	2′-F-CMP
E. coli tRNA^{Asp}	1	0.5	0.4	0.6	0.1
yeast tRNA^{Asp}	1	0.4	0.04	0.04	0.01

[a] Determination by laser scanning densitometry of an autoradiograph of gel electrophoretically anaylyzed [^{32}P]GMP-labeled transcripts. The yield of the full-length *E. coli* tRNA^{Asp} unmodified transcript was 1.7-fold higher than that of the unmodified yeast transcript.

is quite different for the *E. coli* and the yeast transcripts. The yields of the latter are considerably lower than those of the former, particularly when the analogues of the pyrimidine nucleotides are used as substrates. In these instances the yields are decreased approximately 10-fold (Table II). The products of the transcription reactions are in most cases not homogeneous, containing faster as well as slower moving bands. This is also true for the transcription in the presence of the four normal triphosphates (Figure 2, lanes 5 and 6). The occurrence of the faster moving products is presumably the result of premature termination, whereas that of the longer products is not understood at present.

Some transcripts of *E. coli* tRNA^{Asp} were analyzed by partial alkaline hydrolysis to confirm the correct incorporation of the analogues during transcription (Beijer et al., 1990). It has been shown earlier that RNA containing either 2′-fluoro or 2′-amino analogues is resistant to cleavage by alkali (Hobbs et al., 1973; Pieken et al., 1991). Figure 3 shows that 2′-fluoro-2′-deoxyuridines as well as 2′-amino-2′-deoxyuridines are present at the positions expected for the *E. coli* tRNA^{Asp} transcript (Sprinzl et al., 1989).

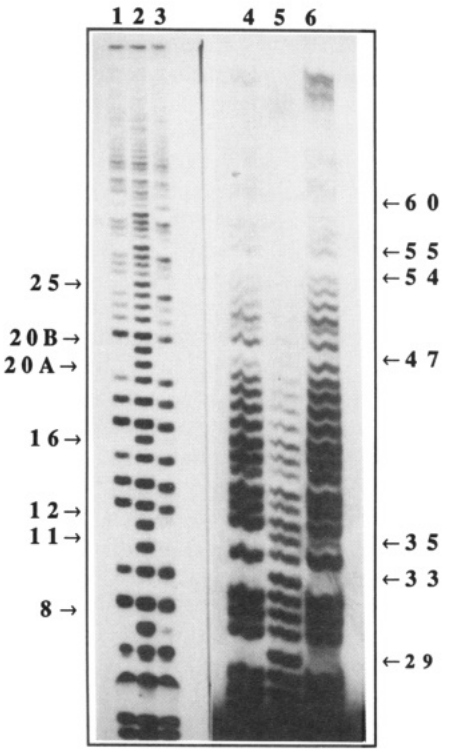

FIGURE 3: Autoradiograph of PAGE of partial alkaline hydrolysis of 5′-labeled *E. coli* $tRNA^{Asp}$ transcripts. 2′-F-UMP-modified transcripts, lanes 1 and 4; unmodified transcript, lanes 2 and 5; 2′-NH_2-UMP-modified transcripts, lanes 3 and 6. Lanes 1–3 were subjected to electrophoresis for 1.5 h; lanes 4–6, for 4 h. Numbers at the side indicate positions of uridines in *E. coli* $tRNA^{Asp}$. Details are as described in Materials and Methods.

DISCUSSION

Our interest in the introduction of 2′-modifications into RNA was stimulated by the observation that several hammerhead ribozymes, particularly those containing 2′-fluoro or 2′-amino derivatives of the pyrimidine nucleosides, are kinetically competent as well as considerably stabilized against degradation by nucleases (Pieken et al., 1991; Olsen et al., 1991; Williams et al., 1992). As these results suggest a number of applications of such modified RNAs, we considered it of interest to explore the possibility of preparing such substituted RNAs by enzymatic transcription. This would facilitate the preparation of much longer RNAs than are presently accessible by chemical synthesis.

The introduction of unnatural nucleotides into RNA or polynucleotides is of long-standing interest. In the past, such modified polymers were mainly prepared to study the influence of such substitutions on structural aspects of polynucleotides with their occasional exploitation in biological systems such as in the induction of interferon (Black et al., 1972; Green et al., 1982; Kakiuchi et al., 1982). The enzymatic polymerization of modified ribonucleoside 5′-diphosphates by polynucleotide phosphorylase has been widely used for the introduction of such modifications into polyribonucleotides. The polymerization of 2′-fluoro-2′-deoxynucleoside 5′-diphosphates by this enzyme has been reported by Janik et al. (1972), Guschlbauer et al. (1977), and Ikehara et al. (1978a,b); that of 2′-chloro-, 2′-amino-, and 2′-azido-2′-deoxynucleoside 5′-diphosphates has been reported by Hobbs et al. (1972, 1973) and Torrence et al. (1972). Polynucleotides containing 2′-*O*-methyl or 2′-*O*-ethyl groups have also been prepared (Bobst et al., 1969; Zmudzka & Shugar, 1970; Kusmierek et al., 1973; Kurshid et al., 1972). In most of these publications the substrate properties were examined only in a qualitative manner and no kinetic constants were reported. Of course polynucleotide phosphorylase is a template-independent enzyme and thus no genetic information can be transcribed into the polynucleotide, which makes this methodology less interesting for the synthesis of modified RNA. At present there is much less data available using template-dependent polymerases. However, one example described the ability of DNA-dependent RNA polymerase from *E. coli* to accept various nucleotide analogues: 2′-fluoro-2′-deoxyuridine 5′-triphosphate was found to be a substrate with a K_m 3 times higher than that of uridine triphosphate and a greatly reduced rate of incorporation with poly[d(A-T)] as template (Pinto et al., 1979). Also, using poly[d(A-T)] as template and *E. coli* RNA polymerase, no incorporation could be observed with 2′-amino-2′-deoxyuridine or 2′-amino-2′-deoxyadenosine triphosphates as substrate (Armstrong & Eckstein, 1976). These compounds were weak, competitive inhibitors.

There is one report in which the incorporation of 2′-fluoro-2′-deoxycytidine into cellular and viral DNA and to a lesser extent into RNA in cells infected with a herpesvirus is described. The interpretation is that the nucleoside was phosphorylated by either the viral or cellular kinases to the triphosphate, which was then used as a substrate by the polymerases (Wohlrab et al., 1985).

The most commonly used template-dependent RNA polymerase for the preparation of transcripts is that of phage T7. Studies with modified nucleotides with this enzyme have been very limited. It has been shown to accept base-modified nucleotides as substrates as long as base pairing, even though not of the Watson–Crick type, can be maintained. Thus, base pairs between isoguanosine and isocytidine and also between xanthosine and diaminopyrimidine nucleosides can be formed using the appropriate template (Switzer et al., 1989; Piccirilli et al., 1990). The only sugar-modified nucleotide analogues which have been examined as substrates for the T7 RNA polymerase are the 2′-deoxynucleoside triphosphates. It was found that in their presence the polymerase will terminate transcription 30% of the time (Milligan & Uhlenbeck, 1989).

We decided to examine the substrate properties of some 2′-modified triphosphates with the T7 RNA polymerase as this is the most suitable polymerase for runoff transcription. The vector pSPT19 digested with *Bst*NI chosen as template directs the synthesis of a transcript of 2500 nucleotides in length. The sequence of the first 10 nucleotides of the transcript is 5′-GGG AGA CCC A-3′. The first CMP appears at position 7 and the first UMP at position 14. It has been shown earlier that the amount and length of abortive initiation products are dependent on the first 8–10 nucleotides in the sequence of the template, which also influences the yield of final reaction product (Milligan & Uhlenbeck, 1989). In particular, it was observed that abortive products are preferentially generated immediately following the incorporation of UMP into the transcript (Martin et al., 1988). Difficulties have been encountered in the determination of K_m values for the elongation process when using a relatively short template where, at least in part, the kinetic parameters are affected by a contribution from the initiation event. As the *Bst*NI-cut plasmid pSPT19 circumvents or minimizes these complications, at least for the pyrimidine nucleosides, it was chosen for this kinetic analysis of incorporation of the 2′-modified pyrimidine nucleosides. However, it should be kept in mind that the kinetic parameters are still a composite of at least three independent parameters, promoter recognition, initiation, and elongation.

As shown in Table I, replacement of the 2′-hydroxyl group by either an amino group or a fluorine atom reduces the catalytic efficiency of these modified triphosphates as substrates for T7 RNA polymerase in comparison with the parent compounds. In the case of the 2′-NH_2-UTP this is mainly due to a reduction in the V_{max} value, whereas for the 2′-fluoro derivatives both a decrease in the V_{max} and an increase in the K_m is observed. The relatively large difference of a factor of 20 between the K_m for the natural substrate and that for the 2′-fluoro nucleotides on the one hand, and the very small difference of only a factor of 2 for the 2′-amino compounds on the other, requires an explanation. Steric hindrance can be discounted as a reason for poor affinity of the analogue, as both of the 2′-modifications have a van der Waals radius smaller than that of the parent hydroxyl group (Guschlbauer & Jankowski, 1980; Wohlrab et al., 1978). A suitable explanation is also difficult when the differences in conformation of the various 2′-modified nucleosides are considered: ribonucleosides exist to the extent of about 60% in the 3′-endo or N-conformation (Saenger 1984; Guschlbauer & Jankowski, 1980). 2′-Fluoro nucleosides have an even higher content of this conformation, namely, 80%. The 2′-amino nucleosides, with only 20% of the N-conformation, are more akin to the 2′-deoxynucleosides with 40% of this conformer. Thus, one would argue that if conformation of the ribose was a determining factor for selection of the substrate, the 2′-fluoro nucleotides should be better substrates than the 2′-amino derivative. A possible explanation might be found in the difference of the substituents at the 2′-position in supporting hydrogen bonds. Obviously the hydroxyl group can act as a hydrogen-bond acceptor as well as a donor, as can the amino group. In contrast, the fluorine atom can only function as a probably weak acceptor. These results are therefore compatible with the idea that the 2′-substituent of the substrate is involved as a hydrogen-bond donor, presumably to the enzyme. This picture would agree with a suggestion put forward by Piccirilli et al. (1991), who, on the basis of results obtained with formycin triphosphate as substrate for the T7 RNA polymerase, speculated that this position of the substrate might interact with the enzyme.

When the *Bst*NI-cut plasmid pSPT19 is used as the template for T7 RNA polymerase-catalyzed transcription of 2′-modified substrates, full-length transcripts of 2500 nucleotides can be obtained for the 2′-fluoro derivatives. But as shown in Figure 1, this product is a very minor one, whereas in the transcription with the normal triphosphates it is the predominant product. Thus it is clear that any modified transcript will have to be purified by gel electrophoresis and properly analyzed before further use. The transcript obtained with the 2′-aminouridine triphosphate is shorter than expected. This product can also be seen when the normal triphosphates and the 2′-fluorocytidine triphosphate are used as substrates (lanes 2 and 5). In these cases, however, transcription continues more efficiently to the full-length product. The reason why the 2′-aminouridine incorporation prevents further elongation is not clear at present. However, premature termination in the presence of this particular triphosphate is not an inherent property, as we have indeed obtained a full-length transcript of 2200 nucleotides in length for the human acetylcholinesterase in the presence of 2′-aminouridine triphosphate (data not shown). Thus it is certain that structural features of the template play an important role in the incorporation of this analogue and presumably of others as well.

In order to see whether shorter full-length transcripts could be obtained with greater ease, we tested the transcription of the genes of *E. coli* and yeast $tRNA^{Asp}$ with expected transcripts of 75 and 77 nucleotides in length, respectively. As can be seen in Figure 2, even using the 2′-fluoro analogues, the full-length transcripts are the main products. This indicates that for such shorter transcripts full-length products can be obtained more easily and that the probability of premature termination increases between a length of 75 and 2500 nucleotides. Where exactly this becomes a problem will have to be determined in each case. However, even for these shorter full-length transcripts the products are not homogeneous in length. This, however, is not peculiar for the analogues but can also be observed in the nonmodified product.

Interestingly, although the kinetic analysis for the incorporation of 2′-fluoro-2′-deoxyadenosine indicated that it was not a substrate, examination of the gels shows clearly that in these experiments full-length product is synthesized. Inspection of the sequence of the first 10 nucleotides of the pSPT19 transcript shows that there are adenosines in positions 4, 6, and 10. It is at least conceivable that these represent a barrier to elongation under the conditions of the kinetic assay.

The large difference in yield obtained with the two different genes for $tRNA^{Asp}$, particularly with the pyrimidine analogues, is puzzling (Table II). It has been reported that for normal transcripts the *E. coli* gene is transcribed better than the yeast gene by a factor of 2 (Milligan & Uhlenbeck, 1987). This is confirmed by our own experience. It is striking that the yields of the yeast $tRNA^{Asp}$ transcripts are considerably lower by 10–15-fold than the corresponding *E. coli* $tRNA^{Asp}$ transcripts, particularly when 2′-modified pyrimidine nucleotides are used as substrate analogues. It is not clear at present why this reduction in transcription yield should be so much affected by the analogues. It might, at least in part, be explained by the difference in position of the pyrimidine nucleotides in the initiation region of the transcript.

It was also investigated whether 2′-deoxycytidine and 2′-azidocytidine could be incorporated into a tRNA transcript even though the kinetic analysis indicated the contrary. However, in these two cases no significant amount of product could be seen on the gel. Thus, one has to conclude that the 2′-fluoro and the 2′-amino modifications are particularly suitable for the synthesis of 2′-modified transcripts.

We have also used 2′-fluoro- and 2′-amino-2′-deoxyuridine triphosphates for the transcription of a 34mer hammerhead ribozyme. The gel indicated the same pattern of product distribution as was seen with all four natural substrates. These enzymatically-prepared, modified ribozymes possessed the same kinetic parameters as those which had been chemically synthesized (Aurup, Olsen, Pieken, and Eckstein, unpublished results; Pieken et al., 1991).

We have not specifically analyzed the nuclease stability of the various modified transcripts in this study. However, the published data on the 2′-modified hammerhead ribozymes gives sufficient evidence for the increased stability when pyrimidine nucleosides are replaced by their 2′-modified counterparts (Pieken et al., 1991; Heidenreich & Eckstein, 1992).

In summary, we show that 2′-fluoro-2′-deoxynucleoside triphosphates and 2′-amino-2′-deoxyuridine triphosphate are substrates for T7 RNA polymerase. This offers the possibility of obtaining modified RNAs which display increased RNase stability. It also facilitates the examination of the importance and the role of the 2′-hydroxyl group in RNAs in a variety of systems. In addition, these analogues might be useful for in vitro selection of RNAs with modified properties (Ellington

& Szostak, 1990; Tuerk & Gold, 1990; Robertson & Joyce, 1990).

ACKNOWLEDGMENT

The excellent technical assistance of F. Benseler and U. Kutzke in the preparation of the substrates is gratefully acknowledged.

REFERENCES

Armstrong, V. W., & Eckstein, F. (1976) *Eur. J. Biochem. 70*, 33–38.
Armstrong, V. W., Yee, D., & Eckstein, F. (1979) *Biochemistry 18*, 4120–4123.
Beijer, B., Sulston, I., Sproat, B. S., Rider, P., Lamond, A. I., & Neuner, P. (1990) *Nucleic Acids Res. 18*, 5143–5151.
Black, D. R., Eckstein, F., Hobbs, J. B., Sternbach, H., & Merigan, T. C. (1972) *Virology 48*, 537–545.
Bobst, A. M., Rottman, F., & Cerutti, P. A. (1969) *J. Mol. Biol. 46*, 221–234.
Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.
Chamberlin, M., & Ring, J. (1973) *J. Biol. Chem. 246*, 2235–2244.
Chamberlin, M., & Ryan, T. (1982) *The Enzymes 15*, 87–105.
Codington, J. F., Doerr, I. L., & Fox, J. J. (1963) *J. Org. Chem. 29*, 558–564.
Davanloo, P., Rosenberg, A. H., Dunn, J. J., & Studier, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2035–2039.
Doerr, I. L., & Fox, J. J. (1967) *J. Org. Chem. 32*, 1462–1471.
Ellington, A. D., & Szostak, J. W. (1990) *Nature 346*, 818–822.
Gait, M., Pritchard, C., & Slim, G. (1991) in *Oligonucleotides and Analogs* (Eckstein, F., Ed.) Oxford University Press, Oxford, England.
Greene, J. J., Alderfer, J. L., Tazawa, I., Tazawa, S., Ts'o, P. O. P., O'Malley, J., & Carter, W. A. (1978), *Biochemistry 17*, 4214–4220.
Guschlbauer, W. (1982) *Nucleic Acids Res. 11*, 113–116.
Guschlbauer, W., & Jankowski, K. (1980) *Nucleic Acids Res. 8*, 1421–1433.
Guschlbauer, W., Blandin, M., Drocourt, J. I., & Thang, M. N. (1977) *Nucleic Acids Res. 4*, 1433–1443.
Heidenreich, O., & Eckstein, F. (1992) *J. Biol. Chem. 267*, 1904–1909.
Hobbs, J., Sternbach, H., Sprinzl, M., & Eckstein, F. (1972) *Biochemistry 11*, 4336–4344.
Hobbs, J., Sternbach, H., Sprinzl, M., & Eckstein, F. (1973) *Biochemistry 12*, 5138–5145.
Ikehara, M., Fukui, T., & Kakiuchi, N. (1978a) *Nucleic Acids Res. 5*, 1877–1887.
Ikehara, M., Kakiuchi, N., & Fukui, T. (1978b) *Nucleic Acids Res. 9*, 3315–3324.
Janik, B., Kotick, M. P., Kreiser, T. H., Reverman, L. F., Sommer, R. G., & Wilson, D. P. (1972) *Biochem. Biophys. Res. Commun. 46*, 1153–1160.
Kakiuchi, N., Marek, C., Rousseau, N., Leng, M., De Clercq, E., & Guschlbauer, W. (1982) *J. Biol. Chem. 257*, 1924–1928.
Khurdhid, M., Khan, A., & Rottman, F. (1972) *FEBS Lett. 28*, 25–28.
King, G. C., Martin, C. T., Pham, T. T., & Coleman, J. E. (1986) *Biochemistry 25*, 36–40.
Kusmierek, J. T., Kielanowska, M., & Sugar, D. (1973) *Biochem. Biophys. Res. Commun. 53*, 406–412.
Li, B. F. L., Reese, C. B., & Swann, P. F. (1987) *Biochemistry 26*, 1086–1093.
Ludwig, J. (1987) in *Biophosphates and their analogues* (Bruzik, K. S., & Stec, W. J., Eds.) Elsevier Science Publishers B. V., Amsterdam.
Ludwig, J., & Eckstein, F. (1988) *J. Org. Chem. 54*, 631–635.
Martin, C. T., Muller, D. K., & Coleman, J. E. (1988) *Biochemistry 27*, 3966–3974.
Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., & Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035–7056.
Milligan, J. F., & Uhlenbeck, O. C. (1989) *Methods Enzymol. 180*, 51–62.
Mungall, W. S., George, G. L., Heavnen, G. A., & Letsinger, R. L. (1975) *J. Org. Chem. 40*, 1659–1662.
Olsen, D. B. O., Benseler, F., Aurup, H., Pieken, W. A., & Eckstein, F. (1991) *Biochemistry 30*, 9735–9741.
Peret, V., Garcia, A., Puglisi, J., Grosjean, H., Ebel, J.-P., Florentz, C., & Griegé, R. (1990) *Biochimie, 72*, 735–744.
Piccirilli, J. A., Krauch, T., Moroney, S. E., & Benner, S. A. (1990) *Nature 343*, 33–37.
Piccirilli, J. A., Moroney, S. E., & Benner, S. A. (1991) *Biochemistry 30*, 10350–10356.
Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H., & Eckstein, F. (1991) *Science 253*, 314–317.
Pinto, D., Sarocchi-Landousy, M.-T., & Guschlbauer, W. (1979) *Nucleic Acids Res. 6*, 1041–1048.
Robertson, D. L., & Joyce, G. F. (1990) *Nature 344*, 467–468.
Saenger, W. (1984) in *Principles of Nucleic Acid Structure* (Saenger, W., Ed.) Springer, New York.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schatz, D., Leberman, R., & Eckstein, F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6132–6136.
Sprinzl, M., Hartmann, T., Weber, J., Blank, J., & Zeidler, R. (1989) *Nucleic Acids Res. 17* (Sequences Suppl.), r18–r19.
Switzer, C., Moroney, S. E., & Benner, S. A. (1989) *J. Am. Chem. Soc. 111*, 8322–8323.
Torrence, P. F., Waters, J. A., & Witkop, B. (1972) *J. Am. Chem. Soc. 94*, 3638–3639.
Tuerk, C., & Gold, L. (1990) *Science 249*, 505–510.
Verheyden, D., Wagner, D., & Moffatt, J. G. (1971) *J. Org. Chem. 36*, 250–254.
Williams, D. M., Benseler, F., & Eckstein, F. (1991) *Biochemistry 30*, 4001–4009.
Williams, D. M., Pieken, W. A., & Eckstein, F. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 918–921.
Wohlrab, F., Haertlé, Tricktinger, T., & Guschlbauer, W. (1978) *Nucleic Acids Res. 5*, 4753–4759.
Wohlrab, F., Jamieson, A. T., Hay, J., Mengel, R., & Guschlbauer, W. (1985) *Biochim. Biophys. Acta 824*, 233–242.
Zmudzka, B., & Sugar, D. (1970) *FEBS Lett. 8*, 52–54.

Registry No. CTP, 65-47-4; UTP, 63-39-8; 2'-F-CTP, 66840-03-7; 2'-F-UTP, 66840-02-6; 2'-NH_2-UTP, 61468-90-4; 2'-F-AMP, 68245-91-0; 2'-F-UMP, 50270-97-8; 2'-NH_2-UMP, 34407-64-2; 2'-F-CMP, 63541-62-8; RNA polymerase, 9014-24-8.