Rychlik, I., Chladek, S., and Zemlicka, J. (1967), *Biochim. Biophys. Acta 138*, 640.
Zemlicka, J., and Chladek, S. (1966), *Collect. Czech. Chem. Commun. 31*, 3775.
Zemlicka, J., and Chladek, S. (1968), *Collect. Czech. Chem. Commun. 33*, 3293.
Zemlicka, J., and Holy, A. (1967), *Collect. Czech. Chem. Commun. 32*, 3159.

Analysis of Transcription *in Vitro* Using Purine Nucleotide Analogs*

J. L. Darlix, P. Fromageot, and E. Reich†

ABSTRACT: The process of DNA transcription by purified *Escherichia coli* RNA polymerase has been studied using the 5′-triphosphates of 8-azaguanosine and formycin. While the rate of polymerization of these analogs is influenced by the choice of template and by the ionic environment, it is at all times lower than that of the normal counterparts. Analysis of the different steps in transcription reveals that, compared with the natural purine nucleotides, (1) the initiation of chains by 8-azaguanosine triphosphate and formycin triphosphate is low; (2) the analogs support a rate of chain elongation indistinguishable from normal; (3) the expected spontaneous release of newly synthesized RNA chains from the ternary transcription complex does not occur with either analog. The differences in the extent of chain initiation and release account quantitatively for the decreased rate of transcription in the presence of the analogs.

The behavior of the nucleotide analogs suggests that the initiation sequences in calf thymus DNA and in T_4 phage DNA are different and heterogeneous.

Stereochemical specificity in transcription appears to be much more exacting for chain initiation and termination than for chain elongation.

Formycin and 8-azaguanosine, structural analogs of adenosine and guanosine, respectively (Roblin *et al.* (1945); Koyama *et al.* (1966)), inhibit nucleic acid synthesis in bacterial and mammalian cells (Shapiro *et al.* (1950); Chantrenne (1964); Hori *et al.* (1964); Ward *et al.* (1969)). The analogs resemble their normal counterparts in H-bonding potential and in the formation of base pairs with complementary template residues, and they are also effectively incorporated into RNA *in vivo* and *in vitro* (Shapiro *et al.* (1950); Smith and Matthews (1957); Matthews (1958); Brockman *et al.* (1959); Caldwell *et al.* (1966); Ward *et al.* (1969)).

Previous observations have indicated that formycin and 8-azaguanosine residues in polynucleotides are likely to produce structural anomalies because both of these nucleosides tend to exist in the *syn* conformation (Ward *et al.* (1969)) under conditions in which the natural nucleosides maintain the *anti* conformation. The analogs could therefore be expected to interfere with some of the steps in RNA synthesis, and the rate of RNA synthesis is indeed depressed significantly when FTP[1] or 8-azaGTP is substituted for ATP or GTP, respectively (Kahan and Hurwitz (1962); Ikehara *et al.* (1968)). However, the mechanism by which the analogs depress RNA synthesis has not been defined, and the relationship between *in vitro* RNA synthesis and cytotoxicity remains unclear. We have therefore reexamined the utilization of the analogs by RNA polymerase. The findings presented below demonstrate that the analogs behave differently in the initiation, elongation, and release of RNA chains; in comparison with the natural substrates, the analogs (1) function poorly in initiation, (2) function normally in chain propagation, and (3) the normal pattern of chain termination and the release of long polynucleotides from ternary complexes is altered when the newly synthesized product contains either base analog.

Materials and Methods

Escherichia coli RNA polymerase was prepared and assayed as previously described (Darlix *et al.*, 1969). The conditions of incubation for individual experiments are given in the figure legends. The ionic conditions were one of the following: (1) Tris-HCl, pH 8.5 $\times 10^{-2}$ M; $MgCl_2$, 5 $\times 10^{-3}$ M; $MnCl_2$, 10^{-3} M; β-mercaptoethanol, 8 $\times 10^{-3}$ M; (2) Tris-HCl, pH 8.5 8 $\times 10^{-2}$ M; $MgCl_2$, 10^{-2} M; KCl, 5 $\times 10^{-2}$ M; β-mercaptoethanol, 5 $\times 10^{-3}$ M; (3) as in 2, but KCl, 0.16 M.

Unless otherwise noted, nucleoside triphosphates were present at 10^{-4} M.

The termination factor, ρ, was extracted from *E. coli* and purified by a modification of the procedure of Roberts (1969).

RNA synthesis was monitored by following the incorporation of the appropriate radioactive nucleotide into material precipitable by 5% trichloroacetic acid using a Millipore filter method. The method of direct filtration through Millipore filters (Sentenac *et al.*, 1968) was also used, particularly for estimating the initiation and release of RNA chains. Measure-

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[1] Abbreviations used are: FTP, formycin triphosphate; 8-azaGTP, 8-azaguanosine triphosphate; MAK, methylated serum albumin kieselguhr.

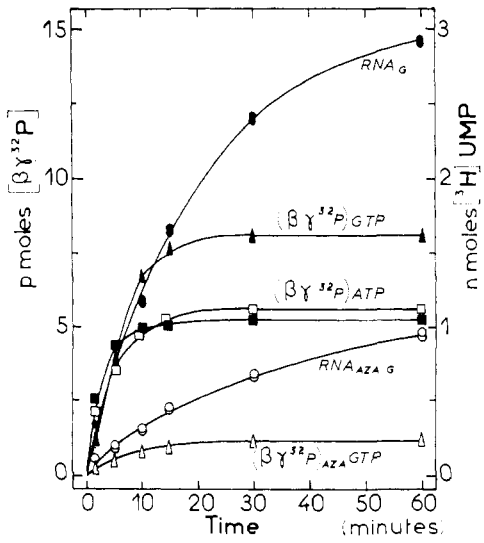

FIGURE 1: Effects of 8-azaGTP on chain initiation by RNA polymerase with calf thymus DNA template. Each of four incubation mixtures contains: calf thymus DNA, 195 μg; RNA polymerase, 300 units (Chamberlin and Berg, 1962); salt conditions I (see Methods); CTP, ATP, [^{3}H]UTP (7600 cpm/nmole), 10^{-4} M, [β,γ-^{32}P]GTP or [β,γ-^{32}P]8-azaGTP, 8×10^{-5} M (150 cpm/nmole). Final volume, 2 ml; 250-μl aliquots were withdrawn at the indicated times and RNA was recovered by direct filtration on Millipore filters: (●—●) incorporation of [^{3}H]UMP with GTP present; (○—○) incorporation of [^{3}H]UMP with 8-azaGTP replacing GTP; (▲—▲) incorporation of [β,γ-^{32}P]GTP in the control; (△—△) incorporation of [β,γ-^{32}P]8-azaGTP with the latter replacing GTP. To test the effect of 8-azaGTP on initiation by ATP, two identical reactions were set up containing [β,γ-^{32}P]ATP (8×10^{-5} M) and either GTP (□—□) or 8-azaGTP (■—■) at 10^{-4} M.

ments of radioactivity were performed using Nuclear-Chicago scintillation counter.

Chromatographic analysis of RNA synthesized *in vitro* was performed with columns of methylated serum albumin (MAK) as already described (Mandell and Hershey, 1960; Darlix *et al.*, 1968), in the presence of total nucleic acids extracted from *E. coli* B as markers.

T_4 phage DNA was kindly donated by Dr. Levine (Gif-sur-Yvette). High molecular weight DNA of calf thymus and *E. coli* were gifts of Dr. Pouyet (Strasbourg).

ATP, FTP, and GTP labeled with ^{32}P in the γ-phosphate were prepared by a modification of the photophosphorylation procedure of Avron (Sentenac *et al.*, 1968), using broken, washed spinach chloroplasts.

[γ-^{32}P]- and [β,γ-^{32}P]azaGTP, GTP, and ATP were synthesized chemically, according to Moffat (1967).

Nucleoside triphosphates and 8-azaGMP were obtained from P-L Biochemicals, Inc., [^{3}H]UTP from the "Service Molécules Marquées" Saclay, and [^{3}H]GTP and [^{3}H]CTP are from Schwarz. Poly(C) was purchased from Sigma Chemical Co.

Results

We have confirmed previous observations (Kahan and Hurwitz, 1962; Ikehara *et al.*, 1968) that the rate and extent of RNA synthesis are decreased when 8-azaGTP and FTP are substituted for GTP or ATP, respectively, and that the K_m for polymerization of 8-azaGTP is higher than that for GTP (2.5×10^{-5} M and 1×10^{-5} M, respectively, under our salt conditions I).

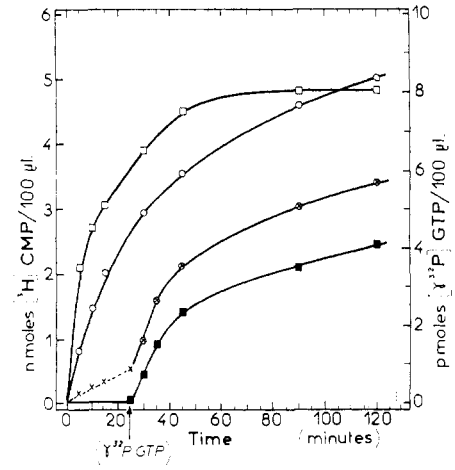

FIGURE 2: RNA chain initiation with GTP in the presence of 8-azaGTP. Two incubation mixtures contain: calf thymus DNA, 120 μg; salt conditions I (see Methods); RNA polymerase, 350 units; UTP, ATP, [^{3}H]CTP (5600 cpm/nmole), 10^{-4} M, and [γ-^{32}P]-GTP, 6×10^{-5} M (1000 cpm/nmole), or 8-azaGTP, 2×10^{-4} M. After 25 min, [γ-^{32}P]GTP, 6×10^{-5} M, was added to the reaction containing 8-azaGTP. Final volume 1 ml; 100-μl aliquots are withdrawn and RNA was recovered by acid precipitation: (○—○) incorporation of [^{3}H]CTP; (□—□) incorporation of [γ-^{32}P]GTP in the control; (×---×) incorporation of [^{3}H]CTP with 8-azaGTP replacing GTP; [⊗—⊗] incorporation of [^{3}H]CTP after delayed (arrow) addition of [γ-^{32}P]GTP; the ^{32}P incorporated is given by the curve (■—■).

I. Chain Initiation by 8-azaGTP and FTP. (*a*) CALF THYMUS DNA TEMPLATE. As originally reported by Kahan and Hurwitz (1962), the replacement of GTP by 8-azaGTP in reactions directed by calf thymus DNA reduces the total synthesis of RNA to 20–25% of that in control reactions. Under these conditions the total number of chains initiated by 8-azaGTP is also much decreased and amounts to only 15% of those initiated by GTP in the control. However, the kinetics of initiation with 8-azaGTP resemble those found with GTP; the final yield in both cases is achieved after 30-min incubation. The presence of 8-azaGTP does not impair chain initiation by ATP (Figure 1). The deficiency in chain initiation by 8-azaGTP is revealed also by the subsequent addition of [γ-^{32}P]GTP to the reaction mixture. As shown in Figure 2, this leads to a round of further initiation by GTP and the number of newly formed chains corresponds exactly to the difference between those normally initiated by GTP and the smaller yield found with 8-azaGTP alone. The behavior of FTP is qualitatively identical with that of 8-azaGTP. Thus, (1) FTP initiates fewer chains than ATP, about two-thirds as many; (2) the remaining chains can be recovered by the subsequent addition of ATP; (3) FTP does not inhibit chain initiation by GTP. The preceding experiments were performed with polymerase preparations known to contain the σ factor (Burgess *et al.*, 1969). Since the specificity of initiation is markedly decreased in the absence of σ with both calf thymus DNA and phage DNA (Sugiura *et al.*, 1970), the experiments were repeated using a polymerase which had been freed of σ. Under these conditions the relative yield of chains initiated by the purine nucleotide analogs was decreased still further. The extent of initiation by 8-azaGTP is less than 10% of that found for GTP, whereas initiation by FTP drops to less than 30% of that observed with ATP.

(*b*) CHAIN INITIATION WITH BACTERIOPHAGE T_4-DNA TEMPLATE. The DNA of bacteriophage T_4 differs in several im-

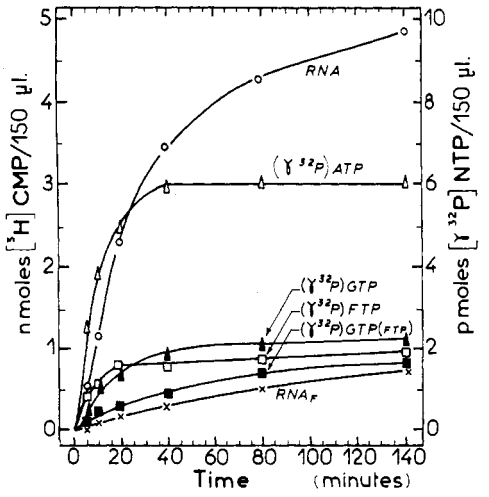

FIGURE 3: Effect of FTP on kinetics of initiation and synthesis of RNA chains with T_4 phage DNA as template. Each of four incubations contains: T_4-DNA, 55 μg; salt conditions 3 (see Methods); RNA polymerase, 600 units; UTP, GTP, [^{3}H]CTP (3500 cpm/nmole), 10^{-4} M, and [γ-^{32}P]ATP (△—△), or [γ-^{32}P]FTP (□—□), or [γ-^{32}P]GTP with ATP present (▲—▲), or [γ-^{32}P]GTP with FTP replacing ATP (■—■). The γ-^{32}P nucleotides were used at 6×10^{-6} M, 700 cpm/pmole. Final volume was 2 ml. Aliquots of 150 μl were removed at the indicated times and the RNA was recovered by acid precipitation. Incorporation of [^{3}H]CTP in the control (○—○), and with FTP replacing ATP (×—×).

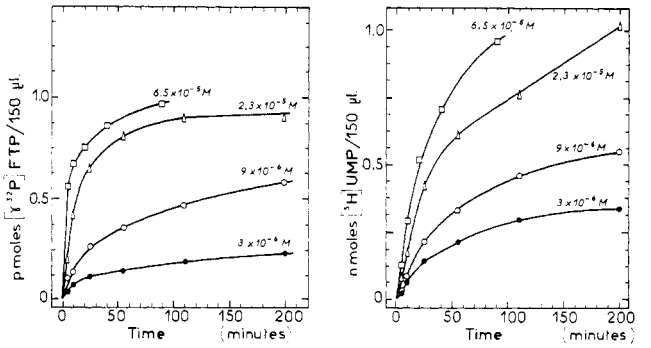

FIGURE 4: Rates of chain initiation and RNA synthesis as functions of FTP concentration. Each of four incubations contains: T_4-DNA, 60 μg; salt conditions 2 (see Methods); RNA polymerase, 300 units; GTP, CTP, [^{3}H]UTP (6000 cpm/nmole), 10^{-4} M, [γ-^{32}P]FTP (900 cpm/pmole) as indicated in the figure. Final volume 1 ml. RNA was recovered by acid precipitation from 150-μl aliquots at the times shown.

portant respects from calf thymus DNA: the G + C content of calf thymus DNA is higher (49%) than that of T_4-DNA (34%); the cytosine residues in T_4-DNA are fully hydroxymethylated and glucosylated, whereas those of calf thymus DNA are not; when RNA synthesis is catalyzed by *E. coli* polymerase the presence of Mn^{2+} ions produces an increased yield with calf thymus DNA template and a decrease with T_4-DNA template (Anthony *et al.*, 1969). The effect of the nucleotide analog substrates with T_4-DNA template also differs somewhat from that described above for calf thymus DNA.

When 8-azaGTP is substituted with T_4-DNA template, RNA synthesis is altered in the following respects.

(1) The initial rate of "initiation" of chains by 8-azaGTP (*i.e.*, chains containing 8-azaGTP at the 5′ terminus) is slowed somewhat, compared with GTP. However, after a short period (about 4 min) the rate of initiation with 8-azaGTP climbs steeply and more rapidly than that observed with GTP. The final yield of chains initiated by 8-azaGTP is approximately twofold greater than that obtained with GTP.

(2) The presence of 8-azaGTP in place of GTP reduces chain initiation with ATP by 50%. No such effects are observed with calf thymus DNA.

(3) In the presence of 8-azaGTP, the total number of chains initiated is about 80% of control, since 8-azaGTP initiates twice as many chains as GTP, while concurrently decreasing initiation by ATP. However, the total yield of nucleotide polymerized into RNA is reduced to one-sixth of normal. This finding is explained by analysis of the reaction products using MAK column chromatography, which reveals that the majority of chains formed in reactions conducted with 8-azaGTP remain as short oligonucleotides and emerge at the solvent front.

All of the above results with 8-azaGTP are observed under conditions of high ionic strength which promote turnover of the polymerase and consequent reinitiation of new chains. The findings obtained when FTP is substituted for ATP under the same conditions are presented in Figure 3. In contrast to 8-azaGTP, FTP initiates far fewer chains than its normal counterpart ATP. On the other hand, FTP reduces both the rate and extent of chain initiation by GTP, just as, in the reciprocal situation described above, 8-azaGTP reduces initiation by ATP.

At low ionic strength, and with the normal nucleotide substrates, RNA polymerase is not released from the ternary synthesizing complex after long RNA chains have been formed. Under these conditions the final yield of RNA is one-half of that in the control reaction shown in Figure 3. In contrast, the final yield of RNA made with FTP is unaffected by the ionic environment since (*vide infra*) the polymerase is in any case not released from ternary complexes whose RNA has been synthesized with the analog. Likewise, the polymerization of normal substrates is ordinarily decreased by the addition of Mn^{2+} ions; no such effect of Mn^{2+} is observed when FTP replaces ATP.

If both ATP and GTP are simultaneously replaced by FTP and 8-azaGTP, the final yield of RNA with calf thymus DNA and with T_4-DNA templates, are, respectively, 3% and 0.2% of the control values. The almost complete suppression of RNA synthesis by the two analogs suggests that virtually no initiation occurs under these conditions.

(c) INITIATION KINETICS AT LOW NUCLEOTIDE ANALOG CONCENTRATIONS. As already known for normal nucleotides (Fromageot and Sentenac, 1969) the initial rate of chain initiation is a function of purine nucleotide concentrations, but the final yield of initiation remains constant. Comparable results are obtained with both FTP (Figure 4) and with 8-azaGTP. At saturating concentrations of FTP, the final yield of initiation is attained rapidly; at limiting concentrations of the analog, the initial rate is lower and the final yield of initiation is delayed. At very low concentrations of FTP, no plateau in the initiation kinetics is achieved, although the ultimate expected level of RNA chains initiated might well have been attained at still later times. The apparent K_m for FTP for chain initiation is 2.5×10^{-5} M and that for chain elongation is 2×10^{-5} M. From identical experiments the corresponding values for 8-azaGTP are 3.5×10^{-5} M (initiation) and 2.5×10^{-5} M (elongation). These values are three- to fourfold higher than those for the normal counterparts ATP and GTP (Anthony *et al.*, 1969).

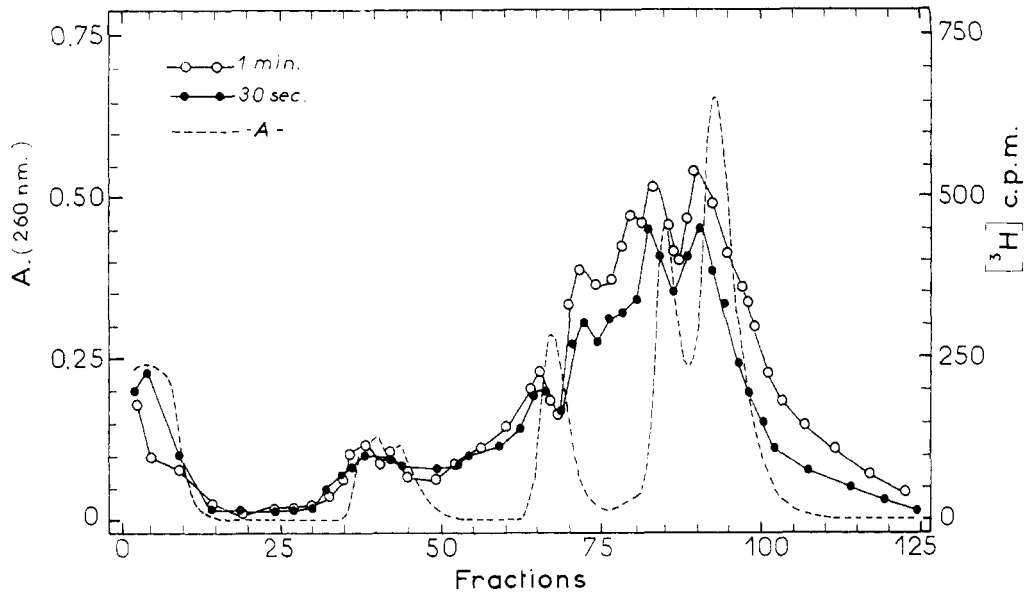

FIGURE 5: MAK column chromatography of the RNA_{azaG} isolated after 30-sec and 1-min synthesis. Each of two incubations contains: calf thymus DNA, 100 μg; salt conditions 1 (see Methods); RNA polymerase, 180 units; CTP, ATP, [^{3}H]UTP, 10^{-4} M, 8-azaGTP, 2×10^{-4} M. Final volume 2 ml: 30-sec synthesis (●—●), [^{3}H]UTP, 51000 cpm/nmole, newly synthesized RNA containing 11500 cpm of incorporated [^{3}H]UMP was adsorbed onto the column; a total of 10000 cpm was eluted; 60-sec synthesis (○—○), [^{3}H]UTP, 46000 cpm/nmole. RNA containing 14000 cpm was adsorbed and 12000 cpm was eluted. The optical density trace is given by the nonradioactive *E. coli* nucleic acids which serve as markers.

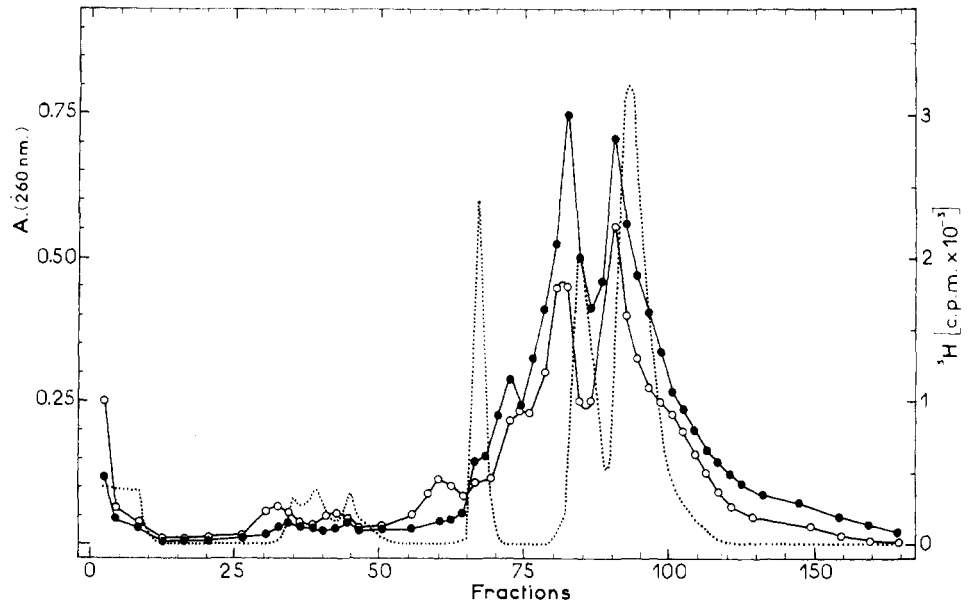

FIGURE 6: Elution profiles of RNA_F formed after periods of 1- and 3-min synthesis. Each of two incubations contains: calf thymus DNA, 100 μg; salt conditions 1 (see Methods); RNA polymerase, 500 units; CTP, UTP, FTP, [^{3}H]GTP. Final volume, 1 ml: 1-min synthesis (○—○), [^{3}H]GTP, 60000 cpm/nmole; adsorbed onto the column, 38000 cpm; recovered, 34500 cpm; 3-min synthesis (●—●) [^{3}H]GTP, 30000 counts/min per nmole; adsorbed onto the column, 49,000 cpm; recovered, 44,000 cpm. *E. coli* B nucleic acids were used as markers.

On the basis of the data presented above, we may calculate the expected overall inhibition of DNA transcription resulting from the decrease in initiation obtained with 8-azaGTP and FTP. The calculated inhibition of RNA synthesis (Table I) for calf thymus DNA is 50% with 8-azaGTP and 15% with FTP. Since the observed inhibition with RNA_{azaG} and RNA_F corresponds to 75–80% and 50–55%, respectively, at least one facet of the transcription process besides initiation must be affected by the analogs.

II. (a) RNA CHAIN GROWTH. The effect of the analogs on chain propagation was analyzed by means of chromatography on columns of methylated serum albumin-Kieselguhr, since this technique separates the products of enzymatic RNA synthesis *in vitro* on the basis of molecular weight. When the reactions are conducted with a mixture of [γ-^{32}P]purine and [^{3}H]pyrimidine nucleotides, the radioactive double labeling of the eluted materials provides an unambiguous measure of the chain length of polynucleotides in the different fractions. For the present experiments calf thymus DNA served as template, and the radioactive substrates were [^{3}H]UTP +

TABLE I: Inhibition of RNA Synthesis Resulting from the Lower Ability of 8-azaGTP and FTP to Initiate Chains, with Calf Thymus DNA as Template.

	ATP and FTP Initiated Chains	GTP and 8-azaGTP Initiated Chains	Total	% Inhibition
Natural NTP	2	3	5	
8-azaGTP	2	0.4	2.4	50
FTP	1.3	3	4.3	15

[γ-^{32}P]ATP + [γ-^{32}P]8-azaGTP; or [^{3}H]GTP + [γ-^{32}P]FTP + [γ-^{32}P]GTP. Aliquots were removed for chromatography after 30-sec, 1-min, 3-min, and 20-min incubation.

The results for one representative experiment in which 8-azaGTP replaced GTP are shown in Figure 5. After 30-sec synthesis, the chains of RNA_{azaG} are distributed into four major fractions, as previously observed for normal reactions with GTP. The longest chains are 2700–2900 nucleotides in length, for which the corresponding average rate of chain growth is 90–100 nucleotides/sec. Identical results were obtained from reactions in which FTP was used in place of ATP, as shown in Figure 6. Thus, initial rates of polymerization with 8-azaGTP or FTP are equal to those obtained with normal substrates.

After 1 min of synthesis, the analysis of control reactions reveals the presence of a new fraction whose chain length is approximately 3500 nucleotides (Darlix *et al.*, 1968). This species results from further elongation of the preexisting fraction with chain length 2700–2900 nucleotides. In contrast the chains of RNA_{azaG} and RNA_F do not elongate beyond 2700–2900 nucleotides at this time; there is merely an increase in the number of chains in each of the size classes.

The results of a series of analyses of this type are summarized in Figure 7. It is apparent that the incorporation of FMP or 8-azaGMP drastically reduces the rate of growth of RNA molecules once these have reached a chain length of 2700–2900 nucleotides. The elongation of these chains resumes only after a relatively long lag period, 10 min in the case of RNA_F.

Roberts (1969) has shown that a protein factor, ρ, prevents the transcription of DNA beyond termination signals. Using MAK column chromatography to analyse the products of transcription of T_4-DNA, we have observed that the addition of ρ prevents chain elongation of chains beyond 2700–2900 nucleotides. This limitation of chain length due to ρ is not altered when either FTP or 8-azaGTP replace their normal counterparts.

(b) PLATEAU KINETICS WITH 8-AZAGTP AND FTP. Previous work demonstrated that the rate of RNA synthesis is not uniform during the growth of individual RNA chains, a fact which may explain the existence of the specific intermediary fractions seen with MAK column chromatography (Darlix *et al.*, 1968; Darlix *et al.*, 1969). Discontinuities in RNA synthesis (plateau kinetics) are readily demonstrated when the rate of polymerization is lowered to 20–25 nucleotides per sec by limiting the concentration of one nucleoside triphosphate to 1×10^{-5} M or less (Darlix *et al.*, 1968; Darlix *et al.*, 1969; J. L. Darlix and P. Fromageot, in preparation). Since elongation of RNA_{azaG} and RNA_F of chain length 2700–2900

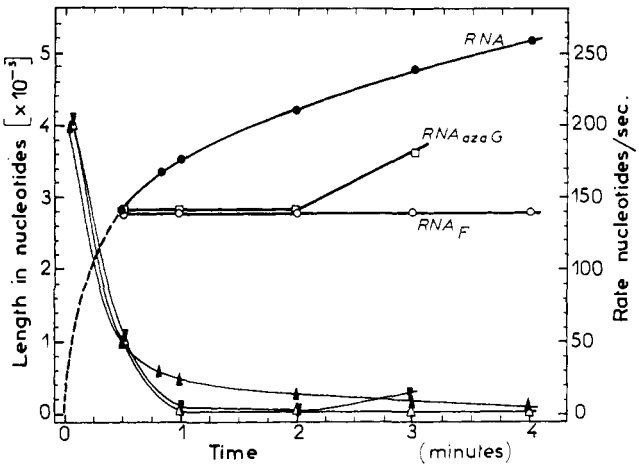

FIGURE 7: Length and chain-growth rate of RNA_{azaG} and RNA_F as a function of time after onset of synthesis. The rate is calculated as the slope of the tangent at the different time: length (●—●) and rate (▲—▲) for control; length (□—□) and rate (▼—▼) for RNA_{azaG}; length (○—○) and rate (△—△) for RNA_F.

nucleotides is delayed for some minutes (Figures 6 and 7), it appears likely that incorporation of 8-azaGMP or FMP into RNA will markedly prolong the plateaus (or stops) observed during kinetics of RNA synthesis. The data in Figure 8 show that this expectation is fulfilled. A reaction containing FTP and limiting UTP shows six plateau periods in 10 min, three of which last 1 min. Control reactions containing ATP (see Figure 18 in Darlix *et al.*, 1968) reveal only two extremely short plateaus during this period. Under conditions which exclude reinitiation by RNA polymerase the kinetics of synthesis of RNA_{azaG} show the same number of plateaus as in Figure 8.

When the concentration of UTP is reduced still further (*i.e.*, to 4×10^{-6} M) the duration of plateaus of synthesis with

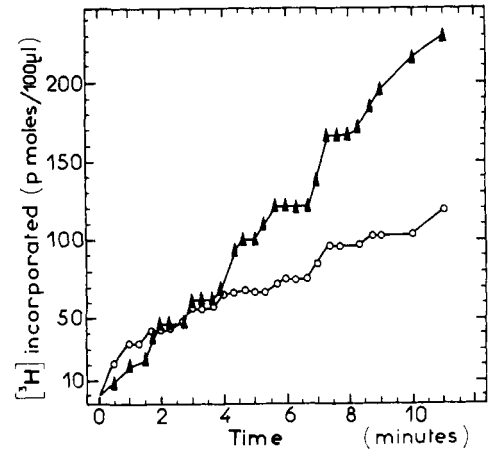

FIGURE 8: Discontinuous synthesis of RNA_F. Each of two incubations contains: calf thymus DNA, 300 μg: salt conditions I (Methods); CTP, GTP, FTP, 10^{-4} M, [^{3}H]UTP, 10^{-5} M. Final volume 3 ml: normal synthesis (▲—▲). The reaction is started by the addition of 600 units of RNA polymerase, [^{3}H]UTP, 34 cpm/pmole; synchronized synthesis (○—○) after a preincubation period of 20 min in the presence of RNA polymerase, 600 units, and without UTP. The reaction is begun by the addition of UTP and the concentration of KCl is raised to 0.6 M to prevent reinitiation by the enzyme, [^{3}H]UTP, 38 cpm/pmole. At the times indicated 100-μl aliquots are withdrawn and RNA recovered on Millipore filters by trichloroacetic acid precipitation.

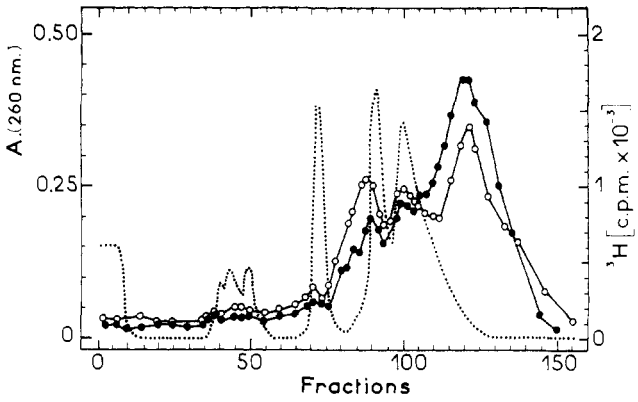

FIGURE 9: MAK column chromatography of the RNA_{azaG} and RNA_F after 1-hr synthesis with T_4-DNA as template: 8-azaGTP: T_4-DNA, 25 μg; salt conditions 3 (Methods); RNA polymerase 300 units; ATP, CTP, UTP, and 8-azaGTP, 10^{-4} M, [^{3}H]UTP, 10000 cpm/nmole. Final volume 1 ml, 1 hr at 37°. Adsorbed onto the column, 39000 cpm; recovered, 37000 cpm (●—●). FTP: as above, CTP, GTP, UTP, FTP, 10^{-4} M; [^{3}H]UTP, 8700 cpm/nmole. Adsorbed onto the column, 44000 cpm; recovered 41000 cpm (○—○).

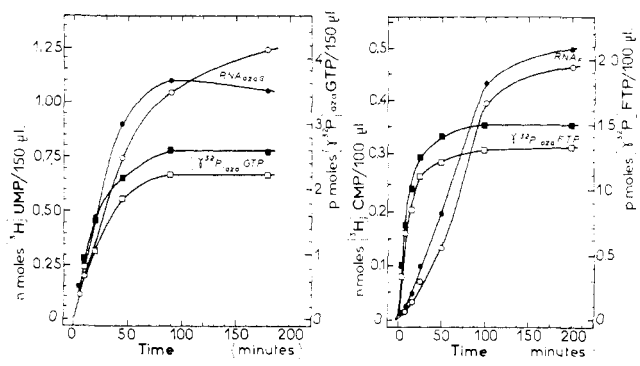

FIGURE 10: Defective release of RNA_{AzaG} and RNA_F chains synthesized with T_4-DNA as template: 8-azaGTP: T_4-DNA, 60 μg; salt conditions 3 (Methods); RNA polymerase, 600 units; ATP, CTP, [^{3}H]UTP (5000 cpm/nmole), 10^{-4} M, [γ-^{32}P]8-azaGTP (300 cpm/nmole), 1.4×10^{-4} M. Final volume 2 ml. RNA is recovered from 150-μl aliquots by trichloroacetic acid precipitation (○—○, □—□) and by direct filtration (●—●, ■—■). FTP: T_4-DNA, 60 μg; salt conditions 3 (Methods); RNA polymerase, 450 units; UTP, GTP, [^{3}H]CTP (5000 cpm/nmole), 10^{-4} M, [γ-^{32}P]FTP (600 cpm/nmole), 6×10^{-5} M. Final volume 1.5 ml. RNA is recovered from 100-μl aliquots by trichloroacetic acid precipitation (○—○, □—□) and by direct filtration (●—●, ■—■).

FTP or 8-azaGTP is increased still further. Since these plateaus represent pauses in synthesis, nucleotide polymerization with the analogs is occurring on the average only during 40% of the total period of incubation. Under comparable conditions the control reactions with normal substrates are incorporating nucleotide during 80% of the incubation time. Thus, the longer periods of interrupted synthesis produced by the analogs reduce the effective duration of polymerization reactions to one-half of that prevailing in controls. These data must be taken into account to explain the inhibition of RNA synthesis by the purine analogs.

III. Effect of Purine Analogs on Chain Release. With T_4 phage DNA as template and under conditions of high ionic strength, the release of newly formed RNA from the ternary transcription complex occurs when a chain length of at least 3000 nucleotides has been reached. This process does not require the ρ factor and the only chains ordinarily released are those initiated by ATP (Darlix *et al.*, 1969; Fromageot and Sentenac, 1969).

After RNA synthesis has been allowed to proceed for 1 hr, 30–40% of the RNA chains formed with either 8-azaGTP or FTP are longer than 3000 nucleotides (Figure 9, fraction IV). The release of chains from the synthesizing complex can be followed by the method of direct filtration through nitrocellulose filters (Sentenac *et al.*, 1968). With this technique, as shown in Figure 10, RNA chains containing incorporated 8-azaGMP or FMP are not released from ternary complexes even after incubation periods of up to 3 hr.

Discussion

The results presented in this paper provide additional insights into possible biochemical mechanisms of action of base analogs and identify some steps in transcription of DNA which may be susceptible to exogenous controls.

Our data show that individual stages in transcription can be differentiated by their response to the base analogs; these stages, which correspond to those previously defined by a variety of independent techniques, are chain initiation, elongation, and termination (and/or release). Of the three processes, the process of chain elongation appears to be the least exacting with respect to substrate specificity, since the decreased number of growing chains grow at normal rates in the presence of either analog.

Chain initiation is quite sensitive to the differences between the analogs and natural substrates. The findings with calf thymus DNA show that the sites normally initiated by both ATP and GTP are heterogeneous, since only a fraction in each class can function with the corresponding analog. Since the deficiency in initiation by the analogs is quantitatively restored on subsequent addition of the natural substrate, it seems likely that the analogs are in fact initiating chains at a restricted proportion of the normal sites.

From their response to the analogs, the initiation sequences in calf thymus DNA and T_4 phage DNA appear to differ significantly. Thus, with calf thymus DNA both analogs fail to initiate as many chains as their normal counterparts, but they do not affect chain initiation by the nonhomologous purine nucleotide (*e.g.*, FTP does not reduce initiation by GTP). In contrast, with T_4 phage DNA, the analogs reduce initiation at both homologous and nonhomologous sites. Since a conformational anomaly—the tendency to assume the syn conformation (Ward and Reich, 1968)—is common to both FTP and 8-azaGTP, this result might imply that the initiation sequences in T_4 phage DNA are more purine rich than those in calf thymus DNA.

Another facet of transcription that is sensitive to the analogs is the process which we provisionally identify as chain termination. We do not know whether the release of chains from ternary synthesizing complexes, or the plateaus corresponding to discontinuities in the rate of chain growth, are normal phenomena related to chain termination under physiological conditions *in vivo*. In any case, both of these parameters are altered by the analogs and could be related to their tendency to induce conformational abnormalities in the newly synthesized RNA (Levin and Litt, 1965; Ward and Reich, 1968). Thus, the incorporation of either analog prevents the release of chains that occurs when RNA is synthesized with natural substrates, and the brief pauses in synthesis which are seen during polymerization of natural nucleotides are greatly accentuated in frequency and duration by the analogs. Taken together, the decrease in initiation and the prolongation of

pauses during chain growth account satisfactorily for the lower rate of enzymatic RNA synthesis caused by these analogs *in vitro*. While it is less certain that similar effects are produced *in vivo*, several observations suggest that related mechanisms may also be operating in intact cells. For example, the incorporation of several analogs selectively blocks the synthesis of ribosomal RNA in mammalian cell cultures (Perry, 1963; Tavitian *et al.*, 1968). The RNA molecules that contain the analogs remain in the nucleus and are never transferred to the cytoplasm. This appears to result from the failure of maturation of the 45S rRNA precursor and might ultimately be due to impaired chain termination.

8-Azaguanine and the adenosine analog 5-bromotubercidin appear to permit selectively the expression of very few early functions of vaccinia virus (Cogniaux-Le Clerc, 1965; B. Brdar, S. Silverstein, and E. Reich, unpublished). This would be consistent with the inhibition of chain initiation or termination for specific classes of sites in the vaccinia genome.

A particularly noteworthy example concerns the synthesis of the viral RNA of Rous sarcoma virus in chick cells. The work of Temin (1969) suggests that the viral RNA is synthesized by transcription of a DNA template. Yet, the synthesis of viral RNA is resistant to two nucleoside analogs which inhibit cellular RNA synthesis almost completely (B. Brdar, D. B. Rifkin, and E. Reich, manuscript in preparation). All of these observations suggest that there are patterns of specificity in the action of analogs on genetic transcription; these might be exploited to achieve selective expression or suppression of particular regions in cellular genomes, or of special categories of RNA molecules.

References

Anthony, D. D., Wu, C. W., and Goldthwait, D. A. (1969), *Biochemistry 8*, 246.

Brockman, R. W., Bennett, L. L., Simpson, M. S., Wilson, A. R., Thomson, J. R., and Skipper, H. E. (1959), *Cancer Res. 19*, 856.

Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969), *Nature (London) 221*, 43.

Caldwell, I. C., Henderson, J. F., and Paterson, A. R. P. (1966), *Proc. Amer. Ass. Cancer Res. 7*, 11.

Chamberlin, M., and Berg, P. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 81.

Chantrenne, H. (1964), *J. Cell. Comp. Physiol. 64, Suppl. 1*, 149.

Cogniaux Le Clerc, J. (1965), *Biochim. Biophys. Acta 108*, 5.

Darlix, J. L., Sentenac, A., and Fromageot, P. (1968), *Biochim. Biophys. Acta 166*, 438.

Darlix, J. L., Sentenac, A., Ruet, A., and Fromageot, P. (1969), *Eur. J. Biochem. 11*, 43.

Fromageot, P., and Sentenac, A. (1969), 1st Lepetit Colloquium on RNA Polymerase and Transcription, Silvestri, L., Ed., North Holland, p 46.

Hori, M., Ito, E., Takita, R., Koyama, G., Takeuchi, T., and Umezawa, H. (1964), *J. Antibiot. Ser. A 17*, 96.

Ikehara, M., Murao, K., Harada, F., and Nishimura, S. (1968), *Biochim. Biophys. Acta 155*, 82.

Kahan, F. M., and Hurwitz, J. (1962), *J. Biol. Chem. 237*, 3378.

Koyama, G., Maeda, K., Umezawa, H., and Itaka, Y. (1966), *Tetrahedron Lett.*, 597.

Levin, D. H., and Litt, M. (1965), *J. Mol. Biol. 14*, 506.

Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 68.

Matthews, R. E. F. (1958), *Pharmacol. Rev. 10*, 359.

Moffat, J. G. (1967), *Methods Enzymol. 12*, 182.

Perry, R. (1963), *Exp. Cell. Res. 29*, 400.

Roberts, J. W. (1969), *Nature (London) 224*, 1168.

Roblin, R. O., Lampen, J. O., English, J. P., Cole, Q. P., and Vaughan, J. R. (1945), *J. Amer. Chem. Soc. 67*, 290.

Sentenac, A., Simon, E. J., and Fromageot, P. (1968), *Biochim. Biophys. Acta 161*, 299.

Shapiro, D., Weiss, R., and Gelhorn, A. (1950), *Cancer 3*, 896.

Smith, J. D., and Matthews, R. E. F. (1957), *Biochem. J. 66*, 323.

Sugiura, M., Okamoto, T., and Takanami, M. (1970), *Nature (London) 225*, 598.

Tavitian, A., Uretsky, S. C., and Acs, G. (1968), *Biochim. Biophys. Acta 157*, 33.

Temin, H. M. (1969), *J. Virology 4*, 554.

Ward, D. C., Cerami, A., Reich, E., Acs, G., and Alterger, L. (1969), *J. Biol. Chem. 244*, 3243.

Ward, D. C., and Reich, E. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1494.