REVERSIBLE BLOCKING OF DNA TRANSCRIPTION OF E. COLI DNA DEPENDENT RNA POLYMERASE WITH THIOPYRIMIDINE NUCLEOTIDES [1]

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SUMMARY

Transcription of DNA templates by E. coli RNA polymerase can be blocked reversibly by 4-thiouridine nucleotide replacing UTP as substrate. Nearest neighbour analysis of the partially synthesized RNA proved that adenosine clusters of the DNA template could not be transcribed with 4-thiouridine in contrast to the natural substrate or 2-thiouridine. Gel filtration permits isolation of a stable complex of RNA polymerase, DNA template and RNA in which the enzyme is fixed in an active state. This complex can serve as a tool for physicochemical studies of the enzyme turnover.

INTRODUCTION

Whereas s^4UTP substitutes for UTP during transcription of poly d-(A-T) (1) it was concluded that poly (d A) or calf thymus DNA may not be transcribed in the presence of this analog. However, further studies with synthetic templates clearly demonstrated that there is no restriction on use of thiopyrimidine nucleotides as substrates by E. coli RNA polymerase if alternating DNA sequences have to be transcribed (2). The present investigation deals with the effect of s^4UTP and s^2UTP on the transcription of calf thymus DNA, a template with a random sequence. The results show that the influence of these two analogs on RNA synthesis is different and an attempt is made to discuss the observed data on a molecular level.

MATERIALS AND METHOD

Enzyme. DNA dependent RNA polymerase was isolated from E. coli MRE 600 as described by Burgess (3), with a specific activity: 800 nmoles

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$[^3H]$ GMP incorporation x 10 min^{-1} x mg $protein^{-1}$. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the enzyme shows only β, β', α, σ bands. One unit of the enzyme incorporates 1 nmole GMP in 10 min of incubation using 10 μg thymus DNA per 100 μl incubation volume as template under standard assay conditions and with ATP, $[^3H]$ GTP, CTP, UTP, 10^{-3} M as substrates.

Assay of RNA Synthesis. The standard incubation mixture contained in a final volume of 100 μl; 4.0 μmoles of Tris·HCl, (pH 7.9), 400 nmoles $MgCl_2$, 100 nmoles of $MnCl_2$, 1.2 μmoles of β-mercaptoethanol, template, RNA polymerase and substrates in concentration as specified in legends to figures.

At various timed intervals 10 μl samples were withdrawn from the incubation mixture and spotted on glass fiber filters (GF/C, Whatman). Acid insoluble radioactivity was precipitated with 5% cold trichloracetic acid. The filters were washed 10 times with 5% trichloracetic acid containing 1% pyrophosphate and finally with alcohol ether (1:1), dried and placed into counting vials. The absorbed radioactivity was counted in standard toluene scintillation fluid.

Template. Highly polymerized calf thymus DNA was a commerical preparation (Boehringer Mannheim GmbH, Mannheim, GFR).

Chemicals. ATP, GTP, CTP and UTP were purchased from Zellstoffabrik Waldhof (Mannheim, GFR), $[8\text{-}^3H]$ GTP (12-13 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England.

The chemical synthese of s^4UTP and s^2UTP have been published elsewhere (4,5). $[\alpha\text{-}^{32}P]$ s^4UTP (1100 cpm/pmole) was synthesized according to (11).

RESULTS

Transcription of calf thymus DNA in the presence of s^4UTP and s^2UTP. If UTP is replaced by one of the two thiopyrimidine nucleotides in the incubation mixture, a drastic depression of RNA synthesis is observed. In the case of s^4UTP the incorporation of $[^3H]$ GMP reaches a plateau

Unusual abbreviations: s^4UTP, 4-thiouridine-5'-triphosphate, s^2UTP, 2-thiouridine-5'-triphosphate; the abbreviations for polynucleotides follow CBN rules see Eur. J. Biochem. 15 (1970) 203.

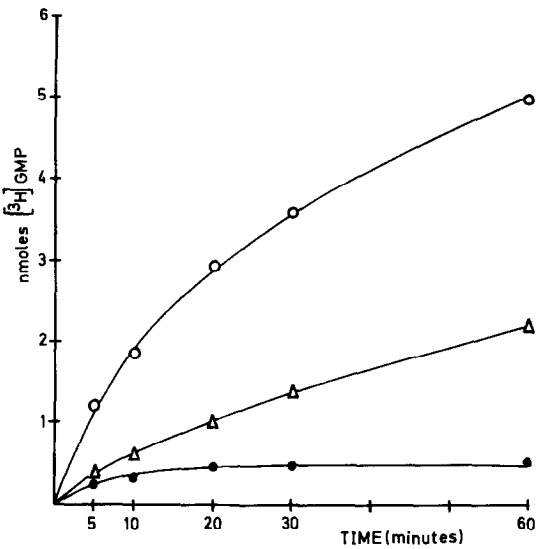

Figure 1

Transcription of calf thymus DNA template with thiouridine nucleotides. Each of the three incubation mixtures contains: calf thymus DNA, 10 µg; RNA polymerase, 2 units (see materials and methods); standard salt conditions; ATP, CTP, [^{3}H] GTP (10 000 cpm/nmole), 10^{-3} M. Final volume 100 µl; 10 µl aliquots were withdrawn at the indicated times for the RNA assay:

o—o incorporation of [^{3}H] GMP with 10^{-3} M UTP present;

Δ—Δ incorporation of [^{3}H] GMP with 10^{-3} M s^2UTP replacing UTP;

•—• incorporation of [^{3}H] GMP with 10^{-3} M s^4UTP replacing UTP;

after about 20 minutes of incubation. In contrast a continuous RNA synthesis is observed after 60 minutes of incubation with s^2UTP (fig 1). From these results the conclusion can be drawn that s^2UTP acts as a normal but less effective substrate compared with UTP for DNA transcription, whereas s^4UTP inhibits RNA synthesis after a definite period of transcription. On the other hand these two analogs have to be present in relatively high concentration to suppress RNA synthesis when the transcription is started with all four natural nucleotides as substrates (Table 1).

To elucidate further the timed inhibition of RNA synthesis by s^4UTP the following experiments and controls were made.

Restart of RNA synthesis after inhibition with s^4UTP.

These experiments are illustrated in fig 2. Under standard assay con-

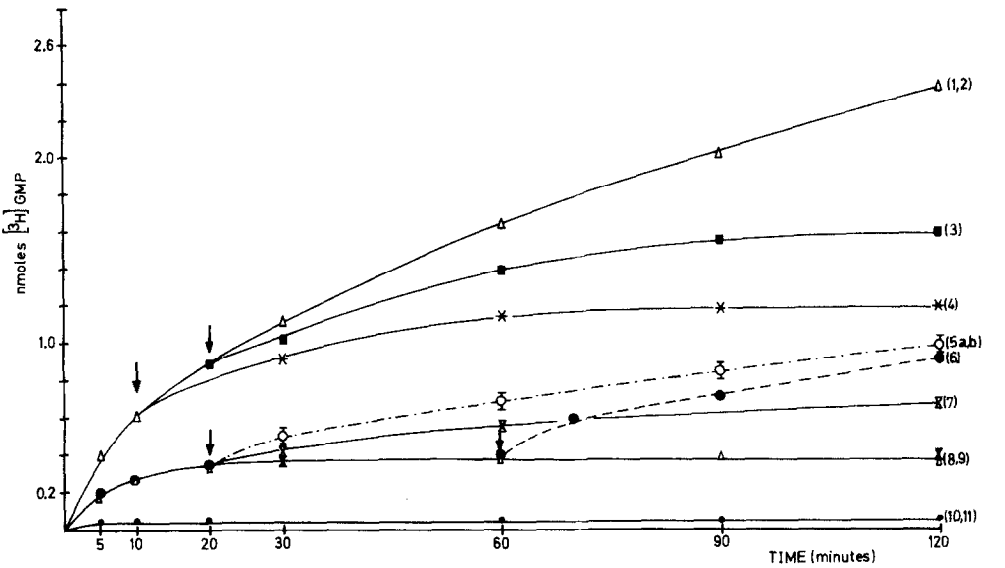

Figure 2

Transcription of calf thymus DNA template in the presence of s^4UTP. Each of the incubation mixtures contains: calf thymus DNA. 10 µg; RNA polymerase, 2 units (see materials and methods); standard salt conditions; ATP, CTP, [^{3}H] GTP (10 000 cmpm/nmole), 10^{-3} M. Final volume 100 µl; 10 µl aliquots were withdrawn at the indicated times for the RNA assay:

Δ—Δ experiment 1, UTP, 5 x 10^{-3} M; experiment 2, UTP, 5 x 10^{-3} M; s^4UTP, 10^{-3} M;

■—■ experiment 3, UTP, 5 x 10^{-3} M; rifampicin, 1 µg was added at the time indicated by arrow;

✳—✳ experiment 4, the same conditions as in experiment 3;

Ø—Ø experiment 5a, s^4UTP, 10^{-3} M; UTP 5 x 10^{-3} M was added at the time indicated by arrow;

experiment 5b, s^4UTP, 10^{-3} M; UTP, 5 x 10^{-3} M and rifampicin, 1 µg, was added at the time indicated by arrow;

●—● experiment 6, conditions the same as in experiment 5a;

⧖—⧖ experiment 7, s^4UTP, 10^{-3} M; additional 3 units RNA polymerase were added at the time indicated by arrow;

⧗—⧗ experiment 8, s^4UTP, 10^{-3} M;

Δ—Δ experiment 9, s^4UTP, 10^{-3} M; additional 10 µg calf thymus DNA were added at the time indicated by arrow;

●—● experiment 10, UTP, 5 x 10^{-3} M; DNA omitted;
experiment 11, UTP 5 x 10^{-3} M; rifampicin, 1 µg; 10 minutes preincubation of rifampicin and all reactants except DNA and [^{3}H] GTP; started with addition of DNA and [^{3}H] GTP.

Table 1

Influence of the RNA synthesis by thiouridine analogs

Added thiouridine (nmoles)	Incorporation of ^{3}H GMP after 10 minutes in the presence of s^4UTP	s^2UTP
100	84 %	90%
200	78 %	80%
300	60 %	65%
400	50 %	60%

The incorporation of [^{3}H]GMP in the absence of thiouridine analogs is taken as 100 %.
Each of the incubation mixtures contains: calf thymus DNA, 10 µg; RNA polymerase, 2 units (see materials and methods); standard salt conditions; ATP, CTP, [^{3}H] GTP (10 000 cpm/nmole), UTP, 10^{-3} M; thiouridine-5′-triphosphate in concentrations as indicated. Final volume 100 µl.

ditions RNA synthesis continues for more than 120 minutes (fig 2, experiment 1) also in the presence of 1 mM s^4UTP (fig 2, experiment 2). If UTP was replaced by s^4UTP, RNA synthesis reached a plateau after 20 minutes (fig 2, experiment 8). On addition of excess UTP after 20 minutes (experiment 5a) or 60 minutes (experiment 6) RNA synthesis could be restarted. The latter experiments show that the complex of RNA polymerase DNA and RNA is still active after this time period. Rifampicin, which is known to suppress only the initiation reaction of RNA polymerase (6) was without influence on the renewed RNA synthesis, from which it follows that primarily elongation of RNA was reassumed (experiment 5b).
An increase in RNA synthesis was also observed by addition of new enzyme after 20 minutes incubation (fig 2, experiment 7); additional DNA shows no effect (fig 2, experiment 9).

The net RNA elongation in the presence of all 4 natural substrates was studied by addition of rifampicin after 20 minutes (experiment 3). 0.6 nmoles GMP were incorporated from 20 to 120 minutes. The same additional incorporation of GMP was observed when RNA synthesis was restarted on addition of UTP to a formerly s^4UTP substituted assay (experiment 5a).

Nearest neighbour analysis of RNA synthesized in the presence of $[\alpha\text{-}^{32}P]$ s^4UTP.

The nearest neighbour analysis was carried out as described by Cramer et al. (1). The standard incubation mixture contained in 200 µl: calf thymus DNA, 20 µg; RNA polymerase, 5 units (see materials and methods); ATP, CTP, GTP, $[\alpha\text{-}^{32}P]s^4$UTP (1100 cpm/pmole), 10^{-3} M. The incubation was performed at 37° for 30 minutes. The distribution of the radioactive label found after alkaline hydrolysis was: AMP 42.8% (2400 cpm), GMP 19.6% (1100 cpm), CMP 37.5% (2100 cpm), s^4UMP 0%. The total radioactivity of the nucleotide spots was taken as 100%, 50 cpm background were substracted.

Restart of RNA synthesis using the isolated complex of RNA polymerase, DNA and RNA.

The standard incubation mixture contained in 100 µl: calf thymus DNA, 10 µg; RNA polymerase, 2 units (see materials and methods); ATP, CTP, s^4UTP, $[^3H]$ GTP (10 000 cpm/nmole) 10^{-3}M. After 30 minutes incubation the mixture was applied on a Sephadex G 100 column (5 x 70 mm), which was equilibrated with the standard incubation buffer. The column was washed with the same buffer at 10° and fractions of 2 drops (60 µl) were collected. 10 µl aliquots were withdrawn from each fraction and the radioactivity was measured. The peak fraction which contains the polymer materials was used for the following experiment. To 50 µl of this fraction 1 µg rifampicin, 500 nmoles UTP, 100 nmoles ATP, CTP, $[^3H]$GTP and buffer were added to give a final volume of 100 µl. After 60 minutes incubation the incorporation of $[^3H]$GMP into acid-insoluble polymer was measured. An additional incorporation of 1.5 nmoles $[^3H]$ GMP was observed. No RNA synthesis could be detected if no substrates were added to the isolated complex.

DISCUSSION

Taking into account the picture which was proposed by Kapuler (7), the

following may be concluded. In principle 3', 5' -phosphodiester bridges between 4-thiouridine and adjacent nucleoside units are possible regardless of whether 4-thiouridine occupies the product terminus site or the substrate binding site of the RNA polymerase molecule. On the other hand the synthesis of the 3',5' -phosphodiester bond between two 4-thiouridine residues seems to be impossible. In this case a stable but not actively synthesizing complex is formed and, as shown with the restart experiments, s^4UTP could be displaced by UTP on the substrate binding site and RNA synthesis start again. The experiments in the presence of rifampicin show that the complex bound enzyme is not inactivated for at least up to 60 minutes in the presence of s^4UTP.

The stabilizing effect of s^4UTP was further demonstrated by isolation of the active complex composed of RNA polymerase, DNA and RNA by Sephadex gel filtration. In the presence of high amounts of rifampicin RNA synthesis could be restarted by addition of substrates. There is no competition between this complex and additional DNA with respect to the enzyme. The used DNA template is not blocked during the transcription in the presence of s^4UTP since additional enzyme is able to synthesize new RNA.

The reported properties of s^4UTP can, we believe, be related to the anomalous reciprocal action of this analog with the DNA template. First evidence for an unusual base pairing of 4-thiouridine and adenosine comes from the work of Saenger et al. (8) who reported the molecular structure of the crystalline complex 1-methyl-4-thiouracil and N^9-methyladenine. These studies revealed a reversed Hoogsteen base pairing involving the 2-ketogroup of the uracil residue instead of the 4-thio-ketogroup. If this is more or less true for the situation on the active site of the enzyme one must conclude that the penultimate normal base pair and a following normal base pair will push a 4-thiouridine residue into the proper position which is needed by the enzyme to built a phosphodiester bond. This will be the case in transcribing alternating sequences. This effect fails if two adjacent base pairs contain 4-thiouridine residues. In this case the RNA synthesis is inhibited.

The impossibility of transcribing adenosine clusters with s^4UTP was shown by nearest neighbour analysis of the synthesized RNA in the presence of

$[\alpha\text{-}^{32}P]s^4UTP$. Since no radioactivity was found with s^4UMP after alkaline hydrolysis no 4-thiouridine clusters were formed during transcription.

The given interpretation is further supported by the finding of Lezius (9) who obtained similar results using DNA polymerase from E. coli and 4-thiothymidine.

The presented data give a possible explanation for the observed cytotoxic effects of thiopyrimidine nucleotides. It was found that the most effective agent was 4-thiouridine (10) which now could be interpreted by its interference with RNA synthesis if adenosine clusters of the DNA template have to be transcribed.

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