THE SYNTHESIS OF THE ALTERNATING COPOLYMER POLY[r (G-s^2 C)] BY RNA POLYMERASE OF *E. COLI*. A POLYNUCLEOTIDE WITH AN EXTREMELY HIGH THERMAL STABILITY†

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1. Introduction

Whereas s^4 U*TP [1] and s^2 UTP may substitute for UPT during RNA polymerase dependent transcription of poly [d(A-T)] quite different properties of these analogs are observed when used as substrates for the transcription of randomly sequenced DNA. In the latter case s^2 UTP acts as a normal substrate whereas s^4 UTP stops transcription specifically at adenosine clusters [3]. The different behavior of s^4 UTP was ascribed to a direct involvement of the 4-thio keto group in Watson–Crick base pairing.

To prove this assumption a similar modification of CTP was studied in order to introduce a different hydrogen bonding site in nucleic acids. Hence this paper describes the transcription of poly [d(G-C)] and poly [d(I-C)] with DNA dependent RNA polymerase substituting s^2 CTP for CTP and the characterisation of the formed RNA poly [r(G-s^{2}C)].

2. Materials and methods

2.1. *Enzymes*

DNA-dependent RNA polymerase (EC 2.7.7.6) was prepared from *E. coli* MRE 600 cells by modification of the methods of Burgess [4] and Zillig [5]. The procedure will be described in detail elsewhere [6]. The purity of the enzyme was greater than 98% based on SDS gel electrophoresis. The specific activity was 950 nmoles [^{3}H]GMP incorporated × 10 min^{-1} × mg protein^{-1} under standard assay conditions [3].

Snake venom phosphodiesterase (EC 3.1.4.1) was supplied by Boehringer, Mannheim, Bovine pancreas, Deoxyribonuclease I (EC 3.1.4.5) was from Worthington.

2.2. *Assay for RNA synthesis*

The assay was described previously [3]. The standard incubation mixture contained in a final volume of 100 µl: 4.0 µmoles Tris–HCl, (pH 7.9), 400 nmoles $MgCl_2$, 100 nmoles $MnCl_2$, 1.2 µmoles β-mercaptoethanol, template, RNA polymerase and substrates as specified in the legends to figures.

2.3. *Templates*

Poly-deoxy-inosinic-deoxy-cytidylic acid, poly [d(I-C)] and poly-deoxy-guanylic-deoxy-cytidylic acid, poly [d(G-C)] were products of Boehringer, Mannheim.

2.4. *Chemicals*

Unlabeled ribonucleoside triphosphates were obtained from Zellstofffabrik Waldhof, Mannheim, and ^{3}H- and ^{32}P-labeled ribonucleoside triphosphates from the Radiochemical Centre, Amersham, England.

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* *Unusual abbreviations:* s^{4}U, 4-thiouridine; s^2 U, 2-thiouridine; s^{2}C, 2-thiocytidine; the abbreviations for polynucleotides follow CBN rules see Eur. J. Biochem (1970) 15, 203, SDS, sodium dodecyl sulfate;

2.4.1. *2-Thiocytidine-5′-triphosphate*

The synthesis of 2-thiocytidine-5′-phosphate has been described [7]. The conversion of 2-thiocytidine-5′-phosphate to 2-thiocytidine-5′-triphosphate was carried out by a standard procedure [8]. The triphosphate was obtained as the amorphous sodium salt in 38% yield. The s^2C/P_i ratio was 1:3.04, using ϵ_{250} = 22300 M^{-1} cm^{-1} in water at pH 7. Ultraviolet absorption (water, pH 7) λ_{max} 270 nm; absorbance ratio A_{250}/A_{270} = 1.31.

2.5. *Spectrophotometric measurements*

UV absorption spectra were recorded with a Hitachi 181 or a Cary 15 spectrophotometer. Thermal transitions were measured spectrophotometrically in thermostated quartz cuvettes. The temperature was determined directly in the cuvette with a calibrated Pt-resistor with digital readout (accuracy of 0.2%). The measuring device was built in our workshop. The temperature of the solution was increased 2–3°C per 10 min and absorption was recorded after temperature equilibration.

2.6. *Synthesis of poly [r(G-s²C)]*

The incubation mixture contained the above described Tris–HCl buffer (1 ml), 0.8 A_{260} units of poly [d(I-C)], 1 μmole [^{3}H]GTP, 1 μmole s^2CTP and 100 units RNA polymerase (see 2.1). The mixture was incubated for 3 hr at 37°C, then 20 μg DNase 1 was added and the mixture was incubated for one more hour. To the incubation mixture were added 150 μl 1% SDS and 100 μl 1 M NaCl. Protein was extracted with chloroform–isoamyl-alcohol (5:2, v/v). The aqueous solution was dialysed 48 hr against two 1 L portions of 1 mM sodium cacodylate, pH 7 and subjected to Sephadex G-100 (superfine) filtration (column size 100 × 15 mm). Sodium cacodylate 1 mM, pH 7 was the eluent.

The fractions in the void volume of the Sephadex column were stored frozen.

The yield was 7.05 A_{250} units poly [r(G-s^2C)].

2.7. *Nearest neighbour analysis*

The polynucleotides were synthesized using the standard incubation mixture and 11 units of RNA polymerase in a final volume of 100 μl. In Expt. 1: [α-^{32}P] GTP (25 000 cpm/nmole), 10^{-3} M; CTP 10^{-3} M and 0.08 A_{260} units poly [d(I-C)]; in Expt. 2: [α-^{32}P]GTP (25 000 cpm/nmole), 10^{-3} M; CTP, 10^{-3} M; 0.075 units A_{260} poly [d(G-C)]; in Expt. 3: [α-^{32}P]GTP (25 000 cpm/nmole), 10^{-3} M; s^2CTP, 10^{-3} M; 0.08 units A_{260} poly [d(I-C)] and in Expt. 4: [α-^{32}P]GTP (25 000 cpm/nmole), 10^{-3} M; s^2CTP, 10^{-3} M; 0.075 units A_{260} poly [d(G-C)] were added.

Incubations were at 37°C for 3 hr. Five 20 μl samples from each incubation mixture were spotted on paper strips (Whatman 3 MM, 2 × 10 cm), and chromatographed in ethanol, 1 M ammonium acetate (1:1, v/v). The starting zone of each strip was cut out

Table 1
Nearest neighbour analysis

Substrate	Template	Radioactivity found after alkaline hydrolysis			
		By paper chromatography*		By chromatography on PEI cellulose**	
		GMP R_f = 0.43	CMP-s^2CMP R_f = 0.6	GMP-s^2CMP R_f = 0.31	CMP R_f = 0.65
		cpm	cpm	cpm	cpm
GTP, CTP	poly[d(I-C)]	–	26 600	–	25 320
GTP, s^2CTP	poly[d(I-C)]	–	17 330	14 210	–
GTP, CTP	poly[d(G-C)]	210	8920	90	3200
GTP, s^2CTP	poly[d(G-C)]	150	7200	140	6600

Details are given in Materials and methods, 50 cpm background were subtracted.
* Solvent: isopropanol–ammonia–water (60:10:30, v/v/v);
** Solvent: 1 M LiCl.

and digested in 3 ml 0.3 M KOH for 18 hr at 25°C. The supernatant liquid was neutralized with 60% $HClO_4$ in the cold and the precipitated $KClO_4$ was removed by centrifugation and the supernatant was evaporated to a volume of about 500 µl. Aliquots of this solution were mixed with unlabeled GMP, CMP and s^2CMP and subjected to descending paper chromatography (Schleicher & Schüll 2040 a), using isopropanol, concentrated ammonia, water (60:10:30, v/v/v) as solvents. In addition chromatography on PEI cellulose (Schleicher & Schüll) with 1 M LiCl as solvent was used. In the first system CMP and s^2 CMP had the same R_f value and were well resolved from GMP. In the second system GMP and s^2 CMP had the same R_f value and were well resolved from CMP.

Following chromatography the nucleotides were spotted by UV light and the spots were cut out or scraped off thin layer plates. Radioactivity was measured in standard toluence scintillation fluid. The results are given in table 1.

2.8. *Sedimentation velocity measurements of poly [r(G-s²C)]*

Sedimentation velocity measurements were performed in a Spinco Model E analytical ultracentrifuge equipped with a RTIC unit and a photoelectric scanner. Double sector cells with 12 nm optical path and sapphire windows were used. The speed was 30 000 rpm and the temperature 20°C. $s_{20,w}$ values were calculated from photoelectric scanner tracings (250 nm) by the moving boundary method and using appropriate corrections. Poly [r(G-s^2C)] had a $s_{20,w} \times 10^{13}$ value of 7.8.

3. Results

3.1. *Kinetics of poly [d(I-C)] and poly [d(G-C)] Transcription*

Poly [d(I-C)] or poly [d(G-C)] directs the incorporation of GMP and CMP into the alternating polynucleotide poly [r(G-C)] by *E. coli* RNA polymerase. Substitution of CTP by s^2 CTP leads to the synthesis of the alternating copolymer poly [r(G-s^2C)] as shown by nearest neighbour analysis (see table 1). The dependency of initial velocity of GMP incorporation on GTP-CTP or GTP-s^2CTP concentrations under saturation conditions with respect to template is

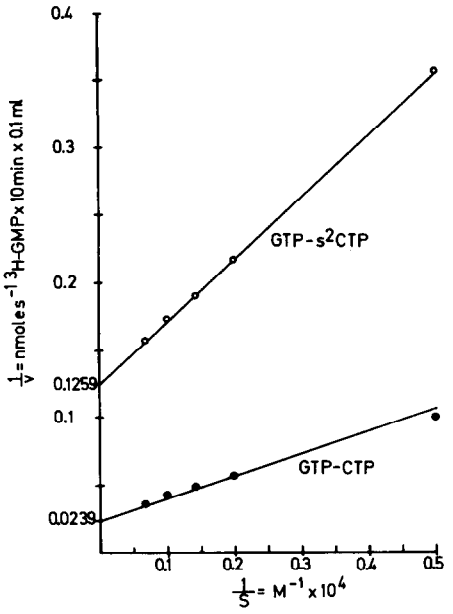

Fig. 1. The kinetics of poly [d(I-C)] transcription with either GTP–CTP or GTP–s^2CTP. The standard incubation mixture (see Materials and methods) contained in addition: 0.08 A_{260} units poly [d(I-C)] and 3 units RNA polymerase (see Materials and methods), and GTP–CTP or GTP–s^2CTP in concentrations given on the abscissa. Final volume: 100 µl. After 10 min at 37°C aliquots of the incubation mixture were assayed for RNA synthesis as described in Materials and methods.

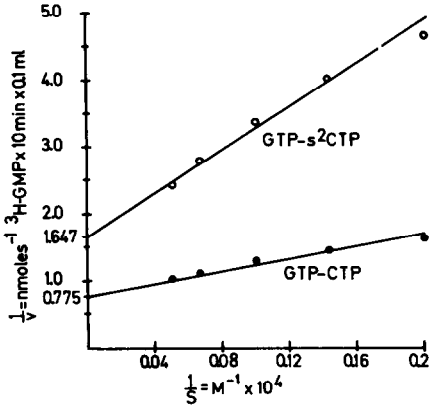

Fig. 2. The kinetics of poly [d(G-C)] transcription with either GTP–CTP or GTP–s^2CTP. The conditions were the same as in fig. 1, except that 0.075 A_{260} units poly [d(G-C)] were used as template.

Table 2
Apparent K_m and V_{max} values

Substrate	Template polymer	V_{max} nmoles GMP × mg^{-1} × min^{-1}	$K_M \times 10^{-4}$ M
GTP–CTP	[d(G-C)]	41	6
GTP–s^2CTP	[d(G-C)]	19	9.9
GTP–CTP	[d(I-C)]	1322	7
GTP–s^2CTP	[d(I-C)]	250	3.6

shown in figs. 1 and 2. Poly [d(G-C)] or poly [d(I-C)] served as templates. The kinetic data and the derived Lineweaver–Burk graphs were calculated with a Wang 702 computer and plotted automatically. The values for K_m and V_{max} are given in table 2.

3.2. *Physical properties of poly [r(G-s^2C)]*

The absorption spectrum of poly [r(G-s^2C)] had a maximum at 246 nm and a shoulder at 270 nm. The weak band at 325 nm may be due to a η-π^* transition [9]. Enzymatic hydrolysis causes to a slight red shift of the maximum to 248 nm and as expected drastic hyperchromocity. The spectra are in fig. 3.

The GMP/s^2CMP ratio of the polynucleotide as derived from the absorption spectra after enzymic hydrolysis was 1.03. The calculation was as follows:

$$A_{270} = \epsilon_{270,s^2C} \cdot c_{s^2C} + \epsilon_{270,G} \cdot c_G; \quad (1)$$

$$A_{250} = \epsilon_{250,s^2C} \cdot c_{s^2C} + \epsilon_{250,G} \cdot c_G; \quad (2)$$

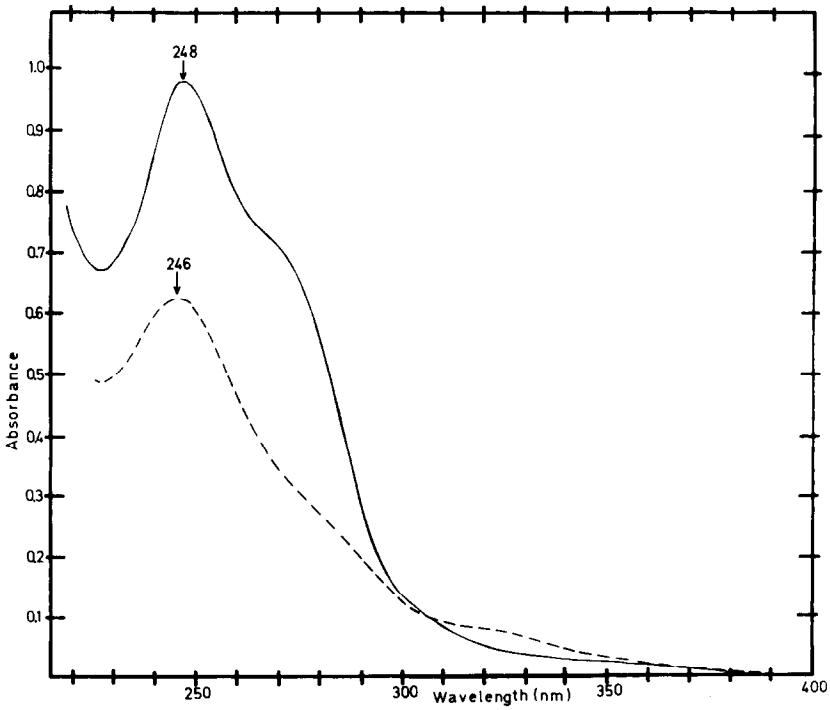

Fig. 3. UV absorption spectrum of poly [r(G-s^2C)]. -------, poly [r(G-s^2C)] in 10^{-3} M Na-cacodylate buffer, pH 7, at 20°C; ———, poly [r(G-s^2C)] after incubation with snake venom phosphodiesterase (5 μg/ml) for 4 hr at 37°C in 10^{-3} M Na-cacodylate buffer, pH 7.0.

s^2C: $\epsilon_{250} = 22.3 \times 10^3\ M^{-1}\ cm^{-1}$, $\epsilon_{270} = 17.1 \times 10^3\ M^{-1}\ cm^{-1}$;
G: $\epsilon_{250} = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$, $\epsilon_{270} = 9.63 \times 10^3\ M^{-1}\ cm^{-1}$;

After enzymatic hydrolysis: $A_{250} = 0.955$; $A_{270} = 0.711$. The poly [r(G-s^2C0] did not undergo thermal transition between 3 to 98°C, even at sodium cacodylate concentrations as low as 10^{-3} M. In order to show helix–coil transitions a more hydrophobic solvent had to be used. In 50% aqueous ethylene glycol a thermal transition was observed with a midpoint of 58° (fig. 4). The thermal transition was however irreversible presumably due to the high ethylene glycol concentration. Reversibility of the transition was achieved by dilution to an ethylene glycol concentration of 25%. This is in agreement with E. L. Duggan's work [10]. The increased temperature interval of the transition is probably due to the higher ethylene glycol concentration. The data are summarized in table 3.

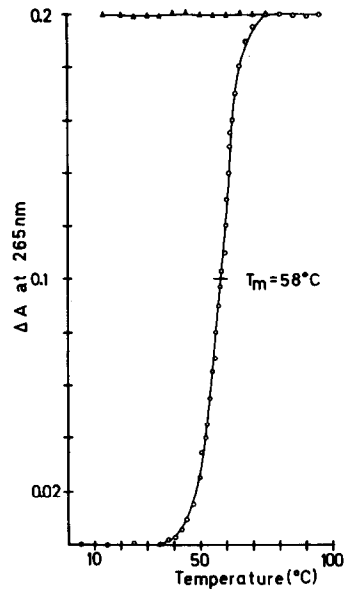

Fig. 4. Temperature profile of poly [r(G-s^2C)]. (o-o-) poly [r(G-s^2C)] in 10^{-3} M Na-cacodylate, 50% ethylene glycol (v/v) buffer, ph 7.0; (– –) poly [r(G-s^2C)], during cooling to 15°C; ΔA is defined as: $A_t o - A_{10} o$ at 265 nm; A_{265} before melting was 0.384.

4. Discussion

The synthesis of poly [2(G-s^2C)] by *E. coli* RNA polymerase either by transcription of poly [d(G-C)] or poly [d(I-C)] was shown by absorption spectra, enzymatic hydrolysis, the nucleotide GMP-s^2 CMP ratio, nearest neighbour analysis and ultracentrifugal analysis. A comparison of the kinetics of GMP incorporation with GTP-CTP and GTP-s^2CTP as substrates in the presence of poly [d(G-C)] or poly [d(I-C)] indicated significant differences only with respect to the V_{max} values. The temperature profile of poly [r(G-s^2C)] absorption did not show thermal transition between 3 to 98°C, indicating an extremely high thermostability of the copolymer. Transition occurred however in a hydrophobic solvent (e.g. 50% aqueous ethylene glycol).

The consequence of replacing the 2-keto group of cytidine by a 2-thioketo group on the stability of the poly (r-I) : poly (r-s^2C) complex was already discussed previously [9]. In this paper the high thermal stability of this complex was interpreted as to indicate

Table 3
Properties of Poly [r(G-s^2C)]

λmax	Total hyperchromicity after enzymatic hydrolysis	Hyperchromicity due to helix–coil transition	$\epsilon_{(P)}$ at 246 nm	Absorbance ratio A_{246}/A_{270}
nm	%	%	1.98×10^4	1.78
246	59 (250 nm)			
270 (shoulder)	85 (265 nm)	52 (265 nm)		

Hyperchromicity was defined as $\Delta A/A_{polymer} \times 100$. $\epsilon_{(P)}$ values (absorbance coefficient per mole phosphorus) were calculated using the following ε-coefficients (water, pH 7): $\epsilon^{GMP}_{250} = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$: $\epsilon^{s^2CMP}_{250} = 22.3 \times 10^3\ M^{-1}\ cm^{-1}$.

enhanced vertical interactions of neighbouring 2-thiocytosine bases. Furthermore it was concluded that vertical interactions between neighbouring 2-thioketo pyrimidine bases stabilize quite generally polynucleotide structures [11]. The question therefore arose whether the stabilizing effect observed with homopolynucleotides and their complexes also exists in polymers with alternating sequences. The data presented here demonstrate this to be the case. The stabilizing effect of the 2-thioketo group was retained also when vertical interactions occured between 2-thioketo pyrimidine and neighbouring purine bases. Since poly [r(G-C)] melts normally at 92.1°C [12] the substitution of 2-thiocytidine for cytidine caused a drastic increase in stability of poly [r(G-s^2C)] with a putative T_m far above 100°C.

Similar results were obtained with alternating polynucleotides containing thiouridine nucleotides [2]. Thus it seems to be established now that 2-thioketopyrimidine bases in polynucleotides stabilize the structure of these polymers. This makes these structures attractive in cases where stable nucleic acid polymers are required.

Acknowledgements

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