Selective in Vitro Transcription of the 5S RNA Genes of a DNA Template†

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ABSTRACT: RNA polymerase III is responsible in vivo for the synthesis of 5S ribosomal RNA. In the present study, transcriptional specificity of partially purified RNA polymerase III in a homologous system containing calf thymus enzyme and calf thymus DNA has been investigated by using DNA complementary to 5S ribosomal RNA (5S cDNA) as a probe and hybridizing RNA synthesized enzymically to this probe. The 5S cDNA probe hybridizes to 5S RNA with a $R_0t_{1/2}$ of 10^{-3} and to calf thymus DNA (in which there are approximately 1500–3000 5S ribosomal RNA genes per haploid genome, 0.005% of the genome) with a $C_0t_{1/2}$ of 2.1. Hybridization of the probe to RNA transcribed by RNA polymerase III from large (10^7 daltons) double-stranded DNA is observed at $R_0t_{1/2}$'s between 0.03 and 0.06, indicating that between 1.6% and 3.5% (2.6 ± 0.88%) of the transcripts are 5S ribosomal RNA and that transcription of the 5S ribosomal RNA genes is up to 700-fold greater than random transcription of the genome. The percentage of 5S ribosomal RNA transcripts is less with smaller DNA as template. Selective transcription is also observed when denatured DNA is used as template. Concordant results are obtained when RNA is synthesized with either Mg^{2+} and/or Mn^{2+} as metal cofactor. Selective transcription of 5S ribosomal RNA genes by calf thymus RNA polymerase III is also observed with salmon sperm DNA as template. RNA transcribed by *Escherichia coli* RNA polymerase in the presence of Mg^{2+} and Mn^{2+} contains respectively 0.3% and 0.02% 5S rRNA. These results suggest that (1) 5S rRNA genes are preferentially transcribed by heterologous (bacterial) as well as homologous polymerase and (2) partially purified calf thymus RNA polymerase III possesses an intrinsic specificity for transcription of 5S ribosomal genes of calf thymus and salmon sperm DNAs.

The multiplicity of eucaryotic RNA polymerases suggests specific functions for each enzyme and evidence has been obtained indicating that, in vivo, polymerase I synthesizes 18S + 28S ribosomal RNA precursor (Reeder & Roeder, 1972), polymerase II synthesizes messenger RNA precursor (Blatti et al., 1970; Zylber & Penman, 1971; Price & Penman, 1972), and polymerase III synthesizes transfer RNA precursor and 5S ribosomal RNA (Marzluff et al., 1974; Weinmann & Roeder, 1974).

The existence of multiple RNA polymerases might suggest, a priori, that each polymerase would selectively recognize a specific class of promotor sites on DNA, and it has recently been demonstrated that yeast RNA polymerase I has the intrinsic ability to selectively transcribe a DNA template (Holland et al., 1977; Van Keulen et al., 1975; Van Keulen & Retel, 1977).

RNA polymerase III is similar to RNA polymerase I in that in vivo it transcribes genes which are present in the genome in multiple copies. It was, therefore, of interest to evaluate in vitro transcription of one of these genes, that for 5S ribosomal RNA. In these studies, DNA complementary to 5S ribosomal RNA has been used as a probe for selective transcription.

Materials and Methods

Calf thymus nuclei were obtained as described previously (Henner et al., 1975). DNA was prepared from nuclei obtained from 15 g of calf thymus. Nuclei were lysed in 100 mL of 10 mM Tris-Cl (pH 7.5) containing 10 mM NaCl, 10 mM EDTA, and 0.1% $NaDodSO_4$ by slow rolling in bottles for 2–3 days at 19 °C. RNases A (1 mg) and T_1 (2500 units) were boiled for 10 min, quick cooled, added to the DNA solution, and slow-rolled for 4 h, after which Pronase (Sigma) (autodigested 30 min at 37 °C) (26 mg) was added and the solution slow rolled for another 4 h. An equal volume of phenol (80%, redistilled) was added, along with NaCl to 500 mM and Tris-Cl (pH 7.5) to 50 mM. The mixture was rolled for 3 h at a time followed by low-speed centrifugation. The organic phase was removed, fresh phenol solution added, and the extraction repeated. After 2 days of repeated extraction, the DNA solution was dialyzed against 1 L of 10 mM Tris-Cl (pH 7.5) containing 10 mM NaCl and 1 mM EDTA for 48 h with four changes of buffer. The DNA was stored at 4 °C. The single-stranded molecular weight of the DNA, as determined by alkaline–sucrose density gradient centrifugation, was 10^7.

In some experiments, calf thymus DNA (Worthington) and salmon sperm DNA (Sigma) obtained commercially were used. These DNAs were further purified by phenol extraction in the presence of 50 mM Tris-Cl (pH 7.5) and 500 mM NaCl. The molecular weight of this calf thymus DNA was 1.3×10^6 (average, single-stranded); salmon sperm DNA averaged 2.5×10^6 daltons. DNA was denatured by incubating DNA at 0 °C in 0.5 N NaOH for 16 h, followed by dialysis against water.

Calf thymus RNA polymerase III was isolated as described previously (Furth et al., 1970; Keshgegian et al., 1975b). Briefly, tissue was homogenized in low salt buffer (50 mM Tris-Cl) and the extract clarified by high-speed centrifugation. RNA polymerase III was precipitated with protamine sulfate, eluted with sodium succinate, concentrated with $(NH_4)_2SO_4$, and chromatographed on phosphocellulose. After again concentrating the enzyme with $(NH_4)_2SO_4$, it was further purified by DEAE-Sephadex chromatography (Keshgegian et al., 1975a).

The specific activity of partially purified RNA polymerase III, which was free of RNA polymerases I and II (Keshgegian et al., 1975a), was approximately 15 units/mg of protein (1 unit of RNA polymerase incorporates 1 nmol of UTP into

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acid-insoluble product in 10 min at 37 °C with native calf thymus DNA as template).

E. coli RNA polymerase, obtained as previously described (Furth & Pizer, 1966), was further purified by DEAE-Sephadex chromatography.

RNA was synthesized in a reaction mixture containing (in 0.5 to 12 mL): 50 mM Tris–maleate (pH 7.7), $MgCl_2$, and/or $MnCl_2$, 5 mM dithiothreitol or mercaptoethanol, $(NH_4)_2SO_4$, or KCl, 320 μM each of ATP, GTP, and UTP, and 60 μM [^{14}C]CTP (12 counts min^{-1} pmol^{-1}); DNA at 0.1–0.6 mg/mL and enzyme at 0.5–3.3 units/mL were added to obtain the desired enzyme:DNA ratio.

Isolation of RNA Synthesized in Vitro. Eighty micrograms of *E. coli* rRNA was added to the reaction mixture, the concentration of $MgCl_2$ was adjusted to 10 mM, and 50 μg/mL DNase I (Worthington) was added. After 30 min at 37 °C, $NaDodSO_4$ was added to 0.1% followed first by phenol:*m*-cresol:H_2O:8-hydroxyquinoline (7:1:2:0.1) and then by chloroform:isoamyl alcohol (25:1) extraction. The RNA was precipitated from the aqueous phase with ethanol and nucleotides were removed by gel filtration (Sephadex G-50) in 0.2 mM EDTA and 2 μg of heparin/mL and lyophilized to dryness.

To eliminate DNA fragments remaining after DNase digestion, the RNA was further purified by chromatography on acetylated *N*-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl]-aminoethyl (DBAE[1])-cellulose (Collaborative Research) by using the procedure of McCutchan et al. (1975), somewhat modified. Lyophilized RNA was resuspended in 0.5 mL of 0.05 M *N*-methylmorpholine (pH 7.7) (Aldrich) containing 0.2 M NaCl, 10 mM $MgCl_2$, and 20% ethanol. RNA was heated at 100 °C for 3 min, cooled in ice water and incubated at 0 °C for 15 min. Insoluble material (which was not RNA) was removed by centrifuging at 15000*g* for 10 min. RNA was then adsorbed to 1.5 mL of DBAE-cellulose (equilibrated with the morpholine containing buffer) at 0 °C at a flow rate of 1 mL/40 min and eluted with 0.05 M sodium acetate (pH 5.1) containing 0.2 M NaCl and 1 mM EDTA. RNA was concentrated with ethanol, resuspended in 0.5 mL of H_2O, and stored at −20 °C.

In a typical preparation, 3 μg of RNA was synthesized enzymically. The reaction mixture contained 1 mg of DNA and 80 μg of *E. coli* ribosomal RNA (added prior to DNA digestion to protect RNA). After DNase digestion and DBAE-cellulose chromatography, the amount of UV-absorbing material that remained was equivalent to the amount of RNA present. In a control experiment with *E. coli* ribosomal RNA omitted, the amount of recoverable UV material was equivalent to the amount in the synthesized RNA, indicating a 75% yield as calculated from recovery of radioactivity. When the bacterial rRNA was present, more than 85% of the radioactivity was recovered. Further, greater than 95% of the recovered UV-absorbing material is alkali labile.

The size of RNA synthesized in vitro was determined by sucrose density gradient centrifugation. Synthesized [^{14}C]-RNA (1000–1500 counts min^{-1}) was applied to 5–30% sucrose gradients containing 0.01 M sodium acetate, 0.2 mM EDTA, and 0.001% $NaDodSO_4$. Gradients were centrifuged at 45000 rpm in the Beckman SW 56 rotor for 15 h at 19 °C. KB ^{3}H-labeled 5S rRNA and yeast tRNA were either mixed with synthesized RNA prior to centrifugation and served as internal markers or run in parallel gradients.

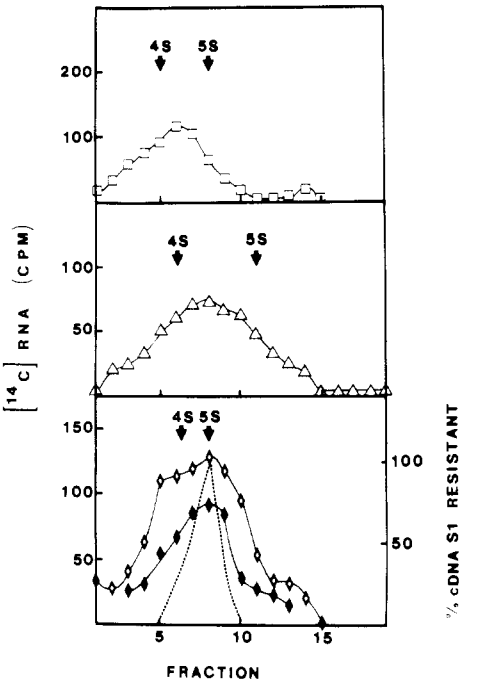

FIGURE 1: Sedimentation pattern of [^{14}C]RNA transcribed from native DNA by RNA polymerase III. RNA was synthesized as described in the legend to Figure 2. In the upper two panels, yeast tRNA and KB ^{3}H-labeled 5S RNA were mixed with the synthesized RNA and served as internal markers; in the lower panel, markers were run in parallel gradients. RNA transcribed from: (□) native DNA (10^7 daltons); (△) denatured DNA (10^7 daltons); (◇) native DNA (1.3 × 10^6 daltons); (---) 5S RNA; (♦) S_1 resistance of cDNA:enzymic RNA hybrid. In this experiment, RNA of each fraction was dialyzed against water, concentrated, and hybridized to the cDNA.

Other Procedures. Described in the accompanying publication (Ackerman et al., 1979) are methods used for the isolation of 5S rRNA, its polyadenylation, synthesis of a cDNA copy, cDNA:DNA annealing, cDNA:RNA hybridization, and T_m analysis of cDNA:RNA hybrids. cDNA (50–100 pg, 500–1000 counts min^{-1}) was hybridized to enzymically synthesized RNA and to 5S rRNA (at 2 μg of RNA/mL) in 50% formamide, 4 × SSC, 0.2 mM EDTA, and 0.01% $NaDodSO_4$ and annealed to DNA at 0.2 mg of DNA/mL as described for RNA hybridization.

Results

Synthesis of RNA from Double-Stranded DNA. RNA synthesized by RNA polymerase III is largely 4 S to 5 S in size (Figure 1). Varying the reaction conditions, such as salt concentration or using Mg^{2+} rather than Mn^{2+}, does not significantly affect the size of the RNA synthesized.

RNA synthesized under optimum conditions, with Mn^{2+} as metal cofactor, was hybridized to the 5S cDNA probe and compared with hybridization of 5S rRNA and annealing of DNA to the 5S cDNA probe. The 5S rRNA (at 2 μg of RNA/mL) hybridizes to the cDNA with a $R_0t_{1/2}$ of 0.001 mol s L^{-1}. RNA synthesized enzymically hybridizes to the cDNA with a $R_0t_{1/2}$ of 0.028, reflecting 3.5% 5S rRNA (Figure 2; experiment 1, Table I). In this experiment, RNA synthesized from smaller DNA (one-eighth the size) hybridizes to the cDNA with a $R_0t_{1/2}$ of 0.11, reflecting 0.9% 5S rRNA.

As shown in Figure 1 (bottom panel), optimal protection of the cDNA from S_1 digestion was observed with RNA 5S

[1] Abbreviations used: cDNA, complementary DNA; 5S cDNA, cDNA complementary to 5S rRNA; C_0t, annealing of DNA to DNA where C_0 is the initial concentration of DNA (in moles of nucleotide/liter) and t is time (in seconds); R_0t, hybridization of RNA to DNA with RNA in vast excess where R_0 is the initial concentration of RNA and t is the time (in seconds); SSC, 0.15 M NaCl containing 0.015 M sodium citrate; DBAE-cellulose, *N*-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose.

Table I: Transcription of 5S RNA Genes of Calf Thymus DNA by RNA Polymerase[a]

					native DNA template				denatured DNA	
					10^7 daltons		1.3×10^6 daltons		10^7 daltons	
expt	polymerase	enzyme to 5S gene ratio[b]	salt[c]	metal[d]	$R_0t_{1/2}$	% 5S	$R_0t_{1/2}$	% 5S	$R_0t_{1/2}$	% 5S
1	calf III	83	$(NH_4)_2SO_4$	Mn^{2+}	0.028	3.5	0.11	0.9	0.028	3.5
2a	calf III	34	$(NH_4)_2SO_4$	Mg^{2+}	0.034	3.0	0.08	1.3	0.08	1.3
2b	calf III	18	$(NH_4)_2SO_4$	Mg^{2+}	0.034	3.0	0.08	1.3	0.08	1.3
3a	*E. coli*	44	$(NH_4)_2SO_4$	Mn^{2+}	4.47	<0.1	>10.0	<0.1	>10.0	<0.1
3b	*E. coli*	44	$(NH_4)_2SO_4$	Mg^{2+}	0.34	0.3				
4a	calf III	10	KCl	$Mn^{2+}Mg^{2+}$	0.028	3.5	0.07	1.4	0.03	3.2
4b	calf III	58	KCl	$Mn^{2+}Mg^{2+}$	0.025	3.2	0.10	0.8	0.025	3.2
4c	calf III	390	KCl	$Mn^{2+}Mg^{2+}$	0.130	0.8				
5	calf III	53	KCl	$Mn^{2+}Mg^{2+}$	0.045	2.2	0.11	0.9	0.053	1.9
6	calf III	30	KCl	Mg^{2+}	0.063	1.6	0.13	0.8	0.063	1.6
7	calf III	18	$(NH_4)_2SO_4$	Mn^{2+}	0.042	2.4	0.32	0.3	0.14	0.7
8	calf III (phosphocellulose fraction)	218	KCl	$Mn^{2+}Mg^{2+}$			0.13	0.8		

[a] RNA synthesized in vitro was isolated as described under Materials and Methods. The % 5S rRNA in the transcripts was calculated from the $R_0t_{1/2}$ of hybridization to 5S cDNA compared with the hybridization of 5S cDNA to 5S RNA run simultaneously. Control annealing of 5S cDNA:calf thymus DNA ($C_0t_{1/2}$ 2.1 ± 0.07) was also run simultaneously. [b] Calculated from the amounts of enzyme and DNA present in the synthetic reaction. The values are estimates of the polymerase molecule:5S rRNA gene ratio, calculated from the molecular weight of polymerase (500 000), and an estimate of the specific activity of pure enzyme (300 units/mg). [c] $(NH_4)_2SO_4$ was added at 50 mM. KCl was added at 125 mM (experiments 5 and 6) or at 200 mM (experiments 4 and 8). [d] $MnCl_2$ was added at 1 mM in experiments 4, 5, 7, and 8 or at 2 mM in experiments 1 and 3. $MgCl_2$ was added at 4 mM in experiments 2, 3, 5, and 6 and at 12 mM in experiments 4 and 8.

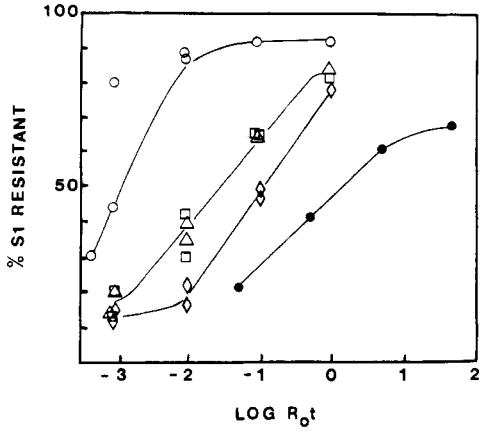

FIGURE 2: Hybridization of RNA transcribed by RNA polymerase III from calf thymus DNA, in the presence of Mn^{2+}, to 5S cDNA (experiment 1, Table I). RNA was synthesized in a reaction volume of 3.75 mL containing 50 mM Tris–maleate (pH 7.7), 2 mM $MnCl_2$, 50 mM $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol, 320 μM ATP, GTP, UTP, 60 μM [^{14}C]CTP (12 counts min^{-1} $pmol^{-1}$), 0.515 mg of each DNA, and 4.2 units of RNA polymerase III, for 90 min at 37 °C. The amounts of RNA synthesized from large native DNA (10^7 daltons) (□), large denatured DNA (△), and small native DNA (1.3×10^6) (◇) were 5.7, 3.9, and 9.5 μg, respectively. (○) The 5S rRNA:cDNA hybridization (●); calf DNA:cDNA annealing.

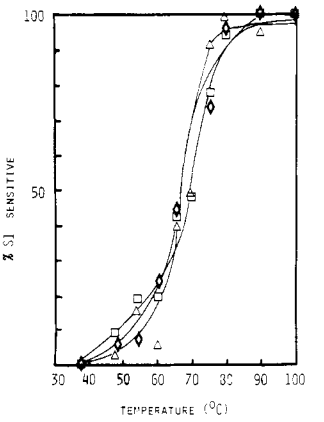

FIGURE 3: Melting profile of the RNA:5S cDNA hybrid. RNA synthesized as described in the legend to Figure 2 was hybridized to 5S cDNA. Results are plotted as relative S_1 resistance. At 37 °C, 20% of the material was degraded; at 100 °C, 95% of the material was degraded. Symbols as in Figure 2.

in size. While RNA the size of 5S protected 75% of the cDNA, RNA one-half or twice the size protected 40% of the cDNA from S_1 digestion.

These results indicate that the proportion of 5S RNA is greatest in the sucrose gradient fraction of the appropriate size. While there are a number of possible explanations, less protection of the cDNA by smaller RNA is most likely due to the presence of RNA chains interrupted during synthesis. Less protection by the larger molecules could be due to the presence of molecules which did not terminate properly or molecules which initiated incorrectly but read through the entire 5S RNA gene.

The T_m of the hybrid formed between the cDNA and the synthesized RNA is 71.5 °C, compared with a T_m of 74 °C for the 5S rRNA:cDNA hybrid (Figure 3). (RNA synthesized from any of the templates used in this study with all the varying reaction conditions have T_m values between 69 and 73 °C.)

Annealing of the cDNA to total DNA (at 200 μg of DNA/mL) results in a $C_0t_{1/2}$ of 2.1 (Ackerman et al., 1979). However, under the conditions used, DNA annealing is more rapid than RNA:DNA hybridization (Melli et al., 1971; Bishop, 1972; Muto, 1977) and the size of the driver and tracer species determines the rate of annealing and hybridization (Hinnebusch et al., 1978). With appropriate correction factors (cf. Young et al., 1974), it can be estimated that there are 1500 5S rRNA genes per haploid calf thymus genome. A value of 3000 is obtained by applying eq 32 of Muto (1977). This compares with estimates of 2000 5S rRNA genes in the HeLa cell genome (haploid) (Hatlen & Attardi, 1971) and 800 5S rRNA genes in the rat genome (Quincey & Wilson, 1969).

Hybridization of RNA synthesized enzymically is not due to the presence of 5S rRNA in the enzyme preparation. The procedure used in purifying RNA polymerase III involves an initial low salt extraction. Chromatin and ribosomes remain intact and are removed by centrifugation. Direct evidence excluding the presence of 5S rRNA was obtained by subjecting the enzyme to the same isolation procedure used in obtaining

Table II: Absence of 5S RNA from DNA and RNA Polymerase III as Determined by Formation of S_1-Resistant Material upon Incubation with cDNA[a]

	S_1-resistant material (%)					
			samples			
	controls		DNA		enzyme	
time (min)	cDNA alone	5S RNA[b]	expt 1[c]	expt 2[d]	expt 3[e]	expt 4[f]
0	12	10	10→15	9→14	12	12
27.5	13	85	8→12	16→14		
275	10	90	13→31	13→13	15	13
2750	11	92	22→16	26→16	16	13

[a] Samples (25 μL) were incubated with cDNA (0.1 ng) as described for DNA:RNA hybridization for the time indicated, and resistance to S_1 nuclease was determined as described under Materials and Methods. [b] 5S RNA (0.1 μg) was incubated with the 5S cDNA. R_0t values attained were: 27.5 min = 10^{-2}; 275 min = 10^{-1}; 2750 min = 1. [c] DNA (0.3 mg) was chromatographed on DBAE-cellulose as in the isolation of enzymically synthesized RNA. Material which eluted from the column was concentrated with ethanol and resuspended in 0.1 mL of water. Aliquots (0.25 μL) were hybridized to cDNA for the time indicated, and S_1 resistance was determined. Arrows point to values obtained when the presumptive RNA was incubated in alkali (0.5 N NaOH for 15 h at 37 °C), neutralized, and then incubated with the cDNA. [d] As in c, except that the DNA was treated with DNase, phenol extracted, and dialyzed against water prior to DBAE-cellulose chromatography. The procedure mimicked that used in isolating enzymically synthesized RNA except that 0.3 mg of DNA (rather than 1 mg) was used and the final material was suspended in 0.1 mL (rather than 0.5 mL). [e] To enzyme (4 μL, 0.3 unit), 2 μL of 5% $NaDodSO_4$ was added followed by the components required for cDNA:RNA hybridization. [f] Enzyme (50 μL, 4 units) was incubated in the standard synthetic reaction (see Materials and Methods) except that unlabeled triphosphates and metal cofactor were omitted. "RNA" was isolated as described under Materials and Methods.

enzymically synthesized RNA. No 5S rRNA was obtained (Table II).

The presence of 5S rRNA in DNA was excluded in part by studies on transcription by *E. coli* RNA polymerase (see later) and by experiments similar to those in which 5S RNA in enzyme was excluded (Table II).

When RNA is synthesized with Mg^{2+} as metal cofactor, essentially similar results are obtained as when Mn^{2+} is the metal cofactor (Figure 4; experiment 2a, Table I). Although total RNA synthesis is less with Mg^{2+} as metal cofactor (Keshgegian et al., 1975a), there is no increase in the relative proportion of 5S rRNA synthesized.

In a number of other experiments, the salt present in the synthetic reaction was changed from $(NH_4)_2SO_4$ to KCl and the concentration of metal cofactor was varied. In these experiments there was no significant change in the relative extent of 5S rRNA transcription (Table I). In all cases, selective transcription was observed. The levels of 5S rRNA transcription from the larger DNA template was consistently between 300- to 700-fold (2.6 ± 0.88%) above random transcription (0.005%, based upon a $R_0t_{1/2}$ of 10^{-3} and a $C_0t_{1/2}$ of 2.1); 5S rRNA transcription from the smaller template was between 160- and 300-fold (0.9 ± 0.32%) greater than random transcription.

The ratio of enzyme to DNA appears to affect the relative amount of 5S rRNA synthesis. As shown in Table I, experiment 4, with a lower enzyme to DNA ratio, selectivity for 5S rRNA synthesis increases. This is consistent with the notion that excess enzyme incorrectly initiates at nicks and gaps, or possibly at promotor regions where initiation does not normally occur in vivo.

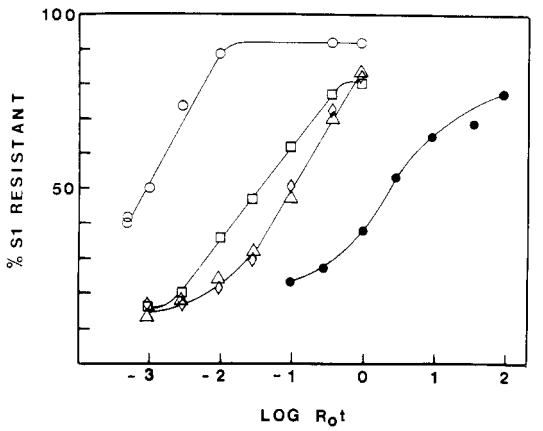

FIGURE 4: Hybridization of RNA transcribed by RNA polymerase III, in the presence of Mg^{2+}, to 5S cDNA (experiment 2a, Table I). RNA was synthesized in a reaction volume of 11.25 mL as described in the legend to Figure 2 with the following modifications: 2.75 mg of each DNA was used, [^{14}C]CTP was at 13 cpm/pmol, 9.4 units of RNA polymerase III was used, and 4 mM $MgCl_2$ replaced $MnCl_2$. The amounts of RNA synthesized from large native DNA (10^7 daltons) (□), large denatured DNA (△), and small native DNA (1.3 × 10^6 daltons) (◇) were 4.5, 3.5, and 3.3 μg, respectively. (○) The 5S rRNA:cDNA hybridization; (●) calf DNA:cDNA annealing.

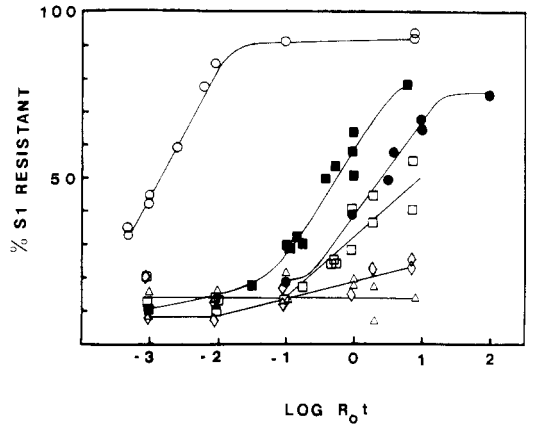

FIGURE 5: Hybridization of RNA transcribed by *E. coli* RNA polymerase to 5S cDNA (experiment 3, Table I). RNA was synthesized in a reaction volume of 0.5 mL (with 2 mM $MnCl_2$) or 2 mL (with 4 mM $MgCl_2$) as described in the legend to Figure 2 with the following modifications: 0.30 mg/mL of each DNA was used, [^{14}C]CTP was at 13 counts min^{-1} $pmol^{-1}$, 13 units of *E. coli* RNA polymerase per mL was used, and the incubation period was 30 min. The amounts of RNA transcribed from large native DNA [$MnCl_2$ (□); $MgCl_2$ (■)]; large denatured DNA [$MnCl_2$ (△)] and small native DNA [$MnCl_2$ (◇)] were 9.8, 5.6, 6.6, and 15 μg, respectively; 5S rRNA:cDNA hybridization (○); calf DNA:cDNA annealing (●).

RNA transcribed from DNA by a less purified fraction of RNA polymerase III also showed selective transcription of 5S rRNA genes. In this experiment (8, Table I), both Mg^{2+} and Mn^{2+} were used. RNA synthesized by this fraction is slightly smaller, mainly 4 S (results not presented; Furth & Austin, 1970). The T_m of the hybrid of the RNA and 5S cDNA is 65 °C (results not shown). The lower T_m is probably due to less complete transcription of the 5S rRNA gene.

In contrast to the results obtained with RNA polymerase III, *E. coli* RNA polymerase, in the presence of Mn^{2+}, shows little selectivity for 5S ribosomal RNA genes (Figure 5; Table I, experiment 3a). While some hybridization is observed at high R_0t values, this occurs at values similar to that observed in 5S cDNA:DNA annealing. These RNAs are 3 S to greater than 10 S in size (data not presented). However, when DNA is transcribed with Mg^{2+} as metal cofactor, RNA is obtained which hybridizes to 5S cDNA with a $R_0t_{1/2}$ of 0.34, reflecting

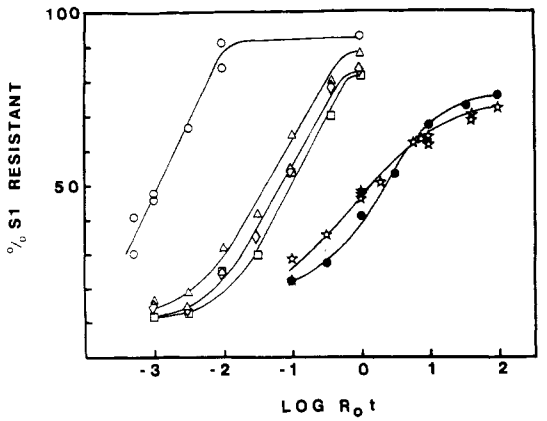

FIGURE 6: Hybridization of RNA transcribed from salmon sperm DNA by calf RNA polymerase III to 5S cDNA. RNA was synthesized as described in the legend to Figure 2 with the following modifications: (□) RNA (4.2 μg) synthesized with $MnCl_2$ (1 mM) and $MgCl_2$ (12 mM) in a reaction volume of 2.5 mL, 1.53 mg of native salmon DNA, and 8.5 units of RNA polymerase III; (△) RNA (2.5 μg) synthesized with $MgCl_2$ (4 mM) in a reaction volume of 2.0 mL, 1.84 mg of native salmon DNA, and 10 units of RNA polymerase III; (◇) RNA (4.1 μg) synthesized with 2 mM $MnCl_2$ in a reaction volume of 3.0 mL, 1.84 mg of salmon DNA, and 10 units of RNA polymerase III. (○) The 5S rRNA:cDNA hybridization; (●) calf DNA:cDNA annealing; (*) salmon DNA:cDNA annealing.

0.3% 5S RNA in the transcripts.

Synthesis of RNA from Denatured DNA. RNA was transcribed from denatured DNA (10^7 daltons) as well as from native DNA. This RNA contains approximately the same proportion of 5S rRNA as transcripts from native DNA (2.1 ± 0.97%) (Figures 2 and 4 and Table I). The size of this RNA is the same as RNA transcribed from native DNA, as is the T_m of cDNA:5S cDNA hybrid formed (Figures 1 and 3).

Transcription of 5S RNA Genes of Salmon Sperm DNA by Calf Thymus RNA Polymerase III. The 5S RNA structural genes of salmon sperm DNA have a similar or identical sequence as 5S RNA structural genes of calf thymus and are present at approximately the same reiteration frequency (Ackerman et al., 1979). RNA synthesized by using 2 mM $MnCl_2$ and 50 mM $(NH_4)_2SO_4$ hybridizes to cDNA with a $R_0t_{1/2}$ of 5×10^{-2}, reflecting 2% 5S rRNA sequences in the transcripts (Figure 6). Changing the metal cofactor to 4 mM $MgCl_2$ does not appreciably affect the results ($R_0t_{1/2} = 3.5 \times 10^{-2}$, 2.8% 5S rRNA in the transcripts), neither does the use of $MgCl_2$ and $MnCl_2$ (12 mM and 1 mM, respectively) as metal cofactors with KCl (200 mM) as salt ($R_0t_{1/2} = 6.7 \times 10^{-2}$, 1.5% 5S rRNA in the transcripts). These results suggest that not only are the base sequence and number of 5S rRNA genes of salmon sperm DNA similar to that of calf thymus DNA but also that the promotor regions may be similar.

Discussion

In a previous study (Atikkan & Furth, 1977) it was demonstrated that the transcription of both DNA and chromatin, in vitro, by RNA polymerase III is not random. While random transcription would have resulted in the same relative proportion of repetitive sequences (40% of calf thymus DNA) in the product, little transcription of very highly repeated DNA was observed. Somewhat surprisingly, in view of studies in many laboratories indicating restricted transcription of chromatin compared with transcription of DNA by both eucaryotic and *E. coli* RNA polymerase, the overall hybridization pattern of RNA transcribed from chromatin was similar to that of RNA transcribed from DNA. On the other hand, comparison of transcription of DNA by RNA polymerase III with the transcription of the same template by *E. coli* RNA polymerase indicated that the eucaryotic enzyme transcribed a greater proportion of moderately repeated DNA.

In the present study, synthesis of one species of RNA, 5S ribosomal RNA, has been investigated, and it has been observed that calf thymus RNA polymerase III selectively synthesizes this species of RNA.

The size of DNA affects the extent of selective transcription. In the present experiments, with smaller DNA as template the proportion of 5S RNA transcripts decreases. Similar observations on selective transcription by RNA polymerase I have been made by Van Keulen et al. (1975). This implies that nicks or single-stranded regions contribute to false starts; elimination of these in the template may increase the degree of selective transcription.

Selective transcription of 5S RNA by RNA polymerase III did not appear to be affected by the choice of metal cofactor used in the synthetic reaction. In contrast to transcription of ribosomal RNA by RNA polymerase I (Holland et al., 1977), Mn^{2+} as well as Mg^{2+} is effective as metal cofactor.

Similar results were obtained with RNA synthesized in the presence of either $(NH_4)_2SO_4$ or KCl. In addition, with denatured DNA as well as with native DNA as template, there is selective transcription of 5S RNA. This suggests that recognition of promotor sites does not require DNA to be in a double-stranded configuration. There is precedent for this. For example, *E. coli* RNA polymerase preferentially transcribes "early" RNA from a denatured T4 DNA template, albeit with less fidelity than it transcribes double-stranded DNA (Brody & Geiduschek, 1970). In this connection it is noteworthy that Falco et al. (1978) have reported that RNA polymerase of the N4 bacteriophage of *E. coli* has a strong preference for denatured N4 DNA in vitro. This DNA is transcribed asymmetrically and selectively by N4 polymerase only when single stranded.

It should be noted that, while selective transcription has been observed, the RNA which hybridizes to cDNA is the expected size, and the T_m of the cDNA:RNA hybrid is as expected, we have not yet established that RNA polymerase III recognizes the precise initiation and termination sequences for 5S rRNA synthesis. Earlier work (Keshgegian et al., 1973) established that RNA synthesis by polymerase III is initiated with the purine nucleoside triphosphates, as would be expected from the initial nucleotide of 5S rRNA. Further, the observation that correct (selective) transcription of salmon 5S rRNA genes occurs with our RNA polymerase suggests that initiation is close to the precise sequence at which initiation occurs in vivo.

In the present experiments, selectivity was 100- to 700-fold above the level of random transcription. While these experiments were in progress, Parker & Roeder (1977) reported that *Xenopus laevis* RNA polymerase III transcribes the 5S rRNA genes of *X. laevis* DNA about 30- to 50-fold above random. In their experiments, 1% of the total transcripts were 5S rRNA. However, Parker & Roeder obtained similar results with *X. laevis* RNA polymerase I, and with *E. coli* RNA polymerase 0.5% of the transcripts were 5S rRNA. (The *Xenopus* genome contains a much larger number of 5S rRNA genes, 0.05% of the genome, while only 0.005% of the calf genome is 5S rRNA genes.) Their results obtained by using the bacterial enzyme indicated about a 10-fold selectivity for the 5S rRNA genes; in the present experiments a 4-fold selectivity was observed with Mn^{2+} as metal cofactor and a 60-fold selectivity was observed with Mg^{2+} as metal cofactor.

Parker & Roeder used purified 5S rDNA prepared from strains of *E. coli* harboring recombinant plasmids containing

5S rDNA, and 5S rRNA synthesis was evaluated by relatively complicated isotope-dilution competition hybridization assays. In our experiments, we have used a probe (DNA complementary to 5S rRNA) which is extremely sensitive in detecting 5S rRNA transcripts and capable of great precision. This cDNA probe does not detect transcription of spacer sequences or transcription of the antisense strand.

The present experiments utilized mammalian enzyme. It is possible that enzyme of calf thymus differs in specificity from amphibian enzyme. Alternatively, it is possible that our preparation of calf thymus RNA polymerase III retains a component which has been lost or modified during the purification of *Xenopus* enzyme.

Approximately 0.005% of the mammalian genome is 5S ribosomal RNA genes and in HeLa cell nuclei about 6% of synthesized RNA is 5S rRNA (Yamamoto & Seifart, 1977). Yamamoto et al. (1977) observed a similar value (6%) for RNA transcribed by RNA polymerase III in chromatin preparations. In the present experiments, up to 3.5% of the transcripts were 5S rRNA. This suggests that the species of RNA synthesized by RNA polymerase III is in part determined by the intrinsic specificity of the polymerase. The observation that preferential transcription of 5S ribosomal RNA genes (i.e., transcription greater than random) is observed with *E. coli* RNA polymerase (Figure 5; Parker & Roeder, 1977) and with RNA polymerase I (Parker & Roeder, 1977) suggests that promotor regions of certain genes are intrinsically capable of acting as sites for the initiation of RNA synthesis. This is consistent with our previous observation that eucaryotic RNA polymerase III preferentially transcribes "early" RNA from a T even bacteriophage template (Furth et al., 1972).

Acknowledgments

We thank Drs. A. A. Keshgegian, L. J. Bello, E.-C. Wang, D. Henner, and R. I. Kelley for many helpful discussions and Nora Zuno for excellent technical assistance. Avian myeloblastosis virus was obtained from the Division of Cancer Cause & Prevention, National Cancer Institute, Bethesda, MD 20014.

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