Fidelity of Ribosomal Ribonucleic Acid Synthesis by Nucleoli and Nucleolar Chromatin†

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ABSTRACT: Isolated nucleoli, nucleolar chromatin, and nucleolar DNA were used as templates for RNA synthesis in appropriately supplemented systems in which RNA polymerases other than RNA polymerase I were blocked by α-amanitin. With the aid of nucleotide analysis, DNA-RNA hybridization, and homochromatography fingerprinting, it was found that isolated nucleoli and nucleolar chromatin serve primarily as templates for synthesis of rRNA. However, the products formed with purified nucleolar DNA as a template do not contain the specific rRNA oligonucleotides nor are they appreciably hybridized to the rDNA region on cesium chloride gradients. These results indicate that whole nucleoli and nucleolar chromatin contain control mechanisms that restrict readouts by RNA polymerase I of nucleolar DNA to rDNA.

As compared with the whole nuclei and nuclear chromatin, the nucleoli and nucleolar chromatin have the advantage that rDNA is virtually the only active gene set in nucleoli and, thus, rRNA is the major product in vivo and in vitro (Busch and Smetana, 1970; Blatti et al., 1970; Beebee and Butterworth, 1975; Grummt and Lindigkeit, 1973; Grummt, 1976; Zylber and Penman, 1971).

In eukaryotes, rRNA is virtually the only product in the nucleolus despite the fact that the rDNA comprises 0.5–1% of the nucleolar DNA (Busch and Smetana, 1970; Quagliarotti et al., 1970). Accordingly, controls apparently exist in the nucleolus that permit the rDNA to transcribe selectively and prevent the other DNA from being transcribed (Busch and Smetana, 1970). To aid in the study of these controls, the present experiments were initiated to determine whether fidelity of rRNA synthesis is retained in chromatin and DNA prepared from isolated nucleoli.

An advantage of this system is that rRNA is distinguishable from many other RNA species by its high GC content; RNA polymerase I which synthesizes rRNA is readily isolated in high purity (Roeder and Rutter, 1968, 1970; Blatti et al., 1970; Chambon et al., 1972), and the unique oligonucleotides resulting from complete or partial T_1 RNase digestion have been identified (Inagaki and Busch, 1972; Galibert et al., 1975; Matsui et al., 1975; Fuke and Busch, 1975) and are absent from other RNA species (Woo et al., 1975). Recently, Grummt (1976) demonstrated by DNA-RNA hybridization experiments that nucleolar transcripts can be competed with 45S pre-rRNA; these experiments provide that the major product of isolated nucleoli is rRNA.

In this study, the following different RNA products were analyzed by DNA-RNA hybridization and homochromatography to determine the fidelity of in vitro transcription: (1) rRNA synthesized in vivo; (2) RNA synthesized by isolated nucleoli; (3) RNA synthesized on nucleolar chromatin; and (4) RNA synthesized on naked nucleolar DNA.

Materials and Methods

Isolation of Nucleoli. Nucleoli were isolated by the sonic oscillation method (Busch and Smetana, 1970) from Novikoff ascites hepatoma cells. The cell suspension from the peritoneal cavity was freed of red blood cells by centrifugation at 7000*g* for 60 min through 2.0 M sucrose–12 mM $MgCl_2$–10 mM Tris[1]-HCl, pH 7.5, in a Sorvall RC-3 centrifuge. The cells were suspended in 0.88 M sucrose–12 mM $MgCl_2$–10 mM Tris-HCl, sonicated until no cells remained intact, and centrifuged at 1100*g* for 20 min. After the supernatant was decanted, the pellet was suspended in 0.88 M sucrose containing 1 mM $MgCl_2$. Approximately 0.4–0.8 g wet weight of nucleoli were obtained from 200 g of Novikoff cells. The nucleoli were stored at −80 °C in 0.88 M sucrose–50% glycerol–10 mM Tris-HCl, pH 7.5, until use.

Preparation of Chromatin. To obtain nucleolar chromatin, the nucleoli were stirred twice in a Vortex mixer at the highest power setting in 50 volumes of 0.075 M NaCl–25 mM EDTA–0.1 mM $PhCH_2SO_2F$ and then twice in 50 volumes of 10 mM Tris-HCl, pH 8.0. Each washing was followed by centrifugation at 7000*g* for 10 min. The final gelatinous pellet, designated "nucleolar chromatin", was used immediately for transcription. Nucleolar DNA was purified according to Sitz et al. (1973).

Purification of RNA Polymerase and Transcription Assay RNA polymerase I was purified from Novikoff hepatoma nucleoli by sonication in a high ionic strength buffer, ammonium sulfate fractionation, and DEAE-Sephadex column chromatography (Figure 1) as described by Roeder and Rutter (1968) and stored at − 80 °C in TGMED without any significant loss of activity for at least 1 month. The purified enzyme from nucleoli (Figure 1) contained little or no RNA polymerase II and III inasmuch as its activity was not inhibited by α-amanitin (Schwartz et al., 1974). One unit of enzyme incorporates 1 nmol of UMP per 10 μg of DNA per 15 min under

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[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NHP, nonhistone chromosomal protein; Preribosomal RNA, 45S precursor of ribosomal RNA; $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride; rDNA, DNA containing sequences coding for rRNA; SSC, 0.15 M NaCl–0.015 M sodium citrate; TGMED, 25% glycerol–5 mM $MgCl_2$–0.1 mM EDTA–0.5 mM dithiothreitol–10 mM Tris-HCl, pH 8.0; Tris, tris(hydroxymethyl)aminomethane; SE, standard error.

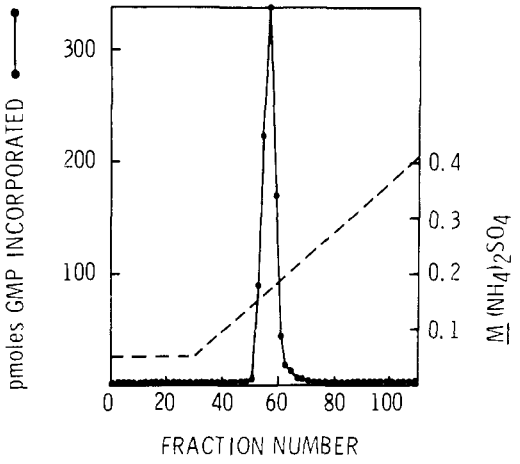

FIGURE 1: DEAE-Sephadex (A-25) chromatography of nucleolar RNA polymerase. Purified Novikoff nucleoli (4.5 g) were sonicated 30 s in TGMED containing 0.3 M $(NH_4)_2SO_4$, and then the sonicate was adjusted to 0.1 M $(NH_4)_2SO_4$. After the chromatin was removed by centrifugation, the supernatant was added with solid $(NH_4)_2SO_4$ to 60% saturation. After 30 min of stirring, the precipitates were collected, dissolved in 10 ml of TGMED containing 50 mM $(NH_4)_2SO_4$, and dialyzed against 2 l. of the same buffer. Insoluble materials were removed and the sample was applied to the DEAE-Sephadex column (2.5 × 18 cm) equilibrated in the same buffer. The column was washed extensively and then the enzyme eluted with linear gradients (300 ml) of $(NH_4)_2SO_4$ (0.05–0.5 M) in TGMED. Fractions (3.0 ml) were collected and 50-μl aliquots analyzed for enzyme activity using the standard assay.

TABLE I: Compositions of Nucleoli and Nucleolar Chromatin.[a]

	DNA	RNA	Protein	
			Acid Soluble[b]	Acid Insoluble[c]
Nucleoli	1.00	1.51	5.60	2.60
Nucleolar chromatin	1.00	0.30	2.65	0.79

[a] Values are relative to DNA and averages of three different preparations. [b] Soluble in 0.25 N HCl. [c] Insoluble in 0.25 N HCl. DNA was determined by a modified diphenylamine reaction (Richards, 1974) and RNA by alkaline hydrolysis (Schmidt and Thanhauser, 1945).

the assay conditions described below. The reaction mixture for the template activity assay contained in 250 μl: 25 μmol of Tris-HCl, pH 8.0, 1.25 μmol of $MgCl_2$, 1.25 μmol of dithiothreitol, 0.25 μmol of $MnCl_2$, 12.5 μmol of $(NH_4)_2SO_4$, 1.25 μmol of NaF, 0.25 μmol each of ATP, GTP, and CTP, 0.025 μmol of [^{3}H]UTP (80 cpm/pmol), and 2.5 μg of α-amanitin. Template activities were determined under conditions where the enzyme and other reaction components were in excess.

For analysis of nucleotide compositions, four incubation tubes were run simultaneously; each contained either 0.2 mM ^{3}H-labeled ATP, GTP, CTP, or UTP and the other three unlabeled nucleoside triphosphates in the same concentrations. Incorporation of radioactivity into RNA was measured by the DEAE filter disc methods of Blatti et al. (1970) or cold Cl_3CCOOH precipitation methods. For homochromatography and hybridization analyses of the in vitro transcripts, the assay volume was scaled up 20 times and contained 250 μCi of [α-^{32}P]GTP (specific activity 500 μCi/μmol) or 100 μCi of [^{3}H]UTP (200 μCi/μmol). An excess of RNA polymerase I was added. After 10 min of incubation, the reaction was stopped by adding sodium dodecyl sulfate to 0.3% and the RNA was extracted by the hot phenol–sodium dodecyl sulfate procedure at pH 5.1 (Steele et al., 1965). The RNA was treated with DNase I (Worthington RNase-free; 50 μg/ml in 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$), reextracted with phenol, and precipitated with ethanol.

Preparation of Labeled rRNA. ^{32}P-labeled rRNA was prepared as described previously (Fuke and Busch, 1975). ^{3}H-labeled rRNA was prepared from a cultured cell line of Novikoff hepatoma which was maintained at 37 °C for 5 days in the SWIM's 67-G medium containing 10% fetal calf serum, 60 μg/ml kanamycin, and 10 μCi/ml [^{3}H]uridine (specific activity 20 Ci/mmol). The medium was replaced by a fresh one containing [^{3}H]uridine every 24 h and the cell density was adjusted to 5×10^5/ml. Cells were harvested, washed in a Dulbecco's saline, and lysed in 0.5% Nonidet P-40, 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5. After nuclei and mitochondrial materials were removed by centrifugation at 10 000 rpm for 10 min, the RNA was extracted from the supernatant by the hot sodium dodecyl sulfate–phenol procedure (Steele et al., 1965) and precipitated with ethanol. Both 18S and 28S RNA were purified by repeated centrifugation on 5–45% sucrose gradients (Quagliarotti et al., 1970) and precipitated with ethanol. Their specific activity was approximately 2×10^5 cpm/μg.

Homochromatography of T_1 RNase Digest. T_1 RNase digestion was carried out as described previously (Brownlee and Sanger, 1969; Fuke and Busch, 1975); $5–10 \times 10^5$ cpm of T_1 RNase digest of ^{32}P-labeled RNA were electrophoresed for 1 h on cellulose acetate (pH 3.5) and then for 18 h on DEAE-cellulose plates (7 × 8 in.) using the homomixture described earlier (Fuke and Busch, 1975). Autoradiograms were obtained on x-ray films exposed to the DEAE-cellulose plates at room temperature in the dark up to 28 days.

Fractionation of Nucleolar DNA on CsCl and Hybridization. The DNA solutions were adjusted to a refractive index of 1.402 with a solid CsCl (Schwarz/Mann; optical grade) in a final volume of 5 ml. Samples containing 50 μg of DNA were centrifuged at 68 700*g* for 72 h at 20 °C in 40 or 50 Ti rotors and 40 fractions were collected from the bottom of the tube. After 0.5 ml of 2 × SSC was added into each tube, the absorbance was determined at 260 nm. The DNA was denatured in 0.1 N NaOH, neutralized with 0.1 volume of 2 M NaH_2PO_4, and immobilized onto nitrocellulose filters (HA Millipore, 25 mm diameter). Each filter was cut into four pieces and hybridized with the RNA samples. The hybridization was carried out at 45 °C for 24–48 h in 2 × SSC containing 30% formamide, 0.1% sodium dodecyl sulfate, 25 mM Hepes (pH 7.5), and 25 000 to 200 000 cpm/ml of labeled RNA. The filters were washed extensively in 2 × SSC, treated with a pancreatic RNase (50 μg/ml)–T_1 RNase (50 units/ml) mixture for 30 min at 37 °C (Sitz et al., 1973), washed in 2 × SSC, dried under an infrared light, and counted. The background level (a blank filter, less than 0.01% of the input counts) was subtracted.

Results

RNA Synthesis by Isolated Nucleoli and Nucleolar Chromatin. The chemical compositions of isolated nucleoli and nucleolar chromatin (Table I) indicated that the preliminary extractions for chromatin preparation removed substantial amounts of RNA and protein from the nucleoli. These values indicate nucleolar chromatin is richer in RNA and acid-soluble

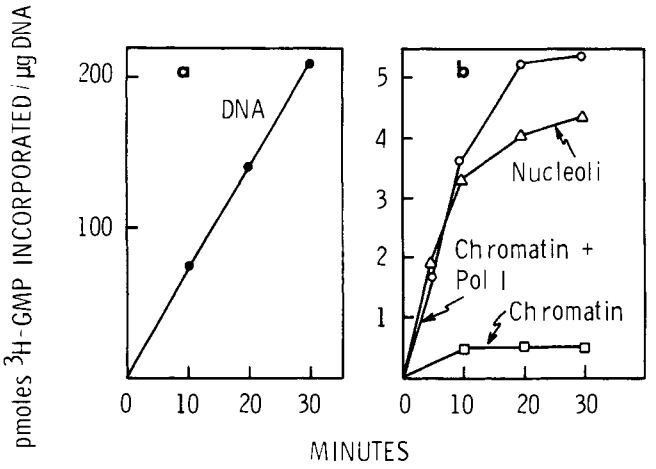

FIGURE 2: Kinetics of RNA synthesis in vitro. (a) RNA synthesis by nucleolar DNA + RNA polymerase I. (b) RNA synthesis by isolated nucleoli (△), chromatin (□), and chromatin + RNA polymerase I (○). When RNA polymerase I was added, the assay was performed under conditions where only the template was rate limiting (see Table II, Materials and Methods). The values are in pmol of [^{3}H]GMP incorporated/µg of DNA.

proteins than whole nuclear chromatin (Busch et al., 1975).

The kinetics of RNA synthesis by isolated nucleoli and nucleolar chromatin and nucleolar DNA (Figure 2) show that, even after extensive washing with 75 mM NaCl–25 mM EDTA and 10 mM Tris-HCl, the nucleolar chromatin retained sufficient RNA polymerase I activity to synthesize RNA at approximately one-tenth the rate of whole nucleoli (Table II). When RNA polymerase I was added, the rate of transcription of chromatin was approximately equal to that of whole nucleoli (Table II and Figure 2). Regardless of the system used, transcription proceeded linearly for up to 10 min, which was used as the standard incubation time.

The RNA synthesized by isolated nucleoli had a lower sedimentation coefficient than that of nucleolar RNA produced in vivo; some [^{3}H]GMP was incorporated into RNA with sedimentation coefficients up to 45 S. The peaks of incorporation were at 18, 20, and 25 S for chromatin, DNA, and nucleolar transcripts, respectively (Figure 3).

Fidelity of in Vitro rRNA Synthesis by Isolated Nucleoli and Nucleolar Chromatin. Three analyses were used for these products, namely, RNA–DNA hybridization, nucleotide composition, and homochromatography fingerprinting of the T_1 RNase digestion products. Nucleotide compositions determined by incorporation of each labeled nucleoside showed that the RNA synthesized by the nucleoli and nucleolar chromatin alone was rich in GMP and CMP; its composition was very similar to that of rRNA or preribosomal RNA (Table III). When RNA polymerase I was added to the nucleolar chromatin, the rate of synthesis was markedly increased; the purine content of the RNA product was essentially the same as that of whole nucleoli or chromatin. However, the pyrimidine content differed in that less CMP and more UMP was incorporated. With DNA as the template (Table III), much more AMP and UMP were incorporated than with whole nucleoli or nucleolar chromatin.

The RNA was treated with DNase I to eliminate trace amounts of contaminating DNA and hybridized to nucleolar DNA (mol wt 10×10^6) which was fractionated on CsCl gradients (Figure 4). The nucleolar transcripts were hybridized mainly to the DNA with a density of 1.715 g/cm^3; little hybridized to the main band DNA (1.692 g/cm^3).

TABLE II: Initial Rate of RNA Synthesis.[a]

Condition	[^{3}H]GMP (pmol) Incorp. µg of DNA^{-1} 10 min^{-1} (± SE)
Nucleoli	3.3 (0.2)
Nucleolar chromatin (N-ch)	0.45 (0.05)
N-ch + RNA polymerase I[b]	3.6 (0.3)
DNA + RNA polymerase I[b]	72.0 (1.2)

[a] Average values based on three experiments. [b] RNA polymerase I (0.5 unit) was used to transcribe chromatin (<20 µg as DNA) or DNA (<2.5 µg). Under the condition employed, the rate-limiting factor for transcription was template availability. After 10 min incubation in the standard assay, the reaction was stopped by adding ice-cold 5% Cl_3CCOOH. Acid precipitates were collected on a Whatman GF/C filter disk, washed with 5% Cl_3CCOOH, ethanol, and ether, and dried. Filters were counted in a toluene-base scintillant.

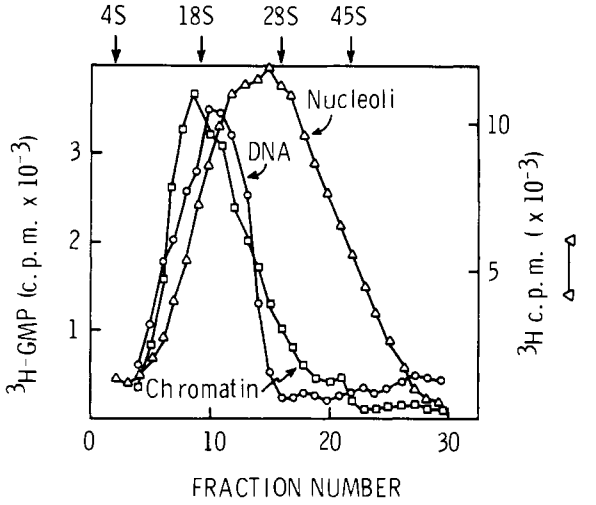

FIGURE 3: Sucrose gradient centrifugation pattern of RNA synthesized in vitro. Samples were run on 30-ml gradients of 5 to 45% sucrose–0.1 M NaCl–0.02 M acetate buffer (pH 5.1)–0.01 M EDTA for 18 h at 85 000*g*; (△) nucleolar transcripts; (□) chromatin transcripts; (○) DNA transcripts.

The presence of rRNA in the nucleolar transcripts was confirmed by homochromatography fingerprinting of T_1 RNase digests of the in vitro transcripts labeled with [α-^{32}P]GTP. Each oligonucleotide resulting from T_1 RNase digestion contains one labeled 3′-Gp except for the 3′ terminus. A 1:1 molar mixture of 18S and 28S rRNA labeled in vivo with [^{32}P]orthophosphate was analyzed (Figure 5a) for comparison of the marker sequences. Figure 5b shows the marker oligonucleotides of the nucleolar transcripts. Several unique oligonucleotides of both 18S and 28S rRNA were present in this pattern.

Insufficient amounts of isotope were incorporated into rRNA by isolated nucleolar chromatin for homochromatography or DNA–RNA hybridization. Specific oligonucleotides were not detected by homochromatography of transcripts from chromatin containing 250 µg of DNA even after exposure for 4 weeks. When RNA polymerase I was added to the same system, 10^6 cpm was incorporated into the RNA; this amount was sufficient for detection of the specific oligonucleotides (Table II, Figure 5). By comparison with the transcripts with

TABLE III: Nucleotide Composition Analysis by Relative Nucleotide Incorporation.

Condition	No. of Determinations	Rel Nucleotide Incorp.[a] AMP	UMP	GMP	CMP	A + U / G + C
Nucleoli	3	13.0	21.0	35.5	30.5	0.52
Nucleolar 45S RNA[b]		14.6	20.5	35.1	29.7	0.54
Chromatin	2[c]	13.4	20.6	37.3	29.7	0.52
Chromatin + polymerase I	4[c]	14.5	24.4	37.9	23.2	0.66
DNA + polymerase I	2	24.9	32.3	21.9	20.9	1.34

[a] Values based on 10 min transcription carried out in the presence of α-amanitin (10 μg/ml). [b] Nucleotide composition of nucleolar 45S RNA determined by ultraviolet spectra analyses (Muramatsu et al., 1966). [c] The endogenous incorporation by chromatin was 0.46 pmol of [^{3}H]GMP per μg of DNA per 10 min. With exogenous enzymes, the incorporation was 3.52 pmol of [^{3}H]GMP per μg of DNA per 10 min.

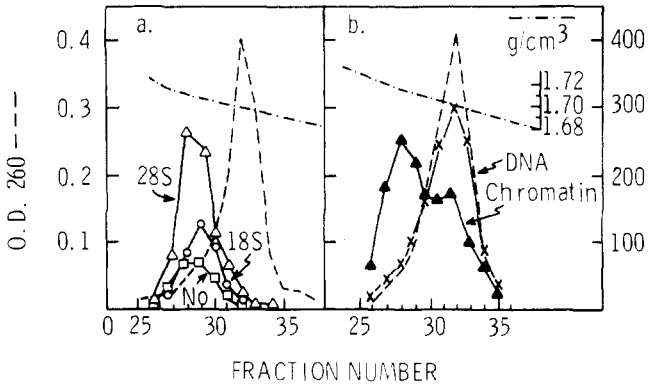

FIGURE 4: Hybridization of rRNA and in vitro transcripts to nucleolar DNA fractionated on CsCl gradients. Samples containing 50 μg of nucleolar DNA (mol wt 10×10^6) were centrifuged in a neutral CsCl gradient (5 ml) at 68 700g for 72 h. The DNA in each fraction was denatured with 0.1 N NaOH, adsorbed to a Millipore filter, and hybridized with the appropriate RNA in 2 × SSC–30% formamide–25 mM Hepes–0.1% sodium dodecyl sulfate at 45 °C for 48 h. (a) Hybridization with ^{3}H-labeled 28S rRNA (△), ^{3}H-labeled 18S rRNA (○), and ^{3}H-labeled nucleolar transcripts (□). In this particular experiment, the incorporation rate was 0.41 pmol of GMP incorporated per μg of DNA per 10 min for endogenous activity and 4.0 pmol of GMP incorporated per μg of DNA per 10 min with exogenous enzyme. Specific activities of rRNA and nucleolar transcripts were 2.06×10^5 and 3.0×10^3 cpm/μg, respectively. Input: 1 μg/ml for rRNA and 15 μg/ml for nucleolar transcripts. (b) Hybridization with ^{3}H-labeled chromatin transcripts with exogenous RNA polymerase I (▲) and ^{3}H-labeled DNA transcripts with exogenous RNA polymerase I (X). Specific activities of chromatin and DNA transcripts were 1.25×10^4 and 1.74×10^6 cpm/μg, respectively. Input: about 0.3 μg/ml.

isolated nucleoli, the size of chromatin transcripts was smaller (Figure 3), i.e., approximately 18 S.

The base composition of these products differed from rRNA or preribosomal RNA (Table III). To determine whether major products represented mainly rRNA or preribosomal RNA, DNA–RNA hybridization was carried out; in addition to the sequences hybridizing to the GC-rich DNA of ribosomal cistrons, the products also contained sequences hybridizing to the main band DNA (Figure 4b). Since neither rRNA nor nucleolar transcripts hybridized to the main band DNA (Figure 4a), the system containing nucleolar chromatin and the exogenous RNA polymerase I permitted other DNA to be transcribed.

To determine whether the nucleolar chromatin transcripts contained oligonucleotides of both 18S and 28S rRNA, homochromatography fingerprinting was carried out (Figure 5c). No oligonucleotides were present in positions other than those of rRNA oligonucleotides. Accordingly, rRNA was the major transcript; the remainder of the transcripts were apparently heterogeneous (Woo et al., 1975).[2]

Template Restriction and Fidelity of rRNA Synthesis. Similar experiments were done to determine the transcripts of purified nucleolar DNA. Interestingly, the size of the product was not significantly different from that of the chromatin transcripts (Figure 3). However, most of the hybridization occurred only with the main band DNA (d = 1.692 g/cm). There was no disproportionate hybridization to the GC-rich rDNA region (Figure 4). Along with the nucleotide compositions (Table III), these results indicate that rDNA is not specifically transcribed in this system. Homochromatography fingerprinting of the T_1 RNase oligonucleotides (Figure 5d) shows that no rRNA-specific oligonucleotides were produced.

Discussion

The present findings provide additional evidence that nucleoli contain controls that limit its transcription to ribosomal cistrons. These results are in agreement with the findings of Beebee and Butterworth (1975) that the exogenous RNA polymerase I transcribes mainly rDNA with isolated nucleoli as a template. Although these controls were partially preserved in purified nucleolar chromatin, they were lost when the nucleolar DNA alone was the template (Figures 4b and 5d). This result indicates that factors other than DNA and RNA polymerase I are necessary for a specific transcription of rDNA. Muramatsu et al. (1975) showed by DNA–RNA competition hybridization experiments that approximately 40% or more of the transcripts primed by nucleolar chromatin were ribosomal sequences but few ribosomal sequences were found in the DNA transcripts.

Assuming that RNA hybridizing to GC-rich DNA represents only rRNA and the sequences hybridizing to main band DNA are nonribosomal (Figure 4b), approximately 60% of the isotope in the RNA synthesized by the nucleolar chromatin hybridized to rDNA under the conditions employed. Several problems exist that limit precise quantitative estimation of the amounts of rRNA produced in isolated nucleoli (Figure 4b, Table III). The presence of unlabeled RNA (0.3 μg/DNA), which is probably mainly rRNA, reduces the specific activity of newly synthesized rRNA; in hybridization analysis, this dilution reduces the estimates of the amount of rRNA sequences produced. On the other hand, hybridizations in this

[2] In preliminary studies, it was found that, when nucleolar chromatin was reconstituted from nucleolar DNA, nonhistone proteins, and histones (1:0.8:1), the pattern of products obtained was the same as that of Figure 5c.

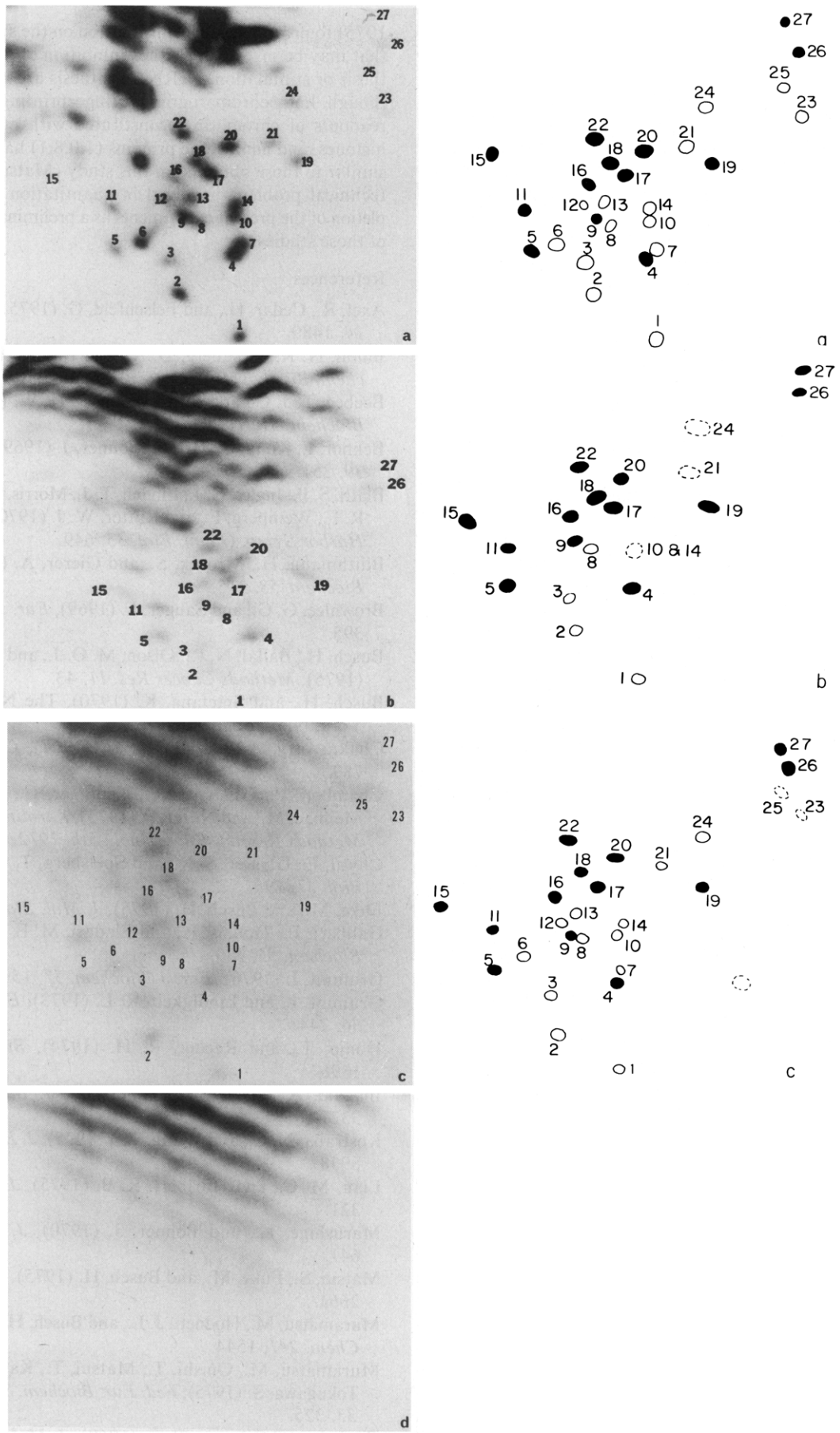

FIGURE 5: Homochromatography fingerprints of T_1 RNase oligonucleotides of rRNA and in vitro transcripts. (Left) Autoradiogram; (right) schematic drawing. 18S rRNA-specific and 28S rRNA-specific oligonucleotides are shown by closed and open circles, respectively. Exposure times: 1–4 weeks. (a) Novikoff hepatoma rRNA (18S + 28S) labeled with [^{32}P]orthophosphate in vivo (Fuke and Busch, 1975). (b) Nucleolar transcripts (specific activity 1.2×10^4 cpm/μg). (c) Chromatin transcripts (specific activity 2.0×10^4 cpm/μg). (d) DNA transcripts (specific activity 8×10^6 cpm/μg). (b–d) Transcripts are labeled with [α-^{32}P]GTP (Materials and Methods).

experiment were carried out only for 24–48 h under conditions which would preferentially hybridize reiterated sequences. Hybridization of transcribed single copy sequences would require longer periods and, accordingly, the data do not permit analysis of less highly reiterated or sparse RNA sequences. The peak of hybrid at the high density on CsCl (Figure 4b) does indicate a highly selective transcription of rDNA among the repetitive sequences in the nucleolar chromatin–RNA polymerase I system.

The presence of specific 18S and 28S rRNA oligonucleotides in homochromatography fingerprints of transcripts digested with RNase T_1 provides the most specific evidence for transcription of rDNA in nucleoli and nucleolar chromatin. Although these spots were readily detectable when nucleolar chromatin was supplemented with RNA polymerase I, with the very low activities in the endogenous transcription of chromatin, specific oligonucleotides of rRNA were not detected.

Reeder (1973) and Honjo and Reeder (1974) reported that, with *E. coli* RNA polymerase, and to a lesser extent with RNA polymerase I, both the "sense" strands and the "nonsense" strands were transcribed. If this occurred to the extent that "nonsense" transcripts were equal in amount of the "sense" transcripts, complementary oligonucleotides might have been detected, but none were found in either the whole nucleolar or nucleolar chromatin transcripts (Figure 5). If other strands were transcribed to less than 10% of the rRNA, the limit of sensitivity of this method could preclude their visualization.

Although the analysis of transcripts from whole nucleoli showed no evidence of RNA other than rRNA, transcripts other than rRNA were clearly made from isolated nucleolar chromatin to which RNA polymerase I was added. The higher content of UMP and lower content of CMP in these transcripts (Table III) as well as the hybridization of 40% of the labeled RNA to main band nucleolar DNA (Figure 4b) indicate that other genes were transcribed. Nucleolar chromatin was prepared from isolated nucleoli by successive extraction with the commonly used reagents 0.075 M NaCl, 0.025 M Na_2EDTA, and 0.01 M Tris buffer (Marushige and Bonner, 1970). These solvents extract approximately 50% of the total nucleolar proteins (Table I) including soluble proteins that bind to DNA (Prestayko et al., 1976). Inasmuch as Kostraba and Wang (1975) reported that gene inactivating proteins include nonhistone proteins, it is not surprising that removal of such proteins releases genes from transcriptional inaccessibility.

Nucleoli and nucleolar chromatin offer a valuable system for analysis of transcription in eukaryotic systems. Thus far, the transcript size in vitro was smaller than that of 45S pre-rRNA in vivo. The endogenous RNase activity contributes to the degradation of RNA synthesized in vitro (Chakravorty and Busch, 1967). In addition, synthesis of RNA in nucleoli or nucleolar chromatin proceeded linearly only for up to 10 min (Figure 2); this may result from the absence of reinitiation of RNA synthesis (Grummt, 1976) in vitro or "piling-up" of the RNA polymerase within the ribosomal cistrons (Liau and Hurlbert, 1975). Another possible explanation for short transcripts is that the rDNA may have been nicked by sonication or traces of DNase.

Despite the problems indicated above, the nucleolar chromatin system offers an opportunity for analysis of factors involved in gene restriction. Such factor(s) may include RNA or nonhistone proteins. Studies on reconstituted systems (Paul and Gilmour, 1968; Stein and Farber, 1972; Axel et al., 1975; Stein et al., 1975a,b; Spelsberg et al., 1971; Chytil et al., 1974; Bekhor et al., 1969; Bliithmann et al., 1975; Matsui et al., 1975) to provide further information on the specific proteins that may be involved in either inhibition of non-rRNA synthesis or stimulation of rRNA synthesis are in progress. Although homochromatography fingerprinting showed that readouts of chromatin reconstituted with nucleolar DNA, histones, and nonhistone proteins (1:0.8:1) had spot patterns similar to those obtained in this study (Matsui et al., 1975), technical problems involved in quantitation required completion of the present experiments as a preliminary to extension of those studies.

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Effect of a Single Cis Double Bond on the Structure of a Phospholipid Bilayer†

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ABSTRACT: The ordering of the hydrocarbon chains and the rates of lipid motion are two independent parameters characterizing the structure and the dynamics, respectively, of a bilayer membrane. In this work, deuterium magnetic resonance has been used to elucidate the influence of a single cis double bond on the hydrocarbon chain *ordering* of a phospholipid bilayer. 1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine was specifically deuterated at various segments of the palmitic acyl chain and at the 9, 10 position of the oleic acyl chain, and the segmental order parameters were deduced from the quadrupole splittings of the unsonicated bilayer phases. The shape of the order profile of the palmitic acyl chain is similar to that observed for the corresponding fully saturated membrane, but the magnitude of the order parameters is distinctly smaller in the unsaturated system. This demonstrates that the presence of a double bond in a membrane causes a more disordered conformation of the hydrocarbon chains. Considering the *relative* flexibility within the palmitic acyl chain, the deuterium resonance data indicate a local stiffening of those segments which are located in the vicinity of the double bond. The membrane *fluidity* was investigated using a nitroxide-labeled stearic acid spin probe. The smaller electron paramagnetic resonance line width in bilayers of 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine demonstrates an increased fluidity compared to bilayers of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine.

The phospholipids found in bacterial and mammalian cell membranes usually contain saturated as well as unsaturated fatty acyl chains. There is a strong positional preference of the two types of fatty acids, with the saturated and unsaturated substituents being localized at position 1 and 2, respectively, of the glycerol part of the molecule. Monounsaturated phospholipids are predominant but lipids containing more than one double bond also occur quite commonly. The incorporation of a double bond introduces a rigid element into the otherwise flexible hydrocarbon chain and, furthermore, since double bonds are usually found to be in the cis conformation, it prevents the chain from assuming a completely extended configuration. Double bonds are important in biological membranes for at least two reasons. Firstly, it is known that the activity of certain membrane-bound enzymes depends critically on the presence of cis double bonds (Rothfield and Romeo, 1971), indicating a specific interaction between enzymatic recognition sites and the cis double bond. Since the replacement of the unsaturated grouping by a *cis*-cyclopropane ring also leads to almost equally active enzymes, it may further be inferred that in these cases the cis double bond plays its activating role not by its chemical nature but by its unique configuration. The second effect of cis double bonds is to control the physiological properties of membranes by a nonspecific, purely thermodynamic mechanism, namely, by modulating the membrane "fluidity". In the case of the bacterium *Escherichia coli,* it has been demonstrated quite convincingly that the proper functioning of the system requires the membrane lipids to be in the "fluid" (liquid crystalline) state (Overath et al., 1970, 1975). The enzymatic activity of membrane-bound proteins is greatly slowed down if the bacterium is cooled to temperatures where the lipids become rigid. It is known from thermal studies with synthetic lipids that the incorporation of cis double bonds into a saturated lipid drastically lowers the gel-to-liquid crystal transition point (Ladbrooke and Chapman, 1969; Phillips et al., 1972), which has been interpreted to suggest that the probability of a membrane to be "fluid" increases with the percentage of double bonds. This has indeed been verified experimentally for *E. coli* membranes using appropriate *E. coli* fatty acid auxotrophs (Overath et al., 1971).

The notion of a "fluid" membrane is, however, not sufficient

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