Secrist, J. A., Barrio, J. R., Leonard, N. J., & Weber, G. (1972) *Biochemistry 11*, 3499.
Suchy, J., Mieyal, J. J., Bantle, G., & Sable, H. Z. (1972) *J. Biol. Chem. 247*, 5905.
Wittorf, J. H., & Gubler, C. J. (1970) *Eur. J. Biochem. 14*, 53.
Zoltewicz, J. A., & Kauffman, G. M. (1977) *J. Am. Chem. Soc. 99*, 3134.

Cloning and Determination of a Putative Promoter Region of a Mouse Ribosomal Deoxyribonucleic Acid Fragment†

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ABSTRACT: An endonuclease *Eco*RI digest of mouse DNA was subjected to molecular cloning, after partial purification with respect to the ribosomal RNA sequence, using λgtWES·λB with an in vitro packaging technique. Twelve positive clones were obtained from approximately 2×10^4 plaques. One of the clones transferred to the plasmid pBR322 (pMrEL-1) was about 14.9 kb long, hybridizing only with 18S rRNA but not with 28S rRNA. Hybridization of restriction fragments and electron microscopic studies of the R-loop confirmed that this fragment carried about half of the 18S rRNA sequences at one end, suggesting that it contained the initiation site for the 45S preribosomal RNA (pre-rRNA). S_1-nuclease protection mapping with hybrids between restriction fragments of the cloned DNA and the 45S pre-rRNA indicated that at least major transcription of the 45S RNA started at a site approximately 4.0 kb upstream from the 5′ end of the 18S rRNA. This was confirmed by electron microscopic observations of these hybrids.

One approach to the study of molecular mechanisms of gene regulation in eukaryotes may be the establishment of an in vitro transcriptional system that mimics the in vivo mechanisms with high fidelity. For this purpose, isolation and characterization of a specific gene are the first prerequisites. The ribosomal RNA gene (rDNA) has several interesting features in that it is arranged in tandem in the order of several hundreds and transcribed in vivo by RNA polymerase I, which is one of the three types of eukaryotic RNA polymerases. Its transcription is affected strongly by protein synthesis and, in some cases, cell growth.

Genes coding for rDNA have been cloned from yeast (Kramer et al., 1976), *Drosophila* (Thomas et al., 1974), *Bombyx mori* (Manning et al., 1978), *Xenopus* (Morrow et al., 1974), chicken (McClements & Shalka, 1977), mouse (Tiemeier et al., 1977), and human cells (Wilson et al., 1978). The mouse rDNA fragment isolated by Tiemeier et al. (1977) was an internal fragment containing both 18S and 28S rRNA gene regions; it did not contain initiation or termination regions.

To study the regulatory mechanisms of rDNA transcription, isolation of a fragment containing the 5′-terminal region of the rDNA was essential. In this paper, we report the isolation of such a fragment from mouse DNA. We also describe attempts to locate the initiation site for 45S pre-rRNA on this fragment with biochemical and electron microscopic methods.

Materials and Methods

Preparation of High Molecular Weight DNA and RPC-5 Column Chromatography. The preparation of DNA from the newborn Balb/c mice was described elsewhere (Kataoka et al., 1979). In brief, the method involved treatment with $NaDodSO_4$[1] and $NaClO_4$, phenol–chloroform extraction followed by RNase treatment, and extensive dialysis against 0.1 × SSC. DNA was digested completely with *Eco*RI, extracted with phenol–chloroform, and precipitated with 2 volumes of ethanol. Forty milligrams of DNA digested with *Eco*RI was chromatographed on an RPC-5 column as described previously (Tiemeier et al., 1977).

Preparation and Screening of the Recombinant Phage and Plasmid. Bacteriophage λgtWES·λB, an EK-2 vector, was propagated in *Escherichia coli* strain ED8656. Introduction of DNA into ED8656 by an in vitro packaging technique was carried out according to Blattner et al. (1978) on the basis of other reports (Becker & Gold, 1975; Hohn & Murray, 1977; Sternberg et al., 1977). Cloning experiments were performed in a P2 facility according to the proposed Japanese Guidelines (1978) and revised NIH Guidelines (1976).

The λgtWES arms were purified by 5–25% sucrose density gradient centrifugation after digestion with *Eco*RI and *Sac*I. The ligation of λgtWES arms and mouse DNA fractionated by the PRC-5 column was carried out according to the method of Tiemeier et al. (1977). Five micrograms of λgtWES arms was ligated with 1.5 μg of DNA in a volume of 0.5 mL at 15 °C for 16 h.

About 2000 plaques on each L-broth supplemented agar plate (Bacto-trypton (10 g), yeast extract (5 g), NaCl (5 g), agar (15 g), and distilled water (1 L)) were transferred to the Millipore filter essentially according to Benton & Davis (1977). Filters were hybridized with ^{32}P-labeled 18S cDNA (see below) and autoradiographed. Positive plaques were propagated as described by Tiemeier et al. (1977) and DNA was extracted.

To re-clone the fragment obtained above, *Eco*RI-digested recombinant DNA was ligated with plasmid pBR322 DNA, which had been digested with the same enzyme and treated

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; SSC, 0.15 M NaCl and 0.015 M sodium citrate; AMV, avian myeloblastosis virus; sarcosyl, sodium dodecyl sarcosinate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); kb, kilobase.

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with alkaline phosphatase. Ligation was performed in two steps by modifying the method of Dugaiczyk et al. (1975). The first step involved linear ligation at high DNA concentrations. pBR322 DNA (4.5 μg) and rDNA (10.5 μg) were ligated in a volume of 0.15 mL of reaction mixture at 15 °C for 1 h. The second step of ligation was circular; the ligation mixture from the first step was diluted 10-fold and further incubated with T_4-ligase at 15 °C for 16 h.

Transformation of ED8656 was done according to Enea et al. (1975). Briefly, bacterial strain ED8656 was grown up to 0.4 A_{650nm}. Cells were harvested at 5000 rpm for 6 min, washed in 10 mM NaCl, the pellet was resuspended in 40 mM sodium acetate (pH 5.6), 30 mM $CaCl_2$, and 70 mM $MnCl_2$, and the mixture was allowed to stand for 20 min at 0 °C. After centrifugation at 5000 rpm for 6 min, the pellet was resuspended in a 1:100 volume of the above solution. The cell suspension was mixed with plasmid DNA and held at 0 °C for 60 min; then the mixture was spread over an L-broth supplemented agar plate containing 40 μg/mL ampicillin. Ampicillin-resistant colonies were then grown on a Millipore filter, which was placed on a 40-μg/mL ampicillin-containing L-broth agar plate. Positive colonies were detected according to the method of Grunstein & Hogness (1975). Positive colonies were propagated as described previously (Clewell & Helinski, 1969).

Preparation of Ribosomal RNA. 18S and 28S rRNA were prepared from ddy mice after isolation of 40S and 60S ribosomal subunits with EDTA. 45S pre-rRNA was prepared from MH134 cells as described previously (Kominami & Muramatsu, 1977).

Radioactive Labels. In this study, several probes were used for the hybridization experiments. ^{125}I-labeled 18S and 28S rRNAs were prepared according to Commerford (1971), except that we used a cellulose (CF-11) column to separate RNA and free iodine. The specific activity was about 10^7 cpm/μg of RNA. ^{32}P- and ^{3}H-labeled DNAs complementary to 18S, 28S, and 45S RNA, termed 18S, 28S, and 45S cDNA, respectively, were prepared according to Taylor et al. (1976) with AMV reverse transcriptase, using [^{32}P]dCTP or [^{3}H]dTTP and -dATP as labeled substrates and DNase-treated calf-thymus DNA as a random primer. The specific activity of ^{32}P-labeled cDNA was on the order of 10^8 cpm/μg. ^{3}H-labeled cDNA complementary to 18S rRNA was purified by incubating to a C_0t of 10^{-2} and fractionating by a hydroxylapatite column. The specific activity was 10^7 cpm/μg. ^{32}P-labeled in vivo 18S rRNA was prepared from cultured cells (MH 134) in a phosphate-free medium as described previously (Kominami & Muramatsu, 1977). The specific activity was 2×10^5 cpm/μg of RNA.

Recovery of DNA from Agarose Gel. Extraction of DNA from agarose gel was effected by the method of Tabak & Flavell (1978). pMrEL-1 DNA was digested with either *Eco*RI or *Sal*I and run on 1% agarose gel. Hydroxylapatite was packed in a trough which had been made adjacent to the DNA band, and the DNA was electrophoresed into the hydroxylapatite, which was taken out from the trough. The hydroxylapatite was poured on the top of Sephadex G-50 and the DNA eluted with 0.4 M phosphate buffer (pH 6.8).

Filter and Liquid Hybridization. DNA samples that were transferred from agarose gel to Millipore filter by the method of Southern (1975) were immobilized and hybridized as described previously (Kataoka et al., 1979).

When ^{125}I-labeled 18S and 28S rRNAs were used as probes, the filter was treated with RNase A (50 μg/mL in 2 × SSC at 37 °C, 30 min), washed with 2 × SSC, dried, and exposed on Fuji film that had been sensitized with a flashlight.

In those cases where probes were cDNA complementary to 18S, 28S, and 45S RNA, filters were washed with 0.1 × SSC containing 0.3% sarcosyl at 65 °C for 30 min.

In the liquid hybridization experiments, DNA samples were denatured at 100 °C for 10 min and incubated with ^{3}H-labeled 18S cDNA at 65 °C for 2 h in 0.6 M NaCl, 0.2 mM EDTA, and 0.02 M Tris-HCl (pH 7.4). After the samples were treated with S_1-nuclease at 43 °C for 30 min, acid-insoluble counts were determined with a liquid scintillation counter.

Enzymes. *Eco*RI and *Sac*I were purified according to the methods of Yoshimori (1971) and R. J. Roberts (unpublished results), respectively. T_4-ligase was a generous gift from Dr. M. Takanami (Kyoto University). Other enzymes were purchased from Bethesda Research Laboratories, Inc.

R-Loop Formation. The formation of an R-loop between 18S rRNA and recombinant DNA digested with *Eco*RI or *Hin*dIII was carried out essentially as described by Thomas et al. (1976). The *Eco*RI or *Hin*dIII-digested fragment (22.8 μg/mL) and 18S rRNA (5.8 μg/mL) were incubated in 70% formamide, 0.04 M Pipes–NaOH (pH 6.8), 0.001 M EDTA, and 0.5 M NaCl at 56 °C for 2 h in a 50-μL sealed capillary tube. Formation of the R-loop between 45S pre-rRNA and the *Sal*I 3.2-kb fragment was carried out as described above.

The mixture was diluted 20-fold with the same buffer containing 91 μg/mL cytochrome *c* and spread onto a hypophase of 10% formamide, 0.1 mM EDTA, and 0.01 M Tris (pH 8.0).

The DNA–RNA hybrid was picked up on a collodion-coated 200 mesh grid, stained with 5×10^{-5} M uranyl acetate, coated with carbon, and shadowed with Pt/Pd. The preparations were viewed with a Hitachi HU-12 electron microscope and electron micrographs were taken.

Detection of the Initiation Site for 45S Pre-rRNA on the rRNA Gene by the S_1-Nuclease Protection Method (Berk & Sharp, 1977). The 5′ end of the *Sal*I 3.2-kb fragment or 14.9-kb rDNA was labeled with polynucleotide kinase and [γ-^{32}P]ATP. 45S pre-rRNA (3.2 μg) was hybridized with ^{32}P-labeled 3.2- or 14.9-kb fragments (~0.1 μg) in 50 μL of 70% formamide, 0.5 M NaCl, 1 mM EDTA, and 0.04 M Pipes (pH 6.8) at 56 °C for 2 h. The solution containing hybrids of 45S pre-rRNA and 3.2- or 14.9-kb fragments was diluted 20-fold with S_1-nuclease buffer (30 mM sodium acetate (pH 4.5), 0.22 mM $ZnSO_4$, and 0.2 M NaCl) and treated with S_1-nuclease followed by ethanol precipitation. The precipitates were subjected to 1% alkaline agarose gel electrophoresis (McDonnell et al., 1977). The gel was dried and autoradiographed as described above.

Results

Partial Purification of a Large rDNA Fragment Containing the 18S rRNA Sequence. Figure 1 depicts diagrammatically the structure of an rRNA gene of the mouse (Arnheim & Southern, 1977). It may be seen that at least three classes of fragments are generated from each rRNA gene repeat when the mouse DNA is digested with endonuclease *Eco*RI. In order to concentrate the DNA fragments containing ribosomal RNA sequences, we digested the DNA prepared from newborn mice with *Eco*RI and fractionated it by RPC-5 column chromatography. Each fraction was tested for the presence of rRNA sequences with blotting hybridization according to Southern (1975), using ^{125}I-labeled 18S and 28S rRNAs as probes.

Figure 2A shows a DNA elution profile of an RPC-5 column tested with 1% agarose gel electrophoresis, and Figure 2B shows the filter hybridization profile after Southern

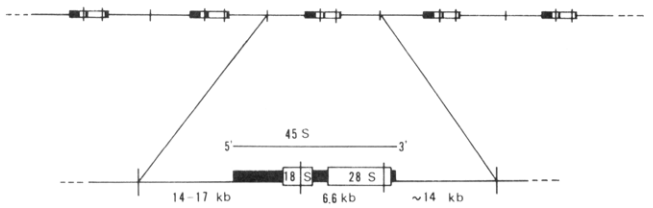

FIGURE 1: Organization of *Eco*RI fragments within repeating units of mouse rRNA gene. The open bars marked as 18S and 28S indicate the 18S and 28S rRNA genes, respectively, and the closed bars indicate the transcribed spacer region of 45S pre-rRNA. The sites of *Eco*RI are indicated by the vertical lines and their molecular lengths are shown as kilobases.

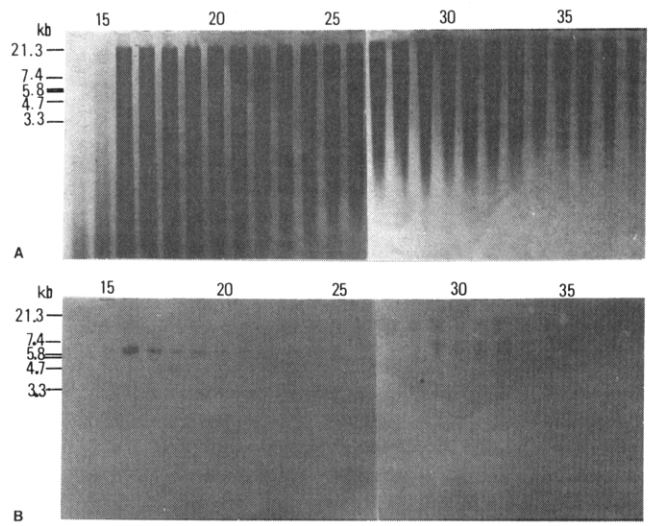

FIGURE 2: Agarose gel electrophoresis pattern of DNAs fractionated with RPC-5 column chromatography and identification of rRNA genes. Mouse DNA was digested with *Eco*RI followed by RPC-5 column chromatography: (A) ethidium bromide staining of DNA of chromatographic fractions 14 through 38 that were run on a 1% agarose gel; (B) autoradiogram of the filter blotted by the method of Southern (1975) and hybridized with ^{125}I-labeled 18S and 28S rRNA. *Eco*RI-digested λCI857 fragments were used as size markers.

blotting. As is evident from the radioactive bands, fractions 15–19 and fractions 29–32 were found to contain rRNA sequences. The 6.6-kb fragment in fractions 15–19 was the segment containing parts of 18S and 28S rRNAs, which had already been cloned by Tiemeier et al. (1977). Fractions 29–32 contained 14–17-kb fragments, which were supposed to contain the initiation site together with a part of the 18S rRNA sequence. Fractions from 29 through 32 were pooled and used for cloning.

Cloning and Screening of the Ribosomal RNA Gene Fragment. The DNA fragments obtained above were ligated with λgtWES arms in vitro by T_4-ligase and transferred into ED8656 as described under Materials and Methods. Screening of clones was carried out essentially according to Benton & Davis (1977). Approximately 20 000 plaques were screened and 12 positive clones were obtained (Mishima et al., 1978). The recombinant phages shown by autoradiography to contain ribosomal sequences were grown on a small scale. DNA was extracted, digested with *Eco*RI, run on an agarose gel, and tested for the presence of the 18S rRNA sequence by Southern's blotting method (1975). Liquid hybridization also confirmed the presence of this sequence (data not shown; see Mishima et al., 1978).

Although all the clones tested were positive using ^{32}P-labeled 18S cDNA, one of the clones (no. 3) was further examined using C_0t purified ^{3}H-labeled 18S cDNA as a probe. Clone no. 3 DNA hybridized with 47.4% of input cDNA, whereas

Table I: Presence of 18S rRNA Sequences in Recombinant DNA[a]

hybridizing nucleic acids	% hybridized
18S RNA	84.2
pMrEL-1 DNA	46.3
pBR322 DNA	1.3

[a] ^{3}H-labeled 18S cDNA (1200 cpm) was hybridized with 18S rRNA, pMrEL-1, or pBR322 DNA as described under Materials and Methods, and digested with S_1-nuclease. S_1-nuclease-resistant radioactivities are expressed as percentages of input counts.

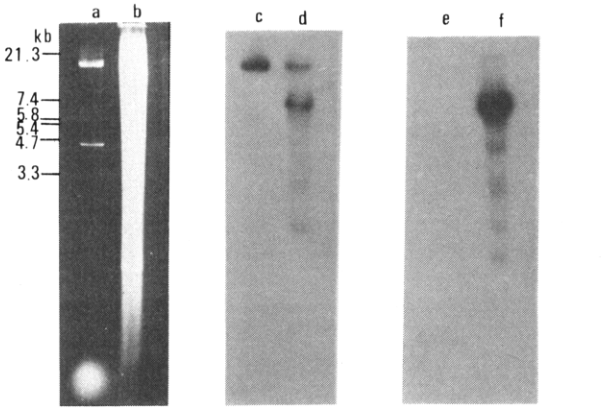

FIGURE 3: Filter hybridization between the pBR322 rDNA recombinant and ^{32}P-labeled 18S cDNA or ^{32}P-labeled 28S cDNA: (a) 1% agarose gel electrophoresis of the recombinant DNA digested with *Eco*RI (also c and e); the 14.9-kb rDNA and 4.3-kb pBR322 DNA fragments are visible; (b) 1% agarose gel electrophoresis of mouse DNA digested with *Eco*RI (also d and f); (c, d) and (e, f) autoradiograms of filter hybridization after Southern blotting using ^{32}P-labeled 18S cDNA and ^{32}P-labeled 28S cDNA as probes, respectively.

clone no. 7 DNA, which had not hybridized originally, did not hybridize at all.

We then transferred this fragment into the plasmid pBR322 for the following reasons. First, a cloned DNA seemed to be more stable in plasmids than in this phage (Grummt et al., 1979). Second, the cloned fragment and the shorter arm of the phage were of similar size, making their separation by gel electrophoresis rather difficult. Third, DNA production efficiency was better in the plasmid than in the phage. A positive clone that was found among 60 colonies tested was examined further. DNA prepared from the recombinant plasmid was examined for the presence of the 18S rRNA sequence.

Table I shows the liquid hybridization experiment in which ^{3}H-labeled 18S cDNA was hybridized with 18S rRNA or pMrEL-1 or pBR322 DNA. The results were compatible with the notion that this recombinant DNA contained about half of the 18S rRNA sequence.

Figures 3a and 3b show the agarose gel patterns of the recombinant DNA and of mouse DNA, respectively, which was digested with *Eco*RI. Figures 3c,d and 3e,f show the filter hybridization patterns with ^{32}P-labeled 18S cDNA and ^{32}P-labeled 28S cDNA as probes, respectively. The results clearly show that the inserted 14–15-kb fragment hybridizes only with ^{32}P-labeled 18S cDNA and not with ^{32}P-labeled 28S cDNA. We designated this clone as pMrEL-1.

Organization of 18S rRNA and Its Preceding Sequences. The 14.9-kb fragment excised from the recombinant plasmid was digested with various restriction enzymes and the fragments were electrophoresed on an agarose gel. Analyses were made of the ethidium bromide stained bands and of labeled bands with Southern blot hybridization using an 18S rRNA

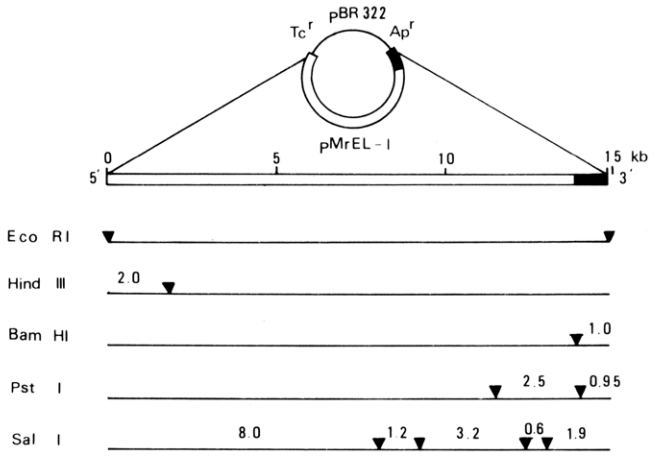

FIGURE 4: Diagram of restriction endonuclease cleavage sites of recombinant rDNA. The upper circle represents the orientation of rDNA in the pBR322 DNA. Open and closed bars show the rDNA fragment and 18S coding region, respectively. The thin line represents the pBR322 DNA, and Tc^r (tetracycline resistant) and Ap^r (ampicillin resistant) genes are shown. Lower lines show the stretched 14.9-kb rDNA fragment and restriction cleavage sites, where sizes are shown as kilobases.

probe (data not shown), and a restriction map was constructed, as shown in Figure 4.

Orientation of rDNA in the plasmid was determined by digestion of the recombinant plasmid DNA with *Hin*dIII. The results indicated that the rDNA was inserted in an orientation such as Ap^r-(3′) 18S rDNA (5′)-Tc^r (Ap^r, ampicillin resistance marker; Tc^r, tetracycline resistance marker).

Electron Microscopic Examination of the R-Loop. To confirm that this cloned fragment contained part of the 18S rRNA sequence at one end, hybrids formed between the 18S rRNA and the fragment were examined under an electron microscope.

Figure 5A shows a typical electron micrograph of hybrids formed by incubation of 18S rRNA and *Eco*RI-digested rDNA. A number of molecules 14.4 ± 0.2 kb (n = 12) in length were seen with a fork at one end; the unequal arms were 0.83 ± 0.03 kb (n = 12) and 1.59 ± 0.02 kb (n = 12), respectively. The fork may be interpreted to represent an 18S DNA–RNA hybrid and the displaced anti-strand, as shown in the diagram (Figure 5). Since this DNA–RNA hybrid was obtained from the *Eco*RI digest, the 18S rRNA sequence present here must represent the 5′ half of its gene region (see Figure 1). Next, the fragment obtained by *Hin*dIII digestion was used for DNA–RNA hybridization. Since the orientation of the recombinant molecule is Ap^r-(3′) 18S rDNA (5′)-Tc^r as shown in Figure 4, the R-loop should be seen at about 12 and 4 kb from both ends of the molecule. This was indeed the case, as seen in Figure 5B. R-loops were observed between 11.23 ± 0.6 kb (n = 5) and 5.29 ± 1.3 kb (n = 5) from both ends. The size of the R-loop was 0.74 ± 0.04 kb (n = 5) in length, in agreement with the *Eco*RI-digested hybrid.

Localization of the Initiation Site for 45S Pre-rRNA on the rRNA Gene. In order to search for the initiation site of the rRNA gene, we first subjected *Sal*I fragments of rDNA to blotting hybridization using ^{32}P-labeled 45S cDNA as a probe. Among the five fragments produced by *Sal*I (Figure 4), 1.9- and 3.2-kb fragments hybridized with 45S cDNA, but 0.6-, 1.2-, and 8.0-kb fragments did not (data not shown). Although it was not clear why the 0.6-kb fragment did not hybridize well with the probe, this does indicate that the 3.2-kb fragment most probably has the initiation site of rDNA transcription.

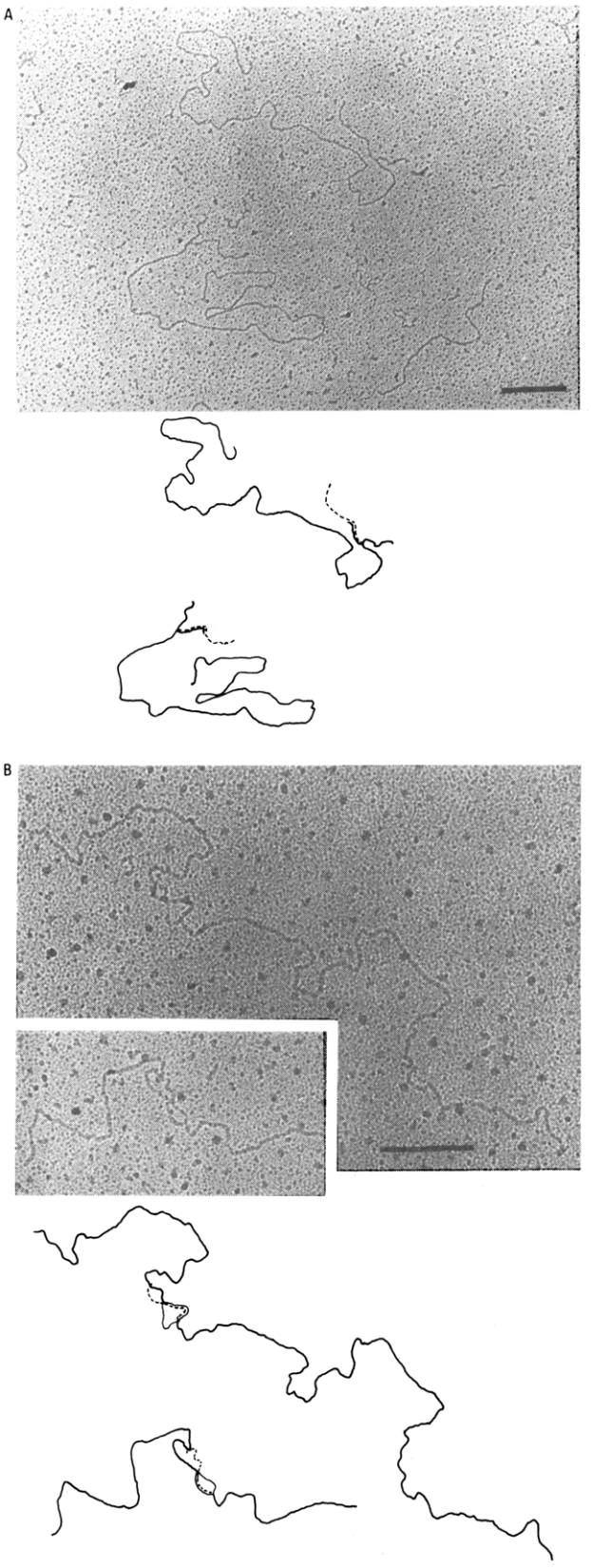

FIGURE 5: Electron micrograph of R-loops between the 18S rRNA and the pMrEL-1 DNA fragments digested with either *Eco*RI (A) or *Hin*dIII (B). Molecules were viewed at 10000-fold magnification in a Hitachi HU-12 electron microscope. The bar indicates 0.5 μm. DNA strands are drawn with continuous lines, whereas 18S rRNA molecules are traced with broken lines.

Next, we examined the location of the initiation site of 45S pre-rRNA by the technique of S_1-nuclease protection mapping (Berk & Sharp, 1977; see Materials and Methods) and by the

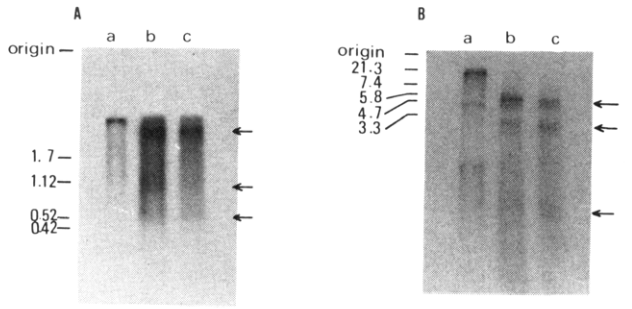

FIGURE 6: S_1-nuclease protection mapping of the 45S RNA initiation region. (A) The hybrids formed between the 45S RNA and ^{32}P-labeled *Sal*I 3.2-kb fragment were treated with S_1-nuclease followed by ethanol precipitation and electrophoresis on a 1% alkaline agarose gel (McDonnell et al., 1977). The gel was neutralized, dried, and autoradiographed. Size markers used are SV-40 *Hin*dIII fragments: (a) nontreated *Sal*I 3.2-kb fragment; (b) 25 U S_1-nuclease treated; (c) 100 U S_1-nuclease treated. (B) The hybrids formed between the 45S RNA and ^{32}P-labeled 14.9-kb rDNA fragment were treated with S_1-nuclease followed by the same procedure as in A. Size markers used are λ*Eco*RI fragments: (a) nontreated 14.9-kb rDNA; (b) 25 units of S_1-nuclease treated; (c) 100 units of S_1-nuclease treated.

electron microscopic examination.

The *Sal*I 3.2-kb or the original 14.9-kb rDNA fragment was labeled with [γ-^{32}P]ATP at the 5′ end and hybridized with 45S pre-rRNA. After the hybrid was treated with S_1-nuclease, the sizes of the protected DNA fragments were examined on an alkaline agarose gel (McDonnell et al., 1977). Figure 6A, lane a, shows the control *Sal*I 3.2-kb fragment, which was not treated with S_1-nuclease, and Figures 6A, lanes b and c, show the DNA fragments that were digested by different doses of S_1-nuclease. Three bands of about 2.4, 0.9, and 0.6 kb (arrow indicated) appeared after treatment with S_1-nuclease (Figure 6A, lanes b and c). The largest 2.4-kb fragment was most probably protected by 45S RNA, indicating that the transcription must have started from somewhere near this region. It is not clear at the present time whether 0.9- and 0.6-kb fragments represent the second or the third initiation site of pre-rRNA or whether they are protected by the partially processed rRNA precursors that are present in the 45S RNA fraction (Kominami & Muramatsu, 1977). Figure 6B, lane a, shows the control 14.9-kb fragment, which was not treated with S_1-nuclease, and Figure 6B, lanes b and c, show the protected DNA bands after S_1-nuclease treatment, respectively. Three bands of about 5.0, 3.0, and 1.0 kb appeared after S_1-nuclease treatment. Presumably, the largest fragment represents the segment protected by 45S RNA. These results strongly suggest that the transcription starts from about 2.4–2.5 kb upstream from the 3′ end of the *Sal*I 3.2-kb fragment.

We tried further to locate the initiation site by electron microscopic examination of the R-loop formed between the *Sal*I 3.2-kb fragment and 45S pre-rRNA. Figure 7 shows typical examples of the hybrids. Two classes of DNA duplexes, 0.9 and 2.5 kb in length, were observed with 2.3- and 0.7-kb tails that were displaced by the hybrids, respectively. This confirms the above biochemical mapping data that the initiation site lies about 2.4 kb upstream from the 3′ end of the *Sal*I 3.2-kb fragment (Figure 6).

Discussion

In this paper, we described the cloning and partial characterization of a fragment of mouse ribosomal DNA containing a putative promoter region.

The R-loop study and the restriction mapping showed that the clone contained the 5′ half of the 18S RNA gene sequence

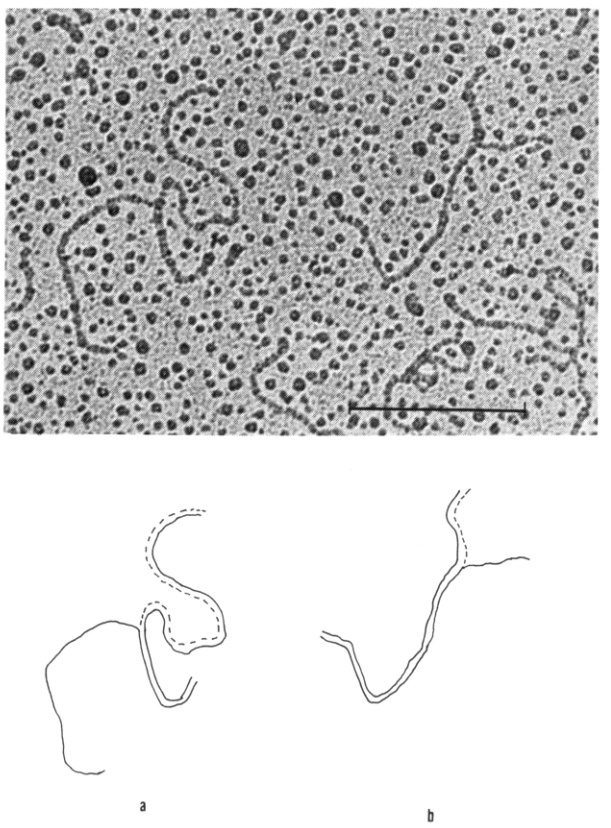

FIGURE 7: Electron micrograph of R-loops between the 45S RNA and the *Sal*I 3.2-kb fragment. The bar indicates 0.5 μm. The DNA strands are drawn with continuous lines, whereas 45S RNA molecules are traced with broken lines.

at one end of the molecule along with the preceding transcribed and nontranscribed spacers. This structure implies the presence of an initiation site for 45S pre-rRNA some distance from the 5′ end of the 18S RNA gene. Indeed, hybridization of restriction fragments with labeled 45S pre-rRNA indicated that the *Sal*I 3.2-kb fragment was the one located the furthest upstream that we could hybridize with this probe, suggesting strongly that the fragment contained the initiation sequence.

While this study was underway, papers by Grummt et al. (1979) and Arnheim (1979) appeared, in which the cloning and structural analysis of a similar fragment of mouse rDNA were described. Our restriction map is very similar but not identical with theirs; e.g., one *Hin*dIII site that is located 2.0 kb from the 5′ end of our cloned fragment was not present in their clones. The absence of the *Hin*dIII site in their clones may be explained in two ways. First, the base sequence in this region may really be different between the clones; either elimination of this *Hin*dIII site or appearance of a new *Eco*RI site slightly downstream of the *Hin*dIII site may be compatible with the difference.

Alternatively, their clone may have lost a portion of the original fragment containing the *Hin*dIII region during cloning and propagation in λWES, since they claim that the rDNA fragment inserted in λ phage is very unstable, causing various deletions during propagation. The slightly smaller size (<12 kb) of their clone than the genomic fragments identified by Southern blotting (~14 kb) favors this idea, although more detailed structural analysis is required to determine this point.

Determination of the hybridizable length by the S_1-nuclease protection mapping showed that 45S RNA hybridized with the DNA sequences up to 2.4 kb from the 3′ end of the *Sal*I 3.2-kb fragment, indicating the presence of the initiation sequence near this region.

This was confirmed by electron microscopic observations of the R-loop formed between 45S pre-rRNA and the *Sal*I 3.2-kb fragment. Major hybrids that were found had a 0.9-kb DNA duplex connected with a 2.3-kb displaced single-stranded and a slightly longer DNA–RNA hybrid, which were separated from each other in a Y shape (Figure 7a). We did not see long tails of 45S RNA, probably because of the contaminating RNase activity during the preparation of the hybrids. S_1 protection mapping revealed two more minor bands of 0.9 and 0.6 kb in length.

Molecules representing these hybrids were also observed by electron microscopy, as shown in Figure 7b. Minor components comprising less than one third of the total hybrid structures usually had a 2.5-kb DNA duplex region with a 0.7-kb DNA–RNA hybrid tail, which was rather consistent with the 0.6-kb band in S_1-nuclease protection experiments (Figure 6A).

Parallel protection experiments with the original 14.9-kb rDNA showed the presence of 5.0-, 3.0-, and 1.0-kb hybrids (Figure 6B). We assume that the 5.0-kb hybrid definitely corresponds to the 2.4-kb hybrid formed with the *Sal*I 3.2-kb fragment and that the 3.0-kb hybrid corresponds to the 0.6-kb hybrid. The 1.0-kb band may have been produced by protection with the 41S preribosomal intermediate that was present in small amounts in the 45S RNA preparation.

In any event, the multiple hybrid bands found in these S_1-nuclease protection experiments may be explained by one of the following three possibilities. First, these bands may have just been produced by partially processed pre-rRNAs with distinct length classes. Although no definite intermediate has yet been described between 45S RNA and the 41S intermediate (Wellauer et al., 1974; Perry, 1976), there is evidence that some kind of processing may be taking place at the 5′ terminus before the former is converted into the latter (Kominami & Muramatsu, 1977). On the other hand, there may be multiple initiation sites on one rDNA, producing various lengths of the primary transcripts. It should be mentioned here that such a case has recently been described for an *E. coli* rRNA gene (de Boer & Nomura, 1979). *Xenopus laevis* rDNA also has multiple promoter-like sequences on one gene repeat (Moss & Birnstiel, 1979; Boseley et al., 1979). Third, the heterogeneity of rDNA repeats may cause the same effect. If there are different classes of rDNA sequences whose initiation sites lie at different sites on the *Sal*I 3.2-kb fragment, 45S RNAs made on different classes of rDNA may have different lengths of homologous region to our cloned rDNA. Indeed, some sequence heterogeneity was found even in gene sequences (R. Kominami and M. Muramatsu, unpublished results) in addition to spacer sequences (Arnheim & Southern, 1977; Cory & Adams, 1977) of mouse rDNA. Which of these possibilities is correct could be answered by extensive sequencing of these regions.

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References

Arnheim, N. (1979) *Gene 7*, 83–96.

Arnheim, N., & Southern, E. M. (1977) *Cell 11*, 363–370.

Becker, A., & Gold, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 581–585.

Benton, W. D., & Davis, R. W. (1977) *Science 196*, 180–182.

Berk, A. J., & Sharp, P. A. (1977) *Cell 12*, 721–732.

Blattner, F. R., Blechl, A. E., Denniston-Thompson, K., Faber, H. E., Richards, J. E., Slighton, J. L., Tucker, P. W., & Smithies, O. (1978) *Science 202*, 1279–1284.

Boseley, P., Moss, T., Machler, M., Portmann, R., & Birnstiel, M. (1979) *Cell 17*, 19–31.

Clewell, D. B., & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 1159–1166.

Commerford, S. L. (1971) *Biochemistry 10*, 1993–2000.

Cory, S., & Adams, J. M. (1977) *Cell 11*, 795–805.

de Boer, H., & Nomura, M. (1979) *J. Biol. Chem. 254*, 5609–5612.

Dugaiczyk, A., Boyer, H. W., & Goodman, H. M. (1975) *J. Mol. Biol. 96*, 171–184.

Enea, B., Vovis, G. F., & Zinder, N. D. (1975) *J. Mol. Biol. 96*, 495–509.

Grummt, I., Soellner, C., & Scholz, I. (1979) *Nucleic Acids Res. 6*, 1351–1369.

Grunstein, M., & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3961–3965.

Hohn, B., & Murray, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3259–3263.

Kataoka, T., Yamawaki-Kataoka, Y., Yamagishi, H., & Honjo, T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4240–4244.

Kominami, R., & Muramatsu, M. (1977) *Nucleic Acids Res. 4*, 229–240.

Kramer, R. A., Cameron, J. R., & Davis, R. W. (1976) *Cell 8*, 227–232.

Manning, R. F., Samols, D. R., & Gage, L. P. (1978) *Gene 4*, 153–166.

McClements, W., & Skalka, A. M. (1977) *Science 196*, 195–197.

McDonnell, M. W., Simon, M. N., & Studier, F. W. (1977) *J. Mol. Biol. 110*, 119–146.

Mishima, Y., Sakai, M., Muramatsu, M., Kataoka, T., & Honjo, T. (1978) *Proc. Jpn. Acad. 54*, 657–662.

Morrow, J. F., Cohen, S. N., Chang, A. C. T., Boyer, H. W., Goodman, H. M., & Helling, R. B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1743–1747.

Moss, T., & Birnstiel, M. (1979) *Nucleic Acids Res. 6*, 3733–3743.

Perry, R. P. (1976) *Annu. Rev. Biochem. 45*, 605–629.

Recombinant DNA Research Guidelines (1976) *Fed. Regist.* 41 (Sept 9).

Recombinant DNA Research Guidelines (1978) *Jpn. Sci. Monthly 31*, 626–647.

Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.

Sternberg, N., Tiemeier, D., & Enquist, L. (1977) *Gene 1*, 255–280.

Tabak, H. F., & Flavell, R. A. (1978) *Nucleic Acids Res. 5*, 2321–2332.

Taylor, J. M., Illmensee, R., & Summers, J. (1976) *Biochim. Biophys. Acta 442*, 324–330.

Thomas, M., Cameron, J., & Davis, R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4579–4583.

Thomas, M., White, R. L., & Davis, R. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2294–2298.

Tiemeier, D. C., Tilghman, S. M., & Leder, P. (1977) *Gene 2*, 173–191.
Wellauer, P. K., Dawid, I. B., Kelley, D. E., & Perry, R. P. (1974) *J. Mol. Biol. 89*, 397–407.
Wilson, G. N., Hollar, B. A., Waterson, J. R., & Schmickel, R. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5367–5371.
Yoshimori, R. N. (1971) Ph.D. Dissertation, University of California, San Francisco.

Magnesium-Dependent Interaction of 30S Ribosomal Subunits with Antibodies to N^6,N^6-Dimethyladenosine†

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ABSTRACT: The modified nucleoside N^6,N^6-dimethyladenosine occurs in *Escherichia coli* 16S ribosomal RNA only in two successive positions near its 3′ end. Antibodies directed against dimethyladenosine were induced with a nucleoside–albumin conjugate. As measured by second antibody precipitation of immune complexes, antidimethyladenosine antibodies bound 30S ribosomal subunits, ribosomal core particles, and ribosomal RNA which contain dimethyladenosine but showed little cross-reactivity with RNA or ribosomal subunits from a kasugamycin-resistant mutant which lacks dimethyladenosine. Antibody binding to ribosomal subunits was strongly influenced by the concentration of magnesium ion in the reaction medium and by the prior treatment of the subunits. Functionally active 30S subunits showed a striking binding optimum at 2–4 mM Mg^{2+}; this optimum disappeared if the subunits were inactivated by dialysis against low concentrations of magnesium ion. Instead, the inactivated subunits showed a gradual increase in antibody binding as the magnesium ion concentration was raised to 20 mM; binding of 16S ribosomal RNA or subribosomal core particles from 30S subunits gave qualitatively similar curves, with no evidence of a low [Mg^{2+}] optimum. The stability of antibody–subunit complexes was also found to depend upon subunit conformation and magnesium ion concentration; the half-life of an inactivated subunit–antibody complex (15 mM Mg^{2+}) averaged 130 min, while active subunit–antibody complexes (3 mM Mg^{2+}) had an average half-life of 70 min. More of the immune complexes with inactivated subunits were found to survive sucrose gradient sedimentation (relative to active subunits), and the concentration of subunits needed to halve antibody binding of [^{3}H]-N^6,N^6-dimethyladenosine was lower with inactivated subunits. The results suggest that the antibody binding optimum seen with active subunits at 2–4 mM Mg^{2+} represents a dynamic aspect of the three-dimensional ribosomal subunit structure; a site near the 3′ end of the RNA is involved, and both the availability of the modified nucleoside to an antibody probe and the stability of the resulting complexes are involved.

The 30S subunit of the *Escherichia coli* ribosome is a complex of 21 proteins and one molecule of RNA. The physical structure of the subunit and its components and the relationships of structure to subunit function have become major areas of research [reviewed by Brimacombe et al. (1978), Cox (1977), and Kurland (1977)]. Work from several laboratories has resulted in the localization of most of the subunit proteins and in the delineation of some of the structural and functional roles of the ribosomal RNA.

Shine & Dalgarno (1974) called particular attention to a nucleotide sequence near the 3′ end of the 16S ribosomal RNA that binds mRNA during the initiation of protein synthesis. The chemical cross-linking of the 3′ end of the RNA to initiation factor IF-3 (Van Duin et al., 1975) and the immunoelectron microscopic localization of the 3′ terminus (Olson & Glitz, 1979) within an initiation neighborhood (Kurland, 1977; Lake, 1978) further implicate this region of the RNA in the initiation step. A modified nucleoside, N^6,N^6-dimethyladenosine (m_2^6Ado),[1] occurs 24 and 25 residues from the 3′ end of the 16S RNA and nowhere else in the molecule (Brosius et al., 1978; Carbon et al., 1978, 1979). We have prepared and characterized antibodies to dimethyladenosine, and, following the techniques pioneered by Lake (Lake et al., 1974; Lake & Kahan, 1975; Lake, 1978) and in the Berlin laboratories (Tischendorf et al., 1974a,b, 1975), we have localized the nucleoside by electron microscopy of an antibody–subunit complex (Politz & Glitz, 1977). This localization is consistent with results which have identified ribosomal components that can interact with IF-3 (Czernilofsky et al., 1975; Heimark et al., 1976; Van Duin et al., 1976) and with the immunoelectron microscopic localization of the derivatized 3′ terminus (Olson & Glitz, 1979; Shatsky et al., 1979).

During the course of the characterization of ribosome–anti-m_2^6Ado interactions, we noted a significant variation in the ability of antibodies to bind different subunit preparations. The variations appeared at least partly related to methods of subunit isolation and storage. In part due to stimulation by conversations with Dr. Pallaiah Thammana and the results of Thammana & Cantor (1978), we have now examined antibody binding as a function of magnesium ion concentration and ribosome conformation. The extent of apparent anti-

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[1] Abbreviations used: m_2^6Ado, N^6,N^6-dimethyladenosine; anti-m_2^6Ado, antibodies to N^6,N^6-dimethyladenosine; rRNA, ribosomal ribonucleic acid.

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