Volume 7 Number 6 1979 Nucleic Acids Research

Fine structure of ribosomal RNA. II. Distribution of methylated sequences within Xenopus laevis rRNA*

Reindert C.Brand and Susan A.Gerbi

Division of Biology and Medicine, Brown University, Providence, RI 02912, USA

Received 21 August 1979

ABSTRACT

The distribution of methyl groups in rRNA from Xenopus laevis was analyzed by hybridization of rRNA to subfragments of either of two cloned rDNA fragments, Xlrl1 and Xlrl2, which together constitute a complete rDNA repeat unit. Using a mixture of ^{3}H-methyl plus ^{32}P-labelled rRNA as probe, the molar yield of methyl groups per rRNA region in hybrid could be calculated. For this calculation the length of the rRNA coding region in each DNA subfragment is needed, which was determined for Xlrl1 subfragments by the nuclease S_1 mapping method of Berk and Sharp. The results show that both in 18S and 28S rRNA the methyl groups are nonrandomly distributed. For 18S rRNA, clustering was found within a 3' terminal fragment of 310 nucleotides. For 28S rRNA, clustering of methyl groups was found within a region of 750 nucleotides in length, which ends 500 nucleotides from the 3' end. In contrast, the 28S rRNA 5' terminal region of 900 nucleotides is clearly undermethylated. The general position of methyl groups in 28S rRNA correlates with the location of evolutionarily conserved sequences in this molecule, as recently determined in our laboratory.

INTRODUCTION

We previously reported that evolutionarily conserved regions are present within eukaryotic 18S and 28S rRNA and suggested that these sequences play an important structural and/or functional role in rRNA [1]. Most of the methylated sites in eukaryotic rRNA have also been found to be highly conserved during evolution [2]. In fact, even the timing of introduction of certain methyl groups during rRNA maturation is the same between different species [3-5]. It is of interest, therefore, to ask whether the sequences which surround methylated sites are among those evolutionarily conserved sequences mentioned above.

In order to answer this question, we had to determine the distribution of methyl groups along the rRNA molecule. Data about the distribution of methyl groups in eukaryotic rRNA is still scarce, unlike the situation in E. coli where the position of nearly all of the 12 methyl groups in 16S rRNA

is known precisely [6, 7] and 5 out of a total of ca. 12 methyl groups in 23S rRNA are mapped in detail [8]. Only the position of the $-m^6_2A-m^6_2A-$ sequence near the 3' end of eukaryotic 18S rRNA is known exactly [9-11]. Recently Maden and Reeder [12] were able to assign nearly all of the 40 and 68 methyl groups present in X. laevis 18S and 28S rRNA respectively to either a short 3' terminal region or the long 5' remaining part of both rRNAs. In this paper we report on a more detailed mapping of methyl groups in X. laevis 28S rRNA as well as the general distribution in 18S rRNA. Our results show that the general position of methyl groups in the 28S rRNA chain correlates with the location of evolutionarily conserved sequences in this molecule, as recently determined in our laboratory (Richard L. Gourse and Susan A. Gerbi, manuscript in preparation).

MATERIALS AND METHODS

Preparation of rRNA

^{32}P-labelled RNA was prepared from X. laevis kidney culture cells grown for 3.5 days in modified Eagle's medium containing 10% dialyzed fetal calf serum, 2.5×10^{-5}M NaH_2PO_4 and 50 µCi/ml [^{32}P]orthophosphate (New England Nuclear, carrier free) [13]. The cells were chased for 3-4 hr in unlabelled medium before removal from the flask by treatment with 10x pancreatin (Grand Island Biological Company) for 2 min at room temperature. The cells were pelleted, resuspended in Kirby D-19 buffer [14] and homogenized on ice in a Ten Broeck hand homogenizer. RNA was extracted twice with phenol/cresol as described by Loening [14], but in the presence of 0.5% (w/v) sodium dodecyl sulfate. The aqueous phase was brought to 0.1M NaCl and the RNA precipitated with 2 vol of 95% ethanol at -20°C.

To prepare [^3H]methyl-labelled rRNA, cells were grown for 48 hr with 5 mCi/ml L-[methyl-^3H]methionine (New England Nuclear, 80 mCi/µmol) in methionine-free Eagle's medium containing 10% dialyzed fetal calf serum, and 10^{-2} M sodium formate as well as 2×10^{-5}M adenosine and guanosine to prevent labelling of the 1-carbon-pool and purine biosynthetic pathway [3]. Before the label was added to the medium, the [methyl-^3H] methionine was dried under N_2-gas for about 7 hr, while cooled in ice, to remove the 70% ethanol in which it was shipped, and then dissolved in the medium to the appropriate concentration. RNA was extracted as described above.

Radioactive rRNA was purified by sucrose gradient sedimentation as described previously [1]. The specific radioactivity for ^{32}P- and [^3H]methyl-labelled rRNA was 70,000-500,000 cpm per µg and about 2,000 cpm per µg respectively.

Unlabelled rRNA, prepared from either whole cell extracts or from X. laevis ovaries was a gift from Richard L. Course.

Preparation of recombinant plasmid DNA

The two Eco RI fragments from an entire rDNA repeat unit have been cloned as recombinant plasmids pXlrl1 and pXlrl2 using ColE1 DNA as a cloning vehicle [15, 16]. Both plasmids were kindly provided by Dr. Igor B. Dawid. Non-radioactive plasmid DNAs were prepared as described by Boseley et al. [17], but with some modifications. Cultures were grown in L-broth (+ Colicin E1) and the plasmid DNA was amplified using chloramphenicol [18]. A cleared lysate was prepared according to Clewell and Helinski [19] and extracted with phenol/chloroform. After ethanol precipitation, RNasing and another deproteinization step, both plasmid DNAs were further purified on CsCl/ethidium bromide gradients (refractive index 1.3910). The closed circular plasmids were collected, extracted with isoamyl alcohol, and, after dilution with 0.01 M Tris-HCl pH 8, precipitated by centrifugation at 26,000 rpm in a SW27 rotor at 15°C for 15 hr. The DNA was dissolved in 0.01 M Tris-HCl pH 8 and stored at 4°C.

In vivo ^{32}P-labelled pXlrl1 DNA was prepared using the conditions for labelling as described by Renkawitz et al. [20]. The specific radioactivity of this DNA was 2 x 10^5 cpm/µg.

Containment conditions

Plasmids containing X. laevis DNA segments were propagated under P2 + EK1 conditions as specified by "National Institutes of Health Recombinant DNA Research Guidelines" (June 23, 1976).

Isolation of restriction fragments from rDNA

To prepare the fragments r11-A to r11-E (see Fig. 1), pXlrl1 DNA was first digested with restriction endonucleases Bam HI plus Hind II in 6.6 mM each of Tris-HCl pH 7.9, β-mercaptoethanol and $MgCl_2$, 60 mM NaCl. To prepare the fragments r12-A and r12-B (see Fig. 1), pXlrl2 DNA was first digested with Bam HI alone using the same buffer. The digests were twice extracted with phenol and the DNA precipitated from the aqueous phase with 0.1 M NaCl and 2 vol 95% ethanol at -20°C. The DNA precipitate was collected by centrifugation for 10 min in an Eppendorf centrifuge, briefly dried in vacuo and dissolved in 0.01 M Tris-HCl pH 8. Next, both sets of DNA fragments were digested with Eco RI in 0.1 M Tris-HCl pH 7.5, 0.05 M NaCl, 0.005 M $MgCl_2$.

The DNA fragments derived from pXlrl1 and pXlrl2 DNA were separated by preparative electrophoresis on a 3 mm thick 2.5% agarose gel in TBE buffer

[21] at 6 V/cm. Gels were photographed after staining with ethidium bromide (1 µg/ml distilled water). DNA was recovered from the gel strips by electro-elution in 0.5 x TBE buffer at 50 V for 24 hr. The eluted DNA was loaded onto a 0.5 x 1 cm column of benzoyl-naphthoyl-DEAE-cellulose (Serva). The column was washed with 0.3 M NaCl, 0.01 M Tris-HCl pH 7.4, 0.001 M EDTA and then the DNA was eluted with a small volume of 1 M NaCl, 15% (w/v) ethanol, 0.01 M Tris-HCl pH 7.4, 0.001 M EDTA. The purified DNA fragments were precipitated with 2 vol 95% ethanol at -20°C and collected by Eppendorf centrifugation. The recovery of DNA after this purification procedure was 60-70%. The purity of fragments was checked by gel blots with ^{32}P-labelled rRNA [22]. All fragments were pure except rll-B for which contamination a correction was made (see Table 1, footnotes c and d).

80% formamide hybridization, nuclease S1 digestion of RNA-DNA hybrids and alkaline gel electrophoresis.

Hybridization of *in vivo* ^{32}P-labelled pXlrll DNA with unlabelled rRNA in a buffer containing 80% formamide and digestion of the hybrid with endonuclease S1 were performed according to Berk and Sharp [23]. The DNA was denatured at 75°C for 10 min and hybridized for 2 hr at 65°C, which we found to be the optimum temperature for hybridization under these conditions (data not shown). The hybrid was incubated with 100 units of endonuclease S1 (Boehringer) per 100 µl reaction mixture. After ethanol precipitation, the samples were electrophoresed on vertical agarose gels (25 cm long, 3 mm thick) in alkaline buffer [24]. Wet gels were covered with Saran wrap and exposed to Kodak XR5 X-ray film at -70°C using Cronex "Lighting Plus" intensifying screens (Dupont). Several controls as described by Renkawitz *et al.* [20] were performed parallel to each experiment. For molecular weight markers on all gels we used the fragments of pBR322 DNA digested with either Hind II *plus* Hind III *plus* Ava I or Hind III *plus* Hinf, which were end-labelled with [γ-^{32}P] ATP and kinase [25]. Molecular weights were rounded off to multiples of 10 nucleotides.

Filter hybridization

Isolated subfragments of pXlrll and pXlrl2 were loaded onto 0.45 µm nitrocellulose filters (Sartorius) and hybridized with subsequent RNase treatment and washing as previously described [1]. The hybridization solution was an equimolar mixture of [^{3}H]methyl- and ^{32}P-labelled rRNA in 2x SSC (SSC = 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0) containing 0.1% (w/v) sodium dodecyl sulfate, with an rRNA concentration of 3 pmoles(= 6.7 µg) / ml. The hybridized filters were counted in Aquasol-2 (New England Nuclear) after dissolving

in 0.5 M HCl and ethyl acetate [26]. A correction was made for 1.2% ^{32}P cpm spillover into the ^{3}H channel.

RESULTS

To determine if the methyl groups along X. laevis rRNA are randomly distributed or clustered, we hybridized labelled rRNA to the rDNA subfragments of a cloned ribosomal repeat unit from X. laevis shown in Fig. 1. When an equimolar mixture of [^{3}H]methyl- plus ^{32}P-labelled rRNA is hybridized to a DNA fragment containing rRNA coding sequences and non-hybridized RNA is removed, the ratio of ^{3}H cpm/^{32}P cpm in hybrid corresponds to the degree of methylation of that particular rRNA region (defined as the number of methyl groups per unit length of rRNA). The total number of methyl groups contained in the rRNA regions that hybridize to the cloned rDNA fragments Xlrl1 and Xlrl4 (which contains identical coding regions as fragment Xlrl2 [16] used in this study) is 66-67 and 43-44 respectively, as shown by fingerprint analysis [2, 12]. Therefore, the absolute number of methyl groups contained in the rRNA regions which hybridize to the DNA subfragments rl1-A to rl1-E and rl2-A and rl2-B can be calculated from the ratio of ^{3}H cpm/^{32}P cpm per hybrid, when the length of the rRNA coding region per DNA subfragment is known. For each of these subfragments the length of its coding region is known from literature data (see legend to Fig. 1) except for 28S rRNA which may or may not be present in subfragment rl1-B. This length was determined using the method described by Berk and Sharp [23]. In this way the length of the other coding regions within Xlrl1 DNA could be analyzed at the same time in order to validate the electron microscopic measurements reported in the literature.

To this end in vivo ^{32}P-labelled pXlrl1 DNA was digested with endonucleases Hind II plus Bam HI plus Eco RI to yield the subfragments rl1-A to rl1-E as well as vector Col El DNA, as is shown after gel electrophoresis under alkaline conditions in Fig. 2, lanes 1 and 6. When these fragments are hybridized with an excess of unlabelled rRNA followed by treatment with nuclease S1, only DNA sequences representing coding regions for rRNA are resistent to digestion and can be detected after separation on alkaline gels. As expected the position in the gel of fragments, rl1-A, -D, and -E has not changed after hybridization with 28S plus 5.8S plus 18S rRNA (Fig. 2, lanes 2 and 7) confirming that these DNA fragments completely represent 28S rRNA coding sequences. In addition this result shows that these rDNA fragments do not contain an intervening sequence as found within this part of the 28S rRNA gene of

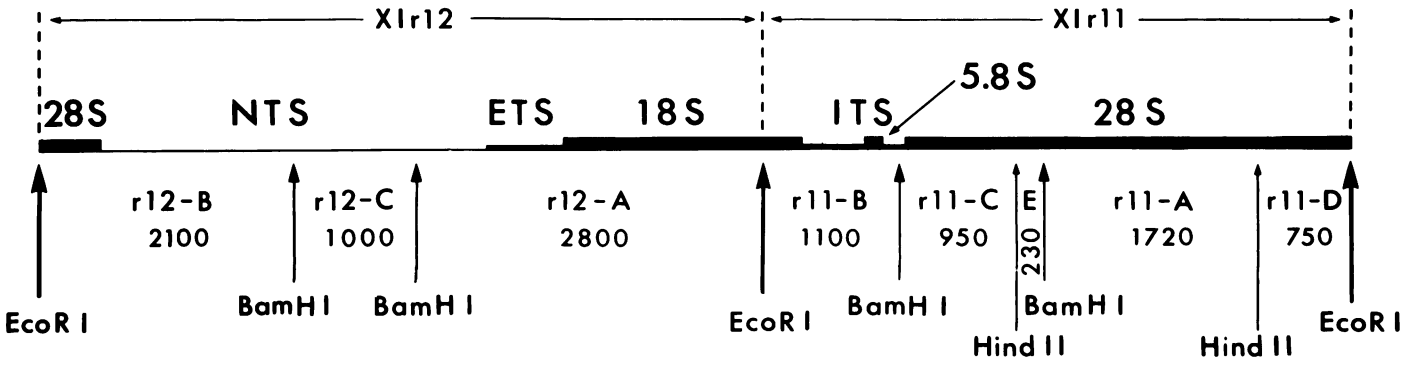

Fig. 1. Summary of structural data for two cloned rDNA fragments, Xlr11 and Xlr12

This figure summarizes existing data from the literature and from gel electrophoretic analysis of restriction fragments reported in this paper. Using the two cleavage sites for restriction endonuclease Eco RI per repeat unit in rDNA from X. laevis the two fragments designated Xlr11 and Xlr12 have been cloned [15, 16]. The physical maps for the digestion products of Xlr11 DNA with Hind II and Bam HI are taken from Boseley et al. [17], which used plasmid pX1212 containing an insert homologous to Xlr11; the map for digestion of Xlr12 DNA with Bam HI is from Botchan et al. [16]. We have corrected the lengths of these restriction fragments from other published values, by using as a molecular weight marker fragments of pBR322 DNA of which the exact length is known [27], see Fig. 2 and also Richard L. Gourse and Susan A. Gerbi, manuscript in preparation. Fragment lengths are shown in the figure in base pairs (bp). NTS and ETS stand for non-transcribed spacer and external transcribed spacer respectively. The initiation site for transcription of the 40S rRNA precursor molecule starts in the Xlr12 DNA fragment at about 2250 bp from the Eco RI site in the 18S rRNA gene [28] (also reported for the homologous rDNA insert X1108 by Boseley et al. [29]). The polarity of the transcript is 5'-18S-5.8S-28S-3' [12, 15, 30, 31]. The length of the 28S rRNA coding sequence in Xlr12 DNA is known from DNA sequence analysis to be 486 bp (Dr. Marco Crippa, personal communication) or about 500 bp [28]; the 18S rRNA coding sequence in this insert is about 1610 bp in length (this paper; see also [32]). The length of the small coding region for 18S rRNA within Xlr11 DNA is 310 bp (this paper; see also [32]), whereas the length of the internal transcribed spacer (ITS) is about 840 bp (this paper; see also [33]). The 5.8S rRNA coding region starts at about 810 bp from the 18S rDNA Eco RI site and by DNA sequence analysis its length has been shown to be 159 bp indicating a deletion of 2 bp from the 5.8S rRNA sequence [17]. In this paper we report that the 28S rRNA gene starts at about 50 bp from the Bam HI site within subfragment r11-C.

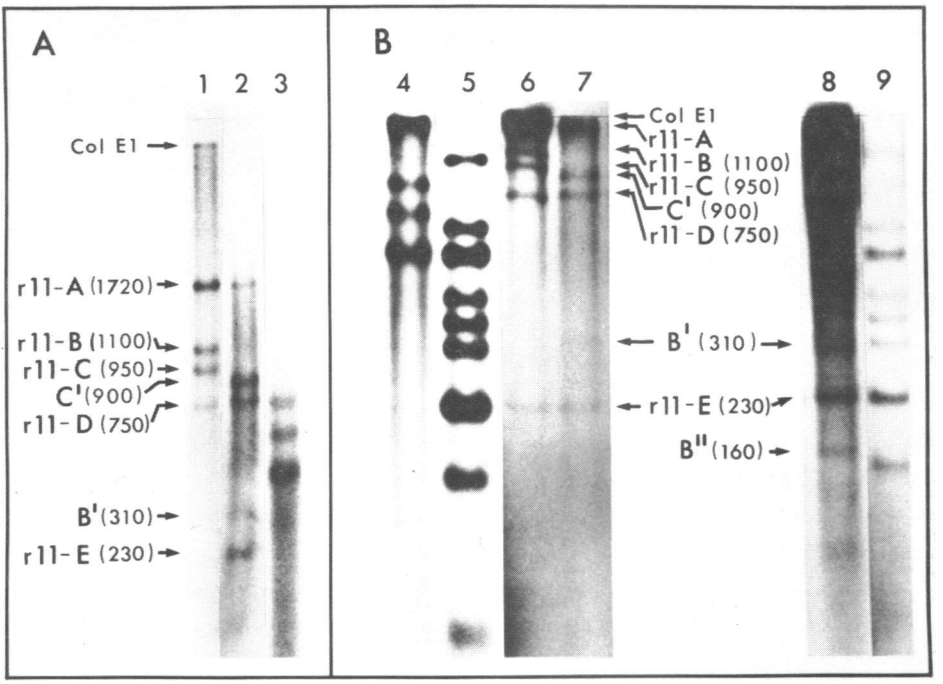

Fig. 2. Alkaline gel electrophoresis of nuclease S1 treated hybrids between rRNA and Xlrll DNA subfragments

0.7-5.4 µg of *in vivo* ^{32}P-labelled pXlrll DNA was digested with Hind II *plus* Bam HI *plus* Eco RI and the fragments were hybridized with 50 µg rRNA in 10 µl for 2 hr at 65°C. After nuclease S_1 treatment the samples were run on 1.2% alkaline agarose gels at 60 V for 13 hr (panel A) or on 3% gels at 70 V for 15 hr (panel B). Lanes (1) and (6): non-hybridized pXlrll DNA fragments, no S_1 treatment, serving as reference; lanes (2) and (7): hybridization to 18S *plus* 28S *plus* 5.8S rRNA; lanes (3) and (4): marker digest of pBR322 DNA with Hind III *plus* Hind II *plus* Ava I; lanes (5) and (9); marker digest of pBR322 DNA with Hind III *plus* Hinf; lane (8): hybridization to 28S *plus* 5.8S rRNA. The lengths are indicated in number of bases. Lanes (4) to (7) and (8) *plus* (9) are from different gels.

some other eukaryotes [34-40]. Fragments rll-B and rll-C (and Col El DNA) have disappeared in these gel lanes, whereas two new fragments of 900 and 310 nucleotides, called C' and B' respectively, can be detected. Because fragment rll-B cannot contain a coding region as long as 900 bp (see Fig. 1), fragment C' must represent the coding sequence of fragment rll-C. Therefore, the 28S rRNA gene starts within fragment rll-C at a position about 50 bp from the Bam HI cut site. If coding sequences for 28S rRNA would have been present

within fragment rll-B, its length could never have exceeded about 130 bp (= 1100 - 810 - 159; see Fig. 1). Therefore, since fragment B' is longer than this, it clearly represents the coding region for 18S rRNA within fragment rll-B. The length of this 18S rRNA coding region, as found here, is about 50 bp less than determined by electron microscopy [32] . Since the length of 18S rRNA is about 1920 nucleotides [41] it now follows that the 18S rRNA coding region within Xlrl2 DNA is about 1610 bp. A fragment corresponding to the 159 bp coding region for 5.8S rRNA [17] should be present after hybridization with 28S plus 5.8S plus 18S rRNA, but could not be detected in lane 7 of Fig. 2, probably because of the low radioactivity in such a fragment. However, after hybridization with 28S plus 5.8S rRNA and autoradiography of the profile of the nuclease S1 digested fragments for a longer time, a fragment of 160 nucleotides called B'' was clearly detectable (Fig. 2, lane 8). The length of the coding regions in fragments rll-A to rll-E, as well as for rl2-A and rl2-B is summarized in Table 1, column 1, and was used to calculate the absolute number of methyl groups in the rRNA regions represented by these rDNA fragments.

The distribution of methyl groups along rRNA was determined by hybridization of the purified subfragments rll-A to rll-E, rl2-A and rl2-B, loaded onto nitrocellulose filters, with an equimolar mixture of [^{3}H]methyl- and ^{32}P-labelled rRNA. High amounts of purified rDNA subfragments need to be loaded onto the filters in order to detect enough ^{3}H counts in hybrid. The concentration of input rRNA in the hybridization mixture that is sufficient for saturation of the coding sequence was determined using linear pXlrll and pXlrl2 DNA on filters. Fig. 3 shows that a plateau is reached at an input rRNA concentration of 3 pmoles/ml hybridization mixture, and such a saturating value of input rRNA was used for further hybridizations with the subfragments of Xlrll and Xlrl2 DNA. After incubation with pancreatic ribonuclease the RNA-DNA hybrids were assayed for ^{3}H- and ^{32}P-radioactivity. Table I summarizes the results of two independent experiments. In the first experiment the number of ^{3}H cpm in the hybrids with fragments rll-B, -C and -E is rather low, but the triplicate values of which the average is shown in Table I are in excellent agreement. In the second experiment the amount of subfragment DNA loaded onto a filter was raised in order to obtain more ^{3}H counts in hybrid. The ^{3}H cpm/^{32}P cpm ratio for a labelled rRNA region in hybrid corresponds directly to the degree of methylation of that region, since use of this ratio corrects for differences in loss of the various DNA subfragments from the filters during hybridization. The ^{3}H cpm/^{32}P cpm ratios found for the rRNA regions hybridized to subfragments rll-B and rll-D are higher than the weight

Table 1. Hybridization of [^{3}H]methyl- plus ^{32}P-labelled rRNA with rDNA fragments

In experiment 1 the following amounts of DNA were loaded onto nitrocellulose filters: in triplicate 2 µg of r11-A, 1 µg each of r11-B, R11-C and r11-D; in duplicate 1.1 µg of r11-E and 1.3 µg of r12-B. In experiment 2 the following amounts of DNA were loaded: 3 µg of r11-A, 1.5 µg of r11-B, 5.7 µg of r11-C, 1.0 µg of r11-D, 8.9 µg of r11-E, 1.0 µg of r12-A, and 1.4 µg of r12-B. Filters were hybridized with a mixture of [^{3}H]methyl- and ^{32}P-labelled rRNA as described in Materials and Methods. The amounts of ^{3}H- and ^{32}P- radioactivity for each hybrid were corrected for background counts on blank filters without DNA (20 and 53 cpm respectively for experiment 1, and 21 and 35 cpm for experiment 2).

rDNA fragment	length of coding region in bp[a]	[^{3}H]methyl cpm in hybrid		^{32}P cpm in hybrid		^{3}H cpm/^{32}P cpm[b] x10^{-2}		molar yield of methyl groups per rRNA region[f]		
		exp 1 ±SE	exp 2	exp 1	exp 2	exp 1	exp 2	exp 1	exp 2	average
r11-A	1720	90 ±3	394	1611	3276	5.6[c]	12.0[d]	18.3	21.1	19.7
-B -18S 310	469	48 ±1	206	351	840	15.1[c]	28.6[d]	13.5	13.7	13.6
-5.8S 159										
-C	900	13 ±2	36	716	1010	1.8	3.6	3.1	3.3	3.2
-D	750	159 ±8	421	819	1269	19.4	33.2	27.7	25.4	26.6
-E	230	7 ±1	21	83	160	8.4	13.1	3.7	3.1	3.4
r11-total	4069					8.6[e]	16.0[e]			
r12-A	1610	----	516	---	3993	---	12.9	---	32.0	
-B	486	108 ±19	70	967	458	11.2	15.3	---	11.4	
r12-total	2096						13.5[e]			

a. See Fig.2 plus accompanying text and legend to Fig.1.

b. The difference in ^{3}H cpm/^{32}P cpm ratios between experiments 1 and 2 reflects the decay of ^{32}P- radioactivity.

c. This value has been corrected for 6 and 5% contamination with the hybrids of fragments r11-A and r11-C respectively.

d. This value has been corrected for 11 and 9% contamination with the hybrids of fragments r11-A and r11-C respectively.

e.
$$\text{weight average} = \frac{\sum_{1}^{n} (^3\text{H cpm}/^{32}\text{P cpm})_i \times \text{length of coding region in fragment}_i}{\sum_{1}^{n} \text{length of coding region in fragment}_i},$$

i denotes each of the n different rDNA subfragments derived from either X1r11 or X1r12 DNA.

f. From the work of Maden et al. [2, 12] the total number of methyl groups in the rRNA regions (abbreviated as methyl$_T$) coded for by X1r11 and X1r12 DNA are known to be 66-67 and 43-44 respectively. Therefore, the molar yield of methyl groups per rRNA fragment (methyl$_F$) coded for by a X1r11 or X1r12 subfragment can be calculated using:

$$\text{methyl}_F = (\text{methyl}_T) \frac{(\text{length in bases}_F)(^3\text{H}_F/^{32}\text{P}_F)}{(\text{length in bases}_T)(^3\text{H}_T/^{32}\text{P}_T)}$$

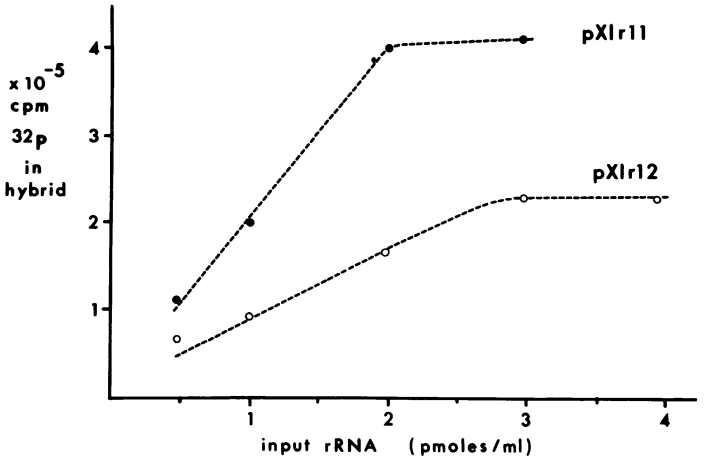

Fig. 3. Saturation curves for hybridization of cloned rDNA fragments from X. laevis with homologous rRNA. pXlr11 and pXlr12 recombinant plasmid DNAs were linearized by digestion with endonuclease Eco RI and 17.8 µg or 17.3 µg of digested DNA (corresponding to 2.3 and 1.4 pmoles respectively) was loaded onto nitrocellulose filters in duplicate. The DNA was hybridized with increasing amounts of ^{32}P-labelled rRNA under the same conditions as described in Materials and Methods for rDNA subfragments. The ^{32}P-labelled rRNA used in this experiment was directly extracted from the cells according to Wolf and Schlessinger [42] by adding per tissue culture flask 2 ml of distilled water and 0.5 ml of 0.5 M Tris-acetate pH 5.4, 0.05 M EDTA, 2.5% (w/v) sodium dodecyl sulfate, containing 100 µg/ml of polyvinylsulfate. Purification of rRNA was the same as described in Materials and Methods. Any traces of DNA contamination were removed as described previously [1]. The specific radioactivity of this rRNA was 2.4 x 10^5 cpm per µg.

average for all rRNA regions coded for by Xlr11 DNA (8.6 and 16.0 for experiments 1 and 2 respectively), whereas the ^{3}H cpm/^{32}P cpm ratio for the rRNA region hybridized to fragment r11-C is much lower. This means that the rRNA regions coded for by r11-B and r11-D DNA contain a large number of methyl groups per unit length of rRNA, whereas few methyl groups are found in the rRNA region coded for by r11-C. In contrast, the ^{3}H cpm/^{32}P cpm ratios found for the rRNA regions coded for by r11-A and r11-E as well as those for r12-A and r12-B DNA are close to the weight averages of the ratios for the hybrids with all Xlr11 and Xlr12 DNA subfragments respectively. The molar yield of methyl groups contained in the rRNA region(s) coded for by each rDNA subfragment could be calculated as shown in Table 1. An average molar yield of 13.6 is found for the methyl groups found in the rRNA regions coded for by subfragment r11-B; of this total number 1.4 is the molar yield of methyl groups in

5.8S rRNA [2] leaving a molar yield of 12.2 for the methyl groups in the 18S rRNA region. The molar yields as calculated for both experiments are in close agreement. The average of both sets of data, therefore, can be considered as a close approximation of the actual number of methyl groups contained in the various rRNA regions.

DISCUSSION

In this paper the partial data of Maden and Reeder on the distribution of methyl groups along 18S and 28S rRNA of X. laevis [12] are confirmed and extended for the four regions of 28S rRNA coded for by the subfragments A, C, D and E of Xlrll DNA. The molar yield of methyl groups calculated for the 18S rRNA regions coded for by the rl2-A and rll-B DNA subfragments as well as for the 28S rRNA region corresponding to subfragment rl2-B (Table 2) agree reasonably well with the molar yields known from fingerprint analysis: 31-32, 8-9 and 12 respectively [12]. This close agreement supports the reliability of the experimental approach used in this paper. Using this method the distribution of methyl groups along 18S and 28S rRNA was found to be non-random (Table 2).

For 18S rRNA, clustering of methyl groups was found within the short 3' terminal sequence corresponding to subfragment rll-B DNA, in which the degree of methylation (expressed as the average number of methyl groups per 100 bases

Table 2. Distribution of methyl groups along various regions of X. laevis rRNA

rRNA region (corresponding rDNA fragment)	(1) molar yield of methyl groups per rRNA region[a]	(2) length of rRNA region in bases[b]	(3) average number of methyl groups per 100 bases of rRNA = column(1)/column(2) x10^{-2}
5' 18S (rl2-A)	32.0	1610	2.0
3' 18S (rll-B)	12.2	310	3.9
	Σ = 44.2	Σ = 1920	wt $\bar{x}$ = 2.3
5.8S (rll-B)	1.4	161	8.7
5' 28S (rll-C)	3.2	900	0.4
28S (rll-E)	3.4	230	1.5
28S (rll-A)	19.7	1720	1.1
28S (rll-D)	26.6	750	3.5
3' 28S (rl2-B)	11.4	486	2.3
	Σ = 64.3	Σ = 4086	wt $\bar{x}$ = 1.6

a. See Table 1; the number for 5.8S rRNA is taken from Khan and Maden [2].
b. See legend to Fig. 1.

of RNA, Table 2, column 3) is two times as high as in the remaining part of 18S rRNA coded for by subfragment r12-A DNA. The molar yield of methyl groups in the 3' terminal sequence includes the 4 methyl groups of the m_2^6A-m_2^6A cluster found in 18 rRNA [12].

For 28S rRNA, clustering of methyl groups was also found within a 3' terminal sequence: especially in the region corresponding to DNA subfragment r11-D but also to r12-B. The degree of methylation for these regions is 2.2 and 1.4 times as high respectively as the average for the whole 28S rRNA sequence. In contrast, the 5' terminal sequence of 28S rRNA coded for by subfragment r11-C DNA is clearly undermethylated: the degree of methylation is four times less than the average for the whole 28S rRNA molecule.

From other studies from our laboratory we know the position of those regions of the 18S and 28S rRNA sequence that are conserved between X. laevis and yeast (Richard L. Gourse and Susan A. Gerbi, manuscript in preparation). Within X. laevis 28S rRNA, the regions corresponding to the DNA subfragment r11-A and r11-D have the highest percentage of evolutionarily conserved sequences, whereas the region coded for by r11-C DNA hardly contains any sequence conservation at all. In addition, the other 28S rRNA regions as well as those of 18S rRNA, listed in Table 2, have only the average percentage of sequence conservation. Thus, when we compare this data with Table 2, the degree of methylation correlates with the extent of sequence conservation for at least the 28S rRNA regions corresponding to the Xlr11 DNA subfragments C, D and E. The fact that this correlation cannot be shown for the other 28S or 18S rRNA regions, does not necessarily exclude that the position of methyl groups and evolutionarily conserved sequences coincide. If the distribution of methyl groups were known for shorter rRNA regions than, for example, the 1720 bases corresponding to the long DNA fragment r11-A, the correlation mentioned above might be found as well.

In addition, the data in Table 3 support the suggestion that the flanking sequences of methylated sites within rRNA are evolutionarily conserved. An equimolar mixture of [^{3}H]methyl- and ^{32}P-labelled rRNA from X. laevis was hybridized with homologous and heterologous rDNA, present as recombinant plasmid DNA containing a complete repeat unit. The increase in the ^{3}H cpm/^{32}P cpm ratio from 13.5 X 10^{-2} in the homologous hybrid with X. laevis rDNA to 22.9 and 24.9 x 10^{-2} in the heterologous hybrids with D. melanogaster and yeast rDNA respectively reflects the high number of methyl groups contained in the rRNA sequences conserved between X. laevis and the other species. Because about 15% of X. laevis rRNA (= 0.15 x 6170 = 925 bases) is conserved within

Table 3. Hybridization of [^{3}H]methyl- plus ^{32}P-labelled rRNA from X. laevis with homologous and heterologous rDNA

The heterologous rDNAs used were the recombinant plasmids cDm103 and pYlrA12 containing a complete ribosomal repeat unit from Drosophila melanogaster [43] and S. cerevisiae [44] respectively. 11-24 µg of linear DNA was loaded onto nitrocellulose filters and hybridized with a mixture of [^{3}H]methyl- and ^{32}P-labelled rRNA from X. laevis as described in Materials and Methods. A correction was made for background counts as described in the legend to Table 1 for experiment 2.

DNA	[^{3}H]methyl cpm in hybrid	^{32}P cpm in hybrid	^{3}H cpm/^{32}P cpm x 10^{-2}
pXlrl1 (Xenopus)	1024	6947	14.7
pXlr12 (Xenopus)	219	1942	11.3
			13.5[a]
cDm103 (Drosophila)	437	1909	22.9
pYlrA12 (yeast)	629	2523	24.9

a. weight average calculated as indicated in Table 1, footnote e.

yeast rRNA (Richard L. Gourse and Susan A. Gerbi, manuscript in preparation), the number of methyl groups within these conserved sequences can be calculated as $110 \times \frac{925}{6170} \times \frac{24.9}{13.5} = 30$ (see legend to Table 1, footnote f). The number of methyl groups per se that is conserved between X. laevis and yeast rRNA is ca. 46 [2, 4, 5, 45-48], so a substantial portion of these conserved methyl groups have flanking sequences that are evolutionarily conserved as well.

As a by-product of our methylation study reported here, we have made some adjustments to the X. laevis rDNA restriction map. We used the method of Berk and Sharp [23] to determine the position of the start of the 28S rRNA gene. This method is more accurate than using electron microscopic measurements to line up the 28S rRNA coding region in Xlrl1 DNA [12, 17]. This explains the small differences between our map positions and the map of Boseley et al. [17]. It is now clear that Xlrl1 subfragment B does not contain the beginning of the 28S rRNA gene. This gene starts in subfragment rl1-C at about 50 bp from the Bam HI cut site. In addition, we found that the 18S rRNA coding sequence within subfragment rll-B is 310 bp in length, slightly less than the length reported by Wellauer and Reeder [32]. Moreover the 28S rRNA gene as localized on Xlrl1 DNA does not contain an intervening sequence, which has been reported to interrupt the 28S (or 25S) rRNA coding region at a position about 2/3 its length downstream in several eukaryotes: Drosophila melanogaster [34-37],

Drosophila virilis [38], Calliphora erythrocephala [39], Tetrahymena pigmentosa [40] and Sciara coprophila (Drs. Renate Renkawitz-Pohl and Lloyd Matsumoto, personal communication). Ultimately, DNA sequencing will further refine the exact lengths of the Xenopus rDNA coding regions and the positions of methylated sites in the transcript.

ACKNOWLEDGEMENTS

We thank Prof. Rudi J. Planta for the initial suggestion that we correlate methylated and evolutionarily conserved regions in rRNA, after a stimulating discussion at the 1975 E.C.B.O. Nucleolus Workshop in Varna, Bulgaria. We thank Richard Gourse for helpful discussions and advice. This work was supported by PHS-GM 20261; S.A.G. is recipient of a Research Career Development Award, PHS-GM 00036. R.C.B. was supported in part by a stipend from the Netherlands Organization for the Advancement of Pure Research (ZWO).

*Paper I in this series is reference 1.

REFERENCES

1. Gerbi, S.A. (1976) J. Mol. Biol. 106, 791-816
2. Khan, M.S.N., Salim, M. and Maden, B.E.H. (1978) Biochem. J. 169, 531-542
3. Maden, B.E.H. and Salim, M. (1974) J. Mol. Biol. 88, 133-164
4. Brand, R.C., Klootwijk, J., Van Steenbergen, T.J.M., De Kok, A.J. and Planta, R.J. (1977) Eur. J. Biochem. 75, 311-318
5. Brand, R.C., Klootwijk, J., Planta, R.J. and Maden, B.E.H. (1978) Biochem. J. 169, 71-77
6. Brosius, J., Palmer, M.L., Kennedy, P.J. and Nolles, H.F. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4801-4805
7. Carbon, P., Ehresmann, C., Ehresmann, B. and Ebel, J.P. (1978) FEBS lett. 94, 152-156
8. Branlant, C., Sri Widada, J., Krol, A. and Ebel, J.P. (1977) Nucl. Acids Res. 4, 4323-4345
9. De Jonge, P. Klootwijk, J. and Planta, R.J. (1977) Nucl. Acids Res. 4, 3655-3663
10. Hagenbüchle, O., Santer, M., Steitz, J.A. and Mans, R.J. (1978) Cell 13, 551-563
11. Alberty, H., Raba, M, and Gross, H.J. (1978) Nucl. Acids Res. 5, 425-434
12. Maden, B.E.H. and Reeder, R.H. (1979) Nucl. Acids Res. 6, 817-830
13. Averner, M.J. and Pace, N.R. (1972) J. Biol. Chem. 247, 4491-4493
14. Loening, U.E. (1969) Biochem. J. 113, 131-138
15. Dawid, I.B. and Wellauer, P.K. (1976) Cell 8, 443-448
16. Botchan, P., Reeder, R.H. and Dawid, I.B. (1977) Cell 11, 599-607
17. Boseley, P.G., Tuyns, A. and Birnstiel, M.L. (1978) Nucl. Acids Res. 5, 1121-1137
18. Clewell, D.B. (1972) J. Bacteriol. 110, 667-676
19. Clewell, D.B. and Helinski, D.R. (1970) Biochemistry 9, 4428-4440
20. Renkawitz, R., Gerbi, S.A. and Glätzer, K.H. (1979) Molec. Gen. Genet. 173, 1-13
21. Peacock, A.C. and Dingman, C.W. (1968) Biochemistry 7, 668-674
22. Alwine, J.C., Kemp, D.J. and Stark, G.R. (1977) Proc. Natl. Acad. Sci., U.S.A. 74, 5350-5354

23. Berk, A.J. and Sharp, P.A. (1977) Cell 12, 721-732
24. McDonell, M.W., Simon, M.N. and Studier, F.W. (1977) J. Mol. Biol. 110, 119-145
25. Egan, J. and Landy, A. (1978) J. Biol. Chem. 253, 3607-3622
26. Kobayashi, Y. and Harris, W.G. (1975) Liquid Scintillation Counting Applications Notes no. 1, New England Nuclear, Boston
27. Sutcliffe, J.G. (1978) Nucl. Acids Res. 5, 2721-2728
28. Sollner-Webb, B. and Reeder, R.H. (1979) Cell, in press
29. Boseley, P., Moss, T. Mächler, M., Portmann, R. and Birnstiel, M. (1979) Cell 17, 19-31
30. Reeder, R.H., Higashinakawaga, T. and Miller, O. (1976) Cell 8, 449-454
31. Reeder, R.H., Sollner-Webb, B. and Wahn, H.L. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5402-5406
32. Wellauer, P.K. and Reeder, R.H. (1975) J. Mol. Biol. 94, 151-161
33. Forsheit, A.B., Davidson, N. and Brown, D.D. (1974) J. Mol. Biol. 90, 301-304
34. Glover, D.M. and Hogness, D.S. (1977) Cell 10, 167-176
35. White, R.L. and Hogness, D.S. (1977) Cell 10, 177-192
36. Wellauer, P.K. and Dawid, I.B. (1977) Cell 10, 193-212
37. Pellegrini, M. Manning, J. and Davidson, N. (1977) Cell 10, 213-224
38. Barnett, T. and Rae, P.M.M. (1979) Cell 16, 763-775
39. Beckingham, K. and White, R. (1979) J. Mol. Biol., in press
40. Wild, M.A. and Gall, J.G. (1979) Cell 16, 565-573
41. Wellauer, P.K. and Dawid I.B. (1974) J. Mol. Biol. 89, 379-395
42. Wolf, S.F. and Schlessinger, D. (1977) Biochemistry 16, 2783-2791
43. Glover, D.M. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4932-4936
44. Petes, T.D., Hereford, L.M. and Skryabin, K. (1978) J. Bacteriol. 134, 295-305
45. Klootwijk, J. and Planta, R.J. (1973) Eur. J. Biochem. 39, 325-333
46. Klootwijk, J., Planta, R.J. and Klein, I. (1973) Mol. Biol. Rep. 1, 187-191
47. Klootwijk, J., Van den Bos, R.C. and Planta, R.J. (1972) FEBS lett. 27, 102-106
48. Maden, B.E.H., Forbes, J., De Jonge, P. and Klootwijk, J. (1975) FEBS lett. 59, 60-63