Volume 10 Number 13 1982 Nucleic Acids Research

Variable methylation of the ribosomal RNA genes of the rat

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Received 5 April 1982; Revised and Accepted 2 June 1982

ABSTRACT

Both the pattern and level of rRNA gene methylation vary in the rat. This variation reflects stages in the maturation process and perhaps the level of gene expression in different tissues. We studied methylation at a common site, the inner cytosine of the sequence CCGG, by hybridizing ^{32}P-rRNA to DNA digests obtained with endonuclease Msp I (which cleaves CCGG and C^{m}CGG) and its isochizomer, Hpa II (which cleaves only CCGG). In the liver, the changing pattern of rRNA gene methylation reflected the late stages of development: the rRNA genes were mostly unmethylated at 14 days gestation; by 18 days gestation, about 30% of them were methylated, and this level persisted into adulthood. In 18-day DNA, the methylation was uniform, but in adult DNA, the methylation pattern was discontinuous, because otherwise methylated genes contaned a demethylated region. Similar developmental changes were observed in brain DNA. In a tissue culture cell line, the change from the continuous to the discontinuous pattern of methylation could be induced by transformation with Kirsten sarcoma virus. And, in adult tissues, the lowest level of rRNA gene methylation was found in rapidly growing jejunal epithelium, and the highest level, in non-growing spermatozoa.

INTRODUCTION

Changes in the level of DNA methylation are believed to be related to eukaryotic gene control mechanisms (1,2; for reviews, see 3,4). In mammals, 4%-5% of all cytosine residues are methylated as 5-methylcytosine (5-mC), with more than 90% of the total 5-mC in the sequence $^{5'}$CG$^{3'}$ (5,6,7). Other cytosines are rarely methylated.

Unlike the overall base composition of DNA, the ratio of 5-mC cytosine varies from tissue to tissue within a single animal. Such variable methylation may be an important developmental mechanism. For example, Holliday and Pugh (1) proposed that selective modification of DNA could direct differentiation and gene induction, by changing the binding of a repressor at a methylated site, or as part of a sequential >clock> mechanism, in which methylation at one site directs later methylation at an adjacent site. Similarly, Sager and Kitchin (2) proposed that methylation silences genes during development. For at least one gene family, the globin genes, recent

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observations on rabbit (8) and human (9) DNA are compatible with these theories: tissues that synthesize globins show less globin gene methylation than tissues that do not synthesize globins; surrounding DNA regions show a high level of methylation regardless of globin gene expression. Not all observations in these studies are consistent with the theories, because DNA from tissue culture cell lines and from placenta also shows reduced levels of globin gene methylation, and these cells probably do not synthesize globins. However, in somatic tissues, the correlation between reduced globin gene methylation and globin gene expression is striking.

Analysis of the rRNA genes in Xenopus laevis has also shown tissue specific changes in DNA methylation patterns (10, 11). In the oocyte, the genes are largely unmethylated, while in somatic DNA, most CG sites are heavily methylated. A recent study has shown that some of the rRNA genes are also methylated in the mouse. In this case, selective degradation of the unmethylated genes was observed when chromatin was digested with DNase I, suggesting that the unmethylated genes are transcriptionally active, and conversely, that the methylated genes are inactive (12). However, the actual demonstration of differential transcription in vivo has not been achieved.

Restriction endonucleases that contain $^{5'}CG^{3'}$ within their cutting site have been extremely useful in the analysis of methylation specificity (8, 10, 13). The paired use of Hpa II and Msp I has been particularly valuable: both cut the common tetranucleotide $^{5'}CCGG^{3'}$; however, Msp I cuts $^{5'}C^{m}CGG^{3'}$ while Hpa II does not (8, 13). When the outer cytosine is methylated, as $^{5'm}CCGG^{3'}$, then the specificity is reversed, and neither cuts $^{5'm}C^{m}CGG^{3'}$ (14). Thus comparison of Hpa II and Msp I digests of the same DNA gives a direct measure of the level of $^{5'm}CG^{3'}$ within the sequence CCGG. When such digests are blotted to nitrocellulose (15) and hybridized to specific gene probes, analysis of the hybridization pattern gives quantitative information about individual endonuclease sites within these genes.

In this study, we showed that there is significant methylation of the rRNA genes of the rat. We emphasized the study of rat liver, a tissue that is easy to follow developmentally and to manipulate experimentally. Although total liver DNA methylation detected by Hpa II sensitivity is at adult level by 14 days' gestation (Kunnath and Locker, submitted for publication), we found that methylation of the rRNA genes occurs later. Moreover, well after methylation is established, the pattern of methylation changes. To evaluate the significance of these changes, we also analyzed DNA from a variety of other adult and embryonic tissues, and two tissue culture cell lines.

MATERIALS AND METHODS

Animals Wistar-Furth rats are maintained in a small breeding colony in our laboratory. Timed pregnant female and other adult Sprague-Dawley rats were obtained from the Holtzman Company, Madison, WI. Gestational age of fetuses was determined from data provided by the company and from developmental landmarks.

Cell lines NRK ("normal rat kidney") cells were provided by R. C. Ting, Biotech Research Labs, Inc., Rockville, MD. NRK cells transformed by Kirsten murine sarcoma virus (KSV-NRK) were provided by W. Kirsten, University of Chicago, Chicago, IL. (16). Cells were maintained in modified Eagle's medium containing a 4-fold concentration of vitamins, nonessential amino acids, and 10% fetal calf serum (GIBCO, Grand Island, N.Y.), as described in Somers and Kirsten (17).

Purification of High-Molecular-Weight DNA Tissue was diced; suspended in 5 ml/g of 100 mM NaCl, 10 mM Tris, pH. 7.6; and homogenized with 10 strokes of a tight Dounce homogenizer. Nuclei were spun out at 3000 rpm for 10 min and resuspended in 5 ml of the following solution/g of tissue: 100 mM NaCl, 10 mM Tris, pH 7.6, 10 mM EDTA. When the nuclei were thoroughly resuspended, 10% Sarkosyl was added to a final concentration of 1% and proteinase K was added to a final concentration of 100 µg/ml. The solution was mixed gently but completely and then incubated at 37° for 2 hours. This highly viscous mixture was extracted several times (30 rpm, 30 min, room temperature) with an equal volume of buffer-equilibrated redistilled phenol, until no more precipitate formed at the interface between the two phases; phases were separated at 9000 g for 10 min. The DNA solution was dialyzed for 24-48 hr against several changes of 10 mM NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA. Pancreatic ribonuclease was added (from a stock solution previously heated to 100°C for 10 min) to a final concentration of 50 µg/ml, and T1 ribonuclease (Calbiochem, San Diego, CA.) to a final concentration of 5 units/ml. The solution was incubated for 2 hours at 37°C; then proteinase K was added to a concentration of 100 µg/ml, and the solution was further incubated for 2 hours at 37°C. The mixture was extracted twice with equilibrated phenol as above, and the DNA was precipitated overnight at - 10°C with two volumes of ethanol. The ethanol precipitate was washed several times with 80% ethanol, drained, partially dried, and gently redissolved in a small volume of 10 mM Tris, pH 7.6, 1 mM EDTA (about 1-2 ml/5 g tissue weight), by incubating 1-2 days at 37°C.

Several modifications were required to purify DNA from rat spermatozoa which were isolated from rat epididymis and vas deferens as described by Shiurba and Nandy (18). Purified spermatozoa from one rat were suspended in 2 ml of 10 mM Tris, pH 7.6, 10 mM NaCl, 10 mM EDTA. Dithiothreitol was added to a concentration of 100 mM, and Sarkosyl, to 1%. After 30 minutes incubation, 100 µg of proteinase

K /ml was added, and the mixture was incubated at 37°C for 3 hours. Sodium p-aminosalicylate was added to a final concentration of 300 mM, and the solution was rotated 30 minutes at 30 rpm at room temperature. The solution was extracted several times with phenol equilibrated with buffer containing 300 mM sodium p-aminosalicylate. Further DNA purification was as described above.

Tissue culture cells were removed from the culture flasks with 1 x trypsin EDTA (GIBCO Laboratories, Grand Island, N.Y.); washed with medium; recentrifuged; suspended in five volumes of 100 mM NaCl, 10 mM, Tris, pH 7.6; homogenized in a Dounce homogenizer; and otherwise treated exactly as tissue peparations.

Jejunal epithelium was separated from the intestinal wall as follows: a 5-cm segment of rat jejunum was opened and rinsed five times with 0.137M NaCl, 0.0026M KCl, 0.0043M Na_2PO_4, 0.001% phenol red. The jejunal segment was then immersed in a few ml of trypsin-EDTA solution and agitated gently; epithelial cell masses rapidly detached from the mucosal surface. After a few minutes, the piece of jejunum was removed from the solution, and the epithelial cells were then treated as described above for tissue culture cells. Cell purity was domonstrated by microscopic analysis.

Some purified DNA preparations were refractory to restriction enzyme digestion. Digestion by Eco R1 was especially inhibited and was used as a criterion for further purification. In general, embryonic liver DNA was strongly inihibitory to Eco R1, and adult kidney DNA was moderately inhibitory. Such DNA preparations with inhibitory activity were rapidly reprecipitated by mixing with one volume of cold ethanol and centrifuging immediately, washed with 70% ethanol, and redissolved as above (19). This procedure resulted in substantial loss of DNA, but the inhibitory material was removed.

Ribosomal RNA purification. rRNA was generally purified from the liver post-nuclear supernatant. The supernatant was recentrifuged at 20,000 rpm for 10 minutes, and the pellet was discarded. A 1:10 volume of 10% Sarkosyl was added and the mixture was extracted several times with an equal volume of equilibrated phenol until no more precipitate formed at the interface. The RNA was then precipitated overnight at -20°C with 2 volumes of ethanol. The precipitate was washed with ethanol, redissolved in electrophoresis buffer, and resolved on 0.6% agarose - 6M urea gels as described by Locker (20). 28S and 18S RNA bands were excised, and the RNA was extracted and then repurified over a second gel. The 28S and 18S rRNA were then reextracted from the gels and precipitated for subsequent labelling (20).

RNA labeling. For a high-specific-activity hybridization probe, RNA was degraded with 0.2 M NaOH for 0.5 hours at 0°C (21), neutralized, and labelled with

polynucleotide kinase and γ^{32}P-ATP (22).

Restriction enzyme digestion of DNA. Hpa II (Bethesda Research Laboratories, Gaitersburg, MD.) digestion was carried out in 20 mM Tris, pH 7.6, 7 mM $MgCl_2$; Msp I (New England Biolabs, Beverly, MA.) digestion in 10 mM Tris, pH 7.6, 10 mM $MgCl_2$, 6 mM KCl. Both enzyme reactions also contained 100 µg/ml BSA and 10 mM β-mercaptoethanol.

Gel electrophoresis and blotting. Electrophoresis of DNA digests was performed in agarose by methods modified from Hayward and Smith (22) and Sugden et al (24).

DNA was blotted to nitrocellulose by the method of Southern (15), according to a protocol described in Desai et al. (25).

Hybridization conditions. Hybridization conditions were modified from those described by Alwine et al. (26). Filters were preincubated at 37°C for 2-24 hours in 50% formamide, 5 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5x Denhardts solution (1 x Denhardt's solution is 0.004% bovine serum albumin, 0.004% polyvinylpyrrolidone, 0.004% Ficoll), 50 mM $NaPO_4$ buffer, pH 6.5, 0.1% sodium dodecyl sulfate, 10% sodium dextran sulfate (Pharmacia, Uppsala, Sweden), 300 µg/ml sonicated denatured Micrococcus DNA, and 300 µg/ml E. coli sRNA.

Hybridization was carried out in the same mixture except that it contained 1 x Denhardt's solution and 20 mM $NaPO_4$ buffer, pH 6.5, and in addition contained 1-5 x 10^6 c/min of ^{32}P-labelled hybridization probe. Nitrocellulose filters were sandwiched between two pieces of Whatman no. 540 paper to lower background; hybridization was carried out in plastic bags in the minimum volume that allowed free liquid in the bag, with vigorous shaking at 37°C for 24-48 hours. Filters were then drained; washed vigorously (1/2-1 hour per wash) at 37°C - 3 times with 2 x SSC, 0.1% SDS, and 3 times with 0.1 x SSC, 0.1% SDS; blotted; dried; and exposed for autoradiography.

Quantitation. Gel photographs and autoradiograms were quantitatively scanned with a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments, Chicago, IL.).

RESULTS

Digest, transfer, and hybridization analysis. Initial experiments were carried out on DNA purified from adult liver. Restriction enzymes were titrated against λDNA; Hpa II and Msp I digestion was generally carried out with 4 units of enzyme/µg DNA for 4 hours (a 16-fold excess). Figure 1 illustrates the results of quantitative digestion and loading experiments. For these digests, as much as 30 µg DNA per channel can be resolved without overloading, although 10-12 µg are generally sufficient. As shown in Figure 1, Msp I cleaves rat liver DNA into smaller pieces than Hpa II does. Figure 1 also illustrates the pattern obtained by hybridizing

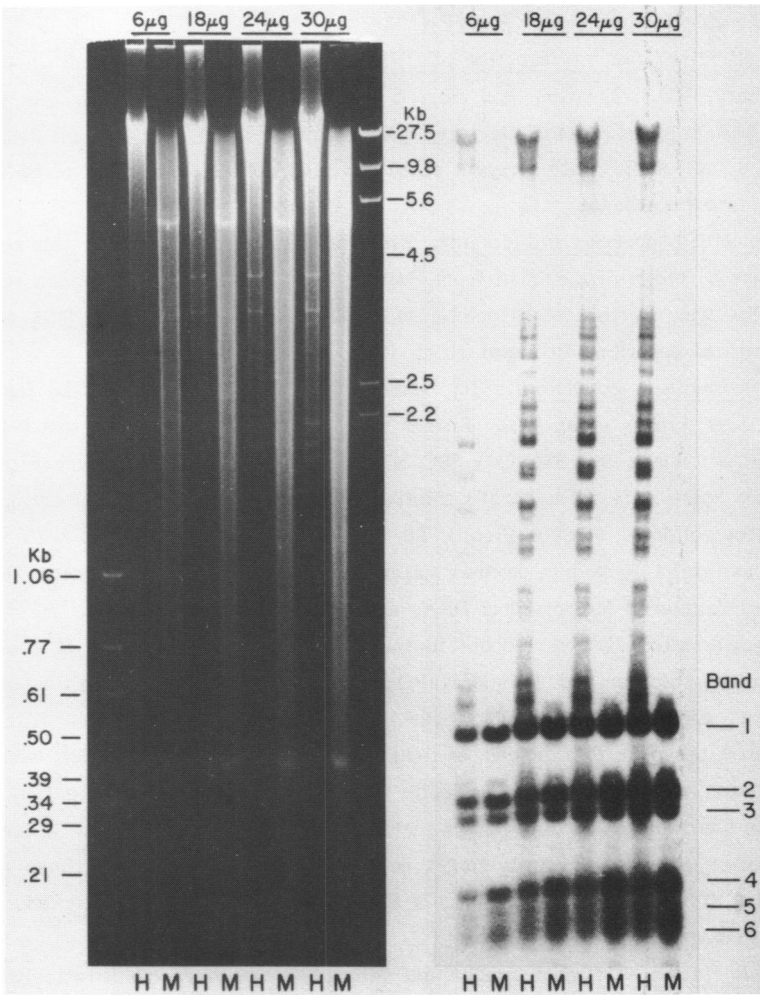

Figure 1. Msp I (M) and Hpa II (H) digests of liver DNA, and 18S rRNA hybridization. Electrophoresis of varying amounts of digested DNA was carried out in 1.5% agarose, in a gel 0.3 cm thick by 40 cm long, for 16 hr at 100V. Molecular weight standards were a Hinc II digest of ΦX174RF DNA (left) and a Hind III digest of λDNA (right). 18S hybridization bands are numbered as in Table I. The upper 30 cm of the gel was blotted to nitrocellulose and hybridized with 1.5 x 10^6 c/min ^{32}P-18S rRNA for 40 hr. Significant overloading of the gel does not occur at 30 μg of DNA/slot. With long exposure, fainter partial digest bands can be demonstrated in the 6 μg slots.

^{32}P-18S rRNA to Hpa II and Msp I digests blotted to nitrocellulose. Hybridization of 28S rRNA gave similar results. The major species detected in Msp I digests by such hybridizations are listed in Table I. Hybridizations to Hpa II digests showed

Table I. Msp I Fragments Hybridizing to 18S and 28S rRNA[1]

18S[2]		28S	
No.	kb	No.	kb
1	.63	1	.65
2	.47	2	.50
3	.42	3	.44
4	.27	4	.33
5	.24	5	.32
6	.20	6	.26
7	.14	7	.22
		8	.17
		9	.14
Total	2.37	Total	3.02

[1]These fragments were resolved on 2% agarose gels, and can account for the entire 18S gene; the number of bands hybridizing to 28S rRNA do not account for an entire 28S gene. The missing 28S bands may be below the limit of resolution of the gel (about 0.1 kb), and/or some of the higher hybridization bands may be doublets
[2]The tentative fragment order for the 18S gene, from partial digests, is 6-1-3-4-5-7-2.

all of the species visible in Msp I hybridizations, at moderately reduced intensity. In addition, many other fainter species of higher molecular weight were detected. A significant fraction was larger than 30 kb (the ribosomal repeat unit is 37 kb in the rat [27]), and thus represents a totally methylated fraction of the ribosomal genes.

Quantification of methylation. Because of idiosyncracies in the hybridization-blot system, the amount of high-molecular-weight DNA is underestimated by hybridization, since, after blotting, a considerable proportion of DNA from this region remains in the gel. The intensity of hybridization to this high-molecular-weight fraction varied considerably in different blots, not only because of inefficient transfer, but also because of its sensitivity to degradation by non-specific endonucleases. But the amount of DNA in most other faint partial digest bands is overestimated in these autoradiograms; quantitative scans of total hybridization patterns show much more hybridization to Hpa II than to Msp I digests, even though they contain identical amounts of DNA. Because of these two problems of quantification, methylation at these rRNA gene Hpa II-Msp I sites was measured by quantifying only the depletion of complete digest bands 1 to 7 in the Hpa II digest compared to the Msp I digest; for example, the loss of intensity of band 1 in Hpa II digests (compared to Msp I digests) occurred because some of the band 1 DNA was present as partial digest

bands (see Table I, below). By this method the total methylation of bands hybridizing to 18S rRNA was 26% in adult liver DNA. Band 3, however, showed no reduction of intensity and, within the limits of resolution, appeared to show no methylation (see below).

Analysis of Msp I partial digests. To analyze the distribution of methylation reflected in Hpa II digests, we simulated a random methylation pattern by incomplete digestion with Msp I. The results for 18S rRNA hybridization are illustrated in Figure 2. The hybridization patterns differ subtly but significantly from those obtained with Hpa II. Each Msp I partial digest shows a continuum of band sizes; e.g., when 50% digestion is achieved, the bands at the bottom of the gel disappear. In contrast, the Hpa II digest shows a discontinuous distribution of band sizes. The bands present at the bottom of the gel indicate that many of the ribosomal genes are unmethylated; a second region of bands in the middle of the gel corresponds to an intermediate level of methylation; and a third region at the top of the gel corresponds to heavily methylated rRNA genes. Thus the Hpa II digest hybridization shows that the rRNA genes exist in 3 different states in these DNA preparations; although 70% of these genes are unmethylated, others are partially or totally methylated.

There is another difference between the Msp I partial digest and the Hpa II digest. Certain Msp I sites are not methylated and the partial digest fragments that include these sites are not present in the Hpa II pattern. A similar experiment carried out with labelled 28S rRNA (Figure 3) showed the same phenomena.

rRNA gene methylation in animal tissues and cell lines. The analysis of adult liver DNA was extended to a variety of other rat tissues (Figure 4); adult kidney, brain jejunal epithelium, and spermatozoa. Three adult tissues, liver, kidney, and brain, represent differentiated, generally non-dividing cell populations derived from endoderm, mesoderm, and ectoderm, respectively. Jejunal epithelium is an adult tissue of endodermal origin which shows a high rate of cell division. Fourteen and eighteen-day embryonic liver and 14-day embryonic brain show moderate mitotic activity. While spermatozoa are germ cells, they are also a differentiated adult tissue which is incapable of cell division and has minimal transcription.

The relationship between growing cell populations and DNA methylation was further evaluated in tissue culture (Figure 5). NRK is a contact-inhibited cell line derived from embryonic rat kidney. KSV-KRK cells grow more rapidly than NRK cells and show an altered morphology. In addition, they are not contact-inhibited and continue to grow when confluent, until they are about 3 layers thick. At that point, however, they do stop growing, and mitotic activity becomes inapparent. For this analysis, DNA was purified from dividing and non-dividing cell populations from both NRK and KSV-NRK cell lines.

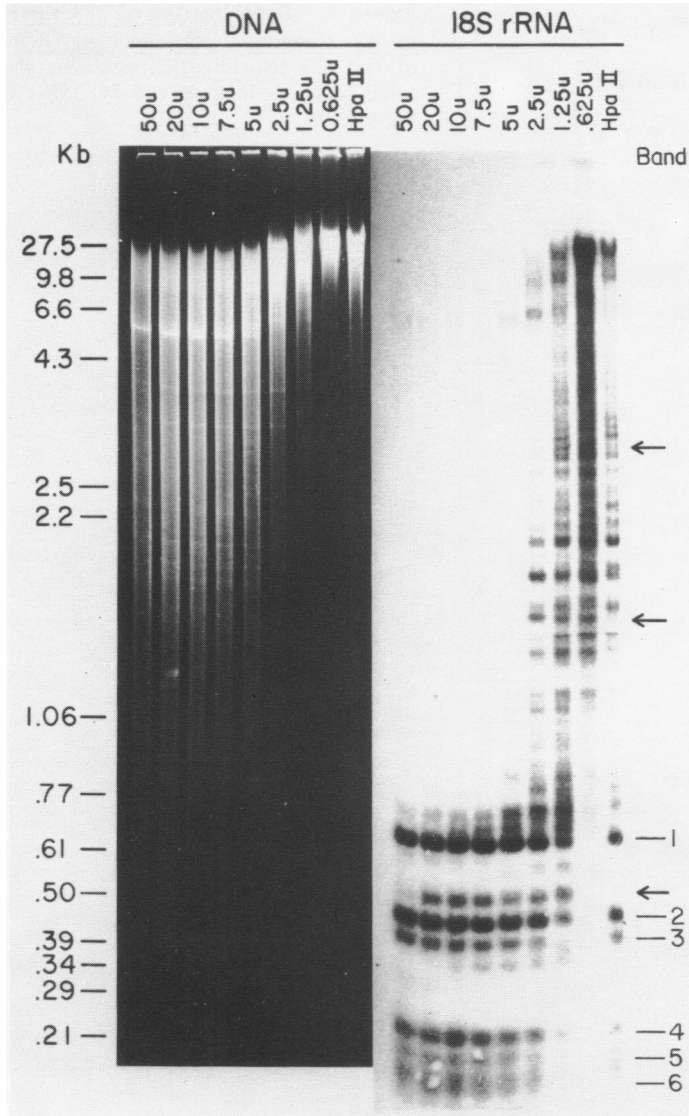

Figure 2. Hybridization of 18S ribosomal RNA to Msp I partial digests of liver DNA. Each gel slot contained a digest of 10 μg of DNA. Digestion was carried out for 1 hr at 37°C with the indicated amounts of enzyme. The Hpa II digest used a large excess of enzyme. Electrophoresis was as described in Figure 1. The stained gel is shown on the left, and the 18S hybridization on the right. The arrows point out some major Msp I partial digest bands not present in the Hpa II digest, indicating that these are not sites of methylation in liver DNA. Each Msp I digest showed a continuous distribution of band sizes, while the Hpa II pattern shows a discontinous distribution of band sizes indicating a mixture of several methylation states.

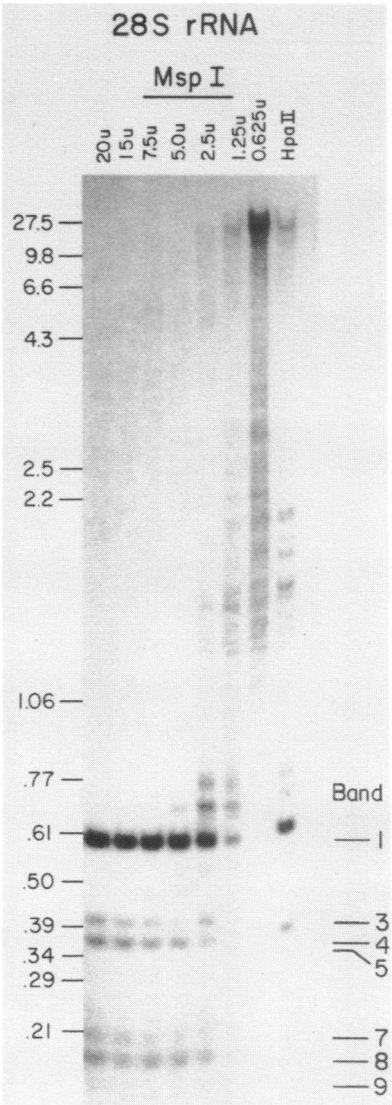

Figure 3. Hybridization of 28S ribosomal RNA to Msp I partial digests of liver DNA. Analysis of the 28S hybridization patterns showed the same phenomena observed for 18S rRNA (see Figure 2).

Methylation of the rRNA genes is low in DNA from 14-day liver or brain (Figure 4 and Table II). In 18-day liver and brain, it is at adult levels. To evaluate the timing of this methylation change, we analyzed the rRNA hybridization to DNA digests from series of livers and brains at different developmental times (not shown). In 14-day tissues, 4/4 livers and 2/2 brains, showed the same low level of rRNA gene methylation; in 18-day tissues, 4/4 livers and 2/2 brains showed the same high level of rRNA gene methylation. And, in a series of 17-day livers, 3/5 showed the

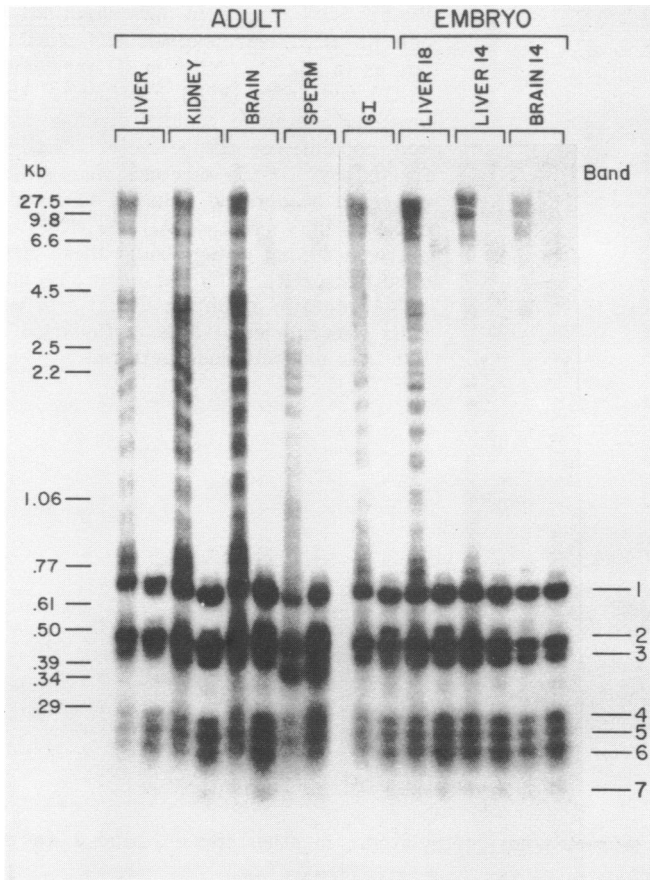

Figure 4. 18S rRNA hybridizations to DNA's from animal tissues. For each pair of digests, the Hpa II digest is on the left, and the Msp I digest is on the right. Each digest contained approximately 10 μg of DNA. Note that the Hpa II digest of sperm DNA is defective because of nonspecific degradation during restriction endonuclease digestion, causing loss of high-molecular-weight bands from the upper part of the hybridization pattern; however, the expected depletion of complete digest bands in the sperm Hpa II digest is clear.

methylated pattern of hybridization, and 2/5 showed the unmethylated pattern. The marked change in rRNA gene methylation is a rather abrupt event, occuring near 17 days gestation, and at about the same time in both liver and brain.

Besides changes in amount of methylation the pattern of rRNA gene methylation also varied (Figure 6). In adult liver, kidney, and brain, methylation resulted in reduced intensity of 18S rRNA hybridization bands 1, 2, 4, 5, 6, and 7 (see Table I). However, the intensity of hybridization band 3 was not reduced, indicating that

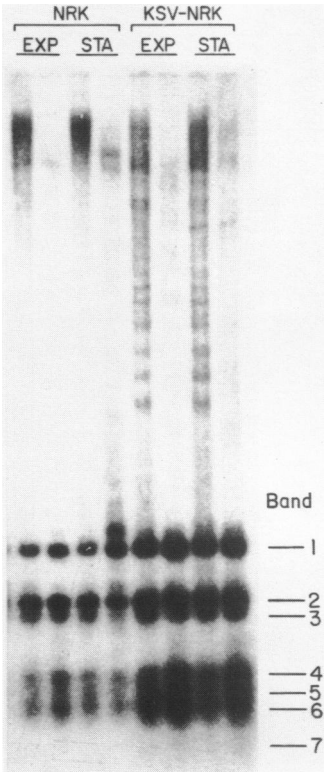

Figure 5. 18S rRNA hybridizations to DNA's from cell lines. Electrophoresis and blotting were carried out as in Fig. 1. NRK cells were collected at 40% (EXP) and 100% (STA) confluence; these cell preparations contained rapidly dividing and nondividing cell populations, respectively. NRK cells transformed by Kirsten sarcoma virus (KSV-NRK) were collected as dividing cells at 40% and as non-dividing cells at 300% confluence. The rRNA hybridization patterns did not change under these different growth conditions; NRK cells had about 30% methylation of rRNA genes in a continuous pattern, while KSV-NRK cells also had about 30% methylation of rRNA genes, but in a discontinuous pattern.

these Msp I sites are not methylated. In other tissues, band 3 was methylated in the same proportion as the rest of the rRNA gene.

The relative methylation of individual bands was difficult to quantitate, so the following band groups were compared: 1, 2+3, and 4+5+6+7. Total methylation was calculated by averaging these three values (Table II). Methylation of band 3 is reflected but overestimated in the value obtained for group 2+3.

Band 3 is less methylated than the other rRNA hybridization bands in three non-growing adult tissues, but methylated at the same level as other rRNA hybridization bands in two growing tissues, jejunal epithelium and 18-day embryonic liver. In the cell lines, band 3 is methylated in transformed cells and unmethylated in non-transformed cells.

DISCUSSION

Methylation of rRNA genes. All rat tissues and cell lines we examined contain at least a small proportion of methylated rRNA genes (8%-60%). In adult liver DNA, hybridization of rRNA to Hpa II complete digests differs from hybridization

Table II. Methylation of 18S rRNA Genes

Tissue	Average Methylation[1]	Methylation of bands 2 + 3[2]	Ratio[3]
Adult:			
Liver	26%	10%	.4
Kidney	27	7	.3
Brain	29	11	.4
Sperm	60	50	.8
GI epithelium	23	21	.9
Embryo:			
18-day liver	27	22	.8
14-day liver	8	5	.6
14-day brain	10	5	.5
Cell lines:			
NRK	29	30	.9
NRK-KSV	33	10	.3

[1]Methylation was quantitated by comparing scans of the relative intensity of complete digest bands 1-7 in Hpa II and Msp I digests as in Figure 6. Methylation was calculated as [1 - (Hpa II band intensity/Msp I band intensity)] x 100. Because of limited resolution, bands were quantitated in three groups, 1, 2+3, and 4+5+6+7, as defined in Table I. The three values were then averaged as total methylation. Values were experimentally repeatable, but only large differences should be considered significant.

[2]Bands 2+3 were measured together; inspection of scans indicated that band 2 is normally methylated while band 3 is often unmethylated.

[3]The ratio of band 2+3 methylation to total methylation. A value of 0.3-0.4 indicates a virtual lack of methylation at the sites defining band 3.

to Msp I partial digests; this difference indicates that there is a set of methylated genes and a set of unmethylated genes, not random methylation among all rRNA genes. Also, hybridization patterns from various tissues indicate that some rRNA genes show a discontinuous pattern of methylation resulting in multiple "partial digest" hybridization bands in the middle of the gel blot, while other rRNA genes show a continuous pattern of methylation, resulting in a dense hybridization band at the top of the gel. Tissues contain varying mixtures of unmethylated, continuously methylated, and discontinuously methylated rRNA genes. We estimate that the discontinuous pattern represents genes that are about 80% methylated at the inner C of CCGG, and the continuous pattern represents genes that are completely methylated at this C. Tissues, of course, are complex mixtures of different cell types, and our data do not clarify whether these different methylation states are present in all cells; however, DNA from homogenous tissue culture cell lines also contains multiple methylation states.

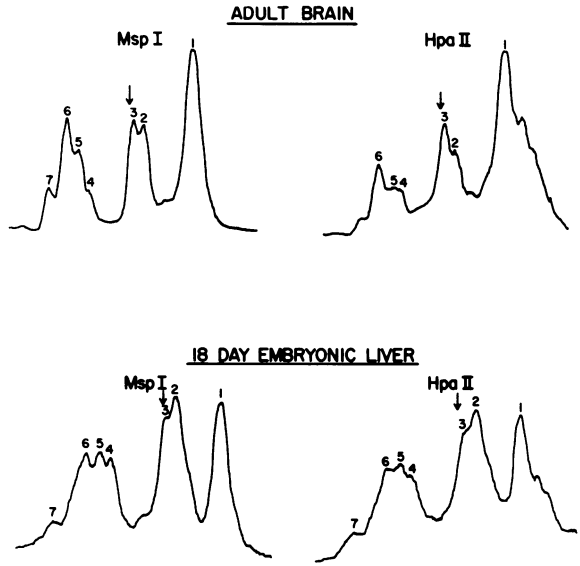

Figure 6. Scans of 18S rRNA hybridization patterns. Scans were obtained from gels such as those shown in Figure 4. In tissues which showed methylation of the 18S rRNA genes, some showed a pattern similar to adult brain (above), in which the sites defining band 3 were selectively under-methylated. Others, like 18-day embryonic liver (below), showed uniform methylation of sites defining all bands. Bands are numbered as in Table I.

The transition from the continuous to the discontinous pattern is caused, at least in part, by a change in methylation of the CCGG sites that flank band 3. If one of these two sites were methylated then band 3 would fade, and a partial digest band would appear. Therefore, the intense band 3 hybridization of adult liver must mean that neither of the 2 consecutive CCGG sites is methylated. These otherwise methylated genes thus contain a demethylated region spanning 2 sites 0.4 kb apart. A similar observation, of a demethylated region within otherwise methylated rRNA genes in Xenopus, was made by Bird and Southern (10).

Methylation and gene activity. Typical adult tissues (e.g. liver, kidney, brain) show methylation of slightly less than 1/3 of their rRNA genes. These cells all have active rRNA transcription, despite this level of methylation. The rat has 3 rRNA gene clusters, on chromosomes 3, 11, and 12 (28), which suggests a possible correlation. Perhaps rRNA gene methylation has chromosomal specificity and is associated with control of a single nucleolus organizer. Results reported by Bird et al. (12) in the mouse are particularly relevant. In their studies, hybridizations showed that mouse DNA contains a fraction of continuously methylated rRNA genes seen

as a dense band at the top of Hpa II hybridization blots. They did not observe the pattern of discontinuous hybridization that predominates in most rat tissues. They were able, however, to demonstrate that the unmethylated genes were in a chromatin region that is more sensitive to DNase I than the region containing the methylated genes. This observation suggests that the unmethylated genes are active in transcription and the methylated genes inactive. On microscopic examination of rat liver (Locker, unpublished results), adult cells generally have two small nucleoli, which suggest activity of 2 nucleolus organizers. In fetal liver (14 and 18 days gestation), there is a single, much larger nucleolus, presumably resulting from the fusion of two or more smaller nucleoli. It appears that two organizers are active and one inactive in the adult, but it is impossible to say how many are active in fetal liver, or when the third turns off. Nonetheless, it is tempting to speculate that the methylation of 1/3 of the rRNA genes occurs after 1 nucleolus organizer turns off. Whatever the relationship to gene activity, there is a clear developmental progression of changes in rRNA gene methylation. In liver, for example, there is little rRNA gene methylation at 14 days gestation. By day 18, 30% of the rRNA genes are methylated in a continuous pattern; still later, the pattern becomes discontinuous. The cell lines show this shift from continuous to discontinuous methylation most clearly. In the two cell lines we used, the overall level of rRNA gene methylation is the same. However, the transformed NRK cells show a discontinuous and the untransformed NRK cells a continuous methylation pattern. Perhaps the region around band 3 in adult liver or transformed NRK cells is the site of a protein complex that interferes with the methylation of that region. Whatever the mechanism, at least two kinds of methylation controls must be present, one that regulates the overall methylation state of an rRNA gene and another that modulates methylation around band 3. These controls result in 3 possible methylation states for an rRNA gene, all of which may be present in a single tissue, and probably within an individual cell.

ACKNOWLEDGEMENTS

We would like to thank Nicholas Cozzarelli, Lucia Rothman-Denes, and Murray Rabinowitz, for critical reading of the manuscript. The work was supported in part by grant 80-10 of the Illinois division of the American Cancer Society, and Public Health Service grants GM-27795 and CA-19264 of the National Institutes of Health. L. K. is a trainee of the Molecular and Cellular Biology Training Grant to the University of Chicago, GM-07183. J.L. is a Junior Faculty Clinical Fellow of the American Cancer Society.

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