Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 489–493, January 1981
Genetics

Amplified ribosomal RNA genes in a rat hepatoma cell line are enriched in 5-methylcytosine

(metaphase chromosomes/immunoperoxidase staining/restriction endonucleases/Southern hybridization)

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Communicated by Seymour Lieberman, October 13, 1980

ABSTRACT **In a rat hepatoma cell line, H4-IIE-C3, a 10-fold excess of 18S and 28S rRNA genes has been found in amplified chromosome regions. Antibodies to 5-methylcytidine bound extensively to the DNA of these regions, indicating a high level of DNA methylation. Most of the amplified rRNA genes were transcriptionally inactive, as shown by their failure to stain with silver. DNAs from the tumor cells and control rat hepatocytes grown with L-[*methyl*-^{14}C]methionine were digested with restriction endonuclease *Eco*RI; the DNA fragments were separated by agarose gel electrophoresis, denatured, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled rRNA or cDNA. Fragments containing the 18S or 28S rRNA coding sequences occurred in three major size classes; all three were rich in 5-methylcytosine. Analysis of *Eco*RI fragments of DNA from the tumor and control cells after digestion with *Hpa* II or *Msp* I endonuclease indicated that the 5′-C-C-G-G-3′ sequences in most of the amplified rRNA genes were methylated. Analysis of the fragments produced by digestion with *Hha* I endonuclease indicated a high degree of methylation within its recognition sequence in the amplified rRNA genes as well. The association of hypermethylation with restricted transcriptional activity suggests that DNA methylation may regulate the activity of the rRNA genes.**

Genes for mammalian 18S and 28S rRNA (rDNA) occur in multiple copies that are clustered at one or more sites per haploid genome. These sites can be visualized by *in situ* hybridization (1, 2) or a silver staining technique (3, 4). However, silver staining is restricted to transcriptionally active rRNA gene clusters or to nucleolus organizer regions (NORs), as shown by studies in interspecific somatic cell hybrids (5–8) and other systems (9–11). A small increase in human rRNA gene multiplicity (gene amplification) has been reported both in oocytes (12) and in diploid somatic cells (13). A much larger (10-fold) increase in the total amount of rDNA per cell has been observed in a rat hepatoma cell line, H4-IIE-C3, or H4 (14), in which the amplified rDNA is located in a series of long, differentially staining regions (DSRs) on the short arms of two NOR-chromosomes, 3 and 11, and two other chromosomes as well. Silver staining was restricted to short segments of the DSRs, indicating that rRNA transcriptional activity is restricted to these sites and suggesting that the great bulk of the amplified rDNA is inactive (14). This is supported by the similarity of the rates of synthesis of the 45S precursor of 18S and 28S rRNA in H4 cells and control hepatocytes (unpublished data).

5-Methylcytosine (5MeCyt) is the only modified base in the DNA of most eukaryotic organisms (15). Preferential methylation of early replicating regulatory sequences that do not code for mRNAs has been suggested (16). DNA methylation has been implicated in a different type of regulation of avian β-globin and egg protein genes (17–19) and of integrated viral genes in mammalian cells (20–22), with the inactive genes highly methylated. Such a simple relationship between DNA methylation and inactivity is not always found (18, 23); for example, in *Xenopus laevis*, both the unmethylated amplified oocyte rDNA and the highly methylated somatic rDNA are transcriptionally active (24). We now present evidence that only the transcriptionally inactive mammalian rRNA genes are highly enriched in 5MeCyt.

MATERIALS AND METHODS

Cell Culture and Chromosome Staining. Cultures of the rat hepatoma line H4-IIE-C3 (H4) and a rat hepatocyte line (K22) were grown in Ham's F-10 medium supplemented with 10% (vol/vol) fetal calf serum. Standard [3:1 (vol/vol)] methanol/acetic acid-fixed, air-dried chromosome spreads were prepared from logarithmically growing cultures. Trypsin/Giemsa banding (25) and silver staining (26) were performed as described. The binding of anti-5-methylcytidine antibodies to denatured chromosomal DNA was detected by an indirect immunoperoxidase technique (27) after denaturing the DNA by UV irradiation (28) or photooxidation (29).

Preparation of DNA. High molecular weight DNA was obtained from cell lysates as described (30, 31). To prepare [*methyl*-^{14}C]DNA, H4 and K22 cells were grown in methionine-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed calf serum and labeled with 2–3 μCi of L-[*methyl*-^{14}C]methionine (specific activity, 40–60 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels; Amersham) per ml of medium for 48 hr with or without HCOONa (1 mg/ml). Even in the absence of HCOONa, 75% of the label was found in 5MeCyt (unpublished data).

Preparation of Radiolabeled rRNA Probes. Cells of the rat fibrosarcoma line XC were grown in Dulbecco's modified medium containing 40 μCi of [^{32}P]orthophosphoric acid (carrier-free; New England Nuclear) per ml for 6 hr and then grown in fresh medium containing 200 μCi [^{32}P]orthophosphate per ml for 24–36 hr. For extraction of unlabeled rRNA, XC cells were grown in Dulbecco's modified medium. In both cases RNA was extracted by a slight modification (32) of the procedure of Glisin *et al.* (33). The pellet was dissolved in 80% dimethyl sulfoxide/20 mM Tris·HCl, pH 8.1/10 mM EDTA/1% $NaDodSO_4$ and boiled for 1 min. The sample was diluted with 20 mM Tris/10 mM EDTA to a final concentration of 20% dimethyl sulfoxide and centrifuged at 20°C in an SW 41 rotor for 16 hr at 24,000 rpm on 15–30% sucrose gradients. The peak fractions of 28S and 18S rRNA were pooled from the sucrose gradients and passed twice through an oligo(dT)-cellulose (Collaborative Re-

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Abbreviations: 5MeCyt, 5-methylcytosine; rDNA, genes for 18S plus 28S rRNA; NOR, nucleolus organizer region; DSR, differentially staining region; kb, kilobase.

search, Waltham, MA) column to remove the poly(A)-containing mRNA. [^{32}P]cDNA to 28S and 18S rRNA was synthesized as described (34). The specific activity of the cDNA was 3–5 $\times 10^8$ DNA.

Gel Electrophoresis and Hybridization. DNA samples of 10–30 μg each were digested with various restriction endonucleases (4 units/μg of DNA) for 2 hr at 37°C in buffers specified by the suppliers (New England BioLabs, Beverly, MA and Bethesda Research Laboratories, Rockville, MD). The digested DNA was extracted with phenol and precipitated with ethanol. Electrophoresis was carried out on 18 × 12 × 0.3 cm vertical slab gels of 1% (wt/vol) or 0.8% agarose in Tris/borate buffer (35) for 16–18 hr at 30 V. The wells were loaded with 5–10 μg of DNA in 20 μl of Tris/borate buffer with bromphenol blue and 1% (wt/vol) Ficoll. Bacteriophage DNA digested with *Hin*dIII restriction endonuclease was run parallel as a marker to determine the molecular weights of various restriction fragments. The DNA was denatured *in situ* and transferred to nitrocellulose filters as described by Southern (36) and modified by Hsu *et al.* (37). Hybridization was carried out in sealed plastic bags for 24 hr at 68°C in 0.6 M NaCl/0.06 M sodium citrate, pH 7, containing Denhardt's buffer (38) and 1 $\times 10^6$ cpm of either ^{32}P-labeled cDNA or rRNA per ml. The filters were washed as described (39, 40), dried, and exposed for 16–24 hr to x-ray film at −70°C with an intensifying screen.

RESULTS AND DISCUSSION

Studies using *in situ* and filter hybridization have shown a 10-fold increase in rRNA gene multiplicity in H4 rat hepatoma cells, with most of the genes in long DSRs in metaphase cells (14). These DSRs showed pale staining by the Giemsa-banding procedure (Fig. 1) and contained highly methylated DNA, as shown by extensive anti-5MeCyt antibody binding after denaturing the DNA by either UV irradiation (which produces pyrimidine dimers) or photooxidation (which modifies guanine residues). The high level of methylation of the DNA in the DSRs was not simply a reflection of the G+C richness of this DNA, because the G+C-rich amplified regions in methotrexate-resistant Chinese and Syrian hamster cell lines (which contain large numbers of active dihydrofolate reductase genes) did not bind anti-5MeCyt after either UV irradiation or photooxidation (unpublished data). Furthermore, it was not a reflection of the amount of rDNA in a single site, because the large NORs of the owl monkey and cat, which contain large blocks of active rRNA genes, did not bind anti-5MeCyt either (41). Silver staining, which identifies transcriptionally active rDNA, was restricted in H4 cells to small sites within the amplified regions (Fig. 2) and showed a pattern reciprocal to that of anti-5MeCyt (i.e., only the secondary constrictions in the DSRs were stained with silver).

These findings are consistent with the idea that transcriptionally inactive amplified rDNA, but not active rDNA, is highly enriched in 5MeCyt residues. However, the DSRs in H4 cells with about 5% of the cells' DNA may contain a large amount of non-rDNA (14). Therefore, the cytological findings, although consistent with the idea that the amplified rDNA itself was highly methylated, cannot prove it. Direct evidence that most of the rDNA in H4 cells was methylated came from biochemical studies.

Digestion of H4 and K22 (control) DNA with restriction endonuclease *Sac* I produced rDNA-containing fragments 15.0 and 3.2 kilobase pairs (kb) long (Fig. 3). Quantitative analysis of these fragments showed a 10-fold increase in the amount of rDNA in H4 DNA fragments of both sizes in comparison with control DNA (Fig. 3*B*). This indicated that denatured fragments of the two sizes were transferred to the filters with equal efficiency and confirmed the level of amplification found earlier. The marked increase in rRNA gene multiplicity in H4 cells was readily apparent with other restriction enzymes as well. For example, *Eco*RI digestion of H4 DNA produced amplified quantities of fragments of three sizes (11.2, 6.6, and 4.6 kb) containing either 18S or 28S rRNA coding sequences, as shown by agarose gel electrophoresis, Southern blotting, and hybridization to either [^{32}P]cDNA (to rRNA) or [^{32}P]rRNA (Fig. 4 *A* and *C*).

In order to look for sites of methylation within the amplified rDNA in H4 cells, we first developed a physical map of the rDNA. To do this, H4 and K22 DNA samples were digested with a series of four restriction endonucleases, singly or in pairs.

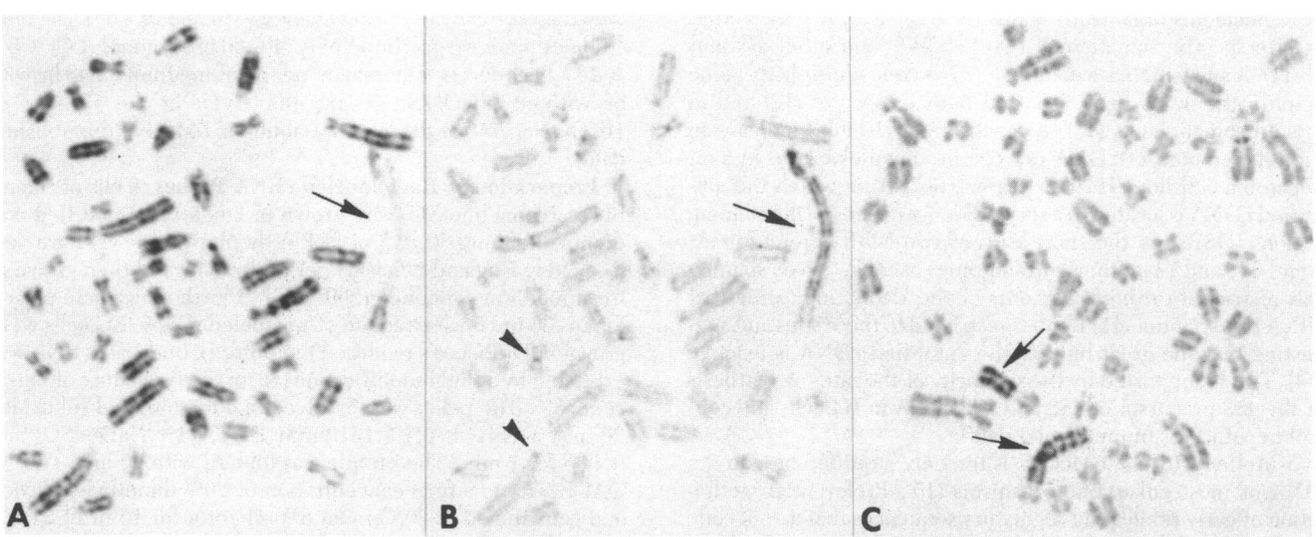

FIG. 1. (*A*) A Giemsa-banded H4 cell with a long rDNA containing DSR (arrow) on one chromosome. Note the multiple unstained regions (secondary constrictions) in the DSR. (*B*) The same cell after destaining in fixative, denaturing the DNA by UV irradiation (total dose 2.3 × 10^6 erg mm^{-2}), exposure to anti-5MeCyt, and indirect immunoperoxidase staining. The DSR (arrow) shows intense binding of anti-5MeCyt except in the secondary constrictions. Some centromeric regions (arrow heads) are also 5MeCyt rich. (*C*) another H4 cell, stained as in B after denaturation of the DNA by photooxidation for 3 hr at 27°C in the presence of methylene blue and oxygen. A 150-W photoflood lamp at 15 cm (6000 foot candles) was used. The DSRs (arrows) show intense anti-5MeCyt binding except to the secondary constrictions.

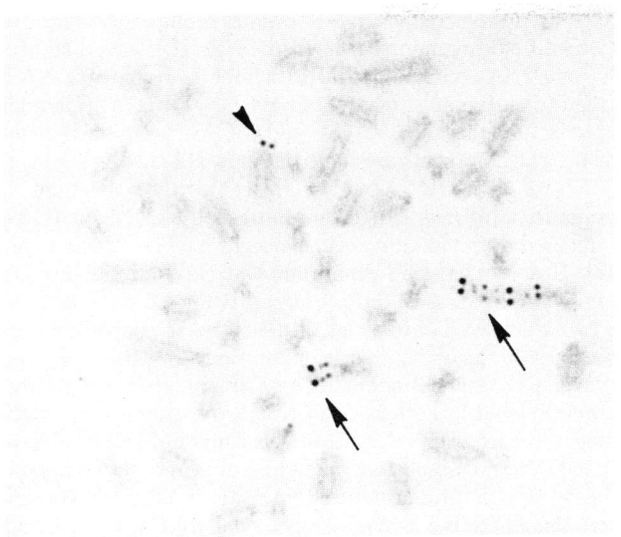

FIG. 2. An H4 cell stained with silver to show the multiple sites of transcriptionally active rDNA within the DSRs (arrows) and one normal site (arrowhead).

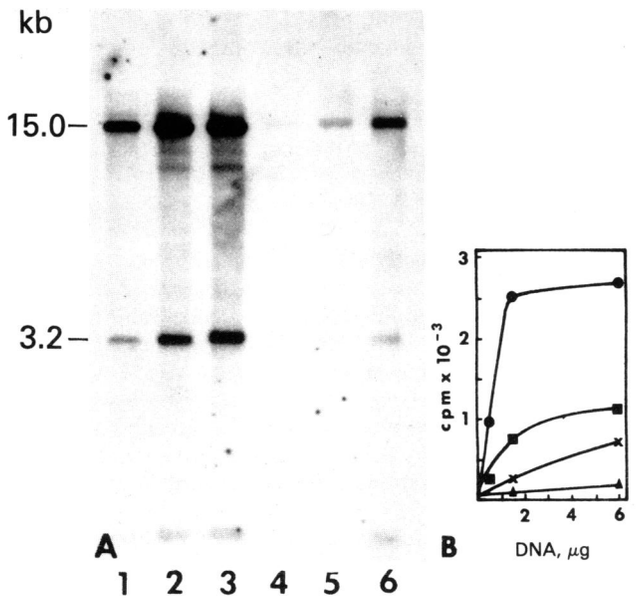

FIG. 3. The relative amounts of 18S plus 28S rDNA in *Sac* I restriction fragments of H4 and control (K22) DNA. (*A*) Autoradiogram showing hybridization of [^{32}P]cDNA (to 18S plus 28S RNA) to a Southern blot of digests of 0.5, 1.5, and 6 μg of H4 DNA (lanes 1–3, respectively) and K22 DNA (lanes 4–6, respectively). (*B*) Quantitative analysis of the amount of [^{32}P]cDNA hybridized to the 15.0- and 3.2-kb fragments, which were cut from the filter and measured in a scintillation counter. H4 DNA shows a 10-fold increase over K22 DNA in the amount of rDNA: 15.0-kb fragments in H4 (●) and K22 (×) DNA; 3.2-kb fragments in H4 (■) and K22 (▲) DNA.

Data obtained with H4 DNA using three of the enzymes are shown in Fig. 4. Most of the rRNA coding sequences in both H4 and K22 DNA were in DNA fragments of the same size, but these were much more abundant in H4 DNA. Because there was limited heterogeneity in the fragment sizes produced by digestion of either H4 or K22 DNA with any of the enzymes used, it was possible to construct a restriction map of the most common repeating units of rDNA in both (Fig. 5). This mapped region was about 22 kb long and contained 18S and 28S coding sequences, the transcribed spacer sequence, and part of the nontranscribed spacer sequence. The complete repeating unit of rat rDNA, as measured by electron microscopy with an R-loop technique, is about 38 kb (42).

The extent and sites of methylation within the rDNA sequences were studied in two ways. First, the general level of methylation of *Eco*RI fragments was assessed autoradiographically after agarose gel electrophoresis of digests of DNA from H4 and K22 cells grown with L-[*methyl*-^{14}C]methionine, both with and without sodium formate as a one-carbon source (to reduce the incorporation of label into DNA bases other than 5MeCyt). Autoradiograms of the dried gels showed that the three fragments (11.2, 6.6, and 4.6 kb) containing the 18S or 28S coding sequences were highly methylated in H4 DNA but showed no methylation in K22 DNA, even when there was

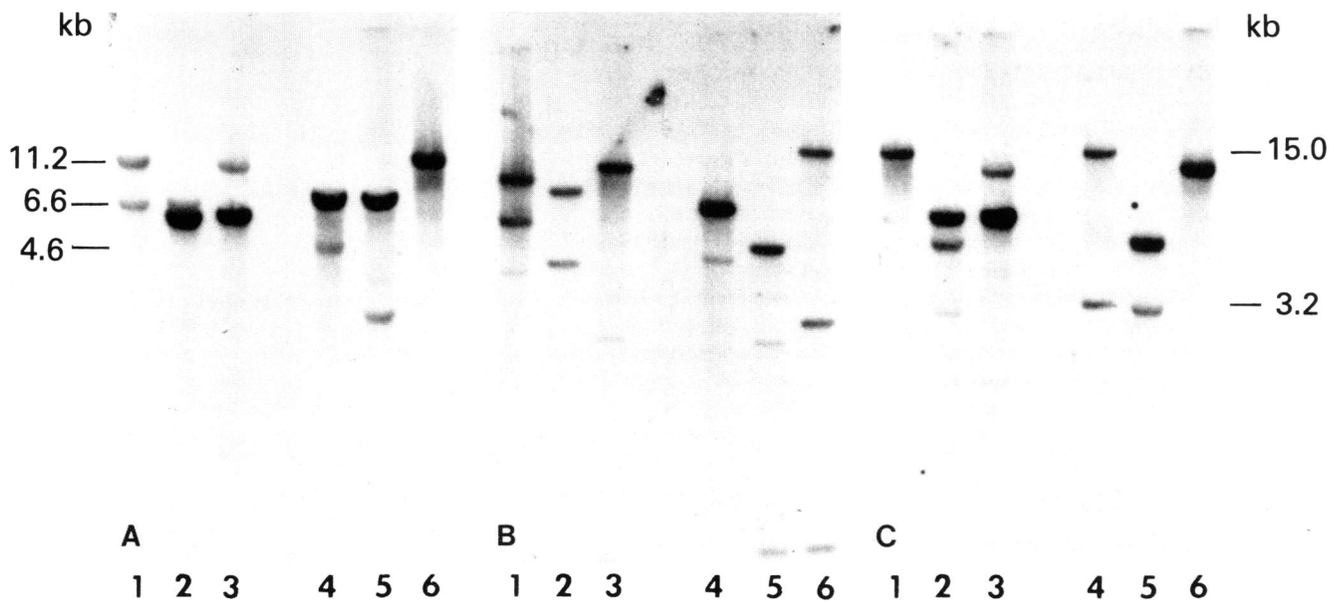

FIG. 4. Southern blot hybridization of ^{32}P-labeled 18S or 28S cDNA to restriction enzyme fragments of H4 DNA. In each case lanes 1–3 represent hybridization to 18S cDNA and lanes 4–6 to 28S cDNA. The middle lane of each group of three shows 18S or 28S rDNA-containing fragments produced by digestion with both enzymes of the pair. (*A*) *Eco*RI and *Hin*dIII restriction fragments. (*B*) *Eco*RI and *Sac* I fragments. (*C*) *Sac* I and *Hin*dIII fragments.

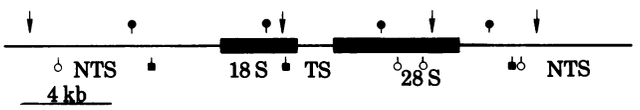

FIG. 5. Restriction map of the most common repeat unit of rat rDNA. NTS, nontranscribed spacer; TS, transcribed spacer; ▼, *Eco*RI; ♁, *Sac* I; ●, *Bam*HI; and ■, *Hin*dIII.

twice as much K22 as H4 rDNA present (Fig. 6).

More precise information concerning the extent and sites of methylation of the amplified rDNA sequences came from studies with methylation sensitive restriction enzymes *Hpa* II, *Hha* I, and *Ava* I, whose recognition sequences are 5′-C-C-G-G-3′, G-C-G-C and C-Pyr-C-G-Pur-G respectively (43–45). In general, rDNA in control cells was cleaved into small fragments by these enzymes; most of the rDNA in H4 cells was not. This was also true for *Xho* I and *Sal* I (data not shown), suggesting that they, too, are methylation sensitive. Their recognition sequences are C-T-C-G-A-G and G-T-C-G-A-C, respectively (46). In some of these studies, H4 and K22 DNA were first digested with *Eco*RI and then with *Hpa* II, *Msp* I, or *Hha* I. *Hpa* II and *Msp* I are isoschizomers that cleave double-stranded DNA in the sequence 5′-C-C-G-G-3′; *Hpa* II will not cleave the DNA if the internal C in this sequence is methylated, but *Msp* I will (43, 44). *Hha* I cleaves DNA in the sequence 5′-G-C-G-C-3′ but does not do so if the internal C is methylated (45). *Msp* I cleaved the major *Eco*RI fragments containing rDNA in both H4 and control DNA (Fig. 7). *Hpa* II cleaved the *Eco*RI fragments of control rDNA to the same extent as *Msp* I. However, *Hpa* II did not cleave most of the major *Eco*RI fragments of H4 rDNA, as shown by the limited reduction in the intensity of hybridization of radioactive ribosomal probe to the 11.2-, 6.6-, and 4.6-kb fragments. *Hha* I, like *Hpa* II, cleaved all of the major rDNA-containing *Eco*RI fragments of control DNA but failed to cleave most of them in H4 DNA (Fig. 7). Therefore, we conclude that the C-C-G-G and G-C-G-C sequences in most of the rDNA repeats are methylated in H4 rDNA.

Not all of the rDNA in H4 cells was highly methylated. For example, after *Hha* I digestion most of the rDNA in H4 cells remained in the high molecular weight fraction (about 15 or 16 kb). However, a small proportion was cleaved, yielding DNA in several size classes, the two largest being 4.5 and 0.8 kb (Fig. 8). *Hha* I cleaved virtually all of the rDNA in control cells, producing two major fragments of the same size as in H4 in approximately equal amounts. These fragments were relatively unmethylated in both H4 and K22; even after a 6-mo autoradiographic exposure of agarose gels containing *Hha* I-digested [^{14}C]DNA, no radioactivity could be detected in that region of the gel (Fig. 8). Because the rRNA genes in K22 cells were transcriptionally active, whereas only a small fraction of those in H4 cells were, it seems likely that the active rRNA genes in each cell line were in the relatively unmethylated compartment of rDNA.

Our results indicate that most of the amplified rRNA genes in rat hepatoma cells were highly enriched in 5-MeCyt residues in and around the 18S and 28S rRNA coding sequences, and this was associated with transcriptional inactivity of most of the gene copies. Similar findings were obtained in the XC rat sarcoma cell line. Cells of this line also had amplified rRNA genes located in highly methylated DSRs that contain two, sometimes three, tiny silver-stained collections of active rRNA genes (unpublished observations). Analysis of the DNA from XC cells indicated that most of its rDNA was highly methylated, although the rDNA on a tiny acrocentric chromosome was not highly methylated and was transcriptionally active (unpublished

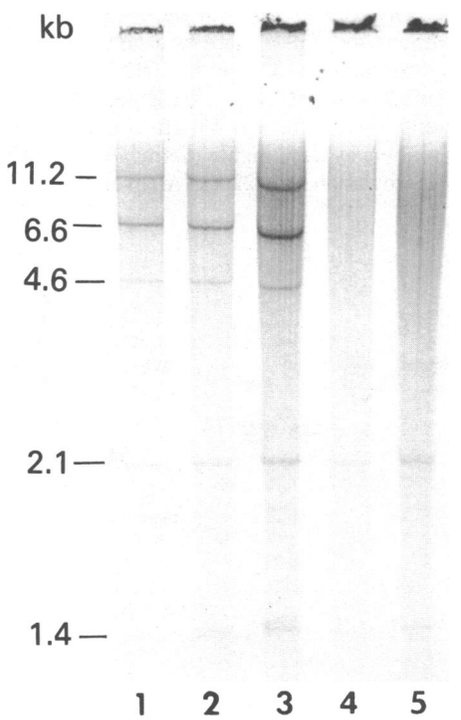

FIG. 6. Autoradiograph (10-day exposure to x-ray film) of *Eco*RI restriction fragments of L-[*methyl*-^{14}C]methionine-labeled H4 and K22 DNA after gel electrophoresis. Lanes 1, 2, and 3 contain 1, 2, and 10 μg of H4 DNA (specific activity, 600 cpm/μg). The 11.2-, 6.6-, and 4.6-kb fragments in H4 DNA are methylated; those in control DNA are not. The 2.1- and 1.4-kb fragments are methylated in both H4 and control DNA. These fragments presumably come from satellite DNA, which is known to be highly methylated (40).

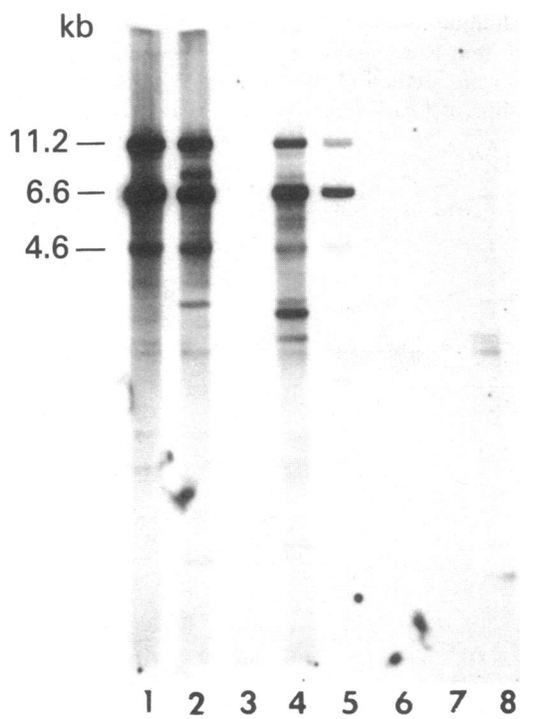

FIG. 7. Southern blot hybridization of ^{32}P-labeled 18S and 28S cDNA to restriction fragments of H4 DNA (lanes 1–4) and K22 DNA (lanes 5–8) produced by *Eco*RI (lanes 1 and 5), *Eco*RI and *Hpa* II (lanes 2 and 6), *Eco*RI and *Msp* I (lanes 3 and 7) and *Eco*RI and *Hha* I (lanes 4 and 8).

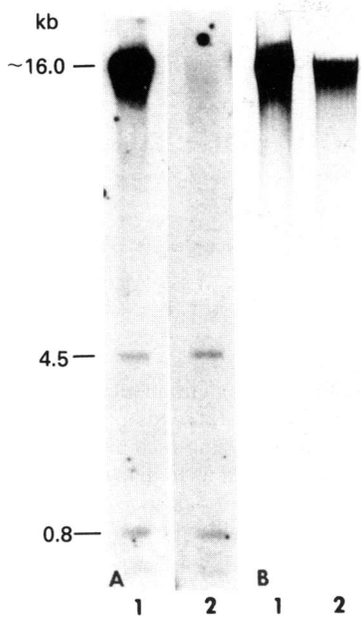

FIG. 8. *Hha* I restriction fragments of equal amounts of H4 and K22 DNA. (*A*) Hybridization to ^{32}P-labeled 18S and 28S cDNA. (*B*) Autoradiogram (6-mo exposure) of 1% agarose gel containing L-[*methyl*-^{14}C]methionine-labeled DNA. Lanes: 1, H4; 2, K22.

data). The DNA in several inherited human DSRs containing amplified and mainly inactive rDNA was also highly enriched in 5MeCyt (41).

DNA methylation appears to be involved in the regulation of integrated viral genes (20–22) and in some aspects of tissue differentiation (47, 48) and gene expression (17–19). The data obtained in the present study support the concept that rRNA genes can be inactivated by a process involving DNA methylation. It remains to be seen whether DNA methylation plays a role in the regulation of rRNA gene expression in other situations than gene amplification—e.g., in interspecific hybrids, in male meiosis, or in terminal differentiation.

H4-IIE-C3 cells were kindly provided by Dr. Henry Pitot, the K22 cells by Dr. I. B. Weinstein, and reverse transcriptase by Dr. J. Beard, through the National Cancer Institute. We wish to thank Dr. B. W. Lubit for purified antibodies to 5-methylcytidine. This work was supported in part by grants from the American Cancer Society (CD-66A), the March of Dimes–Birth Defects Foundation (I-361), and the National Institutes of Health (CA13696, CA27655, AI06860, GM25193). R.V.G. was supported by a contract (1 CP71055) from the National Cancer Institute. U.T. was a predoctoral student supported by a National Institutes of Health Traineeship (GM07088). This report is based on the thesis of U.T. in partial fulfillment of the requirements of the Ph.D. degree.

1. Henderson, A. S., Warburton, D. & Atwood, K. C. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3394–3398.
2. Hsu, T. C., Spirito, S. E. & Pardue, M. L. (1975) *Chromosoma* **53**, 25–36.
3. Goodpasture, C. & Bloom, S. E. (1975) *Chromosoma* **53**, 37–50.
4. Tantravahi, R., Miller, D. A., Dev., V. G. & Miller, O. J. (1976) *Chromosoma* **56**, 15–27.
5. Miller, D. A., Dev., V. G., Tantravahi, R. & Miller, O. J. (1976) *Exp. Cell. Res.* **101**, 235–243.
6. Miller, O. J., Miller, D. A., Dev, V. G., Tantravahi, R. & Croce, C. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4531–4535.
7. Perry, R. P., Kelley, D. E., Schibler, V., Huebner, K. & Croce, C. M. (1979) *J. Cell Physiol.* **98**, 553–559.
8. Tantravahi, R., Miller, D. A., D'Ancona, G., Croce, C. M. & Miller, O. J. (1979) *Exp. Cell Res.* **119**, 387–392.
9. Höfgartner, F. J., Krone, W. & Jain, K. (1979) *Hum. Genet.* **47**, 329–335.
10. Höfgartner, F. J., Schmid, M., Krone, W., Zenzes, M. T. & Engle, W. (1979) *Chromosoma* **71**, 197–217.
11. Schmiady, H., Münke, M. & Sperling, K. (1979) *Exp. Cell Res.* **121**, 425–428.
12. Wolgemuth, D. J., Jagiello, G. M. & Henderson, A. S. (1979) *Exp. Cell Res.* **118**, 181–190.
13. Miller, D. A., Breg, W. R., Warburton, D., Dev, V. G. & Miller, O. J. (1978) *Hum. Genet.* **43**, 289–297.
14. Miller, O. J., Tantravahi, R., Miller, D. A., Yu, L.-C., Szabo, P. & Prensky, W. (1979) *Chromosoma* **71**, 183–195.
15. Vanyushin, B. F., Tkacheva, S. G. & Belozerksy, A. N. (1970) *Nature (London)* **225**, 948–949.
16. Volpe, P. & Eremenko, T. (1974) *FEBS Lett.* **44**, 121–126.
17. McGhee, J. D. & Ginder, G. D. (1979) *Nature (London)* **280**, 419–420.
18. Mandel, J. L. & Chambon, P. (1979) *Nucleic Acids Res.* **7**, 2081–2103.
19. Van der Ploeg, L. H. T. & Flavell, R. A. (1980) *Cell* **19**, 947; 958.
20. Sutter, D. & Doerfler, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 253–256.
21. Guntaka, R. V., Rao, P. Y., Mitsialis, S. A. & Katz, R. (1980) *J. Virol.* **34**, 569–572.
22. Desrosiers, R. C., Mulder, C. & Fleckenstein, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3839–3842.
23. Pollock, J. M., Swihart, M. & Taylor, J. H. (1978) *Nucleic Acids Res.* **5**, 4855–4864.
24. Bird, A. P. & Southern, E. M. (1978) *J. Mol. Biol.* **118**, 27–47.
25. Sun, N. C., Chu, E. H. Y. & Chang, C. C. (1974) *Caryologia* **27**, 315–324.
26. Bloom, S. E. & Goodpasture, C. (1976) *Hum. Genet.* **34**, 199–206.
27. Lubit, B. W., Schreck, R. R., Miller, O. J. & Erlanger, B. F. (1974) *Exp. Cell Res.* **89**, 426–429.
28. Schreck, R. R., Erlanger, B. F. & Miller, O. J. (1974) *Exp. Cell Res.* **88**, 31–39.
29. Schreck, R. R., Warburton, D., Miller, O. J., Beiser, S. M. & Erlanger, B. F. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 804–807.
30. Gross-Bellard, M., Oudet, P. & Chambon, P. (1973) *Eur. J. Biochem.* **36**, 32–38.
31. Varmus, H. E., Bishop, J. M. & Vogt, P. K. (1973) *J. Mol. Biol.* **74**, 613–626.
32. Guntaka, R. V. & Weiner, A. J. (1978) *Nature (London)* **274**, 274–276.
33. Glisin, V., Crkvenjakov, R. & Buys, C. (1974) *Biochemistry* **13**, 2633–2637.
34. Taylor, J. M., Illmensee, R. & Summers, J. (1976) *Biochim. Biophys. Acta* **442**, 324–330.
35. Peacock, A. C. & Dingman, C. W. (1967) *Biochemistry* **6**, 1818–1827.
36. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
37. Hsu, T. W., Guntaka, R. V. & Taylor, J. M. (1978) *J. Virol.* **28**, 1015–1017.
38. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646.
39. Taylor, J. M., Hsu, T. W. & Lai, M. M. C. (1978) *J. Virol.* **26**, 479–484.
40. Shank, P. R., Hughes, S. H., Kung, H.-J., Majors, J. E., Guntaka, R. V., Bishop, M. J. & Varmus, H. E. (1978) *Cell* **15**, 1393–1395.
41. Tantravahi, U., Breg, W. R., Wertelecki, W., Erlanger, B. F. & Miller, O. J. (1980) *Hum. Genet.*, in press.
42. Stumph, W. W., Wu, J. R. & Bonner, J. (1979) *Biochemistry* **18**, 2864–2871.
43. Mann, M. B. & Smith, H. O. (1977) *Nucleic Acids Res.* **4**, 4211–4221.
44. Waalwijk, C. & Flavell, R. A. (1978) *Nucleic Acids Res.* **5**, 3231–3236.
45. Roberts, R. J. (1976) *CRC Crit. Rev. Biochem.* **4**, 123–164.
46. Gingeras, T. R., Myers, P. A., Olson, J. A., Hanberg, F. A. & Roberts, R. J. (1978) *J. Mol. Biol.* **118**, 113–122.
47. Jones, P. A. & Taylor, S. M. (1980) *Cell* **20**, 85–93.
48. Christman, J. K., Weich, N., Schoenbrun, B., Schneiderman, N. & Acs, G. (1980) *J. Cell Biol.* **86**, 366–370.