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Methylation of the 5′ Flanking Sequences of the Ribosomal DNA in Human Cell Lines and in a Human-Hamster Hybrid Cell Line

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Abstract In a human lymphoblastoid cell line (Z83) in which rDNA genes on chromosome 22 are amplified but transcribed at a low level, immunocytological studies with antibodies to 5 methylcytidine provided evidence for hypermethylation of the rDNA. The extent of methylation of the 5′ flanking sequences of the ribosomal DNA was examined by comparing the size of restriction fragments obtained by digestion of genomic DNA with EcoRI and HpaII or EcoRI and MspI. Southern blots indicated hypermethylation of the 5′ flanking sequences of many copies of rRNA genes in these cells, but not in a control lymphoblastoid cell line without rDNA amplification. Results obtained with a somatic hybrid human-hamster cell line, in which the rRNA genes on the single human chromosome 22 are inactive, showed that only a small fraction of the CCGG sites in the 5′ flanking sequences of the transcriptionally silent rRNA genes in this hybrid were methylated. Since inactive rRNA genes can show such a minimal level of methylation, it is likely that the extreme hypermethylation of the amplified rRNA genes in Z83 occurred in association with their inactivation rather than following it.

Key words: rDNA, lymphoblastoid, methylation, hypermethylation, DNA

Transcription of human rRNA genes by polymerase I is modulated by the coordinate binding of trans-acting species-specific factors to cis-acting control elements of the rDNA promoter [Sollner-Webb and Tower, 1986]. In addition, human rDNA is not transcribed in interspecific hybrid cell lines that have lost many human chromosomes but retain some that carry human rDNA [Miller et al., 1976; Perry et al., 1979], due to the absence of species-specific transcription factors [Grummt et al., 1982; Onishi et al., 1984]. In investigations into rDNA methylation in mice, Bird et al. [1981] found that most of the rRNA genes lacked methylated cytosines at sites examined, but some were methylated at most CCGG sites. The hypermethylated rDNA was in a chromatin conformation resistant to DNase I and therefore considered to be inactive. The level of transcription of rDNA estimated from the intensity of silver staining of the nucleolus organizer region (NOR) is generally correlated with the number of ribosomal genes in NOR [Warburton and Henderson, 1979]. Extensive rDNA amplification has been observed in individuals [Miller et al., 1978; Bernstein et al., 1981; Tantravahi et al., 1981a] or mammalian cell lines [Tantravahi et al., 1981b]; in these cases, immunocytological and biochemical procedures have shown that most of the amplified rDNA is extensively methylated and not transcribed. Studies on human lymphocytes suggest DNA methylation is involved in the control of rDNA expression since treatment with 5-azacytidine, an inhibitor of DNA methylation, enhances rDNA transcription [de Capoa et al., 1991]. In addition the promotor region of rDNA is located within a CpG island [Sollner-Webb and Tower, 1986]; in mammals additional methylation of CpG islands is stably transmissible through many cell divisions and is correlated with inactivation of the genes [Bird, 1987].

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Direct examination of the relationship between the state of rDNA methylation and its transcriptional activity may be possible using transfection assays. However, in one such study in which recombinant constructs containing the human rRNA gene promotor were introduced into Chinese hamster cells, which lack human-specific transcription factors for RNA polymerase I, the rDNA was transcribed by RNA polymerase II, rather than remaining transcriptionally silent [Dhar et al., 1987]. Methylation of CpG islands seems to be very unusual: in vivo it has been observed only in genes on the inactive X chromosome [Mohandas et al., 1981], in long dispersed repetitive elements [Crowther et al., 1991], or associated with neoplastic events [De Bustros et al., 1988]. We have therefore raised the question whether the hypermethylation of rDNA in some NORs also involves the promotor region of the rDNA.

In order to answer this question, a study was undertaken using two diploid human cell lines, one of which possesses amplified rRNA genes on chromosome 22, and a somatic hybrid human-hamster cell line possessing a single human chromosome 22.

MATERIALS AND METHODS

Cell Lines

The somatic cell hybrid HY. 137JT22 was obtained as described [Rocchi et al., 1986]. The cytogenetic characterization, using Q-banding, revealed a chromosome 22 as only human contribution. The hybrid was further characterized by fluorescent in situ hybridization using the alphoid probe p82H [Mitchell et al., 1985] which recognizes, at low stringency, all human centromers, and probe pI90.22, specific of chromosome 22 [Rocchi et al., 1991]. Lymphoblastoid cell lines were established from peripheral blood mononuclear cells transformed in vitro by Epstein-Barr virus: one of them was cloned by limiting dilution as previously described [Dante et al., 1992].

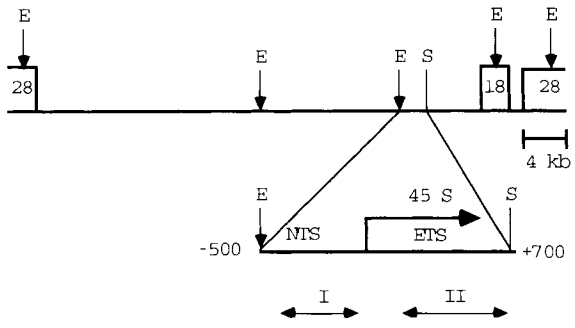
Cytogenetics

Chromosome in standard preparations from the lymphoblastoid Z83 cell line were identified by prior Q-banding for in situ hybridization or for silver staining and by prior R-banding with chromomycin A3/methyl green for immunostaining. Slides were hybridized with ^{3}H-labelled pU 1.2 (pUC plasmid containing the 18S promotor region) and pU 2.5 (pUC plasmid containing the 18S gene) [Wilson et al., 1978]. The hybridization was performed at 39°C for 16 h in 50% formamide, 2 × SSCP (1 × SSCP = 120 mM sodium chloride, 15 mM sodium citrate, 20 mM sodium phosphate, pH 6), 10% dextran sulphate, and 1 mg/ml sonicated herring sperm. The slides were washed at 41°C in 50% formamide-2 × SSC, air dried, and dipped in Ilford K2 nuclear emulsion. Silver staining for NORs was performed (Howell and Black, 1980) at 70°C for about 5 min in 50% $AgNO_3$ in the presence of gelatin (0.5%) and formic acid (0.25%). Immunostaining of UV-denatured chromosomes was performed [Tantravahi et al., 1981a] using monoclonal antibodies directed against 5-methylcytosine.

DNA Analysis

The recombinant plasmid clone pU 1.2 (kindly supplied by James Sylvester) contains a 1.2 kb EcoRI-SalI fragment of rDNA that includes part of the nontranscribed spacer, the promotor, and part of the external transcribed spacer. Probes I and II were restriction fragments of 353 bp and 527 bp in length obtained by HinfI digestion of the 1.2 kb insert (Fig. 1).

Genomic DNAs were extracted [Saluz and Jost, 1990] and digested for 16 h with an excess (10 U/μg) of restriction enzyme (Boehringer, Mannheim) in the buffers recommended by the supplier; completeness of digestion was verified by adding pBR 322 DNA or λ DNA in control experiments. After electrophoresis on standard 12% polyacrylamide gel, DNA fragments were



Human rDNA. E = EcoRI. S = SalI

Fig. 1. The recombinant used in the in situ hybridization was pUC containing a 1.2 kb EcoRI-SalI fragment of human rDNA that includes some non-transcribed spacer (NTS), the promotor, and external transcribed spacer (ETS). Fragments I and II were used as probes for Southern blot shown in Fig. 3.

electrophoretically transferred to nylon membrane (Hybond N^+, Amersham), at 80 V in 36 mM Tris-borate, 0.8 mM EDTA (pH 8.0) for 1 h at 15°C.

After denaturation of DNA in 0.5 M NaOH, 1.5 M NaCl, hybridization was performed at 65°C for 16 h in 5 × SSPE (SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 1% SDS, 100 μg salmon sperm DNA/ml, and nonfat dry milk (0.1 mg/ml). The membranes were then washed twice in 2 × SSPE, 1% SDS at room temperature, twice in 2 × SSPE, 1% SDS at 65°C, and then twice in 0.2 × SSPE, 1% SDS at 65°C. Membranes were exposed at −70°C using Amersham films (Hyperfilm-MP) and intensifying screens.

RESULTS AND DISCUSSION

In situ hybridization of pU 1.2 to chromosome spreads from the human lymphoblastoid cell line Z83 revealed a high concentration of autoradiographic grains on the short arm of chromosome $22p^+$ (Fig. 2). About 60% of the total grains were scored on this chromosome. In order to rule out the possibility of rDNA amplification limited to the non-transcribed spacer (NTS), the experiment was repeated using a probe which includes the 18S gene and part of the external spacer (ETS). Results (not shown) were comparable to those obtained with pU 1.2. These data indicate that the NOR present on the chromosome $22p^+$ includes a relatively high number of ribosomal genes. Silver staining of the metaphase chromosomes has been shown to detect transcriptionally active rDNA clusters [Miller et al., 1976; Perry et al., 1979], with the size of the silver stained region positively correlated with rDNA activity [Warburton and Henderson, 1979]. The NOR located on chromosome $22p^+$ of the Z83 cell line showed little silver staining, the size being in the range of the NORs located on other chromosome of the same cell (Fig. 2), suggesting that most of the copies of amplified rDNA on this chromosome are inactive. Immu-

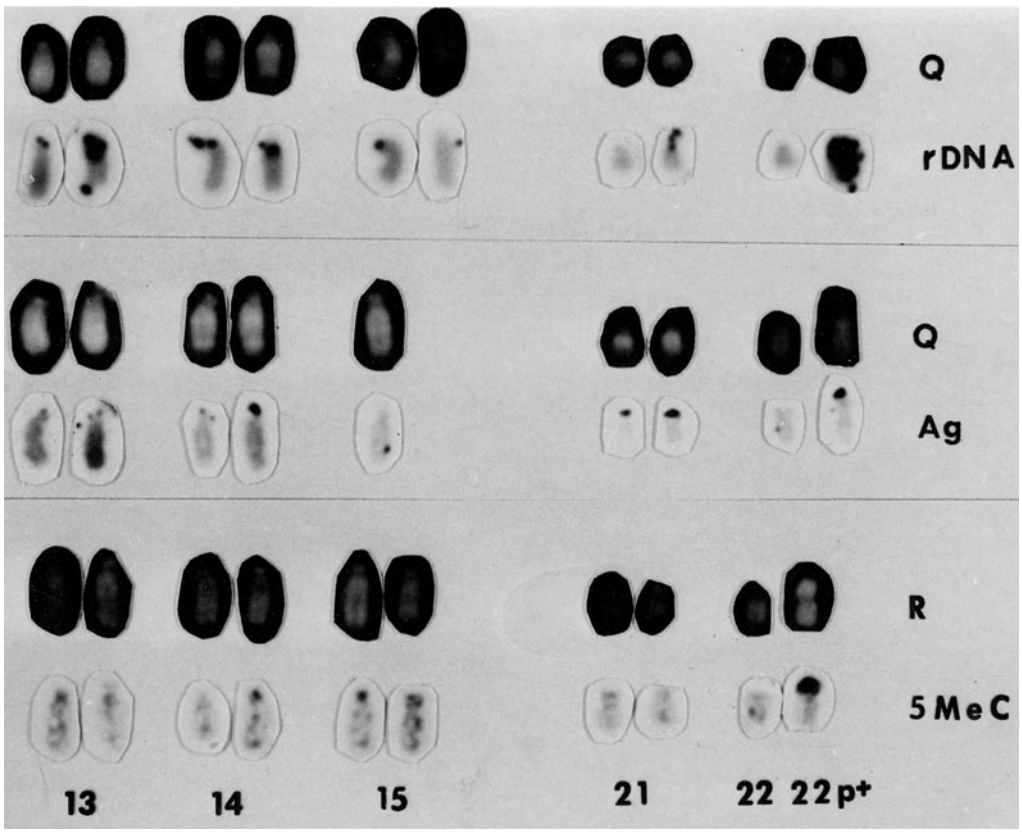


Fig. 2. Partial karyotypes of the Z83 cell line. Metaphase chromosome spreads were treated as follows. Q: Q-banded with quinacrine mustard; rDNA hybridized with ^3H-labelled pU 1.2; Ag: silver stained to show nucleolus organizer regions; R: R-banded with chromomycin A3/methyl green; 5MeC: immunostained with monoclonal antibodies directed against 5-methylcytosine.

nostaining with an antibody directed against 5-methylcytosine revealed a large region, apparently spanning the entire short arm of chromosome 22p$^+$, suggesting that the amplified rDNA is heavily methylated. These results are in good agreement with those of Tantravahi et al. [1981b], who have shown by biochemical procedures and immunocytological staining that amplified rDNA sequences are mostly inactive and are hypermethylated in a rat hepatoma cell line that has a tenfold increase in rRNA genes.

To test the hypothesis that the hypermethylation of the 22p$^+$ rDNA includes the CpG island that is present in the promotor region at the 5′ extremity of each rRNA gene, we examined the 5′ flanking region of rDNA from the Z83 lymphoblastoid cell line. Restriction fragments obtained after digestion of genomic DNA with EcoRI and either HpaII or MspI were separated by polyacrylamide gel electrophoresis, electrotransferred onto a nylon membrane, and hybridized with specific probes within this region (Fig. 3). MspI reduced the rDNA genes in Z83 to small fragments with major bands of about 51 bp for the NTS region (Fig. 3A, lane 6) and 92 bp and 100 bp for the ETS region (Fig. 3B, lane 6). HpaII, the methylation-sensitive isoschizomer of MspI, produced faint bands of the same size, but most of the DNA was in a series of larger bands at the top of the gels (Fig. 3A,B, lane 5), indicating the presence of a high proportion of methylated CCGG sites. In contrast, DNA extracted from lymphoblastoid cell lines established from two healthy donors did not exhibit this rDNA methylation (one is shown in Fig. 3A,B, lanes 3, 4).

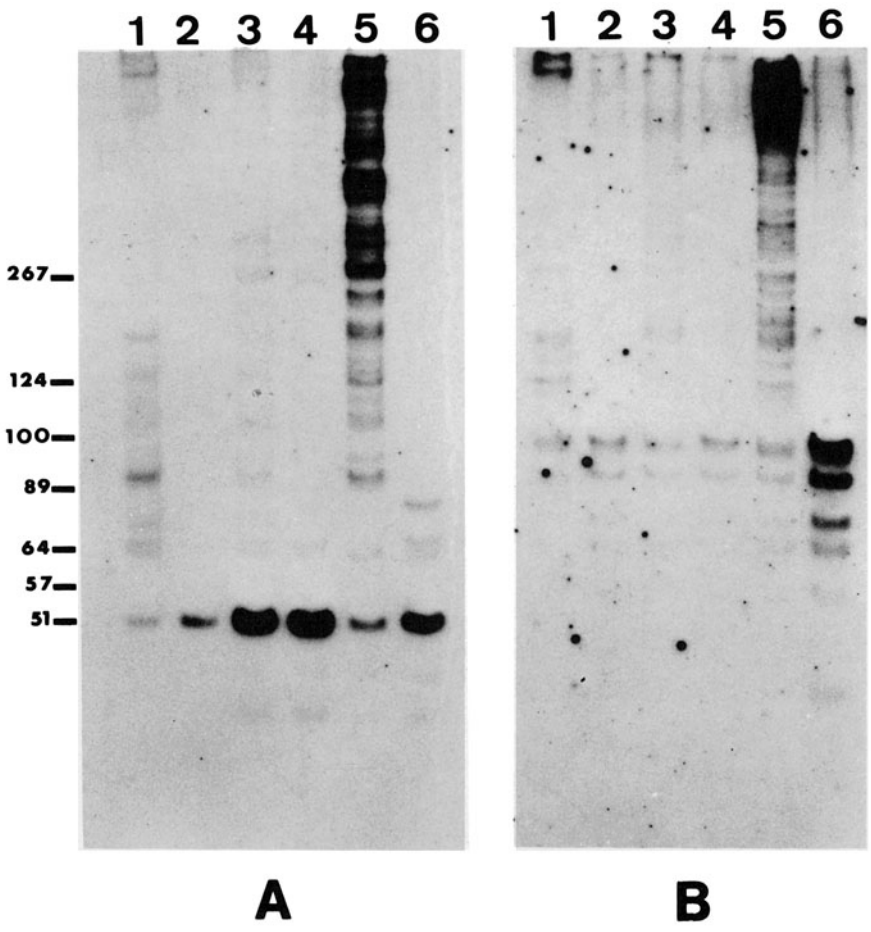


Fig. 3. Southern blot analysis of the methylation of the 5′ flanking sequences of the rRNA genes. Sample DNA (10 μg or 2 μg in B, lanes 3, 4), extracted from a hamster-human cell line containing only one chromosome 22 (lanes 1, 2), a human lymphoblastoid cell line (lanes 3, 4), or the Z83 cell line which contains a 22p+ (lanes 5, 6). The DNA was digested with EcoRI + HpaII (lanes 1, 3, 5) or EcoRI + MspI (lanes 2, 4, 6). The Southern blots were hybridized with (**A**) probe I or (**B**) probe II.

These results indicate that the hypermethylation of the NORs and the low level of rDNA transcription of chromosome $22p^+$ is correlated with the hypermethylation of the promotor region and the external transcribed spacer of the rDNA genes.

Several CpG islands of non-transcribed tissue-specific genes, but not housekeeping genes, become methylated in cell lines [Antequara et al., 1990; Jones et al., 1990], suggesting that methylation of these sequences is actively regulated. The question was therefore raised whether the absence of transcription of human rDNA in rodent-human hybrids with loss of some human chromosomes might allow the methylation of the 5′ flanking regions of the remaining human rRNA genes. In order to test this hypothesis, a study was undertaken using a hamster-human cell line possessing a single human chromosome 22. Silver staining of the metaphase chromosome indicated that the rRNA genes on the two chromosomes 22 were actively transcribed in the parental human lymphocytes used for cell fusion (data not shown), but not on the number 22 in the hybrid cells.

The cloned fragment of the human rDNA promotor region does not cross-hybridize to sequences in Chinese hamster [Dhar et al., 1987] and could therefore be used to analyze this hybrid line. As expected, the pattern obtained after digestion with MspI is similar to those obtained from DNA extracted from both human lymphoblastoid cell lines (Fig. 3A,B, compare lane 2 with lane 4). In the DNA digested by HpaII, some methylated bands containing rDNA were detected. However, the small size of these fragments, 57–200 bp, indicates that only few CCGG sites are methylated, because the regions corresponding to probe I (353 bp long) and to probe II (527 bp long) are considerably larger and contain 13 and 15 MspI/HpaII sites, respectively. It would be interesting to know whether such a low level of methylation has rendered these genes incapable of transcriptional activity. In any case, these results show that some methylation can be detected in the 5′ flanking regions of the rDNA genes that are transcriptionally silent because the absence of human-specific rDNA transcription factors. However, this level of modification is very low when compared to that of the rDNA extracted from the Z83 cell line. Therefore these data strongly suggest that the absence of transcription is not the only factor involved in the regulation of the methylation of these CpG islands. In good agreement with this hypothesis are the recent observations that DNA methylation might be regulated by a combination of demethylation and de novo methylation [Saluz et al., 1986; Antequara et al., 1990; Frank et al., 1991].

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REFERENCES

Antequara F, Bojes J, Bird A (1990): High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 62:503–514.

Bernstein R, Dawson B, Griffiths J (1981): Human inherited marker chromosome 22 short-arm enlargement: Investigation of rRNA gene multiplicity, Ag-band size, and acrocentric association. Hum Genet 58:135–139.

Bird AP (1987): CpG islands as gene marker in the vertebrate nucleus. Trends Genet 3:342–347.

Bird AP, Taggart MH, Gehring CA (1981): Methylated and unmethylated ribosomal RNA genes in the mouse. J Mol Biol 152:1–17.

Crowther PJ, Doherty JP, Linsenmeyer ME, Williamson MR, Woodcock DM (1991): Revised genomic consensus for the hypermethylated CpG island region of the human L1 transposon and integration sites of full length L1 elements from recombinant clones made using methylation-tolerant host strains. Nucleic Acids Res 19:2395–2401.

Dante R, Dante-Paire J, Rigal D, Roizès G (1992): Methylation patterns of long interspersed repeated DNA and alphoid repetitive DNA from human cell lines and tumors. Anticancer Res 12:559–564.

De Bustros A, Nelkin BD, Silverman A, Ehrlich G, Poiesz B, Baylin SB (1988): The short arm of chromosome 11 is a "hot spot" for hypermethylation in human neoplasia. Proc Natl Acad Sci USA 85:5693–5697.

de Capoa A, Aleixandre C, Felli MP, Ravenna L, Costantino MA, Giancotti P, Vincenti O, Poggesi I, Grappelli C, Miller DA (1991): Inheritance of ribosomal gene activity and level of DNA methylation of individual gene clusters in a three generation family. Hum Genet 88:146–152.

Dhar V, Miller DA, Kulkarni AB, Miller OJ (1987): Human ribosomal DNA fragments amplified in hamster cells are transcribed only by RNA polymerase II and are not silver stained. Mol Cell Biol 7:1289–1292.

Frank D, Keshet I, Levine A, Shani M, Razin A, Cedar H (1991): Demethylation of CpG island in embryonic cells. Nature 351:239–241.

Grummt I, Roth E, Paule MR (1982): RNA transcription in vitro is species specific. Nature 296:173–174.

Howell WM, Black DA (1980): Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: A one-step method. Experentia 36:10–14.

Jones PA, Wolkowick MJ, Rideout II WM, Gonzales FA, Mariasz CM, Coetzee SJ, Tapscott SJ (1990): De novo methylation of the *MyoD1* CpG island during the establishment of immortal cell lines. Proc Natl Acad Sci USA 87:6117–6121.

Miller DA, Breg WR, Warburton D, Dev VG, Miller OJ (1978): Regulation of rRNA gene expression in a human familial 14p+, marker chromosome. Hum Genet 43:289–297.

Miller DA, Dev VG, Tantravahi R, Miller OJ (1976): Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. Exp Cell Res 101:235–243.

Mitchell AR, Gosden JR, Miller DA (1985): A cloned sequences, p82H, of the alphoid repeated DNA family found at the centromeres of all human chromosomes. Chromosoma 92:369–377.

Mohandas T, Sparkes RS, Shapiro LJ (1981): Reactivation of an inactive human X-chromosome: Evidence for X-inactivation by DNA methylation. Science 211:393–396.

Onishi T, Berglund C, Reeder RH (1984): On the mechanism of nucleolar dominance in mouse-human somatic cell hybrids. Proc Natl Acad Sci USA 81:484–487.

Perry RP, Kelley DE, Schibler V, Huebner K, Croce C (1979): Selective suppression of the transcription of ribosomal genes in mouse-human hybrid cells. J Cell Physiol 98:553–560.

Rocchi M, Archidiacono N, Carbone R, Bolino A, Shridar V, Ward DC, Baldini A (1991): Isolation of a human chromosome 22-specific alpha satellite clone. HGM11, London 18–22 Aug. 1991, meeting abstract.

Rocchi M, Roncuzzi L, Santamaria R, Archidiacono N, Dente L, Romeo G (1986): Mapping through somatic cell hybrids and cDNA probes of protein C to chromosome 2, factor X to chromosome 13, and alfa1-acid glycoprotein to chromosome 9. Hum Genet 74:30–33.

Saluz HP, Jiricny J, Jost JP (1986): Genomic sequencing reveals a positive correlation between the kinetics of strand-specific DNA demethylation of the overlapping estradiol/glucocorticoid-receptor binding sites and the rate of avian vitellogenin mRNA synthesis. Proc Natl Acad Sci USA 83:7167–7171.

Saluz HP, Jost JP (1990): A Laboratory Guide for In Vivo Studies of DNA Methylation and Protein/DNA Interactions. Birkhäuser, Springer Verlag.

Sollner-Webb B, Tower J (1986): Transcription of cloned eukaryotic ribosomal RNA genes. Annu Rev Biochem 55:801–830.

Tantravahi U, Breg WR, Wertelecki V, Erlanger BF, Miller OJ (1981a): Evidence for methylation of inactive human rRNA genes in amplified regions. Hum Genet 56:315–320.

Tantravahi U, Guntaka R, Erlanger BF, Miller OJ (1981b): Amplified ribosomal RNA genes in a rat hepatoma cell line are enriched in 5-methylcytosine. Proc Natl Acad Sci USA 78:489–493.

Warburton D, Henderson AS (1979): Sequential silver staining and hybridization in situ on nucleolus organizing regions in human cells. Cytogenet Cell Genet 24:168–175.

Wilson GN, Hollar BA, Waterson JR, Schmickel RD (1978): Molecular analysis of cloned human 18S ribosomal DNA segments. Proc Natl Acad Sci USA 75:5367–5371.