Chromatin structure and methylation of rat rRNA genes studied by formaldehyde fixation and psoralen cross-linking

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ABSTRACT

By using formaldehyde cross-linking of histones to DNA and gel retardation assays we show that formaldehyde fixation, similar to previously established psoralen photocross-linking, discriminates between nucleosome- packed (inactive) and nucleosome-free (active) fractions of ribosomal RNA genes. By both cross-linking techniques we were able to purify fragments from agarose gels, corresponding to coding, enhancer and promoter sequences of rRNA genes, which were further investigated with respect to DNA methylation. This approach allows us to analyse independently and in detail methylation patterns of active and inactive rRNA gene copies by the combination of *Hpa*II and *Msp*I restriction enzymes. We found CpG methylation mainly present in enhancer and promoter regions of inactive rRNA gene copies. The methylation of one single *Hpa*II site, located in the promoter region, showed particularly strong correlation with the transcriptional activity.

INTRODUCTION

In cells of higher eukaryotes, two distinct chromatin structures of rDNA coexist (1–4). Their proportion is tissue specific (5) and represents two different transcriptional states—active and inactive ribosomal RNA gene copies (1). In the cells of most eukaryotes the repeated rRNA genes are present in >100 copies per cell, organised in tandem arrays. In the diploid genome of rat, 200 copies are organized in six chromosomal domains (6).

Several techniques have been developed to measure the fraction of actively transcribed ribosomal RNA genes. Muscarella and co-workers (7) took advantage of the preferential association of topoisomerase I with the coding region of transcribed rRNA genes to determine the proportion of active gene copies in normal and aneuploid chicken embryo fibroblasts. Haaf *et al.* (8) described a cytochemical method to estimate the number of active rRNA genes in individual cells. The accessibility of DNA in chromatin to psoralen is probably the most accurate assay to quantify the proportion active and inactive rRNA gene copies (1,2,4,5,9).

We have used an alternative technique to distinguish between the two different classes of ribosomal chromatin, namely formaldehyde cross-linking of histones to DNA. This technique was developed to examine the arrangement and interactions of nucleosome components (10–12). In functional studies, formaldehyde fixation was used to detect the chromatin rearrangement in RNA polymerase II genes (13–15). Here we show that formaldehyde fixation can discriminate successfully between active and inactive rRNA gene copies and allows the purification of the DNA from both classes of ribosomal chromatin for further analyses.

Substantial evidence indicates that DNA methylation may play a role in inactivating gene function and in the propagation of this inactive state in cell generations (for reviews see 16–19). Several studies attempted to correlate DNA methylation and the transcriptional activity of rRNA genes. A loss of rDNA methylation during the onset of rRNA gene transcription was observed in early development of *Xenopus laevis* (20). Furthermore, it was shown that in somatic cells of *Xenopus*, mouse (20,21) and plants (22,23), only a fraction of rDNA is undermethylated at specific sites in the intergenic spacers and in coding regions. The amount of unmethylated genes varies between mouse species and is proportional to the DNAse I sensitive fraction (21). In wheat, cytological measurements suggest that the volume of nucleoli during interphase is related to the size of secondary constrictions in metaphase chromosomes and corresponds to the activity of nucleolar organisers (22). Active nucleoli containing high numbers of transcribed rRNA gene copies with long intergenic spacers (a large number of enhancer repeats) were found preferentially undermethylated (23), compared with rRNA gene copies, with short and highly methylated non-transcribed spacers. Therefore, in wheat a correlation exists between the length of the intergenic spacer and DNA methylation. This approach, however, does not distinguish between active and inactive rRNA gene copies with the same length of intergenic spacers. In differentiated mammalian cells direct evidence is lacking for the role of DNA methylation with respect to rRNA gene transcriptional activity. Since formaldehyde fixation and psoralen photocross-linking allow us to separate and isolate transcriptionally active and silent rRNA gene copies, we analysed the distribution of methylated CCGG sites in different domains along the rat rDNA locus. Our results suggest that in rat cells, there is no particular

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correlation between the intergenic spacer length and rRNA gene activity. Methylated sites are predominantly present in inactive rRNA genes and are not uniformly distributed, but concentrated mainly in the regulatory elements like enhancer and promoter sequences. There is almost no detectable methylation in the coding regions of both active and inactive rRNA gene copies.

MATERIALS AND METHODS

Formaldehyde fixation of nuclei

Nuclei from rat liver and rat cell lines were prepared according to the procedure of Hewish and Burgoyne (24) and cross-linked by formaldehyde using a slightly modified protocol to that of Solomon *et al.* (13). Aliquots of nuclei were washed and resuspended (1.5 $\times$ 10^6 nuclei/ml) in cross-linking buffer containing: 25 mM HEPES pH 7.8, 0.1 M NaCl, 0.15 mM EDTA pH 8 and 20% glycerol. Formaldehyde was added to a final concentration of 1% for 4 h at 4°C with slow agitation. After cross-linking nuclei were pelleted, washed several times in TE (10 mM Tris, pH 8, 1 mM EDTA) and incubated for 4 h at 37°C in TE containing 1% SDS and proteinase K (100 µg/ml). The total DNA–peptide adducts were extracted twice with phenol/chloroform and chloroform and then ethanol precipitated. Cross-linked DNA–peptide complexes were dissolved in TE and then stored at 4°C. Lately, for formaldehyde fixed nuclei of the cell lines the deproteinization step was reduced to one phenol/chloroform extraction.

Psoralen photocross-linking

Nuclei were photocross-linked with thrimethyl psoralen as described previously (4).

Cell lines

N1-S1 Novikoff rat hepatoma cell line was obtained from the ATCC collection (ATCC CRL 1604) and grown as advised in the supporting instructions. C6 rat glyoma was a gift from Prof. U. Suter. C6 cells were cultivated in Dulbecco's modified Eagle's medium supplemented by 10% foetal calf serum.

*Eco*RI digestion of nuclei

Rat liver nuclei (1 ml 1.5 $\times$ 10^6 nuclei/ml per sample) were cross-linked with psoralen or formaldehyde, washed twice with restriction buffer and incubated with 100 U *Eco*RI. After 1 h of digestion the reaction was stopped by EDTA (10 mM final concentration) and total DNA was purified. Each sample was divided into two aliquots. The first aliquot was redigested with *Pst*I and the second one with both *Pst*I and *Eco*RI. All samples were finally ethanol precipitated, redissolved in 1× loading buffer and run in 1% neutral agarose gels without ethidium bromide.

Gel electrophoresis, transfer and hybridization

Cross-linked and untreated DNA were digested using standard Boehringer restriction buffers and enzymes. For cross-linked DNA, three times more enzyme was added than recommended by the supplier. Digested DNA was run in agarose gels.

Enhancer and promoter fragments were eluted from agarose gels using QIAEX II Gel Extraction kit (Qiagene). Alkaline Southern blotting was done on Pall Biodyne B membrane (Pall)

and the filters were hybridized and washed according to the BioRad instruction manual. Rat rDNA probes used for hybridizations, derived from enhancer (pUC/BS), coding (pUC/EB) and promoter sequences (pUC/BH) cloned in pUC 18 (Fig. 1b). The probes were labelled by random priming (Pharmacia). After hybridization (when necessary) the filters were placed in PhosphorImager cassettes and quantified (PhosphorImager; a trade mark of Molecular Dynamics was used with the kind permission of Prof. Ch. Weissman) after several days of exposure.

RESULTS

Formaldehyde fixation and characterisation of ribosomal chromatin

Formaldehyde fixation allows the cross-linking of histones to DNA *in vivo* and *in vitro* (12,13). Formaldehyde added to intact cells or nuclei forms a network of DNA–protein, RNA–protein and protein–protein adducts, which prevents possible rearrangements of the cellular (or nuclear) components. Treatment of DNA–histone complexes with non-specific proteases does not lead to complete digestion of the covalently bound proteins (10,12). After purification of the DNA and DNA–peptide complexes followed by restriction enzyme digestion, DNA–histone peptides adducts migrate in neutral agarose gels with reduced electrophoretic mobility compared with naked DNA (10,12,13). Formaldehyde-mediated cross-linking is reversible by mild temperature treatment and results in peptide-free undamaged DNA (10).

In order to test the reliability of the formaldehyde cross-linking, we performed the experiments in parallel to psoralen photocross-linking, which yields detailed information on the structural organisation of ribosomal chromatin. Similar to formaldehyde fixation, the extent of psoralen cross-linking is detectable in a gel retardation assay. The more a DNA fragment incorporates psoralen, the slower it migrates in a native agarose gel (1).

We used DNA from formaldehyde or psoralen cross-linked intact nuclei to analyse the 6.5 kb *Eco*RI fragment derived from the rat rDNA coding region (Fig. 1a). Purified DNA from untreated, psoralen or formaldehyde cross-linked nuclei was restricted with *Eco*RI. After electrophoresis in 1% native agarose gel, the 6.5 kb *Eco*RI fragment of both formaldehyde- or psoralen-treated samples is resolved as two bands (Fig. 1d and e). As described previously (1), the psoralen cross-linking assay allows one to distinguish between two different classes of rRNA genes coexisting in the same cell population (Fig. 1d). The slowly migrating s-band (DNA more accessible to psoralen) represents the class of nucleosome-free, transcriptionally-active rRNA genes, whereas the less retarded f-band contains nucleosome-packed inactive gene copies (less accessible to psoralen). In purified formaldehyde-treated material the 6.5 kb fragment is also resolved into two bands in neutral agarose gels (Fig. 1e). The shift of the slower band depends on the extent of proteinase K digestion, leading to shortening of the DNA-attached histone peptides (Fig. 1e). After 48 h of digestion the two bands migrate very close to each other. We suggest that the upper band, whose shift depends on the extent of proteinase K treatment, possibly represents nucleosome packed inactive rRNA gene copies. Active rRNA genes (containing no or less cross-linkable histones) accumulate in the band with mobility close to the untreated control DNA. This assumption is tested by the experiments described below.
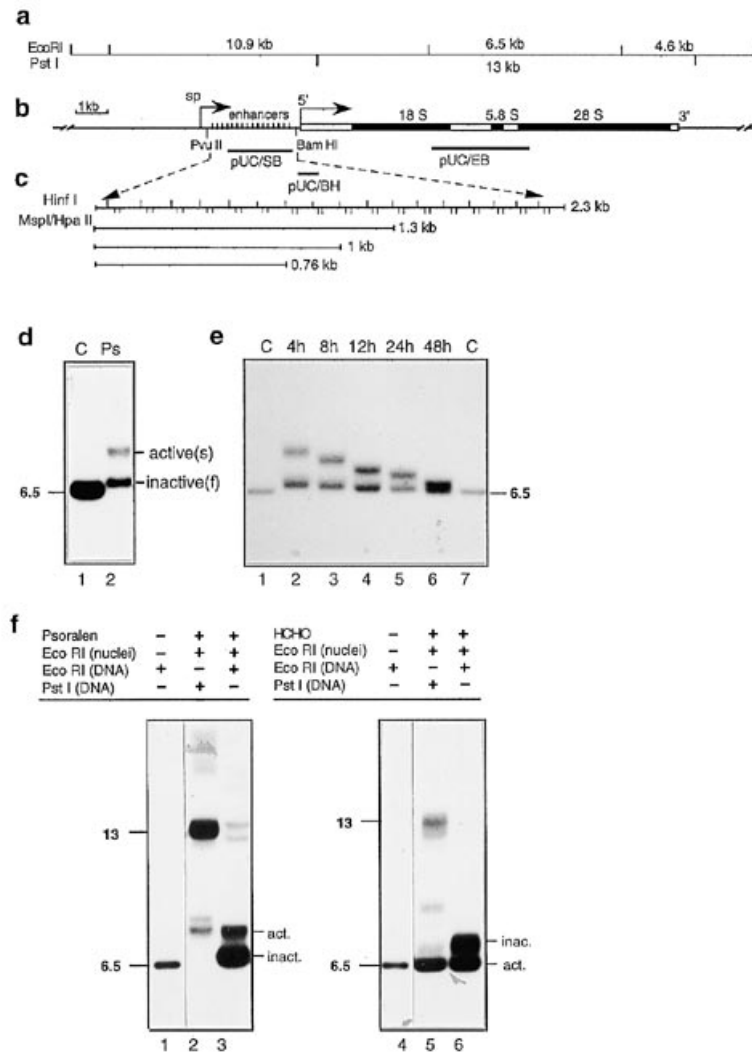
Figure 1. Psoralen and formaldehyde cross-linking of rat liver ribosomal chromatin and its accesibility to *Eco*RI digestion. (a, b and c) Structural organisation of the rat RNA gene unit. (**a**) *Eco*RI and *Pst*I restriction map of rDNA unit. (**b**) Organisation of the enhancer region and 45S rRNA coding sequences. (**c**) Shows the heterogeneity of the enhancer fragments and the cutting sites of *Hin*fI and *Hpa*II restriction enzymes. 2.3, 1.3 and 1 kb enhancer fragments can be observed preferentially in rat liver nuclei and mostly 1.3, 1 and 0.76 kb in the used rat cell lines. The hybridisation probes containing rDNA fragments subcloned in pUC 18 (25), pUC/SB , pUC/EB and pUC/BH are shown below the corresponding regions of the structural map. (**d**) Isolated rat liver nuclei were photoreacted with psoralen and the DNA was purified, digested by *Eco*RI and fractionated in 1% agarose gel (lane 2) alongside the uncrosslinked, *Eco*RI-digested rat liver DNA (lane 1). (**e**) Isolated rat liver nuclei were cross-linked with 1% formaldehyde. Proteinase K digestion time is indicated. DNA, carrying covalently bound short histone peptides, was *Eco*RI-digested and electrophoresed in 1% agarose gel (lanes 2–6) in parallel to DNA from control untreated nuclei (lanes 1 and 7). (**f**) Isolated nuclei were cross-linked with psoralen or formaldehyde, washed with the appropriate restriction buffers and digested with *Eco*RI. *Eco*RI in nuclei is known to have access only to transcriptionally active rRNA gene copies. DNA was purified, digested with *Pst*I and loaded on the gel (lanes 2 and 5) or redigested with *Eco*RI before loading to visualise both populations of inactive and active rRNA gene copies (lanes 3 and 6). DNA from untreated nuclei digested with *Eco*RI (lanes 1 and 4) is shown. The differences in yield in cleaving the active rRNA genes in nuclei after psoralen and formaldehyde cross-linking seen in lanes 2 and 5 were not further investigated.

Accessibility of DNA in nucleosome-free ribosomal chromatin to *Eco*RI

Conconi *et al.* (1) demonstrated that in intact nuclei restriction enzymes preferentially recognise and digest active rRNA genes. When *Eco*RI digested nuclei were psoralen cross-linked, only the slowly migrating band, corresponding to active rRNA genes, could be released. We repeated this assay using rat liver nuclei (Fig. 1f) cross-linked with either psoralen or formaldehyde. Both cross-linked nuclei samples were digested with *Eco*RI. The DNA was purified and half of each sample was additionally redigested with *Eco*RI. All four aliquots were restricted with *Pst*I to reduce the size of uncut DNA and were electrophoresed in agarose gels

alongside *Eco*RI-digested, uncross-linked rat liver control DNA (Fig. 1f, lanes 2–3 for psoralen and 5–6 for formaldehyde cross-linked material, respectively).

After hybridisation with a probe, complementary to the coding rDNA region, *Eco*RI treated nuclei showed a prominent band at ~13 kb corresponding to the *Pst*I fragment, i.e., to the DNA of inactive genes not accessible to *Eco*RI in intact nuclei (Fig. 1f, lanes 2 and 5). In the case of the psoralen cross-linked sample, a band corresponding to the slowly migrating band of the 6.5 kb *Eco*RI doublet was detected (Fig. 1f, lane 2). For the formaldehyde-fixed nuclei, a single band of 6.5 kb is mainly seen (Fig. 1f, lane 5), but here with mobility close to that of untreated control
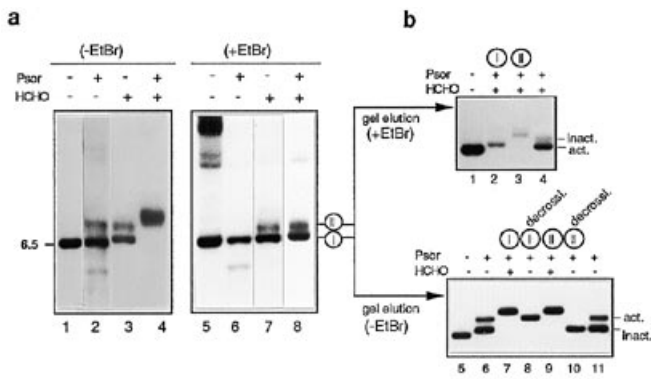
Figure 2. Double, psoralen and formaldehyde cross-linking of ribosomal chromatin. (**a**) Rat liver nuclei were cross-linked with psoralen, formaldehyde or both, and the purified DNA was digested with *Eco*RI to obtain the 6.5 kb fragment of the coding region. Samples were run in a neutral 1% agarose gel without ethidium bromide (lanes 2–4) in parallel to control DNA from untreated nuclei (lane 1), or in a similar 1% agarose gel containing 0.5 µg/ml ethidium bromide (lanes 6–8). Lane 5 is the same control DNA as in lane 1. In presence of ethidium bromide, the psoralen-derived doublet of the 6.5 kb fragment migrates as a single band (compare lane 2 with 6), whereas in the formaldehyde gel retardation assay the doublet is seen in presence and absence of ethidium bromide (compare lane 3 with 7). After double cross-linking with psoralen and formaldehyde (lanes 4 and 8) the 6.5 kb fragment can be visualised as a doublet only in gels containing ethidium bromide (lane 8—for the details see text). (**b**) Eluted DNA from bands I and II was reanalyzed in two parallel gels. In the first one (upper panel), containing ethidium bromide, the samples were loaded directly after gel elution as indicated in lanes 2 and 3 in parallel to the starting material of lane 8 in (a) (lane 4). In the second gel without ethidium bromide (lower panel) the same samples were run before (lanes 7 and 9) and after decross-linking of formaldehyde adducts (lanes 8 and 10). As a reference, *Eco*RI cleaved DNA from psoralen cross-linked nuclei was run in the same gel (lanes 6 and 11).

DNA (Fig. 1f, lane 4). In the aliquots in which the DNA was redigested with *Eco*RI (Fig. 1f, lanes 3 and 6), the doublets were seen as expected, both for the psoralen and the formaldehyde cross-linked nuclei and the 13 kb *Pst*I band disappeared to a large extent. The results of this experiment suggest that, as opposed to psoralen cross-linking, the rDNA from active gene copies migrates faster than that from the inactive rRNA gene copies after formaldehyde treatment of nuclei: in fact, it migrates similarly to the control 6.5 kb *Eco*RI fragment. This indicates that the transcriptionally active rRNA gene copies carry few formaldehyde cross-linkable proteins.

The ratio of nucleosomal and non-nucleosomal 6.5 kb *Eco*RI fragments achieved by formaldehyde fixation and psoralen photocross-linking in the first experiments (Fig. 1d and f) appears to be different. Formaldehyde cross-linking yields more active rRNA gene copies compared with the psoralen assay. We supposed that this discrepancy might be due to the preferential loss of DNA–histone peptide complexes during the deproteinization step. Later, by reducing the phenol extraction we achieved similar values with both cross-linking techniques, but we used mainly psoralen photocross-linking for quantification.

Psoralen and formaldehyde double cross-linking in chromatin

In order to compare the two populations of rDNA fragments which are obtained when nuclei are cross-linked either with psoralen or formaldehyde, a double cross-linking was performed. Rat liver nuclei were first psoralen cross-linked followed by formaldehyde fixation. DNA was purified, digested with *Eco*RI

and electrophoresed either in the presence or in the absence of ethidium bromide. As control, only psoralen or only formaldehyde cross-linked samples were loaded. In absence of ethidium bromide, as shown previously (Fig. 1d and f), the restriction fragments of the formaldehyde or psoralen cross-linked samples were resolved as doublets (Fig. 2a, lanes 2 and 3). However, in the double cross-linked sample, the 6.5 kb rRNA coding fragment migrates as a broad single retarded band (Fig. 2a, lane 4). This result suggests that the slowly migrating fragments from the formaldehyde experiment (inactive rRNA gene copies) comigrate with the heavily psoralen cross-linked DNA fragments (active gene copies). When we rerun the same samples in ethidium bromide containing gels the relative mobility of the formaldehyde derived doublet remained unaffected (Fig. 2a, lane 7), whereas the psoralen cross-linked material is visualised as a single sharp band (Fig. 2a, lane 6). The intercalation of ethidium bromide in DNA appears to mask the difference in mobility between the slightly and heavily psoralen cross-linked rRNA gene populations. The DNA fragments derived from the formaldehyde and psoralen double cross-linked samples are now resolved as a double band in the presence of ethidium bromide (Fig. 2a, lane 8), indicating that in ethidium bromide containing gels the retardation of some of the material leads to visualisation of the two populations of fragments mainly due to the presence of bound peptides.

The two bands of the DNA from double cross-linked nuclei were eluted from agarose gels containing ethidium bromide and reanalysed. In presence of ethidium bromide, aliquots of bands I and II were loaded alongside to double cross-linked material of lane 8 (Fig. 2b, upper panel). Clearly there is no cross-contamination between the two eluted samples. In a second gel without ethidium bromide, aliquots of bands I and II were run after decross-linking of the formaldehyde induced adducts (Fig. 2b, lower panel). As marker, DNA from nuclei cross-linked only with psoralen was loaded (Fig. 2b, lower panel, lanes 6 and 11). As expected, in the absence of ethidium bromide, the DNA from bands I and II of lane 8 in Figure 2a show the same mobility (Fig. 2b, lower panel, lanes 7 and 9). The two decross-linked bands I and II, however, correspond exactly to the material cross-linked only with psoralen (Fig. 2b, lower panel, compare lanes 8 and 10 with 6 and 11). Note that under these conditions (absence of ethidium bromide), the fast migrating band I of lane 8 (Fig. 2a) is retarded more (Fig. 2b, lane 7) than the slowly migrating psoralen band after decross-linking of the formaldehyde adducts (Fig. 2b, lane 8; for explanation see Discussion).

From these results we conclude that formaldehyde fixation as psoralen cross-linking (1) appears to distinguish between nucleosomal and non-nucleosomal rDNA, or based on previous evidence with psoralen cross-linking (1), between inactive and active ribosomal chromatin, respectively. Therefore, formaldehyde fixation can be considered as an alternative approach to investigate transcriptionally active and inactive rRNA gene copies.

Methylation of active and inactive rRNA genes in rat liver nuclei

DNA methylation has been reported to play a role in the repression of some RNA polymerase II transcribed genes, as well in the establishment of the inactive chromatin state (for review see 20). Since we are able to separate DNA fragments from non-nucleosomal, transcriptionally active and nucleosomal, transcrip-

tionally inactive rRNA gene copies, we analysed the relationship between methylation and transcriptional activity of rat rRNA genes applying both the formaldehyde and psoralen assays.

DNA from untreated, psoralen cross-linked or formaldehyde fixed rat liver nuclei was first digested with the appropriate enzymes. Each sample was divided into three aliquots and two of them were redigested with *Hpa*II (sensitive to methylation in position CCmGG) or *Msp*I (insensitive to methylation in the same CCGG site).

When *Eco*RI digested DNA (to obtain the 6.5 kb fragment of the coding region) was incubated with *Hpa*II or *Msp*I, even loading four times more DNA per gel slot than usual, we detected hardly any sequences methylated in all CCGG sites throughout their full length (data not shown, but see Fig. 4a).

Rat rDNA *Pvu*II–*Bam*HI enhancer fragments show a broad length heterogeneity (6,25), consisting of variable numbers of 135 bp repeats (Fig. 3a). In liver nuclei, we found 2.3, 1.3, 1 and some 0.76 kb long enhancer fragments, containing ~16, 10, 8 and 6 135 bp *Hin*fI repeats (Fig. 3a and b, lane 1). Each 135 bp repeat carries two *Hpa*II sites which can be potentially methylated (Fig. 3a). DNA purified from either psoralen or formaldehyde cross-linked samples reveals double bands for the 2.3 and 1.3 kb *Pvu*II–*Bam*HI fragments (in the case of formaldehyde also for the 1 kb fragment; see Fig. 3b, lanes 4 and 7). *Hpa*II-resistant DNA (Fig. 3b, lanes 5 and 8) is detected only for the fragments originating from nucleosomal DNA, i.e., the fast migrating bands in case of psoralen (Fig. 3b, lane 5), or the slowly migrating bands in case of formaldehyde cross-linking (Fig. 3b, lane 8). In the *Msp*I lanes (Fig. 3b, lanes 3, 6 and 9) the DNA appears to be completely digested. These results indicate that methylated CCGG sites are predominantly located in nucleosome-packed, i.e., inactive enhancer regions.

In order to estimate the extent of methylation in nucleosomal and non-nucleosomal enhancer fragments, we eluted the two fractions from agarose gels. We used psoralen cross-linked material to separate and purify the DNA corresponding to the 1.3 kb enhancer bands. Material from several gels was pooled. As control the total population of 1.3 kb enhancers from untreated nuclei was eluted the same way. Eluted samples were redigested with *Hpa*II or *Msp*I and reanalysed in 1.8% agarose gels (Fig. 3c). Since every 135 bp repeat carries two *Hpa*II sites, the lack of methylation in one of them is sufficient to lead to the disappearance of the entire 1.3 kb fragment. After *Hpa*II and *Msp*I treatment most of the material is found as short fragments (Fig. 3c, lanes 2, 3, 5, 6, 8 and 9) of 135 bp (only one site per repeat is accessible to *Hpa*II) or 113 and 22 bp (when both sites are accessible to *Hpa*II and *Msp*I). The short 113–135 bp fragments were not resolved in this type of gel and comigrate as a broad spot (Fig. 3c, lanes 2, 3, 5, 6, 8 and 9). Faint bands of *Hpa*II-resistant material is detected in the control DNA and in the nucleosomal 1.3 kb enhancer fragments (Fig. 3c, lanes 2 and 5). In these nucleosomal 1.3 kb enhancer fragments there are also less *Hpa*II 113–135 bp digestion products compared with the *Msp*I digestion products (Fig. 3c, compare lanes 5 and 6). In the sample corresponding to the non-nucleosomal population of the 1.3 kb enhancers most of the DNA appeared to be digested by *Hpa*II and *Msp*I to the same extent (Fig. 3c, compare lane 8 with 9). These indicate that a small portion of nucleosome-packed enhancers have methylated *Hpa*II sites along the full 1.3 kb length (Fig. 3c, lanes 4–6), whereas in the nucleosome-free fraction (Fig. 3c, lanes 7–9) most of the material is degraded to 113–135 bp
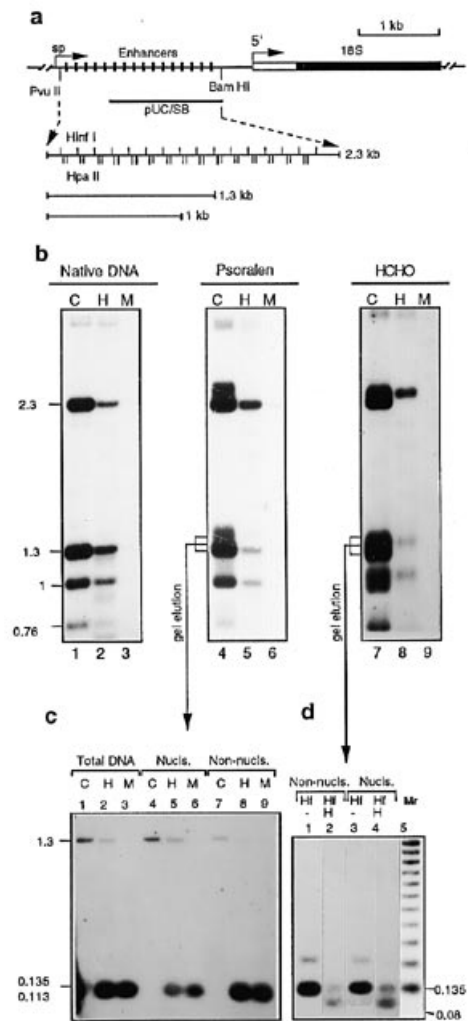

Figure 3. Methylation of rRNA gene enhancers in rat liver nuclei. (**a**) Structural organisation of rat rRNA gene enhancer region. The heterogeneity in length (2.3, 1.3 and 1 kb) of *Bam*HI–*Pvu*II enhancer fragments, composed of 16, 10 or 8 short 135 bp repeats, is shown. The positions of *Hin*fI and *Msp*I restriction sites and the hybridisation probe (pUC/SB) in enhancer repeats are indicated. (**b**) DNA from untreated (lane 1), psoralen (lane 4) or formaldehyde (lane 7) cross-linked rat liver nuclei was digested with *Hpa*II, a CpG methylation sensitive (lanes 2, 5 and 8) or *Msp*I, a methylation-insensitive (lanes 3, 6 and 9) restriction enzyme. (**c**) To analyse the methylation of the full size enhancer regions, the 1.3 kb fragment from untreated nuclei [for reference see lane 1 in (b)] and the DNA from bands corresponding to active and inactive 1.3 kb enhancer fragments from psoralen cross-linked material [for reference see lane 4 in (b)] were gel eluted and redigested with *Hpa*II (lanes 2, 5 and 8) or *Msp*I (lanes 3, 6 and 9). (**d**) Methylation of 135 bp repeats was tested using eluted 1.3 kb fragments from formaldehyde cross-linked sample [for reference see lane 7 in (b)]. The purified DNA was digested with *Hin*fI [lanes 1 and 3; see also (a)] followed by *Hpa*II (lanes 2 and 4—four times more DNA was loaded). As a size marker (lane 7) the BRL 123 bp DNA ladder was used. H and M in (b) are abbreviations of *Hpa*II and *Msp*I restriction enzymes.

fragments both by *Hpa*II and *Msp*I. Around 17% of nucleosomal 1.3 kb enhancer fragments have all the *Hpa*II sites methylated compared with ~0.5% of the non-nucleosomal enhancers.

To determine the proportion of methylated *Hpa*II sites in individual 135 bp enhancer repeats, the purified 1.3 kb bands were first cut with *Hin*fI (Figs 3a and 4c). To obtain the two populations of nucleosomal and non-nucleosomal enhancer
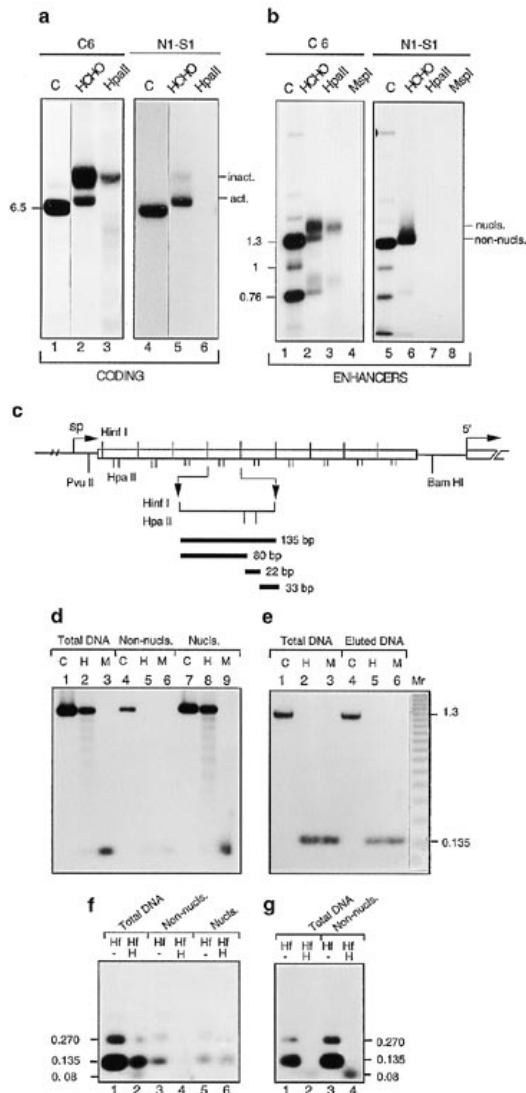
Figure 4. Different methylation patterns of coding and enhancers regions of rat C6 and N1-S1 cell lines. (**a**) Isolated nuclei from C6 and N1-S1 cell lines were formaldehyde cross-linked, the DNA was purified and digested with *Eco*RI. Methylated sequences in the 6.5 kb rDNA coding fragment (lanes 2 and 5) were analysed in both cell lines by *Hpa*II redigestion (lanes 3 and 6). (**b**) For the enhancer region, the DNA purified from cross-linked nuclei was digested with *Bam*HI and *Pvu*II to obtain the 1.3, 1 and 0.76 kb enhancer fragments (lanes 1 and 5; see also Fig. 1). The presence of methylated DNA was determined by *Hpa*II redigestion (lanes 3 and 7) or *Msp*I as a control (lanes 4 and 8). As C (lanes 1 and 5 for the enhancer region and lanes 1 and 4 for coding sequences) is indicated control DNA from uncross-linked nuclei. (**c**) Organisation of rat cell lines enhancer regions. Structural and restriction map of repeated elements is shown. Black thick lines indicate the products of *Hin*fI (135 bp) or *Hin*fI plus *Hpa*II digestion (80, 33 and 22 bp) of 1.3 kb *Pvu*II–*Bam*HI enhancer fragment. (**d**) DNA eluted from the bands corresponding to 1.3 kb non-nucleosomal (lane 4), nucleosomal (lane 7) or to the unfractionated population (uncross-linked nuclei-lane 1) of rRNA gene enhancers in C6 cells (see also Fig. 3) was redigested with *Hpa*II (lanes 2, 5 and 8) or *Msp*I (lanes 3, 6 and 9). (**e**) 1.3 kb enhancer fragments of N1-S1 cell line were eluted from untreated (lane1) or formaldehyde cross-linked nuclei (lane 4) and redigested by *Hpa*II (lanes 2 and 5) or *Msp*I (lanes 3 and 6). Lane 7 is BRL 123 bp ladder size marker. Note that in N1-S1 cell line most of the rRNA genes are active and, therefore, only non-nucleosomal enhancer elements were analysed [see (a) lane 5]. (**f**) 1.3 kb eluted enhancer fragments from C6 cells (see lanes d1, 4 and 7) were digested with *Hin*fI to obtain 135 bp enhancer repeats (lanes 1, 3 and 5) which were further treated with *Hpa*II (lanes 2, 4 and 6). (**g**) The same as (d) but with enhancers derived from N1-S1 cell line.

repeats (Fig. 3d, lanes 1 and 3) we used DNA from formaldehyde cross-linked nuclei, in order to avoid partial digestion of *Hin*fI, which was observed after psoralen cross-linking (not shown). Redigestion of 135 bp *Hin*fI fragments by *Hpa*II demonstrates that in the nucleosome-free repeats most of the material is degraded and some accumulates in a fragment of 80 bp (Fig. 3d, compare lane 1 with 2), whereas nucleosomal repeats are more resistant to *Hpa*II (Fig. 3d, compare lane 3 with 4). The quantification data shows that up to 58% of 135 bp repeats arising from nucleosomal enhancers are methylated at the two possible positions. Although this proportion is rather high according to only 17% of the fully methylated 1.3 kb enhancer fragments, it means that for the rest of the nucleosome-packed enhancers at least one of the 135 bp repeats is unmethylated. In the non-nucleosomal population the amount of methylated 135 bp repeats corresponds to 9%—the data are the average of five independent experiments.

In conclusion, in rat liver nuclei the methylation is prominent mainly in nucleosome-packed enhancers, however not uniformly distributed. Some of the non-nucleosomal enhancer fragments are methylated as well, although to a very low extent.

Methylation of rDNA in rat cell lines

The results described in the previous paragraph are consistent with the suggestion that in the regulatory enhancer elements of rat liver nuclei a correlation exists between the transcriptional activity of rRNA genes and the amount of methylated enhancer sequences. In mammalian cells a certain cell type has a defined number of transcriptionally active and inactive rRNA gene copies (1,5). Since the amount of methylation in rDNA has also been described as cell type and tissue specific (18,21), we wanted to examine if the extent of methylation correlates with the amount of inactive rRNA gene copies. We choose two rat cell lines, which showed a clear difference in the amount of active and inactive rDNA fractions. C6 glyoma cell line shows ~85% of transcriptionally inactive rRNA gene copies (Fig. 4a, lane 2). In contrast, in the N1-S1 hepatoma cell line most (>80%) of the rRNA genes are active (Fig. 4a, lane 5). Aliquots of DNA purified from formaldehyde cross-linked nuclei of both cell lines were digested with *Hpa*II or *Msp*I to analyse the methylation density of enhancer regions and coding sequences.

As can be seen in C6 cells (Fig. 4b, lane 3), the retarded band corresponding to nucleosome organised enhancers is considerably resistant to *Hpa*II digestion. Similar results can be observed even in the coding region (Fig. 4a, lane 3). As described previously, when the bands corresponding to nucleosomal and non-nucleosomal 1.3 kb enhancer *Pvu*II–*Bam*HI fragments were eluted from agarose gels and redigested with *Hpa*II (Fig. 4d), a very low level of undigested material was detected in the enhancers in front of active ribosomal genes (Fig. 4d, lane 5). Only 0.8% of the full length fragment is resistant to *Hpa*II. When the extent of methylation was determined by the combination of *Hin*fI and *Hpa*II, as described above (Fig. 3d), ~1% of single 135 bp repeats derived from non-nucleosomal enhancers remain intact after *Hpa*II digestion (Fig. 4f, lane 4). In contrast, we found ~26% of C6 1.3 kb nucleosomal enhancers methylated along their full length (Fig. 4d, lane 8). A ladder of *Hpa*II digestion products (Fig. 4d, lanes 2 and 8) represents the randomly distributed unmethylated sites on the heavily methylated nucleosomal 1.3 kb C6 fragment. Among the single 135 bp repeats ~81% remain resistant to *Hpa*II

(Fig. 4f, compare lane 5 with 6), which means that they are methylated at both *Hpa*II recognition sites.

In N1-S1 rat glyoma no detectable methylation was observed neither in the coding region (Fig. 4a, lane 6) nor in the corresponding enhancers (Fig. 4b, lane 7, and Fig. 4e and g).

Methylation of a single *Hpa*II site near the rRNA gene promoter

In contrast to the genes transcribed by RNA polymerase II, the analysis of rDNA intergenic spacers in different species did not reveal any consensus sequence near the transcription initiation sites (for review see 26). However, there is a certain similarity in the organisation of regulatory elements like enhancers and transcription terminators in rDNA intergenic spacers of different eukaryotes (6,26,27). In some organisms a single conserved *Hpa*II site can be found close to the transcription initiation site of the rRNA genes. Several reports point out a correlation between the methylation of this site and the transcriptional activity (23,28). Using formaldehyde fixation, we examined the methylation of a single *Hpa*II site located 145 bp upstream from the +1 nucleotide in the rat rRNA gene promoter (Fig. 5a).

We analysed the 405 bp *Bam*HI–*Hin*dIII fragment which carries the rRNA gene promoter region and the transcription initiation site (Fig. 5a). When total genomic DNA, originating from rat liver nuclei or from the two rat cell lines described above after treatment with *Bam*HI and *Hin*dIII, was redigested by *Hpa*II a different amount of *Hpa*II-resistant promoter fragments was observed in samples (Fig. 5b, c and d, compare lane 1 with 2). To confirm that the *Hpa*II-resistant material represents promoters in front of inactive rRNA genes, total DNA purified from formaldehyde cross-linked nuclei was first digested with *Bam*HI and *Pvu*I to obtain a 2.8 kb fragment, which contains the promoter fragment and a part of 45S rRNA precursor coding sequence. After separation of nucleosomal and non-nucleosomal rDNA sequences in 1% agarose gels and separate elution of the two bands, the DNA was redigested with *Hin*dIII to discard the coding sequences from the promoter region. *Hpa*II or control *Msp*I digestion of the purified promoter fragments shows no detectable methylation of this single site in fragments corresponding to non-nucleosomal promoters in liver and in C6 cell nuclei (Fig. 5b and c, lane 7). In contrast, we found nucleosome-packed promoters methylated to a large extent (see the resistant band in Fig. 5b and c, lane 5).

A similar experiment was performed with the N1-S1 cell line (Fig. 5d). Here we used uncross-linked total DNA, since we have estimated that ~90% of rRNA genes in this cell line are active (Fig. 4a, lane 5). After *Hpa*II digestion only a small proportion of the 405 bp fragment remains intact, which correlates to the amount of inactive N1-S1 rRNA genes (Fig. 5d, lane 2). In conclusion, the lack of methylation in this particular *Hpa*II site of active rDNA promoters can be observed in all three cases analysed here.

For both cell lines and liver nuclei the quantification of the 405 bp fragments resistant to *Hpa*II digestion in comparison to the control undigested lane, revealed values close to those calculated for the proportion of active and inactive rRNA genes. Therefore, in these cases, the amount of *Hpa*II resistant promoter fragments can be used as a simple assay to estimate the active and inactive rRNA gene copies.
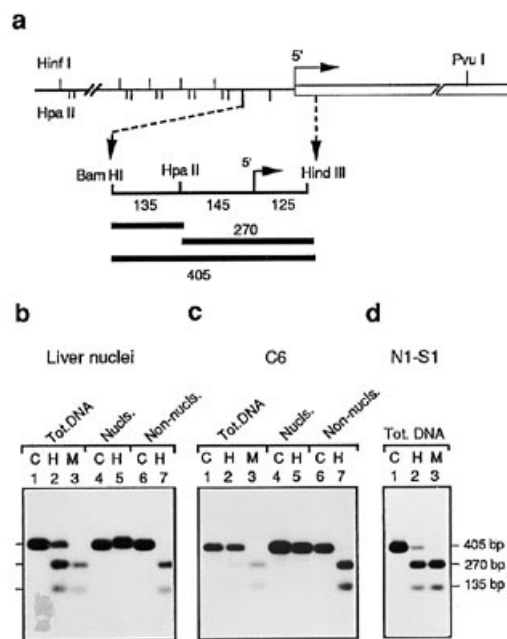

Figure 5. Methylation of a single *Hpa*II recognition site at position –145 in the rDNA promoter region. (**a**) Structural and restriction map of rat the rDNA region 5′ around the transcription start site. To purify the promoter fragments from formaldehyde cross-linked nuclei the DNA was first digested with *Bam*HI and *Pvu*I (1.3 kb downstream of the transcription start site) to obtain a 1.7 kb fragment, which can be resolved as two bands in the formaldehyde gel retardation assay using 1.6% agarose gels. After elution, the samples were redigested with *Hin*dIII to discard the majority of the coding sequences from promoter region. Hybridisation probe spans along the whole 405 bp *Bam*HI–*Hin*dIII promoter fragment. Restriction fragments, which are expected to be visualised after Southern hybridisation of total genomic DNA or eluted fragments digested with the three restriction enzymes *Bam*HI, *Hin*dIII and *Hpa*II (or *Msp*I) are indicated with black bars. (**b**) *Bam*HI–*Hin*dIII promoter fragments from untreated nuclei or formaldehyde cross-linked rat liver nuclei (lanes 1, 4 and 6) were analysed by *Hpa*II (lanes 2, 5 and 7) or *Msp*I digestion (lane 3). (**c**) The same procedure as in (b) using the C6 cell line. (**d**) Methylation at position –145 in the N1-S1 cell line in DNA from untreated nuclei (lane 1) was detected by *Hpa*II (lane 2) or *Msp*I (lane 3) digestion. In liver nuclei and in both cell lines the amount of *Hpa*II-resistant promoter fragments corresponds to the observed amount of inactive rRNA gene copies (for details see the text). Samples treated with *Hpa*II are indicated as H and *Msp*I digested as M in (b–d). C stands for control undigested *Bam*HI–*Hin*dIII promoter fragments [lanes 1 in (b–d)].

DISCUSSION

Formaldehyde fixation of rat ribosomal chromatin

The proportion of active and inactive rRNA genes in vertebrates is cell type specific (1,4,5), whereas in simple eukaryotes, like yeast, it can be modulated according to the growth conditions (2,3). Since this ratio is distinct for tissue-specific cells (1,5) and is maintained stable, perhaps some of the multiple rRNA gene copies in higher eukaryotes become transcriptionally inactive during cell differentiation. Stimuli which modulate the transcriptional activity of the cell do not significantly affect the proportion of active rRNA genes (5). Until now, few reports have been available concerning mainly the factors involved in the transcriptional repression of rRNA genes in growth arrested cells (29,30). The onset and mechanisms of maintenance of inactive state of rRNA

genes, however, is poorly investigated. Growing evidence indicates that several repressive components, such as protein factors, DNA methylation and chromatin structure can contribute simultaneously for rRNA genes inactivation (16,19).

In order to examine the involvement of methylation in the transcriptional repression of rRNA genes we applied formaldehyde fixation of histone proteins to DNA and compared the results with the previously established psoralen cross-linking assay. Our data demonstrate that formaldehyde fixation results in a doublet of bands in a gel retardation assay and by this can distinguish, like the psoralen assay, between the transcriptionally active and inactive rRNA genes coexisting in the same cell population. Through peptide residues remaining attached to the DNA after protease digestion, the DNA–histone peptides adducts migrate slower in a native agarose gel than naked DNA. When formaldehyde fixation was made in presence of heparin which strips histones from the DNA (31), no band-shift was observed (data not shown). This control experiment was consistent with the previous data of Solomon *et al.* (12) suggesting that formaldehyde preferentially cross-links histones, but not other proteins to DNA.

The experiment shown in Figure 1f confirms the observation (1) that treatment of intact nuclei with *Eco*RI leads to cleavage exclusively in the active, non-nucleosomal rRNA gene copies. In case of formaldehyde cross-linking, however, only the fast migrating band of the doublet is seen, which strongly supports the interpretation that in the case of formaldehyde cross-linking, the slowly migrating band of the doublet originates from the inactive, nucleosomal chromatin with cross-linked histone peptides, whereas the fast migrating band from the doublet represents active, non-nucleosomal gene copies. This interpretation is also consistent with the experiment in Figure 2b in which DNA from nuclei which had been cross-linked with both formaldehyde and psoralen was analysed. However, the mobility of 6.5 kb *Eco*RI fragment in agarose gels after double cross-linking of nuclei with both psoralen and formaldehyde was different from that after formaldehyde or psoralen cross-linking alone. We interpret that the DNA has to be fully intercalated with ethidium bromide, in order to visualize gel retardation only due to more or less peptides cross-linked to the DNA. When the fast migrating band of Figure 2a, lane 8, originating from the non-nucleosomal, active rDNA chromatin after double cross-linking was isolated and rerun in the absence of ethidium bromide, the gel retardation was stronger than that of the active band of psoralen cross-linking alone. The additional band-shift is most likely due to the cross-linked peptides, since the mobility of this band increases to the same extent as that of psoralen cross-linking alone, when the formaldehyde adducts of the double cross-linked material were reversed (Fig. 2b, lower panel). We cannot completely exclude the possibility that active rRNA copies are still in contact with some subset of histones. Two possibilities remain open: either there are very few (if any) histones in active rRNA genes, or they are modified in such a way that prevents efficient formaldehyde cross-linking. Histone acetylation is an event that can modify the histone–DNA contacts in transcribed chromatin (32,33).

The two cross-linking techniques described complement each other. Both allow us to separate and purify the two rDNA fractions from agarose gels, corresponding to differently packed gene copies. We made use of the advantages of each technique. For the quantification of both chromatin populations of rRNA genes we used mainly psoralen cross-linking; however, in some cases, when the psoralen cross-linking interferes with restriction

enzyme cutting, it results in variable amount of partial digests. Formaldehyde fixation, however, does not affect the restriction enzyme digestion and after reversing the formaldehyde-mediated adducts the DNA can be used for further analysis. The main disadvantage of the second technique is that it does not allow accurate quantification of nucleosomal and non-nucleosomal fractions.

Methylation pattern of rat rRNA genes

It has been proposed that methylation-mediated gene repression can occur by at least two different mechanisms: either by directly abolishing the binding of transcription factors to their recognition sites or indirectly, through methylated CpG-binding proteins, which block access of the transcription factors to the template (16,19). There is evidence that remethylation after passage of the replication fork can act as a component of the cell memory, whereas *de novo* methylation supports formation of inactive chromatin (for reviews see 16,19). In transcriptional repression, the proteins like MeCP-2 (34,35) and MDBP-2 (36) which bind methylated DNA, probably play a significant role, since it has been demonstrated that in many cases methylation *per se* is not sufficient to abolish transcription (19).

The early investigations of rDNA methylation in higher eucaryotes (37,38) failed to define a correlation between rDNA methylation and transcriptional activity of rRNA genes. *In vivo*, an undermethylated region corresponding to *X.laevis* rDNA enhancers was found in all *Xenopus* somatic cells, whereas it was heavily methylated in sperm DNA (37). Demethylation occurs in early mid-blastula shortly before rRNA synthesis can be detected (37). However, transcription was not abolished when the same fully methylated *Xenopus* sperm rDNA (37) or cloned rRNA genes methylated by *Hpa*II methylase *in vitro* (38) were injected into *Xenopus* oocyte nuclei. These studies and analysis of methylation patterns in mouse culture cells and individual tissues (21), led to the conclusion that methylation does not dramatically affect transcription of rRNA genes, in contrast to many polymerase II genes (19). To the contrary, in wheat the relative activity of individual nucleolar organisers can be determined morphologically by the volume of the corresponding nucleolus (22). Large, presumably active, nucleoli carry rDNA loci with long (22) and undermethylated intergenic spacers (23). On the other hand, rDNA clusters with lower transcriptional activity contain rRNA gene copies with shorter and predominantly methylated intergenic spacers (23). Similar observations were made for other plant species (28). These studies obviously correlate rRNA genes activity to the extent of DNA methylation, however, do not clearly exclude that the active nucleoli could also contain inactive rRNA gene copies.

The formaldehyde and psoralen band-shift assays used here show that in rat cells, the longer rRNA gene enhancer fragments do not necessarily indicate higher number of transcriptionally active rRNA gene copies (data not shown, but see Fig. 3). Non-nucleosomal and nucleosomal enhancers are distributed between all the variations of length of *Bam*HI–*Pvu*II enhancer fragments when several individual rat liver nuclei samples were analysed (data not shown). Experiments shown in Figures 3 and 4 demonstrate that a high density of methylated *Hpa*II sites can be detected mainly in nucleosomal enhancers upstream of inactive rRNA genes. This correlation is even more prominent in the promoter region, where all promoters of inactive genes are

methylated in a single conserved *Hpa*II site. In contrast, the amount of methylated DNA in the regulatory elements of active genes as well in the coding sequences is almost neglectable. These data are consistent with previous observations that undermethylated rDNA sequences can be found in regions hypersensitive to DNAse I in mouse tissues (21).

Recent *in vitro* studies show that transcription of a *Xenopus* rRNA minigene construct is suppressed by methylation of several CpGs in the rRNA gene promoter (39). Footprinting experiments (methylation interference footprinting) demonstrate that xUBF binding is not affected by methylation (40). However, an indirect mechanism of inactivation was proposed by a putative mCpG recognising protein, which abolishes xUBF binding to the recognition sites (39). In rat ribosomal RNA genes we found, in agreement with some previous observations (21,23,28), a single *Hpa*II site methylated in all promoters associated with inactive rRNA gene copies. It is tempting to speculate that for the repression of rRNA transcription in early cell differentiation the crucial step might be the methylation of CpGs in the promoter, which would allow binding of meCpG recognising protein(s). These complexes would abolish the access of transcription factors and thus support the formation of nucleosome-packed inactive chromatin. Methylation might contribute to a stable maintenance of the proportion of active and inactive rRNA gene copies in differentiated cells of higher eukaryotes. On the other hand, lower eucaryotes, like yeast, which lack CpG methylation, can modulate the number of actively transcribed gene copies depending on the growth conditions (2).

In conclusion, there is increasing evidence that DNA methylation plays an important role in inactivation of rRNA genes transcription in vertebrates and plants. The identification of mCpG binding proteins interacting with methylated promoter and enhancer sequences might allow one to reconstitute *in vitro* the mechanism of the inactivation event.

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