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DNA methylation patterns in the 5S DNAs of *Xenopus laevis*

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ABSTRACT

The frequency of cytosine methylation at specific sites in the somatic 5S DNA (Xls) and trace oocyte 5S DNA (Xlt) of X. laevis has been determined using restriction enzymes that are inhibited by the presence of 5-methylcytosine (5^mC) within their cleavage sequences. 5S DNA methylation patterns were determined in genomic DNA from mature red blood cells, which express neither type of 5S gene, and from liver, which expresses only Xls. All the sites examined in Xlt are greater than 95% methylated in red cells and liver. In the Xls of red cells all the sites examined are methylated in greater than 95% of repeats, while in liver some sites are modified in only 90% of repeats. Repeats containing unmethylated sites are randomly distributed throughout the tandem arrays in both red cells and liver. The high levels of methylation for Xls are in marked contrast to the situation with other Xenopus genes which do have sites of significant undermethylation in tissues where they are active. Thus, undermethylation in active genetic regions may not be a general feature for all classes of eukaryotic genes.

INTRODUCTION

5-Methylcytosine (5^mC) occurs in animal DNA as a result of post-replicational methylation of C residues, primarily at CpG dinucleotide sequences (1-3). Restriction enzymes that are inhibited by the presence of 5^mC within their recognition sequences have made it possible to determine the extent of methylation at specific sites in genomic DNA (3,4). In general, a region containing a protein-coding gene has high levels of 5^mC in tissues where the gene is inactive and significantly lower levels in tissues where the gene is expressed (4-8). Several studies have shown that "active" chromosomal regions, as defined by DNase I sensitivity, are extensively undermethylated (9-11). Treatment of cells with 5-azacytidine, which inhibits DNA methylation, results in the activation of some previously repressed genes (12,13), but a strict causal relationship between undermethylation and gene activity has not yet been demonstrated.

The ribosomal RNA gene regions (rDNA) in somatic tissues of many vertebrates are substantially undermethylated relative to the rest of the genome

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(4). In the somatic tissues of X. laevis the rDNA is very highly methylated, except for an undermethylated region in the spacer near the 5' end of the transcription unit (14). This region is fully methylated in sperm rDNA, and becomes undermethylated during early embryonic development, when rRNA synthesis is initiated (14). In X. borealis, however, the analogous region is undermethylated at all stages of development (15). Thus, while methylation in vertebrate genes transcribed by either RNA polymerase I (rDNA) or II (protein-coding genes) has been examined, there have not been any studies concerning $5^{m}C$ patterns in regions containing active 5S RNA genes, which are transcribed by polymerase III.

In most of the previous studies, methylation was examined at only one genetic region in a variety of tissues. Thus, the tissue specific differences observed could be due to variations in the bulk genomic methylation levels from tissue to tissue, and not be related to a particular gene's expression. The dual 5S RNA gene (5S DNA) system in Xenopus laevis allows the study of methylation patterns for both active and inactive structural RNA genes in the same tissue. X. laevis has three kinds of 5S DNA, each comprising a multigene family with repeating units (gene plus characteristic spacer) reiterated in a tandem array (16-18). Two of these 5S DNAs, major oocyte (Xlo) and trace oocyte (Xlt), are expressed only in oocytes. Somatic 5S DNA (Xls) is expressed in all cell types, except mature erythrocytes where no 5S RNA is synthesized (18-20). Xlo, Xlt and Xls are present in 20,000, 1300, and 400 copies, respectively, per haploid genome (17).

Clones of each of the 5S DNAs have been fully sequenced (17,21). No CpG dinucleotides in Xlo occur in methylation-sensitive restriction sites. However, about 20% of the CpGs in both Xls and Xlt are located where their extent of methylation can be assayed with an appropriate restriction enzyme (22). This percentage of sites is twice that examined in studies of other genetic regions (6-8). All the sites examined in Xlt are methylated in greater that 95% of the repeats in both red cells and liver. While all the sites examined in Xls also have an extremely high degree of methylation, several sites are very slightly undermethylated in liver compared to red cell. The data suggest that DNA methylation at C residues is not involved in the control of 5S gene expression in X. laevis.

MATERIALS AND METHODS

DNA Isolation

DNA was purified from the nuclei of either red blood cells or liver cells of adult Xenopus laevis females. All procedures were carried out at 4°C

unless otherwise stated. Two milliliters of blood were obtained by cardiac puncture and diluted to 40 ml in buffer A (60mM KCl, 15mM NaCl, 0.15 mM spermine, 0.5mM spermidine, 0.5 mM DTT, 0.1 mM PMSF, 2.0 mM EDTA, 0.5 mM EGTA, 15mM Tris-HCl, pH 7.5) with 5 U/ml heparin. Blood cells were collected by centrifugation at 4500 rpm for 5 min. in a Sorvall HB-4 rotor, resuspended in 40 ml of buffer A plus heparin, and pelleted again. The cells were lysed by resuspension in 20 ml of buffer A containing 0.34 M sucrose and 0.05% NP-40. After one minute cell lysis was complete and 20 ml of buffer A, 0.34 M sucrose were added to the suspension. Twenty milliliters of the lysate were layered over 10 ml of buffer A (1 mM EDTA, no EGTA), 1.37 M sucrose in each of two tubes and centrifuged at 6000 rpm for 20 min. in an HB-4 rotor. The nuclei were washed by repeating the last procedure.

The liver from an exsanguinated animal was minced and stirred for 5 min. in 40 ml buffer B (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3mM $MgCl_2$, 0.1 mM PMSF, 1.0 mM PCMB, 0.5 mM DTT) with 5 U/ml heparin. Three more such washes removed virtually all residual blood cells. The minced liver was homogenized in 30 ml buffer B, 0.34 M sucrose (homogenization buffer) by 10 strokes with a Dounce (Wheaton) loose pestle, filtered through cheesecloth, and given 3 more strokes with a tight pestle. It was then filtered through cheesecloth again and brought to a volume of 40 ml in homogenization buffer. Ten milliliters of the homogenate were layered over 10 ml buffer B, 1.0 M sucrose, 0.025% NP-40 in each of four tubes and centrifuged at 2000 rpm for 10 min. in an HB-4 rotor. The nuclei were washed by repeating this last step twice, the second time without detergent. Contamination of liver cell nuclei suspensions with erythrocyte nuclei was estimated to be less than 10% by microscopic examination.

Purified nuclei were lysed in 1x SET (150 mM NaCl, 50 mM Tris-HCl, pH 7.9, 1 mM EDTA) containing 0.5% SDS and treated with proteinase K (100 µg/ml) at 37°C overnight. The mixture was extracted once with chloroform:isoamyl alcohol (24: 1, v/v) and once with equal volumes of chloroform: isoamyl alcohol and phenol. The solution was then dialyzed against 0.2xSET and digested with RNAse A (50 µg/ml) for three hours at 37°C. RNAse was removed by proteinase K (100 µg/ml) treatment for at least three hours in 0.5% SDS at 37°C, and the protease was removed by a repetition of the organic extractions. The DNA was dialyzed against 0.2 x SET, ethanol-precipitated and resuspended in 0.2 x SET at a concentration of 800–1000 µg/ml. Before the final resuspension, liver DNA was spermine precipitated (23).

Restriction Digests

The restriction enzymes Hind III, Ava II, Hha I and Hpa II were purchased from Bethesda Research Laboratories. Ava I and Msp I were purchased from New England Biolabs. All digestions were carried out using the supplier's recommended buffers with a 4- to 8-fold excess of enzyme and a 2-fold excess of digestion time. These conditions provided complete digests of unmethylated DNA, as judged by the full digestion of a small amount of pBR322 DNA included in the reactions. Digests were stopped by addition of SDS and EDTA to final concentrations of 0.5% and 10 mM respectively. The DNA fragments from each digest were fractionated on 1.5% or 2.3% agarose gels with restriction digests of pBR322 DNA, run in parallel, serving as size markers. DNA was transferred from the gels to nitrocellulose filters by the method of Southern (24).

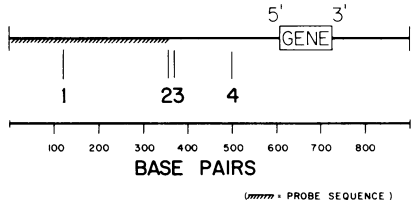
Probes and Filter Hybridization

Probes specific for Xls and Xlt have already been described (17). The vitellogenin cDNA clone pXlvc 23 (25) was provided by I. Dawid. All probes were ^{32}P-labelled by nick translation (26). Filter hybridizations and subsequent washes were done according to our previously described methods (27), except that the final washes were at 60°C in 0.1x SSC. Hybridized filters were autoradiographed on Kodak XAR X-ray film with intensifying screens for periods ranging from three hours to eight days. Microdensitometric (Joyce-Loebl) tracings of the autoradiographs were quantitated by determination of peak areas with a planimeter. Because of the wide range of band intensities, by the time some bands were detected, others had already saturated the film. Thus, several different exposures of each filter were made so that all quantitations could be done within the range of intensities where we found the film response to be linear. In comparing intensities from two different exposure periods, correction for ^{32}P-label decay was made. Autoradiographs used in the figures are those which clearly show the faintest bands. The many steps in these experiments make the values finally determined only indicative, but they were reproducible from one experiment to another.

RESULTS

Methylation in Xls

One repeating unit of Xls is shown in Figure 1, indicating the positions of the four restriction sites used in this study. These sites are the only ones where CpG sequences occur within methylation-sensitive restriction sites, and they account for 16% of the total CpGs in Xls (17).



EXTENT OF UNDERMETHYLATION

SITE	RBC	LIVER
1-Hha I	1 %	3-4 %
2-Hpa II	<1 %	<1 %
3-Ava I	2-5%	2-4 %
4-Hpa II	2-3%	5-8 %

EXTENT OF METHYLATION

	RBC	LIVER
2-Msp I	<1 %	4-6%
4-Msp I	n.d.	75-80%

Figure 1. Cytosine methylation at specific sites in Xls. The 882 bp repeating unit produced by Hind III is shown, indicating the location of the 4 methylation-sensitive restriction sites (17). The region homologous to the Xls-specific probe is also shown. Extent of undermethylation refers to the percentage of Xls repeats which are unmodified at a particular site. The level of methylation at the external cytosine of Msp I sites is referred to as extent of methylation. Values were determined from quantitative assessments of the degree of digestion at each particular site (See Materials and Methods and Figure 2). Any values below 1% were considered unreliable and are simply indicated <1%. The very low levels of digestion by Hpa II at site 2 allowed the extent of digestion by Hpa II at site 4 to be determined directly. The extent of the resistance of site 4 to Msp I digestion in liver (i.e., frequency of methylation of the site's 5' cytosine) was determined by the expression $P_{882} = f_2 \times f_4$, where P_{882} is the fraction of total Xls left as an 882 bp fragment after Msp I digestion and f_2 and f_4 are the methylation frequencies of sites 2 and 4, respectively. This may lead to an overestimate of the extent of methylation if f_2 and f_4 are not independent of each other. All values represent the range of 2-3 independent determinations. n.d. = not determined (see text).

Since HindIII cleaves Xls once per 882 bp repeating unit (17), DNA was cut to completion with this enzyme prior to being digested with one of the methylation-sensitive enzymes. The extent of digestion at a particular site by the second enzyme is then a direct measure of the percentage of Xls repeats which are unmodified at that site. Figure 2 is an example of such digests with red cell and liver DNAs. The indicated fragments of sizes 764, 500, 367 or 357 bp are the result of cleavage by the appropriate enzyme at sites 1, 4, 3 or 2 respectively (Figure 1). All other fragments are from pBR322 included in each reaction to monitor completeness of diges-

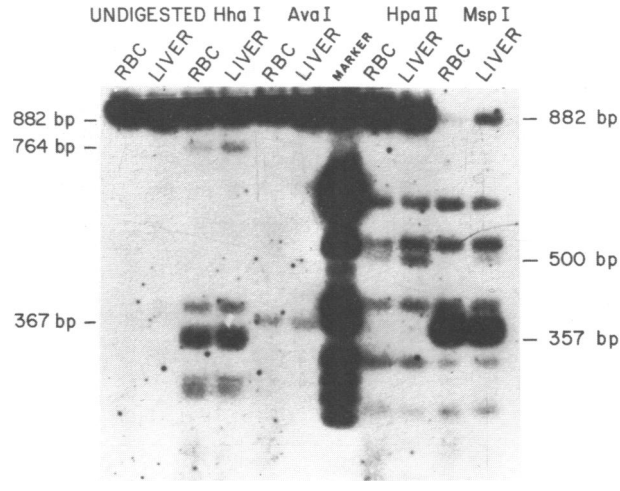


Figure 2. Digestion of Xls by methylation-sensitive restriction enzymes. DNA from red blood cells (RBC) or liver was digested to completion with HindIII prior to being cleaved with one of the four enzymes indicated. Digests were fractionated on a 2.3% agarose gel, blotted to nitrocellulose and hybridized with the Xls-specific probe as described in Materials and Methods. The Xls repeating unit is 882 bp. The indicated fragments of sizes 764, 500, 367 or 357 bp are the result of cleavage by the appropriate enzyme at sites 1, 4, 3 or 2 respectively (17, Figure 1). All other fragments are from the pBR322 included in each reaction to monitor completeness of digestion. Undigested = DNA digested with Hind III alone. Marker = a mixture of Taq I and Alu I digests of pBR322, discrete bands of which are well resolved on shorter exposures.

tion. The results from several experiments are summarized in Figure 1.

It is immediately obvious that all the sites examined in Xls are very highly methylated (>90%). Sites 1 and 4 are slightly, but consistently, undermethylated in liver compared to red cells, while site 3 is methylated to the same high extent in both issues. Hpa II digests of liver DNA, but not of red cell DNA, reproducibly produced a faint 357 bp fragment at a level still too low (<1%) to accurately quantitate. Thus, site 2 is also very slightly undermethylated in liver compared to red cells. The very low level of digestion by Hpa II at site 2 (proximal to the probe) allows the extent of digestion by Hpa II at site 4 (more distal to the probe) to be determined directly.

While Msp I will cleave at CCGG when the internal cytosine is methylated, it will not cleave at that site when the external cytosine is methylated (28). The level of modification at the external cytosine of Msp I sites is referred to as "extent of methylation" in Figure 1. This 5'

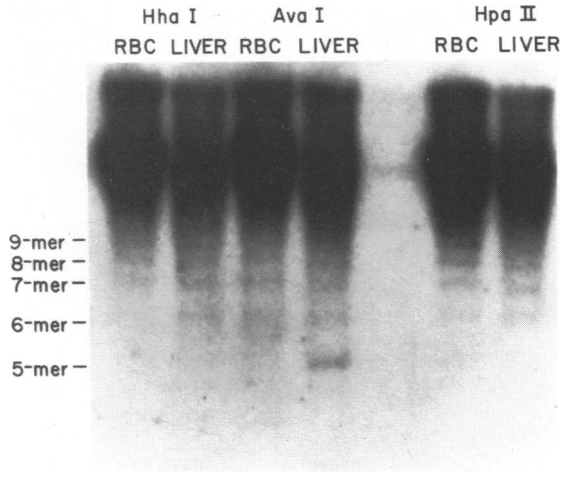
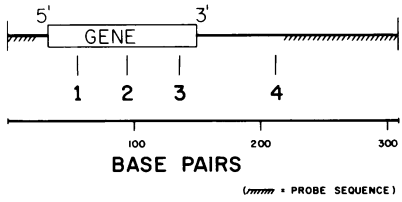


Figure 3. Dispersed arrangement of Xls repeats containing unmethylated sites. Red blood cell or liver DNA was digested with the indicated enzymes, fractionated on a 1.5% agarose gel, blotted to nitrocellulose and hybridized with the Xls-specific probe. As indicated, only bands which are higher multimers of the 882 bp Xls repeat were obtained.

cytosine is methylated at site 2 in 4-6% of Xls repeats in liver, but in <1% of repeats in red cells (Figure 1). Thus, site 2 is methylated predominantly only at the internal cytosine. The presence of an 882 bp fragment and the lack of any detectable 500 bp fragment in Msp I digests of red cell DNA (Figure 2) makes it likely that site 4 is highly insensitive to Msp I, but this could not be quantitated due to the very efficient digestion at site 2 by Msp I. In liver, where the measurement could be made, site 4 was found to have its 5' cytosine methylated in 75-80% of repeats (Figure 1). Some 10% of Xls repeats are methylated only at the internal cytosine of site 4, as judged by the greater extent of digestion at this site by Msp I compared to Hpa II. However, we cannot determine whether the sequence is also methylated at the internal cytosine when the 5' cytosine is modified (7). Thus, either or both of the cytosines could be undermethylated in liver.

The extremely high levels of methylation detected in Xls, particularly at site 2, make it very unlikely that any one repeating unit will have all of its sites unmethylated. To determine whether repeats containing unmethylated sites are clustered in the tandem arrays, we performed single digests of red cell and liver DNA with the various enzymes (Figure 3). The collections of higher multimers obtained in all cases indicates that re-



EXTENT OF UNDERMETHYLATION

SITE	RBC	LIVER
1-Hha I	<1 %	<1 %
2-Hpa II	3 %	1-2 %
3-Hpa II	1 %	<1 %
4-Hha I	<1 %	<1 %

EXTENT OF METHYLATION

	RBC	LIVER
2-Msp I	40-60%	35-50%
3-Msp I	12-16%	10-15%

Figure 4. Cytosine methylation at specific sites in Xlt. The 312 bp repeating unit produced by Ava II is shown, indicating the location of the 4 methylation-sensitive restriction sites (17). The region homologous to the Xlt-specific probe is also shown. Methods for determining the values listed were analogous to those described in Figure 1. Extent of undermethylation refers to the percentage of Xlt repeats which are unmodified at a particular site. The level of methylation at the external cytosine of Msp I sites is referred to as extent of methylation. All values represent the range of 2-3 independent determinations.

peats with unmethylated sites are essentially randomly dispersed. Repeats with Msp I sites methylated at the 5' cytosine are also randomly dispersed (Data not shown).

Methylation in Xlt

Figure 4 contains a map of one Xlt repeating unit showing the positions of the methylation sites analyzed in this study. These four CpG sites are the only ones that occur within methylation-sensitive restriction sites, and account for 24% of the total CpGs in the Xlt. Since trace oocyte 5S DNA is cut once per 312 repeating unit by Ava II (17), DNA was cut to completion with this enzyme prior to being digested with one of the methylation sensitive enzymes. The extent of methylation at each site could then be determined as described above and in the figure legends (Figures 1, 4, 5). Figure 5 is an example of such digests with red cell and liver DNAs, and Figure 4 tabulates the results.

All the sites examined in Xlt are methylated in 97% or more of the repeats. The same results were obtained even when an 8-fold excess of the re-

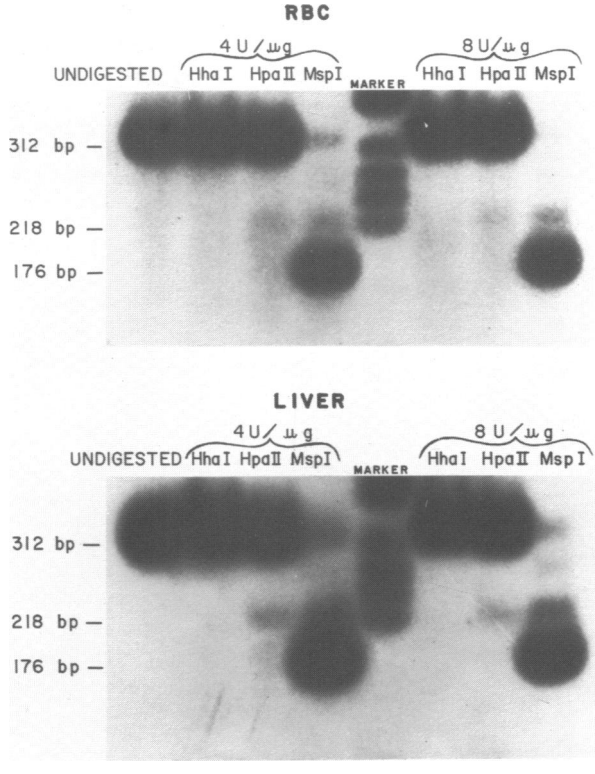


Figure 5. Digestion of Xlt by methylation-sensitive restriction enzymes. DNA from red blood cells (RBC) or liver was digested to completion with Ava II and then cleaved with one of the three enzymes indicated, using either 4 or 8 units of enzyme per μg of DNA. Digests were fractionated on a 2.5% agarose gel, blotted to nitrocellulose and hybridized with the Xlt-specific probe. The Xlt repeating unit is 312 bp. The indicated fragments of sizes 218 and 176 bp are the result of cleavage by Hpa II or Msp I at sites 2 and 3 respectively. Cleavage by Hha I at sites 1 or 4 would produce detectable fragments of sizes 255 bp or 103 bp respectively (15, Figure 4). Undigested = DNA digested with Ava II alone. Marker = a mixture of Taq I and Alu I digests of pBR322.

striction enzymes were used (Figure 5). There was no detectable difference in these methylation levels between red cells and liver, and they were consistently higher than that found in Xls of either tissue (Figures 1 and 4). As was found in Xls, Xlt's Msp I sites also have substantial levels of methylation of the 5' cytosine (Figures 4 and 5). However, in this case there was no significant difference in the extent of such modification between red cells and liver. In about 50% of repeats site 2 is methylated only at the internal C, while the rest of the time the 5' C, and perhaps both C resi-

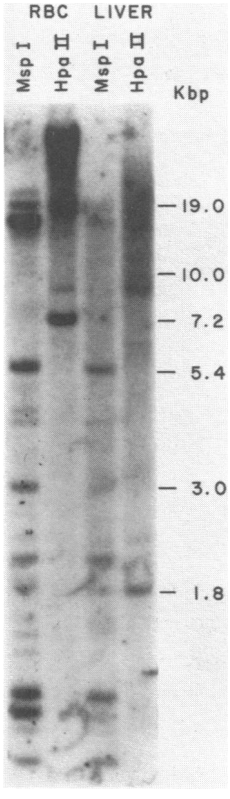


Figure 6. Undermethylation of the vitellogenin gene in liver relative to red blood cells. DNA from red blood cells (RBC) or liver was digested with the enzymes indicated, run on a 1.5% agarose gel, blotted to nitrocellulose and hybridized with the Xlvc 23 probe. Fragment sizes are indicated in kilobase pairs (Kbp). Note the 7.2 Kbp Hpa II fragment in RBC that is missing in liver and the 1.8 Kbp Hpa II fragment in liver that is lacking in RBC.

dues, are modified. The methylation at site 3 is confined almost entirely to the internal cytosine.

Methylation in the Vitellogenin Gene

To determine whether protein-coding genes in Xenopus laevis are methylated to the same high levels as that found in the 5S DNAs, we examined the methylation pattern in a portion of the A1 vitellogenin gene. This gene is actively expressed in liver, but is inactive in blood cells (29). As can be seen in Figure 6 there is substantial methylation at CpG sites in this gene, since few of the gene fragments produced by Msp I digestion are present in Hpa II digests of either DNA. However, there is a 1.8 Kbp fragment in Msp I digests that is also present in Hpa II digests of liver DNA, but is totally lacking in Hpa II digests of red cell DNA. This thus represents a region in the vitellogenin gene which is significantly undermethylated in liver compared to red cell. The 7.2 Kbp fragment in Hpa II digests of red cell DNA is totally lacking in liver Hpa II digests. This may repre-

sent the same or other sites in the vitellogenin gene which are methylated to dramatically different extents in red cells and liver. The high molecular weight fragments and lack of molar ratios between various fragments in Msp I digests of both tissue DNAs suggests that the vitellogenin gene also has Msp I sites in which the 5' cytosine is significantly methylated.

DISCUSSION

The frequency of cytosine methylation is greater than 90% at all the sites examined in the somatic and trace oocyte 5S DNAs of both red cells and liver. While the methylation pattern of Xlt is the same in both liver and erythrocyte DNA, several sites in Xls are slightly undermethylated in liver with respect to red cells.

One explanation for the low level of digestion at all sites examined could be that the methylation-sensitive restriction sites used are only present in a very small percentage of the 5S DNA repeats. This would be the case if the clones originally sequenced (17) were variants not representative of the majority of repeats. However, several Xlt repeats were sequenced (17) without finding any variation in the four sites examined. The sequences at Xls sites 1 and 4 are conserved even in the somatic 5S DNA of another Xenopus species (17). Moreover, the Msp I digests of both 5S DNAs indicate that the Hpa II sites are present in a substantial number of, if not all, repeats. It is also unlikely that tissue-specific alterations in 5S DNA sequences are responsible for the reproducible differences between tissues in extents of digestion at Xls sites. Thus, we conclude that our restriction digests accurately reflect the degree of cytosine methylation at specific positions in the 5S DNAs.

The slight undermethylation of Xls in liver compared to red cells was consistently seen in DNAs from several different individuals. This difference is not likely to be the result of tissue-specific variations in bulk genomic methylation levels, since Xlt has the same methylation pattern in both tissues. Moreover, within each tissue, Xls is slightly undermethylated with respect to Xlt (Figures 1 and 4). These differences are not large enough, however, to suggest a role for DNA methylation in the control of Xls expression. There are virtually no Xls repeats which are unmethylated at all sites, and even if all sites were functionally equivalent, with lack of methylation at any one of them being sufficient for gene activation, the vast majority of Xls repeats in liver would still be inactive. In contrast, our preliminary findings (H. Hoyle and J. Doering, unpublished

results) indicate that essentially all the Xls genes in liver are in a transcriptionally competent chromatin conformation. The Xls repeats containing unmethylated sites are clearly not all clustered in "active" chromosomal regions, nor does a small subpopulation of cells have all its repeats in the unmethylated state (Figure 3).

In addition to methylation at CpG sequences, both types of 5S DNA and the vitellogenin gene contain significant levels of methylation at the 5' cytosine of Msp I sites (CCGG). Recently, this latter type of modification has also been found in mammalian and chicken DNAs (7,28,30), suggesting that in most eucaryotes $5^{m}C$ is not confined to CpG dinucleotides. These external cytosines are not normally methylated in active genetic regions (6-8, 30), but we find that the 5' cytosine at site 4 in Xls of liver is very highly methylated, and at site 2 is more methylated in liver than in red cells.

It is possible that there are significantly undermethylated sequences in active Xls which occur at positions other than those we were able to examine with methylation-sensitive restriction enzymes. However, both coding regions and adjacent spacers are normally undermethylated at active genetic loci (5-8); if a similar situation existed for Xls, it would probably have been detected at the spacer sites studied. This is especially likely given the fact that the extent of methylation was determined for 16% of the CpGs in Xls, a much higher proportion of sites than examined in previous studies of other genetic regions (6-8).

The highly methylated sites of Xls occur in regions known to be DNase I sensitive in liver nuclei but insensitive in red cell nuclei (H. Hoyle and J. Doering, unpublished results). A lack of correlation between DNase I sensitivity and undermethylation has also been reported for the X. borealis rDNA (15) and the chick collagen gene (30). In those cases, undermethylated regions are found which are not DNase I sensitive. Our results with Xls provide a clear indication that undermethylation in a genetic region is not required in order for it to have an "active" chromatin conformation.

We have found that the X. laevis vitellogenin gene contains regions which, though highly methylated in a tissue where the gene is inactive, are significantly undermethylated in a tissue where the gene is expressed. Thus, it would appear that 5S DNAs, but not all X. laevis genes, are an exception to the correlation between undermethylation and gene activity. In fact, all previously reported exceptions to this correlation have involved hypomethylated genes which were still inactive (7,15,30-32). Xls

is the first example of an active genetic region that remains highly methylated. It may be, therefore, that hypomethylation is one of the necessary preconditions for transcription of protein-coding genes and rDNA (15,30), but that 5S DNA expression does not have this requirement. Several studies have provided direct evidence that this may in fact be the case. Viral protein genes, methylated to completion by Hpa II methylase, and injected into X. laevis oocytes are transcriptionally inactive, while the unmethylated genes are transcribed normally (33). Similar in vitro methylation of the hamster aprt gene prevents its expression after being transformed into mouse cells, even though it is integrated into the host genome with the same efficiency as the unmethylated gene which is expressed (34). Despite the fact that in somatic tissues Xlo is highly methylated at every CpG sequence (21), and Xls and Xlt are also heavily methylated (Figures 2 and 4), each of these 5S DNAs can be normally transcribed when injected into oocyte nuclei as either naked somatic cell DNA (18,35) or as intact somatic nuclei (36). Such transcription is no less efficient than when cloned, unmethylated copies of each of the 5S DNAs are used (37). Thus, DNA methylation does not appear to play a role in controlling the developmental expression of any of the X. laevis 5S DNA families. Undermethylation is apparently needed for transcription of other types of genes, and may be due to some hypomethylated substrate requirement by RNA polymerases I and II which is not shared by polymerase III.

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