The methylation pattern of tRNA genes in *Xenopus laevis*

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ABSTRACT

The methylation sensitive restriction enzymes Hha I and Hpa II were used to analyse the methylation pattern of a tRNA gene cluster in germ line and somatic DNA of *Xenopus laevis*. A single tDNA repeat containing 8 tRNA genes was studied and all copies were found to be fully modified in sperm DNA. In the DNA from erythroid cells, hepatocytes, kidney and brain, most tDNA repeats were found to be fully modified. However, in a fraction of the repeats, specific demethylated sites can be detected, giving rise to a pattern which does not vary significantly from one tissue to another. Although our results do not allow a straightforward correlation between hypomethylation and tRNA gene transcription, they are in agreement with the observation that hypomethylation accompanies differentiation and development.

INTRODUCTION

With few exceptions the genomes of eukaryotes contain the modified base 5-methylcytosine (5mC) resulting from the post-replicative modification of CpG dinucleotide sequences in DNA (1-3). The use of restriction enzymes whose activity is inhibited by the presence of 5mC within their recognition sites has led to observations of tissue-specific methylation patterns in chicken globin genes (4,5) and ovalbumin gene (6), the rRNA genes in *X. laevis* embryos (7) and the developmentally regulated β -like globin gene locus in mammals (8,9). In some cases, hypomethylated genes have also been found to be organised into a chromatin structure which is DNase I sensitive (5,10,11). Such data are in agreement with the proposition that specific demethylation events may be necessary for gene expression during development and differentiation. However, most of the investigations have been carried out on genes transcribed by either RNA polymerase I or II and considerably less is known about the methylation patterns of genes transcribed by RNA polymerase III.

The tRNA genes of *X. laevis* are reiterated 8,000 times in toto in the haploid genome (12) and due to their clustered repetitive organization, they

offer a favourable system in which to study specific changes in the inheritance of methylation patterns during gene activation and cell differentiation.

Here, we describe the methylation pattern of one particular tRNA gene cluster in germ line and somatic tissues of *X. laevis*. This cluster consists of tandemly repeated 3.18 kb tDNA fragments, each repeat unit (t210) containing 2 tRNA^{Met} genes and single tRNA genes specific for alanine, asparagine, leucine, lysine, phenylalanine and tyrosine (13, 14). The complete 3.18 kb unit is repeated about 150 times per haploid genome and has been completely sequenced (15).

Virtually every CpG enzyme site has been analysed in the germ line and in somatic tissues at various stages of differentiation. In sperm DNA, Hha I and Hpa II sites are modified in all repeats. In somatic tissues in which tRNA genes are actively transcribed, most repeats are modified but a fraction of hypomethylated repeats can be detected. This pattern of methylation is found early in development and is retained in transcriptionally inactive tissues such as erythrocytes.

MATERIALS AND METHODS

DNA isolation

For isolation of DNA, nuclei were prepared from purified cells. Tadpole erythrocytes were obtained by amputating the tail of a large tadpole, sperm and erythrocytes were removed from a single male *Xenopus* while immature erythrocytes were obtained through rendering a large female *Xenopus* anaemic by subcutaneous injections of phenylhydrazine on consecutive days (16). All procedures were carried out at 4°C unless otherwise stated. Blood was removed by cardiac puncture and diluted into APBS (130 mM NaCl, 5 mM EDTA, 10 mM NaH₂PO₄, pH 7.4). Cells were collected by centrifugation at 500 g for 5 min. in an MSE Chilspin bench centrifuge, resuspended in APBS, pelleted again and purified on Percoll gradients of starting density 1.08 g/ml. Purified cells were resuspended and lysed in buffer A (70 mM KCl, 2 mM EDTA, 1 mM DTT, 50 mM Tris-HCl pH 7.9, 10% Glycerol) containing 0.05% Triton X-100. After cell lysis was complete, nuclei were pelleted at 1,000 g for 5 min., resuspended and washed several times in buffer A to remove residual cytoplasm. Nuclei from hepatocytes and cultured kidney cells were prepared similarly. Purified nuclei were lysed in TEN (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 200 mM NaCl) containing 0.5% SDS, and treated with Proteinase K (100 µg/ml) over-

night at 37°C. The lysate was extracted twice with phenol : chloroform : isoamylalcohol (25 : 24 : 1), twice with chloroform : isoamylalcohol (24 : 1) and once with water saturated ether. The DNA was precipitated with ethanol twice and resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Sperm DNA was similarly prepared from spermatids which were purified on Percoll (17).

Restriction Digests

The restriction enzymes Hha I and Hpa II were purchased from the Radiochemical Centre, Amersham and Msp I from Bethesda Research Laboratories. Digestions were carried out in the buffers recommended by the manufacturers with or without prior digestion of DNA with RNase, using a 4 fold excess of enzyme and digestion at 37°C overnight. Complete digestion was verified by addition of an aliquot of the reaction mixture to cloned plasmid DNA and incubation at 37°C followed by electrophoresis to monitor the expected cleavage products. Reactions were stopped with EDTA to 10 mM and 5 - 10 µg DNA/slot was electrophoresed on 20 x 20 x 0.5 mm thick 1% agarose gels with appropriate size markers. DNA was transferred to Schleicher and Schuell nitrocellulose filters by the method of Southern (18).

Filter Hybridisation

Filters were cut into strips and hybridised to the Xenopus tDNA clone pxt210 (13) which was ³²P-labelled by nick translation to a specific activity of 10⁷ - 10⁸ cpm/µg (19). Filter hybridisations and subsequent washes were performed according to Jeffreys (20) at 65°C with a final stringent wash at 0.1 x SSPE (1 x SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM NaH₂PO₄ pH 7.0). Autoradiographic exposure was carried out using Kodak XR-5 film with Dupont Cronex intensifying screens for a limited period so that the blackening of the autoradiogram was still linearly related to the radioactivity in a band (21). Figures presented are typical and were reproducible from one experiment to another.

RESULTS

The plasmid pxt210 consists of a single 3.18 kb repeat unit, containing 8 tRNA genes, inserted into the Hind III site of pBR322 (Fig. 1). In all, there are twelve Hpa II sites and seventeen Hha I sites in the cloned repeat which has been completely sequenced (15). The CpG dinucleotides recognised by methylation sensitive restriction enzymes comprise about 19% of the total number of CpG residues in the repeat. We first analysed the Msp I cleavage pattern by digesting Xenopus DNA, blotting onto nitrocellulose and hybridis-

Fig. 1: Arrangement of tRNA genes in pxt210. The 3.18 kb unit bordered by Hind III sites is shown, together with the location of CCGG and GCGC sites. Arrows denote direction of transcription.

ation with pxt210 which had been labelled *in vitro* by nick translation. The autoradiograph (Fig. 2) shows that several fragments are produced, ranging from 1.8 kb to 0.6 kb. To ensure that digestion was complete, we included a cloned plasmid DNA as an internal control and monitored the appearance of the expected final cleavage products. Were Msp I to recognise and cleave all CCGG sequences in all repeats, most fragments would be less than 250 base pairs in length and would therefore transfer inefficiently and hybridise poorly (18). This is clearly not the case since the stoichiometry of the bands and the sum of fragment lengths suggests that not all sites are uniformly cut.

In comparison, Hpa II is largely unable to cleave tDNA (Fig. 2 lane 3) although a faint ladder can be seen in which the monomer fragment comigrates with the 3.2 kb repeat. Similarly, Hha I (Fig. 2 lane 4) recognises some unmodified sites within tDNA to generate a faint ladder in which the shortest fragment is the same size as the repeat. This suggests that in a fraction of repeats, one Hpa II and one Hha I site is undermethylated. This situation does not apply to sperm DNA (Fig. 2 lanes 8 and 9) in which all repeats appear to be modified.

To investigate this point in detail, DNA from a wide variety of tissues was first digested with EcoRI which defines the 3.2 kb repeat and then secondly cleaved with one of the methylation-sensitive restriction enzymes. In this way, the methylation of specific restriction sites within well-defined DNA fragments can be analysed accurately. The results are shown in Fig. 2. In somatic tissues, although most repeats are fully modified, a small fraction of repeats is found to be undermethylated giving rise to sub-fragments of 2.85, 2.4, 2.1, 1.8, 1.4, 1.0, 0.15 and 0.6 kb in Hha I digests of DNA from erythrocytes (lane 6), cultured kidney cells (lane 15) and whole kidneys

Fig. 2: Methylation of tRNA gene in germ line and somatic tissues. (A) ethidium bromide pattern, (B) autoradiogram. DNA from erythrocytes (lanes 2-7), sperm (8-12) cultured kidney cells (13,15,17 and 19) or whole kidneys (14,16,18 and 20) was cleaved with Msp I (lane 2), Hpa II (lanes 3 and 8), Hha I (lanes 4 and 9), EcoRI (lanes 5,10,13 and 14) or with EcoRI in combination with Hha I (lanes 6,11,15 and 16), Hpa II (lanes 7,12,19 and 20), or Msp (lanes 17 and 18). DNA was electrophoresed on a 1.2% agarose gel and blot-hybridised to pxt210. Lane 18, in comparison with lane 17, is an incomplete digest. Lane M: molecular weight markers

Fig. 3: tDNA methylation in erythroid tissues. pxt210 was hybridized to DNA from tadpole erythrocytes (lanes 1-6), adult erythroblasts (lanes 7-10), adult erythrocytes (11 and 12), brain (13 and 14) or liver (16-19). Restriction was with EcoRI alone (lanes 7,13 and 16) or with EcoRI in combination with Msp I (lanes 1,2,8, and 17) Hpa II (lanes 3,4,9,11,14 and 18) and Hha I (lanes 5,6,10,12 and 19). Lanes 1,3,5 and 2,4,6 represent separate digests.

(lane 16). Similarly, some repeats are cleaved into fragments of 2.8, 1.9, 1.7, 1.5 and 1.26 kb in Hpa II digests of DNA from cultured kidney cells (lane 19) and whole kidneys (lane 20). These fragments are also present as faint bands in Hpa II digests of erythrocyte DNA (lane 7). Only a single faint sub-fragment is found when sperm DNA is digested with Hha (lane 8) or Hpa II (lane 12). Interestingly, DNA from cultured kidney cells shows the same methylation pattern in this region as that from whole kidneys (compare lane 15 with 16 and 19 with 20). Although there are variations in the intensities of sub-bands, qualitatively the pattern is very similar. These studies have been extended to other tissues in different stages of development and differentiation (Fig. 3). Genomic DNA from tadpole erythrocytes (lanes 1-6), adult erythroblasts (lanes 7-10), adult erythrocytes (lanes 11 and 12), brain (lanes 13 and 14) and liver (lanes 16-19) is similar in its pattern of methylation at Hha I and Hpa II sites in the tDNA locus. Although the results are made complicated by the presence of an additional 1.8 kb tDNA EcoRI repeat, they nevertheless indicate the presence of unmodified sites within regions

of DNA encoding tRNA genes. These sites have not been mapped any further. In Msp I digests of tadpole erythrocyte DNA (lanes 1 and 2) a fraction of remaining hybridisation at the position of the original 3.2 kb fragment indicates that besides the C^MCGG type of modification, C^M₂CGG modification exists.

DISCUSSION

We believe that this is the first report of the methylation of tRNA genes in eukaryotic tissues. Both germ line and somatic tissues have been found to be extensively methylated in at least one tRNA gene locus. At the same time, a fraction of tDNA repeats is found to be undermethylated at some Hha I and Hpa II sites in all somatic tissues examined. It is unlikely that DNA modification by methylation is directly involved in the rapid modulation of transcription of individual tRNA genes since in the absence of cell division, methylation appears to be an irreversible process. In support of this view, mature erythrocytes which undergo a loss in RNA polymerase III activity (22) and do not synthesize tRNA, retain the methylation pattern of tRNA genes seen in immature erythroid cells which are certainly active in tRNA transcription (Talwar *et al.*, unpublished observations). The same pattern appears to be found in tadpole erythrocytes. It is at present an unresolved question as to whether tadpole erythrocytes switch to synthesizing adult globin chains or whether adult erythrocytes are derived from a completely different population of stem cells (Discussion, 16). Whatever the mechanisms may be this study shows that methylation patterns are remarkably conserved during differentiation and development. We conclude that only a few specific sites in the tRNA repeating unit are unmethylated in the erythroid cells of tadpoles, anaemic adults and normal adults and this apparently uniform pattern holds true despite the considerable differences in transcriptional activity which characterise these cells.

The significance of the precise distribution of undermethylated sites for tRNA genes is not immediately obvious and mechanisms to account for these patterns are lacking. It is certainly difficult to ascribe a role for DNA methylation in the control of every tRNA gene or even of every repeat unit. Even if a single hypomethylated site were sufficient for transcription, the vast majority of repeats would not be expressed. A corollary to this is that not all copies of the repeat unit are transcribed. It is conceivable that the specific bands seen in somatic tissues represent undermethylated sites in the

fraction of repeats that are (or were) transcribed during erythroid development.

The method we used to analyse methylation of tRNA genes limits the number of testable sites to those which fall within the recognition sequence of a methylation-sensitive restriction enzyme. Therefore, we cannot exclude the possibility that in probing tDNA, we missed hypomethylation of important cytosines not contained within a restriction site. However, most authors analysing methylation in different genes have reported expression specific hypomethylation in Hha I and Hpa II sites.

It might also be suspected that these results could be partially due to incomplete digestion of DNA and that many other genes would also fail to be restricted with Hha I or Hpa II in such conditions. However, we believe that our restriction digests accurately reflect the degree of cytosine methylation within a tDNA region since hybridisation of identical filters to genes present in only a few copies in the haploid genome has shown that in erythrocytes, the adult major globin locus is hypomethylated whereas the vitellogenin gene is not (Talwar *et al.*, unpublished results, 23).

Because tRNA genes encode housekeeping functions, constitutively expressed in all cell types, it makes sense that these genes have an invariant pattern of methylation. In contrast to tissue-specific genes, their expression would not be modulated during development. Indeed it is difficult to imagine how methylation is involved in the rapid modulation of transcription of each of the eight tRNA genes present in t210. Therefore, methyl variations during development may be restricted to a small proportion of the expressed genome.

Like the 5S RNA genes (24), satellite DNA (25) and repetitive sequences in general, tRNA genes have been found to be heavily methylated in many tissues. The temporal determination of methylation patterns during the differentiation of stem cells into erythroid cells is presently unknown, but the process is analogous to the retention of an "active" chromatin structure in the absence of transcription. Both globin and tRNA genes are found to be in a state sensitive to both DNase I and endogenous nuclease during erythroid differentiation, irrespective of the transcriptional competence of erythroid chromatin (Talwar *et al.*, unpublished results). Our results therefore indicate that undermethylation does not correlate with preferential nuclease sensitivity of tRNA genes. A similar lack of correction was found for the Xenopus 5S DNAs (24) ribosomal DNA (26) and the chicken collagen gene (27).

Thus hypomethylation appears to be necessary but not sufficient for the expression of genes transcribed by RNA polymerase I and II, a requirement which may not be shared by RNA polymerase III. Whatever the significance of these methylation patterns may be, their conservation through development is remarkable.

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