Volume 15 Number 8 1987 Nucleic Acids Research

# DNA methylation pattern and restriction endonuclease accessibility in chromatin of a germ-line specific gene, the rainbow trout protamine gene

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Received September 9, 1986; Revised and Accepted March 3, 1987

ABSTRACT

The chromatin structure of a germ-line specific gene, the TPG-3 gene, one of the rainbow trout protamine genes was analyzed in various tissues. The protamine genes are expressed in early stage testis but not in late stage testis, liver or erythrocyte. Five potential CpG methylation sites in the coding and flanking regions of the TPG-3 protamine gene were monitored in early and late stage testis, nucleoprotamine, liver and erythrocyte. In all cases the patterns of methylation were identical with only one CpG site at position -740 being methylated. Thus, the methylation pattern of this protamine gene remained the same independently of the expression of the gene. Two Msp I sites at positions -293 and/or -275 and +155 were accessible to the enzyme in the TPG-3 chromatin of early stage testis. Since the Msp I site at position -293 and/or -275 was also present in the TPG-3 chromatin of liver, only the site at position +155 within the transcribed region correlated with the expression of the protamine gene.

## INTRODUCTION

Five-methylcytosine is the only modified base present in vertebrate DNA and it is found almost exclusively in the dinucleotide sequence CpG which, perhaps significantly, is underrepresentated (about 4 or 5 times lower than expected) in the genome of vertebrates (see 1 and 2 for reviews). The methylation patterns of more than 30 gene sequences have been analyzed using methylation sensitive restriction endonucleases (Hpa II and Hha I) (1). Although these studies suggest that, in general, active genes are undermethylated compared to inactive genes, some genes have been found to show no clear correlation between their methylation pattern and their expression (1,3). The only feature which has been consistently observed is that no gene is less methylated in sperm DNA than in somatic DNA.

Spermatogenesis in vertebrate testis is a complex developmental process during which five major germ cell types are sequentially generated: the spermatogonium, the primary and secondary spermatocytes, the spermatid and the spermatozoan. Spermatogenesis in rat, mouse and rooster is a "steady

state" system with equivalent amounts of each cell type present at any given time. In contrast, trout spermatogenesis is a fairly synchronous process with one of the cell types being predominant and representative of the maturation stage of the testis (4,5). Each trout testis which is a thread of a few milligrams at the begining of spermatogenesis reaches a weight of 10 g or more at the end of the process. During the later stages of trout spermatogenesis, histones are replaced by small, very basic proteins, the protamines, which condense the DNA for packaging into the sperm head (4,6). The protamines are synthesized in the middle spermatid cells but their genes are transcribed at an earlier stage of development, in the primary spermatocyte cells (6-8) and the mRNAs are stored as inactive ribonucleoprotein particles in the cytoplasm until the middle spermatid stage where they are translated (7,8). The protamines are encoded by a small family of genes which do not have intervening sequences and show considerable sequence homology in their coding and immediate 5' and 3'-flanking regions (9,10). Gregory et al. (11) have cloned and sequenced one of the protamine genes, TPG-3. A striking feature of the coding region is the high content of CpGs which occur at a frequency about 10 times higher than in bulk DNA (fig. 1).

Using S1-nuclease mapping and primer extension of in vivo synthesized trout testis polyA+-mRNA, Gregory et al. (11) localized the mRNA start site of the TPG-3 protamine gene at 14 nucleotides from the start of the

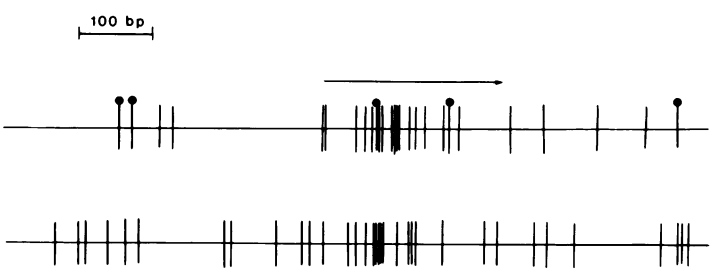


Figure 1 Distribution of CpGs in the coding and immediate flanking regions of the TPG-3 protamine gene

Each vertical line represents a CpG doublet (upper map) or a GpC doublet (lower map). The horizontal arrow indicates the transcribed region of the gene as well as the direction of transcription. (●) indicates the CpG sites which could be monitored for methylation by the restriction endonucleases Hpa II and Hha I. The coding region of the TPG-3 protamine gene can be assigned as a HTF-like region as defined by Bird (2) since the G+C content is over 50% (58%) and the number of CpGs (16) roughly equals the number of GpCs (13).

protein-coding sequence. However, since the coding region of the protamine genes is highly conserved, it is not possible to demonsrate whether the transcript originated from the TPG-3 gene or from one of the other protamine genes. But, it should be noted that the TPG-3 gene does not have any abnormalities in the coding and regulatory sequences, and this gene is transcribed when transfected into Hela cells (12).

In this report, we have studied the DNA methylation pattern and the accessibility in chromatin to the restriction endonuclease Msp I of a germ-line specific gene, the TPG-3 gene, one of the trout protamine genes.

## MATERIALS AND METHODS

### Tissues

Testes and livers were obtained from rainbow trout (Salmo gairdnerii) at the Sun Valley Trout Farm, Mission, British Columbia. Testes were separated according to their weight and color. A pink testis weighing between 1.0 and 1.7 g was considered as an early stage testis while a white testis weighing more than 10 g was classified as a late stage testis.

### DNA preparation and base analysis

DNA preparations and base analysis were done as described by Davie and Saunders (13).

### Analysis of DNA methylation by restriction digestions

DNA isolated from the various sources was digested with the appropriate restriction endonuclease(s) (see fig. 4). A restriction map of the subclones pPA2, pPB2, pPC23 and pPD4 was obtained for the restriction endonucleases Hpa II and Hha I. These sites are shown on the map in figure 4.

For nucleoprotamine preparation, late stage testis nuclei at a concentration of 50 A260/ml (2.5 mg/ml) were digested with 50 A260 units/ml of micrococcal nuclease (Sigma) in buffer A (10 mM PIPES, pH 7.5, 2 mM $MgCl_2$, 30 mM Na butyrate, 1 M hexylene glycol) containing 1 mM $CaCl_2$ for 20 min at 37°C. The suspension was made 10 mM in EDTA and 0.6 M in NaCl to terminate the digestion and to extract nucleohistone. After 20 min on ice, the sample was centrifuged at 5000 rpm in a Sorvall SS-34 rotor for 20 min. The nucleoprotamine which remained with the pellet was extracted again with 0.6 M NaCl and 10 mM EDTA to remove all the nucleohistone and the DNA in the pellet was then purified.

### Msp I digestion of testis, liver and erythrocyte nuclei

Trout testis tissues were homogenized in TMK (50 mM Tris-Cl, pH 7.5, 25 mM KCl, 2 mM $MgCl_2$, 30 mM Na butyrate and 1% (v/v) thiodiglycol)

containing 10 mM iodoacetamide and 1 mM PMSF and washed with TMK containing 0.2% (w/v) Nonidet P-40 and 1 mM PMSF.

Liver nuclei were isolated in the same fashion, except that 0.2% (w/v) Nonidet P-40 was also included in the homogenization step. Erythrocyte cells were homogenized in buffer A containing 10 mM iodoacetamide and 1 mM PMSF. Testis, liver and erythrocyte nuclei were resuspended in buffer B (100 mM NaCl, 50 mM Tris-Cl, pH 8.0, 3 mM $MgCl_2$, 5 mM Na butyrate and 1 mM 2-mercaptoethanol) containing 1 mM PMSF at a final concentration of 20 $A_{260}$/ml (1 mg/ml). Nuclei were prewarmed 5 min at 37°C and digested by Msp I (40 units/ml) for 30 min at 37°C. The digestion was stopped by addition of EDTA (8 mM), Na N-lauroylsarcosine (1.8% w/v), NaCl (0.12 M) and pronase (400 ug/ml).

DNA hybridizations

After restriction digestions, DNA was electrophoresed on a 1% agarose gel and transferred onto MSI Magna nylon 66 membranes (Fisher). Hybridizations to 32P-labeled probes were done in 6 X SSC (20 X SSC: 3 M NaCl, 0.3 M Na citrate), 1% SDS, 200 ug/ml heparin, 0.1% Na pyrophosphate and 10 mM EDTA, overnight at 68°C (method adapted from Singh and Jones (14)). Washes were done as previously described (15).

Isolation of nuclear RNA

Liver and early testis nuclei were isolated as described above in TMKS (TMK and 0.25 M sucrose) containing 1 mM PMSF and 2 mM vanadyl ribonucleoside complex (VRC). Nuclear RNA was isolated essentially as described by Fey *et al.* (16). The nuclei (approximately 30 $A_{260}$ units/ml) were digested with deoxyribonuclease I (DNAase I, Sigma, 30 ug/ml) in RSB (10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 10 mM NaCl, 5 mM Na butyrate, 1 mM PMSF, 2 mM VRC) at 37°C for 20 min. The digestion was terminated by the addition of ammonium sulfate to 0.25 M. The insoluble nuclear material was collected by centrifugation and the pellet was resuspended in 50 mM Na acetate, pH 5.1, 100 mM NaCl, 10 mM EDTA. The suspension was made 0.5% SDS, extracted with phenol/chloroform and ethanol precipitated. The pellet was resuspended in 50 mM Na acetate, pH 6.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, digested with DNAase I (RNAase free, BRL, 30 ug/ml) at 37°C for 20 min, twice phenol extracted, and ethanol precipitated. The RNA was resuspended in water. The concentrations of the RNA isolated from liver and testis were determined by the staining method described by Bulow and Link (17) and by ethidium bromide staining on 1.2% agarose gels containing formaldehyde (15). Equivalent amounts of RNA from liver and testis were made 4.6 M formaldehyde, 7.5 X SSC and heated 15 min at 65°C.

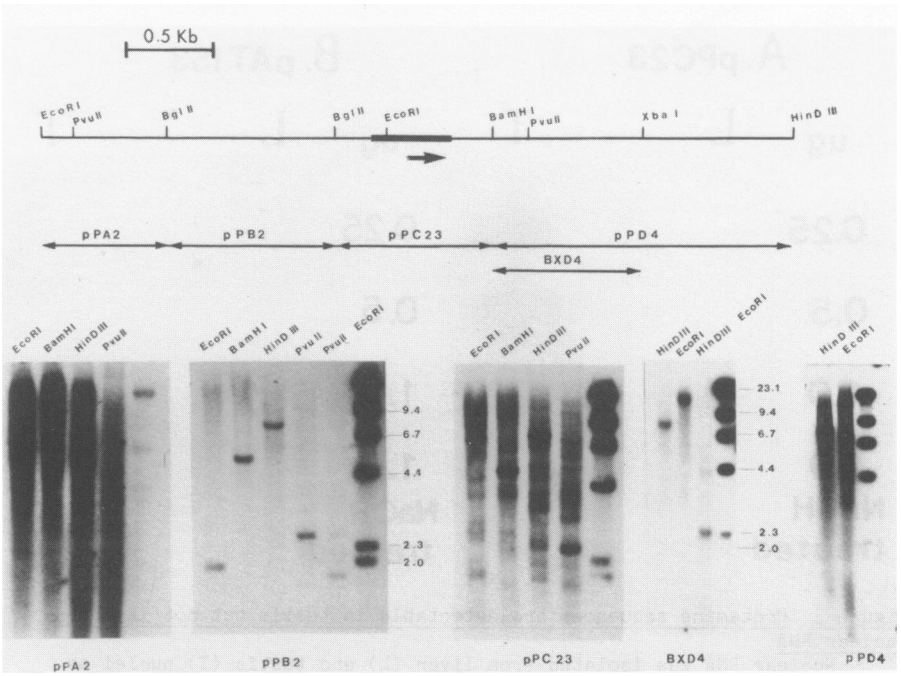


Figure 2 Characterization of subclones of the TPG-3 protamine gene

On top, a map of the TPG-3A24 clone is shown. The bold area indicates the region of homology between all the protamine genes (9) and the arrow represents the transcribed portion of the gene as well as the direction of transcription. Below the map, the probes used in the hybridizations are shown. The DNA was isolated from trout testis, digested with a restriction endonuclease(s) as indicated, separated on a 1% agarose gel, transferred onto Magna paper and hybridized to each $^{32}$P-labeled probe. Hind III fragments of phage λ were used as markers and their sizes are indicated in kb.

The RNA sample was spotted onto nitrocellulose filters by using the slot-blot apparatus from Schleicher and Schuell. In some cases, the RNA was hydrolyzed in 0.3 M NaOH for 1h at 65°C before being spotted onto the nitrocellulose. Hybridization to $^{32}$P-labeled probes and washes were performed as described by Thomas (18).

## RESULTS

### Characterization of the subclones of the TPG-3 protamine gene

The genomic clone pTPG-3A24 contains the complete protamine coding region of the TPG-3 gene plus 2 kb of flanking sequence on either side (11, fig. 2). Five subclones were prepared: pPA2, pPB2, pPC23, pPD4 and BXD4.

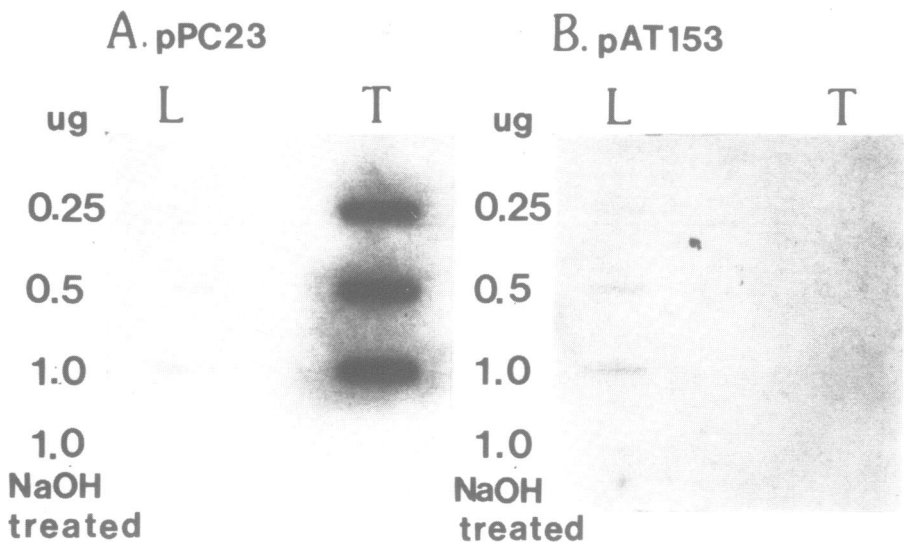


Figure 3 Protamine sequences are detectable in testis but not in liver nuclear RNA

Nuclear RNA was isolated from liver (L) and testis (T) nuclei as described under "Materials and Methods". Varying amounts of the hnRNA samples were spotted onto nitrocellulose by using the slot-blot apparatus from Schleicher and Schuell. The filters were hybridized with 32P-pPC23 (protamine) (A) or 32P-pAT153 (B). The latter probe tested for nonspecific hybridization.

Genomic Southern hybridization blots using each of these five subclones as probes are shown in fig. 2. Both subclones pPA2 and pPD4 contain elements of repetitive DNA while pPB2 and BXD4 do not. This analysis demonstrates that pPB2 and BXD4 are suitable probes to monitor the chromatin structure of the TPG-3 gene.

It should be noted that the prominent Eco RI fragment visualized by the pPB2 probe has a size of 2.0 kb. This is a smaller fragment than expected since no Eco RI sites are present in the 4.4 kb TPG-3A24 clone (11). We think that this is due to a polymorphism in the fish population. This hypothesis is supported by mapping experiments (not shown) which localized an EcoRI site about 100 bp upstream from the transcription initiation site. This fits very well with the presence in the TPG-3A24 clone of a sequence TAATTC (-126 to -120) which differs at only one base pair from the EcoRI recognition site (GAATTC).

The subclone pPC23 recognizes the coding region of the protamine gene

TABLE I Five-Methylcytosine Content in DNA from Testis and Liver

| Sample | 5-methylcytosine content Mol % of total cytosine |
|---|---|
| early stage testis | 6.45 ± 0.71 (n=6) |
| late stage testis | 6.18 ± 0.38 (n=6) |
| liver | 5.50 ± 0.28 (n=6) |

DNA isolation and hydrolysis was performed as described under "Materials and Methods" and the bases were resolved on a Partisil 10 SCX (Whatman) column.

and therefore, hybridizes to all of the protamine genes. According to the number of bands detected by pPC23 for each of the restriction digestions, it appears that there may be more copies of the protamine gene in the trout genome than the six copies which have been cloned and sequenced (9). The actual number of protamine genes present in the trout is difficult to evaluate because of the presence of polymorphisms in the fish population.

Expression of the TPG-3 protamine gene in testis

The results from Dixon's laboratory suggest the protamine genes are not expressed in the somatic tissue, liver (8,19). This conclusion is based on their inability to detect liver transcripts which hybridize to a protamine probe. We confirmed their results by an analysis of hnRNA from liver and early stage testis nuclei. Increasing amounts of each hnRNA sample were spotted onto nitrocellulose. As shown in figure 3, a strong signal was obtained when early stage trout testis hnRNA was hybridized with pPC23, demonstrating that the protamine genes are transcribed in early stage testis. The signal disappeared when the hnRNA sample was hydrolyzed in alkaline conditions. This rules out the possibility of a DNA contamination of our hnRNA preparations. When the liver hnRNA sample was hybridized with pPC23, it only gave a faint signal, no more intense than the one obtained with the pAT153 vector. An increase in the intensity of the hybridization signal with pPC23 was not observed at loadings of liver hnRNA 20X greater than that of the highest loading shown in figure 3. Thus, from this analysis of the steady state hnRNA our results indicate that the protamine genes are not expressed in liver.

Quantification of 5-methylcytosine in DNA from testis and liver

Base analysis by high performance liquid chromatography showed that, as for other vertebrates, the only modified base present in trout DNA is

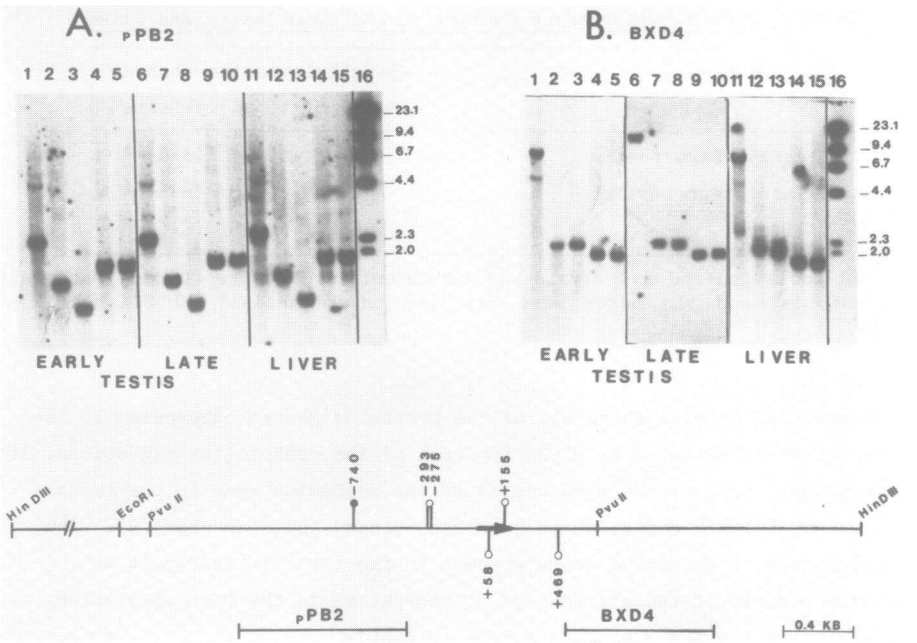


Figure 4 DNA methylation of the TPG-3 protamine gene

In panel A, DNA was isolated and digested with Pvu II (lanes 1,6,11), Pvu II-Hpa II (lanes 2,7,12), Pvu II-Msp I (lanes 3,8,13), Pvu II-Hha I (lanes 4,9,14) and Pvu II-Cfo I (lanes 5,10,15), separated on a 1% agarose gel and blot-hybridized to the 32P-labeled probe pPB2. In panel B, after isolation DNA was digested with Hind III (lanes 1,6,11), Hind III-Hpa II (lanes 2,7,12), Hind III-Msp I (lanes 3,8,13), Hind III-Hha I (lanes 4,9,14) and Hind III-Cfo I (lanes 5,10,15), separated on a 1% agarose gel and blot-hybridized to the 32P-labeled probe BXD4. Hind III fragments of λ were used as markers, their sizes are indicated in kb. It should be noted that lanes 6 to 10 on panel B come from a different gel which was run a bit longer than the others shown. The two probes pPB2 and BXD4 are shown under the map. The arrow on the map represents the transcribed region of the TPG-3 gene as well as the direction of transcription. A line going up represents a Hpa II/Msp I site, a line going down indicates a Hha I/Cfo I site. A methylated site is designated as (●) while a site that is not methylated is designated as (O). Only one circle is used for the two sites at positions -293 and -275 since these two sites are too close to each other to be distinguished.

5-methylcytosine. The content of 5-methylcytosine in early and late stage testis and in liver is shown in Table I. Within the limits of the sensitivity of this assay, the 5-methylcytosine content of the DNA did not detectably change as the testis matures. Liver DNA is slightly less methylated.

DNA methylation of the TPG-3 protamine gene

The methylation state of specific CpG sites in the protamine gene TPG-3 has been determined by using the methylation sensitive restriction endonucleases Hpa II, Msp I, Hha I and Cfo I. Both Hpa II and Msp I recognize the sequence CCGG but Hpa II cuts the DNA only if neither of the Cs is methylated while Msp I cuts CCGG independently of the methylation state of the internal C (20). Hha I and Cfo I recognize the sequence GCGC but Hha I does not cut this sequence if either of the Cs is methylated (20). It has been reported that Cfo I does not cleave the DNA if both Cs are methylated (21) but apparently the methylation sensitivity of this enzyme has not been further investigated. We decided to use it since there was a possibility that unlike Hha I it could cut the DNA if only one of the Cs was methylated.

As shown in figure 4, the TPG-3 gene presents the same methylation pattern in early and late stage testis and in liver. The same pattern was also observed in erythrocyte and in nucleoprotamine (data not shown). Only one of the CpG sites analyzed was found to be methylated (-740, fig. 4). When the probe pPB2 was used (fig. 4, panel A), the Pvu II-Hpa II digestion produced a 1.65 kb fragment while the Pvu II-Msp I digestion gave a 1.2 kb fragment indicating that the CpG site at position -740 is methylated but that the CpG sites at positions -293 and/or -275 are not methylated (these two sites are too close to each other to be able to distinguish between them on a Southern blot hybridization autoradiogram). The Pvu II-Hha I digestion produced a 2.0 kb fragment showing that the CpG site at position +58 is not methylated. When the probe BXD4 was used (fig. 4, panel B), both Hind III-Hpa II and Hind III-Msp I digestions led to the appearance of a 2.1 kb fragment showing that the CpG site at position +155 is not methylated. Neither is the CpG site at position +469 since a Hind III-Hha I digestion produced a 1.8 kb fragment.

Accessibility of the TPG-3 protamine gene chromatin to the restriction endonuclease Msp I

Restriction endonucleases have been used to detect alterations in the chromatin structure of a gene, for example for the chicken adult β-globin gene (22), the mouse thymidine kinase gene (23), the goat γ-, βc- and $\beta^A$-globin genes (24), the chicken thymidine kinase gene (25) and the avian retrovirus ev-3 (25).

We have used the restriction endonuclease Msp I to monitor the chromatin structure of the TPG-3 protamine gene. Nuclei isolated from early and late stage testis, liver and erythrocyte were digested with Msp I. Four

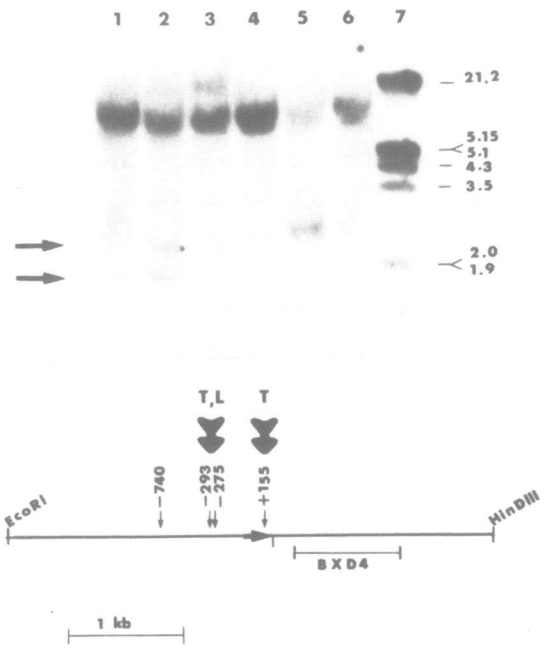


Figure 5 Accessibility to Msp I of the TPG-3 protamine gene chromatin
Nuclei from early (lane 2) and late (lanes 3,4) stage testis, liver (lane 5) and erythrocyte (lane 6) were digested with 40 units/ml of Msp I for 30 min at 37°C. DNA was isolated, digested with Hind III, electrophoresed on a 1% agarose gel and blot-hybridized to the $^{32}$P-labeled BXD4 probe. Lane 1 is a control where nuclei from early stage testis were incubated for 30 min at 37°C without addition of the enzyme. Lane 7 shows Eco RI-Hind III fragments of phage λ whose sizes are indicated in kb. The two arrows at the left of the figure point to the fragments corresponding to the sites accessible to Msp I in nuclei. These sites are also shown on the map by two vertical arrows. T and L represent testis and liver, respectively. The horizontal arrow on the map represents the transcribed region of the TPG-3 gene as well as the direction of transcription.

sites at positions -740, -293, -275 and +155 in the DNA of the TPG-3 gene and its flanking regions can be digested by Msp I. The results shown in figure 5 demonstrate that the TPG-3 gene chromatin in early stage testis has two (or three) sites which are accessible to nuclease attack. One site (-275 and/or -293) which gives rise to the 2.55 kb Hind III-Msp I fragment is in the 5'-flanking region of the gene and is also present in liver. The other site (+155) which produces the 2.1 kb Hind III-Msp I fragment is present only in early stage trout testis where the protamine genes are expressed. None of the sites appear to be present in late stage testis or erythrocyte chromatin.

DISCUSSION

The analysis of the methylation state of the TPG-3 protamine gene by the methylation sensitive restriction enzymes Hpa II and Hha I revealed that only one CpG site at position -740 is methylated. The other CpG sites at positions -293 and/or -275, +58, +155 and +469 are not. This pattern of methylation is the same in all the tissues studied: early and late stage testis, nucleoprotamine, liver and erythrocyte. Thus, we do not observe a correlation between transcriptional activity of the gene and the absence of DNA methylation.

The undermethylated state of the protamine gene TPG-3 may be due to the primary sequence of the protein. Twenty-one out of the 32 amino acids which constitute the protamine protein are arginine residues. Arginine is coded for by 6 different triplets out of which 4 start with CpG. So the high level of arginine in protamines explains why the coding region of the genes contains such a clustering of CpGs and presents a HTF-like character (2, Fig. 1). Since 5-methylcytosine tends to deaminate to thymidine (see 2), HTF-like regions are not expected to be methylated in germ cells at least, or else these regions would be lost over the generations, as a consequence of the mutation of CpGs to TpGs. Bird's hypothesis (2) is that HTF islands identify sequences like regulation sequences for housekeeping genes which must be available for transcription in all cell types. There is evidence that a HTF island must be demethylated for the associated gene to be transcribed. Bird's model is that the availability of HTF islands is achieved by the cooperative binding of proteins which have a high affinity for sequences rich in non-methylated CpGs. These bound factors would constitute a steric obstruction to the methylase and therefore protect DNA against methylation. It is possible that the protamine TPG-3 gene is kept undermethylated in all cell types by the action of such tissue non-specific factors which would recognize the HTF-like character of the coding region.

When nuclei isolated from the various tissues were digested with Msp I, two sites in the TPG-3 protamine gene chromatin at positions -293 and/or -275 and +155 were found to be accessible to the enzyme in early stage testis where the protamine genes are expressed. These sites are not present in late stage testis where more than 97% of the DNA is packaged into nucleoprotamine. However, one of the accessible sites at the 5' end of the gene (-293 and/or -275) is also present in liver. Thus, the TPG-3 protamine gene has one Msp I site localized in the 3' end of the transcribed region whose presence correlates with the expression of the gene. The reason why there is a Msp I

site so accessible in liver chromatin is not known since our results indicate that the protamine genes are not expressed in liver. Such an intriguing situation has also been reported for the rat preproinsulin II gene. This gene has a DNAase I hypersensitive site at its 5' end only in a pancreatic β-cell tumor that secretes insulin. Liver cells do not have this hypersensitive site at the 5' end of the gene but they have another hypersensitive site within the coding region which is close to the 3' end of the gene (26).

ACKNOWLEDGEMENTS

We wish to thank Dr. P.H.W. Butterworth for sending us the pTPG-3A24 clone. We also thank Dianne Konkin for technical assistance. This project was supported by grants from the Manitoba Health Research Council, the National Cancer Institute of Canada and by the award of a Medical Research Council Scholarship to J.R. Davie.

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