Tissue- and developmental-stage-specific methylation in the two kidney promoters of the rat γ -glutamyl transpeptidase gene

Ja-Hyun BAIK, Sylvie SIEGRIST,* Galicia GIULI, Olivier LAHUNA, Frédérique BULLE and Georges GUELLAËN†

Unité 99 INSERM, Hôpital Henri Monodor, 94010 Créteil, France

We have investigated, using DNA methylation patterning, the site-specific methylation of promoters I and II of the rat γ -glutamyl transpeptidase gene. This analysis was done in fetal, newborn and adult rat kidney, in which promoters I and II are progressively active during development, as well as in rat liver, which never expresses mRNAs from these two promoters. During kidney development, a progressive demethylation occurs in the promoter I and II region, specially at the level of the most proximal *MspI* site of promoter II. A progressive reorganization of the methylated sites within the 5' end of the gene also occurs during liver development.

INTRODUCTION

γ -Glutamyl transpeptidase (GGT) is a plasma-membrane-bound heterodimeric glycoprotein which initiates the hydrolysis of glutathione [1–3]. In adult tissues, the highest GGT activity is detected in the kidney, and a 300-fold lower level is found in the liver. During ontogeny, there is a 10-fold increase of the GGT activity in the kidney, whereas in the liver, the enzymatic activity drops at least 30-fold [4]. By using a rat kidney GGT cDNA [5] we demonstrated that these changes occur mainly at the transcriptional level [6]. Until now, three rat GGT mRNAs (I, II and III [7,8]) have been characterized. They have identical coding sequences, but they differ in their extreme 5' untranslated regions [7]. These mRNAs are transcribed from three different promoters [9] located on a single gene [10]. The mRNAs I and II, specific for the kidney, are co-ordinately regulated during ontogeny [7,11]. Changes in DNA methylation have been proposed as one of the mechanisms involved in the control of gene transcription [12]. Although some discrepant results have been reported [13], an inverse correlation between extent of methylation and gene expression is usually shown [14,15]. In a previous report [16] we demonstrated a clear correlation between the hypomethylation status of the GGT gene and its expression during liver development. In the present study we investigated whether DNA methylation would also participate in the regulation of the GGT gene expression during kidney development.

MATERIALS AND METHODS

Isolation of DNA and digestion with restriction enzymes

High-*M_r* DNA was extracted from rat kidney and liver as described previously [9]. Genomic DNA (20 μ g) was digested overnight at 37 °C with *HpaII*, *MspI* or *HhaI* restriction enzymes (4 units/ μ g of DNA) and Southern blots were performed as previously reported [8].

Probes

Two probes, specific for the rat kidney mRNA I and II (probe

I, Fig. 1 below, and probe II, Fig. 2 below, respectively) were labelled to a specific radioactivity of 5–10⁹ d.p.m./ μ g using [³²P]dCTP (3000 Ci/mmol) by PCR as previously reported [11].

Blot hybridization

The blots were prehybridized and hybridized as already described [16] and washed twice for 1 h in 0.5 $\times$ SSC (1 $\times$ SSC is 0.15 M-NaCl/0.015 M-sodium citrate)/0.1 % SDS at 68 °C. The blot was subjected to autoradiography at –80 °C with an intensifying screen on Hyperfilm (Amersham).

RESULTS

The methylation pattern of the rat GGT gene was determined by restriction-endonuclease analysis using cytosine-methylation-sensitive enzymes. The two isochizomers *HpaII* and *MspI* cleave at CCGG sequences, but only *MspI* is efficient when the internal cytosine of this sequence is methylated. The enzyme *HhaI* recognizes the sequence GCGC as long as the internal cytosine is not methylated.

DNA methylation pattern of the kidney-specific GGT promoter I

The methylation of the promoter I region was assessed in kidney and liver at different stages of development. To this end, genomic DNA was prepared from fetal, newborn and adult kidney and liver. These DNA preparations were hydrolysed with the restriction enzymes *MspI*, *HpaII* and *HhaI*. The size of the restriction fragments corresponding to the leader exon was determined by Southern-blot hybridization with probe I, specific for mRNA I (Fig. 1*b*).

In kidney, a single 0.7 kb band was observed after *MspI* digestion, whatever the stage of development (Fig. 1*a*, kidney, *MspI*). This band has the expected size deduced from the location of the two *MspI* sites, M1 and M2 (Fig. 1*b*). With *HpaII* restriction enzyme, at least 11 bands were observed in fetal kidney, ranging from 0.7 to 9 kb, with most of the signal in the high-molecular-mass bands (6.4 to 9 kb) (Fig. 1*a*, kidney, *HpaII*). At birth, the same number of bands was still detectable. In adult kidney there was a decrease in the intensity of the high-molecular-mass bands and an increase in the intensity of the 0.7 kb band.

Abbreviations used: GGT, γ -glutamyl transpeptidase; 1 $\times$ SSC, 0.15 M-NaCl/0.015 M-sodium citrate.

* To whom correspondence should be sent.

† To whom requests for reprints should be sent.

Fig. 1. DNA methylation pattern of kidney and liver DNA in the promoter I region of the GGT gene

(a) Southern-blot hybridization analysis of kidney and liver DNA at different stages of development: 18 days gestation fetuses (F); 2-day-old neonatal animals (NB); and adult (A). Samples of DNA were digested with *MspI*, *HpaII* or *HhaI*, blotted and hybridized with probe I as designed in (b). (b) Restriction map of the 5' region of the GGT gene, including promoter I and promoter II (redrawn from [6]). Map distances are given in bp. M and H represent the restriction sites of the enzymes *MspI*/*HpaII* and *HhaI* respectively. Exons are shown as boxes: □, common 5' untranslated sequence shared by GGT mRNA I and II; ▤, 5' untranslated sequence specific for GGT mRNA I; ▨, 5' untranslated sequence specific for GGT mRNA II. The thick bar represents the position of probe I.

This result reveals that, after birth, there is a demethylation of the M1 and/or M2 sites (Fig. 1b). With *HhaI* restriction enzyme, an intense signal was observed in fetal kidney at 14 kb and one discrete band at 5.8 kb (Fig. 1a, kidney, *HhaI*). At birth there was no change. In the adult there was a decrease in the intensity of the high-molecular-mass bands, with the appearance of the 2.75 and 1.9 kb bands corresponding to H3–H1 and H2–H1 fragments respectively (Fig. 1b). This pattern reveals that, after birth, there is a demethylation of H1, H2 and/or H3 sites.

In liver, the expected 0.7 kb band was determined after *MspI* cleavage at all the developmental stages (Fig. 1a, liver, *MspI*). With *HpaII* restriction enzyme, seven bands were discriminated in fetal liver from 3.05 to 14 kb, including four intense bands within the range 7.4–14 kb (Fig. 1a, liver, *HpaII*). At birth, there were no striking changes in the methylation pattern. In adult liver, only one band (7.4 kb) remained detectable. This result shows that, whatever the stages of development, at least M1 and/or M2 are fully methylated, since no 0.7 kb band was observed with this enzyme. After birth, there is a methylation for all but two *MspI* sites within the 7.4 kb range that framed the first exon specific for mRNA I (Fig. 1b). With *HhaI* restriction enzyme, at least seven bands were detected in fetal liver, within

the range of 1.9–14 kb (Fig. 1a, liver, *HhaI*). The 1.9 kb band corresponds to the one detected in the adult kidney resulting from cleavages at H1 and H2. At birth, the only change is a complete disappearance of the 1.9 kb band, indicating a methylation of H1 and/or H2 (Fig. 1b). In adult liver, four bands are visible between 6.4 and 14 kb, with a more intense signal of the 6.4 kb band (Fig. 1a, liver, *HhaI*).

DNA methylation pattern of the kidney-specific GGT promoter II

A similar analysis was done, using probe II corresponding to the leader exon specific for mRNA II, to analyse the extent of methylation of the promoter II region (Fig. 2b).

In kidney, *MspI* generated a fragment of 1.6 kb whatever the stages of development (Fig. 2a, kidney, *MspI*). It corresponds to the one framed by M4 and M5 sites (Fig. 2b). With the isoschizomer *HpaII*, at least 11 bands were detected in fetal kidney within the range of 1.75–14 kb, with only three bands of 1.75, 2 and 2.2 kb intensively labelled. At birth, three bands were present, of respective size 1.6, 1.85 and 2.05 kb, with some faint bands in the high-molecular-mass range. The decrease in size (150 bp) of these three bands suggests that, in fetal kidney, M6

Fig. 2. DNA methylation pattern of kidney and liver DNA in the promoter II region of the GGT gene

(a) Same legend as Fig. 1, except that the Southern blots were hybridized with probe II as designed in (b). (b) Same legend as Fig. 1, except that the thick bar represents the position of probe II.

is demethylated and that, at birth, there is a complete and rapid demethylation of M5 sites. In adult kidney, only the 1.6 kb band was clearly detected (Fig. 2a, kidney, *HpaII*), indicating that M4 and M5 sites are almost completely demethylated (Fig. 2b). The *HhaI* restriction enzyme revealed four bands in fetal kidney of size 0.85, 1.1, 4.3, and 14 kb (Fig. 2a, kidney, *HhaI*). The two smaller bands correspond to fragments H3–H2 and H4–H2 respectively (Fig. 2b). At birth, there was no striking changes in the methylation pattern. In adult kidney the 0.85 kb band increased, the 1.1 kb band decreased, and a 2.75 kb band appeared. This last band corresponds to fragment H3–H1. These modifications correspond to a demethylation at H3 site.

In liver, the expected 1.6 kb band was observed after cleavage with *MspI* (Fig. 2a, liver, *MspI*). When *HpaII* was used, 14 bands were analysed in fetal liver, within the range of 1.75–22 kb (Fig. 2a, liver, *HpaII*). Two groups of bands are intensively labelled: one group above 9 kb and three bands at 1.75, 2 and 2.2 kb similar to those observed in fetal kidney. This pattern progressively evolved, in adult liver, to a series of three faint bands

and one intensively labelled at 7.4 kb. Thus, in liver, M5 is methylated at all stages (Fig. 2b). As for probe I, there is in the adult a 7.4 kb band that contains the promoter II region, indicating a progressive methylation of the M6, M4, M3 and/or M2 sites. With *HhaI* at least seven bands were visible in fetal liver, within the range 0.85–22 kb. Three species were identical with those detected in kidney (0.85, 1.1 and 4.3 kb). No significant modifications were observed at birth. In adult liver, the 0.85 and 3.8 kb bands were no longer detected and the 1.1 kb band decreased in intensity (Fig. 2a, liver, *HhaI*). These changes are compatible with a methylation at H4, H3 and/or H2 sites that surround promoter II (Fig. 2b).

DISCUSSION

In the present paper we investigated whether DNA methylation, in the 5' region of the rat GGT gene, correlates with gene

transcription from its promoters I and II. This study was done in kidney, in which both promoters are progressively activated during ontogeny [7], and in liver, in which these two promoters are always silent [11].

In kidney, the analysis of the GGT gene methylation patterns, using probes close to promoter I and promoter II, reveals a progressive demethylation during development. In fetal and newborn kidney, there is a complex DNA methylation pattern that reflects a heterogeneous CpG methylation status. The only clear event is a rapid and almost complete demethylation of M5 sites. In adult kidney, the methylation pattern reflects a demethylation of the CpG sites located in the 5' region of the GGT gene. This demethylation is not complete, since some high-molecular-mass bands are still present, in particular for the promoter I region. The heterogeneous patterns observed at all the developmental stages might reflect the cellular distribution of GGT mRNAs I and II. As a matter of fact, both mRNAs are barely detectable in the fetus and heavily expressed in the adult, and we have shown by hybridization studies *in situ* that, at both stages, they are expressed only in the distal part of the proximal convoluted tubules [7]. Altogether, these results apparently argue for a correlation between demethylation in the 5' end of the GGT gene and expression of GGT mRNA I and II during kidney development. Such an observation has already been made for other genes [12]. As an example, Peek *et al.* [14] clearly show that DNA demethylation of the promoter region is the determining step in the developmental-stage-specific expression of the rat γ -crystallin genes.

In liver, the analysis of the GGT gene methylation exhibits also heterogeneous patterns, as well as a progressive reorganization of the methylated sites within the 5' end of the gene during development. Indeed, both promoter I and II regions are progressively methylated within a region of roughly 7.4 kb. Since mRNAs I and II have never been detected at either stage, this methylation is not relevant to a specific regulation of the transcriptional activity of the GGT gene at these two promoters. It can be postulated either that the methylation at all stages is sufficient to block the expression of mRNAs I and II or that specific transcription factors are involved in the regulation of the expression of both mRNAs. This second hypothesis is strengthened by three observations. First, the methylation patterns within promoter I were found similar in fetal liver and kidney, although there is no expression of mRNA I in the hepatic tissues. Secondly, even after a complete demethylation of the DNA after 5-azacytidine treatment of cells of hepatic origin, Fao, we did not observe the synthesis of mRNAs I and II [17]. Thirdly, transfection experiments with demethylated promoter I exhibit cell specificity [9]. Such a lack of correlation between gene expression during development and DNA methylation has already been reported for the expression of α -foetoprotein, as well as for albumin [18,19].

In a recent study, Coloma & Garcia-Jimeno [20], using genomic probes, observed a methylation of the 5' end region of the rat GGT gene in the liver and a demethylation in the kidney. They concluded that DNA methylation would correlate with a tissue-specific expression of the GGT activity in liver and kidney. Their

results, in agreement with ours, do not support their conclusions. In fact, the hepatic GGT activity is not related to promoter I and II, but it results from the transcription initiated on a third promoter located at least 3.4 kb upstream of promoter II [21]. Thus the increase in the DNA methylation, around promoter I and II in the liver, cannot be relevant to the diminution of GGT activity in this tissue.

In conclusion, our results suggest that DNA demethylation could be responsible for GGT promoter I and II activation during kidney development, but both promoters must be under the control of specific factors, which are necessary to explain their relative activity in liver and kidney.

We thank L. Rosario for expert secretarial assistance and Dr. Y. Laperche, Dr. R. Barouki and Dr. A. Pawlak for the critical reading of the manuscript before its submission. This work was supported by the Mutuelle Générale de l'Education Nationale.

REFERENCES

- Griffith, O. W. & Meister, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5606–5610
- Curthoys, N. P. (1983) *Miner. Electrolyte Metab.* **9**, 236–245
- Meister, A. & Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52**, 711–760
- Albert, Z., Rzuclido, Z. & Starzyk, H. (1970) *Acta Histochem.* **37**, 34–39
- Laperche, Y., Bulle, F., Aissani, T., Chobert, M. N., Aggerbeck, M., Hanoune, J. & Guellaën, G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 937–941
- Jacquemin, E., Bulle, F., Bernaudin, J. F., Wellman, M., Hugon, R. N., Guellaën, G. & Hadchouel, M. (1990) *J. Pediatr. Gastroenterol. Nutr.* **11**, 89–95
- Chobert, M. N., Lahuna, O., Lebargy, F., Kurauchi, O., Darbouy, M., Bernaudin, J. F., Guellaën, G., Barouki, R. & Laperche, Y. (1990) *J. Biol. Chem.* **265**, 2352–2357
- Power, C. A., Griffiths, S. A., Simpson, J. L., Laperche, Y., Guellaën, G. & Manson, M. M. (1987) *Carcinogenesis* **8**, 737–740
- Kurauchi, O., Lahuna, O., Darbouy, M., Aggerbeck, M., Chobert, M. N. & Laperche, Y. (1991) *Biochemistry* **30**, 1618–1623
- Pawlak, A., Lahuna, O., Bulle, F., Suzuki, A., Ferry, N., Siegrist, S., Chikhi, N., Chobert, M. N., Guellaën, G. & Laperche, Y. (1988) *J. Biol. Chem.* **263**, 9913–9916
- Darbouy, M., Chobert, M. N., Lahuna, O., Okamoto, T., Bonvalet, J. P., Farman, N. & Laperche, Y. (1991) *Am. J. Physiol.* **261**, 1130–1137
- Razin, A. & Cedar, H. (1991) *Microbiol. Rev.* **55**, 451–458
- Gellersen, B. & Kempf, R. (1991) *Mol. Endocrinol.* **5**, 1874–1886
- Peek, R., Niessen, R., Schoenmakers, J. & Lubben, N. (1991) *Nucleic Acids Res.* **19**, 1–77
- Tratner, I., Nahon, J. L., Sala-Trepat, J. M. & Venetianer, A. (1987) *Mol. Cell. Biol.* **7**, 1856–1864
- Baik, J. H., Griffiths, S., Giuilli, G., Manson, M., Siegrist, S. & Guellaën, G. (1991) *Carcinogenesis* **12**, 1035–1039
- Baik, J. H., Chikhi, N., Bulle, F., Guili, G., Guellaën, G. & Siegrist, S. (1992) *J. Cell. Physiol.*, in the press
- Kunnath, L. & Locker, J. (1983) *Embo J.* **2**, 317–324
- Vedel, M., Gomez-Garcia, M., Sala, M. & Sala-Trepat, J. (1983) *Nucleic Acids Res.* **13**, 4335–4354
- Coloma, J. & Garcia-Jimeno, A. (1991) *Biochem. Biophys. Res. Commun.* **177**, 229–234
- Lahuna, O., Brouillet, A., Chobert, M. N., Darbouy, M., Okamoto, T. & Laperche, Y. (1992) *Biochemistry*, in the press