The Glucocorticoid Response Element II Is Functionally Homologous in Rat and Human Insulin-like Growth Factor-binding Protein-1 Promoters*

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In vivo, insulin-like growth factor-binding protein-1 (IGFBP-1) modulates the IGFs’ bioavailability and may contribute to their delivery to peripheral tissues. In rat and human hepatocytes, glucocorticoids stimulate IGFBP-1 gene transcription through homologous glucocorticoid response units (GRU). Transfection experiments have shown that one of these, GRU2 (nucleotide nt –121 to –85 and nt –111 to –74 in human and rat promoters, respectively), was on its own able to mediate the glucocorticoid response in rat but not in human species (Suwanichkul, A., Allander, S., Morris, S. L. & Powell, D. R. (1994) J. Biol. Chem. 269, 30835–30841, Goswami, R., Laeson, R., Yang, E., Sam, R. & Unterman, T. (1994) Endocrinology 134, 736–743, and Suh, D. S., Ooi, G. T. & Rechler, M. M. (1994) Mol. Endocrinol. 8, 794–805). A close comparison of GRU2 sequences has pointed out a C to A transition in the underlying GREII, which creates a GATC tetranucleotide in rat species. This tetranucleotide is submitted to adenosyl methylation (dam methylation) in most Escherichia coli bacterial strains, but not in eucaryotic cells. We showed (i) that on its own, the unmethylated rat GRU2 (propagated in dam E. coli strains) was inactive, as is the case for its human counterpart (nonsignificant glucocorticoid inductions, 1.48 ± 0.23 and 1.7 ± 0.35-fold in Chinese hamster ovary cells, respectively) and (ii) that its adenosyl methylation in standard dam+E. coli strains yielded a functional GRU (6.5 ± 1.1 and 13.1 ± 3.9-fold glucocorticoid inductions in Chinese hamster ovary and HepG2 cells, respectively). Transient transfection in HepG2 hepatoma cells clearly showed that the interaction of liver-enriched trans-acting factor(s) with the 5'-overlapping insulin response element does not enable the unmethylated rat GRU2 or the human GRU2 to become responsive to glucocorticoids (nonsignificant 2.21 ± 0.48 and 1.20 ± 0.06-fold induction, respectively). Furthermore, we have correlated these functional data with in vitro DNA-protein interaction studies: the dam methylated rat GREII displayed a 2.8-fold higher affinity for the glucocorticoid receptor than its unmethylated counterpart.

Insulin-like growth factor-binding protein-1 (IGFBP-1) belongs to a family of six related proteins that bind the IGFS (IGF-I and -II) with high affinity and thus modulate their bioavailability both in the serum and in extracellular fluids, inhibit or potentiate their actions and possibly confer tissue specificity (1, 2) (also reviewed in Ref. 3).

In vivo, serum IGFBP-1 is thought to play an important role in the short term modulation of IGFS’ bioavailability. Accordingly, its abundance is rapidly regulated and may vary by more than 10-fold in normal subjects (4). In addition, perfusion studies point out a role for circulating IGFBP-1 to deliver IGFS to peripheral tissues (5). During the perinatal period and in adults, serum IGFBP-1 is primarily synthesized in hepatocytes (6–9). In vivo and in vitro, the hepatic production of IGFBP-1 is regulated by multiple factors (glucocorticoids, cAMP agonists, insulin, and growth hormone) (10–12) and appears to be correlated to the abundance of its mRNA; IGFBP-1 transcripts increase after glucocorticoid treatment and in diabetic animals and decrease after insulin treatment (13–15).

Regulation of IGFBP-1 and of its mRNA by glucocorticoids, cAMP, and insulin have also been observed in rat (H4II) and human (HepG2) hepatoma cell lines (16–20). In these cell lines, as well as in hepatocytes, the modulation of IGFBP-1 gene expression has been shown to be regulated at the transcriptional level (13, 14, 21), and accordingly, hormone response elements (HREs) have been identified in both rat and human IGFBP-1 5'-flanking sequences (19, 20, 22–25).

One glucocorticoid response unit (GRU2 hereafter) is highly homologous in the human and rat IGFBP-1 promoters. It consists of a composite cts-element, in which the glucocorticoid response element (GRE) and the insulin response element (IRE) are closely intricated (22–25) and interact with the glucocorticoid receptor (GR) and with liver-enriched trans-acting factors, respectively (23, 26–28).

Transfection experiments have shown that the rat GRU2, but not its human counterpart, is on its own able to mediate the glucocorticoid response (23–25). A second GRE, located in a more remote 5'-position, is absolutely required for glucocorticoids to stimulate human IGFBP-1 promoter activity (22).

The different abilities of rat and human GRU2 to enhance IGFBP-1 promoter activity in transfected hepatoma cells was unexpected since their nucleotide sequence is highly conserved between both species. However a C to A transition (third base

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§ The abbreviations used are: IGFBP-1, insulin-like growth factor-binding protein-1; GRU, glucocorticoid response unit; IGF, insulin-like growth factor; HRE, hormone response element; GRE, glucocorticoid response element; IRE, insulin response element; GR, glucocorticoid receptor; nt, nucleotide(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; ds, double-stranded; CHO, Chinese hamster ovary.
pair in the 3'-half of the GREII imperfect palindromes) creates a GATC tetranucleotide in the rat promoter (GCTC in the human GREII), a tetranucleotide that undergoes deoxyadenosine methylation (N6 position of the adenine residue; *dam* methylation hereafter) in most *E. coli* bacterial strains. In addition, *dam* methylation of GRE imperfect palindromes that encompass the GATC tetranucleotide confers responsiveness to glucocorticoids in gene transfer experiments (29).

Because *dam* methylation does not occur in eucaryotic cells, we wondered whether the ability of the transfected rat GRU2 to enhance transcription in the presence of glucocorticoids could be due to its methylation in *dam*+ *E. coli* strains. We thus compared the ability of the rat GRU2 propagated in *dam*+ and *dam*− *E. coli* strains to mediate the glucocorticoid response and showed that in the absence of bacterial methylation, the rat GRU2 was unable to sustain a significant glucocorticoid increase in promoter activity, as is the case for its human counterpart. Furthermore, we correlated this differential activity on transcription with the ability of the GR to interact with the methylated or unmethylated rat GREII in *vivo*. EXPONENTIAL PROCEDURES

Oligonucleotides—The double-stranded oligonucleotides consisted of rat (nt -111 to -74) and human (nt -121 to -85) sequences from the IGFBP-1 promoter (i.e. encompassing the GREII and IRE cis-elements; pIGFBP-1-GRU2 hereafter). They were flanked by HindIII (5′-) and SalI (3′-) cohesive ends. Sequences were as follows: pHGFBP-1-GRU2, 5′-AGCT/TAGCAGAACAATTTGGAACTCCTGCTAGTCG-3′; unmethylated rIGFBP-1-GRU2, 5′-(AGCTT)AGGAAACAAACT-TATTGTTAAGAGGATTTCGACGTCGGA-3′; dam methylated rIGFBP-1-GRU2, upper strand, 5′-(AGCTT)AGGAAACAAACT-TATTGTTAAGAGGATTTCGACGTCGGA-3′; lower strand, 5′-TCGACGTCGACGAAATAATTTCTTGCTTA-3′. In some instances, dam methylation of the unmethylated rIGFBP-1-GRU2 was performed in *vivo* using recombinant *dam* methylase (New England Biolabs) and S′-adenosyl-methionine according to the manufacturer's instructions.

Plasmids—Rat (nt -111 to -74) and human (nt -121 to -85) IGFBP-1-GRU2 were inserted as cohesive, double-stranded oligonucleotides (30). The unique HindIII/SalI restriction sites of pBLCAT2, an eucaryotic expression vector in which CAT gene expression is directed by promoter sequences (nt -105 to +51) of the herpes simplex virus thymidine kinase gene (31). This yielded prIGFBP-1-GRU2 and pHGFBP-1-GRU2, respectively, the sequences of which were checked by dyeoxy sequencing.

pRShGRα, a hGR expression vector, and pRSVLuc, an ubiquitous and constitutive luciferase expression plasmid (Rous sarcoma virus long terminal repeat upstream of the luciferase cDNA) have been previously described by Hollenberg et al. (31) and de Wet et al. (32), respectively.

Plasmid Preparation—Large scale preparations of the plasmids were obtained from *E. coli* HB101 (dam+) transformed bacteria. A dam mutant strain (JM110, Stratagene) was also used to prepare prIGFBP-1-GRU2 unmethylated at dam+ methylation sites.

Methylation Analysis—NdeI-Xhol DNA fragments of 444 and 442 bp were excised from prIGFBP-1-GRU2 and pHGFBP-1-GRU2, respectively and purified by agarose gel electrophoresis. The fragments were restricted with MboI (10 units/µg DNA) (Roche Molecular Biochemicals) at 37 °C for 2 h. This enzyme recognizes GATC palindromes and cleaves exclusively the unmethylated sequence (33). The restriction fragments were then end-labeled with 32P-ATP in the presence of T4-polymerase kinase. The digestion products were resolved by 12% polyacrylamide gel electrophoresis and detected by autoradiography.

Cell Culture and Transfection—Human hepatoma (HepG2) and Chinese hamster ovary (CHO-IR) cells were grown in Dulbecco's modified Eagle's medium. Rat H4II hepatoma cells were grown in Coon's F12. The culture medium was supplemented with 10% (HepG2, CHO-IR cells) or 5% (H4II cells) fetal calf serum, 2 mM t-glutamine, and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin), and the cells were maintained in a humidified atmosphere of 5% CO2 in air.

For transfection experiments, 5 × 105 CHO-IR or 105 HepG2 cells were plated into 60-mm Petri dishes the day before, and the medium was renewed 3 h before transfection. Co-transfection (10 µg of pHGFBP-1-GRU2 or of pRSV-Luc and 0.125–1 µg of pRSV-HGα) was performed using calcium phosphate-DNA co-precipitation (34). 24 h post-transfection, the culture medium was replaced by serum-free Dulbecco's modified Eagle's medium, and the cells were incubated for an additional 18 h in the absence or presence of 10−6 M dexamethasone.

CAT and luciferase activities were assayed according to standard procedures (32, 35). CAT activities were always normalized relative to those of luciferase (an internal monitor of transfection efficiency), and the following CAT/luciferase ratios were used to compute the glucocorticoid induction.

**glucocorticoid induction**

| Normalized CAT activity in cells | co-transfected in the presence of Dex | Normalized CAT activity in cells | co-transfected in the absence of Dex |
|--------------------------------|-------------------------------------|--------------------------------|-------------------------------------|

**Preparation of Nuclear Extracts Containing hGRα**—Growth and infection of SF9 cells were performed as described previously (36). 1 µM of triamcinolone acetonide was added to the culture medium 1 h before harvesting the cells, and crude nuclear extracts were prepared under high ionic strength conditions (36–38). The recombinant glucocorticoid receptor (BachGRα) recovered in the crude nuclear extracts was analyzed for its molecular weight, and its ability to bind glucocorticoids was assessed by the hydroxyapatite method (39). The binding capacities of the crude nuclear extracts used hereafter were 3,800 pmol/ml (specific activity, 576 pmol/mg protein) and 1,710 pmol/ml (specific activity, 123 pmol/mg protein).

**Electrophoretic Mobility Shift Assay (EMSA)**—The rat (unmethylated or *dam* methylated) and human IGFBP-1-GRU2 (ds-oligonucleotides or inserts isolated from prIGFBP-1-GRU2 and from pHGFBP-1-GRU2) were end-labeled by filling in using the Klenow fragment of *E. coli* DNA polymerase 1, and either [α-32P]dATP or [α-32P]dATP and [α-32P]dCTP or [α-32P]dCTP. The radiolabeled IGFBP-1-GRU2 were purified by 12% polyacrylamide gel electrophoresis.

1 µl of crude nuclear extracts (1.71 or 3.80 pmol of BachGRα, as estimated by steroid binding) was mixed with 2 µg of sonicated salmon or herring sperm DNA (final volume, 12 µl) and incubated at 0 °C for 15 min. 32P- or 32P-labeled rat or human IGFBP-1-GRU2 (final concentrations, 0.016 units/ml; 69% of the signal in lane 1) was then added alone or with a 6–120-fold molar excess of unlabeled competitor oligonucleotide (TAT-GREII, unmethylated or *dam* methylated rat IGFBP-1-GRU2 or human IGFBP-1-GRU2). The final reaction mixture (18 µl: 20 mM Tris-HCl, pH = 7.4, 2.2 mM Hepes, 1 mM EDTA, 40–60 mM NaCl, 0.125 mM MgCl2, 4 mM diethiothreitol, 10% glycerol, 0.05% bovine serum albumin) was incubated for an additional 60 min at 25 °C, then analyzed on a 5% acrylamide/agarose gel in recirculating 0.25× TB at 0–4 °C. Gels were dried and DNA-protein interactions were visualized by autoradiography (1 h).

The radioactivity of the bands corresponding to both DNA-bound GR (homodimer) and the free probe was quantified using an Instant Imager (Packard Instrument Co.) or a Berthold linear analyzer.

When supershift experiments were to be performed, the 60-min incubation at 25 °C was extended for an additional 20 min, after the addition of a polyclonal anti-GR antibody in the reaction mixture (1 or 2 µl) (40).

**Southern Blot Analyses**—Genomic DNA was prepared from confluent monolayers of H4II rat hepatoma cells, and 20-µg aliquots were digested either by PstI (5 units/µg DNA) or by MboI (5 units/µg DNA). The restriction fragments were separated by agarose gel electrophoresis (2% agarose). The 340-bp HindIII/SalI restriction fragment isolated from pHGFBP-1-GRU2 (i.e. the human IGFBP-1 promoter) was run in parallel as a positive control for hybridization. After electrophoresis, the DNA was transferred to a nitrocellulose membrane and hybridized (18 h, 68 °C) with a random-labeled probe that encompassed 3-flanking sequences from the human IGFBP-1 promoter (nt -340 to +1; 69% sequence identity with the rat IGFBP-1 promoter) (specific activity, 2.7 × 106 cpm/ng; 3 × 106 cpm/ml hybridization buffer). After two washes (15 min at room temperature then 15 min at 50 °C) in 2× SSC, 0.1% SDS, the membranes were analyzed using an Instant Imager (Packard Instrument Co.).

**RESULTS**

The rat (nt -111 to -74) and human (nt -121 to -85) GRU2 are composite cis-elements, in which both the GRE and the IRE (Fig. 1A) are crucial for promoter activity to be enhanced by

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**GREII in Rat and Human IGFBP-1 Promoter**
glucocorticoids (22–25). On the basis of transfection experiments, Goswami et al. (23) and Suh et al. (25) have reported that the rat GRU2 is functional on its own (23–25). This is in contrast with the data reported by Suwanichkul et al. (22) for the human GRU2; it is inactive by itself (22). This discrepancy would imply that the human and rat promoters are not functionally homologous with regard to the glucocorticoid response, despite high sequence similarity between the two GRUs. The IRE (100% of sequence similarity between the rat and human IGFBP-1 promoters) cannot account for this discrepancy (Fig. 1A). On the contrary the GREIIs differ by 4 bp; two are localized in the 3-bp spacer that separates the half-palindromes, the two others are at positions +1 and +3 (A to G and C to A transitions, respectively) (Fig. 1B). Both positions are contacted by the glucocorticoid receptor (GR), one of which (+3) is essential for high affinity GR-GRE interaction (reviewed in Ref. 41). Interestingly, the C to A transition (+3) creates a GATC tetranucleotide in the rat GREII (boxed in Fig. 1B). This sequence is methylated on the adenosine residue (N6 position) in most E. coli strains and such dam methylation has been shown to generate artificial GREs (29).

The Rat Proximal GREII Is Methylated When prIGFBP-1-GRU2 Is Propagated in dam E. coli Strains—prIGFBP-1-GRU2 was propagated in either dam+ (JM110) or dam– (HB101) E. coli strains. In each case, the Ndel-XhoI fragment (444 bp) was excised, purified, then restricted with MboI, an enzyme that cleaves exclusively the unmethylated GATC sequence, and the restriction fragments were end-labeled (Fig. 2). When excised from prIGFBP-1-GRU2 propagated in JM110 the Ndel-XhoI fragment was cleaved by MboI and yielded 172-, 157-, 92-, and 23-bp bands (Fig. 2). These bands were generated by enzymatic cleavage at the three available GATC sequences (one located in the 3′-half-palindrome of rat GREII sequences, the other two being localized in pBLCAT2 vector DNA). On the contrary, when prIGFBP-1-GRU2 was propagated in HB101,
the NdeI-XhoI fragment remained uncleaved after MboI digestion. These results demonstrated that the adenine residue at position +3 of the rat GREII was methylated when pHGFBP-1-GRU2 was propagated in a dam⁻ E. coli strain.

**dam Methylation Is Responsible for the Ability of the Rat GREII to Mediate Glucocorticoid Responsiveness in Mammalian Cells**—When pHGFBP-1-GRU2 was prepared from the widely used HB101 E. coli strain (dam⁺), then co-transfected in CHO cells with an expression vector encoding the human GR, the rat GRU2 increased CAT reporter gene expression by 6.50 ± 1.05-fold in the presence of glucocorticoids in the culture medium. GRU2 was thus able to enhance transcription from the heterologous thymidine kinase promoter (Fig. 3, A and B, GAmTC). On the contrary, when pHGFBP-1-GRU2 was prepared from a dam⁻ bacterial strain (JM110), the hormonal inducibility of CAT reporter gene expression was completely abolished; the slight difference in CAT activities between untreated and dexamethasone-treated cells (1.48 ± 0.23-fold) was not statistically significant (Fig. 3, A and B, GATC). These results allowed the conclusions that only the dam methylated pHGFBP-1-GRU2 mediated significant glucocorticoid induction and that such a hormonal inducibility was dependent on adenosyl methylation of the GATC motif of the rat GREII. As a matter of fact, glucocorticoid induction was not obtained with pHGFBP-1-GRU2 (vide infra), which does not contain any GATC in the human GREII but encompasses the GATCs present in pHLCAT2 DNA, the latter being methylated after propagation in the HB101 (dam⁺) E. coli strain (the NdeI-XhoI fragment excised from pHGFBP-1-GRU2 was not restricted by MboI; Fig. 2).

When such co-transfection experiments were performed in the presence of varying amounts of pHShGRα (range 0.125 to 1 µg), the magnitude of glucocorticoid effect was dose-dependent, pointing to GR dependence and was always greater in the dam methylated series (Fig. 4).

Because the rat GRU2 encompasses an IRE that, as is the case for its human counterpart (27), interacts with liver-specific trans-acting factors (26), we wondered whether these factors would cooperate with the GR and thus allow glucocorticoid inducibility from the unmethylated rat GREII in cells from hepatic origin. To address this question, pHGFBP-1-GRU2 was prepared from dam⁻ or dam⁻ E. coli strains, then co-transfected with pHShGRα in HepG2 human hepatoma cells (Fig. 5). In this cell line, the glucocorticoid-induced increase in CAT reporter gene expression was observed when the dam methylated pHGFBP-1-GRU2 was used as a reporter plasmid (13.3 ± 3.9-fold) (Fig. 5, A and B, GAmTC). In contrast, when pHGFBP-1-GRU2 had been prepared from dam⁻ bacteria, the slight difference in CAT activities (dexamethasone versus vehicle; 2.21 ± 0.48-fold) was not statistically significant (Fig. 5, A and B, GATC).

**The Human GREII Is not Functional in CHO and HepG2 Cells**—The human GREII is also degenerated, compared with the consensus GRE sequence (Fig. 1B). When inserted upstream of the heterologous thymidine kinase promoter in pHGFBP-1-GRU2, and co-transfected with pHShGRα in either CHO (which do not contain liver-enriched trans-acting factors) or HepG2 cells, it turns out to be unable to mediate any glucocorticoid response (1.70 ± 0.35 and 1.02 ± 0.06-fold induction in CHO and HepG2 cells, respectively) (Figs. 3 and 5; A and B, GATC).

Altogether, these data show that the human GRU2 is thus functionally homologous to the unmethylated rat GRU2 (the actual methylation status of the rat GRU2 in vivo; cf. results below and under “Discussion”).

**dam Methylation of the GATC Motif of the Rat GREII In-**
increases its affinity toward the GR—Unmethylated or dam methylated rIGFBP-1-GRU2 were excised from prIGFBP-1-GRU2 (HindIII-SalI restriction fragments) that had been propagated in dam- or dam+ E. coli strains, respectively. They were end-labeled and incubated with crude nuclear extracts prepared from Sf9 cells overexpressing hGRa.

Both unmethylated or dam methylated rIGFBP-1-GRU2 yielded identical patterns in EMSA, with one retarded band, (GR)b, corresponding to GR homodimers (Fig. 6A). That (GR)b corresponded to bona fide GR-GRE interactions was concluded from two additional observations. This band was extinguished in the presence of unlabeled TAT-GREII in the incubation mixture (25-fold molar excess) and was super-shifted in the presence of anti-GR antibody (Fig. 6A).

When rat GRU2 ds-oligonucleotides were used in EMSA, the intensity of the (GR)b band was increased 3.74-fold when the rat GREII had previously been dam methylated in vitro using recombinant dam methylase (Fig. 6B, left panel, lanes GATC; Fig. 6C, left panel, lanes GATC). The intensity of the (GR)b band was also increased by 2.72-fold when the rat GRU2 was excised from dam methylated versus unmethylated prIGFBP-1-GRU2 (i.e. propagated in dam+ and dam- strains of E. coli, respectively) (Fig. 6B, right panel; and Fig. 6C, right panel).

EMSAs were also carried out with a 32P-labeled rat GRU2 oligonucleotide that had been synthesized in its dam methylated form, introducing a N\(^{6}\)-methyl-adenine instead of an adenine within the GATC tetranucleotide (Eurogentec). Competition experiments were performed in the presence of 6–120-fold molar excesses either of the homologous oligonucleotide or of unmethylated rat GRU2 or of human GRU2. In the presence of the homologous nucleotide 50% competition (EC\(_{50}\)) was achieved in the presence of a 33.8-fold molar excess of unlabeled competitor (Fig. 7, A, top row; and B). Competition was 2.78 times less efficient in the presence of unmethylated rat GRU2 (EC\(_{50}\) of 94.2-fold molar excess) (Fig. 7, A, middle row; and B).

That the affinity of the GR for the rat GRU2 was increased after dam methylation could also be inferred from the further observation that <<sham>> dam methylation of the human GRU2 oligonucleotide (devoid of GATC tetranucleotide in the GREII) in vitro (i.e. using recombinant dam methylase) did not
yield any significant increase in the amount of GR-GRE complexes (Fig. 6, B and C; middle panels). Moreover, competition experiments in EMSA showed that the affinity of the human GRU2-GRE interaction was close to that observed for the unmethylated rat GRU2 and was 1.94 times lower than that of dam methylated rat GRU2 (EC_{50} of 65.5-fold molar excesses) (Fig. 7, A, middle row; and B).

The Rat GREII Is Not Methylated in Vivo—To check what the functional status of the rat GRU2 in vitro, we examined whether or not the GATC tetranucleotides were methylated at the IGFBP-1 locus. H4II rat hepatoma cells, an IGFBP-1-producing cell line in which IGFBP-1 gene expression is stimulated by glucocorticoids, was used as a model system (18). Genomic DNA was prepared from H4II cells, digested either by FokI alone or by FokI and MboI, then run on agarose gel. The human IGFBP-1 promoter (nt −340 to +1; an internal standard for hybridization efficiency) was run in parallel on the same gel (Fig. 8, lane 1). The restriction fragments and the IGFBP-1 promoter fragment were analyzed by Southern blotting, using a 32P-labeled probe that encompassed the human IGFBP-1 promoter (nt −340 to +1). FokI cleaves IGFBP-1 genomic DNA in the 5' flanking sequences (between nt −755 and −754) and in exon 1 (between nt +133 and +134), and a restriction fragment of 887-bp was indeed obtained by Southern blot analyses (Fig. 8, lane 2). MboI cleaves twice the IGFBP-1 gene in the promoter region (between nt −353/−352 and −82/−81), exclusively if the GATCs are unmethylated. If such is the case in H4II hepatoma cells, restriction by both FokI and MboI should yield three fragments of 600 (nt −754 to −155), 72 (nt −154 to −82), and 215 (nt −81 to +133) bp. However, under our experimental conditions (stringency of the washes necessary to get rid of non-specific hybridizations, length of the overlap between the restriction fragment and the probe), only the 600-bp fragment, which overlaps 187-bp fragment of the random-labeled probe, should be detectable on the blot. As can be seen on Fig. 8 (lane 3) the 887-bp fragment observed after FokI digestion was detected no more after digestion of H4II genomic DNA by FokI and MboI. Moreover, digestion by FokI and MboI yielded a 600-bp fragment. These results strongly support the conclusion that the rat GRU2 is not methylated in H4II cells.
that the GATCs (including that of GREII) were unmethylated at the IGFBP-1 locus in IGFBP-1-expressing H4II hepatoma cells.

**DISCUSSION**

The 340-bp fragment upstream of rat and human IGFBP-1 genes' cap sites constitute highly tissue-specific promoters that encompass both liver-specific (HNF1, HNF3, DBP) (9, 26–28, 42, 43) and ubiquitous (GRE, CRE, HMGI) cis-elements. These are required for efficient gene transcription in hepatocytes (9, 42, 44), and for strict hormonal regulation of IGFBP-1 gene expression (19, 20, 22–25).

In rat and human IGFBP-1 promoters, the GRU and cAMP response units, respectively, consist of overlapping cis-elements; one of these corresponds to the hormone response element (GRE or CRE), and the other(s) to the recognition sequence(s) for liver-specific trans-acting factors (HNF3 and DBP) (26–28). The rat and human IGFBP-1 promoters contain three GREs; GREI, the most 5'-GRE, spans nt −186 to −172 (rat) and nt −198 to −173 (human); GREII overlaps the insulin response element (rat, nt −91 to −77; human, −110 to −84), and GREIII maps either in the 5'-untranslated sequences of the transcript (rat, nt +41 to +56) or close to the cap site (human, nt −52 to −25). However, progressive 5'-truncations of the rat and human IGFBP-1 promoters yielded discrepant functional results in transient transfection experiments. Deletion of the human promoter sequences located upstream of nt −140 (i.e. eliminating GREI) abolished promoter stimulation by dexamethasone. Accordingly, an internal deletion or mutation of GREI sequences within the 1.2-kilobase promoter fragment, led to the inability of dexamethasone to enhance transcription (22). By contrast, truncation of the rat promoter to nt −154 and even −92 (just 5' to GREII) does not abolish the ability of dexamethasone to stimulate promoter activity (23, 25). This may result from low versus high affinity GR binding to the human and rat GREII, respectively. As a matter of fact, replacement of GREII by the rat tyrosine aminotransferase GREII (a potent GRE) in human IGFBP-1 promoter sequences yielded glucocorticoid responsiveness, whether GREI was mutated or not (22).

Rat and human IGFBP-1-GREIIIs differ by 4 bp and one of these, a C to A transition at position +3, generates a GATC tetranucleotide in the 3'-half palindromic of the rat GREII. This sequence is a target for the bacterial dam methylase, and previous studies have shown that its adenosyl methylation in standard dam- strains used for large scale preparation of reporter plasmids, may alter promoter and/or enhancer activity when these plasmids are used in transient transfection experiments in eucaryotic cells. For instance, the HNF1 cis-element of the rat albumin promoter contains a GATC sequence, and its function is abolished when methylated in dam+ bacterial strains (45). Conversely, artificial steroid hormone response elements (GRE/PRE) are created by dam methylation (29), and adenine methylation at dam sites has been shown to increase transient gene expression in plant cells (46).

In this study, we demonstrate that the functional status of the rat IGFBP-1-GREII is also dependent on N6-adenine (dam) methylation of its GATC tetranucleotide. Even though the unmethylated rat IGFBP-1-GREII does not mediate any significant glucocorticoid stimulation of reporter gene expression in transient transfection experiments, its adenosyl methylation in standard dam- bacterial strains yielded a functional GRE. The unmethylated rat GREII thus behaves as the human IGFBP-1-GREII (GCTC instead of GATC), which, per se, is unable to mediate any glucocorticoid induction, irrespective of the dam phenotype of the bacterial strain.

These functional data are supported by DNA-protein interaction studies; dam methylation of the rat GREII increases its affinity by ~3-fold for the glucocorticoid receptor. This is probably related to the fact that the methyl group at position N6 of adenine can mimic the 5'-methyl group of the thymine (i.e. establish hydrophobic interaction with residue Val482 of the glucocorticoid receptor) (47) and enables specific interaction of the GR with the major groove, although the positions of these methyl groups differ in the major groove (29). Also in agreement with the functional data is the ~2 times lower affinity of the human GREII (relative to that of the dam methylated rat GREII) for the glucocorticoid receptor; it is close to that measured for the unmethylated rat GREII. As far as we are aware, dam methylation has only been detected in bacteria (e.g. cyanobacteria and in the group of related families of Enterobacteriaceae, e.g. E. coli, Parovacti- riaeae, and Vibrionaceae) (48) and has never been reported in vertebrates. In mammalian cells, genomic DNA is methylated at the 5th position of the cytosine residue in CpG dinucleotides. This is the only chemical modification that genomic DNA allows under physiological conditions (for review, see Ref. 49), and such CpG methylation may cause promoters and/or enhancers to be oblivious to transcription factors, as is the case for the silent allele of imprinted genes (for recent reviews, see Refs. 50 and 51).

In this connection, our results clearly show that the GATC tetranucleotides are not methylated at the rat IGFBP-1 locus in H4II rat hepatoma cells, a cell line that expresses IGFBP-1, and in which its expression is regulated by glucocorticoids (22). The lack of dam methylation of the endogenous IGFBP-1 promoter region strongly supports the conclusion that, in vivo, the rat GREII is not by itself able to enhance IGFBP-1 gene expression in the presence of glucocorticoids and thus behaves as its human homologue. Altogether these data may explain the functional discrepancy previously noticed between rat and human species (22, 23, 25), and allow speculation that in both species, the promoters are functionally homologous with regard to the glucocorticoid response.2

Finally, our data clearly show that the interaction of liver-enriched trans-acting factor (HNF3 and/or DBP) with the 5'-

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2 G. Schweizer-Groyer, work in progress.
overlapping IRE cis-element does not enable the unmethylated rat GRU2 or the human GRU2 to become responsive to glucocorticoids and thus does not allow the GR to form committed rapid start complexes with the basal transcriptional machinery (for review see Ref. 52).

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