Functional role of a novel *cis*-acting element (GAGA box) in human type-1 angiotensin II receptor gene transcription

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ABSTRACT

GH/growth factors have been shown to increase angiotensin type 1 receptor expression. In the present study we determined the *cis*-acting regulatory region controlling GH-induced transcription of the human type-1 angiotensin receptor (hAT₁). In human proximal tubule cells transfected with a chloramphenicol acetyl transferase (CAT) reporter plasmid under the control of the hAT₁ promoter, GH induced CAT activity. Serial deletions of the hAT₁ promoter region indicated that an area between -314 bp and -70 bp upstream of the 5'-end of the cDNA sequence was essential for this activation to occur. Although sequence analysis identified putative multiple nuclear protein binding sites in this region, we determined that a 12 bp sequence (5'-GAGAGGGAGGAG-3', GAGA box) located between -161 bp and -149 bp was important for GH-mediated activation. Using mobility shift assays we demonstrated increased DNA binding activity to the labeled GAGA box in

nuclear extracts treated with GH, suggesting this sequence is a GH response element. Southwestern analysis identified an 18 kDa GAGA box-binding protein (GAGA-BP). GH-induced activity of the GAGA-BP occurred within 2.5 min and reached a maximum at 5 min. Activation did not require *de novo* protein synthesis. Removal of the GAGA box abolished GH-induced transcription as well as basal transcription of the hAT₁ gene. Additional studies demonstrated that epidermal growth factor, platelet-derived growth factor and insulin activate the GAGA-BP, suggesting these growth factors can also regulate the transcription of the hAT₁ gene through the GAGA box. Our data show that the GAGA-BP acts as a *trans*-acting factor binding to the *cis*-acting regulatory element in the hAT₁ promoter, which is necessary for the basal and growth factor(s)-mediated transcriptional activation of the hAT₁ gene.

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INTRODUCTION

The renin–angiotensin system (RAS) is an important regulator of many physiological functions and pathophysiological conditions, including catecholamine release, aldosterone synthesis, sodium retention and organ development as well as vascular and cardiac hypertrophy (Raizada *et al.* 1996). The main effector peptide of the RAS is angiotensin II (AngII). In humans, AngII actions are mediated by two pharmacologically defined cell surface receptors, angiotensin II type 1 (AT₁) and type 2. In the adult kidney the AT₁ receptor is the predominant subtype, which regulates hemodynamics, tubular transport and growth (Raizada *et al.* 1996). AngII influences renal hemodynamics

by preferentially constricting the efferent arteriole thereby increasing glomerular hydrostatic pressure and glomerular filtration rate. It also acts directly through proximal tubule AT₁ receptors, and indirectly through aldosterone release to increase sodium reabsorption. Therefore, under normal physiological conditions, the AT₁ receptor is very important in maintaining kidney function. Factors or events that alter AT₁ gene expression will therefore disrupt normal kidney function.

Initiation of mRNA synthesis is a primary control point in the regulation of gene expression. Cells respond to intra- and extracellular signals by turning specific genes on and off and by altering the extent of transcription of active genes (Maniatis *et al.* 1987). Although the mechanism and

biochemical pathways involved are still largely unknown, it is clear that the initiation of mRNA synthesis requires the interaction of proteins with specific DNA sequence(s) in the gene 5'-regulating region. In this manuscript, we address the regulation of human AT_1 (h AT_1) mRNA in the kidney at the transcriptional level. Previous studies of the transcriptional regulation of the AT_1 receptor have principally utilized the rat or mouse as models (Regitz-Zagrosek *et al.* 1996). In those studies a variety of vasoactive peptides, hormones and growth factors have been shown to alter AT_1 receptor levels by either enhancing or repressing the transcriptional activity of the gene (Guo *et al.* 1995, Regitz-Zagrosek *et al.* 1996). We investigated growth factors and, in particular, growth hormone (GH) regulation of h AT_1 gene transcription in human proximal tubule cells for several reasons. First, GH alters sodium balance and cardiovascular homeostasis in humans (Hoffman *et al.* 1996) and animals (Wyse *et al.* 1993). GH-induced changes in the rat are associated with alterations in RAS activity and, in particular, the AT_1 receptor density of the kidney, adrenal and liver *in vivo* (Wyse *et al.* 1993). Secondly, *in vitro*, GH directly regulates rat AT_{1A} receptors in primary astrocyte cultures by altering transcription (Wyse & Sernia 1997). Finally, GH and AngII receptors are expressed in the proximal tubule, the nephron segment in which the majority of filtered sodium is reabsorbed (Mujais *et al.* 1986, Chin *et al.* 1992).

The goal, therefore, of this study was to elucidate associations between GH and the h AT_1 gene. We demonstrate that GH alters the rate of transcription of the h AT_1 gene through a 12 bp (GAGA box) sequence. This GAGA box was shown to act as a bifunctional element that is necessary for both basal and GH/growth factor(s)-mediated activation of h AT_1 transcription. We also identified a novel 18 kDa nuclear protein that is specifically activated by GH/growth factor(s) and interacts with the GAGA box. Therefore, we propose that binding of GH/growth factor(s) to receptors activates a novel *trans*-acting nuclear factor which specifically binds to a *cis*-acting element of the promoter region of h AT_1 receptor to increase its rate of transcription.

MATERIALS AND METHODS

Materials

LRCC8, an SV40-transformed human kidney proximal tubule cell line was kindly provided by Dr Lorraine Racusen (The Johns Hopkins University School of Medicine, Baltimore, MD, USA) (Racusen *et al.* 1997). Dulbecco's Modified Eagle's

Medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, trypsin/EDTA and lipofectamine Plus reagents were from Life Technologies (Gaithersburg, MD, USA); [α - 32 P]dGTP, [α - 32 P]dATP and [α - 35 S]dATP were from Dupont-New England Nuclear (Boston, MA, USA); pCAT reporter plasmids and pSV- β -galactosidase expression plasmids and restriction enzymes were from Promega Co. (Madison, WI, USA); sequencing kits were from the United States Biochemical Co. (Cleveland, OH, USA); Taq polymerase was from Perkin-Elmer Co. (Norwalk, CT, USA); Tube-O Dialyzer was from Geno-Technology (St Louis, MO, USA); NICK (Sephadex G-50) columns were from Pharmacia Biotech (Uppsala, Sweden); electrophoresis reagents, human GH (hGH) and all other chemicals were from Bio-Rad (Richmond, CA, USA) and Sigma Chemical Co. (St Louis, MO, USA). MacVector 6.5 sequence analysis software was from Oxford Molecular Ltd (Madison, WI, USA) and GraphPad Prism statistical analysis software was from GraphPad Software Inc. (San Diego, CA, USA).

Isolation of the h AT_1 receptor genomic clone and the construction of the expression vectors

To isolate the 5'-regulatory region of the h AT_1 receptor a human placental genomic library (Clontech, Palo Alto, CA, USA) was screened using an end-labeled oligonucleotide (5'-GACCCAGG CAGCAGCGAGTGACAGG-3') that corresponds to the 5'-end of the published h AT_1 cDNA (Bergsma *et al.* 1992). Nine positive clones were isolated. Southern blot analysis, using the oligonucleotide corresponding to the 5'-end of the h AT_1 receptor hybridized specifically to seven clones confirming their identity as h AT_1 receptor gene coding for the 5'-sequence. Using one of these clones, HARG9, as a template for PCR and oligonucleotides from a published sequence of the promoter region (Guo *et al.* 1994), fragments of varying length were amplified. The oligonucleotide corresponding to exon 1 (+49 bp to +70 bp) of the h AT_1 receptor (5'-CCGCCGCGGCCCGCAGAGCTG-3') was used as the antisense primer for each reaction. The varying sense primers (hp1: 5'-GAGGAAGTTCCTATTCCTAG-3'; hp2: 5'-CCTCACTTGACCATGGTGTATAGTC-3'; hp3: 5'-GAACACGAATCTCCGCAG-3'; hp4: 5'-GCA GCAACGCCCTCACT-3') yielded fragments of 1348, 845, 384 and 141 bp in size respectively. Each fragment was sub-cloned into the EcoRV site of pBluescript II KS (Stratagene, La Jolla, CA, USA). These clones were then digested with SalI and XbaI to release the fragments that were consequently

inserted into the pCAT-Basic plasmid. Removal of the GAGA box (–161 bp to –149 bp; 5'-GAGA GGGAGGAG-3') and TATA box (–54 bp to –49 bp; TATAA) was achieved by PCR amplifying the flanking regions of the respective sites separately (Thomas *et al.* 1995). The resultant DNA fragments were ligated and then inserted into pCAT-Basic to generate pCATHp3-GA and pCATHp3-TA respectively. The authenticity of clones was confirmed by restriction analysis and dideoxy sequencing as described previously (Thomas *et al.* 1995).

Cell culture and transfection of reporter gene constructs

LRCC8 cells were maintained in DMEM/F12 media supplemented with 5% FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin (complete medium). Transfection experiments involved growing cells to 70–80% confluence in six-well plates in complete medium. Four micrograms DNA (2 µg reporter constructs, 2 µg pSV-β-galactosidase expression construct) were transiently transfected into cells using the lipofectamine Plus reagent method as described by the manufacturer (Thomas *et al.* 1995). Cells were then grown for 24 h in complete medium.

Chloramphenicol acetyl transferase (CAT) assay

The CAT assays were performed as described previously with minor modifications (Bhat *et al.* 1995). Briefly, 24 h serum-depleted transfected cells were treated with and without hGH. After 18 h the cells were washed three times with PBS at 4 °C and collected by scraping. Cells were then centrifuged and the resultant pellet was resuspended in 70 µl 0.25 M Tris-HCl, pH 7.8. Extracts were prepared by sonication (10 s at 4 °C), and sedimented by centrifugation (17 530 *g* for 5 min at 4 °C). Thirty microliters of the supernatant were removed to determine β-galactosidase activity. The remaining supernatant was incubated at 70 °C for 10 min to inactivate endogenous acetylases and centrifuged. The CAT assay involved the addition of 20 µl cellular extract to 130 µl CAT assay buffer (140 mM Tris-HCl, pH 8.0, 0.2 µCi [14 C]chloramphenicol, 4 mM acetyl-coenzyme A). The reaction mixture was incubated at 37 °C for 1 h and extracted with ethyl acetate. The organic phase was removed and vacuum dried. The resultant pellet was resuspended in 30 µl ethyl acetate and the labeled chloramphenicol and acetylated derivatives were separated by ascending thin-layer chromatography. The radioactivity was visualized by autoradiography and its

intensity of density (IOD) quantitated by image analysis. CAT activity (IOD) was normalized with respect to levels of β-galactosidase activity, which were measured according to a previously published method (Sambrook *et al.* 1989).

Preparation of cytosolic and nuclear extracts

Cells were grown (100 mm plate) to 80–90% confluence in complete medium. Forty-eight hour serum-depleted cells were treated with or without hGH (100 ng/ml), epidermal growth factor (EGF) (200 ng/ml), platelet-derived growth factor (PDGF) (100 ng/ml) or insulin (30 ng/ml) for indicated times. Alternatively, cells were treated prior to hGH treatment with cyclohexamide (1 µg/ml) for 15 min. The cells were washed (PBS, 4 °C) and scraped into the same buffer. Nuclear and cytosolic extracts were made as previously described (Bhat *et al.* 1994). Briefly, harvested cells were resuspended in 1 ml hypotonic solution (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT)) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin and 10 µg/ml aprotinin), Dounce homogenized and sedimented by centrifugation (1000 *g* for 10 min). The cytoplasmic extract (supernatant) was placed at –70 °C for use later. The nuclei (pellet) were resuspended in a high-salt buffer (20 mM Hepes pH 7.9, 25% glycerol, 400 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors (see above), dialyzed (membrane molecular weight cut off of 8 kDa) overnight in a buffer containing 20 mM Tris-HCl pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol and 50 µg/ml PMSF and stored at –70 °C for later use. Protein concentration was determined for each extract according to Bradford (1976).

Electrophoretic mobility shift assay

Gel mobility shift assays were performed as previously described (Bhat *et al.* 1994). pCATHp3 fragments with 5'-overhangs were generated by restriction enzyme (SalI/EcoRI) digestion and isolated by gel purification. In addition, a double-stranded GAGA box-containing sequence with a 5'-overhang was generated by annealing two oligonucleotides (sense primer; 5'-GAGAGGG AGGAG-3', antisense primer; 5'-CTCCTCC CTC-3'). The double-stranded GAGA box was labeled by filling the overhang with Klenow in the presence of [α ³²P]dTTP and [α ³²P]dCTP. Nuclear extracts from hGH-treated and untreated cells were incubated with 1 µg poly(dI-dC) at room temperature for 20 min in a total volume of 20 µl containing

100 mM Tris-HCl, pH 8.0, 50% glycerol, 500 mM KCl, 10 mM EDTA, and 10 mM DTT supplemented with either 2 µl proteinase K, varying concentrations (0 to 50 times) of unlabeled double-stranded GAGA box, 50-fold excess of unlabeled double-stranded mutant GAGA (Mut1: 5'-GAG ATTTAGGAG-3'; Mut2: 5'-AAAAGGGAG GAG-3'; Mut3: 5'-GAGAGGGAAAAA-3'; Mut4: 5'-AAAATTTAGGAG-3') or water. Labeled probe (200 000 c.p.m.) was then added and the incubation continued for a further 10 min. The DNA-protein complex was electrophoresed on a 4% native polyacrylamide gel containing $0.5 \times$ Tris-borate EDTA buffer (25 mM Tris-HCl, 25 mM boric acid, 0.5 mM EDTA). Gels were run at 240 V at 4 °C, dried and exposed to Fuji RX-film at -70 °C.

Southwestern analysis

Nuclear and cytosolic extracts were fractionated through a 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes as described previously (Bhat *et al.* 1994). Membranes were then washed in PBS, prehybridized in a buffer containing 10 mM Tris-HCl (pH 8.0), 5% glycerol, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 500 ng/ml salmon sperm for 30 min and then incubated with ³²P-labeled double-stranded GAGA box or pCATHp3 for 30 min. To remove non-specific DNA binding, DNase I was added (2 units, 15 min) at 37 °C. The membranes were washed (PBS, 22 °C) twice and exposed to X-ray film at -70 °C with intensifying screens.

Computer analysis and statistical methods

Sequence analyses and alignment were performed using MacVector 6.5 sequence analysis software. Transcription factors search and analysis were performed using TESS (Transcription Element Search Software) on the World Wide Web (URL: <http://www.cbil.upenn.edu/tess/index.html>). Statistical differences between two experimental groups were tested by Student's *t*-test for unpaired samples using the computer software GraphPad Prism.

RESULTS

Cloning of the hAT₁ gene promoter and its introduction to reporter gene constructs

The authenticity of our isolated genomic clone, HARG9, was confirmed by sequence analysis using primers corresponding to the published hAT₁

promoter. Computer analysis of the hAT₁ promoter region indicated putative binding sites including TATA, specificity protein 1 (Sp1) and numerous activator protein 1 (AP1) within the first 1000 bp upstream of the transcription start site (Guo *et al.* 1994). In this study, we investigated the involvement of the -1278 bp to +70 bp fragment in the regulation of the hAT₁ gene. To define the role of specific DNA sequences within this region, various sized deletion constructs were created and placed upstream of the CAT reporter gene expression vector (Fig. 1).

Measurement of hAT₁ transcription

Reporter pCAT constructs were transiently transfected into LRCC8 cells and treated with or without hGH (100 ng/ml). GH significantly increased CAT activity in cells expressing pCATHp1 (382 ± 156 IOD (S.E.M); $P < 0.05$), pCATHp2 (427 ± 140 IOD; $P < 0.05$) and pCATHp3 (520 ± 230 IOD; $P < 0.05$) above their relative basal levels (pCATHp1: 61 ± 25 IOD; pCATHp2: 41 ± 28 IOD; pCATHp3: 95 ± 48 IOD) (Fig. 2). CAT activity of cells expressing pCATHp4 was unchanged with hGH treatment (4 ± 1) compared with basal levels (9 ± 7). These results suggest that the DNA sequence required for hGH-induced transcription of the hAT₁ is located between -314 bp (pCATHp3) and -71 bp (pCATHp4). hGH produced a dose-dependent increase of CAT activity in cells transfected with pCATHp3 (Fig. 3). Maximal response was observed at an hGH dose of 100 ng/ml (5.3 ± 2.1 -fold increase over basal), which was maintained at 200 ng/ml (5.4 ± 2.3 -fold increase over basal).

Identification of hGH-induced *cis*-acting element on the hAT₁ gene

Computer analysis indicated a number of putative binding sites located in the region between primers hp3 and hp4 (Fig. 1). A 12 bp sequence (5'-GAG AGGGAGGAG-3') found within -161 bp to -149 bp of the hAT₁ gene showed 92% homology with a GH regulatory element (GAGA box) located on the rat serine protease inhibitor 2.1 (rSpi2.1) gene (Le Cam *et al.* 1994). Thus, we refer to this sequence as the hAT₁-GAGA box. To confirm the ability of the hAT₁-GAGA box to specifically bind hGH-induced nuclear protein(s), a mobility shift assay was performed using proximal tubule cell nuclear extracts and labeled hAT₁-GAGA as the probe. Nuclear extracts from cells treated with hGH showed a mobility shift of the labeled GAGA box (Fig. 4A, lane 2). There was no specific binding of the labeled hAT₁-GAGA probe in nuclear

FIGURE 1. The 5'-regulatory region of the hAT_1 gene. (A) A schematic representation of the pCAT reporter expression vector containing serially deleted 5'-promoter region of the hAT_1 gene. pCATThp1 is labeled with a number of computer-generated putative transcription binding sites. (B) DNA sequence of the 314 bp fragment of the hAT_1 promoter. Primer sequences used in the construction of DNA fragments pCATThp3 and pCATThp4 are shown by a dotted line. The TATA, GAGA and Sp1 sites are boxed. +1 refers to the 5'-end of the cDNA clone obtained by Bergsma *et al.* (1992). The arrow indicates the transcription start site shown in Curnow *et al.* (1995).

extracts from untreated cells under these conditions (lane 4). Pre-treatment with proteinase K abolished the mobility shift of the hAT_1 -GAGA probe (lane 3) indicating the formation of an hGH-induced protein-DNA complex. The presence of increasing

concentrations (0–50 times) of unlabeled hAT_1 -GAGA probe progressively abolished the protein-DNA complex formation, demonstrating the specificity of the protein binding to the hAT_1 -GAGA box (Fig. 4B). To identify the essential

FIGURE 2. Human GH-induced CAT activity of hAT₁ reporter gene constructs. CAT activity for the serially deleted promoter fragments in pCAT vector was determined. Bars represent the CAT activity for each serially deleted promoter fragment in cells treated with (closed bars) or without (shaded bars) hGH (100 ng/ml) for 18 h. 'n' represents the number of experiments. Values are the mean ± s.e.m. *P<0.05.

DNA sequences within the GAGA motif necessary for nuclear factor(s) to bind GAGA-DNA, mobility shift assays were performed with GH-treated nuclear extracts in the presence of 50-fold excess of unlabeled mutant GAGA-DNA. Figure 5 shows that the 5'-GAGAGGG-3' portion of the hAT1-GAGA is required for protein(s) to bind,

FIGURE 3. Dose-response of hGH to CAT activity of pCAThp3. LRCC8 cells were transfected with pCAThp3, treated with hGH (0–200 ng/ml) and CAT activity measured. hGH-induced activity is expressed as a fold increase relative to the activity of untreated cells. Data shown are representative of multiple experiments (n=4).

FIGURE 4. Identification of specific protein binding activity, in nuclear extracts from LRCC8 cells, to the GAGA box of the hAT₁ promoter. The mobility shift assay was performed as described in Materials and Methods. ³²P-labeled GAGA probe was incubated with nuclear extracts (10 µg) from LRCC8 cells. Samples were analyzed after electrophoresis on a 4% non-denaturing polyacrylamide gel and autoradiography. (A) Labeled probe in the absence of extract (lane 1), in the presence of hGH (100 ng/ml)-treated extract (lane 2), in the presence of GH-treated extract with proteinase K (lane 3), and in the presence of hGH-untreated extract (lane 4). (B) A mobility shift assay was performed using the labeled GAGA probe in the presence of hGH-treated nuclear extracts and increasing concentrations of unlabeled GAGA probe, as indicated. The arrow indicates the position of the protein–DNA complex.

FIGURE 5. Determination of specific DNA sequences within the GAGA necessary for nuclear factor to bind GAGA-DNA. The mobility shift assay was performed with hGH-treated nuclear extracts in the presence of unlabeled double-stranded mutant GAGA-DNA. Samples were analyzed after electrophoresis on a 4% non-denaturing polyacrylamide gel and autoradiography. Lane 1, nuclear extract (10 μ g) incubated with 32 P-labeled double-stranded GAGA probe (5'-GAGAG GGAGGAG-3'); lane 2, nuclear extract incubated with 32 P-labeled GAGA to which 50-fold excess of unlabeled GAGA was added as a competitor; lanes 3-6, nuclear extract incubated with 32 P-labeled GAGA to which 50-fold excess of unlabeled double-stranded mutant GAGA (lane 3; 5'-GAGATTTAGGAG-3', lane 4; 5'-AAAAGGGAGGAG-3', lane 5; 5'-GAGA GGGAAAA-3', lane 6; 5'-AAAATTTAGGAG-3') was added as competitor. Underlined sequences indicate the mutated bases. The arrow indicates the position of the protein-GAGA-DNA complex.

although certain mutations within the 5'-end or 3'-end of this sequence have no effect and allow effective competition with the wild-type sequence.

Identification of *trans*-acting factor

To determine the direct interaction between the GAGA box and the nuclear protein(s), Southwestern analysis was performed. Figure 6 indicates

FIGURE 6. Demonstration of direct binding of an 18 kDa protein to the GAGA sequence of the hAT₁ gene. Southwestern analysis was performed to identify the direct interaction between the GAGA sequence and its binding protein. Cytosolic (10 μ g) and nuclear (10 μ g) extracts from LRCC8 cells were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane and hybridized with 32 P-labeled GAGA probe. (A) The time course of hGH (100 ng/ml) activation of an 18 kDa protein (cytosolic and nuclear, as indicated). The arrow indicates the position of the GAGA-BP. (B) Densitometric analysis of the blot, plotted for the hGH-induced activation over time (■ nuclear, ▲ cytosolic). This is an autoradiograph representative of four experiments.

that an 18 kDa DNA-binding protein interacts directly with the hAT₁-GAGA box. The pCATHp3 DNA fragment also recognized the 18 kDa binding protein with the same specificity as the GAGA probe (data not shown). This GAGA box-binding protein (GAGA-BP) was principally present in the nuclear fraction. Treatment of cells with hGH for varying times indicated that the 18 kDa protein was activated within 2.5 min reaching a maximum at 5 min. The observed activity of this protein was maintained up to 60 min after hGH treatment. Addition of excess unlabeled GAGA-DNA successfully competed with the binding of the GAGA probe to the 18 kDa protein (data not shown). The immediate activation, as early as 2.5 min, of the

FIGURE 7. Determination of whether GAGA-BP activation requires *de novo* protein synthesis. Nuclear extracts (10 µg) from LRCC8 cells that were untreated (SF), treated with 100 ng/ml hGH (GH) for 5 min or treated with cycloheximide (1 µg/ml) and hGH (CHX+GH) underwent Southwestern analysis using the 32 P-labeled double-stranded GAGA probe. Data shown are representative of multiple experiments ($n=4$). The arrow indicates the position of GAGA-BP.

18 kDa protein by hGH suggests that *de novo* synthesis is not required for its activation. To confirm this, cells were pre-treated with a protein synthesis inhibitor cycloheximide (1 µg/ml) and hGH-induced activity was measured (Fig. 7). Our results show that inhibition of protein synthesis did not reduce hGH-induced activity of the 18 kDa protein to bind the hAT₁-GAGA box.

Functional analysis of the hAT₁-GAGA box

To determine whether the GAGA box was responsible for hGH-induced transcription of the hAT₁ receptor, we constructed a pCAThp3 mutant in which the GAGA box (pCAThp3-GA) was selectively removed. When pCAThp3-GA was transfected into LRCC8 both hGH-induced transcription and basal transcription were inhibited (Fig. 8). To further investigate basal transcription of the hAT₁ gene, the putative TATA sequence was selectively deleted from pCAThp3. This deletion (pCAThp3-TA) did not abolish basal transcription and had no effect on hGH-induced transcription. These results indicate that the GAGA box and not the putative TATA sequence is responsible for the hGH-induced as well as basal transcription of the hAT₁ gene.

FIGURE 8. CAT activity of LRCC8 cells expressing pCAThp3 mutants. pCAThp3-GA and pCAThp3-TA mutants were constructed, transfected and treated with and without hGH (100 ng/ml). Bars represent CAT activity for each fragment relative to the activity of untreated cells expressing the pCAThp3 fragment. Data shown are means $\pm$ S.E.M. of four separate experiments.

hAT₁-GAGA box is a *cis*-acting element for other growth factors

To determine if growth factors which have similar signaling pathways to hGH can activate the GAGA-BP, cells were treated for 5 min with either EGF (200 ng/ml), PDGF (100 ng/ml) or insulin (30 ng/ml). Southwestern analysis indicated that nuclear extracts from PDGF-, EGF- and insulin-treated cells increased the binding activity of the GAGA-BP (Fig. 9). This result indicates that the GAGA box may function as a common *cis*-acting element for growth factor(s)-induced *trans*-acting factor (GAGA-BP) in hAT₁ gene transcription.

DISCUSSION

The transcription of hAT₁ receptors is a complex process, as indicated by differences in tissue-specific expression and developmental regulation. The recent cloning and identification of the 5'-regulatory region of hAT₁ enabled us to investigate regulation of hAT₁ transcription. In this study, we have identified a novel regulatory element of the hAT₁ gene that is between -161 bp and -149 bp (hAT₁-GAGA box) upstream of the 5'-end of the cDNA sequence identified by Bergsma *et al.* (1992). This element is involved in growth factor(s)-mediated as well as basal transcription of the hAT₁ receptor gene. Yet the lack of endogenous hAT₁ receptor mRNA and protein expression in immortalized cell lines, including the one we used in this study, makes it difficult to address the correlation

FIGURE 9. Determination of growth factor involvement in GAGA-BP activation. Southwestern analysis (using the ^{32}P -labeled GAGA probe) of nuclear extracts (10 μg) from LRCC8 cells that were untreated (SF) or treated with insulin (30 ng/ml), PDGF (100 ng/ml) or EGF (100 ng/ml) for 5 min. The arrow indicates the position of GAGA-BP. This blot is representative of three experiments.

between the GH and the endogenous hAT_1 . However, we have previously shown in rat studies that a deficiency in GH is associated with a decrease in AT_{1A} receptor (in all organs measured) and infusion of GH increased AT_{1A} receptors towards normal (Wyse *et al.* 1993). In addition, we have shown in primary astrocytes culture cells that GH selectively upregulates the endogenous AT_{1A} receptor mRNA at the transcriptional level (Wyse & Sernia 1997). Computer analysis of the rat AT_1 receptor's 5'-regulatory region indicates that the AT_{1A} and not the AT_{1B} possesses a similar GAGA box to that found in the hAT_1 receptor (Fig. 10). This observation suggests GH-mediated transcriptional regulation of rat AT_{1A} and hAT_1 receptors may utilize similar mechanisms. Functional GAGA boxes have been identified in the promoter regions of a number of genes in different species including the *Drosophila Kruppel* (Kerrigan *et al.* 1991) and heat shock proteins (Lee *et al.* 1992, Lu *et al.* 1993), rSpi2·1 (Le Cam *et al.* 1994), human $\alpha 1(V)$ collagen (Lee & Greenspan 1995) and murine p44 mitogen-activated protein kinase (Pages *et al.* 1995). These GAGA boxes possess GA repeats; however, the overall sequence differs between the genes and species suggesting gene- and species-specific regulation. The closest sequence homology to the hAT_1 -GAGA is the rSpi2·1-GAGA box (Le Cam *et al.* 1994) that shows a 92% homology. The rSpi2·1-GAGA box contains two GAGGAG repeats separated by a G. The difference between the hAT_1 -GAGA and the rSpi2·1-GAGA is a single base (G) deletion in the middle of one of the

GAGGAG repeats. Interestingly, like the hAT_1 -GAGA box, to our knowledge, the rSpi2·1-GAGA box is the only other GAGA box that mediates basal and growth factor-dependent gene transcription (Legraverend *et al.* 1996).

The interaction between TATA-binding proteins (TBP) and the TATA element is generally the first step in the initiation of RNA polymerase II-dependent transcription. In the absence of a functional TATA element, other *cis*-acting elements are also capable of promoting transcription initiation (Bossone *et al.* 1992). Promoter-specific transcription by RNA polymerase II is often dictated by binding of activator proteins to upstream DNA elements. However, the mechanism by which activator proteins stimulate promoter-specific transcription remains unclear. The use of a pCATHp3 mutation (pCATHp3-GA) showed that selective removal of the hAT_1 -GAGA box blocked the action of hGH to induce hAT_1 transcription. In addition, basal promoter activity was abolished suggesting that the hAT_1 -GAGA box acts as a bifunctional *cis*-acting element in that it is important in controlling both basal as well as growth factor-induced transcription of the hAT_1 gene. Selective removal of the TATA did not alter the basal promoter activity or the ability of hGH to induce the hAT_1 transcription. These results suggest the hAT_1 -GAGA box and not the TATA element is essential for basal transcription of the hAT_1 gene. However, two studies indicate that the hAT_1 TATA box is functional based on the observation that initiation starts downstream of the

FIGURE 10. Promoter sequence alignment of the human and rat *AT₁* receptors. Comparison of the putative regulatory region of the hAT₁ (top line), the rat AT_{1A} (middle line) and the rat AT_{1B} (bottom line). The shaded box highlights the GAGA motif and the open boxes highlight the Sp1 and TATA motifs. Their relative locations with respect to the putative transcription start site of each gene are indicated with an asterisk (Guo & Inagami 1994, Guo *et al.* 1994, Takeuchi *et al.* 1993). Sequences common to hAT₁ and rat AT_{1A} or hAT₁ and rat AT_{1B} are indicated in bold.

TATA motif (Su *et al.* 1994, Takayanagi *et al.* 1994). In contrast, a more recent study suggests that the transcription initiation site is located on the last A residue of the putative TATA element (Curnow *et al.* 1995). If this later observation is correct, it would be necessary for the activator proteins or TBP to bind upstream of the TATA motif in order for RNA polymerase to initiate transcription from the TATA element. Our data that the hAT₁-GAGA box is necessary for basal as well as hGH-induced transcription support this contention. The hAT₁-GAGA box may be used as an accessible region either for basic transcriptional machinery in the presence of a functional TATA box, or as an essential site for the transcription initiation and interaction of other activator DNA-binding proteins (in the presence of a non-functional TATA motif). Numerous studies have demonstrated that a GAGA box can be involved in transcription initiation with or without the presence of a functional TATA element. For example, in the hsp70 promoter of *Drosophila melanogaster*, the GAGA box is required for TBP recruitment to its TATA motif (Weber *et al.* 1997). Alternatively, a number of GAGA boxes found in TATA-less promoters of other genes have been shown to be

necessary for the initiation of transcription (Bossone *et al.* 1992). Although it is possible that the hAT₁ gene may possess alternate initiation sites that are determined by the presence or absence of GAGA-BP(s), the mechanism by which the receptor can choose the alternate sites to initiate transcription remains to be determined.

We have identified an hGH/growth factor(s)-induced 18 kDa protein, which acts as a transcription factor by binding to the hAT₁-GAGA box of the hAT₁ gene. The GAGA-BP was found in the cell cytoplasm as well as in the nucleus. Although the presence of low levels of active GAGA-BP in the cytosolic fraction may be a result of contamination with nuclear proteins (during the preparation), consistency in our observation suggests that the GAGA-BP is translocated to the nucleus before binding to the hAT₁-GAGA box. Cyclohexamide, a protein synthesis inhibitor, did not reduce growth factor activation of the GAGA-BP, suggesting *de novo* synthesis is not required for the regulatory effects of hGH/growth factor(s). The observation that cyclohexamide treatment resulted in super-induction of the GAGA-BP suggests that *de novo* protein synthesis is involved in the stability of GAGA-BP and/or that cyclohexamide blocks a

factor(s) which inhibits GAGA-BP activation. The mechanism by which the GAGA-BP is activated in the nucleus by hGH/growth factor(s) requires further investigation. There are a number of known transcription factors that are activated by GH-induced phosphorylation. These include signal transducers and activators of transcription (STAT) proteins, insulin receptor substrates 1 and 2 and c-fos and c-Jun (Ogbourne & Antalis 1998). Our data suggest the GAGA-BP is a novel transcription factor induced by hGH, as the observed size of GAGA-BP (18 kDa) differs from the size of known hGH-induced transcription factors. In addition, the hAT₁-GAGA box is not a known binding site for many growth factor-induced transcription factors such as STATs, nuclear factor- κ B, c-fos and c-Jun. There have been a number of GAGA-BPs identified, although none has a similar size to hAT₁-GAGA-BP. For instance MAZ, a human zinc finger protein, binds to the GA box of the c-MYC gene and regulates the initiation of its transcription (Bossone *et al.* 1992). MAZ is a 52.5 kDa protein that is found abundantly in numerous tissues with the exception of the kidney. The hAT₁-GAGA-BP identified in this study may be related to MAZ in function but its size and tissue distribution differ from the hAT₁-GAGA-BP. Our data suggest that the hAT₁-GAGA-BP is both a novel hGH transcription factor as well as a novel GAGA-BP. The observation that PDGF, EGF and insulin activate GAGA-BP indicates that the 18 kDa protein is a common transcription factor for numerous growth factors and may be activated by shared signaling pathways. This is supported by Legraverend *et al.* (1996) who showed that both GH and interleukin-6 increased the transcription of the rSpi2.1 through its GAGA box. While those investigators demonstrated that activation of the rSpi2.1-GAGA-BP was via a Janus kinase 2-dependent, STAT-independent pathway, they did not identify the transcription factor(s) involved. Since the rSpi2.1- and hAT₁-GAGA boxes are 92% sequence homologous, we believe that the hAT₁-GAGA-BP may be the same protein involved in the transcriptional regulation of both genes. It is possible that there will be additional proteins involved in the overall regulation of hAT₁ gene transcription through the hAT₁-GAGA box. These would not be detected by Southwestern analysis, as the denaturing SDS-PAGE conditions would prevent interaction between these additional proteins and the hAT₁-GAGA-BP. It is worth noting that a prolonged exposure of a Southwestern gel revealed other higher molecular weight proteins bound to the labeled hAT₁-GAGA probe (data not shown). These were of relative low intensity

compared with the GAGA-BP. These findings indicate that the GAGA-BP preferentially binds to the hAT₁-GAGA box but other proteins may have binding affinity for DNA sequences found within the hAT₁-GAGA box of the hAT₁ 5'-regulatory region.

In summary, our studies show that hAT₁ gene transcription can be activated by hGH as well as other growth factors. This activation is mediated by a *cis*-acting element that consists of a GAGA sequence located in the 5'-regulatory region of the gene. Furthermore, we identified an hGH/growth factor(s) activating an 18 kDa DNA-binding *trans*-acting factor that specifically recognizes the hAT₁-GAGA sequence. The formation of the complex between the hGH-induced GAGA-BP and the hAT₁-GAGA box is responsible for the hGH-mediated increase in hAT₁ gene transcription *in vitro*. The existence of this relationship *in vivo* is difficult to address in humans; however, studies in the rat show that the GH-mediated transcription of the rat Spi2.1 gene via the rat GAGA box *in vitro* is transposed to an *in vivo* model (hypophysectomized rats) (Simar-Blanchet *et al.* 1998). If the hGH-mediated increase in hAT₁ gene transcription occurs *in vivo*, some of the metabolic effects of altered hGH levels in humans (sodium and fluid retention) may be explained. Isolation of the GAGA-BP will allow an understanding of the molecular mechanisms by which hGH and other growth factors influence hAT₁ gene transcription.

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