Differential transcriptional regulation of the *human thrombin receptor* gene by the Sp family of transcription factors in human endothelial cells

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The mitogenic effects of thrombin are mediated by a G-protein-coupled receptor. Because the effects of thrombin are strongly influenced by the expression of its receptor, an understanding of its regulatory mechanisms is essential. To identify mechanisms of *human thrombin receptor (HTR)* gene regulation, a series of *HTR*-promoter-luciferase constructs were made and transfected into human microvascular endothelial cells for analysis. Deletion from bp –303 to –164 abolished reporter gene expression. Dimethyl sulphate treatment *in vivo* and DNase I footprinting *in vitro* demonstrated that a cluster of three GC box consensus sites was occupied, and electrophoretic mobility-shift assays established that Sp1 and Sp3 both bind to this 3' GC box cluster. We mutated each of the three GC boxes individually and all three

collectively within this 3' cluster. Basal promoter activity was decreased to 46%, 78% and 29% of control for each of the GC boxes mutated individually, and to 6% when the three were mutated collectively. To test the individual abilities of Sp1 and Sp3 to activate or repress *HTR* transcription, we conducted co-transfection experiments with wild-type or mutated *HTR*-promoter-luciferase constructs. Co-transfection with Sp1 significantly augmented wild-type *HTR* promoter activity. Sp3 alone did not affect activity, and inhibited Sp1-mediated activation. Competition for shared binding sites by Sp1 and Sp3 might differentially regulate *HTR* expression in vascular endothelial cells.

INTRODUCTION

The diverse cellular and haemostatic effects mediated by thrombin (reviewed in [1]) can be accounted for by the serine protease activity of this molecule. Thrombin cleaves fibrinogen to generate fibrin monomers that polymerize to form a stable clot [2]. In addition, thrombin exerts its cellular effects through its actions on at least one thrombin receptor [3,4]. This G-protein-coupled receptor is activated by proteolytic cleavage of the N-terminus by thrombin, releasing a 'tethered ligand' that initiates receptor signalling [3].

Targeted disruption of the thrombin receptor gene in mice has demonstrated a critical physiological role for the thrombin receptor in embryonic development and in cellular responsiveness to thrombin [5]. Embryonic death occurs in one-half of mice homozygous for thrombin receptor deletion, whereas mice that survive gestation are grossly normal. Loss of the thrombin receptor attenuates the Ca²⁺ response to thrombin in fibroblasts [5] and blocks thrombin-induced mitogenesis [6]. In contrast, platelet responsiveness to thrombin is not altered in mice deficient in thrombin receptor expression [5].

Given the demonstrated function of thrombin in platelet aggregation, vasoconstriction and mitogenesis of vascular cells [7–12], a significant role for the thrombin receptor in vascular pathology has been postulated [1]. The thrombin receptor is expressed at low levels in normal arteries but is up-regulated after vascular injury in animals [13]. In addition, thrombin receptor expression is increased in humans after percutaneous transluminal angioplasty and in atherosclerotic lesions [14–17]. It is speculated that under these circumstances the thrombin receptor contributes to vascular lesion progression. Activation of the thrombin receptor might enhance vascular smooth-muscle cell growth [18]; blockade of the thrombin receptor with a neutralizing antibody has been shown to inhibit arterial throm-

basis in an African green monkey model of vascular injury [19]. Understanding the mechanisms that regulate the thrombin receptor in vascular cells might therefore provide insight into the pathogenesis of vascular disease.

As a first step in defining the mechanisms of human thrombin receptor (*HTR*) expression, we [20] and others [21] have cloned and characterized the *HTR* gene. Transcription of the *HTR* gene is initiated from multiple start sites, and functional analysis of *HTR* 5' flanking sequence has shown that multiple regulatory elements mediate its promoter activity. Here we define elements that regulate *HTR* proximal promoter activity. Promoter activity is diminished when sequences from bp –303 to –164 are deleted. Within this region, a 3' GC box cluster is bound by both Sp1 and Sp3, and mutation of this cluster virtually abolishes *HTR* promoter activity. In co-transfection experiments, Sp1 trans-activates the *HTR* promoter, whereas Sp3 blocks this effect. Titration of Sp1 and Sp3 dosages might provide a mechanism for differential regulation of *HTR* through the same element.

EXPERIMENTAL

Cell culture

Primary-culture human umbilical-vein endothelial cells (HUVEC) and human aortic smooth-muscle cells were obtained from Clonetics Corp. (San Diego, CA, U.S.A.) and were grown in EGM and SmGM medium respectively (Clonetics). Transformed human microvascular endothelial cells (HMEC) were obtained as a gift from Dr. S. W. Caughman and were grown in EBM (Clonetics) with 2% (v/v) fetal bovine serum, 10 ng/ml epidermal growth factor and 1 µg/ml hydrocortisone. Rat aortic smooth-muscle cells were isolated from the thoracic aortas of 200–250 g male Sprague-Dawley rats by enzymic digestion, as described previously [22]. Cells were grown in DME supplemented with 10% (v/v) heat-inactivated fetal bovine serum,

Abbreviations used: DMS, dimethyl sulphate; EMSA, electrophoretic mobility-shift assay; HMEC, human microvascular endothelial cells; HTR, human thrombin receptor; HUVEC, human umbilical-vein endothelial cells; LM-PCR, ligation-mediated PCR.

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100 i.u./ml penicillin and 100 µg/ml streptomycin. The human promyelocytic cell line HL60 and the human erythroleukaemia cell line HEL were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). HL60 cells were grown in RPMI 1640 supplemented with 5% (v/v) iron-fortified calf serum, 1 µg/ml transferrin and 20 nM sodium selenite, and HEL cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum. Primary-culture cells were passaged every 4–6 days and experiments were performed on cells 3–6 passages from primary culture. Nuclear extracts were prepared as described [23]; protein concentrations in nuclear extracts were measured with the Bio-Rad (Richmond, CA, U.S.A.) protein assay system.

Construction of promoter–luciferase plasmids

Poluc-HTR/–0.5, Poluc-HTR/–0.4, Poluc-HTR/–0.3 and Poluc-HTR/–0.15 were constructed by inserting the 542 bp *Sall/SmaI*, 412 bp *HindIII/SmaI*, 297 bp *DdeI/SmaI* and 158 bp *EcoR47III/SmaI* fragments of the *HTR* promoter into the Poluc plasmid in the appropriate orientation.

Site-directed mutagenesis of the 3' GC box cluster located between bp –202 to –173 of the *HTR* promoter was performed by PCR as described by Higushi et al. [24]. A DNA fragment containing the 542 bp *Sall/SmaI* fragment was used as a template. Amplified fragments were inserted into Poluc and the presence of mutations (in bold below) was confirmed by sequencing. The sequence of the mutant oligonucleotides and the constructs generated were as follows: GC.1mut, 5'-GTCCCGTTCGCC-CCGCTAACCGCCCCAGA-3'; GC.2mut, 5'-GTCCCGCCCGTTCCGCTAACCGCCCCAGA-3'; GC.3mut, 5'-GTCCCGCCCCGCTAACCGTTCCAGA-3'; GC.1,2,3mut, 5'-GTCCCGTTCCGTTCCGCTAACCGTTCCAGA-3'.

Transfections

HMEC grown in six-well plates were transfected by the Lipofection method (Life Technologies, Gaithersburg, MD, U.S.A.) as described previously [20]. HMEC were used for transfection experiments because of their higher transfection efficiency compared with primary culture endothelial cells. The appropriate reporter construct (3 µg) was transfected along with 1 µg of the alkaline phosphatase expression vector pSV2Aap to correct for variability in transfection efficiency. Cell extracts were prepared 48 h after transfection by a detergent lysis method (Promega). Luciferase activity was measured in duplicate for all samples with a Packard top-count scintillation counter and the Promega Luciferase Assay system (Madison, WI, U.S.A.). The ratio of luciferase activity to alkaline phosphatase activity in each sample served as a measure of the normalized luciferase activity. For co-transfection experiments, stated amounts of CMV-Sp1 or CMV-Sp3 were co-transfected with 3 µg of reporter plasmid and an appropriate amount of pCDM8 to equalize the amount of DNA in each transfection, and luciferase activity was normalized to the protein concentration of cell extracts before analysis on the basis of the method of Hagen et al. [25]. Each construct was transfected at least six times, and results for each construct are presented as means ± S.E.M. Relative luciferase activity among constructs was compared by a factorial analysis of variance followed by Fisher's least-significant-difference test. Statistical significance was accepted at $P < 0.05$.

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed as described previously [26]. The probe consisted of annealed synthetic 36 bp complementary oligonucleotides corresponding to bp –360 to –325 (5' GC box

cluster) or 30 bp oligonucleotides corresponding to bp –202 to –173 (3' GC box cluster) of the published *HTR* sequence [20]. Before annealing, the oligonucleotides were labelled with [γ - 32 P]ATP by using polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN, U.S.A.). A typical binding reaction contained 20000 c.p.m. of DNA probe, 0.5 µg of poly(dI-dC)·poly(dI-dC), 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 5% (v/v) glycerol and 10 µg of nuclear extract in a final volume of 25 µl. The reaction mixture was incubated at room temperature for 20 min and fractionated on a 5% (w/v) native polyacrylamide gel in 0.5×TBE buffer (22 mM Tris base/22 mM boric acid/0.5 mM EDTA). To determine the specificity of the DNA–protein complexes, we performed competition assays with 100-fold molar excess of an unlabelled double-stranded specific competitor DNA. To characterize specific DNA-binding proteins, we incubated nuclear extracts with polyclonal anti-human Sp1, Sp3 or egr-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 3 h at 4 °C before addition of probe.

Footprinting *in vivo* by ligation-mediated PCR (LM-PCR)

Dimethyl sulphate (DMS) treatment *in vivo* was performed by the method of Mueller and Wold [27] with modifications. For methylation, cells were treated with 0.1% (v/v) DMS at room temperature for 3–5 min, washed and deproteinized in lysis buffer (300 mM NaCl/50 mM Tris/HCl/25 mM EDTA/0.2% SDS/0.2 mg/ml proteinase K) at 37 °C for 4 h. For methylation of genomic DNA *in vitro*, naked DNA was treated with 0.125% (v/v) DMS for 2 min. Methylated DNA was subsequently cleaved with piperidine.

LM-PCR was performed by the method of Garrity and Wold [28], with the exception that a mixture of 3:1 7-deaza-2'-dGTP:dGTP was used in place of dGTP during primer extension and PCR. A set of primers specific for the *HTR* gene was designed in accordance with the criteria of Mueller et al. [29] to evaluate the region between –303 to –164: primer 1, 5'-CACCCTCTCTCTCCTGACTTCT-3' (–59/–80); primer 2, 5'-AGAGCGCAGGGTGCCACGGGTAA-3' (–112/–135); primer 3, 5'-GAGCGCAGGGTGCCACGGGTAAAGATCA-3' (–113/–140). Numbers in parentheses indicate positions of the starting and ending nucleotides for each primer relative to the translation initiation site. For PCR, DNA was denatured at 95.5 °C for 1 min, annealed at 65 °C for 2 min, and extended at 76 °C for 3 min with Vent polymerase (New England Biolabs, Beverly, MA, U.S.A.) for 20 cycles. G ladders were generated by Maxam–Gilbert cleavage followed by LM-PCR with the same primer sets. Samples obtained by LM-PCR were separated on 6% (w/v) denaturing polyacrylamide gels and detected by autoradiography.

DNase I footprinting *in vitro*

DNA fragments containing bp –303 to –6 of the *HTR* gene were labelled with [α - 32 P]dCTP and [α - 32 P]dATP by using the Klenow fragment of DNA polymerase I. Approx. 10000 c.p.m. (30 pg) of the labelled DNA fragment were incubated with 20 µg of nuclear extract or BSA and 1 µg of poly(dI-dC)·poly(dI-dC) for 20 min on ice and then for 2 min at room temperature. Samples were treated with increasing doses of DNase I (0.005 Kunitz unit with BSA and 0.05 Kunitz unit with nuclear extract) at room temperature for 2 min. Samples were analysed with a 6% (w/v) denaturing polyacrylamide/urea gel. For comparison, a G ladder of the same end-labelled DNA fragment was generated by Maxam–Gilbert chemistry.

RESULTS

Functional analysis of the proximal *HTR* promoter

We have previously cloned and characterized the genomic organization of the *HTR* gene [20]. Functional analysis of the *HTR* promoter in transient transfection assays demonstrated that the deletion of 5' flanking sequence from bp -548 to -164 relative to the translation start site decreased promoter activity to near background levels. To define the functional elements responsible for this activity we constructed a series of luciferase reporter plasmids containing serial 5' deletions through this region (Figure 1). These plasmid constructs in Poluc were co-transfected into HMEC with pSV2Apap (to correct for differences in transfection efficiency), and luciferase activity was normalized to alkaline phosphatase activity. Deletion from bp -548 to -303 did not produce any important differences in *HTR* promoter activity (Table 1), implying that elements in this region are not necessary for basal activity of the *HTR* promoter. Deletion of sequences between bp -303 and -164 significantly decreased *HTR* promoter activity essentially to background levels ($P < 0.05$ compared with Poluc-HTR/-0.3), indicating that most *HTR* promoter activity resides in this fragment.

DNA-protein interactions with the *HTR* promoter

We performed DMS footprinting *in vivo* by LM-PCR [28,29] to determine which DNA sequences of the *HTR* promoter might be constitutively bound by nuclear proteins to account for the

Figure 1 Structure of the *HTR* 5' flanking sequence

A map of the 5' flanking sequence of *HTR* 5' flanking sequence with previously identified transcription start sites (arrows) and potential regulatory motifs. Promoter constructs and EMSA probes used in these experiments are shown.

Table 1 Deletion analysis of *HTR* promoter in HMEC

5' deletions of *HTR* promoter-luciferase reporter constructs were transfected into HMEC by lipofection. Constructs were co-transfected with pSV2Apap to correct for transfection efficiency, and luciferase activity is expressed as a percentage of Poluc activity. The relative positions of constructs are shown in Figure 1. * $P < 0.05$ compared with Poluc-HTR/-0.3.

Plasmid	Luciferase activity (% of Poluc)
Poluc	1.00 ± 0.10
Poluc-HTR/-0.5	17.03 ± 0.24
Poluc-HTR/-0.4	13.07 ± 0.82
Poluc-HTR/-0.3	18.07 ± 1.47
Poluc-HTR/-0.15	2.87 ± 0.27*

Figure 2 Nuclear proteins binding to the *HTR* promoter

(A) DMS footprinting of the *HTR* promoter *in vivo* by LM-PCR. HUVEC were treated with DMS *in vivo*; for comparison, HUVEC genomic DNA was treated with DMS *in vitro*. LM-PCR was performed on DMS-treated DNA with primer sets specific for the *HTR* 5' flanking sequence. Rightward arrows indicate bands that are protected *in vivo*; leftward arrows indicate bands that are hypersensitive *in vivo*. GC 1-3 denote the position of GC boxes in this region. (B) Interactions of nuclear proteins with the *HTR* promoter defined by DNase I footprinting *in vitro*. The bp -303 to -6 DNA fragment of the *HTR* promoter was used to assess DNA-protein interactions on the non-coding strand by DNase I footprinting analysis *in vitro*. The fragments were incubated with BSA or nuclear extract (NE) and were incubated with DNase I. Rightward arrows denote protected guanine residues; leftward arrows mark hypersensitive residues. The positions of consensus GC box elements are shown at the right. Numbers represent the positions of nucleotides in relationship to the translation initiation site.

functional activity of sequences between bp -303 and -164. Compared with HUVEC genomic DNA treated with DMS *in vitro*, protection from DMS modification was observed on G residues at -227, -214, -195, -190 and -178 when HUVEC were treated with DMS *in vivo* (Figure 2a); in addition, G residues at -208, -206, -185 and -172 were reproducibly hypersensitive. No differences in protection from methylation were observed upstream of these residues (results not shown). This pattern of protection and hypersensitivity suggests that nuclear proteins might bind these sequences *in vivo*. When compared with the published *HTR* sequence [20], protected sequences correspond to one of two clusters of three consensus GC box sequences in the *HTR* 5' flanking sequence [30], and to sequences immediately 5' of this cluster. This GC box cluster, which is the more 3' of the two (Figure 1), is located immediately 5' of two major transcription start sites previously identified at positions -173 and -166 relative to the initiation methionine codon [20]. GC boxes might be bound by members of the Sp family of transcription factors [30].

Figure 3 Analysis of nuclear proteins binding the *HTR* 3' GC box cluster by EMSA

A radiolabelled probe corresponding in sequence to the 3' GC box cluster was incubated with nuclear proteins from the indicated cell types. Specific complexes identified are noted as A and B. For competition experiments, binding reactions were performed in the presence of a 100-fold excess of non-radioactive Sp1 consensus sequence, with a 100-fold excess of non-radioactive specific competitor (S) or with a 100-fold excess of a non-specific competitor (NS). Abbreviations: HASM, human aortic smooth-muscle cells; RASM, rat aortic smooth-muscle cells.

Probably because of the high GC content of the *HTR* 5' flanking region, we were unable to footprint the non-coding strand adequately. Therefore, to confirm these results, we performed DNase I footprinting assays *in vitro* with a labelled fragment from bp -303 to -6 of the *HTR* promoter as a probe to disclose interactions protecting the non-coding strand. Protected and hypersensitive residues determined by this method corresponded to the location of the 3' GC box cluster (Figure 2b), similar to the results of footprinting *in vivo*. Two additional protected residues were also observed immediately 5' of the GC box cluster; the significance of these protected residues is uncertain but because they correspond to similar protected patterns identified *in vivo* (Figure 2a), they might reflect additional nuclear proteins interacting with the *HTR* promoter immediately upstream of the 3' GC box cluster. On the basis of the results of footprinting *in vivo* and *in vitro*, we chose to examine further the 3' GC box cluster as a potential regulatory element between bp -303 and -164.

Nuclear proteins bind the 3' GC box cluster

To determine the nuclear proteins bound to the sequences protected by footprinting *in vivo* and *in vitro*, we performed EMSA with an oligonucleotide probe corresponding to bp -202 to -173 of the *HTR* 5' flanking sequence, which contains the 3' GC box cluster. After incubation of the radiolabelled probe with nuclear extract from HUVEC, two shifted bands (A and B) were observed (Figure 3). That the nuclear protein(s) binding this probe are broadly expressed was demonstrated by the fact that a similar or identical binding pattern was observed with nuclear extract from diverse cell lines (HUVEC, HMEC, HEL, HL60, rat aortic smooth-muscle cells and human aortic smooth-muscle cells). Binding could be competed away by a 100-fold excess of unlabelled specific probe but not by a non-specific competitor, in-

Figure 4 Determination of nuclear proteins binding the *HTR* 3' GC box cluster by supershift analysis

The 3' GC cluster probe was incubated with HUVEC nuclear extract (NE) in the presence (+) or absence (-) of competitor. To ascertain the nuclear proteins present in extracts, experiments were performed in which extracts were preincubated with antibodies to Sp1, Sp3 or egr-1. Specific complexes A and B and the supershifted complexes are indicated by arrows.

dicating that the shifted bands are specific. In addition, binding could be competed away by an excess of unlabelled, unrelated GC box consensus oligonucleotide, indicating that binding to the labelled probe is likely to occur via the GC box motifs (Figure 3).

We performed supershift assays to define further the nuclear proteins interacting with the probe. Incubation of the probe with HUVEC nuclear extract produced the two primary complexes, A and B, which were competed away by unlabelled specific competitor oligonucleotide (Figure 4). (Note that weaker complexes migrating slightly faster than complex B are seen in Figures 3 and 4. These complexes were present intermittently in our nuclear extracts and might represent degradation products or non-specific interactions.) When nuclear extract was preincubated with a specific anti-Sp1 antibody before the binding reaction, formation of complex A but not B was partly inhibited and a retarded (supershifted) band appeared, indicating that complex A contains immunoreactive Sp1 protein. Similarly, preincubation of nuclear extract with a specific anti-Sp3 antibody abolished formation of complex B but not A, and also resulted in a supershifted band. In contrast, preincubating HUVEC nuclear extract with an antibody recognizing egr-1, a related zinc finger-containing nuclear protein, did not alter the formation of either complex or result in a supershifted band, demonstrating specificity of the supershift reactions with Sp1 and Sp3 antibodies. Taken together with the results presented in Figure 3, we conclude that both Sp1 and Sp3 are capable of binding the 3' GC box cluster of the *HTR* gene, with Sp1 participating in complex A in this assay and Sp3 participating in the faster-migrating complex B.

The 3' GC box cluster is a functional element of the *HTR* promoter

To determine the functional role of the 3' GC box cluster in *HTR* gene regulation, we mutated each of the GC boxes within the cluster, individually and collectively, to generate the luciferase reporter constructs Poluc-*HTR*/-0.5-GC.1mut, Poluc-*HTR*/-0.5-GC.2mut, Poluc-*HTR*/-0.5-GC.3mut and Poluc-*HTR*/-0.5-GC.1,2,3mut (Figure 1). The mutations were specifically

Table 2 Effect of mutation of GC box consensus sites within the 3' cluster on reporter gene expression

Individual GC box consensus sites within the 3' cluster were mutated individually or collectively in plasmid Poluc-HTR/—0.5 as shown in Figure 1, and the effect of mutations was examined in transient transfection assays. Relative luciferase activity for each mutated construct is expressed as a percentage of Poluc-HTR/—0.5 activity. * $P < 0.05$ compared with Poluc-HTR/—0.5.

Plasmid	Luciferase activity (% of Poluc)
Poluc-HTR/—0.5	100.00 $\pm$ 1.40
Poluc-HTR/—0.5-GC.1MUT	46.10 $\pm$ 1.34*
Poluc-HTR/—0.5-GC.2MUT	78.10 $\pm$ 5.63*
Poluc-HTR/—0.5-GC.3MUT	29.00 $\pm$ 10.69*
Poluc-HTR/—0.5-GC.1,2,3MUT	6.13 $\pm$ 1.23*

Table 3 Trans-activation of the *HTR* promoter by Sp family member proteins

The reporter plasmids Poluc-HTR/—0.5 or Poluc-HTR/—0.5-GC.1,2,3MUT were co-transfected with expression plasmids encoding Sp1, Sp3, both, or empty vector control into HMEC; luciferase activity was measured. Values are presented as percentages of Poluc-HTR/—0.5 transfected with control vector. * $P < 0.05$ compared with Poluc-HTR/—0.5; † $P < 0.05$ compared with Poluc-HTR/—0.5-GC.1,2,3MUT.

Reporter	Trans-activator	Luciferase activity (% of Poluc-HTR/—0.5)
Poluc-HTR/—0.5	—	100.00 $\pm$ 4.70
Poluc-HTR/—0.5	Sp1	435.90 $\pm$ 11.50*
Poluc-HTR/—0.5	Sp3	102.20 $\pm$ 3.69
Poluc-HTR/—0.5	Sp1 + Sp3	91.70 $\pm$ 14.70
Poluc-HTR/—0.5-GC.1,2,3MUT	—	6.10 $\pm$ 0.60
Poluc-HTR/—0.5-GC.1,2,3MUT	Sp1	14.00 $\pm$ 1.30†
Poluc-HTR/—0.5-GC.1,2,3MUT	Sp3	4.65 $\pm$ 0.40
Poluc-HTR/—0.5-GC.1,2,3MUT	Sp1 + Sp3	6.40 $\pm$ 0.40

targeted to alter nucleotides important for Sp1 binding to the GC box [30] and did not compete for Sp1 binding to the native sequence in EMSA experiments (results not shown). Reporter constructs were transiently transfected into HMEC, and activity was compared with that of Poluc-HTR/—0.5, containing native *HTR* 5' flanking sequence. A 2 bp mutation within the distal GC box in the 3' cluster decreased the promoter activity of Poluc-HTR/—0.5 by 54 % (Table 2). Similar 2 bp mutations in the middle and proximal GC boxes decreased promoter activity by 22 % and 71 % respectively. To our surprise, when 2 bp mutations were introduced into all three GC boxes simultaneously, promoter activity was decreased by 94 %, to 6 % of the activity of the native Poluc-HTR/—0.5 construct, essentially abolishing the positive effect of sequences from bp —303 to —164 on transcription. Results of this targeted mutation analysis, in conjunction with the deletion analysis, demonstrate that the 3' GC box cluster is necessary for the complete activation of the *HTR* promoter, and that when this GC box cluster is mutated or deleted, the majority of *HTR* promoter activity is lost. In addition, the results suggest that the level of transcriptional activation derived from the 3' GC box cluster is established in an additive manner through the binding of nuclear proteins to multiple GC boxes, with the proximal of the three sites examined having the greatest effect.

Differential effects of Sp1 and Sp3 on *HTR* promoter activity

Having determined that the GC boxes in the 3' cluster were functionally important and that they bound both Sp1 and Sp3, we next analysed the respective roles of Sp1 and Sp3 in the transcriptional activation of *HTR*. Eukaryotic expression plasmids coding for either Sp1 or Sp3 (or empty vector as a control) were transiently transfected alone or together with Poluc-HTR/—0.5 in HMEC. In comparison with Poluc-HTR/—0.5 transfected with empty vector, transfection with CMV-Sp1 resulted in a significant increase in reporter gene activity (Table 3), with 2 μ g of co-transfected plasmid resulting in a 4.4-fold increase in relative luciferase activity. In contrast, co-transfection of Poluc-HTR/—0.5 with CMV-Sp3 (2 μ g) resulted in no significant change in promoter activity. Interestingly, co-transfection of Sp3 simultaneously with Sp1 abolished the ability of Sp1 to trans-activate the *HTR* promoter, indicating that within the context of the *HTR* promoter, Sp3 might have competitive or partial agonist effects.

If Sp family proteins act entirely through the 3' GC box cluster of the *HTR* promoter, then they should not be able to trans-activate constructs in which this cluster is mutated. Alternatively, the activity of GC boxes in the 5' cluster might be functionally revealed by testing for trans-activation when activity mediated through the 3' cluster is abolished. To decide between these two possibilities we examined the effect of Sp1 and Sp3 on the trans-activation of Poluc-HTR/—0.5-GC.1,2,3mut. As expected, the activity of Poluc-HTR/—0.5-GC.1,2,3mut when co-transfected with empty vector was very low in comparison with the native Poluc-HTR/—0.5 construct (Table 3). In spite of the low level of transcriptional activity of the mutated construct, Sp1 also trans-activated this construct, although to a smaller degree than it did the native construct (2.3-fold compared with 4.4-fold). In addition, Sp3 similarly blocked the effect of Sp1 on Poluc-HTR/—0.5-GC.1,2,3mut activity. Thus, although the 3' GC cluster is essential for maximal *HTR* promoter activity, other Sp1-binding sites are also likely to be operative in the regulation of this gene.

DISCUSSION

In the present paper we have delineated specific DNA elements and nuclear proteins that regulate the transcriptional activation of the *HTR* promoter. A positive regulatory element between bp —303 and —164 has a marked effect on promoter activity in transient transfection assays. Within this element we show by targeted mutagenesis that the 3' cluster of GC boxes is necessary for the activity of this element and that full transcriptional effects from this cluster are mediated through interactions with the GC consensus sites. In addition, we show that both Sp1 and Sp3 bind to this 3' GC box cluster and that these two nuclear proteins have differential effects on *HTR* promoter activity. Differential interactions of Sp family members with this site, in addition to functional interactions of other nuclear proteins with Sp1 and/or Sp3 bound to the 3' GC box cluster, provide a likely mechanism for the regulation of *HTR*.

The effect of Sp3 overexpression on basal transcription and on Sp1-mediated trans-activation in transient transfection assays has not consistently been one of transcriptional repression. In some systems Sp3 expression induces transcriptional activation [31–33], whereas in others it has little effect on basal transcription but blocks Sp1-mediated trans-activation [25,34–36]. On the basis of our results, and in contrast with observations from the *SIS/PDGF-B* and *p21^{CIP/WAF1}* genes [31,32], effects of Sp3 and Sp1 on the *HTR* promoter would fall in the latter category. We

observed no significant differences in activity of *HTR* promoter construct Poluc-*HTR*/−0.5 when Sp3 was overexpressed, but found that the expression of Sp3 neutralized the trans-activation of this construct induced by Sp1. Because Sp3 did not decrease unstimulated expression from the *HTR* promoter, it is unlikely that Sp3 functions in this context either as a pure transcriptional repressor or as a trans-activationally inert competitor with endogenous Sp1 for shared binding sites. It is also difficult to explain why Sp3 might activate promoters under some circumstances yet serve as a repressor of Sp1 under others.

It has recently been demonstrated that Sp3 contains both a transcriptional inhibitory domain and glutamine-rich activation domains, the latter being similar, but not functionally interchangeable, with similar domains in Sp1 [25,34]. In addition, the DNA-binding domain of Sp3 is necessary for its ability to block Sp1-induced trans-activation [25]. It is therefore plausible that Sp3 has partial agonist/antagonist activity and that this activity is regulated both by the relative dosages of Sp3 and Sp1 and by differential usage of the activation and inhibitory domains depending on promoter context. Because both Sp1 and Sp3 might interact functionally with other nuclear proteins [33,37], it is possible that some of these interactions might serve to block preferentially either the activation or inhibition potential of Sp3, as has been proposed previously [33,34]. Alternatively, differentially translated or post-translationally modified forms of Sp3 could be generated *in vivo* to account for the different effects.

The precise role of the Sp family members in *HTR* regulation *in vivo* remains to be determined. Although Sp1 has primarily been considered a transcription factor for housekeeping genes, convincing evidence suggests that it is functionally regulated [38,39] and participates in cell type-specific gene expression [40–42]. Studies have demonstrated that the pattern of Sp1 expression during mouse development overlaps with that of the thrombin receptor [43,44]. Although the level of resolution and stage of examination of the two genes vary between the studies, both are developmentally regulated and highly expressed in similar tissues, including the developing heart, nervous system, haemopoietic system and epithelium. Compellingly, Sp1 and the thrombin receptor are expressed in the fetal liver later than day 12.5 after conception, but in both cases expression is restricted to embryonic haemopoietic cells and is not seen in adjacent hepatocytes. It is plausible, then, that Sp1 might participate not only in the constitutive expression of *HTR* but also in its developmental regulation. If this is so, Sp1 is likely to interact functionally with other transcription factors, as is true for developmentally regulated erythroid genes [45].

Plasmid CMV-Sp1 was provided by Dr. Robert Tjian and plasmid CMV-Sp3 was provided by Dr. Guntram Suske. We thank Joann Aaron for editorial assistance, and Li-Yan Yin and Chris Horaist for technical assistance. This work was supported by Grant HL03658 from the National Heart, Lung, and Blood Institute and a Grant-In-Aid from the American Heart Association, Texas Affiliate (to C.P.), and by Grant HL48667 from the National Heart, Lung, and Blood Institute and a grant from the Clayton Foundation for Research (to M.S.R.).

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