A distal Sp1-element is necessary for maximal activity of the human gastrin gene promoter

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Abstract Studies of transgenic mice have shown that transcriptional control of the gastrin gene exhibits significant species differences. Transfection of the human gastrin promoter in murine cells have depicted proximal Sp1, E-box and CACC elements as the major determinants of transcription. We have examined *cis*-regulatory elements of the human promoter in a human gastrin expressing cell line and find that a distal −135 to −142 Sp1 element is necessary for maximal activity. Alignment of the mouse and human promoters shows that the proximal human Sp1 and CACC elements are not conserved, whereas the E-box element is retained. The distal Sp1 element is present in mouse but exhibits a C to T transition in the core that is likely to reduce binding affinity of Sp1. We conclude that gastrin gene transcription is regulated by distinct elements in man and rodents.

Key words: Transcription factor; Sp1; Gastrin promoter; Gastric adenocarcinoma

1. Introduction

The peptide hormone gastrin is an important regulator of gastric acid secretion and growth of the gastrointestinal mucosa [1]. In fetal life gastrin is expressed in the pancreas [2] and the colon [3], whereas antral G-cells are the main site of synthesis in adults [1]. Hence, gastrin expression is tissue specific and developmentally regulated.

Studies of transgenic mice have indicated that transcriptional control of the gastrin gene exhibits significant species differences and may be controlled by distinct promoter elements in man and rodents. In these experiments the human gastrin promoter did not direct transcription to antral G-cells, but was only active in the liver [4] and the pancreatic islets [4,5]. In contrast, the rat gastrin promoter directed expression to the G cells, while expression in fetal pancreas was absent [5]. Previous studies of the human gastrin promoter in murine cell lines have revealed both positive and negative regulatory *cis*-elements in the proximal upstream regulatory domain [6–9]. In two rat insulinoma cell lines, maximal activity of the promoter was mediated by the concerted action of an Sp1, E-box and a CACC element within 111-bp upstream of the transcription initiation site (Fig. 1).

In this study, we have depicted *cis*-regulatory elements in the human gastrin promoter by deletion analysis in human gastric adenocarcinoma AGS cells. We report the presence of a novel distal Sp1 element necessary for maximal activity of the proximal part of the gastrin promoter. Moreover, we have determined the sequence of the mouse gastrin gene promoter to compare *cis*-regulatory elements between rodents and man.

2. Materials and methods

2.1. Materials

The gastric adenocarcinoma cells, AGS, were obtained from American Type Culture Collection (Rockville, Maryland, USA). pCAT-basic and recombinant human Sp1 transcription factor were from Promega (USA). Rabbit polyclonal Sp1 antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). Control rabbit polyclonal antiserum, c-fos(Ab-2), was from Oncogene Science. The GASCAT1300 plasmid, which contains sequence −1300 to +57 relative to the cap-site [10], was a kind gift from Dr. Jens Vuust, Copenhagen, Denmark.

2.2. Isolation of the mouse gastrin gene promoter

The mouse gastrin gene was isolated from a 129 SVJ mouse genomic library (Stratagene). The library was screened with a mouse gastrin gene specific probe, that had been amplified by PCR using mouse genomic DNA and primers specific for rat gastrin. These primers were complementary to positions 46–59 and 347–356 of the rat gastrin cDNA [11]. The gastrin promoter sequence is reported in this paper, whereas the gene sequence will be reported elsewhere.

2.3. Plasmid constructions

Deletion constructs of the 5′ upstream regulatory region of the human gastrin gene were generated by PCR using GASCAT1300 as template [10]. The 3′ PCR primer encompassed the *Pst*I-site located at position 3241 of the human gastrin gene in exon 1 [12], whereas the 5′ primers were specific for upstream promoter sequences and contained a *Hin*dIII site to facilitate cloning in the p0CAT vector. The p0CAT vector is a pCAT-basic derivative, where the original *Bam*HI site has been removed and a *Hin*dIII site added to the multiple cloning site. The GASCAT142 with a mutated Sp1-site was constructed with the mutant primer: 5′-TAT AAA AGC TTA AGT TCG GGG CAG GGT GAT GGG CTG-3′.

2.4. Cell culture and transfections

AGS cells were cultured at 5% CO_2 and 37°C in Ham's F-12 supplemented with 10% fetal calf serum, 1% glutamine and 1% penicillin-streptomycin. One day prior to transfection, cells were split and 1.5×10^6 cells seeded in 100-mm Petri dishes. Cells were transfected with 20 μg of DNA from the deletion constructs using the modified calcium phosphate method of Chen and Okayama [13] as previously described [14]. To normalize CAT activity cells were co-transfected with 5 μg pCH110, a vector which expresses the β-galactosidase gene [15]. Two days after transfection, cells were harvested and stored at −80°C. The chloramphenicol acetyl-transferase and β-galactosidase assays were performed essentially as described [15,16].

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared essentially as described [14,17]. Approximately 1.5×10^9 cells were harvested, washed and resuspended in 4 ml 10 mM Hepes, pH 7.8, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 2.8 μg/ml aprotinin (buffer A). After 15 min at 0°C the cells were homogenized with a Dounce homogenizer and centrifuged at $4000 \times g$ for 10 min at 4°C. The pellet was resuspended in 3 ml of

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buffer A per 10^9 cells, NaCl added to a final concentration of 450 mM and the extract gently shaken for 30 min at 0°C. After centrifugation at 16,000 × *g* for 60 min at 4°C the supernatant was recovered and $(NH_4)SO_2$ added to a final concentration of 300 mg/ml. The extract was incubated for 60 min at 0°C with gentle stirring. After centrifugation at 16,000 × *g* for 15 min at 4°C, the pellet was resuspended in 0.4 ml of 50 mM Hepes, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF with 10% glycerol, per 10^9 cells and dialyzed against the same buffer. Finally, the extract was centrifuged at 18,000 × *g* for 15 min and the supernatant aliquoted and stored at −80°C. The protein content of the extract was determined using the Bradford method [18].

2.6. Gel mobility shift assay

Approximately 10 fmol of a double-stranded oligonucleotide gastrin Sp1 (gSp1) (5′-AAT GAA AGG GCG GGG CAG GGT GAT GGG C-3′), was labeled with [γ-^{32}P]ATP and incubated with 5 μg of nuclear extract in the presence or absence of unlabelled competitor in 25 mM Hepes, pH 7.9, 150 mM KCl, 10% glycerol, 5 mM DTT at 0°C for 30 min. A double-stranded Sp1 consensus binding site oligonucleotide, (Sp1) (5′-GAT CCC TTG GTG GGG GCG GGG CCT AAG CTG CGC AT-3′), was used as a competitor. Loading buffer with 5% glycerol was added to the samples which were applied to a 4% non-denaturing polyacrylamide gel.

In the supershift assay, the mixtures of labeled oligonucleotide and nuclear extracts were incubated with 0.1–1 μM antibody for 2 h at room temperature. These samples underwent electrophoresis on a 1% agarose gel in 0.5 × TBE which was fixed in 10% trichloroacetic acid and dried in a slab dryer (Bio-Rad) prior to autoradiography.

3. Results and discussion

3.1. Identification of 5′ upstream cis-regulatory elements

Cis-regulatory elements in the human promoter were examined by transient transfection of AGS cells with a series of promoter deletions fused to the *cat* reporter gene (Fig. 2). The gastrin promoter is highly active in AGS cells and exhibits similar activity as the Rous sarcoma virus promoter (data not shown). The proximal 142 bp of the promoter exhibit about 50% of the activity of the 1300-bp region, whereas the proximal 82 bp only provides 25% of maximal activity. Positive elements are located within the proximal 82 bp and in the −84 to −115 and −135 to −142 regions. Putative negative elements are localized in the −82 to −84 and −115 to −135 regions, and upstream of −142 bp. The proximal 82-bp region includes an Sp1 site and an E-box motif (Fig. 1) which is the binding site of transcription factors of the helix-loop-helix family. the −84 to −115 region contains the recently described CACC element that binds a 70-kDa *trans*-acting factor [9]. Maximal activity of the proximal promoter requires the inclusion of bp −135 to −142. Since this stretch contains a putative Sp1 binding site, we examined the binding of this factor.

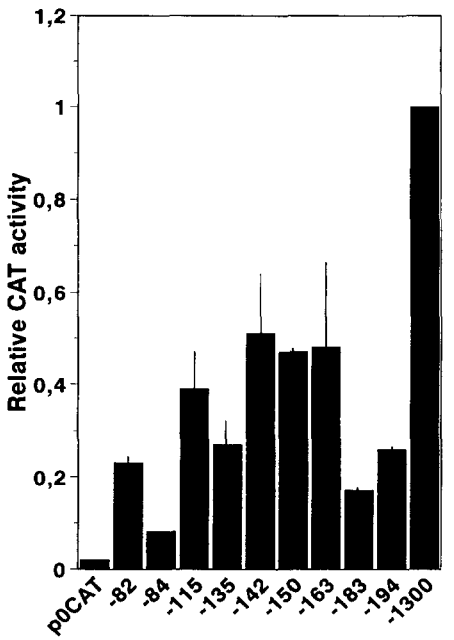

Fig. 2. Transient expression of gastrin gene promoter-*cat* fusion constructs in human AGS cells. Data are expressed relatively to the activity of the −1300 promoter region and are mean ± S.D. of three independent experiments.

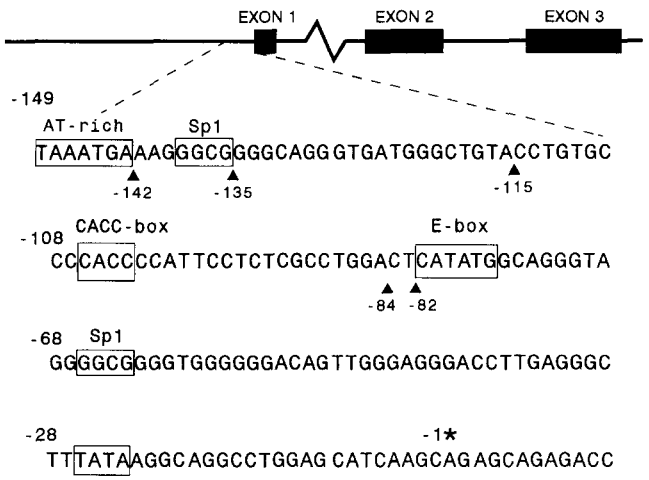

Fig. 1. Structure of the human gastrin gene and sequence of the proximal promoter region. The gene consists of three exons of which exon one comprises the 5′ untranslated mRNA. The blow out shows the sequence of the region −149 to +12 relatively to the cap-site, marked with an asterisk [10]. Previously reported *cis*-elements are boxed, and deletion points of the promoter constructs are marked with arrows.

Recombinant Sp1 bound to the promoter element and comigrate with the major *trans*-acting factor in nuclear extracts (Fig. 3). Binding was competed with 100-fold excess of unlabelled

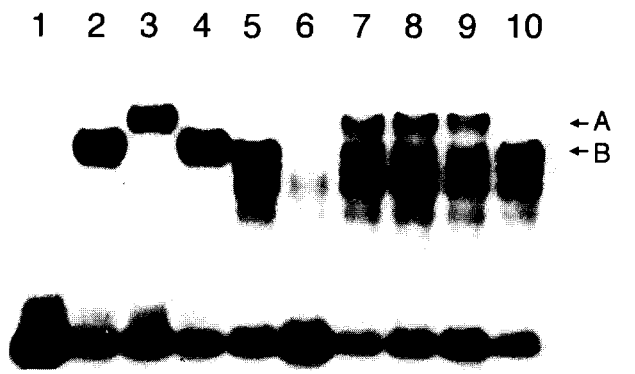

Fig. 3. Gel mobility shift assay of the −135 to −142 Sp1 element. gSp1 was used as labelled probe (lane 1) and added the following: lane 2: recombinant Sp1 (rSp1); lane 3: rSp1 and Sp1 antibody; lane 4: rSp1 and control antiserum; lane 5: AGS nuclear extract; lane 6: nuclear extract and 100-fold excess unlabelled Sp1 probe; lane 7–9: nuclear extract and increasing amounts of Sp1 antibody; lane 10: nuclear extract and control antiserum. The arrow marked B indicates the bound probe, whereas A marks the supershifted band.

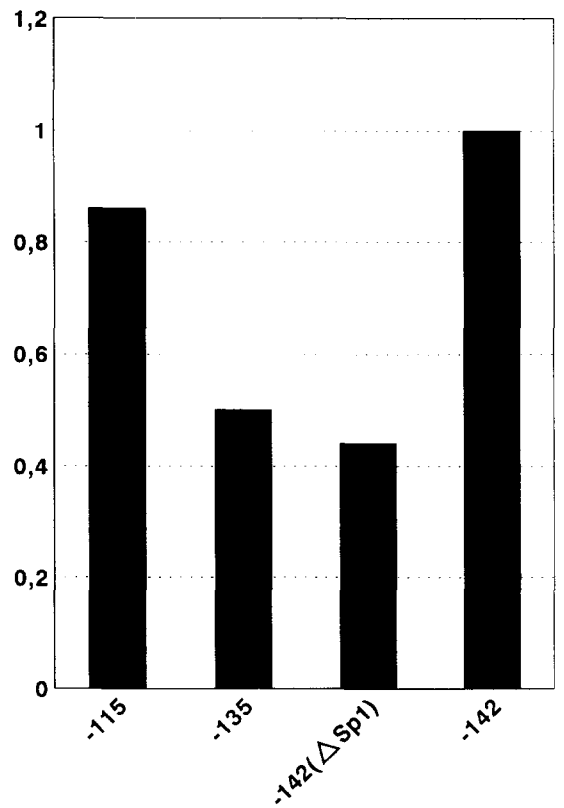

Fig. 4. Mutational effect of the −135 to −142 Sp1 binding site. CAT activities of AGS cells transfected with human gastrin promoter deletions and the mutant −142(ΔSp1), in which the GGCG core of the Sp1 site has been altered to TTCG.

Sp1 consensus probe, and Sp1 specific antibodies supershifted the *trans*-acting factor in nuclear extract. In the samples with nuclear extract two additional bands are apparent in the gel mobility shift assay. Binding was competed with 100-fold excess of Sp1 probe indicating that the factors represent isoforms of Sp1, e.g. the phosphorylated form, and/or interactions with other factors. To examine the functional significance of the Sp1 element the cells were transfected with constructs including a mutation in the Sp1 binding site. A double mutation in the core consensus sequence reduces promoter activity to the level of the −135 construct (Fig. 4), indicating that Sp1 binds a distal site in the gastrin promoter and is necessary for maximal activity of this region.

Sp1 is a general transcription factor involved in the control of a variety of genes. It has been suggested, that Sp1 is normally present in limiting amounts which may increase in response to physiological stimuli [19]. Moreover, Sp1 levels varies up to 100-fold with the highest expression in cells undergoing late stages of differentiation, which includes antral gastrin expressing cells [19]. Therefore Sp1 is likely to be involved in both basal and regulated of gastrin gene transcription.

3.2. Comparison of human and mouse cis-regulatory elements

To examine the conservation of the respective *cis*-regulatory elements, we cloned and sequenced the proximal part of the mouse gastrin gene promoter (Fig. 5). When comparing the proximal 300 bp of the mouse and human gastrin promoters they were found to possess an overall homology of 72% with the region immediately 5′ of the cap-site, including the TATA-box, being highly conserved. Similarly, the E-box motif of the human promoter (Fig. 1, 5′-CATATG-3′), which is the binding site of transcription factors of helix-loop-helix family and enhances transcription in murine cells [6], is conserved in the mouse. However, only the consensus element of the E-box is conserved (5′-CANNTG-3′). This suggests an important role of the E-box in regulation of gastrin gene expression.

In this study, we have demonstrated the importance of a distal Sp1 binding site that has also recently been reported to be active in murine cells [20]. This element is also conserved in the mouse promoter. However, the 5′-GGCG-3′ core of the Sp1 binding site has been altered to 5′-GGTG-3′. Though this constitutes a putative Sp1 binding, the substitution reduces binding affinity of Sp1 6-fold compared to that of the ideal Sp1 consensus [21].

Apart from these *cis*-regulatory elements, there are substantial differences between regulatory elements in the human promoter and putative elements in the mouse promoter. For instance, enhancement of gastrin transcription by EGF has been proposed to be mediated by a Sp1 binding site at positions −68 to −53 of the human promoter [22], however this element is not present in the same region of the mouse promoter. Similarly, the CACC-box proposed to be the binding site of a 70-kDa activator is not conserved except as a part of the mouse E-box. In the mouse the presence of an element equivalent to an AT-rich region in −149 to −143 bp of the human promoter is uncertain. This element was recently proposed to be the binding site of a regulator of expression in insulinoma cells [20]. In the

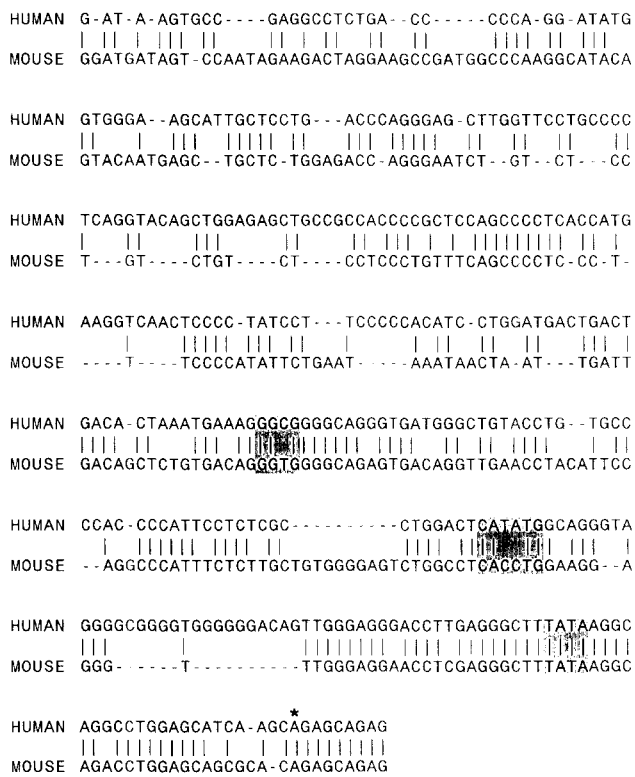

Fig. 5. Comparison of the human and mouse gastrin promoters. The sequence from −327 to +9 of the human promoter and from −301 to +9 of the mouse promoter were aligned. The conserved *cis*-regulatory elements are shaded in grey. The cap-site is marked by an asterisk.

mouse, an AT-rich stretch is located nearby in the mouse promoter, but it is not clear if the factor described in murine cell extracts will bind to this element.

In line with the previous observations in transgenic mice [4,5], our data suggest that there are important differences in the transcriptional regulation of gastrin expression in man and rodents. Hence, these differences should be taken into account in the analysis of transcriptional activation of the gastrin gene.

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