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GATA-6 DNA binding protein expressed in human gastric adenocarcinoma MKN45 cells

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Abstract **A cDNA for the GATA-6 (GATA-GT1) DNA binding protein was cloned from a library of the human gastric adenocarcinoma cell line MKN45. The deduced amino acid sequence (449 residues) indicates that the primary structure of human GATA-6 is highly homologous to that of the rat protein. The potential phosphorylation site for protein kinases (A and C), and histidine and alanine clusters are conserved. Whereas the rat H^+/K^+-ATPase α and β subunit genes have two and three GATA protein binding sites in their promoter regions, respectively, the human α subunit gene has only one binding site [Maeda, M., Kubo, K., Nishi, T. and Futai, M. (1996) J. Exp. Biol. 199, 513–520]. We cloned the 5′-upstream region of the human H^+/K^+-ATPase β subunit gene by genome walking and found that it also has a single GATA protein binding site near the TATA box. The GATA sites of the human α and β subunit genes are recognized by the zinc finger domain of human GATA-6. The conservation of the GATA protein binding sites suggests that they are important for the gene regulation of the human and rat H^+/K^+-ATPase.**

Key words: GATA factor; DNA binding protein; MKN45 cells; Gastric carcinoma; Parietal cells; Transcriptional regulation

1. Introduction

A GATA DNA binding protein family with a zinc finger domain [$CysX_2CysX_{17}CysX_2Cys$-X_{29}-$CysX_2CysX_{17}CysX_2$-Cys] plays important roles in tissue and cell specific transcription [1,2]. We reported that gastric GATA proteins (GATA-6 and GATA-4, previously named GATA-GT1 and GATA-GT2, respectively) are transcribed in the rat gastrointestinal tract [3]. They are suggested to play roles in the expression of H^+/K^+-ATPase in parietal cells [4], since the mRNAs of gastric GATA proteins have been detected in the same rat cells [5]. Actually, the [(G/C)PuPu(G/C)NGAT(A/T)PuPy] sequences located immediately upstream of the TATA boxes of the rat H^+/K^+-ATPase α and β subunit genes are recognized by GATA binding proteins [6,7]. Furthermore, the above binding sequence in the rat β subunit gene [6] contains typical GATA motifs [(A/T)GATA(A/G)] [1].

To elucidate the function of GATA proteins in human parietal cells, we were interested in whether or not GATA protein(s) are expressed in the human gastric adenocarcinoma cell line MKN45. This cell line retains the parietal cell function [8]; MKN45 cells express the H_2 receptor and respond to histamine to increase the cytoplasmic cAMP concentration [9], although H^+/K^+-ATPase cannot be detected. In this study, we found GATA-6 mRNA in MKN45 cells and deduced the primary structure of human GATA-6 from the cloned cDNA. We also found that a single GATA site was located in the promoter of the human β subunit gene similar to in the human α subunit gene [3], whereas the rat α and β subunit genes have tandem GATA sites in their promoters [4]. We discuss the transcription of H^+/K^+-ATPase genes from the viewpoints of the similarities and differences between human and rat.

2. Materials and methods

2.1. cDNA cloning of human GATA-6

A poorly differentiated human gastric adenocarcinoma cell line, MKN45 (JCRB0254) [10], was cultured at 37°C in RPMI 1640 medium containing 10% fetal calf serum. Total cellular RNA was extracted by the guanidine thiocyanate-CsCl method [11], and poly(A) RNA was isolated using Oligotex-dT30 <Super> (Daiichi Chemical, Tokyo). Single-stranded cDNAs were prepared from poly(A) RNA with a Pharmacia First-Strand cDNA Synthesis Kit. About 5 ng was subjected to PCR [12] together with degenerate primers corresponding to the conserved regions of the zinc finger domain of GATA binding proteins [3]. A cDNA library of MKN45 cells was constructed from poly(A) RNA with a Pharmacia TimeSaver cDNA Synthesis Kit using λgt10 as a vector. Replica filters (Schleicher and Schüell, Dassel, Germany) were hybridized [11] with DNA fragments labeled with a Random Primed DNA Labelling Kit (Boehringer Mannheim, Germany) or an ECL Random Prime Labelling System (Amersham Life Science). The positive clones were subcloned into pBluescript SKII$^-$, and both strands were sequenced with the dideoxy chain termination method [13] using a Silver SEQUENCE DNA Sequencing System (Promega, WI).

2.2. PCR amplification of the 5′-upstream region of the human H^+/K^+-ATPase β subunit gene

Human liver chromosomal DNA (1 μg) [7] was digested with restriction enzyme *Eco*RI and ligated with an *Eco*RI cassette (0.1 μg), and then used as the template for PCR amplification according to the manual from Takara (Kyoto). The PCR primers used were cassette specific primers (C_1, 5′-GTACATATTGTCGTTAGAACGCG, and C_2, 5′-TAATACGACTCACTATAGGGAGA) and gene specific primers ($HKhB_1$, corresponding to 61–81 bp (sense strand) of human H^+/K^+-ATPase β subunit cDNA, and $HKhB_2$, corresponding to 36–59 bp (sense strand) of the cDNA [14]). They were synthesized with a Pharmacia LKB Gene Assembler Plus. The reaction mixture (40 μl), comprising 50 ng chromosomal DNA, 1 μM C_1 and $HKhB_1$ primers,

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Abbreviations: GST, glutathione *S*-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline (10 mM sodium phosphate buffer (pH 7.2), 137 mM NaCl, 3 mM KCl); PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends

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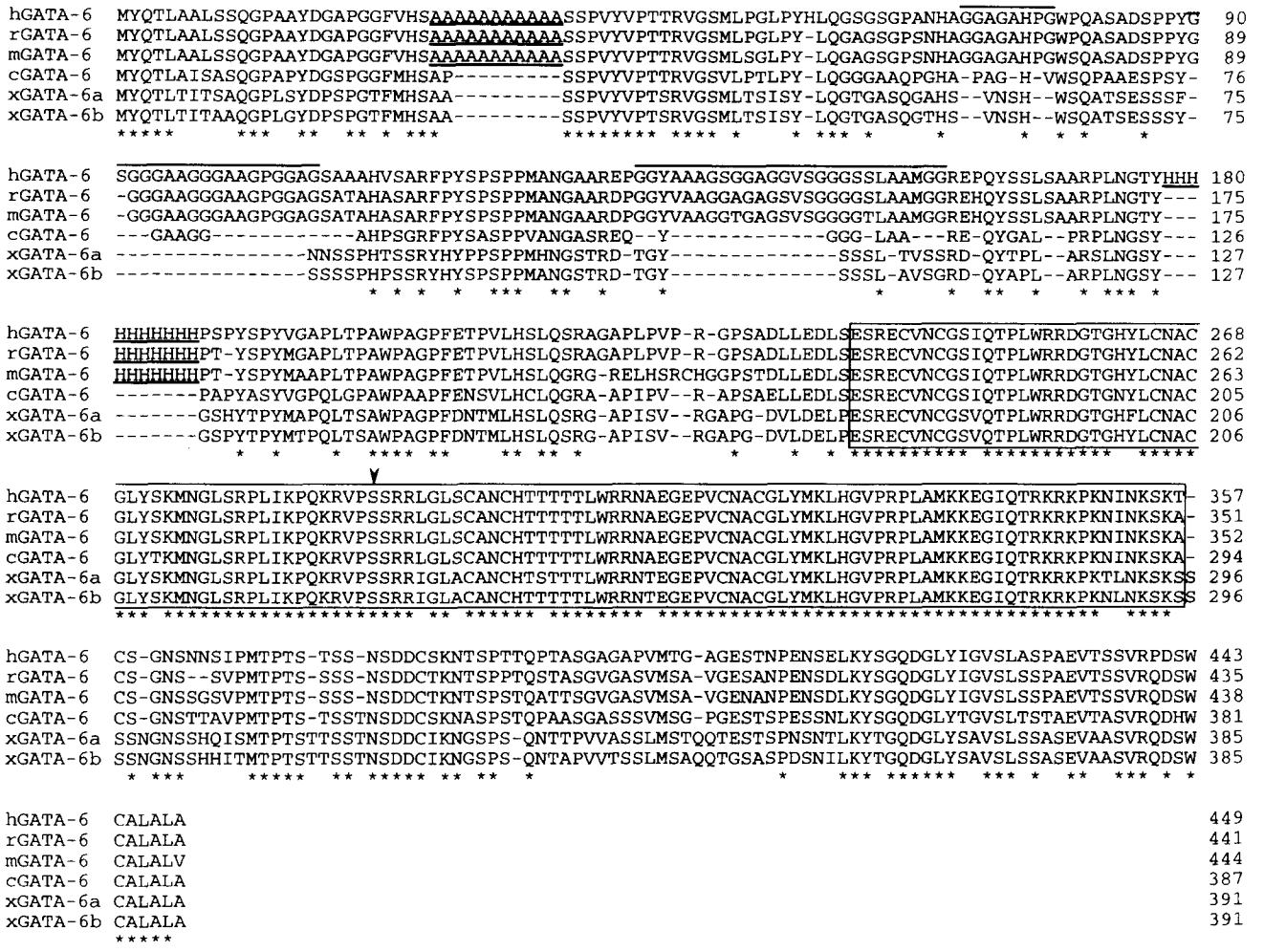

Fig. 1. Sequence comparison of GATA-6 proteins. The amino acid sequences of the human (this study), rat [3], mouse [20], chicken [19] and *Xenopus* [21,22] GATA-6 proteins are aligned. The zinc finger domain [2] is boxed. We define a $(CX_2C)X_{17}(CX_2C)$ sequence as a zinc finger segment. Alanine and histidine clusters are underlined. The regions rich in Gly residues are overlined. The potential phosphorylation site [3,4] for cAMP-dependent protein kinase A [LysArgXX(Ser/Thr)] and protein kinase C [ArgXX(Ser/Thr)XArg] is indicated by an arrowhead. Asterisks and hyphens show identical residues and gaps, respectively. The rat sequence extends 50 residues further upstream compared with that in the previous report [3]; around the *Pvu*II-*Sac*II region (−70–45 bp) of the original rat clone was re-sequenced and the nucleotide sequence revised with corrections. The 5′-upstream sequence of the mouse clone has a termination codon in frame from the codon for the potential initiator methionine (Met^1) [20]. During the preparation of this article, the human GATA-6 sequence was submitted to GenBank (U66075) [34]. The nucleotide sequence comparison indicated that three and two residues in the 5′- and 3′-noncoding regions, respectively, are substituted. The third letter of the Gly^{92} codon is also substituted. These changes could be ascribed to the fact that the specimens were from different races.

0.2 mM each dNTP and 1 unit *Taq* DNA polymerase (Perkin-Elmer Cetus), was treated as follows: preheating (94°C, 2 min), followed by 25 cycles of denaturation (94°C, 0.5 min), annealing (55°C, 2 min) and extension (72°C, 2 min). The second PCR was performed using 1 μl of the first product, and primers C_2 and $HKhB_2$. The amplified fragment was electroeluted from the polyacrylamide gel, treated with T4 polynucleotide kinase and the Klenow fragment, and then ligated into the *Eco*RV site of pBluescript $SKII^+$. The transcriptional start site was determined by means of the 5′-RACE technique. Single-stranded cDNAs derived from human stomach mRNA (purified from autopsy material supplied by Dr. Y. Miyazaki, Faculty of Medicine, Osaka University) were subjected to PCR with a 5′-RACE System (Gibco BRL) using a primer corresponding to 81–64 bp of the β subunit cDNA [14]. Both strands of the amplified fragments were sequenced with the dideoxy chain termination method.

2.3. Gel mobility shift assay

The zinc finger domain of human GATA-6 (Glu^{240}–Thr^{357}) was fused with GST: the PCR-amplified cDNA moiety of the zinc finger domain was inserted between the *Bam*HI and *Eco*RI sites of pGEX-2T. The plasmid construct (pGEX-hZf) was introduced into *E. coli* Top10F′ (Invitrogen), and a fusion protein (GST-Zf) was induced for 2 h at 30°C in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside. A cell lysate was prepared and the GST-Zf was affinity-purified on glutathione Sepharose 4B (Pharmacia) [15]. GST was purified similarly from *E. coli* carrying pGEX-2T. An aliquot (0.4 μg) was incubated with a digoxigenin-labeled DNA probe (DIG Gel Shift Kit; Boehringer Mannheim) encoding the GATA site of the human H^+/K^+-ATPase β subunit gene (−111 to −89 bp). The competitors used are indicated in the text.

2.4. Expression of human GATA-6 in COS-1 cells

pME-hGT1 was constructed by inserting the cDNA (−132 to 1659 bp) for human GATA-6 between the *Xho*I and *Spe*I sites of pME18S [16], and then introduced into COS-1 cells (5×10^5 cells plated on a 3.5 cm diameter dish) by the DEAE-Dextran method [11]. The cells were cultured for 48 h in Dulbecco's modified Eagle medium containing 7% fetal calf serum at 37°C. The translated GATA-6 was detected by Western blotting after SDS polyacrylamide gel electrophoresis, using rabbit antibodies recognizing the carboxyl terminal region of rat GATA-6 (Thr^{383}–Ala^{411}) [17]. Protein concentrations were determined with a Bio-Rad assay kit using bovine serum albumin (Fraction V; Sigma) as a standard [18].

2.5. Chemicals

Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, calf

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human  GAATTCCTCCATGGACACAAAAAAATCCTAAAGGGCCCTCGGTGCCACTGCCCTCTGGGGATCTAAGAGGCTATTGCTGCTCTGGGTGAA  -600

                          P1                                                            P2
human  TCTGGGAGGCAGAGGAGTCCCCGGGGACCCCTGTGTTCCCTGCCAACCCAGCTCCAGCTGCCAGGCTCACCCTTCCACTGGGTGACCCAG  -510

                                                                              D
human  AATCGGGGCGGCCGCAGCGGGGGGCCTAGGGAGCCGGTCGCCCCTCACTCGCCTGCCTCACGCCCTCCCAGGCCAGGGATGCGGCCCCAG  -420

human  CTGGCTCAGCCCAAGGTCGTGGACACAGTGGGTCCAGGCCGGCTCTGCTCTGACCTCAATCTCCCTCCTGAAGGAGAGGAGGGAAGGGGA  -330

human  AGGGCCTGTGAGTGCTGCTCACCCCGGAACGTGCCCTCACCGCAATCAGAGCAGACAAAGCTG-CAGCCAAGGGCGTCCCAA-GAGCAGC  -242
rat                                                              AttAAGtTGtCAGCCAAGGGCacCCCAAgGAcCA--  -178
                    P3       P4         P5
human  AGGGTGAGGAAAGCGGGCCTGGCCCGCCCCAGGGGCAACAGCCGGGCTGCACCCAGC---CCCTGGTGTTTGGTGAGGCGATGGCCG-GC  -156
rat    Ac--TGA--------cttCTGGgaCagtggAGGacagAtAGCacGcaaGC-CCCAGCcctCCCTtaTGTTTataGAGGCGATaGCgGaGa  -98

human  GCTGATAAACGGCCCTGGCCTCTCCCTGGGATCCTTCGCCTGCGATGCCTTTGACCTCTGGCAGAGGAAACTATAAAGCCCAGAGGTGGG  -66
rat    aCTGATAgctGG---------------------TTC-----tGATGCCTTTGACCTCacaCAGAGGAgACTATAAgcCCCAGAGGacGc  -35
                                                              D                +1
human  CATCTGGCCTCAGTCTGGGCGTAGAGGGTGCAGGGAGCAGACGGGAGGATCTCAGGCCAGGGACG   ATGGCGGCTCTGCAGGAGAAG  +21
rat    tccCTGGgCcCAGTCcaGGC-------------aAGCAG--GaGAGGAc---------------   ATGGCaGCcCTGCAGGAGAAG  +21
                                                                           M  A  A  L  Q  E  K
human  AAGACGTGTGGCCAGCGCATG
rat    AAGtCaTGcaGCCAGCGCATG
       K  T  C  G  Q  R  M
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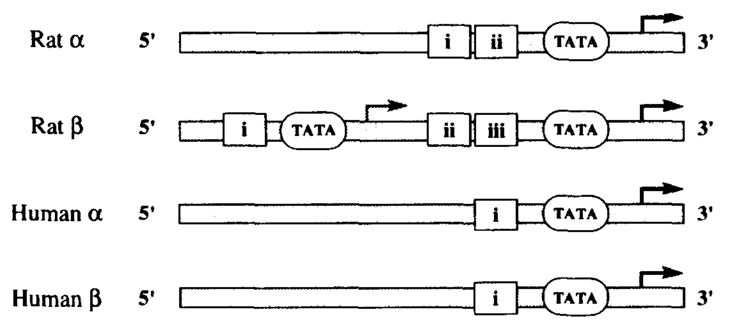
Fig. 2. Nucleotide sequence of the 5′-upstream region of the human gastric H^+/K^+-ATPase β subunit gene. The nucleotide sequence of the human H^+/K^+-ATPase β subunit gene amplified by PCR is shown together with the rat sequence [6]. Nucleotides are numbered on the right of each line from the translational start site. Nucleotide residues of the rat gene identical to those of the human are indicated by capital letters and different residues by small letters. In the coding region, amino acid residues are also indicated. The transcription start site of the human gene (this study) is indicated by a thick arrow. The potential binding sites for basal transcription factor TFIID [TATA box, consensus TATA(T/A)T(T/A)] and Sp1 (GC box, GGGCGG or CCGCCC), and CACCC box (CACCC and GGGTG) [24] are shown by boxes, wavy underlines and underlines, respectively. GATA sites are double underlined. Direct repeats (D) with more than 10 residues and palindromes (P1–P5) with continuously identical residues (more than 5) are also indicated.

A)



B)

	GATA protein binding site	TATA-box	Transcriptional start site
Rat α	i CAGCTGATTAT (-103 ~ -113) ii CAGCTGATTAC (-93 ~ -103)	-65 ~ -59	-31
Rat β	i GGACAGATAGC (-213 ~ -203)	-175 ~ -170	-91
	ii GAGGCGATAGC (-171 ~ -161) iii GAACTGATAGC (-157 ~ -147)	-54 ~ -48	-23
Human α	i CAGCTGATTAC (-100 ~ -110)	-59 ~ -53	-30
Human β	i GCGCTGATAAA (-157 ~ -147)	-84 ~ -79	-54

Fig. 3. Locations of the GATA sites in the 5′-upstream regions of the H^+/K^+- ATPase α and β subunit genes. A: The locations of the GATA protein binding sites in the 5′-upstream regions of the α and β subunit genes for the human and rat H^+/K^+-ATPase [6,7,24] are shown schematically together with the TATA boxes. Arrows indicate the transcription start sites (this study, [25,35]). The minor start site for the rat β subunit gene [25] is also indicated by a gray arrow. B: The positions of the GATA protein binding sites, TATA boxes and transcriptional start sites. The nucleotide residue numbers are calculated from the first letter of the initiation codon.

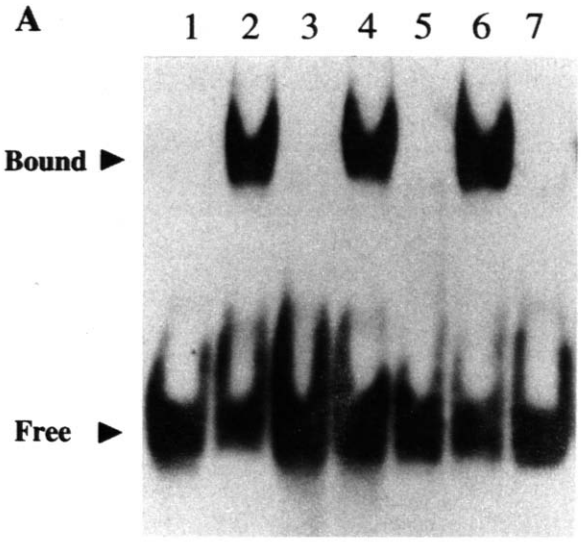


Fig. 4. Binding of the zinc finger domain of human GATA-6 to the GATA motif. The DNA binding of GATA-6 was examined by means of a gel mobility shift assay. A: The affinity-purified fusion protein (GST-Zf) (0.2 μg) was incubated with 0.4 pmol of probe β [a digoxigenin-labeled DNA probe encoding the human H^+/K^+-ATPase β subunit gene (−111 to −89 bp from the first letter of initiation codon)]. Lane 1 (left side), without GST-Zf; lanes 2–6, with GST-Zf; lane 7, GST. Excess competitors (200 pmol) [unlabeled β, βμ (mutant version of β), α (portion of the human H^+/K^+-ATPase α subunit gene (−67 to −89 bp) and αμ (mutant version of α)] were added (lanes 3–6). B: The nucleotide sequences of oligonucleotides β, βμ, α and αμ are shown. The GATA sites are underlined.

intestine phosphatase and the Klenow fragment were obtained from Takara Shuzo (Kyoto), Toyobo (Osaka), New England BioLabs (Beverly, MA), or Nippon Gene (Toyama). [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) was from the Radiochemical Center, Amersham. Sera and medium were purchased from Gibco BRL. All other chemicals used were of the highest grade commercially available.

3. Results

3.1. Cloning of human GATA-6 cDNA

The cDNAs synthesized from poly(A) RNA of MKN45 cells were subjected to PCR using common primers to amplify messages for GATA family proteins [3], and the amplified 194 bp DNA fragments were cloned. Interestingly, all 10 clones randomly sequenced encoded the zinc finger domain of the GATA-6 protein, suggesting that the GATA-6 messages could be dominant among the GATA family members [19] in MKN45 cells. The amplified segment was used for screening a cDNA library derived from MKN45 cells. Six partial clones were isolated from among 7×10^5 plaques, and then a full-length clone was obtained from among 3×10^5 plaques by hybridization with the *Nco*I fragment (between codons 157 and 330) of a partial clone. Sequence determination demonstrated that human GATA-6 comprises 449 amino acid residues (Fig. 1).

3.2. Structure of human GATA-6

Pairwise comparisons of the primary sequence of human GATA-6 with those of rat [3], mouse [20], chicken [19], and *Xenopus* (GATA-6a and GATA-6b) [21,22] indicated that 414, 398, 317, 263 and 274 residues, respectively, were identical. The zinc finger domains [2] of the six GATA-6 proteins, and the Ser residue between the two zinc finger segments potentially phosphorylated by protein kinases A and C were conserved (Fig. 1). The Ala and His clusters were conserved in mammalian GATA-6, but both were deleted in the chicken and *Xenopus* proteins. Furthermore, many Gly residues (overlines in Fig. 1) were conserved in the mammalian proteins. These regions only found in the mammalian GATA-6 may play unique roles in the interaction with proteins necessary for formation of the mammalian transcriptional initiation complex. The initiator methionine was determined from the molecular size of the GATA-6 protein expressed transiently in COS-1 cells (not shown).

3.3. 5′-Upstream region of the human β subunit gene

We reported the potential role of GATA protein(s) in the transcriptional regulation of rat gastric H^+/K^+-ATPase subunit genes: the tandem GATA sites in the promoter regions of the rat α and β subunit genes are critical for transcriptional activation [23]. However, the human α subunit gene has a single GATA site [4,24]. To determine how many GATA sites exist in the human β subunit gene, we amplified the 5′-upstream region of the gene by PCR genome walking. As shown in Fig. 2, a typical GATA motif was located immediately upstream (63 bp) of the TATA box. 5′-RACE suggested that the transcription actually starts 25 bp downstream of this TATA box. Sequence comparison between the 5′-upstream regions of the human and rat β subunit genes suggested that the nucleotide sequences around the TATA box and GATA site are highly conserved (Fig. 2). Thus, these conserved regions of the human β gene may be important for transcription initiation similar to those of the rat genes [23,25].

3.4. Binding of GATA-6 to the GATA site of the human β subunit gene

The sequences of the GATA sites of the human and rat subunit genes were compared, and their positions are schematically shown in Fig. 3. Comparison of these sequences, which are located 30–110 bp upstream of TATA boxes, suggested that the consensus sequence could be (G/C)-APuCTGAT(A/T)PuC. It must be noted that a single GATA site and the TATA box of the rat β subunit gene function together in vivo, since minor transcription occurs from downstream of this TATA box, as shown in Fig. 3A (the gray arrow) [25].

The gel mobility shift assay demonstrated that the GATA site in the human β subunit gene was recognized by the zinc finger domain of human GATA-6 (Glu^{240}–Thr^{357}) (Fig. 4, lanes 1 and 2). The shifted band due to protein binding to the probe disappeared in the presence of excess amounts of the unlabeled probe (lane 3). The oligonucleotide carrying the GATA site of the human α subunit gene was also an effective competitor, whereas those with mutations in the GATA sites of the human α and β subunit genes did not compete (lanes 4–6). The GST moiety did not interact with the probe DNA (lane 7). These results clearly indicate that human GATA-6

could bind to the GATA sites near the TATA boxes of the human α and β subunit genes.

4. Discussion

The present results suggest that the genes for the human and rat H^+/K^+-ATPase subunits could be regulated by GATA protein(s). The fragments carrying the GATA site and TATA box of the human α and β subunit genes (−622–+4 and −689–+42, respectively) could confer the promoter activity responsive to the GATA-6 protein in a reporter gene assay system (data not shown). The MKN45 cells show the properties of gastric parietal cells, since not only the H_2 receptor [9] but also GATA-6 messages are transcribed, as shown in this study. However, specific antibodies failed to detect H^+/K^+-ATPase in these cells, possibly due to the absence of regulator(s) important for transcriptional activation with GATA-6. In this regard, GATA-1 functions with other transcriptional factors such as Sp-1 and EKLF [26].

Furthermore, we speculate, from the different numbers of GATA sites in the human and rat genes, that the modes of transcription in these two species could be slightly different. The finding that tandem GATA sites are essential for transcriptional activation of the rat subunit genes [23] suggests that interaction with an enhancer carrying binding sites for the GATA protein and/or other factors [27] could be critically important for transcription of the human subunit genes. Thus, we have special interest in identifying parietal cell-specific enhancers and co-regulators with gastric GATA proteins. The distribution of gastric GATA proteins in the heart as well as the gastrointestinal tract [3,19–22,28–33] could also be explained by the idea that they may function in the interactions with different tissue specific factors.

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