J. Biochem. **135**, 639–650 (2004)
DOI: 10.1093/jb/mvh077

A Unique Role of an Amino Terminal 16-Residue Region of Long-Type GATA-6

Mika Takeda, Kanako Obayashi, Ayako Kobayashi and Masatomo Maeda*

Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871

Received March 12, 2004; accepted April 2, 2004

Human GATA-6 mRNA utilizes two Met-codons *in frame* as translational initiation codons in cultured mammalian cells. An internal ribosome entry site (IRES) is not present in front of the coding region for short-type GATA-6. The 5′-upstream sequence with a short upstream open reading frame (uORF) did not affect the production of either long- or short-type GATA-6. Introduction of a canonical Kozak sequence around the upstream Met-codon resulted in predominant synthesis of long-type GATA-6, suggesting that the translation of short-type GATA-6 could be due to leaky scanning of the Met-codon by ribosomes. We found that at least the sequence comprising the 90th to 139th nucleotide bases from the first letter of the upstream Met-codon plays a positive role in the expression of long-type GATA-6. This was confirmed by insertion of the corresponding sequence in frame at the site of deletion (the 38th to 304th nucleotide residues). However, insertion of the sequence comprising the 92nd to 141st bases did not suppress the negative effect of the deletion. These results suggest that the translation of this region (Glu-31–Cys-46) could be critical for the apparent production of long-type GATA-6. We also demonstrated that long-type GATA-6 is potentially more active than the short-type.

Key words: DNA binding protein, GATA-6, GATA factor, Kozak sequence, protein synthesis, translation.

Abbreviations: bp, base pairs; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine-tetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; IF, intrinsic factor; IRES, internal ribosome entry site; PBS, phosphate-buffered saline [10 mM sodium phosphate buffer (pH 7.2), 137 mM NaCl, 3 mM KCl]; PBS-T, PBS containing 0.1% (v/v) Tween 20; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; TAE, Tris-acetate [40 mM Tris-acetate, 1 mM EDTA (pH 8.0)]; TBS, Tris-buffered saline [140 mM NaCl, 3 mM KCl , 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.9 mM Na_2HPO_4, 25 mM Tris-HCl (pH 7.4)]; Tris, Tris (hydroxyl)aminomethane; uORF, upstream open reading frame.

Transcription factor GATA (*1*, *2*) with conserved tandem zinc fingers, (CVNC-X_{17}-CNAC)-X_{29}-(CXNC-X_{17}-CNAC) (*3*, *4*), binds to a canonical DNA motif (G/A)GATA(A/T) (*5–8*), and regulates the expression of various genes required for developmental processes and tissue-specific functions (*1–3*). Mammalian GATA factors are classified into two groups; GATA-1/-2/-3 (*1*) function in haematopoietic cells, whereas GATA-4/-5/-6 (*2*) function in mesoderm- and endoderm-derived organs such as the heart and gastrointestinal tract, respectively. It is believed that GATA factors other than GATA-5 are essential for mammalian development and mutually indispensable, since disruption of these genes caused embryonic lethality in mouse (*9–16*).

GATA-6 was cloned from a rat stomach cDNA library together with GATA-4, the clones being as GATA-GT1 and GATA-GT2, respectively (*17*), and then from mouse (*18*) and man (*19–21*). Human GATA-6 was expressed in fetal heart, gastrointestinal tract and lungs, and in adult heart, lungs, liver, kidneys, pancreas, spleen and small intestine (*19*, *20*). Mouse GATA-6 was expressed in the arterial system, lungs and urogenital tract (*18*). GATA-6 transcripts were detected in human and rat vascular myocytes (*19*), and rat gastric corpus mucosal cells (*22*). Consistent with the tissue distribution of its mRNA, GATA-6 transactivated the promoter-enhancers of genes in heterologous cells, such as those of cardiac troponin C, atrial natriuretic factor, B-type natriuretic peptide, surfactant proteins A and C, thyroid transcription factor 1, WNT7b, lactase-phrolizin hydrolase, H^+/K^+-ATPase, trefoil peptides, Dab2 and smooth muscle-myosin heavy chain (*18*, *23–32*).

Co-expression of GATA-6 and GATA-4 in the heart suggests their functionally redundant roles in this organ, and the two GATA factors mutually interact and synergistically activate cardiac-specific genes (*23*). Gene disruption experiments further demonstrated cross-talk between GATA-6 and GATA-4 gene expression (*15*). However, GATA-6 showed distinct properties; the promoter of the Dab2 gene was activated by GATA-6, but not by GATA-4 (*31*), and GATA-6 and GATA-4 are distributed differently in the gonads (*33*). The development of lung buds and gene expression in the lung epithelial cells seem to be exclusively regulated by GATA-6 (*15*, *24–27*).

*To whom correspondence should be addressed. Tel: +81-6-6879-8185, Fax: +81-6-6879-8189, E-mail: mmaeda@phs.osaka-u.ac.jp

It is now recognized that GATA-6 is an important regulator of cell cycle progression, as was shown by a transient decrease in GATA-6 transcripts in vascular myocytes upon growth response (*19*), and by the inhibition of S-phase entry through induction of the Cdk inhibitor p21 (*34*).

Although its functional significance has become apparent, little is known about the *in vivo* translational product of GATA-6 mRNA, possibly due to the limitation of protein detection (*16*). The primary structure of GATA-6 was initially thought to be similar in length to those of other GATA factors (*3*, *21*) from the sequence similarity with those of GATA-4 and GATA-5. However, the subsequently cloned cDNA has an additional Met-codon *in frame* in its 5′-upstream region (*20*). The *in vitro* reticulocyte translation system revealed that both Met-codons are used as initiator codons, resulting in the synthesis of long-type and short-type GATA-6 proteins, respectively (*35*). Thus, it would be interesting to determine the protein size of GATA-6 and to study its behavior at the cellular level.

In this study, we addressed how these two initiator codons are selected in cells. Analysis of GATA-6 transiently expressed from cDNA suggested that the downstream codon is used as a result of leaky ribosome scanning. Furthermore, the protein sequence between the two Met codons affected the production of long-type GATA-6. Considering the importance of this transcription factor in the processes of embryogenesis and specific gene expression, the intrinsic properties of GATA-6 should shed light on the novel regulatory mechanism underlying GATA-6 function(s).

MATERIALS AND METHODS

Transfection for Analysis of GATA-6 Protein—Cos-1 cells (ATCC) were grown in DMEM (GIBCO) supplemented with 7% (v/v) FBS (JRH Biosciences). An expression plasmid was introduced into these cells by means of the DEAE-dextran method (*36*); 5×10^5 cells were plated onto a 60 mm-diameter dish in 5 ml medium. The culture dishes were coated with gelatin (cell matrix type I-C, Nitta Gelatin). After 20 h' incubation in a CO_2 incubator at 37°C, the medium was discarded and the dish was washed with TBS (3 ml). A DNA solution (1 ml TBS) containing 2.5 μg expression plasmid for GATA-6, 2.5 μg salmon sperm DNA (Wako), and 110 μl DEAE-dextran (Sigma) solution was added, and then the dish was incubated for 30 min. The cells were further incubated for 3 h in fresh medium (2 ml) containing serum and 100 μM chloroquin (Sigma). After a TBS (3 ml) wash, 2 ml medium [serum plus 20% (v/v) glycerol] was added, and then the dish was kept for precisely 4 min at room temperature and then washed with TBS (3 ml). Cells were grown for 48 h before harvesting in 5 ml of the culture medium. Protease inhibitors (20 μM MG115, 1 mM PMSF and 50 μM E-64d) were added at 24 h before harvesting as a DMSO solution (10, 25 and 25 μl/5 ml medium, respectively).

In each well of a 6-well culture plate, 1×10^5 CHO-K1 cells (*37*) were seeded in 2 ml F12 medium (GIBCO) containing 7% (v/v) FBS. Lipofectamine™ (Invitrogen) was used for the transfection of an expression plasmid (0.6 μg/well), according to the manual supplied by the manufacturer, except that the volume was quadrupled using F12 medium. HeLa cells (*29*) were similarly treated with DMEM.

SDS–Polyacrylamide Gel-Electrophoresis and Western Blotting—A nuclear extract was prepared by the published method (*38*). Briefly transfected cells were washed with PBS, and then scraped into 1 ml PBS with a rubber policeman. Cells were precipitated in a micro-centrifuge (3,000 rpm) for 2 min at 4°C, and then suspended in 200 μl Buffer A [10 mM HEPES-KOH (pH 7.6), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF]. After incubation for 10 min at 4°C, the cell suspension was mixed vigorously (10 s with a Vortex mixer), and then centrifuged (3,000 rpm) for 10 min at 4°C. The nuclear pellet was suspended in 50 μl Buffer C [20 mM HEPES-KOH (pH 7.6), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM DTT, 0.2 mM PMSF] and then kept on ice for 20 min. Aliquots of the supernatant obtained on centrifugation at 15 k rpm for 2 min at 4°C were stored at –80°C until use. A whole cell extract was also prepared after similar treatment of cells. Cells were collected in 1 ml PBS and precipitated. They were suspended in 150 μl water containing 1 mM PMSF, and then sheered 20–30 times with a 25G needle. Protein concentrations were determined with a BCA Protein Assay (Pierce) using BSA (Fraction V, Sigma) as a standard (*39*).

Proteins (10 μg for the nuclear extract and 20 μg for the whole cell extract) were subjected to SDS-polyacrylamide gel [7.5% or 10% (w/v), 1 mm thickness] electrophoresis (*40*), and then electro-blotted (200 mA, 90 min, ATTO Model-AE6675) onto an Immobilon™-P membrane [Millipore PVDF membrane (0.45 μm), IPVH00010] (*41*). The membrane was blocked overnight at 4°C with PBS-T containing 3% (w/v) BSA. Rabbit site-specific polyclonal antibodies, hGATA-6N, recognizing human GATA-6 (Leu^{59}–Gln^{217}) (*42*) were used as the first antibodies (×500 diluted) at 25°C; 2 ml solution was layered onto parafilm (7 × 9 cm) covering the glass, and the side of the membrane onto which proteins had been blotted was carefully placed in contact with the solution. The second antibodies, horseradish peroxidase-linked donkey anti-rabbit immunoglobulin (×4,000 diluted) (Amersham-Pharmacia-Biotech), were similarly used. An enhanced chemiluminescence Western blotting kit (Amersham-Pharmacia-Biotech) was used according to the manual supplied by the manufacturer. Usually we exposed the film for 1–60 min before development. When we used antibodies (hGATA-6C) recognizing human GATA-6 (Cys^{358}–Ala^{449}) (H-92; Santa Cruz), the blotted membrane was blocked for 1 h and then reacted with the antibodies (×10,000 diluted) overnight at 25°C. Reprobing was carried out as follows; the membrane was treated with a buffer [2% (w/v) SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.7)] for 30 min at 50°C, blocked overnight at 4°C, and then reacted with antibodies in the same way as for hGATA-6N. The amino acid residue numbers are based on those of S-type GATA-6 (*21*).

Cloning of the 5′-Upstream Region of Human GATA-6 mRNA—Total RNA of MKN-45 cells (*21*) was treated with RNase-free DNase I (TaKaRa). 5′-RACE was carried out with an aliquot (1 μg) using a 5′-RACE System for

Table 1. **PCR primers used in this study.**

MT003	5′-AGG AG*C TC*T ACC AGA CCC T-3′
MT004	5′-TA*G* AG*C* TC*C* TCC GGC TGC T-3′
MT005	5′-AT*C* TC*G* AGT TCC GAC CCA CAG CCT GGC AC-3′
MT006	5′-*GCC ACC* ATG GCC TTG ACT GAC G-3′
MT007	5′-GG*C CAT GGT GGC* TCC GGC GCC GCT CCA-3′
MT010	5′-AGG CGC GGA GAG AAG CCG AG-3′
MT011	5′-ACT GCA GCC TGG GCT CCT GAT TG-3′
MT012	5′-GG*C TA*G CCT TGA CTG ACG GC-3′
MT013	5′-GCT AGC CAC GGT CCG GCG CC-3′
RN016	5′-CCC G*CT* CGA GGA GCT AGA CGT CA-3′
RN017	5′-AGC ATG GAA CCC ACG CGG GT-3′
AYA007	5′-AGA GCG GCG AGG GTC TGG TA-3′
hGT1-1	5′-GCA GCA AGT CCT CCC AGC T-3′
dAs	5′-CCG GGG AGC CCT CCA CGC CGC CTT CCC CCA TCT CTT CCT CGT CCT CCT CCT GCT-3′
dAa	5′-CCG GAG CAG GAG GAG GAC GAG GAA GAG ATG GGG GAA GGC GGC GTG GAG GGC TCC-3′
dA(-2)s	5′-CCG GAG CCC TCC ACG CCG CCT TCC CCC ATC TCT TCC TCG TCC TCC TCC TGC TCC-3′
dA(-2)a	5′-CCG GGG AGC AGG AGG AGG ACG AGG AAG AGA TGG GGG AAG GCG GCG TGG AGG GCT-3′
CTGm1s	5′-CCG GGG GCA AC*T* TGT CGA GCT GGG AGG ACT TGC T*C*C T*C*T TCA CTG ACC TC-3′
CTGm1a	5′-TTG GTC GAG GTC AGT GAA *G*AG *G*AG CAA GTC CTC CCA GCT CGA CA*A* GTT GCC C-3′
CTGm2s	5′-GAC CAA GCC GCG ACC GCC AGC AAG CT*C* CT*C* TGG TCC *TCG* CG*A* GGC GCC AAG T-3′
CTGm2a	5′-TCA ACT TGG CGC *CTC GCG A*GG ACC A*G*A G*G*A GCT TGC TGG CGG TCG CGG C-3′
AAP	5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′
UAP(-)	5′-GGC CAC GCG TCG ACT AGT AC-3
M13-F	5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′
M13-R	5′-GAG CGG ATA ACA ATT TCA CAC AGG-3′
pGL3-F	5′-TTC AGG GGG AGG TGT GGG AG-3′
pGL3-R	5′-GAC GAT AGT CAT GCC CCG CG-3′

The introduced restriction enzyme sites are underlined. Bold italic letters indicate the nucleotide substitutions.

Rapid Amplifaication of cDNA Ends (GIBCO). The first strand cDNA was synthesized with a primer, MT010 (all the PCR primers used in this study are listed in Table 1), and then subjected to PCR (*43*); the primer pairs for the 1st and 2nd PCR were MT010/AAP and MT011/UAP(–), respectively. The PCR conditions were 35 cycles of denaturation (94°C, 0.5 min), annealing (55°C, 0.5 min), and extension (72°C, 0.5 min). The products were analyzed by agarose gel electrophoresis [1.0% (w/v) agarose (TaKaRa L03) in TAE-buffer] (*36*), and visualized by ethidium bromide staining. The amplified ~200 bp fragment was eluted with Geneclean III (Bio 101), and then ligated into the pGEM T-Easy vector (Promega). To determine the transcription start site, the cloned cDNA was sequenced by the dideoxy chain-termination method (*44*) using an ABI PRISM TM310 Genetic Analyzer with a Big DyeTM Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems) and sequence primers (M13 forward and reverse).

To extend the 5′-side of GATA-6 cDNA, we amplified the 5′-upstream sequence from the above cDNA by successive PCR with primer pairs MT005/RN017 and MT005/AYA007 in the presence of 10% (v/v) DMSO under the same PCR conditions. The amplified fragments were cloned and sequenced. The resulting clone without a deletion was named pTA5-7. The *Xho*I–*Aat*II fragment of pME-hGT1L was substituted with the corresponding fragment of pTA5-7 to construct pME-hGT1L5′.

The GATA-6 mRNA transiently expressed from the expression plasmid in Cos-1 cells was also analyzed. Total cellular RNA was extracted with Isogen (Wako Chemicals) (*45*). The first strand cDNA was synthesized from 5 μg RNA with primer RN017 as above, and then PCR was carried out with primer pair RN016/RN017 in the presence of 10% (v/v) DMSO. The PCR conditions were 35 cycles of denaturation (96°C, 0.5 min), annealing (58°C, 0.5 min), and extension (72°C, 1 min). The products were cloned and sequenced. The cDNA portion amplified from MKN-45 cell RNA with RN016/RN017 was also inserted into the pGEM T-Easy vector (pTA4-4).

Construction of Various Plasmds for GATA-6 Expression—The *Apa*I–*Dra*I fragment of our human GATA-6 cDNA (*21*) was inserted into the *Apa*I–*Eco*RV sites of pGEM5Zf(+). The *Apa*I (Klenow-treated)- and *Spe*I-digested fragment was ligated into the *Xho*I (Klenow-treated) and *Spe*I sites of pME18S. The resulting plasmid was named pME-hGT1S [designated as pME-hGT1 previously (*21*)]. The *Xho*I–*Not*I fragment of pTA4-4 was substituted with the corresponding fragment of pME-hGT1S to construct pME-hGT1L. The downstream Met codon of pME-hGT1L was substituted with a Leu (CTC) codon; the plasmid template was subjected to PCR [25 cycles of denaturation (94°C, 1 min), annealing (57°C, 0.5 min), and extension (72°C, 3 min)] with primer pairs RN016/MT004 and MT003/RN017, and both amplified fragments with a *Sac*I site were ligated into the pGEM T-Easy vector. The *Xho*I–*Sac*I and *Sac*I–*Bst*XI fragments, respectively, were inserted between the *Xho*I and *Bst*XI sites of pME-hGT1L to produce pME-hGT1LdL. The upstream Met codon of pME-hGT1L5′ was substituted with a Leu (CTC) codon; plasmid template pTA5-7 was subjected to PCR [30 cycles of denaturation (95°C, 0.5 min), annealing (55°C, 0.5 min), and extension (72°C, 0.5 min)] with primer pairs MT005/MT013 and MT012/

RN017, respectively, and both amplified fragments with a *Nhe*I site were ligated into the pGEM T-Easy vector. The *Xho*I–*Nhe*I and *Nhe*I–*Acc*I fragments, respectively, were inserted between the *Xho*I and *Acc*I sites of pME-hGT1L to produce pME-hGT1L5′uL. pME-hGT1L5′ΔEXuL was similarly constructed from pTA4-4ΔEX (see below).

pTA4-4 was digested with *Xma*I or *BpU*1102I, and then filled-in with the Klenow enzyme. After *Eco*47III or *Sma*I digestion, each large fragment was self-ligated. The resulting plasmids were named pTA4-4ΔEX and pTA4-4ΔSB, respectively. To construct pME-hGT1LΔEX and pMEhGT1LΔSB, the *Xho*I–*Acc*I segment of each plasmid was ligated between the corresponding sites of pME-hGT1L. The *Xho*I–*Aat*II segment was excised from pTA5-7, and then substituted with the corresponding part of pME-hGT1LΔEX and pME-hGT1LΔSB to construct pME-hGT1L5′ΔEX and pME-hGT1L5′ΔSB, respectively. Synthetic oligonucleotide pairs dAs/dAa and dA(-2)s/dA(-2)a were 5′ phosphorylated and annealed. They were inserted into the *Xma*I site of pTA4-4ΔEX, and then the *Xho*I–*Acc*I segment of each plasmid was ligated between the corresponding sites of pME-hGT1L. The resulting plasmids were named pME-hGT1LΔA and pME-hGT1LΔA(-2), respectively. The CTG codons located between the *Xma*I and *BpU*1102I sites were substituted with CTC or TTG codons by cassette mutagenesis; synthetic oligonucleotide pairs CTGm1s/CTGm1a and CTGm2s/CTGm2a were 5′-phosphorylated and annealed. They were tandemly introduced between the *Xma*I and *BpU*1102I sites of pTA4-4ΔEX. The *Xho*I–*Acc*I fragment was ligated between the corresponding sites of pME-hGT1LΔEX to produce pME-hGT1LΔEX/CTGm.

Further deletion plasmids (pME-hGT1L5′d2 and pME-hGT1L5′d19) were constructed; pTA5-7 was digested with *Xma*I, and nucleotides were deleted by Exonuclease III treatment. Following Mung bean nuclease and Klenow enzyme treatment, plasmid DNA of ~150 bp shorter in size than the original one was separated by agarose gel electrophoresis and then self ligated. The *Xho*I–*BpU*1102I fragment derived from the deletion construct and the *BpU*1102I–*Acc*I fragment derived from pTA4-4 were ligated to the large *Xho*I-*Acc*I fragment from pME-hGT1L.

A Kozak sequence was introduced around the upstream initiation codon of pME-hGT1L5′ and pME-hGT1L5′ΔEX; the plasmid template was subjected to PCR in the presence of 5% (v/v) DMSO [30 cycles of denaturation (96°C, 0.5 min), annealing (58°C and 48°C, 0.5 min), and extension (72°C, 0.5 min)] with primer pairs MT005/MT007 and MT006/RN017, respectively, and both amplified fragments with a *Nco*I site were cloned into the pGEM T-Easy vector. The *Xho*I–*Nco*I and *Nco*I–*Acc*I fragments derived from the respective clones were ligated to the large *Xho*I–*Acc*I fragment derived from pME-hGT1L. The resulting plasmids were named pME-hGT1LK5′ and pME-hGT1LΔEK5′, respectively. The molecular biological manipulations were performed by the published methods (*36*). The DNA sequence after each manipulation step was confirmed by the dideoxy chain-termination method (*23*).

Construction of Reporter Plasmids—The 5′-upstream *Nhe*I–*Eco*RI (Klenow-treated) fragment (–1257–+50 bp, numbered from the first letter of the initiation codon) of the rat H^+/K^+-ATPase β subunit gene (*46*) was cloned between the *Nhe*I and *Sma*I sites of the luciferase reporter gene plasmid, pGL3 basic vector (prHKβ/GL3) (*47*). Complementary oligonucleotides carrying tandem GATA sites derived from Rβ2 [rat H^+/K^+-ATPase β subunit gene corresponding to –157/–143 bp (*46*)] with a *Pst*I site on both sides were annealed, and then their 5′-sides were phosphorylated with T4 kinase and treated with ligase (*36*). An aliquot was mixed with *Pst*I-digested pBluescriptSKII(+), followed by ligation. A clone with three tandem repeats (pBS-6GATA) was isolated. The Klenow-treated *Nco*I–*Eco*47III fragment derived from the rat intrinsic factor gene (*48*) was inserted into the *Eco*RV site of pBS-6GATA. The *Sac*I–*Apa*LI fragment derived from the resulting plasmid (pBS-6GATA-rIF) was inserted between the *Sac*I and *Hin*dIII sites of pUC18 (pUC-8GATA). Although the *Apa*LI and *Hin*dIII sites were treated with the Klenow enzyme, the *Hin*dIII site was regenerated in the plasmid construct. The *Eco*RI (Klenow-treated)–*Hin*dIII fragment or *Sma*I–*Hin*dIII fragment of pUC-8GATA was inserted between the *Sma*I and *Hin*dIII sites of pGL3. The resulting plasmids (prIF/GL3 and p8GATA/GL3, respectively) were used for the present study.

Reporter Gene Assay—In each well of a 6-well culture plate, 1×10^5 CHO-K1 cells were seeded in 2 ml F12 medium containing 7% (v/v) FBS. Lipofectamine™ (Invitrogen) was used for transfection (duplicate) of plasmid DNA, reporter plasmid (1 μg), GATA-6 expression plasmid (0.6 μg), and pSV-β-Gal (0.1 μg) (*47*) per well, according to the manual supplied by the manufacturer, except that the volume was quadrupled using F12 medium. Lysis buffer (90 μl) was added at 48–53 hrs after the start of transfection, and then a cell lysate was prepared (12,000 ×*g*, 10 min at 4°C). An aliquot (20 μl and 5 μl) of the supernatant was used for measurement of luciferase and β-galactosidase activities, respectively (*47*). To prepare nuclear proteins, the pellet was washed with 30 μl Buffer C as described above. The nuclear extracts of duplicate samples were combined and subjected to Western-blotting analysis for GATA-6 using antibodies for hGATA-6N.

Chemicals—Restriction enzymes were purchased from NEB, MBI, TaKaRa and Toyobo. T4 DNA ligase was supplied by Stratagene or TaKaRa. T4 DNA polymerase Exonuclease III, mung bean nuclease and RNaseH were from TaKaRa. The Klenow enzyme, T4 polynucleotide kinase and calf intestine phosphatase were obtained from NEB. *Ampli Taq* was from Applied Biosystems. Oligonucleotides were purchased from Invitrogen. MG115 and E-64d, and PMSF were from the Peptide Institute and Sigma, respectively. All other chemicals used were of the highest grade commercially available.

RESULTS

Detection of GATA-6 Proteins Expressed in Cos-1 Cells Transiently—The amino terminal sequence of short-type GATA-6 (S-type) (*21*) is similar to those of GATA-4 (*17*) and GATA-5 (*49*). In addition to the S-type, GATA-6 mRNA encodes a long type product (L-type) that extends 146 amino acid residues in frame further upstream of the

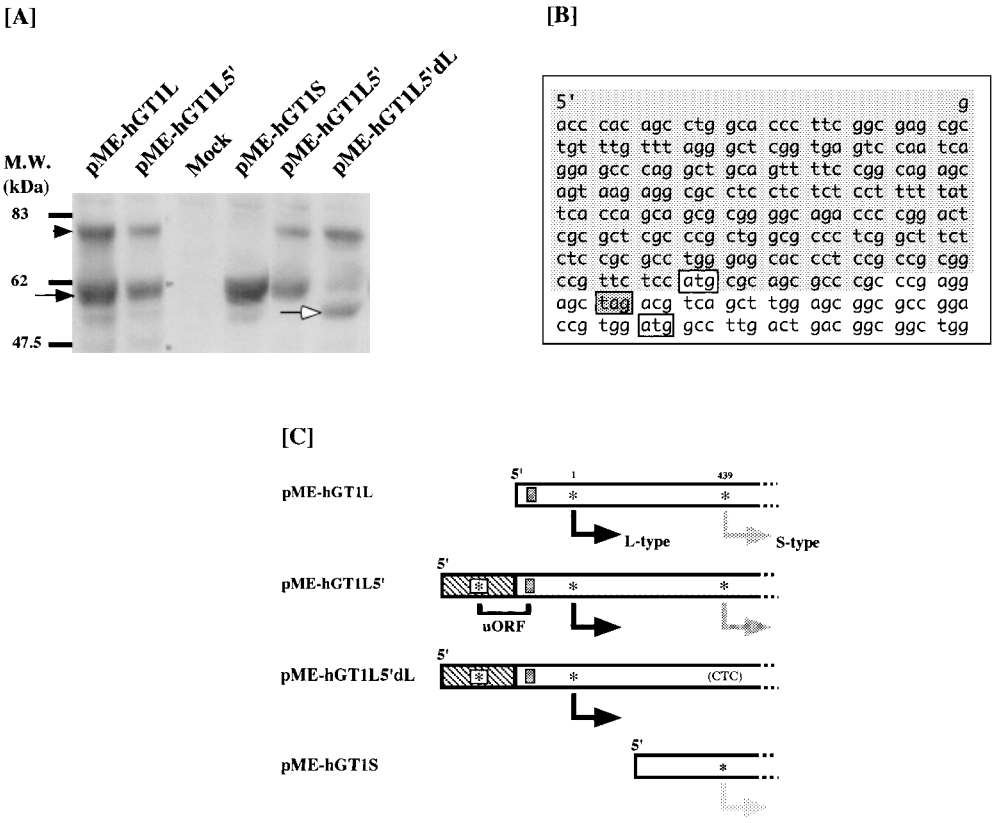

Fig. 1. **Detection of GATA-6 in transiently transfected Cos-1 cells.** Cos-1 cells were transfected with either pME-hGT1L, pME-hGT1L5′, pME-hGT1S, ME-hGT1L5′dL, or pME18S (as mock transfection). After two days, a nuclear extract was prepared. An aliquot was subjected to SDS–polyacrylamide gel-electrophoresis (10%) and then analyzed by Western-blotting with hGATA-6N antibodies [A]. The positions of L-type and S-type GATA-6 are indicated by the closed arrowhead and closed arrow, respectively, and that of GATA-6(Δ50) by an open arrow. The extremely 5′-upstream sequence is shown in [B]. The upstream open reading frame starts from the first "atg" codon and ends at the "tag" codon. The L-type GATA-6 starts from the second "atg" codon. The expression plasmids and translation pattern are schematically shown in [C]. The position of an initiation codon is indicated by an asterisk together with the nucleotide residue number. The uORF is also indicated. The hatched box corresponds to the sequence with the gray background in [B]. The termination codon for uORF is boxed in gray.

S-type initiation methionine (*20*, *35*). In this study, we examined which type of GATA-6 is translated at the cellular level with a transient transfection system and what signal in GATA-6 mRNA or protein affects the production of the two types of GATA-6. The expression of GATA-6 was detected with antibodies upon transfection of expression plasmids into Cos-1 cells, as shown in Fig. 1A.

When an expression plasmid with two initiation codons for the L- and S-types (pME-hGT1L) was introduced, two specific bands corresponding to apparent molecular sizes of 70k and 56k, respectively, appeared, as determined with antibodies for GATA-6. The 56k band corresponded to the GATA-6 protein expressed from the S-type expression plasmid (pME-hGT1S).

Effect of the 5′-Upstream Sequence on the Expression of GATA-6—The above observations do not exclude the possibility that the 5′-upstream sequence has a critical role in selection of the initiation codon. Since our initial expression plasmid (pME-hGT1L) has only a portion of the upstream sequence of GATA-6 cDNA (*20*), we added a further upstream sequence proximal to the 5′-cap site (*51*) to the expression plasmid. To do so, we first determined the 5′ end point by 5′-RACE using mRNA of human gastric adenocarcinoma MKN-45 cells (*21*). As shown in Fig. 1B, the 5′-upstream sequence was essentially the same as the reported end of cDNA (*20*). Interestingly, there is a short open reading frame (uORF) in the entire upstream sequence. Such an uORF often affects the expression of a downstream open reading frame positively or negatively (*51*, *52*). So we amplified the upstream sequence by PCR, and a plasmid expressing the entire 5′-upstream sequence was constructed (pME-hGT1L5′). Plasmids pME-hGT1L and pME-hGT1L5′ were introduced into Cos-1 cells, and then the expression of L-type and S-type GATA-6 was compared. However, the expression patterns of the two types of GATA-6 were essentially the same (Fig. 1A), indicating that the 5′-upstream sequence proximal to the cap-site or the short uORF encoding 8 amino acid residues has no effect on the production of either translational isoform.

When the initiation codon (downstream) for the S-type was changed to a Leu codon (ATG→CTC) (pME-hGT1L5′dL) (Fig. 1C), the S-type band disappeared but the 70k band was still visible, confirming that the latter corresponds to the L-type. The lower molecular weight product than S-type GATA-6 detectable upon substitution of the downstream Met codon was similar in size to that of rat GATA-6(Δ50) (*50*), suggesting that a further downstream Met codon could be used.

Deletion of the Sequence between the Two Initiation Codons—It is also possible that the internal ribosome entry site (IRES) between the two Met codons stimulates the translation from the downstream initiation codon (*53*). To examine this possibility, we deleted a portion between the upstream and downstream initiation codons of expression plasmid pME-hGT1L, and then GATA-6 proteins were analyzed. Two plasmid constructs (pME-hGT1LΔEX and pME-hGT1LΔSB) were expected to express similar sized L-types, since 268 (upstream half,

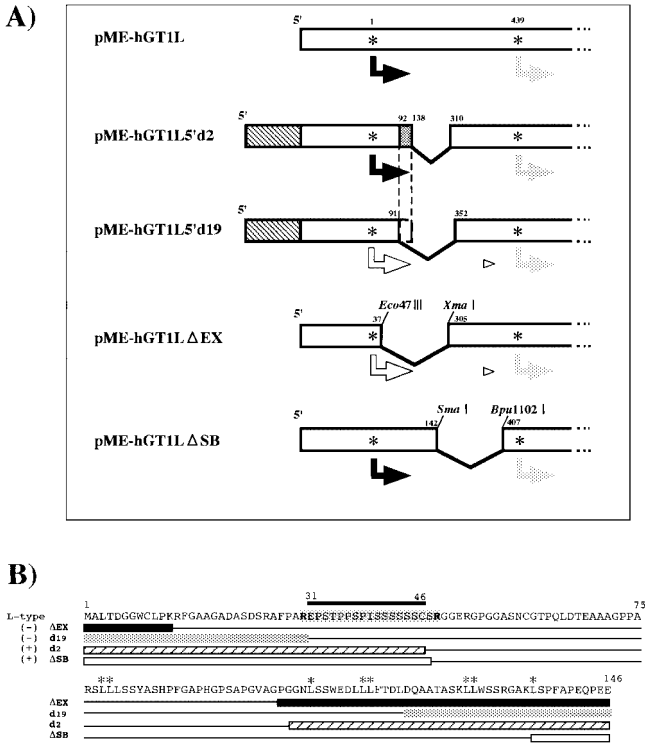

Fig. 2. **Schematic representation of expression plasmids for various amino terminal deletions of GATA-6, and the results of deletion.** (A) Expression plasmids for amino terminal truncated GATA-6 are schematically shown. Asterisks indicate the initiation codons for L- and S-type GATA-6 (1st and 439th codons, respectively). All the plasmids produced S-type GATA-6 (gray arrows), while two of them (d19 and ΔEX) did not apparently produce the L-type (open arrows) in contrast to d2 and ΔSB (black arrows), but a GATA-6 slightly larger in size than the S-type was detected (small open arrowheads). The nucleotide residue numbers for the ligated regions of d2, d19, ΔEX and ΔSB are indicated. The extremely 5′-upstream sequences (Fig. 1, B and C) shown for pME-hGT1L5′d2 and pME-hGT1L5′d19 were also connected to pME-hGT1L, pME-hGT1LΔEX and pME-hGT1LΔSB, as described under "MATERIALS AND METHODS." (B) The translated regions of the corresponding L-type GATA-6 derived from expression plasmids (indicated in A) are shown as boxes, while the deleted regions are indicated by lines. Comparison of the translated products and deleted sequences suggested the region with the overline (92nd and 138th nucleotide residues corresponding to Glu-31 and Cys-46) was important for the producction of L-type GATA-6. The L-type specific amino acid sequence is shown with asterisks on the Leu residues encoded by CTG codons.

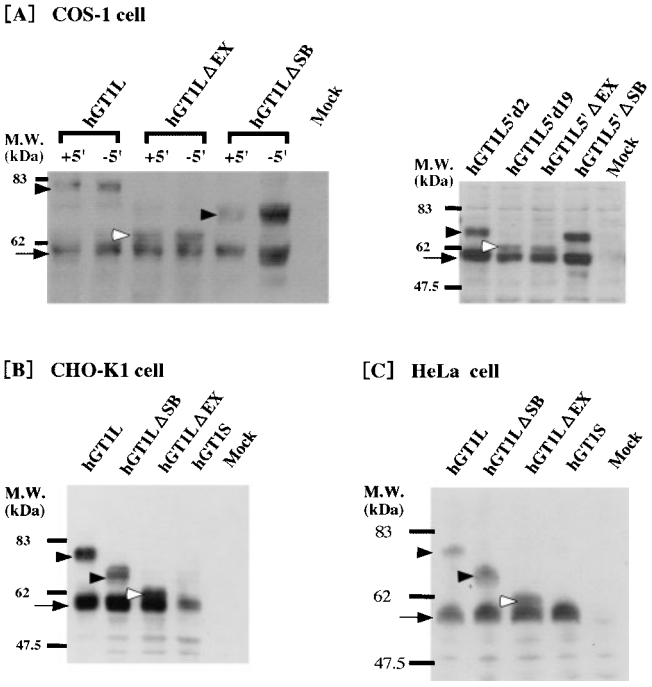

Fig. 3. **Effects of deletion of the nucleotide residues between the initiator Met codons.** Cos-1 cells [A; left], CHO-K1 cells [B], and HeLa cells [C] were transfected with either pME-hGT1L, pME-hGT1LΔEX, pME-hGT1LΔSB, pME-hGT1S or pME18S (as mock transfection). An extremely 5′-upstream sequence was present in the plasmid used in experiment [A; right] (pME-hGT1L5′d2, pME-hGT1L5′d19, pME-hGT1L5′ΔEX or pME-hGT1L5′ΔSB). After two days, a nuclear extract [A and B] or whole cell extract [C] was prepared. They were analyzed by Western-blotting with hGATA-6N ([A] left, [B] and [C]) or hGATA-6C ([A] right) antibodies after SDS–polyacrylamide (10% for [A], and 7.5% for [B] and [C]) gel-electrophoresis. The positions of L-type and S-type GATA-6 are indicated by closed arrowheads and closed arrows, respectively. The open arrowheads indicate an unusually sized product. The expression plasmids introduced are schematically shown in Fig. 2A.

Δ38–304) and 265 (downstrean half, Δ143–406) nucleotide fragments were deleted, respectively (Fig. 2A).

As shown in Fig. 3A (left), an L-type GATA-6, with the size expected from the deletion, was produced from the latter plasmid, although its molecular weight was smaller than that of the native L-type. However, surprisingly, a corresponding L-type band could not be detected for the former plasmid. Furthermore, the addition of the 5′-upstream sequence (pME-hGT1L5′ΔEX and pME-hGT1L5′ΔSB) did not affect the expression pattern in the case of pME-hGT1LΔEX or pME-hGT1LΔSB. These results suggest that the unexpected expression pattern could be primarily due to the mRNA sequence between the two initiation codons. Such a phenomenon together with translation of both the L- and S-types were reproducibly observed with not only Cos-1 cells but also with CHO-K1 and HeLa cells (Fig. 3, B and C), suggesting that it might be a general phenomenon in cultured mammalian cells, consistent with the previously reported *in vitro* translation system (*35*).

To further confirm this observation, we constructed a deletion plasmid by a different route and determined the important region for expression of L-type GATA-6. Manipulation with nuclease generated plasmids pME-hGT1L5′d2 and pME-hGT1L5′d19, which have 171 and 261 bp deletions (Δ139–309 and Δ92–352, respectively) (Fig. 2A). The Δ139–309 deletion produced an L-type GATA-6 similar to pME-hGT1L5′ΔSB, whereas the Δ92–352 deletion showed a similar expression pattern to pME-hGT1L5′ΔEX (Fig. 3A, right). These results suggest that the nucleotide residues between the 92nd and 138th (or Glu-31–Cys-46) positions could play a critical role in the expression of L-type GATA-6 (Fig. 2B).

Effect of the Internal Sequence—We next determined the effect of inserting this nucleotide sequence into expression plasmid pME-hGT1LΔEX. We inserted two 50 base sequences, which have identical residues except for

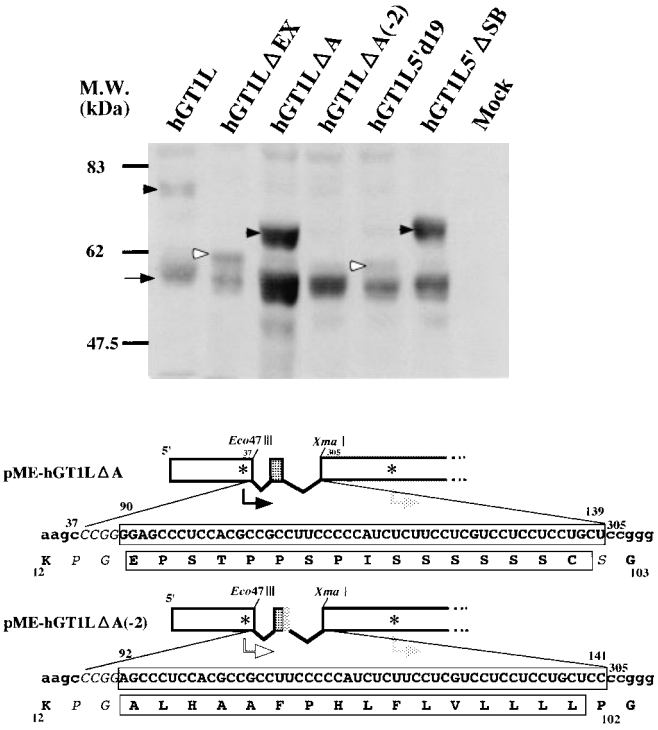

Fig. 4. **Effect of re-introduction of the 50-bp nucleotide residues on the expression of L-type GATA-6.** Cos-1 cells were transfected with the plasmid carrying the re-introduced 50 bp nucleotide residues [pME-hGT1LΔA or pME-hGT1LΔA(-2)] or with either pME-hGT1L, pME-hGT1LΔEX, pME-hGT1L5′d19, pME-hGT1LΔSB, pME-hGT1S or pME18S. After two days, a nuclear extract was prepared. An aliquot was subjected to SDS-polyacrylamide gel electrophoresis (10%) and then analyzed by Western-blotting with hGATA-6N antibodies. The positions of L-type and S-type GATA-6 are indicated by closed arrowheads and the closed arrow, respectively. The open arrowheads indicate an unusually sized product. The inserted nucleotides and translated sequences are shown in the lower part. Italic letters indicate unrelated nucleotide residues and amino acid residues generated at the inserted edges. Plasmid pME-hGT1LΔA produced both L-type (closed arrow) and S-type GATA-6 (gray arrow), while pME-hGT1LΔA(-2) only produced the S-type.

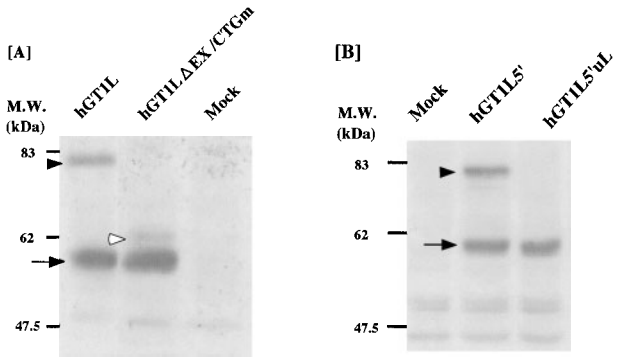

Fig. 5. **Effects of nucleotide changes for Leu codons and the upstream initiation codon on expression of the unusually sized product.** Cos-1 cells were transfected with expression plasmid pME-hGT1L, pME-hGT1LΔEX/CTGm (carrying altered Leu codons), or pME18S [A], or with pME-hGT1L5′, pME-hGT1L5′uL (carrying a CTC codon instead of the upstream initiation codon), or pME18S [B] (see Fig. 6C). After two days, a nuclear extract was prepared. An aliquot was subjected to SDS-polyacrylamide gel electrophoresis (7.5% for [A] and 10% for [B]) and then analyzed by Western-blotting with hGATA-6N antibodies. The positions of L-type and S-type GATA-6 are indicated by closed arrowheads and closed arrows, respectively. The open arrowhead indicates an unusually sized product.

two each at the 5′ and 3′ ends. Although these sequences are essentially the same at the nucleotide level, the translated sequences are completely different. Thus, we attempted to determine which is important for the production of L-type GATA-6, the nucleotide or the amino acid sequence. The results demonstrated that the nucleotide sequence that produces the correct amino acid sequence (pME-hGT1LΔA) restored the expression pattern (Fig. 4), while pME-hGT1LΔA(-2), which does not produce the correct amino acid sequence, did not. All these results strongly suggest that the amino acid residues (Glu-31–Cys-46) encoded by the 92nd to 138th nucleotide residues but not the nucleotide residues *per se* are essential for the formation of L-type GATA-6 (Fig. 2B).

Evaluation of the Translation from the CUG Codons between the Two Met-Codons—An unusually sized product, whose molecular weight was slightly higher than that of S-type GATA-6, was evident, especially upon transfection of pME-hGT1LΔEX, pME-hGT1L5′ΔEX and pME-hGT1L5′d19, although it was apparently not detected with pME-hGT1LΔA(-2), possibly due to the extremely hydrophobic nature of the peptide sequence translated from the insertion (Figs. 3 and 4). When we studied the time course of GATA-6 accumulation in transfected cells, the expression patterns at 24, 36 and 48 h after transfection were found to be essentially the same, and we could not detect any L-type production immediately after transfection. We also did not observe cryptic splicing products in the cells transfected with pME-hGT1LΔEX, pME-hGT1LΔA(-2), and pME-hGT1LΔSB (data not shown). This may suggest that the appearance of the unusual protein band on focusing is not due to the post-translational proteolysis of full-length L-type GATA-6 after completion of its biosynthesis or the altered splicing of its mRNA.

It is well known that a non-AUG codon such as CUG is often used for oncoproteins, growth factors and transcription factors (*51*). Actually, many CUG codons are distributed between the upstream and downstream Met-codons of GATA-6 mRNA, and most of these codons (6 out of 8) are located between the *Xma*I and *BpU*1102I sites of pME-hGT1ΔEX, with only one in pME-hGT1LΔSB from the 5′ side (Fig. 2B). To determine whether or not the translation starts from these CTG codons in pME-hGT1LΔEX, we substituted all these CTG codons with CTC or TTG. However, the resulting plasmid, pME-hGT1LΔEX/CTGm, still produced the slightly higher molecular weight protein together with S-type GATA-6 (Fig. 5A), suggesting that the internal CUG codons could not function as translational initiation sites. Although the higher molecular weight product (from pME-hGT1L5′ΔEX and pME-hGT1L5′d19) was similarly detected in the presence of inhibitors for typical proteases such as MG115 (proteasome), PMSF (serine protease), and E-64d (cystein protease) (data not shown),

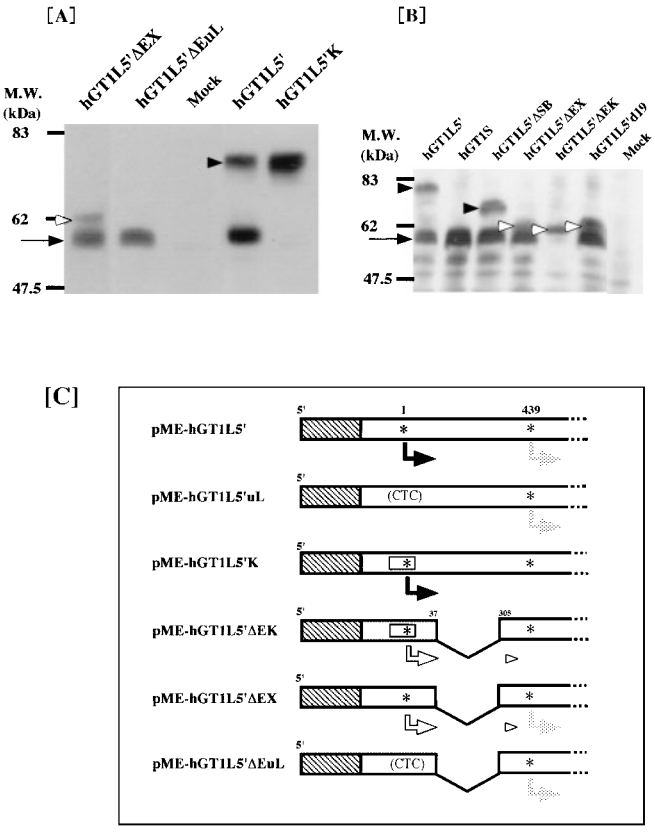

Fig. 6. **Effects of nucleotide changes for the upstream initiation codon and to a Kozak sequence.** Cos-1 cells were transfected with expression plasmid pME-hGT1L5′, pME-hGT1L5′ΔEX, pME-hGT1L5′ΔEuL, pME-hGT1L5′K or pME18S [A], or with pME-hGT1L5′, pME-hGT1S, pME-hGT1L5′ΔSB, pME-hGT1L5′ΔEX, pME-hGT1L5′ΔEK, pME-hGT1L5′d19 or pME18S [B]. After two days, a nuclear extract ([A]) or whole cell extract ([B]) was prepared. They were analyzed by Western-blotting with hGATA-6C ([A]) or hGATA-6N ([B]) antibodies after SDS-polyacrylamide (7.5%) gel-electrophoresis. The positions of L-type and S-type GATA-6 are indicated by closed arrowheads and closed arrows, respectively. The open arrowheads indicate an unusually sized product. The expression plasmids introduced (except for pME-hGT1S and pME-hGT1L5′d19; see Figs. 1 and 2.) are schematically shown in [C]. The hatched box corresponds to the sequence with the gray background in Fig. 1B. In [C], L-type GATA-6, S-type GATA-6, and the product slightly larger in size than the S-type are indicated by black arrows, gray arrows and open arrowheads, respectively. The open arrows indicate that the L-type was apparently not produced. Asterisks indicate the initiation codons for L- and S-type GATA-6 (1st and 439th codons, respectively). The upstream initiation codon with a Kozak sequence is boxed. The upstream initiation codon was also substituted with CTC in pME-hGT1L5′uL and pME-hGT1L5′ΔEuL.

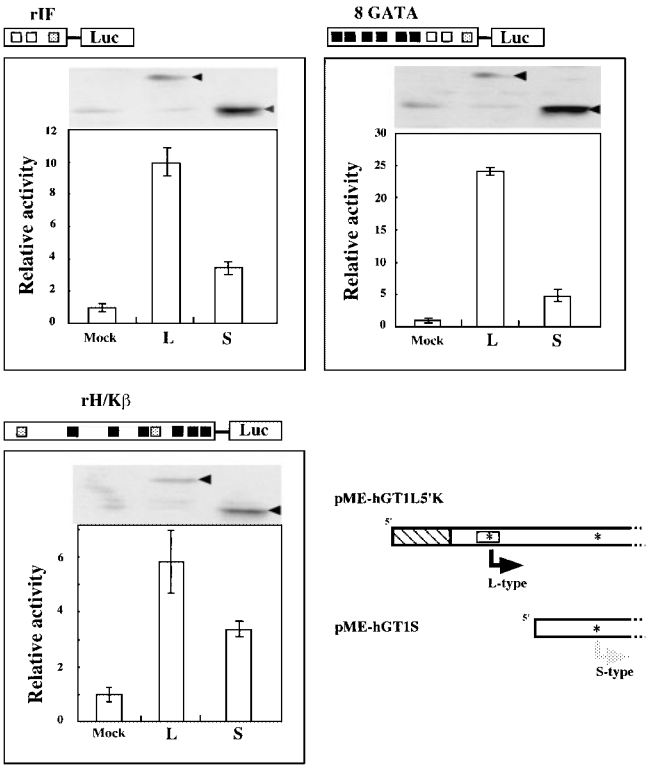

Fig. 7. **Comparison of the transcriptional activities of L- and S-type GATA-6 with various GATA-responsive promoters.** Expression plasmids for L- and S-type GATA-6 (pME-hGT1L5′K and pME-hGT1S, respectively) were introduced into CHO-K1 cells together with either of the reporter gene plasmids (prIF/GL3, prHKβ/GL3 or p8GATA/GL3) plus the β-galactosidase expression plasmid, as described under "MATERIALS AND METHODS." Luciferase activities were normalized as to those of the β-galactosidase. The relative activities as to mock transfection (pME18S instead of expression plasmids for GATA-6) are shown with deviation for duplicate measurements. Similar tendencies were observed in independent transfection experiments. A nuclear extract was also prepared and then an aliquot was subjected to Western blotting with hGATA-6N antibodies. The closed arrowheads indicate L- and S-type GATA-6. The positions of "GATA" sequences in the promoter regions of the IF and H^+/K^+ ATPase β subunit genes are schematically shown by open and closed boxes, respectively. The gray boxes show the GATT sequences recognized by GATA-6 (*8*). The asterisks in the expression plasmids correspond to the initiation codons.

this experimental evidence suggests that the nascent L-type protein would be unstable without the region at least between Glu-31 and Cys-46, and would probably be partly degraded.

We further constructed an expression plasmid with substitution of the upstream Met codon with a Leu codon (CTA). The resulting plasmid (pME-hGT1L5′uL) did not produce L-type GATA-6 or the intermediate size product larger than the S-type (Fig. 5B). It was also demonstrated that pME-hGT1L5′ΔEuL did not produce such an unusual intermediate product (Fig. 6A). These results again support the above suggestion that L-type GATA-6 would be labile during synthesis without the region between Glu-31 and Cys-46.

Effect of a Kozak Sequence around the Upstream Initiation Codon—The sequences around both the upstream (for L-type) and downstream (for S-type) initiation codons of GATA-6 mRNA (C**C**GUGG<u>aug</u>**G** and **G**AG**G**A-G<u>aug</u>U, respectively) do not match the Kozak sequence [GCC(A/G)CC<u>aug</u>G] well (*51*). Considering that position "–3" is most critical, and position "+4" is the next most critical, the S-type seems to be more favorably translated. So we next changed the sequence around the upstream initiation codon to a Kozak sequence (GCCAC-C<u>aug</u>G). Introduction of the resulting plasmid, pME-hGT1L5′K, demonstrated that only L-type was detected (Fig. 6A), suggesting that L-type GATA-6 is translated exclusively from the upstream initiation codon with a

Kozak sequence. This was again observed not only in Cos-1 cells but also in CHO-K1 and HeLa cells (data not shown). Probably, scanning by ribosomes (*49*, *50*) could be leaky in the case of native GATA-6 mRNA, and a less suitable initiation codon for the L-type would often be passed and a more suitable one for the S-type be recognized. Substitution of the downstream Met codon with CTC (Fig. 1) did not alter the expression pattern except for disappearance of the S-type and the concomitant appearance of GATA-6 (Δ50), indicating that ribosomes seem to scan further and start initiation from the Met codon for GATA-6 (Δ50) (*21*).

Analysis with a whole cell extract gave essentially the same results (Fig. 6B), indicating that the results were not due to alteration of the cytoplasmic localization of GATA-6. Actually, it has been demonstrated that the nuclear localization signal of GATA-6 is present in the sequence of GATA-6 (Δ50) (*50*). We further constructed a deletion version of the 92nd to 138th nucleotide residues (pME-hGT1L5′ΔEK) from pME-hGT1L5′K, and determined the expression pattern of GATA-6. Interestingly, typical L- and S-types were not produced from this construct, while only a slightly larger band than that of the S-type, similar to in the case of pME-hGT1L5′ΔEX and pME-hGT1L5′d19, was detected (Fig. 6B). Potentially, translation starting exclusively from the upstream initiation codon with a Kozak sequence would be affected in the absence of the amino acid sequence from the 92nd to 138th nucleotide residues, and the size of L-type could be decreased.

Molecular Activities of L- and S-Type GATA-6—L-type GATA-6 was stably translated from its initiation codon in the Kozak sequence without the production of S-type GATA-6 (Fig. 6), which prompted us to examine the molecular activities of L- and S-type GATA-6. The expression plasmid for the L-type (pME-hGT1L5′K) or S-type (pME-hGT1S) was introduced into CHO-K1 cells together with either a reporter plasmid (prIF/GL3, prHKβ/GL3 or p8GATA/GL3) carrying a GATA-responsive promoter [rat intrinsic factor gene (*48*), rat H^+/K^+-ATPase β subunit gene (*46*), or the combination of the intrinsic factor gene and the GATA binding motif of the rat H^+/K^+-ATPase β subunit gene (this study), respectively] upstream of the luciferase reporter gene. As shown in Fig. 7, the introduction of expression plasmids for GATA-6 enhanced the luciferase activity driven by GATA-responsive promoters. The luciferase activity on expression of the L-type was higher than that of the S-type. Western blotting analysis demonstrated that a rather high amount of the S-type was detected compared with that of the L-type in all the experimental groups, suggesting that L-type GATA-6 could have higher molecular activity to transactivate GATA-responsive genes.

The difference in the activation by L- and S-type GATA-6 was small with the promoter of the H^+/K^+-ATPase β subunit gene. This might be explained by the presence of various negative elements in addition to positive GATA-binding sites in the long cloned 1,300 bp region. The higher activity with p8GATA/GL3 can be ascribed to the presence of a number of GATA binding sites, since a cluster of binding sites could stimulate the transcription (*54*).

DISCUSSION

Determination of the 5′-terminal sequence of human GATA-6 mRNA revealed an *in frame* upstream initiation codon (*20*). From such mRNA with a 5′-upstream sequence, two GATA-6 proteins are translated. In this study, we constructed various expression plasmids for human GATA-6, and examined in detail the effects of gene manipulation on the expression of translational isoforms at the cellular level, because little is known about the molecular mechanisms of GATA-6 regulation in spite of its importance in embryogenesis and tissue-specific gene expression (*15*, *18*, *23–32*). The expression plasmid produced two isoforms (L-type and S-type) of GATA-6 on transient introduction into Cos-1 cells, HeLa cells and CHO-K1 cells, suggesting that such a mode of translation would be a general phenomenon, at least in cultured cells. Ribosomal scanning from 5′-CAP of mRNA is suggested to be a mechanism of selection for initiation codons (*51*, *52*). The introduction of a Kozak sequence (*51*) around the upstream AUG codon stopped the expression of the S-type. Furthermore, substitution of the downstream AUG codon with CUC not only stopped the expression of the S-type but also produced GATA-6(Δ50). These results suggest that the context of the upstream initiation codon of inherent mRNA for GATA-6 is not favorable and that the downstream AUG codon for the S-type [or GATA-6(Δ50)] is recognized on leaky ribosome scanning (*51*, *52*).

An IRES sequence to which a ribosome binds and starts translation is known in virus-derived mRNAs (*53*). An IRES sequence promotes translation from a downstream initiation codon. However, we could not find such an IRES sequence between the upstream and downstream AUG codons in deletion experiments. A short uORF located on the extremely 5′-side of mRNA often stimulates or inhibits translation from the immediately downstream initiation codon (*52*). The 5′-upstream sequence of GATA-6 mRNA determined by 5′-RACE with mRNA of MKN-45 cells revealed a uORF consisting of 8 codons. The expression levels of L- and S-type GATA-6 were essentially the same in the presence and absence of this uORF, suggesting that the uORF does not function in regulation of the translation of L-type GATA-6.

An interesting finding is that deletion of the 267 bp region between the ATG codons for L- and S-type GATA-6 surprisingly abolished the expression of the L-type without affecting that of the S-type. Only the 50 bp region re-introduced into the 267 bp-deleted sequence caused production of the L-type again. However, introduction of essentially the same nucleotide sequence with a shifted frame did not restore the expression of the L-type, indicating that the protein sequence in its own might be important for the apparent synthesis of L-type GATA-6. Furthermore, interestingly, the identified protein sequence was a typical PEST sequence (*55*) with a Pro-rich amino terminal half and a poly-Ser carboxyl terminal half between the Arg residues. This PEST sequence is hardly expected to function as a degradation signal, at least under the present experimental conditions. Rather it would likely prevent potential degradation of nascent polypeptide chains, probably through interaction with other proteins. The expression of amino

terminal truncated GATA-6 with this PEST sequence (161 amino acid residues) did not restore the production of the L-type from pME-hGT1LΔEX or pME-hGT1L5′ΔEX (not shown), suggesting that the PEST sequence is *cis*-regulatory. Furthermore, introduction of a Kozak sequence around the upstream AUG codon could not overcome the effect of removal of the PEST sequence. The present results strongly indicate a novel mechanism by which the unique sequence of the extended N-terminal region of GATA-6 participates in the stable expression of the L-type.

Our expression plasmid carrying the 5′-upstream region of the mRNA of GATA-6 produces mRNA from exon 1a, but a truncated version from exon 2. Various organs synthesize mRNAs starting from exon 1a and exon 1b in a similar ratio through alternative splicing, and both mRNAs translate L- and S-type GATA-6 in an *in vitro* system (*35*). Furthermore, the present results demonstrated that two isoforms of GATA-6 are produced with or without exon 1a. Considering these observations, it is clear that exon 1 does not play a specific role in the production of the L- and S-types. It must also be noted that both the L- and S-types are produced from mouse GATA-6 cDNA, suggesting that this phenomenon could be conserved in mammals (*35*). Furthermore, the L-type is detected much more than the S-type in an *in vitro* system, although the cDNA sequence has been manipulated (*35*). However, the amounts of the L-type were slightly lower compared with those of the S-type in cultured cells.

The N-terminal sequence of S-type GATA-6 exhibits similarity to those of GATA-4 and GATA-5 (*17*, *21*, *49*), suggesting that the S-type would be a prototype of GATA-6, and it seems likely that the evolutionarily added long 5′-upstream sequence would have further put an ATG codon *in frame*. The resulting L-type might have been selected, since it has a stronger effect on transcriptional activation. As for the effect of GATA-6 on growth, mRNA of GATA-6 decreases upon stimulation of human and rat endothelial cells by a growth factor (*19*), suggesting that GATA-6 may function in the cell cycle similar to p53 (*34*). However, GATA-6 is expresseed in a region in which undifferentiated and actively dividing cells are dominant in chick intestinal villi (*56*), and its over-expression in *Xenopus* embryos blocks the differentiation of heart precursors (*57*). Analysis of the GATA-6 protein is required to determine whether the paradoxical modes of expression and function could be due to the selection of translational isoforms (L-type and S-type) or the sequence divergence between mammals and other vertebrates (*21*).

The functional significance of the two forms of GATA-6 is not known at present. However, fine-tuning of GATA-1-dependent gene expression is proposed for alternative translation products of GATA-1 with different transcriptional activities, although the L-type of GATA-1 corresponds to the S-type of GATA-6 (*58*). C/EBPβ is an example of which the biological significance in gene regulation is fairly well known (*59*, *60*). The L-type of C/EBPβ is a transcriptional activator, whereas the S-type is an inhibitor. They are generated *in frame* from a single mRNA. The ratio of these two types is suggested to be important for differentiation of the liver and mammary glands. Thus, analysis of GATA-6 in the tissues in which GATA-6 is specifically expressed and a study on the role of the PEST sequence from the viewpoint of protein-protein interactions could provide valuable information on the mechanism and regulation of GATA-6 at the molecular level.

This research was supported in part by grants from MEXT [Grant-in-Aid for Scientific Research B (14370744)], and the Takeda Science Foundation.

REFERENCES

1. Orkin, S.H. (1992) GATA-binding transcription factors in hematopoietic cells. *Blood* **80**, 575–581
2. Molkentin, J.D. (2000) The zinc finger-containing transcription factors GATA-4, -5, and -6; ubiquitously expressed regulators of tissue-specific gene expression. *J. Biol. Chem.* **275**, 38949–38952
3. Maeda, M., Kubo, K., Nishi, T., and Futai, M. (1996) Roles of gastric GATA DNA-binding proteins. *J. Exp. Biol.* **199**, 513–520
4. Teakle, G.R. and Gilmartin, P.M. (1998) Two forms of type IV zinc-finger motif and their kingdom-specific distribution between the flora, fauna and fungi. *Trends Biochem. Sci.* **23**, 100–102
5. Merika, M. and Orkin, S.H. (1993) *Mol. Cell. Biol.* **13**, 3999–4010
6. Ko, L.J. and Engel, J.D. (1993) *Mol. Cell. Biol.* **13**, 4011–4022
7. Whyatt, D.J., deBoer, E., and Grosveld, F. (1993) The two zinc finger-like domains of GATA-1 have different DNA binding specificities. *EMBO J.* **12**, 4993–5005
8. Sakai, Y., Nakagawa, R., Sato, R., and Maeda, M. (1998) Selection of DNA binding sites for human transcriptional regulator GATA-6. *Biochem. Biophys. Res. Commun.* **250**, 682–688
9. Pevny, L., Simon, M.C., Robertson, E., Klein, W.H., Tsai, S.-F., D'Agati, V., Orkin, S.H., and Costantini, F. (1991) Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**, 257–260
10. Tsai, F.-Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W., and Orkin, S.H. (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221–226
11. Lim, K.-C., Lakshmanan, G., Crawford, S.E., Gu, Y., Grosveld, F., and Engel, J.D. (2000) Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. *Nat. Genet.* **25**, 209–212
12. Molkentin, J.D., Lin, Q., Duncan, S.A., and Olson, E.N. (1997) Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061–1072
13. Kuo, C.T. , Morrisey, E.E., Anandappa, R., Sigrist, K., Lu, M.M., Parmacek. M.S., Soudais, C., and Leiden, J.M. (1997) GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048–1060
14. Molkentin, J.D., Tymitz, K.M., Richardson, J.A., and Olson, E.N. (2000) Abnormalities of the genitourinary tract in female mice lacking GATA5. *Mol. Cell. Biol.* **20**, 5256–5260
15. Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S., and Parmacek. M.S. (1998) GATA-6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12**, 3579–3590
16. Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R., and Grosveld, F. (1999) The transcription factor GATA6 is essential for early extraembryonic development. *Development* **126**, 723–732
17. Tamura, S., Wang, X.-H., Maeda, M., and Futai, M. (1993) Novel gastric DNA binding proteins recognize upstream sequence motifs of parietal cell-specific genes. *Proc. Natl Acad. Sci. USA.* **90**, 10876–10880
18. Morrisey, E.E., Ip, H.S., Lu, M.M., and Parmacek, M.S. (1996) GATA-6: a zinc finger transcription factor that is expressed in

multiple cell lineages derived from lateral mesoderm. *Dev. Biol.* **177**, 309–322
19. Suzuki, E., Evans, T., Lowry, J., Truong, L., Bell, D.H., Testa, J.R., and Walsh, K. (1996) The human GATA 6 gene: structure, chromosomal location, and regulation of expression by tissue-specific and mitogen-responsive signals. *Genomics* **38**, 283–290
20. Huggon, I.C., Davies, A., Gove, C., Moscoso, G., Moniz, C., Foss, Y., Farzaneh, F., and Towner, P. (1997) Molecular cloning of human GATA-6 DNA binding protein: high levels of expression in heart and gut. *Biochim. Biophys. Acta* **1353**, 98–102
21. Yoshida, T., Sato, R., Mahmood, S., Kawasaki, S., Futai, M., and Maeda, M. (1997) GATA-6 DNA binding protein expressed in human gastric adenocarcinoma MKN45 cells. *FEBS Lett.* **414**, 333–337
22. Dimaline, R., Campbell, B.J., Watson, F., Sansvik, A.K., Struthers, J., and Noble, P.-J. (1997) Regulated expression of GATA-6 transcription factor in gastric endocrine cells. *Gastroenterology* **112**, 1559–1567
23. Charron, F., Paradis, P., Bronchain, O., Nemer, G., and Nemer, M. (1999) Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression. *Mol. Cell. Biol.* **19**, 4355–4365
24. Bruno, M.D., Korfhagen, T.R., Liu, C., Morrisey, E.E., and Whitsett, J.A. (2000) GATA-6 activates transcription of surfactant protein A.*J. Biol. Chem.* **275**, 1043–1049
25. Liu, C., Glasser, S.W., Wan, H., and Whitsett, J.A. (2002) GATA-6 and thyroid transcription factor-1 directly interact and regulate surfactant protein-C gene expression. *J. Biol. Chem.* **277**, 4519–4525
26. Shaw-White, J.R., Bruno, M.D., and Whitsett, J.A. (1999) GATA-6 activates transcription of thyroid transcription factor-1. *J. Biol. Chem.* **274**, 2658–2664
27. Weidenfeld, J., Shu, W., Zhang, L., Millar, S.E., and Morrisey, E.E. (2002) The WNT7b promoter is regulated by TTF-1, GATA-6, and Foxa2 in lung epithelium. *J. Biol. Chem.* **277**, 21061–21070
28. Fitzgerald, K., Bazar, L., and Avigan, M.I. (1998) GATA-6 stimulates a cell line-specific activation element in the human lactase promoter. *Amer. J. Physiol.* **274**, G314–324
29. Nishi, T., Kubo, K., Hasebe, M., Maeda, M., and Futai, M. (1997) Transcriptional activation of H^+/K^+-ATPase genes by gastric GATA binding proteins. *J. Biochem.* **121**, 922–929
30. Al-azzeh, E., Fegert, P., Blin, N., and Gött, P. (2000) Transcription factor GATA-6 activates expression of gastroprotective trefoil genes TFF1 and TFF2. *Biochim. Biophys. Acta* **1490**, 324–332
31. Morrisey, E.E., Musco, S., Chen, M.Y.Z., Lu, M.M., Leiden, M., and Parmacek, M.S. (2000) The gene encoding the mitogen-responsive phosphoprotein Dab2 is differentially regulated by GATA-6 and GATA-4 in the visceral endoderm. *J. Biol. Chem.* **275**, 19949–19954
32. Wada, H., Hasegawa, K., Morimoto, T., Kakita, T., Yanazume, T., and Sasayama, S. (2000) A p300 protein as a coactivator of GATA-6 in the transcription of the smooth muscle-myosin heavy chain gene. *J. Biol. Chem.* **275**, 25330–25335
33. Heikinheimo, M., Ermolaeva, M., Bielinska, M., Rahman, N.A., Narita, N., Huhtaniemi, I.T., Tapanainen, J.S., and Wilson, D.B. (1997) Expression and hormonal regulation of transcription factor GATA-4 and GATA-6 in the mouse ovary. *Endocrinology* **138**, 3505–3514
34. Perlman, H., Suzuki, E., Simonson, M., Smith, R.C., and Walsh, K. (1998) GATA-6 induces p21Cip1 expression and G1 cell cycle arrest. *J. Biol. Chem.* **273**, 13713–13718
35. Brewer, A., Gove, C., Davies, A., McNulty, C., Barrow, D., Koutsourakis, M., Farzaneh, F., Pizzey, J., Bomford, A., and Patient, R. (1999) The human and mouse GATA-6 genes utilize two promoters and two initiation codons. *J. Biol. Chem.* **274**, 38004–38016
36. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y
37. Maeda, M., Nishijima, M., Akamatsu, Y., and Sakakibara, Y. (1985) Alteration in the characters of CDP-choline synthetase and phospholipid- choline exchange enzyme upon choline starvation in Chinese hamster ovary cells. *J. Biol. Chem.* **260**, 5925–5930
38. Andrews, N.C. and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19**, 2499
39. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of proteins using bicinchroninic acid. *Anal. Biochem.* **150**, 76–85
40. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **227**, 680–685
41. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA.* **76**, 4350–4354
42. Nakagawa, R., Sakai, Y., Takashima, A., Terada, T., Kobayashi, A., and Maeda, M. (2001) GATA DNA-binding protein expressed in mouse I-10 Leydig testicular tumor cells. *Biochem. Biophys. Res. Commun.* **283**, 412–416
43. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491
44. Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. (1980) Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* **143**, 161–178
45. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
46. Maeda, M., Oshiman, K., Tamura, S., Kaya, S., Mahmood, S., Reuben, M.A., Lasater, L.S., Sachs, G., and Futai, M. (1991) The rat H_+/K_+-ATPase β subunit gene and recognition of its control region by gastric DNA binding protein. *J. Biol. Chem.* **266**, 21584–21588
47. Sato, R., Miyamoto, W., Inoue, J., Terada, T., Imanaka, T., and Maeda, M. (1999) Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. *J. Biol. Chem.* **274**, 24714–24720
48. Maeda, M., Asahara, S., Nishi, T., Mushiake, S., Oka, T., Shimada, S., Chiba, T., Tohyama, M., and Futai, M. (1995) The rat intrinsic factor gene: its 5′-upstream region and chief cell-specific transcription. *J. Biochem.* **117**, 1305–1311
49. Morrisey, E.E., Ip, H.S., Tang, Z., Lu, M.M., and Parmacek, M.S. (1997) GATA-5: a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development. *Dev. Biol.* **183**, 21–36
50. Nakagawa, R., Sato, R., Futai, M., Yokosawa, H., and Maeda, M. (1997) Gastric GATA-6 DNA-binding protein: proteolysis induced by cAMP. *FEBS Lett.* **408**, 301–305
51. Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266**, 19867–19870
52. Geballe, A.P. and Morris, D.R. (1994) Initiation codons within 5′-leaders of mRNAs as regulators of translation. *Trends Biochem. Sci.* **19**, 159–164
53. Kozak, M. (2001) New ways of initiating translation in eukaryotes? *Mol. Cell. Biol.* **21**, 1899–1907
54. Ko, L.J., Yamamoto, M., Leonard, M.W., George, K.M., Ting, P., and Engel, J.D. (1991) Murine and human T-lymphocyte GATA-3 factors mediate transcription through a *cis*-regulatory element within the human T-cell receptor δ gene enhancer. *Mol. Cell. Biol.* **11**, 2778–2784
55. Rogers, S., Wells, R., and Rechsteiner, M. (1986) Amino acid sequence common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364–368
56. Gao, X., Sedgwick, T., Shi, Y.-B., and Evans, T. (1998) Distinctions are implicated for the GATA-4, –5, and –6 transcription

factors in the regulation of intestine epithelial cell differentiation. *Mol. Cell. Biol.* **18**, 2901–2911
57. Gove, C., Walmsley, M., Nijjar, S., Bertwistle, D., Guille, M., Partington, G., Bomford, A., and Patient, R. (1997) Over-expression of GATA-6 in *Xenopus* embryos blocks differentiation of heart precursors. *EMBO J.* **16**, 355–368
58. Calligaris, R., Bottardi, S., Cogoi, S., Apezteguia, I., and Santoro, C. (1995) Alternative translation site usage results in two functionally distinct forms of the GATA-1 transcription factor. *Proc. Natl Acad. Sci. USA.* **92**, 11598–11602
59. Descombes, P. and Schibler, U. (1991) A liver-enriched transcription activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67**, 569–579
60. Hirai, Y., Radisky D., Boudreau, R., Simian, M., Stevens, M.E., Oka, Y., Takebe, K., Niwa, S., and Bissell, M. (2001) Epimorphin mediates mammary luminal morphogenesis through control of C/EBPβ. *J. Cell Biol.* **153**, 785–794