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Amino-terminal extension of 146 residues of L-type GATA-6 is required for transcriptional activation but not for self-association

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ABSTRACT

Transcription factor GATA-6 plays essential roles in developmental processes and tissue specific functions through regulation of gene expression. GATA-6 mRNA utilizes two Met-codons *in frame* as translational initiation codons. Deletion of the nucleotide sequence encoding the PEST sequence (Glu^{31}–Cys^{46}) between the two initiation codons unusually reduced the protein molecular size on SDS–polyacrylamide gel-electrophoresis, and re-introduction of this sequence reversed this change. The long-type (L-type) GATA-6 containing this PEST sequence self-associated similarly to the short-type (S-type) GATA-6, as determined on co-immunoprecipitation of Myc-tagged GATA-6 with HA-tagged GATA-6. The L-type and S-type GATA-6 also interacted mutually. The L-type GATA-6 without the PEST sequence also self-associated and interacted with the S-type GATA-6. The transcriptional activation potential of L-type GATA-6 is higher than that of S-type GATA-6. When the PEST sequence (Glu^{31}–Cys^{46}) was inserted into the L-type GATA-6 without Arg^{13}–Gly^{101}, the resultant recombinant protein showed significantly higher transcriptional activity, while the construct with an unrelated sequence exhibited lower activity. These results suggest that the Glu^{31}–Cys^{46} segment plays an important role in the transcriptional activation, although it does not participate in the self-association.

1. Introduction

Transcription factor GATA-6 containing tandem zinc fingers (CVNC-X17-CNAC)-X29-(CXNC-X17-CNAC) recognizes a canonical DNA motif, (T/A)GATA(A/G) [1,2], and regulates the expression of various genes required for developmental processes and tissue-specific functions [3,4]. Among mammalian GATA factors, GATA-6 is distinct in that it has a 146 extra-amino terminal extension compared with the other five members [3,5]. Interestingly, both L-type and S-type GATA-6 are translated from a single mRNA [5,6] due to the leaky scanning of Met codons by ribosomes; the introduction of a Kozak sequence, 5′GCCACCaugG3′ [7], around the upstream initiation codon 5′CCGUGGaugG3′ by means of site-directed mutagenesis produced only L-type GATA-6 [5].

GATA-6 has been identified as an essential gene since a knock-out mouse showed embryonic lethality [8,9]. However, the roles of translational isoforms of GATA-6 have not been examined although both L-type and S-type GATA-6 are produced under *in vivo* conditions [10,11], and the molecular activity of L-type GATA-6 is suggested to be higher than that of the S-type [5]. Similar to other GATA factors, GATA-6 might have a function in regulation of cell type-specification or determination through unique interactions with other semi-restricted transcription factors [2]. Actually, GATA-6 was shown to physically interact with a coactivator, p300/CBP, which results in transcriptional synergy for the smooth muscle-myosin heavy chain gene and maintenance of the differentiated phenotype in vascular smooth muscle cells [12]. Furthermore, CHF1/Hey2 interacts directly with GATA-6 and prevents triggering of the smooth muscle-heavy chain transcription upon de-differentiation of smooth muscle cells [13]. In the lungs,

Abbreviations: HA, human influenza hemagglutinin; PBS, 10 mM sodium phosphate buffer (pH 7.2), 137 mM NaCl, 3 mM KCl; PBS(+), PBS containing 0.1% (v/v) Tween 20; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TNE buffer, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 10 μg/μl leupeptin, 10 μg/μl pepstatin A; TNE(+ NP40), TNE buffer containing 1% (w/v) NP-40; Tris, tris(hydroxymethyl)aminomethane.

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GATA-6 physically interacts with homeobox factor TTF1 and activates the surfactant protein-C gene [14]. It has also been demonstrated that cooperative interaction between GATA-4 and GATA-6 affects transcription of atrial natriuretic factor and B-type natriuretic peptide genes in cardiomyocytes positively [15].

Although such heterotypical interaction with other regulatory proteins has been described, a homotypic interaction has not been demonstrated for GATA-6. As for GATA-1, self-association synergistically stimulated transcription [16]. In this study, we examined whether GATA-6 interacts mutually and whether such an interaction may be affected by the presence of the amino-terminal 146 residues in the L-type or not. Here we show that GATA-6 self-associates, although the L-type specific sequence does not affect the self-association of GATA-6. Furthermore, we demonstrated that a PEST sequence [17] in the amino-terminal extension causes the higher transcriptional activity of L-type GATA-6.

2. Materials and methods

2.1. Expression plasmids for GATA-6 with a Myc-tag or HA-tag

The construction of expression plasmids for GATA-6 with a Myc-tag is shown in Fig. S1. The resulting expression plasmids for L-type GATA-6 with a carboxyl-terminal Myc-tag with or without the sequence between Arg^{13} and Gly^{101} (Fig. 1) were named pME-hGT1L5′KMyc and pME-hGT1L5′ΔEKMyc, respectively. An expression plasmid for S-type GATA-6 (pME-hGT1SMyc) was similarly constructed. All the expression plasmids were derivatives of pME18S [18]. The expression plasmids for L-type GATA-6 with an amino-terminal HA-tag were constructed by introduction of a CTC codon instead of an ATG codon corresponding to the S-type initiation codon (Fig. S2). The DNA sequence was confirmed by the dideoxy chain-termination method [19]. The molecular biological techniques were performed by published methods [20].

2.2. Expression plasmids for GATA-6 to measure luciferase activity

Expression plasmid, pME-hGT1S, pME-hGT1L5′K and pME-hGT1L5′ΔEK were described previously [5]. pME-hGT1L5′ΔAK and pME-hGT1L5′ΔA(−2)K were constructed in this study (Fig. S3). In the latter plasmids, nucleotide sequences encoding the PEST sequence and an unrelated sequence, respectively (Fig. 1), were introduced into pME-hGT1L5′ΔEX together with a Kozak sequence, GCCACCatgG [7], around the upstream initiation codon for L-type GATA-6. The introduction of a Kozak sequence results in inhibition of leaky scanning by ribosomes and the production of only L-type GATA-6 [5].

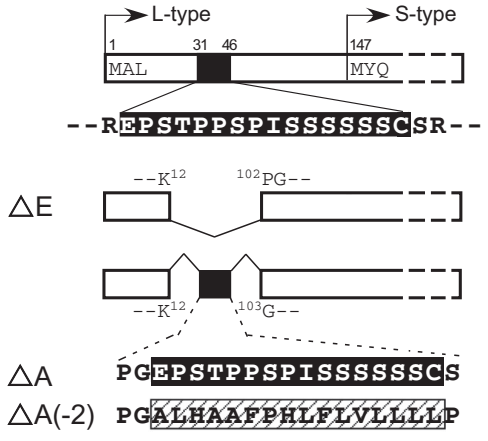

Fig. 1. Schematic representation of human GATA-6 expressed in this study. L-type GATA-6 has an extension of 146 amino acid residues in front of S-type GATA-6. Numericals indicate the amino acid residue numbers from the amino terminus of L-type GATA-6. The 16 residue segment between Glu^{31} and Cys^{46} was determined to affect the mobility of GATA-6 on SDS–polyacrylamide gel-electrophoresis [5]. The ΔE protein was derived from L-type GATA-6 by deletion of the region between Arg^{13} and Gly^{101}. The ΔA protein was constructed by addition of the DNA segment encoding Glu^{31}–Cys^{46}. The ΔA(−2) protein was constructed by adding essentially the same DNA segment to produce a frame shift of two bases.

2.3. Expression of GATA-6 tagged with a Myc-tag or HA-tag, and its immunoprecipitation

Cos-1 cells (ATCC) were grown in Dulbecco's modified Eagle medium (GIBCO). An expression plasmid was introduced into the cells by means of the diethylaminoethyl-dextran method, as described previously [5], and then cells were grown for 48 h before harvest. Protease inhibitors [20 μM benzyloxycarbonyl-Leu-Leu-norvalinal (Peptide Institute), 1 mM PMSF (Sigma), and 50 μM L-trans-epoxysuccinyl-Leu-3-methylbutylamide-ethyl ester (Peptide Institute)] were added at 24 h before harvesting as a dimethyl sulfoxide solution (10, 25 and 25 μl/5 ml medium, respectively). All the media were supplemented with 7% (v/v) fetal bovine serum (JRH Biosciences).

A cell extract was prepared by the published method [10,21]. Briefly cells from two dishes (Φ 10 cm) were collected in 1 ml of ice-cold TNE (+ NP-40) and kept on ice for 30 min. After sheering the suspension 10 times through a 25G needle, a supernatant (12,000*g*, 30 min) was obtained. Protein G Sepharose beads (GE Healthcare) were prewashed with TNE (+ NP-40). The cell extract (1 ml) was treated with the prewashed Protein G Sepharose beads (30 μl bed volume) for 1 h, and then centrifuged (2000*g*, 5 min). The supernatant was treated with 5 μl of anti-HA (F-7) antibodies (Santa Cruz) for 1 h, and further incubated with Protein G Sepharose beads (50 μl bed volume) for 1 h. The beads were precipitated (2000*g*, 5 min) and then washed five times with 200 μl of TNE buffer without NP-40. The recovered immuno-complex was heated at 95 °C for 5 min after the addition of 10 μl of 2× sample buffer [22]. The solubilized protein was subjected to SDS–polyacrylamide gel-electrophoresis, and then to Western blotting.

2.4. SDS–polyacrylamide gel-electrophoresis and Western blotting

A cell extract (20 μg protein) or immuno-precipitate (25 μl) was subjected to SDS–polyacrylamide gel-electrophoresis [7.5% (w/v), 1 mm thickness], and then electro-blotted (200 mA, 90 min.; ATTO Model-AE6675) onto an Immobilon™-P membrane [Millipore PVDF membrane (0.45 μm), IPVH00010] [5]. The membrane was washed with PBS, and then blocked for 1 h at 4 °C with PBS(+) containing 5% (w/v) skim milk (DIFCO). The Myc-tag was reacted with the peroxidase-linked mouse anti-c-Myc antibodies (MC045, Nacarai Tesque) (8000× diluted). Chemiluminescence was detected with an ECL Western blotting kit (GE Healthcare) using Scientific Imaging Film (KODAK).

Reprobing was carried out as follows. The membrane was treated with the buffer [2% (w/v) SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris–HCl (pH 6.7)] for 30 min at 50 °C, and then blocked overnight at 4 °C. The HA-tag was detected with HA-7 (10,000× diluted) (Sigma), followed by horseradish peroxidase-linked anti mouse IgG (GE Healthcare) (4000× diluted) as the second antibody. Protein concentrations were determined with a BioRad Protein Assay Kit using bovine serum albumin (Fraction V, Sigma) as a standard [23].

2.5. Reporter gene assay

The reporter gene assay was carried out as described previously [5]. Into each well of a 6-well culture plate, 1×10^5 CHO-K1 cells were seeded. Lipofectamine™ (Invitrogen) was used for

transfection (duplicate) of plasmid DNA mix, a reporter plasmid p8GATA/GL3 responsive to GATA-6 DNA-binding protein (1 μg), a GATA-6 expression plasmid (0.6 μg of pME-hGT1S, pME-hGT1L5′K, pME-hGT1L5′ΔEK, pME-hGT1L5′ΔAK, pME-hGT1L5′ΔA(−2)K or pME18S), and pSV-β-Gal (0.5 μg). Lysis buffer (90 μl) was added at 53 h after the start of transfection, and a cell lysate was prepared (12,000*g*, 10 min at 4 °C). An aliquot (20 μl and 10 μl) of the supernatant was used for measurement of the luciferase and β-galactosidase activities, respectively. The luciferase activity was normalized as to the β-galactosidase activity.

A nuclear extract was prepared from the 12,000*g* pellet as described previously [5]. An aliquot of the extract (5 μg protein) was subjected to SDS–polyacrylamide gel-electrophoresis and Western blotting. Rabbit site-specific polyclonal antibodies, hGAT-A-6N [18], were used as the first antibodies (1000× diluted). The second antibodies, horseradish peroxidase-linked donkey anti-rabbit immunoglobulin (4000× diluted) (Amersham-Pharmacia-Biotech), were similarly used.

2.6. Chemicals

Restriction enzymes were purchased from NEB and Toyobo. T4 DNA ligase and Agarose-LE Classic Type were supplied by TaKaRa. T4 polynucleotide kinase and calf intestine phosphatase were obtained from NEB. Klenow enzyme and *Ampli Taq* were from Toyobo and Roche, respectively. Oligonucleotides were purchased from Gene Design Inc., leupeptin and pepstatin A were provided by Sigma. All other chemicals used were of the highest grade commercially available.

3. Results

3.1. Mutual interaction of the L-type and S-type GATA-6 proteins

Expression plasmids for L-type and S-type GATA-6 with a HA- or Myc-tag were introduced into Cos-1 cells in three combinations (pME-hGT1L5′KMyc and pME-hGT1LmHA, pME-hGT1SMyc and pME-hGT1SHA, and pME-hGT1SMyc and pME-hGT1LmHA), and mutual interaction was examined by means of the immuno-precipitation method (Fig. 2).

L-type GATA-6 with a HA-tag was immuno-precipitated with antibodies to HA, and then the precipitate was analyzed by Western blotting with antibodies to Myc. As shown in Fig. 2A (right), L-type GATA-6 with a Myc-tag was effectively recovered in the HA-tag immuno-precipitate. The same was true for the combination of S-type GATA-6 with a HA-tag and that with a Myc-tag (Fig. 2B, right). Furthermore, S-type GATA-6 with a Myc-tag was similarly recovered in the immuno-precipitate of L-type GATA-6 with a HA-tag (Fig. 2C, right). These results indicate that the GATA-6 molecule interacts mutually independent of the presence of the L-type specific amino-terminal 146 residues.

We also excluded the possibility that the antibodies to HA-tag bind to GATA-6 with a Myc-tag and precipitate it; they did not recognize or precipitate GATA-6 with a Myc-tag (Fig. 2, center). Furthermore, antibodies to Myc-tag did not recognize GATA-6 with a HA-tag (Fig. 2, left).

3.2. Mutual interaction of L-type GATA-6 without the region between Arg^{13} and Gly^{101} containing the PEST sequence

Next we examined the interaction of GATA-6 without the region between Arg^{13} and Gly^{101} containing the PEST sequence (Fig. 3A). The expression plasmid, pME-hGT1LΔEmHA, was expressed together with pME-hGT1L5′ΔEKMyc or pME-hGT1SMyc. The results showed that the L-type without the region between Arg^{13} and Gly^{101} could interact mutually and with S-type GATA-6 (Fig. 3A and B, right). The amounts of recovered GATA-6 with a Myc-tag were less than those in Fig. 2. This may partly be explained by the low expression level of deleted L-type GATA-6 without Arg^{13} and Gly^{101} (ΔE).

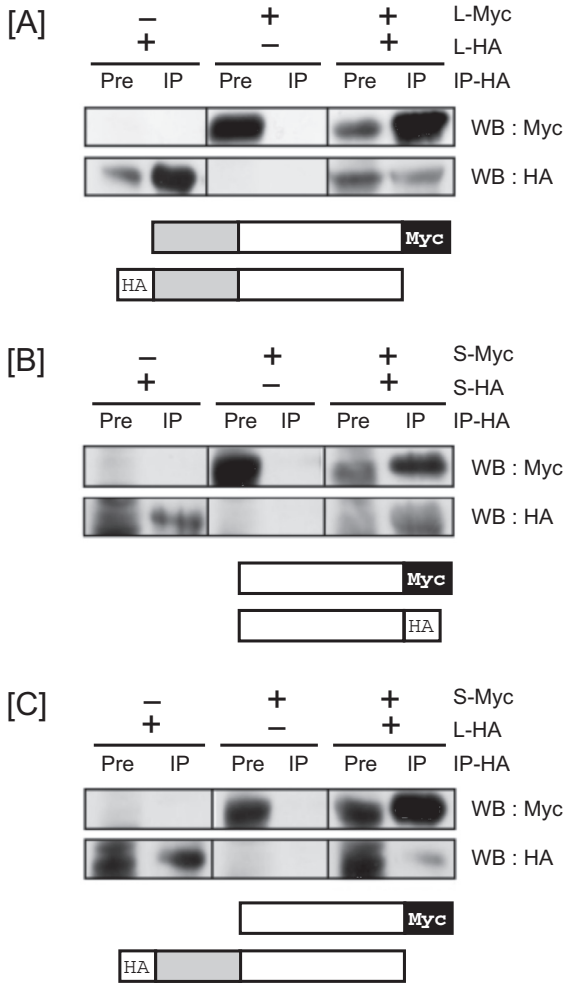

Fig. 2. Association of L-type and S-type GATA-6 molecules. Cos-1 cells were transfected with expression plasmids in three combinations. (A) pME-hGT1L5′KMyc and pME-hGT1LmHA; (B) pME-hGT1SMyc and pME-hGT1SHA; and (C) pME-hGT1SMyc and pME-hGT1LmHA. A cell extract was treated with anti-HA antibodies, and then a precipitate was collected with Protein G-Sepharose beads. It was analyzed by SDS–polyacrylamide gel-electrophoresis, followed by Western blotting with anti-Myc antibodies. The membrane was reprobed with anti-HA antibodies. A control experiment involving expression of only HA-tagged GATA-6 or Myc-tagged GATA-6 was also carried out (left and center, respectively).

3.3. Effect of the PEST sequence on transcriptional activation of L-type GATA-6

Plasmid constructs with the Kozak sequence around the initiator Met-codon for L-type GATA-6 (pME-hGT1L5′K and pME-hGT1L5′ΔEK) produced only the L-type due to inhibition of leaky ribosomal scanning [5]. Thus, we examined whether or not the L-type GATA-6 with or without the PEST sequence functions differently in terms of transcriptional activation of the GATA-responsive reporter gene. As shown in Fig. 4A, L-type GATA-6 from pME-hGT1L5′K showed stronger activation of the reporter gene compared with that of the S-type from pME-hGT1S, which is evident from the expression level (Fig. 4B).

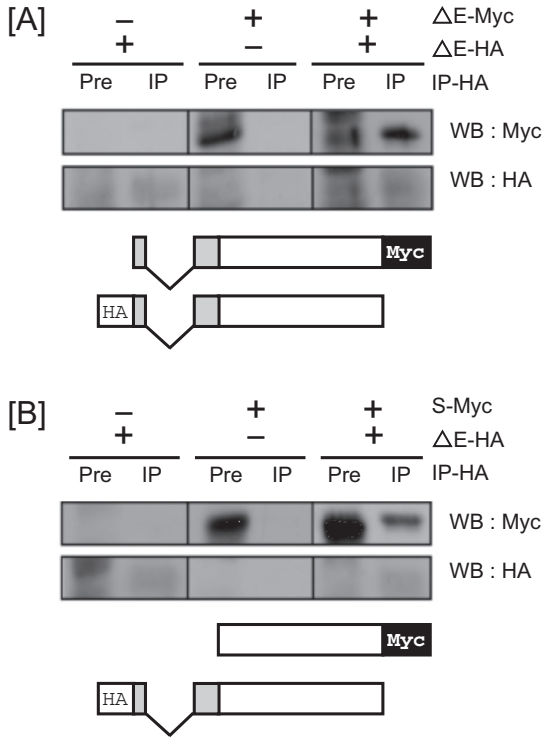

Fig. 3. Association of L-type GATA-6 with a deletion between Arg^{13} and Gly^{101}. Cos-1 cells were transfected with expression plasmids in two combinations. (A) pME-hGT1L5′ΔEKMyc and pME-hGT1LmΔEHA; (B) pME-hGT1SMyc and pME-hGT1LmΔEHA. The GATA-6 proteins with a Myc-tag or a HA-tag were analyzed as described in the legend to Fig. 2.

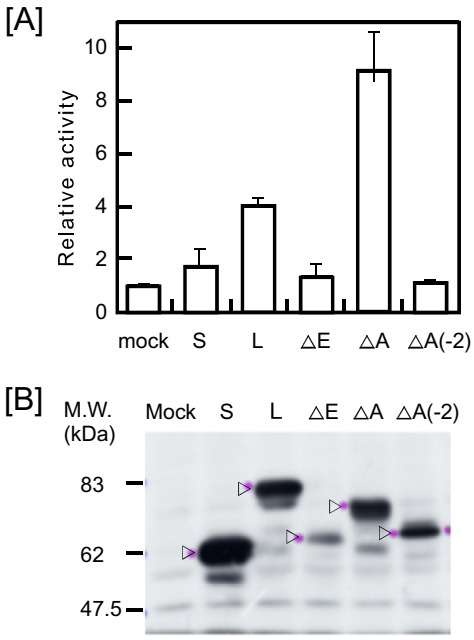

Fig. 4. Transcriptional activity of L-type GATA-6 with or without the PEST sequence. (A) Expression plasmids for L-type GATA-6 with or without the PEST sequence, and for S-type GATA-6 were introduced into CHO-K1 cells together with a reporter gene plasmid (p8GATA/GL3) plus a β-galactosidase expression plasmid [5]. Luciferase activities were normalized as to those of β-galactosidase. The relative activities as to mock transfection (pME18S instead of an expression plasmid for GATA-6) are shown with the standard deviation for two independent experiments. The average values for pME18S (Mock) were 6.68 (±0.05) × 10^5 and 1.85 (±0.04) × 10^5 (RLU/mU). S, S-type GATA-6 expression from pME-hGT1S; L, full-length L-type expression from pME-hGT1L5′K; ΔE, expression of the L-type without Arg^{13}–Gly^{101} from pME-hGT1L5′ΔEK; ΔA, expression of ΔE with the PEST sequence inserted from pME-hGT1L5′ΔAK; and ΔA(−2), expression of ΔE with the frame-shifted sequence inserted from pME-hGT1L5′ΔA(−2)K. The inserted sequences for ΔA and ΔA(−2) are shown in Fig. 1. (B) Expression levels of GATA-6 in transfectants with various expression plasmids. A nuclear extract (5 μg protein) was separated by SDS–polyacrylamide gel-electrophoresis, and then subjected to Western blotting with antibodies for hGATA-6N [18].

Interestingly, the L-type without the PEST sequence (ΔE) from pME-hGT1L5′ΔEK did not activate the reporter gene significantly, the activation level being comparable to that with the S-type. Addition of the PEST sequence (Glu^{31}–Cys^{46}) enhanced the transcription significantly, while GATA-6 with an unrelated sequence instead of the PEST sequence showed lower activity (compare ΔA and ΔA(−2)). Since the expression level of ΔA was similar to that of ΔA(−2) (Fig. 4B), the PEST sequence may have a stimulatory effect on transcription. It is also likely that the Arg^{13}–Arg^{30} and Arg^{48}–Gly^{101} regions may have inhibitory effects on transcription because the activity of the L-type is lower than that of ΔA without these two regions. The expression level of ΔE was lower (Fig. 4B, and Refs. and 10), possibly due to its unstable nature without the PEST sequence *in vivo* and/or during preparation. However, the present results clearly indicate that the PEST sequence positively affects the transcriptional activity of L-type GATA-6.

4. Discussion

In this study, mutual interaction of GATA-6 was examined by immuno-precipitation methods. We compared the precipitation of L-type and S-type GATA-6, because the translational isoforms are synthesized from a single mRNA through leaky ribosomal scanning [5]. To compare the molecular properties of L-type and S-type GATA-6 precisely, we changed the nucleotide sequence around the upstream initiation codon to the canonical Kozak sequence. This substitution stopped the leaky ribosomal scanning and resulted in the start of translation from the first Met codon effectively, since only L-type GATA-6 was predominantly produced from the transiently introduced expression plasmid (Fig. 4B in this study and Refs. and 10).

As for the structural basis of the self-association, X-ray structural analysis was carried out for the GATA-3 zinc finger region containing both an N-finger and a C-finger [24], the dimerization interfaces on tandem GATA sites being determined; one between the two C-terminal ^{351}NRPLT^{355} (^{332}PRPLA^{336} for S-type human GATA-6 [25]), and the other minor one between the C-terminal basic tail of C-finger K^{357}–Arg^{366} and the end of the recognition helix of N-finger Leu^{347}–Ile^{350} (Lys^{338}–Arg^{347} and Leu^{328}–Val^{331} for S-type human GATA-6, respectively). These results are consistent with those for GATA-1 self-association [16] and GATA-6 (this study), because the L-type specific sequence of 146 amino acid residues did not apparently participate in the self-association.

Since we first reported that GATA-6 (GATA-GT1) together with GATA-4 (GATA-GT2) in the stomach [1], both have been found widely in various tissues and organs of endoderm and mesoderm origins [25,26]. The essential role of GATA-6 in developmental processes and functional diversity for gene expression [27] could be attributable to properties of the GATA-6 protein and its binding sites on DNA. GATA-6 binds not only the canonical GATA-motif of the GATA family, but also to slightly diverse motifs [28]. Self-association of GATA-6 together with other regulatory factors may open the chromatin structure, and thereby serve to bring together and stabilize loops between distant regulatory elements, as suggested for GATA-1 self-association in the globin locus control region and downstream globin promoters [16]. From the reporter gene assay results (Fig. 4), it is likely that inhibitory and activating co-factors may bind not only to zinc fingers of GATA-6 but also to the L-type specific region. The PEST sequence seems to have a rather stabilizating effect on the expressed protein compared with

the protein without this sequence. Thus, it would be interesting to identify proteins that interact with the L-type specific sequence especially the PEST sequence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.019.

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