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Identification and Characterization of Positive Regulatory Elements in the Human Glyceraldehyde 3-Phosphate Dehydrogenase Gene Promoter

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The gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is expressed at high levels in almost all tissues. However, the molecular mechanism which sustains high-level expression of this house-keeping enzyme is still unknown. Here we show that transcriptional activity is reduced by deletion of the nucleotides from −181 to −144 (relative to the transcriptional start site) in the promoter of human GAPDH gene, both in CHO (derived from Chinese hamster ovary) and HepG2 (derived from human hepatoma) cells. Gel retardation assays revealed that at least two nuclear factors, termed GAPBF1 and GAPBF2, bind to this region. Mutations in the GAPBF1 binding site (−178 to −169) or the GAPBF2 binding site (−168 to −163) reduced this promoter activity *in vivo*, showing that these two sites contribute to the activity of the GAPDH gene promoter. Since mutations in the region from −162 to −146 also reduced the promoter activity, this region seemed to function as an added *cis*-element, although we failed to find a factor that interacted specifically with this region *in vitro*. Accordingly, we propose that there are multiple *cis*-elements in the region from −181 to −144, each of which contributes to the promoter activity of GAPDH gene; the GAPBF1 binding site has the unique feature of having a stretch of repeated A nucleotides.

Key words: A-stretch site, *cis*-elements, GC-rich site, glyceraldehyde 3-phosphate dehydrogenase, *trans*-acting factors.

Transcriptional regulation of genes is one of the most critical steps in the regulation of cellular events triggered by internal or external stimuli (*1*). Mechanisms of regulation have been studied intensively with genes involved in development, differentiation, and signal transduction, while less attention has been paid to metabolic genes, especially those encoding glycolytic enzymes, probably because the glycolytic genes are expressed relatively constitutively. Generally, glycolytic genes are highly expressed by mechanisms that have not yet been understood. Recent studies have identified several *trans*-acting factors for expression of glycolytic genes in the liver. Nuclear factor-I (NF-I), CCAAT/enhancer binding protein (C/EBP), albumin D-element binding protein (DBP), and hepatocyte nuclear factors (HNFs) are such transcription factors that play important roles in the expression of the glycolytic genes (*2*). For example, a ubiquitous nuclear factor, NF-I, and a liver-specific factor HNF-3 bind to several sites in the liver-type promoter of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene *in vitro* (*3, 4*). A footprinting experiment has shown the promoter of aldolase B gene to contain C/EBP and HNF-3 binding sites (*5*), and over-expressions of C/EBP and some HNF family proteins have revealed that these factors not only bind to this promoter, but also play important roles in expression of this gene *in vivo* (*6*). An *in vitro* analysis of the promoter of liver-type pyruvate kinase gene has revealed that it contains the binding sites for NF-I, HNF-1, and HNF-4 (*7*).

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12, GAPDH) catalyzes a reversible step in the glycolytic pathway. It consists of four identical subunits. Although there are many sequences related to the GAPDH gene in the human chromosome, most of them are pseudogenes, and only a single copy is functional; unlike most other glycolytic enzymes, GAPDH does not have isozymes (*8*). It has been reported that GAPDH gene expression is regulated hormonally and nutritionally to some extent (*9*), although the expression is maintained at a relatively stable level. It has already been shown in *in vitro* experiments that several transcription factors bind to the GAPDH promoter: AP-1, which is a heterodimer of c-*fos* and c-*jun*, binds to the region from −1070 to −1040 (relative to the transcription start site), and C/EBP binds to the site located between −365 and −315 (*10*). However, it has not yet been established whether or not these factors contribute to the transcription of this gene *in vivo*. Nasrin *et al.* created deletion promoter mutants in an attempt to find insulin-responsive elements in this promoter, and mapped two insulin response elements (IRE-A and B) in the regions from −488 to −408 and −408 to −269 (*9*). They screened an IRE-A binding protein from an expression library and identified it as SRY (sex determining region Y gene) protein-related protein, but the role of this factor in the

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

insulin signaling remains obscure (*11*).

To investigate the mechanism by which GAPDH gene is expressed at a high level in almost all tissues, positive regulatory elements in this promoter were surveyed. We show here that the region from −181 to −144 plays a crucial role in transcription of the GAPDH gene in both CHO (derived from Chinese hamster ovary) and HepG2 (derived from human hepatoma) cells and that at least two nuclear factors bind to this region.

MATERIALS AND METHODS

Construction of Plasmids—The promoter (−489 to +21) of human glyceraldehyde 3-phosphate dehydrogenase gene was selectively amplified by polymerase chain reaction (PCR) with the primer X (5′-ACTCTCGAGCAGGAGGAGCAGAGAGCGAAGCGG-3′) corresponding to the region from −3 to +30, and primer P (5′-GCAAAGCTTCGAGGAGAAGTTCCCCAACTTTCC-3′) corresponding to the region from −496 to −464. In these primers, the nucleotides shown in boldface letters have been mutated to afford newly created restriction sites. The resulting *Xho*I and *Hin*dIII sites are indicated with underlines. PCR was carried out with these primers and 500 ng of genomic DNA prepared from HepG2 cells using Ex-*taq* DNA polymerase (TaKaRa, Kyoto). Incubation conditions in the PCR were 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C, repeated for 30 cycles. After PCR, the product was digested with *Xho*I and *Hin*dIII and cloned into the multicloning site of a vector pBLCAT6, which contains the chloramphenicol acetyltransferase (CAT) gene (*12*). The resulting plasmid was designated as pGAP489CAT. Three independent clones were sequenced and the obtained sequence data perfectly matched the previously reported sequence (*9*), except for the existence of an additional C between the formerly numbered −237 and −236. Since this additional C was found in all of three independent clones, we used one of them for further analysis. The numbering of nucleotides in this report was on the basis of the present result. To create reporter plasmids which contain a deletion mutant of the GAPDH promoter, each PCR was carried out similarly for 15 cycles with pGAP489CAT as the template, and primer X and one of the following primers, A-D. The sequence and position in the promoter of the respective primers were: primer A (5′-GGCGGATCCGGAAAAAAAAAAGCGG-3′, −189 to −165), primer B (5′-CCCGGATCCTAGCGGTTTTACGGGC-3′, −151 to −126), primer C (5′-GGCGGATCCTGGCGGGAGGCGGGGT-3′, −89 to −65), primer D (5′-CCCGGATCCTATAAATTGAGCCCGC-3′, −39 to −15). Each primer had mutations that are shown in boldface letters; *Bam*HI sites were created by these mutations (underlined). The resulting PCR products were digested with *Xho*I and *Bam*HI, and cloned into pBLCAT6. The constructed plasmids were designated as pGAP181-CAT, pGAP143CAT, pGAP82CAT, and pGAP31CAT, respectively. To construct reporter plasmids containing deleted and/or mutated derivatives of GAPDH promoter, we synthesized various oligonucleotides, which corresponded to the region from −181 to −144 or from −181 to −166. Figures 2A and 4A show the sequences of these oligonucleotides, all of which carried the additional tetranucleotide, GATC at each 5′-end for ligation. After annealing of these oligonucleotides with the respective synthetic complementary oligonucleotides, they were inserted into the *Bam*HI site of pGAP143CAT, which contains the GAPDH promoter from −143 to +21, in the correct orientation. All inserted sequences of the constructed plasmids were confirmed by dideoxy sequencing using an automated DNA sequencer (Applied Biosystems, USA, model 373A) or TTH sequencing kit (Toyobo) with [α-^{32}P]-dCTP (3,000 Ci/mmol, Amersham, UK).

Cell Culture and Transient Transfection Assay—CHO cells were grown in Ham's F12 medium (Nissui, Tokyo) supplemented with 10% fetal bovine serum (GIBCO, USA). HepG2 cells were grown in Dulbecco's modified minimum essential medium (Nissui) supplemented with 10% fetal bovine serum. All cell culture was performed at 37°C in a 5% CO_2 atmosphere. The transfection was carried out by the calcium phosphate co-precipitation method (*13*) when cells were grown to 50% confluence on 60 mm diameter plates. The cells on each plate were co-transfected with 5 μg of a reporter plasmid and 5 μg of pAD-β-galactosidase plasmid (Clontech, USA), the latter serving as the internal standard for normalization of variable transfection efficiency. All the plasmids used for the transfection assay were purified by QIAGEN-tip solid-phase anion-exchange columns (QIAGEN, Germany). Cell lysates (100 μl) were prepared by three cycles of freeze-thawing and assayed for CAT and β-galactosidase activities according to the standard methods (*14*). In brief, CAT activity was determined by incubation of the cell lysate for 10 to 60 min in a buffer [0.25 M Tris-HCl, (pH 7.8)] with 4 mM acetyl CoA (Boehringer, Germany), and 0.1 μCi of [^{14}C]chloramphenicol (Amersham, UK). Acetylated chloramphenicol was separated on a TLC plate (silica gel 60F, Merck, USA) by development with a mixture of chloroform and methyl alcohol (95:5). The radioactivity of the product spot was measured with a BAS-1000 Bioimaging analyzer (Fuji Films, Tokyo). β-Galactosidase activity was determined by incubation of 10 to 30 μl of each cell extract for 10 to 180 min at 37°C in 300 μl of 66 mM sodium phosphate buffer (pH 7.5) containing 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, and 0.88 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The reaction was stopped by adding 500 μl of 1 M Na_2CO_3, and the absorbance at 420 nm, of the reaction mixture was measured.

Gel Retardation Assay—Nuclear extracts were prepared from cells using the methods of Dignam *et al.* (*15*) with slight modifications. All the manipulations described below were performed at 4°C. CHO or HepG2 cells confluent on a 150 mm dish were washed with PBS, then scraped off and suspended in 5 ml of PBS. The cells were collected by centrifugation at 1,500 rpm for 5 min, then the cell pellet was suspended in 5 ml of buffer A [10 mM HEPES-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF], left for 10 min, and homogenized by 20 strokes with a type B pestle of a glass Dounce homogenizer (Wheaton, USA). The homogenate was centrifuged for 5 min at 2,000 rpm to precipitate the cell nuclei. The nuclei thus obtained were resuspended in 1 ml of buffer B [20 mM HEPES-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 0.33 M ammonium sulfate, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF] and rocked for 30 min. After centrifugation of the suspension at 20,000 rpm for 15 min, the supernatant was recovered and dialyzed against 500 ml of buffer C [20 mM HEPES-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 0.1 M KCl, 20%

glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF] for 5 h. The dialyzed sample was centrifuged at 15,000 rpm for 15 min. The resulting supernatant was designated as the nuclear extract. Protein concentration of the nuclear extract was determined by using a Bio-Rad protein assay kit (Bio-Rad, UK). Gel retardation assay was performed with synthetic DNAs whose sequences are indicated in Figs. 2A and 4A. The DNAs having native promoter sequences are used as probes; they were labeled by Klenow fragment with [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham). The nuclear extract was incubated with 2 ng of a labeled probe (about 10,000 cpm) and 1 μg of poly(dI-dC)·poly(dI-dC) or poly(dA)·poly(dT) in 20 μl of binding buffer [20 mM HEPES-NaOH (pH 7.9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 0.5 mM EDTA, 1 mM DTT, 8% glycerol] for 15 min at room temperature. Then, the reaction mixtures were subjected to electrophoresis on a 6% nondenaturing polyacrylamide gel in TAE buffer [6.7 mM Tris-HCl (pH 7.9), 3.3 mM sodium acetate, and 1 mM EDTA]. For antibody supershift assay, 0.1 or 0.5 μg of rabbit IgG (Zymed Laboratories, USA) or rabbit anti-Sp1 polyclonal antibody (Santa Cruz Biotechnology, USA) was added to the nuclear extract and incubated for 1 h at 4°C prior to incubation with a probe DNA. The dried gels were analyzed with a BAS-1000 Bio-imaging analyzer or exposed to X-ray film at −70°C with an intensifying screen.

RESULTS

Deletion Analysis of the GAPDH Gene Promoter—To search for positive regulatory elements in the promoter of the GAPDH gene, we isolated the DNA fragment containing the GAPDH gene promoter (−489 to +21) from human genomic DNA and constructed the reporter plasmid, pGAP489CAT (see "MATERIALS AND METHODS"). When CHO and HepG2 cells were transfected with this construct, these cells developed CAT activity, showing that transcription of the CAT gene was driven by the GAPDH gene promoter. Since the promoter activity of this GAPDH gene promoter was nearly equal to that of SV40 early promoter (data not shown), it is likely that some potent *cis*-elements reside in the region (−489 to +21). To define the positive *cis*-elements in this region, we assayed CAT activity derived from several constructs harboring differently truncated GAPDH promoters. In both CHO and HepG2 cells, CAT activity driven by the promoter truncated at −181 (pGAP181CAT) was almost equal to that with pGAP489CAT. On the other hand, an appreciably reduced CAT activity was detected when the construct with the fragment from −143 to +21 (pGAP143CAT) was used (Fig. 1, columns 1-3). Further deletion to −82 still more reduced the promoter activity, indicating that positive *cis*-element(s) should also be located in the region between −143 and −83. Essentially the same result was obtained with L6 cells (derived from rat skeletal muscle) and HeLa cells (derived from a human uterine tumor) (data not shown). These results suggest that the function of the region from −181 to −144 as *cis*-element(s) is conserved among various species and tissue types. They also suggest that the region from −489 to −182 may not be crucial for the basal activity, although two insulin-responsive elements and a C/EBP binding site were reportedly identified in this region (*9, 10*).

Identification of Nuclear Factors That Bind to the Region from −181 to −144—To search for factor(s) which selectively bind to the region from −181 to −144, gel retardation assay was carried out using a synthetic DNA corresponding to this region (WT1 in Fig. 2A) as the probe. When we used poly(dI-dC)·poly(dI-dC) to suppress nonspecific bindings of nuclear proteins to the probe DNA, two bands became discernible with the nuclear extract from CHO cells (Fig. 2B, bands A and A′), suggesting that one or more factors bind to this region. To define the binding site of the factors, competition study was performed using several mutated oligonucleotides, as shown in Fig. 2A. The presence of the non-labeled competitor oligonucleotide with mutation of the A-stretch site (M5) had no effect on the band pattern obtained in the absence of any competitor,

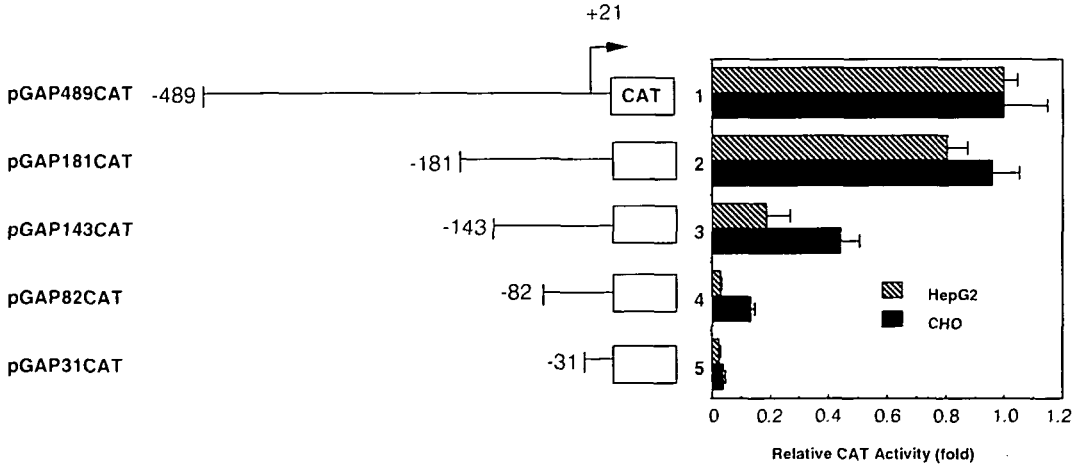


Fig. 1. **Activities of 5′-truncated promoters of GAPDH gene.** The 5′-truncated promoter of human GAPDH gene is schematically presented and CAT activities driven by these promoters in CHO or HepG2 cells are shown. Transfection was performed with a reporter plasmid (5 μg) and pAD-β-galactosidase (5 μg), as the internal standard. CAT activity was measured in the cell lysate with a fixed amount of β-galactosidase activity. Then the relative CAT activity was calculated by setting the CAT activity from pGAP489CAT as unity. Data are presented as average values and standard deviations of four samples. Similar results were obtained in two other separate experiments.

whereas either of the other oligonucleotides, WT1 or M1-4, reduced the intensity of the two bands. This result suggests that the factor(s) recognize the A-stretch site; we termed the factor(s) GAPBF1. A similar factor, with regard to sequence-specificity, was also found in the nuclear extract from HepG2 cells (Fig. 2B, band B). When we performed

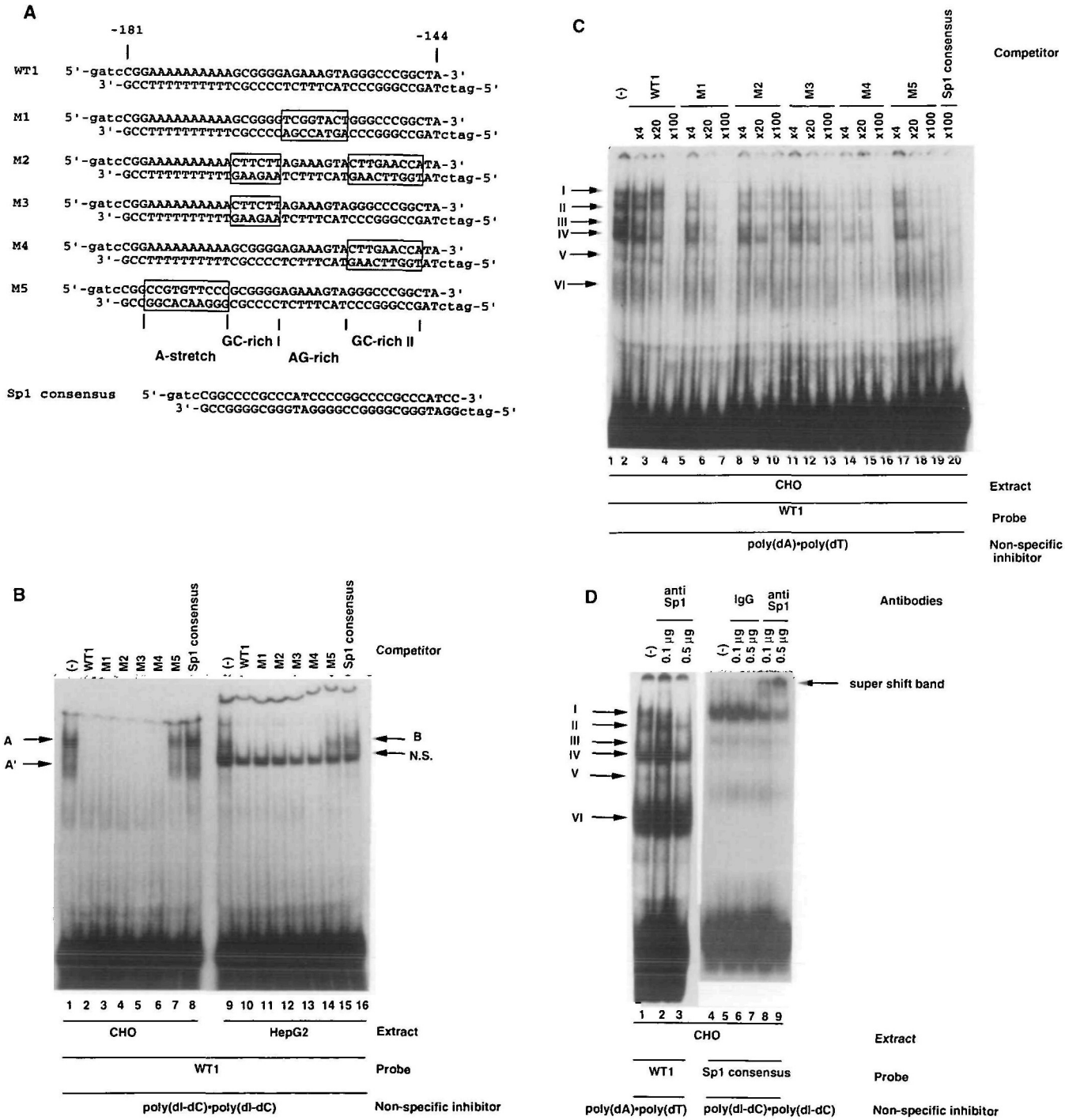


Fig. 2. **Identification of the factors which bind to the sequence from −181 to −144 by gel retardation assay.** (A) Sequences of oligonucleotides used as probes or competitor DNAs in gel retardation assays. Each oligonucleotide contained an additional GATC sequence, which are indicated with small letters, at the 5′ end. Mutated nucleotides are boxed. The Sp1 consensus sequence was adapted from that of the herpes simplex virus immediate-early 3 gene promoter (*25*). (B, C, and D) Eight micrograms of the nuclear extract prepared from CHO or HepG2 cells was incubated with ^{32}P-labeled probe DNA, and 1 μg of poly (dI-dC)·poly(dI-dC) (panel B) or poly(dA)·poly(dT) (panels C and D). The synthetic DNA shown as WT1 in panel A was used as the probe and all the DNAs in panel A were used as competitors. For competition assay, a 4-, 20-, or 100-fold mass excess of an indicated competitor was added to the binding reaction mixture. For antibody supershift assay (panel D), an indicated amount of IgG or anti-Sp1 antibody was added as described in "MATERIALS AND METHODS." The retarded bands are indicated with the symbols, A, A′, B, and I through VI. A non-specific band is indicated by N.S.

gel retardation assays with the CHO nuclear extract using poly(dA)·poly(dT) as a non-specific inhibitor, we observed a band pattern different from the one obtained using poly-(dI-dC)·poly (dI-dC) (Fig. 2, C and D). Several bands, including the six bands (I-VI) marked with arrows, were detected. Among them, the band marked II seemed to reflect the complex formation and so was studied first. On the other hand, bands I, III, and V were not always reproducible and band VI was too diffuse to represent a specific complex formation. Band IV was diminished by addition of any other competitor listed, except Sp1 consensus. Band II was only slightly affected by addition of the oligonucleotide M2 or M3 to the reaction mixture although the addition of either WT1, M1, M4, or M5 diminished these bands. This suggests that band II represents the specificity of the factor(s) for GC-rich site I (Fig. 2A). All six bands, including bands II, were also diminished by the addition of an Sp1 consensus sequence (Fig. 2A), which is also highly GC-rich, as is the competitor (Fig. 2C, lane 20). Thus, band II might reflect an interaction of the probe DNA with Sp1. To examine this possibility, we performed an antibody supershift assay. When we used Sp1 consensus sequence as the probe DNA, addition of anti-Sp1 antibody to the protein-DNA complex reaction buffer reduced the mobility of the shifted band while addition of IgG did not. On the other hand, when we used WT1 as the probe DNA, addition of anti Sp1 antibodies did not affect the mobility of band II (Fig. 2D). These results suggest that band II did not reflect the interaction of the probe with Sp1, but rather the interaction with some other factor(s). Thus, we assigned the name GAPBF2 to the factor responsible for band II. When band III was observed, it behaved similarly to band II. Thus, band III may be due to a proteolytic product of the factor for band II or may represent a different nucleoprotein complex of the same factor involving other protein components. These explanations are alternatives to two distinct factors being responsible for the two bands. We also performed gel retardation assays with the nuclear extract from HepG2 cells, but strong signals from non-specific bands made it difficult to evaluate the data (not shown).

Characterization of Positive Regulatory Elements in the Region from −181 to −144—To investigate the function of the GAPBF1 and GAPBF2 binding sites, we performed transfection experiments with several plasmids that were constructed by fusion of synthetic DNAs to the GAPDH promoter truncated at −143. The results are summarized in Fig. 3. The CAT activity from pGAP143CATWT1 that carried a wild-type sequence with an inserted ligation pad of four base pairs long was about twice that from pGAP143-CAT in both cells. Mutation of GC-rich site I reduced the promoter activity in both CHO and HepG2 cells and mutation in the A-stretch site also reduced the activity in HepG2 cells and very slightly reduced that in CHO cells (Fig. 3, columns 5 and 7). Therefore, these two sites seemed to be positive elements for the activity of the GAPDH gene promoter. Although we failed to demonstrate any factors that bind to GC-rich site II or the AG-rich site in the gel retardation assay, mutations in these regions also reduced the promoter activity, indicating that other *cis*-elements may reside in the region from −162 to −144 (Fig. 3, columns 3 and 6).

Characterization of A-Stretch Site and Its Binding Factor, GAPBF1—Further characterization of the DNA recognition property of GAPBF1 was performed by gel retardation competition analysis. As shown in Fig. 4B, in the nuclear extract from CHO cells, the competitor DNA with the mutation of the original CGG sequence (M6)

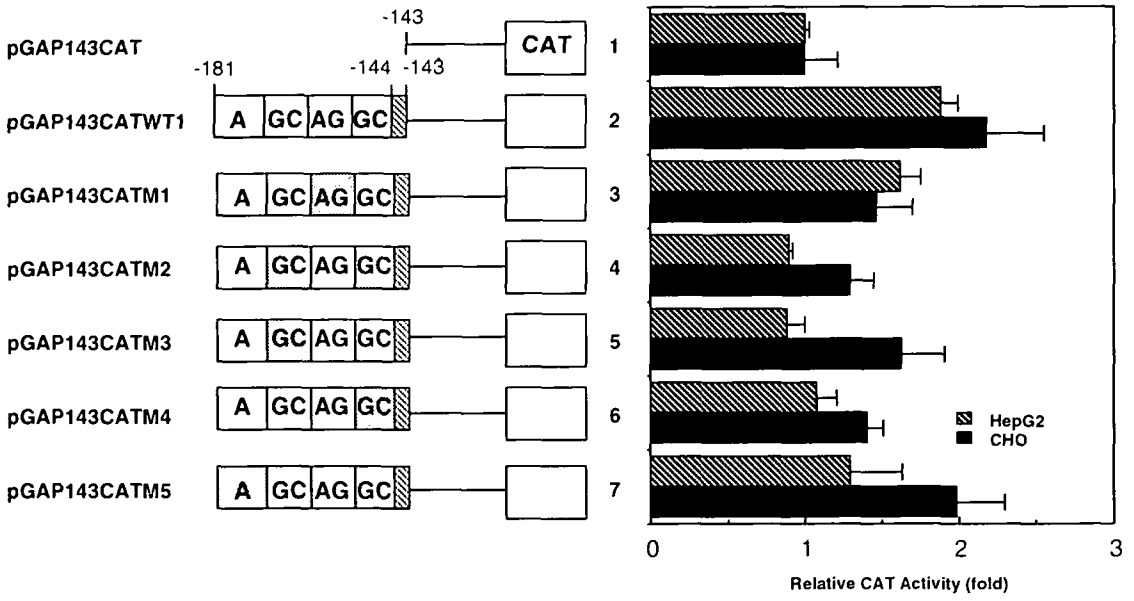


Fig. 3. **Identification of positive *cis*-elements in the region from −181 to −144.** The reporter plasmid constructs that carry mutations in the human GAPDH gene promoter are schematically presented and CAT activities driven by these promoters in CHO or HepG2 cells are shown. Dotted boxes indicate mutated sites. Hatched boxes indicate the insertion of an artificial GATC sequence. These plasmids were transiently transfected into CHO or HepG2 cells. CAT activities were determined as described in the legend to Fig. 1 and the relative activities are shown based upon the activity from pGAP143-CAT as unity. Data are presented as averages and standard deviations of four samples. Similar results were obtained in two other separate experiments.

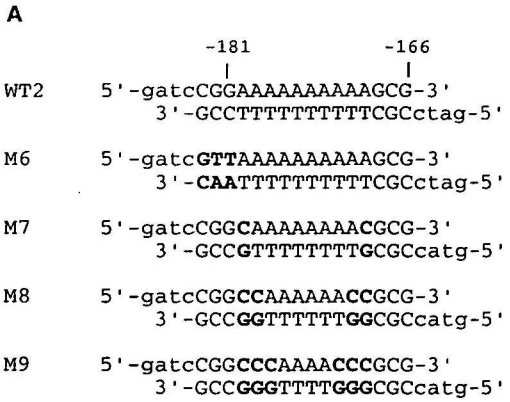
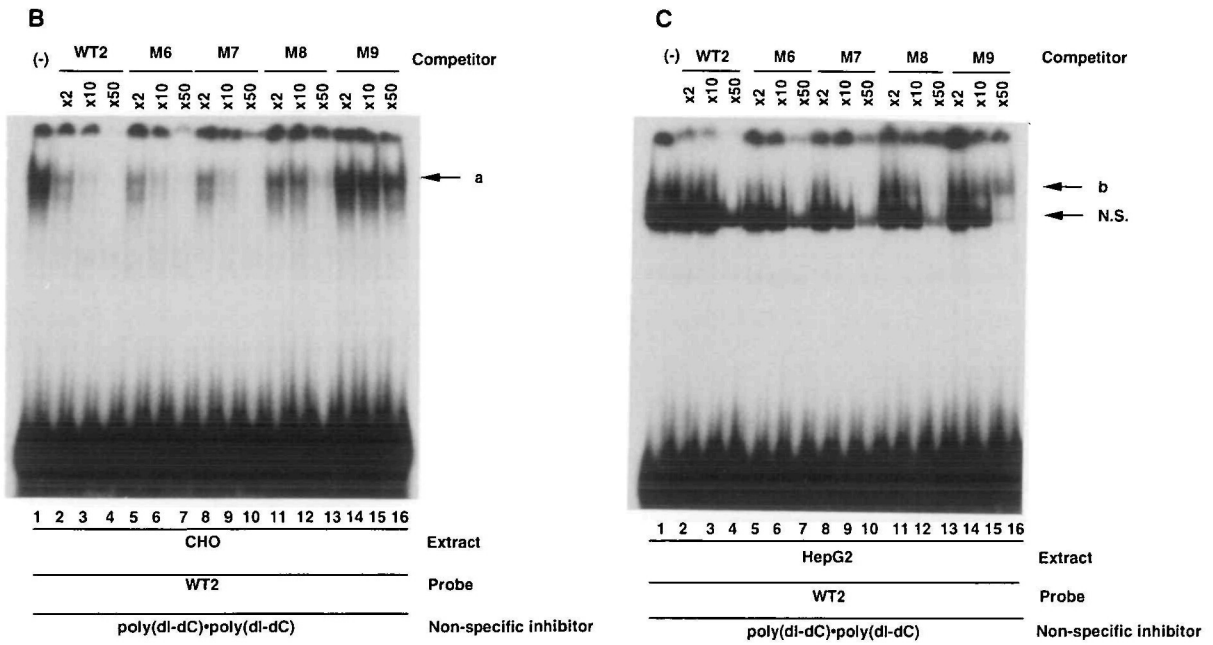


Fig. 4. **Characterization of GAPBF1 which binds to the A-stretch site by gel retardation assay.** (A) Sequences of oligonucleotides used as probes or competitors in gel retardation assays. Each oligonucleotide contained the GATC sequence, indicated with small letters, at the 5′ end. (B and C) Eight micrograms of nuclear extract prepared from CHO (panel B) or HepG2 (panel C) cells was incubated with the ^{32}P-labeled probe DNA and 1 μg of poly(dI-dC)·poly(dI-dC). The synthetic DNA shown as WT2 in panel A was used as the probe and all the listed DNAs were used as competitors. For competition assay, a 2-, 10-, or 50-fold mass excess of an indicated competitor oligonucleotide was added in the binding reaction buffer. The positions of the retarded bands are indicated by a and b. A non-specific band is indicated by N.S.



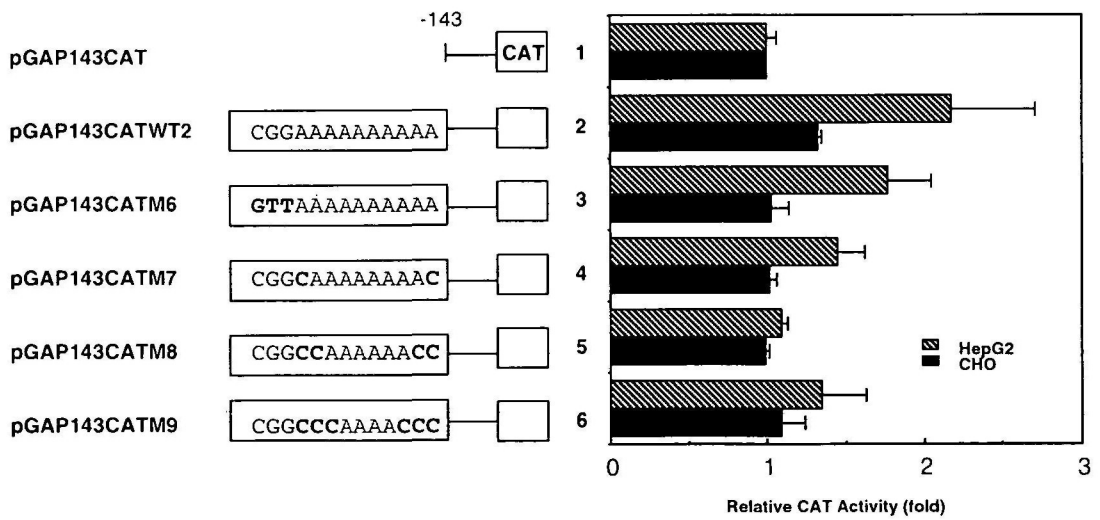


Fig. 5. **A-stretch site as a positive regulatory element.** The reporter plasmid constructs for analysis of the function of the A-stretch site in the human GAPDH gene promoter are schematically presented. Boldface letters indicate mutated nucleotides. These plasmids and pAD-β-galactosidase were transiently transfected into CHO or HepG2 cells and relative CAT activities were determined as described in the legend to Fig. 3. Data are presented as averages and standard deviations of four (HepG2) or three (CHO) samples. Similar results were obtained in two other separate experiments.

competed for the protein-DNA complex formation as effectively as the wild-type oligonucleotide (WT2) (Fig. 4B, lanes 1-7). This and the previous result that the mutation of GC-rich site I did not affect the DNA-binding activity of GAPBF1 (Fig. 2) indicate that GAPBF1 does not recognize the CGG sequence fringing the A-stretch, but recognizes the A-stretch itself. To determine the minimum repeat of A nucleotides for effective binding of the factor, gel retardation assay was performed using several oligonucleotides which contained decreasing numbers of A in the A-stretch, as competitor DNAs. The competitor DNA with an A-stretch of 8 base-repeats (M7) inhibited the complex formation between the probe DNA and GAPBF1 as effectively as WT2, but M8, which possessed 6 A-repeats showed a reduced ability as a competitor, and M9 of 4 A-repeats did not affect the complex formation (Fig. 4B, lanes 5-16). These results indicate that the straight A-repeat of 8 bases is sufficient for the DNA-binding of GAPBF1. GAPBF1 also seemed to bind to the straight A-repeat of 6 bases with a reduced affinity, but does not bind to that of 4 bases. Very similar results were obtained with the nuclear extract from HepG2 cells (Fig. 4C). A transfection experiment was carried out to examine the correlation between DNA recognition of GAPBF1 *in vitro* and activity of the GAPBF1-binding site as a *cis*-element *in vivo*. As shown in Fig. 5, fusion of the wild-type fragment (WT2; −181 to −166), which contained the A-stretch site, to the *Bam*HI site of pGAP143CAT increased the CAT activity by about 2-fold in HepG2 cells and 1.3-fold in CHO cells. In HepG2 cells, mutations of the CGG sequence, which adjoins the A-stretch site, might decrease the CAT activity (Fig. 5, column 3), although the observed effect was within the standard error in this experiment. Reduction of the number of A-repeats resulted in a gradual decrease of CAT activity in HepG2 cells, although reduction from 6 to 4 bases seemed to cause a slight increase of the CAT activity (Fig. 5, columns 4-6). These results suggest that the length of the A-stretch plays a crucial role in interaction with GAPBF1 to enhance the transcription. A similar tendency might also exist with CHO cells, but the effect was small, as seen in Fig. 3. The indicated correlation between DNA recognition of GAPBF1 *in vitro* and the activity of the A-stretch site also suggests that GAPBF1 may function as a *trans*-acting factor *in vivo*.

DISCUSSION

In this report, we demonstrate that the region of the GAPDH promoter from −181 to −144 is a positive regulatory region in CHO and HepG2 cells, and that two factors named GAPBF1 and GAPBF2 bind to two distinct sites in this region. Mutation of either site affects the activity to enhance transcription. In addition, since mutations in the region from −160 to −146 also affect the promoter activity, there may be some factor(s) which bind to this region, although we did not detect such factors in our gel retardation assay. It is likely, therefore, that interactions of multiple *cis*-elements in this region and their binding proteins contribute to enhanced expression of the GAPDH gene. These sets of *cis*-elements and *trans*-acting factors seemed to be conserved among mammals and functional in a non-tissue specific manner, since similar results were obtained with cells of different origins, CHO and HepG2 cells, and with cells of other origins as well.

The A-stretch site binding protein, GAPBF1 is unique in its DNA recognition. As shown in the gel retardation assay, this factor recognized only A (T) nucleotides in binding to DNA. Reduction of the repeat number of A nucleotides decreased the binding activity of GAPBF1 to the A-stretch site *in vitro* and also decreased the activity to enhance the transcription *in vivo*. These well-correlated tendencies represent convincing evidence that GAPBF1 detected in gel retardation assay also functions as a transcriptional factor *in vivo*. The A-stretch sequence has some intriguing features; one helical turn for A-stretch consists of only 10.0 base pairs while it consists of 10.5 base pairs for random sequences (*16*). Probably because of this feature, A-stretch sequence is not preferably accommodated in the nucleosome (*17*). In the yeast RNA polymerase I promoter, the T-rich region works as a positive regulatory element (*18*) and in yeast *pet*56 or *his*3 gene promoter, an A-rich sequence is suggested to be required for the constitutive expression of these genes, presumably because the sequence prevents nucleosome formation (*19*). However, the putative factors that bind specifically to these regions have not been found yet. To our knowledge, no factor has been reported to bind to an A-stretch sequence, although some AT-rich sequence binding proteins are known, including TATA-box binding protein (TBP) and myocyte enhancer-2 binding protein (MEF2) (*20*). Thus, GAPBF1 seemed to be a novel transcription factor although further analysis is necessary to characterize its function more precisely.

We also found another factor, GAPBF2 that binds to GCGGGG sequence (GC-rich site I). Although GAPBF2 recognized the highly GC-rich sequence, this factor is distinct from Sp1, which is one of the most-well documented transcription factors binding to GC-rich sequences. Recent studies revealed that Sp1 belongs to a family whose members bind to GC-rich motifs (*21*). There are also factors, such as *Krox*-20 protein (*22*), that bind to GC-rich sequences, but do not belong to the Sp1 family. Therefore, the relationship between GAPBF2 and other GC-rich sequence binding factors remains to be elucidated.

In CHO cells the CAT activity from pGAP143CATWT1, harboring the reconstructed promoter was almost equal to that from pGAP181CAT, harboring the native promoter. But in HepG2 cells, the CAT activity from pGAP181CAT was almost twofold higher than that from pGAP143-CATWT1, as indicated by a comparison of the promoter activity of pGAP181CAT with that of pGAP143CAT (Fig. 1) and the relative activity of pGAP143CATWT1 in Fig. 3. Since pGAP143CATWT1 carried an additional GATC between −144 and −143 as the ligation pad, it is likely that this insertion exerts an unspecified effect upon a *cis*-element that may function only in HepG2 cells. Indeed, by gel retardation assay, we have found a factor which recognizes the nucleotide sequence from −149 to −137 (CGGCTAC-TAGCGG) (unpublished data). A palindromic sequence (GCTACTAGC) is found in this site. Many transcription factors, including the thyroid and steroid receptor superfamily (*23*) and GAL4 (*24*), bind to palindromic sequences. Further analysis of this *cis*-element and the *trans*-acting factor are in progress.

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