Functional Interactions of Transcription Factor Human GA-binding Protein Subunits*

(Received for publication, April 27, 1998, and in revised form, August 18, 1998)

Fumihiko Suzuki, Masahide Goto‡, Chika Sawa, Seiichiro Ito, Hajime Watanabe,
Jun-ichi Sawada, and Hiroshi Handa§

From the Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku,
Yokohama 226-8501, Japan

The transcription factor human GA-binding protein (hGABP) is composed of two subunits, the Ets-related hGABPα, which binds to a specific DNA sequence, and either one of two hGABPβ-associated subunits, hGABPβ or hGABPγ. The DNA-binding protein hGABPα cannot affect transcription by itself, but can modify hGABP-dependent transcription in vitro and in vivo in the presence of its associated subunits. In this study, co-transfection assays showed that the ratio of hGABPβ to hGABPγ affected transcription from a promoter containing hGABP binding sites. Biochemical analysis showed that they bind to hGABPα competitively, indicating that the ratio of hGABPβ to hGABPγ is important for hGABP complex formation. Kinetic analysis of the protein-protein interaction using the surface plasmon resonance system showed that hGABPα binds to hGABPβ or hGABPγ with similar equilibrium constants. Kinetic analysis of the DNA-hGABP interaction showed that the binding of hGABPγ to hGABPα stabilized the interaction of hGABPα with its DNA binding site. In addition, the kinetic analysis revealed that this was due to a slower dissociation of the protein complex from the DNA. These results suggest that hGABPα-associated subunits influence the DNA binding stability of hGABPα and regulate hGABP-mediated transcription by competing with each other.

Transcription initiation by RNA polymerase II is regulated by various promoter-specific activators (1), which bind to specific DNA sequences where they interact with various components of the transcriptional machinery. To understand the molecular basis of transcription regulation, it is important to know the effects of complex formation of these factors on transcription regulation.

Human GABP (hGABP)† was originally identified in HeLa cell nuclear extract under the name E4TF1 and was shown to be one of the transcription factors responsible for adenovirus early region 4 gene transcription (2). Later, it was revealed to be a human homologue of murine GABP, which was identified from rat liver cells as a factor capable of binding to the herpes simplex virus immediate early gene promoter (3, 4). In recent years, hGABP (or GABP) was also reported to be a transcription factor responsible for the expression of various cellular proteins such as the leukocyte-specific adhesion molecule CD18 (β2 leukocyte integrin) (5), the tumor suppressor retinoblastoma protein (6), the cytochrome c oxidase subunit IV and Vb proteins (7, 8), and male-specific steroid 16α-hydroxylase protein (9).

Biological studies of hGABP revealed that hGABP was composed of a DNA-binding subunit hGABPα and either one of two associated subunits, hGABPβ or hGABPγ (10). The genes for hGABPα and hGABPβ have been mapped on human chromosome 21q21.2-p21.3 and 7q11.21, respectively (11, 12). hGABPα is an Ets-related DNA-binding protein that recognizes the specific sequence 5'-CGAAGTG-3', but is unable to stimulate transcription by itself (10, 13, 14). It can form a heterocomplex with either hGABPβ or hGABPγ (14). hGABPβ and hGABPγ share a common amino acid sequence in their amino-terminal regions including four tandem repeats homologous to the Notch/ιkrin repeat motif (10, 13), which is responsible for heterodimerization. hGABPβ contains a leucine zipper-like motif in its carboxyl terminus region which is necessary for its homodimerization and capacity to activate transcription (14, 15). Via these dimerization domains, hGABPα and hGABPβ can form a αβαβ heterotetrameric complex, which is able to stimulate transcription efficiently in vitro and in vivo (14, 15). On the other hand, the other associated subunit, hGABPγ, can form heterodimers, but not heterotetramers, with hGABPα because it lacks the leucine zipper-like motif. Also, this heterodimer cannot mediate transactivation (14, 15). hGABPβ was reported to specifically recognize only hGABPα, but not other Ets family proteins (16). These findings suggest that hGABPβ and hGABPγ play important roles both in the complex formation and transcriptional regulation of hGABPα.

In recent years, it has been revealed that some promoter specific activators with DNA binding activity need co-activators for transactivation. Therefore, it is possible that co-activators having different transactivation potentials competitively interact with a DNA-binding factor to precisely control the transcription of certain target genes. Transcriptional regulation by hGABP seems to be a good model system to study such controls mediated by competitive co-activators. In order to expand our understanding of the transcription regulation of hGABP complexes, we examined the influence of hGABPβ and hGABPγ on hGABP-mediated transcription regulation and determined the kinetics of the interactions between the different hGABP subunits and between hGABP and DNA.

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

*This research was supported by Research Grant from Core Research for Evolutionary Science and Technology (CREST) of Japan Science and Technology Corporation (JST) and Research Fellowships from Japan Society for the Promotion of Science for Young Scientists and Suzuki Scholarship Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The present address: Molecular Medicine Laboratories Institute for Drug Discovery Research Yamanouchi Pharmaceutical Co., Ltd., 21, Miyukigaoka, Tsukuba-shi Ibaraki 305-8585, Japan.

§ To whom correspondence should be addressed. Tel.: 81-45-924-5797; Fax: 81-45-924-5834, E-mail: hhanda@bio.titech.ac.jp.

The abbreviations used are: hGABP, human GA-binding protein; SPR, surface plasmon resonance; RU, resonance unit; EDC, N-ethyl-N’-(3-diethylaminopropyl)-carbodiimide; PAGE, polyacrylamide gel electrophoresis.

This paper is available on line at http://www.jbc.org
**Experimental Procedures**

**Western Blotting Assay**—Whole cell extracts of variable cell lines were prepared according to Manley et al.’s (17) method. Nuclear extracts were prepared from 1 × 10⁶ co-transfected cells as described previously (18). Whole cell extracts and equivalent amounts of nuclear extracts were loaded onto an 8% SDS-Polyacrylamide gel. After electrophoresis, proteins were transferred to Immobilon transfer membranes (Millipore). Human and Mouse proteins were detected using the ECL detection kit for rabbit antibody (Amersham Pharmacia Biotech) and polyclonal antibody against hGABPβ and hGABPγ.

**Co-transfection Assay, Luciferase Assay, and β-Galactosidase Assay**—Co-transfection assays, luciferase assays, and β-galactosidase assays were carried out as described previously (6) except that the transfection scales were reduced to one third.

**Preparation of hGABP Subunit Polyepetides**—hGABPα, hGABPβ, and hGABPγ were expressed individually in Esherichia coli BL21 (DE3) as described previously (13). hGABPα was purified from the lysate using latex particles bound to DNA containing the hGABP-binding sequence (19–21). hGABPβ and hGABPγ were prepared from the insoluble fraction of E. coli lysates. Insoluble fractions containing hGABPβ or hGABPγ were dissolved and denatured by the addition of 0.50TgEDN (50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiobisitol, 20% glycerol, 50 mM KCl, 0.1% Nonidet P-40) with 6 μM guanidine hydrochloride. The proteins were then renatured by dialysis for 6 h against 0.50TgEDN followed by another 6 h against 0.03TgEDN (30 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiobisitol, 30 mM KCl, 0.005% Tween 20). The concentrations of the hGABP subunits were determined by the Bradford assay (Bio-Rad). The renaturation efficiency of hGABPγ was determined by comparing the degree of complex formation of renatured proteins with hGABPα to that of native proteins with hGABPα using the gel-shift assay. The native proteins were co-purified with hGABPα by affinity chromatography on latex particles containing DNA with the hGABP-binding sequence.

**Gel-shift Assay and Data Evaluation**—Gel-shift assays were performed as described (10, 14) except that polyacrylamide gel electrophoresis was carried out for 30 min at 4 °C. The mixture of protein-bound and free reactants separated by nondenaturing PAGE was carried out for 30 min at 4 °C. The amounts of protein-bound and free reactants were measured using the AMBIS system (AMBIS). The equilibrium protein-bound and free reactants were measured using the AMBIS system (AMBIS). The amounts of protein-bound and free reactants were separated by nondenaturing PAGE and amounts of protein-bound and free reactants were separated by nondenaturing PAGE and amounts of protein-bound and free reactants were separated by nondenaturing PAGE were measured using the AMBIS system (AMBIS). The equilibrium dissociation constant (Kd) of hGABPα for its DNA binding site was calculated by Lineweaver-Burk plot analysis. Various equilibrium states were obtained by the addition of increasing amounts of unlabeled probe DNA to the binding reactions containing either a constant amount of hGABPα alone or hGABPβ and hGABPγ. The slope of the plots, 1/[DNA·hGABPα]_equilibrium against 1/[DNA]_equilibrium, represents Kd/hGABPα_inbound. For the analysis of the dissociation rate constant (kd) of hGABPα for its binding site, the 32P-labeled probe was incubated with hGABPα for 30 min at 30 °C followed by the addition of a 150-fold excess of unlabeled probe DNA to the reaction. After 0–90 min, protein–DNA complexes were separated by free DNA by PAGE and amounts of protein–DNA complexes were quantified by staining the radioactive DNA by each band. A single-exponential dissociation rate equation [DNA·hGABPα] = [DNA·hGABPα]₀ e⁻kt was used to estimate the dissociation constant (kd), which corresponded to the slope of the semi-logarithmic plots of [DNA·hGABPα]/[DNA·hGABPα]₀ against time, where k₀ and t represent the time 0 and t after the addition of the unlabeled probe.

**Binding Detected by the SPR-based Biosensor System**—The SPR-based biosensor system, BIAcore2000 system (BIACORE), was used to detect in real-time the association and dissociation reactions between hGABP and DNA or between hGABP and hGABP subunits. A continuous flow of running buffer 1 (30 mM Tris-HCl (pH 7.9), 30 mM KCl, 3.4 mM EDTA, 0.005% Tween 20) was maintained at 15 μl/min for all of the experiments. hGABP subunits as analytes were diluted to various concentrations with running buffer 1 and were then injected so that they passed over the sensor chip surface containing the immobilized ligands. Running buffer 1 was automatically replaced with the analyte solution with no intermediate delay. The sensor chip surfaces were regenerated at the end of each detection by injecting a pulse of 2.0 mM KCl for immobilized oligonucleotides or 6.0 mM guanidine HCl for immobilized hGABP subunits. The data was described in the form of sensorgrams (plots of resonance units versus time), and could be used to estimate association and dissociation kinetics.

**Preparation of the Sensor Surface**—A Sensor Chip SA5 (BIACORE) containing streptavidin covalently preimmobilized to dextran was used for the immobilization of the biotinylated oligonucleotides. The oligonucleotides containing the core DNA sequence of the hGABP binding site, 5′-CGGAAAGGCTG-3′ (DNA1), or mutated DNA sequences (DNA2) as shown in Table 1 were synthesized by an Oligo 1000 μM DNA synthesizer (Beckman). The complementary oligonucleotides were annealed prior to biotinylilation with Biotin-21-UTP (CLONTECH) and Kloneov fragment. Nonincorporated Biotin-21-UTP was removed by passing through a Nick Column (Amersham Pharmacia Biotech). They were diluted to 0.02 mg/ml in 20 mM Tris-HCl (pH 7.9), 0.3 mM NaCl, 0.5 mM EDTA and applied to the sensor surface at a constant flow rate of 10 μl/min. The sensor surface could capture the canonical or mutant oligonucleotides via the biotin-streptavidin interaction. For the immobilization of hGABP subunits to the sensor surface of a Sensor Chip CM5 (BIACORE), the amine-coupling kit (BIACORE) was used according to manufacturer’s directions. Briefly, carboxymethyl dextran on the sensor surface was activated by injecting 30 μl of a mixture of 0.05 mM EDC and 0.2 mM N-hydroxysuccinimide, and then hGABP subunits, which were diluted to 20 μg/ml with 10 mM sodium citrate (pH 3.6), were applied to the sensor surface to create covalent links with carboxymethyl dextran. The remaining active esters were blocked and deactivated by the additional injection of 35 μl of 1.0 M ethanolamine.

**Data Evaluation of the SPR System**—The rate constants of DNA-protein and protein-protein interactions were calculated by a nonlinear analysis of the association and dissociation curves using the SPR kinetic evaluation software BIAevaluation 2.1 (BIACORE). The kinetic data were interpreted in the context of a first order kinetic model: A + B = AB (22, 23). From the analysis of the sensorgrams of the dissociation phase, dissociation rate constants (kd) were calculated: ln(R/R₀) = −kt, where R and R₀ represent the SPR signal expressed in RU at time t and at the starting time of dissociation, respectively. The slope value in a plot of ln(R/R₀) against t is expressed as −kd. Based on this value, the association rate constant (ka) was calculated: ka = k₀/C, where C denotes the concentration of analyte. The value of the equilibrium dissociation constant was calculated from the value of the association rate constant and a single averaged dissociation rate constant was derived according to the thermodynamic relationship: KD = k₀/kd. The self-consistency of the data obtained from the SPR system was confirmed when these Kd values were found to be approximately equal to those of the equilibrium constants, Kd, evaluated by fitting data of the steady state response level, R, to the equation: 1/R = 1/(Kd + C)+1/Rmax, where Rmax denotes the SPR signal corresponding to the complete saturation of immobilized acceptor with analyte.

**Results**

**hGABPβ and hGABPγ Are Co-expressed in Various Cell Lines at Different Ratios**—We previously reported the cloning of two types of cDNAs for hGABPα-associated factors, hGABPβ and hGABPγ, from a HeLa cDNA library. To examine whether hGABPβ and hGABPγ are co-expressed in various cell lines, Western blotting analysis was carried out using polyclonal antibody against each factor. As shown in Fig. 1, all of the extracts from HeLa, Jurkat, and 293 cells contained both hGABPβ and hGABPγ. However, the ratio of hGABPβ to hGABPγ varied depending on the cell type. Duplex bands of hGABPβ and hGABPγ correspond to variants of these proteins containing a 12-amino acid insert at a position after 195 amino acid...
Transcription Regulation of hGABP

FIG. 2. The ratio of hGABPβ to hGABPγ influences hGABP-mediated transcription activity. A, co-transfection assays were performed as described under “Experimental Procedures.” SL2 cells were transfected with 0.6 μg of pE4-luciferase reporter plasmid (lanes 1–14), 0.3 μg of hGABPα expression plasmid (except for lane 1), along with the indicated amounts (μg) of expression plasmids for hGABPβ and hGABPγ. All co-transfection assays were repeated three times with three independent plates per sample. The results shown are averages of the values obtained from these assays. B, Western blotting analysis of nuclear extracts using polyclonal antibody against hGABPβ and hGABPγ. Nuclear extracts used in lanes 4 and 5–16 correspond to those of lanes 1 and 3–14 in panel A, respectively. In lanes 1–3, the same amounts of recombinant hGABPβ and hGABPγ, 0.1, 0.3, and 1.0 ng, respectively, were loaded onto an 8% SDS-polyacrylamide gel. Each band detected is indicated on the left by an arrowhead.

Effect of the Quantitative Ratio of hGABP to hGABPγ on hGABP-mediated Transcription—Our previous study using in vitro and in vivo assays showed that the hGABPγ/hGABPβ heterotetramer was able to stimulate transcription, whereas the hGABPα/hGABPγ heterodimer was not. To examine the possibility that the ratio of hGABPβ to hGABPγ plays a role in the control of transcription from promoters with hGABP binding sites, co-transfection assays were performed using Drosophila melanogaster Schneider line 2 (SL2) cells (Fig. 2A). The plasmid pE4-luciferase has the luciferase gene under the control of the adenovirus early 4 promoter which contains a transcription initiation site. A variety of assays were performed using the pE4-luciferase reporter plasmid (Fig. 2B). The results suggested that hGABPβ and hGABPγ bind to hGABPα competitively, and that hGABPγ can drive all of the hGABPα/hGABPβ heterotetramer complex into the hGABPα/hGABPγ heterodimer complex, and vice versa. Furthermore, luciferase activities were correlated with the amount of the hGABPα/hGABPβ heterotetramer. Taken together, these results suggested that hGABP-mediated transcription activation was controlled by the quantitative ratio of hGABPβ to hGABPγ in the cells.

Measurement of the Kinetic Parameters of the Interactions between hGABP Subunits—Previous studies of the interactions between hGABPα and its two associated subunits hGABPβ and hGABPγ were performed using gel-shift assays (10). However, no kinetic estimates of the affinities involved in these interactions were obtained using this technique. To examine for an eventual difference in the affinity between hGABPβ and hGABPγ for hGABPα, we employed the SPR system. First, using hGABPγ immobilized on the sensor chip surface, the binding interaction between hGABPα and hGABPγ was studied by injecting hGABPαa at various concentrations (Fig. 4A). The values of the $k_{d}$, $k_{a}$, and $K_{D}$ were estimated from the experimental curves as being $3.6 \pm 0.86 \times 10^{-4}$ s$^{-1}$, $5.7 \pm 0.18 \times 10^{5}$ M$^{-1}$ s$^{-1}$, and $6.4 \pm 1.7 \times 10^{-10}$ M, respectively (Fig. 4B). The data used to construct the curves was corrected by subtracting the responses of an empty sensor chip surface from the responses of a hGABPγ-immobilized sensor chip surface. Kinetic values were also calculated using a sensor surface with immobilized hGABPα. Analysis of the experimental curves yielded kinetic constants of $6.5 \pm 0.97 \times 10^{-4}$ s$^{-1}$, $4.0 \pm 0.60 \times 10^{5}$ M$^{-1}$ s$^{-1}$, and $17 \pm 5.0 \times 10^{-10}$ M for the $k_{d}$, $k_{a}$, and $K_{D}$, respectively.
concentrations were passed over the chips at a flow rate 15 μl/min. Associated factors enhance the stability of the interaction between hGABP and DNA, so the SPR system was also analyzed using the SPR system. The kinetic constants were determined to be 4.3 ± 0.14 × 10^−4 s^−1, 5.5 ± 0.26 × 10^5 M^−1 s^−1, and 7.8 ± 0.63 × 10^−18 s for the $k_{a1}$, $k_{d1}$, and $K_D$, respectively (Table II). These results suggest that hGABP and hGABPγ bind to hGABPα with similar affinities.

Analysis of the Role of hGABPγ in hGABPα-hGABPγ Complex Binding to DNA—To examine whether the binding of hGABPβ or hGABPγ to hGABPα affects the DNA binding activity of hGABPα, gel-shift assays were performed. When increasing amounts of hGABPβ or hGABPγ were added to the binding reactions containing constant amounts of 32P-labeled DNA probe and hGABPα protein, the level of free DNA was lowered compared with when hGABPα-associated factors were not added (Fig. 5). This suggests that the binding of hGABPα-associated factors enhances the stability of the interaction between hGABPα and DNA. To further analyze the phenomenon kinetically, the dissociation rates of both hGABPα-hGABPβ and hGABPα-hGABPγ complexes from the probe were measured and compared with that of hGABPα by the gel-shift assay. In both cases, about a 10-fold stabilization was observed in the dissociation rate constant compared with the hGABPα-DNA interaction whose equilibrium dissociation constant ($K_D$) and dissociation rate constant ($k_{d}$) were found to be 2.310^−10 M and 1.910^−3 s^−1 (Fig. 6), respectively.

To further confirm that hGABPα-associated factors enhance the affinity of hGABPα for DNA, the SPR system was also employed to measure the dissociation rate constant, as rebinding reactions between hGABP complex and DNA and between hGABP subunits can be avoided using this system. The effect of hGABPγ on hGABPα-DNA binding was taken to be representative of those of hGABPβ and hGABPγ, as they share the same binding mechanism and affinity for hGABPα. Oligonucleotides with the hGABP-binding sequence (DNA1; Table I) or mutated sequence (DNA2; Table I) were immobilized on the surface of a sensor chip SA5 via biotin-avidin interactions. First, repetitive injections of various concentrations of hGABPα were carried out for precise estimations of the kinetic parameters (Fig. 7A). The response was only detected when the sensor surface contained DNA1, while there was no response using sensor surfaces lacking oligonucleotides or containing DNA2. The analyses of the experimental curves gave a dissociation rate constant ($k_d$), an association rate constant ($k_a$), and an equilibrium dissociation constant ($K_D$) between hGABPα and its DNA binding site of 4.4 ± 0.64 × 10^−3 s^−1, 3.0 ± 0.090 × 10^5 M^−1 s^−1, and 15 ± 2.6 × 10^−10 M, respectively. These values were similar to those evaluated by the gel-shift assay. Next, the analyte mixtures containing a constant concentration of hGABPα and increasing concentrations of hGABPγ were passed over the DNA1 immobilized on the sensor surface (Fig. 7C). In the dissociation phase of each of the sensorgrams, the response level was normalized at the point where the dissociation reaction began (Fig. 7D). The results showed that the dissociation rate of hGABPα from the immobilized DNA became gradually lower as the concentration of hGABPγ was increased in the analyte mixture. This result is consistent with the results obtained by the gel-shift assay. Using these results, we estimated the dissociation rate constant of the interaction between the hGABP binding site and the hGABPα-hGABPγ complex. We used the experimental curve of dissociation obtained by injecting a mixture of 20 nM hGABPα and 80 nM hGABPγ because factors binding to DNA were thought to contain little hGABPγ alone at the beginning of dissociation. We analyzed this curve as the sum of two dissociation modes. One dissociation pathway was that of the hGABPα-hGABPγ complex from the DNA. The other one was the dissociation pathway of hGABPγ from hGABPα and the following dissociation of hGABPα from the DNA. The dissociation rate constant of hGABPα-hGABPγ complex from the DNA was calculated to be 1.6 ± 0.022 × 10^−3 s^−1, which is about 2.8-fold less than the $k_d$ value of hGABPα alone. We confirmed that the experimental dissociation curves almost coincided with ideal interaction...
The indicated amounts of nonradiolabeled DNA were used with 0.5 ng of 32P-labeled DNA in each reaction containing 2 ng (lanes 1–5) or 5 ng (lanes 6–10) of hGABP (except for lane 11). B, Lineweaver-Bark plot of the gel-shift assay shown in panel A. The radioactivity in each band corresponding to both free DNA and DNA-hGABPα complex was measured by the AMBIS system (AMBIS) and plotted. 2 ng (open circles) or 5 ng (closed diamonds) of hGABPα were used in the assay. C, gel-shift assays were performed as described under “Experimental Procedures.” 5 ng of hGABPα and 0.2 ng of 32P-labeled DNA probe were mixed. After the incubation, 30 ng of nonradiolabeled DNA probe was added to the reaction (lanes 2–6). After the indicated time, the reaction mixtures were applied to PAGE. D, semi-log plots of the relative amounts of the DNA-hGABPα complex. After the gel-shift assay shown in panel C, the radioactivity of each band corresponding to the DNA-hGABPα complex was measured by the AMBIS system (AMBIS) and plotted. The slope represents $-k_{-2}$. 2 ng (open squares) or 5 ng (closed diamonds) of hGABPα were used in the gel-shift assay.

**FIG. 6.** Analysis of the interaction between hGABPα and DNA using the gel-shift assay. A, the gel-shift assay was performed as described under “Experimental Procedures.” The indicated amounts of nonradiolabeled DNA were used with 0.5 ng of 32P-labeled DNA in each reaction containing 2 ng (lanes 1–5) or 5 ng (lanes 6–10) of hGABP (except for lane 11). B, Lineweaver-Bark plot of the gel-shift assay shown in panel A. The radioactivity in each band corresponding to both free DNA and DNA-hGABPα complex was measured by the AMBIS system (AMBIS) and plotted. 2 ng (open circles) or 5 ng (closed diamonds) of hGABPα were used in the assay. C, gel-shift assays were performed as described under “Experimental Procedures.” 5 ng of hGABPα and 0.2 ng of 32P-labeled DNA probe were mixed. After the incubation, 30 ng of nonradiolabeled DNA probe was added to the reaction (lanes 2–6). After the indicated time, the reaction mixtures were applied to PAGE. D, semi-log plots of the relative amounts of the DNA-hGABPα complex. After the gel-shift assay shown in panel C, the radioactivity of each band corresponding to the DNA-hGABPα complex was measured by the AMBIS system (AMBIS) and plotted. The slope represents $-k_{-2}$. 2 ng (open squares) or 5 ng (closed diamonds) of hGABPα were used in the gel-shift assay.

**TABLE I**

| Oligonucleotides and sequences |
|-------------------------------|
| DNA1 | 5'-AAATTTTCTTAAATGGGAGGATGTTACGTTACCTCTCAATGCATTGCACCCGTTTTACCGAAAGTGACGATT-3' |
| DNA2 | 5'-AAATTTTCTTAAATGGGAGGATGTTACGTTACCTCTCAATGCATTGCACCCGTTTTACCGAAAGTGACGATT-3' |

curves which were simulated with kinetic constants. The result suggests that the binding of hGABPγ to hGABPα stabilizes the DNA-hGABPα interaction by imparting a lower dissociation rate constant.

**DISCUSSION**

In this report, we studied the role of hGABPα-associated proteins hGABPβ and hGABPγ in hGABP-mediated transcriptional activation and their effect on the DNA binding activity of hGABPα. We also sought to determine the interaction affinity of hGABPα for hGABPβ, hGABPγ, and DNA.

Many Ets family proteins have been shown to possess associated factors that result in the formation of large complexes with various levels of transcription activity (25, 26). We previously showed that hGABPβ and hGABPγ are functional partners of Ets-related hGABPα. Here, we show that they have similar affinities for hGABPα and that they compete with one another for binding to hGABPα to regulate transcription activity. These findings are supported by functional domain analysis (14), which has revealed that hGABPβ and hGABPγ have identical domains required for binding to hGABPα. The similar $K_d$ values of hGABPβ and hGABPγ for hGABPα would also appear to reflect the ratio of the hGABPα-hGABPβ complex to the hGABPα-hGABPγ complex in cells which is dependent on the respective intracellular concentrations of hGABPβ and hGABPγ. The transcription activation of hGABP is dependent on the ratio of hGABPβ to hGABPγ. These hGABP complexes have been shown to have different transcription activities in vivo and in vitro, depending on whether they are made up of hGABPα-hGABPβ complex or the hGABPα-hGABPγ complex (14, 15). Our results by Northern blot assay show that the expression levels of hGABPγ mRNA were varied in human tissues examined, compared with that of hGABPβ (data not shown). Therefore, the ratio of the hGABPγ mRNA to hGABPβ mRNA was different in various human tissues. For example, the ratios in skeletal muscle, liver and testis were 0.26, 0.43, and 1.2, respectively. This result is reminiscent of a similar observation previously reported by M. Marchioni et al. (32) who found that the pattern of immunologically related hGABPβ subunit expression changes during Xenopus embryonic development while the pattern of hGABPα-like protein expression remains almost the same. These suggest that the ratio of hGABPβ to hGABPγ may contribute to the characters and functions of the tissues. Taken together, the results of the co-transfection assays and the kinetic studies suggest that the quantitative ratio of hGABPβ to hGABPγ directly influences the relative amounts of hGABPα-hGABPβ complex and...
Transcription Regulation of hGABP

hGABPα-hGABPγ complex in the cell, resulting in fine regulation of promoters having binding sites for the hGABP complex. However, certain promoters may have a mechanism to select for the binding of either the hGABPα-hGABPβ complex or the hGABPα-hGABPγ complex, where other DNA-binding factors on other regions of the promoter may play an important role in recruiting either hGABP complex on the promoter as a transcriptional regulation partner. Such a mechanism of transcription regulation is analogous to that of the positive factor TFIIA and the repressors, Dr1 and Dr2. These factors bind to the basic region of the TATA box-binding protein competitively, resulting in transcription regulation (27, 28). As in the case of hGABPγ and the hGABP complex, some gene-specific transcription factors with DNA binding activity, including Ets family proteins, may have partners which antagonize their interaction with associated factors necessary for their transactivation activity.

The $K_D$ value of hGABPβ and hGABPγ for hGABPα leads to the interpretation that most of the hGABPα protein present in the cell forms a complex with hGABPβ or hGABPγ, if their intracellular concentrations are taken into account. This notion is supported by the observation that these three factors can be co-purified from HeLa nuclear extracts using Sepharose beads bound to DNA containing the hGABP-binding sequence (10), and that hGABPβ and hGABPγ are responsible for efficient hGABPα migration into the nucleus as observed by an immunofluorescence assay of the co-transfected cells (15).

The DNA-protein interactions of hGABP were also characterized kinetically using the SPR system and the gel-shift assay as summarized in Table II. The binding affinity of hGABPα for its target sequence ($K_D$ of $15 \times 2.6 \times 10^{-10}$ M) is equivalent to that of p42/Ets-1 ($K_D$ of $36 \times 10^{-10}$ M) reported previously (29). This may reflect the conservation of the highly homologous DNA binding domain, termed the Ets domain, in both proteins. The DNA binding affinity of gene-specific transcription factors generally ranges from $10^{-8}$ M to $10^{-11}$ M (equilibrium dissociation constant). The affinity of their Ets domains for target sequences is lower than that of the D. melanogaster transcription factor, fushi-tarazu, which has a homeodomain for DNA binding, and whose $K_D$ was reported to be approximately $2.5 \times 10^{-11}$ M by the gel-shift assay (30). We have also shown that the binding of hGABPγ to hGABPα stabilizes the DNA binding affinity of hGABPα because it imparts a slower dissociation rate. The interaction may lead to conformational change(s) in hGABPα. We observed that the binding did not result in faster association of hGABPα with its target.

### TABLE II

| Ligand   | Analyte | $k_a$ | $k_d$ | $K_D$  |
|----------|---------|-------|-------|--------|
| DNA1     | hGABPα  | $30 \pm 0.90$ | $44 \pm 6.4$ | $15 \pm 2.6$ |
| DNA1     | hGABPγ  | $16 \pm 0.22$ | $3.6 \pm 0.86$ | $6.4 \pm 1.7$ |
| hGABPβ   | hGABPα  | $5.5 \pm 0.26$ | $4.3 \pm 0.14$ | $7.8 \pm 0.63$ |
| hGABPβ   | hGABPγ  | $4.0 \pm 0.60$ | $6.5 \pm 0.97$ | $17 \pm 5.0$ |
| DNA1     | hGABPγ  | $8.3^{a}$ | $19^{a}$ | $23^{a}$ |

*These values are determined by gel-shift assay.*
sequence. Recently, Batchelor et al. (31) reported structural analysis of the GABPα and GABPβ complex and showed that Lys69 of GABPβ makes an indirect contact with the DNA sugar-phosphate backbone via Glu321 of GABPα. This may explain the slower dissociation rate. As the result of such stability the hGABP complex may reside for longer periods at promoters containing its recognition site than hGABPα alone. These findings suggest the importance of the stability of DNA binding activity in addition to interactions between the transactivation domain and the RNA polymerase machinery for transcription activation.

Acknowledgments—We are grateful to Drs. H. Yasui, S. Hashimoto, T. Natsume, T. Wada, and T. Imai for helpful discussions. The contribution of M. Ikeda and K. Tamai to the production of polyclonal antibodies against hGABPβ is gratefully acknowledged. We also thank all of our colleagues in the laboratory for helpful comments.

REFERENCES
1. Burley, S. K., and Roeder, R. G. (1996) Annu. Rev. Biochem. 65, 769–799
2. Watanabe, H., Imai, T., Sharp, P. A., and Handa, H. (1988) Mol. Cell. Biol. 8, 1290–1300
3. Thompson, C. C., Brown, T. A., and McKnight, S. L. (1991) Science 253, 762–768
4. Watanabe, H., Imai, T., Sharp, P. A., and Handa, H. (1988) Mol. Cell. Biol. 8, 1290–1300
5. LaMarco, K. L., Thompson, C. C., Byers, B. P., Walton, E. M., and McKnight, S. L. (1991) Science 253, 762–768
6. Rosmarin, A. G., Caprio, D. G., Kirsch, D. G., Handa, H., and Simkevich, C. P. (1995) J. Biol. Chem. 270, 23627–23633
7. Savoysky, E., Mizuno, T., Sowa, Y., Watanabe, H., Sawada, J-i., Nomura, H., Ohsugi, Y., Handa, H., and Sakai. T. (1994) Oncogene 9, 1839–1846
8. Virbasius, J. V., Virbasiu, C. A., and Scarpulla, R. C. (1993) Genes Dev. 7, 380–392
9. Bachman, N. J., Yang, T. L., Dasen, J. S., Ernst, R. E., and Lomax, M. I. (1996) Arch. Biochem. Biophys. 323, 152–162
10. Watanabe, H., Wada, T., and Handa, H. (1990) EMBO J. 9, 841–847
11. Sawada, J-i., Goto, M., Watanabe, H., Handa, H., and Yoshida, M. C. (1995) Jpn. J. Cancer Res. 86, 10–12
12. Goto, M., Shimizu, T., Sawada, J-i., Sawa, C., Watanabe, H., Ichikawa, H., Ohira, M., Okhi, M., and Handa, H. (1995) Gene (Amst.) 166, 337–338
13. Watanabe, H., Sawada, J., Yano, K., Yamaguchi, K., Goto, M., and Handa, H. (1993) Mol. Cell. Biol. 13, 1385–1391
14. Sawada, J-i., Goto, M., Sawa, C., Watanabe, H., and Handa, H. (1994) EMBO J. 13, 1396–1402
15. Sawa, C., Goto, M., Suzuki, F., Watanabe, H., Sawada, J-i., and Handa, H. (1996) Nucleic Acids Res. 24, 4954–4961
16. Brown, T. A., and McKnight, S. L. (1992) Genes Dev. 6, 2502–2512
17. Manley, J. L., Fire, A., Cano, A., Sharp, P. A., and Gefter, M. L. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3855–3859
18. Andrews, N. C., and Fuller, D. V. (1991) Nucleic Acids Res. 19, 2499
19. Kawaguchi, H., Asai, A., Ohtuka, Y., Watanabe, H., Wada, T., and Handa, H. (1989) Nucleic Acids Res. 17, 6229–6240
20. Inomata, Y., Kawaguchi, H., Hiramote, M., Wada, T., and Handa, H. (1992) Anal. Biochem. 206, 109–114
21. Inomata, Y., Wada, T., Handa, H., Fujimoto, K., and Kawaguchi, H. (1994) J. Biomater. Sci. Polym. Ed. 5, 293–302
22. Karlsson, R., Michaelsson, A., and Mattson, L. (1991) J. Immunol. Methods 145, 229–240
23. O’Shannessy, D. J., Bringham-Burke, M., Soneson, K. K., Hensley, P., and Brookes, I. (1993) Anal. Biochem. 212, 457–468
24. Gugneja, S., Virbasius, J. V., and Scarpulla, R. C. (1995) Mol. Cell. Biol. 15, 102–111
25. Wasylyk, B., Hahn, S. L., and Giovane, A. (1993) Eur. J. Biochem. 211, 7–18
26. Janknecht, R., and Nordheim, A. (1993) Biochem. Biophys. Acta 1155, 346–356
27. Inostroza, J. A., Mermelstein, F. H., Ilho, H., William, S. L., and Reinberg, D. (1992) Cell 70, 477–489
28. Mermo, A., Madder, K. R., Lane, W. S., Champoux, J. J., and Reinberg, D. (1994) Nature 365, 227–232
29. Fisher, B. J., Fivash, M., Casas-Finet, J., Erickson, J. W., Kendeh, A., Bladen, S. V., Fischer, C., Watson, D., and Papas, T. (1994) Protein Sci. 3, 257–266
30. Florence, B., Handrow, R., and Laughon, A. (1991) Mol. Cell. Biol. 11, 3613–3623
31. Batchelor, A. H., Parker, D. E., Brouss, F. C., McKnight, S. L., and Wolberger, C. (1998) Science 270, 1037–1041
32. Marchioni, M., Morabito, S., Salvati, A. L., Becari, E., and Carnevali, F. (1993) Mol. Cell. Biol. 13, 6479–6489