Synergistic Transcriptional Activation by hGABP and Select Members of the Activation Transcription Factor/cAMP Response Element-binding Protein Family*

(Received for publication, May 10, 1999, and in revised form, August 24, 1999)

Jun-ichi Sawada, Noriaki Simizu†, Fumihiko Suzukiš, Chika Sawa, Masahide Goto§, Makoto Hasegawa, Takeshi Imai, Hajime Watanabe‡, and Hiroshi Handa¶

From the Research Function for Biotechnology, Frontier Collaborative Research Center, and 3Molecular Medical Engineering, Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 2539 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

The Ets-related DNA-binding protein human GA-binding protein (hGABP) α interacts with the four ankyrin-type repeats of hGABPβ to form an hGABP tetrameric complex that stimulates transcription through the adenovirus early 4 (E4) promoter. Using co-transfection assays, this study demonstrated that the hGABP complex mediated efficient activation of transcription from E4 promoters in a synergistic manner with activating transcription factor (ATF) 1 or cAMP response element-binding protein (CREB), but not ATF2/CRE-BP1. This synergy also partially occurred when hGABPa was used alone in place of the combination of hGABPa and hGABPβ. hGABP activated an artificial promoter containing only ATF/CRE/CRE-binding sites under coexistence of ATF1 or CREB. Consistent with these results, physical interactions of hGABPa with ATF1 or CREB were observed in vitro. Functional domain analyses of the physical interactions revealed that the amino-terminal region of hGABPa bound to the DNA-binding domain of ATF1, which resulted in the formation of ternary complexes composed of ATF1, hGABPa, and hGABPβ. In contrast to hGABPa, hGABPβ did not significantly interact with ATF1 and CREB. Taken together, these results indicate that hGABP functionally interacts with selective members of the ATF/CREB family, and also suggest that synergy results from multiple interactions which mediate stabilization of large complexes within the regulatory elements of the promoter region, including DNA-binding and non-DNA-binding factors.

In eukaryotes, the control of gene expression often involves regulated interactions of gene-specific transcription factors with promoters and enhancer regions. The regulatory proprieties of DNA-binding proteins are often modulated in a combinatorial fashion by interactions among them (1). hGABP1 has been identified as the transcription factor E4TF1 in HeLa cells because of its ability to activate transcription within the adenovirus early 4 (E4) promoter (2, 3). Characterization of cDNAs of E4TF1 subunits (4) revealed that the subunits are highly homologous to the respective rat transcription factor GABP (GA-binding protein) subunits. The GABP subunits bind to the cis-regulatory DNA sequence important for immediate early gene activation of herpes simplex virus type-1 (5, 6). Therefore, E4TF1 has been re-designated as hGABP (human GABP), as described in Ref. 7. The Ets-related protein hGABPa can bind by itself to the DNA sequence 5′-CGGAAGTG-3′ in the E4 promoter, but has no effect on in vitro or in vivo transcription (3, 7, 8). By itself, hGABPβ, which contains four notch/ankyrin-type repeats, neither binds to a specific sequence nor stimulates transcription, but has homodimerization activity via the carboxyl-terminal leucine zipper-like domain. However, the four ankyrin repeats mediate the association of hGABPβ with hGABPa, leading to the formation of an α2β2 heterotetrameric complex on the DNA, resulting in in vitro and in vivo transcriptional activation (3, 7–9). Certain transcription factors, such as NRF-2, EF-1A, Xrpf1, and RBF-1, which have been independently studied as transcription factors involved in cellular or viral gene expression, have been found to be immunologically related to GABP or hGABP (10–13). In particular, subunits of NRF-2 are identical to those of hGABP at the level of cDNA (14). The genes for the hGABP subunits, hGABPa and hGABPβ, have been mapped to human chromosome 21q 21.2-q21.3 and 7q11.21, respectively (15, 16).

Members of the Ets family of DNA-binding proteins contain about an 85-amino acid region of similarity called the ETS domain, which is sufficient for direct DNA binding to a sequence containing a common 5′-GGA(A/T)-3′ core motif (17, 18). Recently, partnerships between the Ets-related proteins and transcription factors belonging to other structural families have been reported, and their functional protein-protein interactions were shown to be important for regulation of gene expression (19–21). The promoter of the adenovirus E4 contains not only an hGABP-binding site, but also several binding sites for the ATF/CREB family which has been revealed to be...

* This work was supported by a Research Grant from Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation (JST), a grant-in-aid for Scientific Research on Priority Areas from The Ministry of Education, Science, Sports and Culture, a grant of R and D Projects in Cooperation with Academic Institutions from New Energy and Industrial Technology Development Organization (NEDO), and research fellowships from the Japan Society for the Promotion of Science for Young Scientists. 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.

† Present address: Molecular Medicine Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba 305-8585, Japan.

‡ To whom correspondence should be addressed: Research Function for Biotechnology, Frontier Collaborative Research Center, Tokyo Institute of Technology, 2539 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan. Tel.: 81-45-924-5797; Fax: 81-45-924-5834; E-mail: hhanda@bio.titech.ac.jp.
Synergistic Transactivation by hGABP and ATF1/CREB

important for its activity by deletion analysis of the promoter (22). Transcription factors belonging to the ATF/CREB family were found to be required for its efficient transcriptional activation in vitro (23). But it is unclear how hGABP and members of ATF/CREB family regulate the transcription.

In order to gain further insight into the transcriptional activator complex involved with the Ets-related protein hGABP, and to better understand the molecular basis of synergistic transcriptional regulation, we used co-transfection and biochemical assays to examine the possibility that hGABP can cooperate with some members of ATF/CREB family. Based on the studies presented here, we propose that hGABP functions as a transcriptional partner of ATF1 or CREB, leading to efficient transcription activation. Our data suggest that a functional synergy between these factors results from a multitude of DNA-protein and protein-protein interactions which stabilize the large activator complex on promoter/enhancer elements.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—Luciferase reporter plasmid p4(hGABP-CRE)luc was constructed to insert the DNA fragment obtained by polymerase chain reaction using pTF1–4(C2AT) (3) as a template into the PicaGene PGV-B plasmid (Toyo-ink), the polymerase chain reaction product which contains four tandem repeats of 5\'AACCGGAATTGAC-GAA-3\' and TATA box sequence, derived from the adenovirus E4 promoter. Luciferase reporter plasmids p4(hGABP-CREmt)luc, p4(hGABP-CREmt)luc, and p4(hGABP-CREmt)luc were constructed to substitute the tandem repeats of p4(hGABP-CRE)luc for four tandem repeats of 5\'AACCGGAATTGAC-GAA-3\', 5\'AACCGGAATTGAC-GAA-3\', and 5\'AACCGGAATTGAC-GAA-3\', respectively.

The pGE\(x\)hGABP plasmid, which encodes hGABP fused with glutathione S-transferase (GST) to its amino terminus (designated GST/hGABP), was constructed by the insertion of a BamHI-digested DNA fragment from hGABP cDNA into pGE\(x\)/p(15). The DNA fragment was constructed using available restriction sites or polymerase chain reaction.

GST fusion proteins to the amino terminus in the GST/hGABP plasmid was described previously (4) into the bacterial expression plasmid pGEX-ATF1 was described previously (3) with EcoRI and HindIII. The GST-hGABP plasmid cDNA was digested with EcoRI and HindIII-digested pGEX-2T. Restriction enzyme-digested DNA fragments encoding the full-length of hGABP and hGABP\(\beta\), which encode various hGABP mutants, which encode various DNA fragments from pET53 (4) into the Smal site of the GST fusion vector pGE\(x\)-2T. The pGE\(x\)/hGABP\(\beta\) plasmid, which encodes hGABP\(\beta\) fused with GST to their amino terminus (designated GST/hGABP\(\beta\)), were constructed by the insertion of Klenow polymerase-treated DNA fragments from pET53 (4) into the Smal site of the GST fusion vector pGE\(x\)-2T. Restriction enzyme-digested DNA fragments encoding the full-length of hGABP and hGABP\(\beta\) were cloned into NdeI-BamHI-digested pKA, a plasmid designed for the expression of a histidine-stretch and the phosphorylation signal sequence from pET60 (4) into the large activator complex on promoter/enhancer elements.

DNA-protein and protein-protein interactions which stabilize a multitude of hGABP and members of ATF/CREB family were purified from HeLa nuclear extract using the hGABP-binding site-immobilized latex beads as described previously (9).

**Cell Line and Immunoprecipitations**—N173 cells were constructed by transfection of pSV2neo and an HA-tagged hGABP plasmid pCHA/E4TFP1–60 which was constructed to insert HA-tagged hGABP into mammalian expression plasmid pCAGGS (29), and following two isolations of a colony in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum and 100 \(\mu\)g/ml G418. The cell line was described previously (29). The cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum and 50 \(\mu\)g/ml G418. N173 and HeLa monolayer cells were harvested and washed twice with ice-cold phosphate-buffered saline, and cell extracts were prepared as previous described (30) with slight modification. The nuclear fraction and cytoplasmic fraction were mixed and dialyzed against 0.1 M Tris(HCl pH 7.9, 10% glycerol, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40). hGABP was further purified by size exclusion chromatography using a Superdex 200 3.2/30 column on SMART system (Amersham Pharmacia Biotech) to separate the hGABP\(\alpha\) and hGABP\(\beta\) complex from the hGABP\(\alpha\) and hGABP\(\beta\) complex.

**GST fusion proteins were expressed in E. coli, BL21(DE3).** The cells were suspended in lysis buffer (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 0.5 mM NaCl, 0.5% Nonidet P-40, 5% sucrose), lysed by sonication, and subsequently centrifuged at 12,000 \(\times\)g at 4 \(^\circ\)C for 10 min to remove cell debris. The supernatants were stored at −80 \(^\circ\)C until they were used for in vitro protein binding assays. hGABP\(\alpha\) and hGABP\(\beta\) with six histidine residues and a PKK phosphorylation site fused to their amino terminus, which were used in Figs. 5 and 6, and also expressed in E. coli BL21(DE3). These fused proteins were purified from supernatants on His-Bind Resin and dialyzed against 0.05 TGKEDN. They were stocked at −80 \(^\circ\)C until they were used as \({\text{32}}^{\text{P}}\)-labeled proteins for GST pull-down assays. Recombinant hGABP\(\alpha\) intact form used in Fig. 5B was expressed in E. coli BL21(DE3) and purified from the bacteria extract using the hGABP-binding site-immobilized latex beads as described previously (9).

**Cell Shift Assay**—The binding reaction was performed as described previously by Watanabe et al. (3). Electrophoresis was performed using a 1% agarose gel containing 2.5% glycerol and TGE buffer (50 mM Tris-HCl, pH 7.9, 380 mM glycerol, 2 mM EDTA) at 4 \(^\circ\)C and 5 V/cm for 4 h. The DNA probes derived from adenosine virus early 4 promoter was prepared by digesting pA5C with EcoRI (3) with EcoRI. The DNA probe was used to perform purification using a Nick column (Amersham Pharmacia Biotech). About 2 ng of the DNA probe was used for the binding reactions.

**Cell Culture and Transfections**—SL2 cells were maintained as described previously (7). Cells were plated onto 35-mm polystyrene dishes at a density of 1 \(\times\) 10\(^5\) cells/ml of medium per dish 5–10 h prior to transfection. DNA/CaPO\(\scriptscriptstyle 4\) precipitates were formed by the dropwise addition of 100 \(\mu\)l of 25% CaCl\(_2\) containing the DNA to 100 \(\mu\)l of 2 \(\times\) HBS (42 mM Hepes, pH 7.1, 275 mM NaCl, 1.4 mM Na\(_2\)HPO\(_4\)) and added once with 200 \(\mu\)l of 100 mM glycine, pH 2.5. The cell lines were plated in 100 \(\mu\)l of 0.1 TGKEDNP buffer, and antigens were released by treatment with Klenow polymerase in the presence of [\(\alpha{}^{32}\text{P}\)]ATP, followed by purification using a Nick column (Amersham Pharmacia Biotech). About 2 ng of the DNA probe was used for the binding reactions.

**Expression Plasmids for full-length hGABP subunits in Drosophila melanogaster** Schneider line 2 (SL2) cells (25) were constructed as described previously (7). To facilitate the construction of expression plasmids for full-length ATF1, pBS/ATF1 was constructed by insertion of an EcoRI-HindIII DNA fragment containing ATF1 cDNA isolated from pGEM-ATF1 (gift of H. C. Hurst) (26) into the sites of EcoRI and HindIII of pBlueScriptII SK+.

The DNA fragment encoding ATF1 was isolated from pBS/ATF1 by digestion with KpnI and EcoRI, and subcloned into the sites of KpnI and EcoRI of pSAC\(_d\) to create an ATF1 expression plasmid, pSAC\(_d\)ATF1. The coding region for CREB was obtained by polymerase chain reaction using pT7\(\scriptscriptstyle 4\)CREB (gift of H. C. Hurst) (26) as template, and the polymerase chain reaction product was then ligated into the site of Smal of pUC119 to create pUC119/CREB.

The CREB expression plasmid, pSAC\(_d\)CREB was constructed by insertion of an EcoRI-BamHI DNA fragment containing CREB cDNA isolated from pUC119/CREB into the site of BamHI and EcoRI of pSAC\(_d\).
onto a Nick column (Amersham Pharmacia Biotech) to remove [γ-32P]ATP and imidazole. For the in vitro binding assay, equal amounts (about 5 μg) of GST fusion proteins were immobilized on 3 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) using the corresponding volumes of the supernatants containing GST fusion proteins. The GST-immobilized beads were equilibrated in binding buffer (50 mM Tris-HCl, pH 7.9, 10% glycerol, 50 mM KCl, 1 mM EDTA, 10 mM MgCl2, 1 mM CaCl2, 1 mM dithiothreitol, 0.01–0.05% Nonidet P-40). The beads were then incubated with about 1 ng of [32P]labeled protein at 4 °C for 2 h and packed in a 1-ml syringe as an affinity column and then washed with 300 μl of binding buffer. Bound proteins were eluted by boiling the beads in 25 μl of SDS sample buffer. Released proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis. After staining with Coomassie Blue, the gels were dried and subjected to autoradiography.

Synergistic Transactivation by hGABP and ATF1/CREB Family—To study which members of the ATF/CREB family can bind to the E4 promoter simultaneously with sequence-specific interaction. To further examine whether the slowest migrated DNA-protein complex by HeLa nuclear extract contains hGABP and ATF/CREB family, antibodies were used in gel shift assay. The most slowly migrated band was obviously reacted against both anti-hGABP and anti-CREB antibodies (lanes 13 and 15). These results show that hGABP and some members of the ATF/CREB family simultaneously bind to the adenovirus E4 promoter in a sequence-specific manner in vitro.

RESULTS

Proteins of the hGABP and ATF/CREB Family Bind Simultaneously to Promoter DNA—The E4 promoter has ATF/CREB-binding sites which interact with hGABP-binding site. To investigate whether hGABP and ATF/CREB family proteins exist on the promoter simultaneously to regulate its activity, gel shift assays were performed with probes derived from the E4 promoter (Fig. 1). This probe DNA contains an hGABP-binding site and a major and a minor recognition site for ATF/CREB family. As shown in Fig. 1, purified hGABP, containing both α and β subunits, formed α5β2 heterotetramers with the probe as described previously (lanes 1 and 8). Members of the ATF/CREB family were prepared by purification from HeLa nuclear extract using DNA affinity beads (28), and ATF/CREB family proteins formed DNA-protein complexes with higher mobility (lane 2). When hGABPα/hGABPβ heterotetramers were incubated with purified ATF/CREB family proteins, a new complex with the least mobility appeared (lane 3). Three retarded bands appeared when HeLa nuclear extract was used (lane 7). The mobility of the three bands were similar to those of the three bands obtained by combination use of the purified proteins of hGABP and ATF/CREB family. In either case when the purified proteins or HeLa nuclear extract were used, the DNA-protein complex with the least mobility was competed out with both cold DNA fragments containing hGABP and ATF/CREB family recognition sequences, while the two complex with faster mobility disappeared by adding either competitor containing hGABP or ATF/CREB-binding sites (lanes 4, 5, 8, and 9). This result indicates that hGABP and the ATF/CREB family can bind to the E4 promoter simultaneously with sequence-specific interaction.
columns). In contrast, co-transfections of increasing amounts of transfected ATF1 with constant amounts of hGABPα and hGABPβ led to a marked synergistic transactivation in a dose-dependent manner (Fig. 2A, white columns). Qualitatively similar results were obtained with the use of transfected CREB as substitute for transfected ATF1 (Fig. 2B). But ATF2/CRE-BP1 transfected together with hGABP did not lead to a synergistic activation of transcription (Fig. 2C). These results show that hGABP functionally interacts with ATF1 and CREB, but not ATF2/CRE-BP1, resulting in stimulation of synergistic transactivation.

**Functional Interaction of hGABP with ATF1 and CREB**—To explore whether the synergy between hGABP and ATF1 or CREB entirely depends on the interactions to their binding sites on DNA, we used four luciferase reporter gene constructs. The p4(hGABP-CRE)luc reporter plasmid was constructed to be under control of an artificial promoter including four tandem repeats of an E4 promoter-derived DNA sequence containing both an hGABP-binding site and a CRE site (ATF/CREB family binding site). It is noted that the CRE sequence 5'-TGACGAAA-3' is not a very good CRE site with some deference compared with the consensus one 5'-TGACGTCA-3'. The p4(hGABPmt-CRE)luc, p4(hGABP-CREmt)luc, and p4(hGABPmt-CREmt)luc reporter plasmids were also constructed to have mutations in their hGABP-binding sites, CREs, or both sites, respectively, resulting in the inability of each factor to bind to their reporter genes. Lack of the hGABP-binding motif resulted in no activation by transfected hGABPα and hGABPβ, even when large amounts of the expression plasmids were used (Fig. 3A, compare solid columns 2–5 with white columns 2–5). But p4(hGABPmt-CRE)luc did not abrogate the ability of hGABP to synergistically activate transcription in the presence of co-transfected ATF1 or CREB (Fig. 3A, solid columns 6–15). Importantly, the magnitude of the synergistic effect was reduced when compared with the reporter constructs p4(hGABP-CRE)luc (Fig. 3A, compare solid columns 6–15 with white columns 6–15). In the case of the reporter constructs p4(hGABP-CREmt)luc, lack of a CRE motif did not abolish the ability of ATF1 or CREB to synergistically transactivate in the presence of co-transfected hGABPα and hGABPβ (Fig. 3B, lanes 1–7). However, the synergy was not observed when p4(hGABPmt-CREmt)luc was used as a reporter gene (Fig. 3B, lanes 8–12). These results suggest that the synergism requires the binding of proteines to their corresponding binding sites on the promoter DNA.

To further investigate which subunit of hGABP effects synergistic transactivation, either hGABPα or hGABPβ was co-transfected with ATF1 (Fig. 4A). Although synergistic transactivation was not detected when hGABPβ was co-transfected with ATF1 (solid columns), transfected hGABPα synergistically activated the transcription from the E4 promoter in the presence of ATF1 (white columns). When CREB was substituted for ATF1 as shown in Fig. 4B, transfected hGABPα similarly showed synergistic transactivation with CREB. A weak synergistic transactivation was detected when a large amount of hGABPβ was co-transfected together with CREB. The magnitude of the synergy of hGABPα with ATF1 or CREB decreased compared with that of the synergy of when hGABPα and hGABPβ were used in combination (compare Figs. 4, A and B, with 2, A and B).

**Physical Interaction between hGABPα and ATF1**—The results of our co-transfection assay could be explained if hGABP somehow interacted with ATF1 and CREB on the promoter. To study potential physical interaction between hGABP and ATF1 or CREB, we performed co-immunoprecipitation experiments using the whole cell extract prepared from a HeLa cell transformant N173 cell line which constitutively expressed HA-tagged hGABPα. The immunoprecipitate with anti-HA monoclonal antibody was loaded on 10% SDS-PAGE and examined by immunoblotting assay with antibodies against hGABPα, hGABPβ, and CREB. Immunoreacted bands corresponding to hGABPβ and CREB were present in the immunoprecipitate as well as that to HA-tagged hGABPα, whereas they were not detected in an immunoprecipitate from HeLa cell extract using anti-HA antibody (Fig. 5A). We also did not detect DNA-binding transcription factor Sp1 in the immunoprecipitate by HA-antibody (data not shown).

Synergism and in vitro association of hGABPα with CREB indicated that hGABPα may interact directly with ATF1 and CREB. We examined this possibility by in vitro binding assays using GST fused proteins. When 32P-labeled recombinant ATF1 and CREB were mixed and captured with GST-hGABP subunits bound to glutathione-coated Sepharose beads, about
FIG. 3. hGABP and either ATF1 or CREB affect transcription from artificial promoters containing four tandem repeats of both the hGABP-binding site and CRE or CRE alone (A), the hGABP-binding site alone (B), but not the binding site. A, SL2 cells were transfected with 0.6 \( \mu g \) of either p4(hGABP-CRE)Luc or p4(hGABP-CRE)mtLuc, each 0.03, 0.1, 0.3, and 1.0 \( \mu g \) (columns 2–5, 7–10, and 12–15, respectively), of hGABP \( b \) or p4(hGABP-CRE)mtLuc, 0.03, 0.1, and 0.3 \( \mu g \) of ATF1 (columns 2–4, respectively), of CREB (columns 11–15) expression plasmid. B, SL2 cells were transfected with 0.6 \( \mu g \) of p4(hGABP-CRE)mtLuc, 0.03, 0.1, and 0.3 \( \mu g \) of ATF1 (columns 2–4, respectively) or CREB (columns 5–7, respectively) expression plasmid, along with/without each 0.3 \( \mu g \) of hGABP \( b \) and hGABP \( b \) expression plasmids (lanes 1–7). SL2 cells were transfected with 0.6 \( \mu g \) of p4(hGABP-CRE)mtLuc, 0.3 and 1.0 \( \mu g \) of ATF1 (columns 8 and 9, respectively) or CREB (columns 11 and 12, respectively) expression plasmid, along with/without each 0.3 \( \mu g \) of hGABP \( b \) and hGABP \( b \) expression plasmids (lanes 8–12). Co-transfection assays and their analyses were carried out as described in the legend to Fig. 2. All results shown represent mean ± S.E. of three separate experiments.

FIG. 4. hGABP \( b \) stimulates transcription synergistically with ATF1 or CREB. SL2 cells were transfected with 0.6 \( \mu g \) of the reporter plasmid pE4-luc, 0.03, 0.1, and 0.3 \( \mu g \) (columns 2–4, respectively) of ATF1 (A) or CREB (B) expression plasmid with/without 0.3 \( \mu g \) of either hGABP \( b \) or hGABP \( b \) expression plasmid. Co-transfection assays and their analyses were carried out as described in the legend to Fig. 2. All results shown represent mean ± S.E. of three separate experiments.

8% of input ATF1 and CREB bound to GST-hGABP \( b \) beads (Fig. 5B, lanes 5 and 6), and they were not retained to a significant degree when GST-hGABP \( b \) immobilized beads were used (lanes 8 and 9). GST-hGABP \( b \) used in the assay appeared to retain their native structure as judged by their ability to bind to hGABP \( b \) (lane 7). To test if the observed interaction of hGABP \( b \) with ATF1 and CREB depends on contaminated DNA, the in vitro interaction assays were performed in the presence of ethidium bromide (34). When \( ^{32}P \)-labeled recombinant hGABP \( b \) was mixed and captured with GST-ATF1 bound to glutathione-coated Sepharose beads, about 15% input of hGABP \( b \) was retained on GST-ATF1 beads, either in the presence or absence of 100 \( \mu g \)/ml ethidium bromide (Fig. 5A, lanes 10 and 11). On the other hand, hGABP \( b \) was not bound to a significant degree onto GST immobilized beads (lanes 12 and 13).

The physical interaction of hGABP \( b \) with ATF1 or CREB was hereafter studied using the binding activity of ATF1 as a representative of the ATF/CREB family because the primary structures of these proteins are very similar to each other. To study the kinetics of the interaction between hGABP \( b \) and ATF1, an in vitro binding assay was performed with the BIACORE system (Biacore AB), using surface plasmon resonance (SPR), a technique able to direct protein-protein interactions in real-time manner. A GST-ATF1 fusion protein that had been immobilized to the sensor surface, via anti-GST antibody coupled to the surface via their amino group, was exposed to purified recombinant hGABP \( b \), and the association and dissociation of hGABP \( b \) were recorded in real time. As shown in Fig. 5C, the interaction of hGABP \( b \) with GST-ATF1 was detected in a dose-dependent manner when increasing concentrations of hGABP \( b \) were used. As a result of analyzing the sensorgrams, the dissociation and association rate constants of the interaction were measured as \( 2.4 \times 10^{-3} \) s\(^{-1} \) and \( 1.2 \times 10^{5} \) M\(^{-1}\) s\(^{-1} \), respectively, which indicates the dissociation constant as \( 2.0 \times 10^{-3} \) M. These results show that ATF1 and CREB interact physically with hGABP \( b \) in cell extract and in vitro, but not with hGABP \( b \) or hGABP \( b \).

Complex of hGABP and ATF1—We examined the possibility that hGABP \( b \) could influence the ATF1-hGABP \( b \) interaction, for hGABP \( b \) was originally copurified with hGABP \( b \) from HeLa cell nuclear extract (3, 4). hGABP \( b \) was mixed alone, or in the presence of hGABP \( b \) with GST-ATF1 fusion protein immobilized on beads, and the captured proteins were detected by autoradiography (Fig. 5D). Approximately 8% of the input hGABP \( b \) was bound to the beads in the absence of hGABP \( b \), the same result as in Fig. 5 (lane 3). When increasing amounts of hGABP \( b \) were mixed together with constant amounts of hGABP \( b \), the amounts of the captured hGABP \( b \) progressively increased (lanes 4–6). hGABP \( b \) was also progressively captured, together with hGABP \( b \), via an hGABP \( b \)-hGABP \( b \) interaction (lanes 4–6), while hGABP \( b \) were not captured to a significant degree by the beads in the absence of hGABP \( b \), as a negative control (lanes 7–9). These results show that the ATF1-hGABP \( b \) and hGABP \( b \)-hGABP \( b \) interactions were mutually permissible, and that the association of hGABP \( b \) with hGABP \( b \) led to a more stable interaction of hGABP \( b \) with ATF1.

hGABP \( b \) Interacts with the DNA-binding Domain of...
ATF1—To define the domains responsible for complex formation between hGABPα and ATF1, a series of ATF1 deletion mutants, with GST fused to their amino termini, were used for the in vitro binding assay (Fig. 6A). Roughly equal amounts of GST-ATF1 deletion mutants bound to glutathione-coated beads were incubated with 32P-labeled hGABPα. Fig. 6B shows the result of the deletion analysis performed to identify the hGABPα interaction region of ATF1. Incubation with GST alone served as a negative control (Fig. 6B, lane 7). The results show that the amino-terminal deletion mutants tested were sufficient to interact with hGABPα (lanes 5 and 6). Removal of the basic region-leucine zipper (bZip) domain, known as a DNA-binding motif, led to a complete loss of the interaction activity with hGABPα (lanes 2–4). These results demonstrate that hGABPα interacts with the DNA-binding bZip domain of ATF1.

ATF1 Interacts with the Amino-terminal Region of hGABPα—To map the ATF1-binding region of hGABPα, GST-hGABPα mutants as shown in Fig. 6C were applied to the in vitro binding analysis using GST fused proteins. As shown in Fig. 6D, deletion of the amino-terminal portion (amino acids 1–399) of hGABPα had a severe impact on its ability to bind to ATF1 (lane 5). The amino-terminal region (amino acids 1–319) and the Ets DNA-binding domain (amino acids 294–406) of hGABPα were sufficient to form a complex with ATF1 (lanes 3 and 4). Similar results were obtained by analyses using the BIACORE system and indicated that the GST fused N-region (1–319) and Ets domain (294–406) bound to ATF1 with low efficiency compared with GST-hGABPα, while the GST fused C-region detected the same change of SPR as when the GST protein was used alone (results not shown). These results appear that ATF1 associates with the amino-terminal region (amino acids 1–399) of hGABPα, which is not always necessary for the interaction with hGABPβ (8). These results are consistent with the previous results that ATF1-hGABPα and hGABPα-hGABPβ interactions are not exclusive to each other (Fig. 5D).

DISCUSSION

In this report, we have demonstrated that hGABP interacts functionally with selective members of the ATF/CREB family, ATF1 and CREB, which results from formation of a large transcriptional activator complex on the E4 promoter. In this study, the adenovirus E4 promoter was used as model promoters to demonstrate the synergy of hGABP with ATF1 and CREB. We do not have any direct evidence that the syn...
Synergistic Transactivation by hGABP and ATF1/CREB

Fig. 6. Mapping of the protein interaction domains in vitro. A, schematic structures of GST-fused ATF1 variants. B, in vitro binding assay using GST-ATF1 variants. An arrowhead indicates the bound hGABPα to GST-fused ATF1 variant immobilized on glutathione- Sepharose. Binding assays and the analysis were carried out as described in the legend to Fig. 5. Coomassie Blue staining and autoradiography of the same gel are shown. C, schematic structures of GST-fused hGABPα variants. D, identification of hGABPα domain that interacts with ATF1 in vitro. Binding assays using GST-hGABPα variants were performed as in panel B. An arrowhead indicates the bound 32P-labeled ATF1 to GST-hGABPα variants immobilized on glutathione-Sepharose. Twenty percent of the total 32P-labeled ATF1 protein input (IN) are shown in lane 1. Coomassie Blue staining and autoradiography of the same gel are shown.

Energy functions on the promoters in vivo, and need additional experiments to study which gene expression the synergy functions for in cells. Our transient transfection assays also demonstrated the synergistic transcription activation on minimal promoter of retinoblastoma gene (data not shown). The likelihood of synergy contributing to adenovirus E4 gene and retinoblastoma gene expression appears high, based on our results presented here (Figs. 1 and 2) and our previous report (2, 7, 12, 35, 36).

Models for the Mechanism of the Synergistic Transactivation—An important question in understanding the role of the promoter/enhancer region is how gene-specific transcription factors can regulate transcription efficiency. Our results indicated an architecture of gene-specific activators in the context of the adenovirus E4 promoter, and we suggest a feasible model for synergistic transcription activation. The theory is that the synergistic effect is a result of a large number of individual contacts between, at least, promoter DNA, hGABP subunits, and ATF1 (or CREB). Direct interaction between hGABPα and ATF1 (or CREB) may contribute to the stability of their binding to the promoters. Furthermore, this possible effect could increase since hGABPα binding to hGABPα enhances the affinity of the interaction between hGABPα and ATF1 as shown in Fig. 5D. However, the direct interaction between hGABPα and ATF1 was not so stable that the transcription factors would not form a complex without tethering them on the promoter DNA in vivo. Studies of the mechanism by which ATF1 and CREB activate transcription reveals that the CREB-binding protein (CBP) act as their coactivator and their interactions are in a phosphorylation dependent manner (37, 38). Recently, Bannert (39) reported that hGABPα control the interleukin 16 promoter activity in concert with CBP which interacted with hGABPα. In the case of the simultaneous and stable existence of ATF1 and hGABP on the promoter, the complex can have multiple CBP-docking sites, which contribute to a great advantage to competition for limiting the level of CBP, the subsequent recruitment onto the promoter, and the maintenance of CBP on the promoter. This would allow the formation of a large activator complex that would be important for the stimulation of transcription. For synergistic transcription regulation, it is understandable that CBP have multiple interfaces to be occupied directly and simultaneously by different DNA-binding activators in some unique fashions (40). Another possible mechanism that explains the synergistic transactivation, although neither negated by the model described above nor substantiated by our results, is that the interaction of hGABPα with ATF1 and CREB may facilitate phosphorylation of them. It was written previously that ATF1 and CREB must be phosphorylated to interact with CBP (37, 38). This possibility was supported by our preliminary findings that hGABPα enhanced the phosphorylation of ATF1 in vitro using HeLa nuclear extract as a source of kinases and that hGABPα preferred to interact with unphosphorylated ATF1 to the phosphorylated form by protein kinase A in vitro (results not shown). This model is different from the previously reported model, which suggests that multiple direct interactions of activators with specific TAFs, components of the RNA polymerase II complex, may account for synergism and proper gene expression (41). In any case, multiple binding of these activators appears to lead to multiple contact with cofactors or basal transcription factors. This enables coordination and efficient recruitment of the complex containing RNA polymerase II to form a stable and active initiation complex. Although we did not show here any evidence that CBP plays a functional role in the synergism, this proposal should stimulate further experimental tests to study the possibility and which step makes the most important contribution to the synergistic transactivation in vivo.

The Transcription Factor Network between Ets and bZip Family Transcription Factors—As reviewed in the Introduction, some of Ets family members are often found as subunits of larger transcription complexes and are involved in the regulation of viral and cellular function via promoter/enhancer sequences. In particular, bZip family transcription factors which include the ATF/CREB subfamily and the Fos/Jun subfamily, were previously reported to act as the interaction partners of Ets proteins. Our results show a large transcription enhancer complex including Ets transcription factor hGABPα and strengthen the notion that the Ets family proteins functionally and physically interact with bZip family proteins to regulate gene expression. Transcriptional activity within the Ets/bZip transcription factor network represents both repression, in the case of MafB and Ets-1 for erythroid differentiation (20), and stimulation, in the case of the Ets protein Pointed and Jun in R7 Drosophila eye cells (19). Also, Ets-1 and ATF2/CRE-BP1 promotes activity through the TCRα enhancer (21). Synergistic activity of hGABP with ATF1 and CREB within the Ets/bZip network is an example of the latter case. It is possible that a selective partnership generating synergistic transcriptional activity does not depend only on the degree of the interaction affinity among Ets proteins and bZip proteins, cooperative DNA binding activity, a common coactivator and recruitment of a kinase (discussed above), but on unknown important regula-
tions at several points. It is likely that elucidation of such signal pathways which regulate the formation and activity of the large transcription complex will establish a general idea for gene expression control.

Acknowledgments—We thank Dr. H. C. Hurst (Imperial Cancer Research Fund) for plasmids pGEM-ATF1 and pT7-CREB1. The contribution of Dr. M. Ikeda and Dr. K. Tamai (Medical & Biological Laboratories Co., Ltd.) to the production of polyclonal antibody against hGABPα and hGABPβ is gratefully acknowledged. We also thank all members of the Handa laboratory for helpful comments and technical help throughout the project.

REFERENCES

1. Thanos, D., and Maniatis, T. (1995) Cell 83, 1091–1100
2. Watanabe, H., Imai, T., Sharp, P. A., and Handa, H. (1988) Mol. Cell. Biol. 8, 1290–1309
3. Watanabe, H., Wada, T., and Handa, H. (1990) EMBO J. 9, 841–847
4. Watanabe, H., Sawada, J., Satoh, K., Yamaguchi, K., Goto, M., and Handa, H. (1993) Mol. Cell. Biol. 13, 1385–1391
5. LaMarco, K. L., Thompson, C. C., Byers, B. P., Walton, E. M., and McKnight, S. L. (1991) Science 253, 789–792
6. Thompson, C. C., Brown, T. A., and McKnight, S. L. (1991) Science 253, 762–768
7. Sawada, J., Goto, M., Suzuki, F., Watanabe, H., Sawada, J.-i., and Handa, H. (1996) Nucleic Acids Res. 24, 4954–4961
8. Sawada, J.-i., Goto, M., Sawada, J., Watanabe, H., and Handa, H. (1994) EMBO J. 13, 1396–1402
9. Suzuki, F., Goto, M., Sawada, C., Ito, S., Watanabe, H., Sawada, J., and Handa, H. (1998) J. Biol. Chem. 273, 29302–29308
10. Bolwig, G. M., Bruider, J. T., and Hearing, P. (1992) Nucleic Acids Res. 20, 655–664
11. Marchion, M., Morimoto, S., Salvati, A. L., Becetti, E., and Carnevali, F. (1990) Mol. Cell. Biol. 10, 6479–6489
12. Sawada, J., Goto, M., Watanabe, H., Wada, T., and Handa, H. (1995) Mol. Cell. Biol. 15, 102–111
13. Gugnaj, S., Virbasius, J. V., and Scarpulla, R. C. (1993) Genes Dev. 7, 380–392
14. Gugnaj, S., Virbasius, J. V., and Scarpulla, R. C. (1995) Mol. Cell. Biol. 15, 102–111

15. Sawada, J.-i., Goto, M., Watanabe, H., Handa, H., and Yoshida, M. C. (1995) Jpn. J. Cancer Res. 86, 10–12
16. Goto, M., Shimizu, T., Sawada, J.-i., Sawa, C., Watanabe, H., Ichikawa, H., Ohira, M., Okhi, M., and Handa, H. (1996) Gene (Amst.) 166, 337–338
17. Janneke, R., and Nordheim, A. (1993) Biochem. Biophys. Acta 1155, 346–356
18. Wasylyk, B., Hahn, S. L., and Giovane, A. (1993) Eur. J. Biochem. 211, 7–18
19. Treier, M., Bohmann, D., and Mlodzik, M. (1995) Cell 83, 753–760
20. Sieweke, M. H., Kekel, H., Frampton, J., and Graf, T. (1996) Cell 83, 49–60
21. Giese, K., Kinga, C., Krahnen, J. R., and Grosschedl, R. (1995) Genes Dev. 9, 995–1008
22. Ooyama, S., Imai, T., Hanaka, S., and Handa, H. (1989) EMBO J. 8, 653–656
23. Hai, T., Horikoshi, M., Roeder, R. G., and Green, M. R. (1988) Cell 54, 1043–1051
24. Wada, T., Takagi, T., Yamaguchi, Y., Kawase, H., Hiro, M., and Ueda, T., Takayama, M., Lee, K. A., Hurst, H. C., and Handa, H. (1996) Nucleic Acids Res. 24, 876–884
25. Schneider, I. (1972) J. Embryol. Exp. Morphol. 27, 353–365
26. Hurst, H. C., Totty, N. F., and Jones, C. (1991) Nucleic Acids Res. 19, 4603–4609
27. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
28. Inomata, Y., Goto, M., Hiro, M., and Ueda, T., Takayama, M., Lee, K. A., Hurst, H. C., and Handa, H. (1997) Anal. Biochem. 260, 109–114
29. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Gene (Amst.) 108, 193–199
30. Schreiber, E. M., Muller, M. M., and Schffner, W. (1989) Nucleic Acids Res. 17, 6419
31. Hai, T., Liu, F., Coukos, W. J., and Green, M. R. (1989) Genes Dev. 3, 2083–2090
32. Gonzalez, G. A., Yamamoto, K. K., Fisher, W. H., Karr, D., Menzel, P., Biggs, W., Vale, W. W., and Montminy, M. R. (1989) Nature 337, 749–752
33. Maekawa, T., Ootsu, K., Kakei-Ishii, C., Sudo, T., Yoshimura, T., Fujisawa, J., Yoshida, M., and Ishii. S. (1989) EMBO J. 8, 2925–2930
34. Lai, J.-S., and Herr, W. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 6958–6962
35. Takayama, M., Lee, K. A., Hurst, H. C., and Handa, H. (1996) J. Biol. Chem. 271, 17957–17960
36. Sowa, Y., Siro, Y., Fujita, T., Matsumoto, T., Okuyama, Y., Kato, D., Inoue, J., Sawada, J.-i., Goto, M., Watanabe, H., Handa, H., and Sakai, T. (1997) Cancer Res. 57, 3145–3148
37. Chu, C.-L., Kwok, R. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodmam, R. H. (1993) Nature 365, 855–859
38. Shimomura, A., Ogawa, Y., Kita, T., Fujisawa, H., and Hagiwara, M. (1996) J. Biol. Chem. 271, 17857–17860
39. Bannert, N., Avots, A., Baier, M., Serfling, E., and Kurth, R. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 1541–1546
40. Shikama, N., Lyon, J., and Thangue, N. B. L. (1997) Trends Cell Biol. 7, 230–236
41. Sauer, F., Hansen, S. K., and Tjian, R. (1995) Science 270, 1783–1788