4954–4961 *Nucleic Acids Research, 1996, Vol. 24, No. 24*

Functional domains of transcription factor hGABPβ1/E4TF1-53 required for nuclear localization and transcription activation

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Received August 13, 1996; Revised and Accepted October 31, 1996

ABSTRACT

Transcription factor E4TF1 is the human homolog of GABP and has been renamed hGABP (human GABP). hGABP is composed of two types of subunits; hGABP-β1/E4TF1-53 and the ets-related protein hGABP-α/E4TF1-60. Both bind together to form an $(\alpha)_2(\beta1)_2$ heterotetrameric complex on DNA and activate transcription at specific promoters *in vitro*. Tetramer formation depends on two regions of hGABPβ1; the N-terminal region containing the Notch/ankyrin-type repeats is necessary for binding to hGABPα and the C-terminal region is necessary for homodimerization. In this report, we constructed various deletion mutants of hGABPβ1 in order to delimit the functional regions required for nuclear localization and transcription activity. We found that hGABPβ1 localization in the nucleus is dependent on a region located between amino acids 243 and 330 and that the presence of hGABPβ1 influences the efficiency of hGABPα transport into the nucleus. Next, we demonstrated that the hGABP complex composed of α and β1 subunits activates transcription from the adenovirus early 4 promoter *in vivo*. This transcription activation needs the C-terminal region of hGABPβ1 and is consistent with results obtained with the *in vitro* assay. Furthermore, site-directed mutagenesis analysis of the C-terminal region reveals that the α-helix structure and the leucine residues are important for formation of a heterotetrameric complex with hGABPα *in vitro* and for transcription activation *in vivo*. These results suggest that hGABPβ1 stimulates transcription as part of a heterotetrameric complex with hGABPα *in vivo*.

INTRODUCTION

In eukaryotes, many gene-specific transcription factors regulate transcription initiation by RNA polymerase II. These transcription factors function cooperatively by forming large complexes with one another at promoter sequences. Within these complexes protein–protein interactions between DNA binding transcription factors and non-DNA binding transcription factors have been shown to be responsible in most instances for transcriptional regulation of gene expression. Small regions termed activation domains were found to interact with certain general transcription factors and/or co-activators and to be both necessary and sufficient for transcription activation.

Transcription factor E4TF1 was originally purified to homogeneity from HeLa cells on the basis of its ability to bind to and stimulate transcription from the adenovirus early 4 (E4) promoter (1,2). Further characterization of E4TF1 revealed the presence of two distinct subunits, an ets-related DNA binding protein, E4TF1-60, and a non-DNA binding factor, E4TF1-53. The N-terminal region of the latter contains four tandemly repeated motifs homologous to Notch/ankyrin. Both subunits interacted with one another to form the E4TF1 heterotetrameric complex $(E4TF1\text{-}60)_2(E4TF1\text{-}53)_2$ on the specific DNA sequence 5′-CGGAAGTG-3′. This was shown to result in efficient activation of transcription *in vitro* (2–5). Another E4TF1 subunit, E4TF1-47, is identical to E4TF1-53 in the N-terminal region but contains a distinct C-terminal 15 amino acid sequence. It can complex with E4TF1-60 but the complex does not stimulate transcription *in vitro* as efficiently as E4TF1 complexes made up of E4TF1-53 (6). Sequence data of cDNA clones from a HeLa cDNA library showed that E4TF1-60, E4TF1-53 and E4TF1-47 are highly homologous to GABPα, GABPβ1-1 and GABPβ1-2 respectively. For this reason, E4TF1 is thought to be the human homolog of rat GA binding protein (GABP), which was purified as a factor that binds to a *cis*-regulatory DNA sequence important for herpes simplex virus type 1 (HSV-1) immediate early (IE) gene activation (8–10,13). E4TF1 and its subunits have thus been renamed according to the human GABP nomenclature (hGABP). hGABPα, hGABPβ1 and hGABPγ1 correspond to E4TF1-60, E4TF1-53 and E4TF1-47, as shown in Figure 1A. The genes for the hGABP subunits, hGABPα and hGABPβ1, were mapped to human chromosomes 21.q21.2–q21.3 and 7.q11.21 respectively, while the genes for the mouse GABP subunits, GABPα and β, were mapped to mouse chromosomes 6 and 2 respectively (11–13). Recently, the transcription factors EF-1A (14), NRF-2

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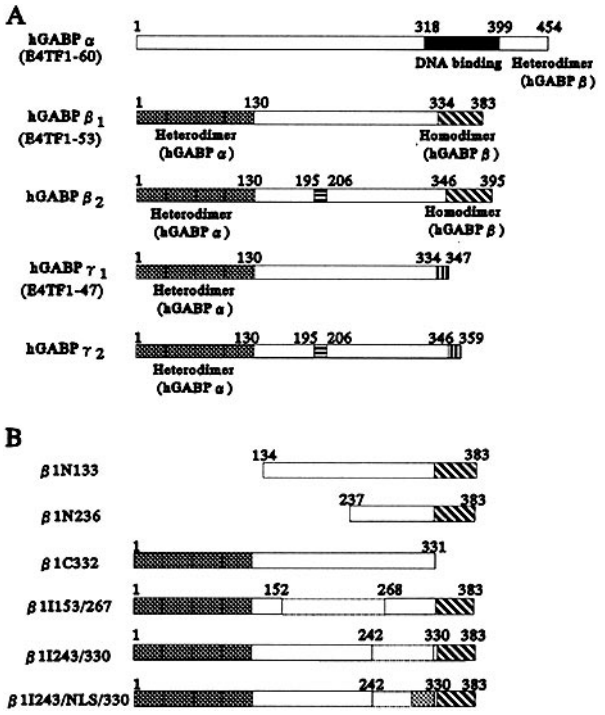

Figure 1. (**A**) Schematic structures of hGABP subunits. The black region indicates the ets-related region and the stippled regions indicate four Notch/ankyrin-type repeats. Hatched regions indicate the hGABPβ-specific regions. Cross-hatched regions indicate the insertion of 12 amino acids in hGABPβ2 and hGABPγ2. (**B**) Schematic structures of hGABPβ1 mutants. The regions surrounded by broken lines indicate the internal deletion region of hGABPβ1 mutants and the broad cross-hatched region indicates a nuclear localization signal derived from the SV40 large T antigen.

(15), XrpFI (16), RBF-1 (17) and β factor (18) have been found to be immunologically related to GABP. This is especially the case for the NRF-2 subunits α, β2 and γ2, which are identical to hGABPα, hGABPβ1 and hGABPγ1 respectively at the level of cDNA. The NRF-2 β1 and γ1 subunits are variants of β2 and γ2 possessing an additional 12 amino acid insertion (19). GABP has been shown to be involved in the expression of certain cellular genes, for instance the male-specific steroid 16α-hydroxylase gene (20) and the leukocyte-specific adhesion molecule CD18 (β2 leukocyte integrin; 21).

In this report, we demonstrate that an hGABP complex composed of hGABPα and hGABPβ1 can activate transcription in a transient transfection assay while an hGABP complex composed of hGABPα and hGABPγ1 cannot. To gain further insights into the mechanism by which the non-DNA binding factor hGABPβ1 functions as a transcriptional activator *in vivo*, we undertook a structure–function analysis of hGABPβ1 to identify the domains involved in nuclear localization and transcription activation.

MATERIALS AND METHODS

Plasmid construction

pET53 (6) was completely digested with *Sac*I and partially digested with *Pst*I to generate an 879 bp fragment that carries a partial cDNA of hGABPβ1. In order to generate plasmids pETβ1QK339GT and pETβ1GL341GT expressing β1QK339GT and β1GL341GT respectively (Fig. 4), the DNA fragments encoding the N-terminal flanking region of the mutants 339-N and 341-N were synthesized and annealed. The DNA fragments encoding the C-terminal flanking regions of the mutants were amplified using two primers, βBamHI and 339-C or 341-C respectively. The PCR products were digested with *Kpn*I and *Bam*HI and purified using agarose gel electrophoresis. The two fragments above and the 879 bp fragment were inserted into the *Sac*I and *Bam*HI sites of pET53. To generate plasmids pETβ1KL369GT and pETβ1EA371GT expressing β1KL369GT and β1EA371GT, the DNA fragments encoding the C-terminal flanking regions of mutants 369-C and 371-C were synthesized and annealed. The DNA fragments encoding the N-terminal flanking regions of the mutants were amplified using two primers, βsppstI and 369-N or 371-N respectively. The PCR products were digested with *Pst*I and *Kpn*I and purified by agarose gel electrophoresis. The two fragments above and the 879 bp fragment were inserted into the *Sac*I and *Bam*HI sites of pET53. The other plasmids that express hGABPβ site-directed mutants in *Escherichia coli* were constructed as follows. The DNA fragment encoding the N-terminal flanking region of the mutant was amplified using two primers, Number-N and βsppstI. The DNA fragment encoding the C-terminal flanking region was amplified by PCR using two primers, Number-C and βsppstI. The PCR products were digested with *Kpn*I and *Bam*HI or *Pst*I and purified by agarose gel electrophoresis. The two PCR fragments and the 879 bp fragment were inserted into the *Sac*I and *Bam*HI sites of pET53. The construction of hGABPβ1 deletion mutant expression vectors in *E.coli* was as described previously (7).

All plasmids that expressed hGABPβ1 mutants in *Drosophila melanogaster* Schneider line 2 (SL2) cells (22) were constructed as follows. pETβ mutants were digested with *Bam*HI and *Bgl*II. The DNA fragments containing the region that codes for the hGABPβ mutants were subcloned into the *Bam*HI site of A5CΔP, which contains the *Drosophila* actin 5C promoter (23).

All plasmids that expressed mutant hGABPβ1 in COS-1 cells were constructed as follows. pETβ mutant plasmids were all digested with *Bam*HI and *Bgl*II. The DNA fragments containing the coding regions of the corresponding mutants were subcloned into the *Bgl*II site of the mammalian expression vector pCAGGS (24).

The pETβ1I243/NLS/330 plasmid was constructed as follows. The C-terminal flanking region was amplified using two primers, βBamHI and NLS-243, which contains the nuclear localization signal (NLS) sequence of the SV40 large T antigen. The PCR product was digested with *Bam*HI and *Pst*I and purified by agarose gel electrophoresis. The DNA fragment coding for a part of hGABPβ was prepared by digesting pET53 with *Sac*I and *Pst*I and purifying the DNA fragment by agarose gel electrophoresis. Then, the PCR product and the fragment produced by *Sac*I and *Pst*I digestion were subcloned into the *Sac*I and *Bam*HI sites of pET53. Next, to construct the expression vector for SL2 and COS-1 cells, pETβ1I243/NLS/330 was digested with *Bam*HI and *Bgl*II and the fragment was subcloned into the *Bam*HI site of A5CΔP and the *Bgl*II site of the pCAGGS vector.

All synthesized oligonucleotides to construct the plasmids are shown in Table 2.

All DNAs of hGABPβ mutants were sequenced using a 373A-18 sequencer with a fluorescence detection system (Applied Biosystems).

Table 1. The sequence of synthetic DNA fragments and PCR primers

Antibody & Location / Transfected DNA	hGABP α			hGABP β1		
	N	N / C	C	N	N / C	C
αwt/-	2	28	-			
-/β1wt				30	-	-
αwt/β1wt	30	-	-	30	-	-
αwt/γ1wt	25	5	-	20	10	-
αwt/β1N133	-	30	-	30	-	-
αwt/β1C332	30	-	-	30	-	-
αwt/β1N236	-	30	-	30	-	-
αwt/β1I153/267	30	-	-	28	-	2
αwt/β1I243/330	1	28	1	1	-	29
αwt/β1I243/NLS/330	30	-	-	30	-	-

The table shows the data obtained from 30 transfected COS-1 cells. N, nucleus; N/C, nucleus and cytoplasm; C, cytoplasm.

Table 2. DNA fragment and PCR primer of sequence

Name	primer
339-N	5' GAAATAGAAGAGAGAGAAGCTCTTGGTAC 3' 3' ACGTCTTTATCTTCTCTCTCTTCGAGAAC 5'
341-N	5' GAAATAGAAGAGAGAGAAGCTCTTCAGAAAGGTAC 3' 3' ACGTCTTTATCTTCTTCTCTCTTCGAGAAGTCTTTC 5'
369-C	5' CGAAGCTATGACTCGTCTTCAGACTAATAAAGAAGCTGTTTAAG 3' 3' CATGGCTTCGATACTGAGCAGAAGTCTGATTATTTCTTCGACAAATTCCTAG 5'
371-C	5' CCATGACTCGTCTTCAGACTAATAAAGAAGCTGTTG 3' 3' CATGGTACTGAGCAGAAGTCTGATTATTTCTTCGACAACCTAG 5'
βspPstI	5' GGTGGAATCTGCAGAAATAG 3'
βBamHI	5' TCAGGATCCTTAAACAGCTTCTTTATTAGTC 3'
339-C	5' GGTACCCAGCTGGATGAAGCAAATCG 3'
341-C	5' GGTACCGATGAAGCAATCGAGAAGC 3'
343-C	5' GGTACCGCAAATCGAGAAGCACAAAAAT 3'
345-C	5' GGTACCCGAGAAGCACAAAAATATCG 3'
347-C	5' GGTACCGCACAAAAATATCGACAGCAG 3'
349-C	5' GGTACCAAATATCGACAGCAGCTCCT 3'
351-C	5' GGTACCCGACAGCAGCTCCTAAAGAA 3'
353-C	5' GGTACCCAGCTCCTAAAGAAAGAACAG 3'
355-C	5' GGTACCCTAAAGAAAGAACAGGAAGCA 3'
357-C	5' GGTACCAAAGAACAGGAAGCAGGGCC 3'
359-C	5' GGTACCCAGGAAGCAGAGGCCTACAG 3'
361-C	5' GGTACCGCAGAGGCCTACAGACAGAA 3'
363-C	5' GGTACCGCCTACAGACAGAAGTTGGAA 3'
365-C	5' GGTACCAGACAGAAGTTGGAAGCTATG 3'
367-C	5' GGTACCAAGTTGGAAGCTATGACTCG 3'
343-N	5' GGTACCCAGCTGTTTCTGAAGAGCTT 3'
345-N	5' GGTACCTTCATCCAGCTGTTTCTGAAG 3'
347-N	5' GGTACCATTTGCTTCATCCAGCTGTT 3'
349-N	5' GGTACCTTCTCGATTTGCTTCATCCAG 3'
351-N	5' GGTACCTTGTGCTTCTCGATTTGCTT 3'
353-N	5' GGTAVVATATTTTTGTGCTTCTCGATT 3'
355-N	5' GGTACCCTGTCGATATTTTTGTGCTTC 3'
357-N	5' GGTACCGAGCTGGCTGTCGATATTTTTG 3'
359-N	5' GGTACCCTTTAGGAGCTGCTGTCGAT 3'
361-N	5' GGTACCTTCTTTCTTTAGGAGCTGCT 3'
363-N	5' GGTACCTTCCTGTTCTTTCTTTAGGAG 3'
365-N	5' GGTACCCTCTGCTTCCTGTTCTTTCTT 3'
367-N	5' GGTACCGTAGGCCTCTGCTTCCTGTT 3'
369-N	5' GGTACCCTGTCTGTAGGCCTCTGCTT 3'
371-N	5' GGTACCCAACTTCTGTCTGTAGGCCT 3'
NLS-243	5'GCCCATGGCACCACCAAAGAAGAAGCGAAAGGTTTCCCTGGYAGATTTGGGAAA3'

Immunofluorescence assay

Transfected COS-1 cells were placed onto a micro cover glass. The cells were washed twice with sterile PBS(–) and fixed in 3.7% formalin/PBS(–) for 10 min. The cells were washed with PBS(–) and 0.1% Triton X-100/PBS(–) was added for 10 min to permeabilize the cells. After blocking with 1% skimmed milk/PBS(–) for 10 min, the first antibody (anti-hGABP monoclonal antibody) was added for 1 h. The monoclonal antibodies were 3A4G7G3H11 and 4F3HF12E12, directed against hGABP-β, and 5B8A12D7C12, directed against hGABPα. The cells were washed with 0.1% NP-40/PBS(–) three times for 5 min with agitation, followed by treatment with 5 μg/ml TRITC-conjugated anti-mouse secondary antibody (Chemicon) for 20 min in a dark box. They were subsequently washed with 0.1% NP-40/PBS(–) three times for 5 min with agitation. Cells were stained with 0.1 mg/ml diaminophenolindole (DAPI) for 5 min and washed twice with PBS(–) and mounted in 90% glycerol, 10 mg/ml *p*-phenylenediamine, 50 mM Na_2CO_3–$NaHCO_3$, pH 8.0. Samples were examined and photographed using a Carl Zeiss microscope equipped for fluorescence photomicroscopy and a Fuji NEOPAN 400. All procedures were carried out at room temperature.

Cell maintenance and transfection

SL2 cells were maintained in tissue culture flasks containing Schneider's *Drosophila* Medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 50 mg/ml streptomycin sulfate and 50 U/ml penicillin G. At 2–3 h before transfection, the cells were replated onto 60 mm polystyrene dishes at a density of 3×10^6 cells/5 ml medium/dish. Transfections were carried out by the calcium phosphate method (25). The cells received variable amounts of hGABP expression vectors, the luciferase reporter vector, the β-galactosidase vector and A5CΔP DNA, so that the total concentration of DNA was 12 μg/dish. After addition of DNA, cells were incubated at 27°C and left undisturbed until the time of harvest 40 h later.

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% FBS. Transfections were carried out by electroporation. A total of 6 μg DNA was transfected into a volume of 250 ml containing ~1×10^6 cells using a Bio-Rad Gene Pulser set at 220 V and 960 μF. The width of the cuvette was 0.4 cm. Transfected cells were plated onto a micro cover glass in a 60 mm tissue culture dish containing 5 ml medium and the dishes were incubated at 37°C with 5% CO_2 until harvesting 40 h later.

Luciferase and β-galactosidase assays

Cell extracts were prepared as follows. Transfected SL2 cells grown in 60 mm dishes were washed three times with PBS(–) and then lysed by the addition of 400 ml cell lysis buffer (Toyo-ink PGK-L-500). Cell lysates were collected in 1.5 ml tubes and centrifuged at 12 000 *g* for 5 min at 4°C. The supernatant of the cell lysate was diluted 1:10 in cell lysis buffer containing 1 mg/ml bovine serum albumin fraction V. For the luciferase assay, 20 ml diluted cell lysate were mixed with 100 ml luminescence reagent (Toyo-ink) and luciferase activity was measured in a Lumat LB

9501 luminometer (Berthod). For the β-galactosidase assay, 20 ml diluted cell lysate were mixed with 500 ml buffer Z (10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, 0.1 M $NaHPO_4$–Na_2HPO_4, pH 7.5) and 5 ml chlorophenyl red β-D-galactopyranoside (Boehringer Mannheim) and then incubated at 30°C for ~20 min. The absorbency of each sample was measured at 574 nm. The luciferase activity in each assay was standardized against the corresponding β-galactosidase activity.

Western blotting assay

The cell lysates were loaded onto 10% SDS–PAGE gels. After electrophoresis proteins were transferred to immobilon transfer membranes (Millipore) and the hGABP protein was detected using a blotting detection kit for mouse antibodies (Amersham) and the monoclonal antibody anti-hGABP antibody.

Expression and purification of hGABP subunit polypeptides

Proteins were expressed in *E.coli* BL21(DE3). Purification and renaturation of hGABP subunits and hGABPβ1 mutant polypeptides were performed as previously described (6). The concentration of each renatured protein was determined by silver staining of SDS–PAGE gels.

Gel shift assay

Gel shift assays were performed as previously described (2) except that gel electrophoresis was carried out at 4°C. The DNA probe for this assay contained a single hGABP recognition site and was prepared as previously described (7). About 1 ng DNA probe was used for the binding reactions.

RESULTS

Nuclear localization of hGABP

We isolated two cDNA clones coding for the two newly described subunits of hGABP (19). They contain an additional 12 amino acid insertion into hGABPβ1 and hGABPγ1. These subunits have therefore been termed hGABPβ2 and hGABPγ2, as indicated in Figure 1A.

Before examining the transcription activation domain of the hGABP complex, it was essential first of all to identify the region of the hGABP complex required for transport into the nucleus. We constructed expression vectors of hGABPα, β1, γ1 and the various hGABPβ1 mutant constructs (Fig. 1) and transfected them into COS-1 cells. Their location in the cell was detected by an immunofluorescent assay using specific monoclonal antibodies as described in Materials and Methods. hGABPα was found to be localized in the entire cell when expressed alone (Fig. 2a and s). However, hGABPα became localized in the nucleus upon co-transfection with the expression vectors for hGABPβ1 or hGABPγ1 (Fig. 2b and c). On the other hand, full-length hGABPβ1 was observed only in the nucleus, whether expressed alone or co-expressed with hGABPα (Fig. 2j and k).

Deletion of the N-terminal amino acids of hGABPβ1 in β1N133 and β1N236, as well as deletion of an internal sequence in β1I153/267, did not affect nuclear localization (Fig. 2m, o and q). Likewise, deletion of the C-terminal amino acids in β1C332 did not affect its nuclear localization (Fig. 2n). However,

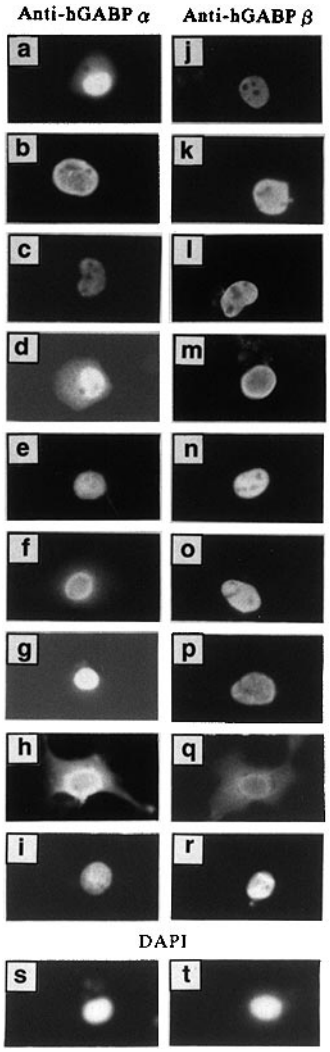

Figure 2. Localization of full-length of hGABP subunits and various mutants of hGABPβ1. Localization analysis was performed by immunofluorescence assay. COS-1 cells were transiently transfected with expression vectors of (**a**, **s**) hGABPα, (**j**) hGABPβ1, (**b**, **k**) hGABPα and hGABPβ1, (**c**, **l**) hGABPα and hGABPγ1, (**d**, **m**) hGABPα and β1N133, (**e**, **n**) hGABPα and β1C332, (**f**, **o**) hGABPα and β1N236, (**g**, **p**) hGABPα and β1I153/263, (**h**, **q**, **t**) hGABPα and β1I243/330, (**i**, **r**) hGABPα and β1I243/NLS/330. Staining of the cells in (a)–(i) and (j)–(r) was with anti-hGABPα antibody and anti-hGABPβ antibody respectively. Cells in (s) and (t) were counter stained with the DNA-specific dye DAPI to visualize the position of nuclei in the same cells shown in (a) and (q) respectively.

the β1I243/330 mutant protein was detected only in the cytoplasm, regardless of expression of hGABPα (Fig. 2h, q and t). These results (summarized on Table 1) demonstrate that the hGABP complex composed of hGABPα and hGABPβ1 is localized in the nucleus and that nuclear localization depends on a region of hGABPβ1 situated between amino acids 268 and 330.

Transcription activation by hGABP *in vivo*

To examine whether the hGABP complex can stimulate transcription *in vivo*, we carried out transient transfection assays with *D.melanogaster* SL2 cells (22) as described in Material and Methods. We chose these cells because they are highly responsive

to exogenous transcription factors, in contrast to mammalian cells (26,27). The luciferase reporter plasmid used in these experiments contains an intact (–324 to +39) adenovirus E4 promoter (Fig. 3A). As shown in Figure 3B, no activation was observed when each subunit was present alone (lanes 2–4), consistent with our previous results *in vitro* (2). However, strong activation of transcription from the E4 promoter was observed when hGABPα was co-expressed with increasing amounts of hGABPβ1 or β2 (Fig. 3B, lanes 7–12). In this assay, hGABPβ1 was more efficient in activation of transcription compared with hGABPβ2. This was not due to differences in the amount of expressed protein, as demonstrated by a quantitative Western blot assay (data not shown). On the other hand, transcription activation was not observed when hGABPα was co-expressed with hGABPγ1 or γ2. These results indicate that the hGABP complexes composed of hGABPα and hGABPβ1 or hGABPβ2 are transcriptional activators and that transcription activation requires the non-DNA binding hGABPβ1 or β2 subunits, but not the hGABPγ1 or γ2 subunits.

Analysis of the transcription activation domain of hGABPβ1

To analyze further the region of hGABPβ1 necessary for transcription activation from the E4 promoter *in vivo*, a series of expression vectors containing deletions in hGABPβ1 (Fig. 1B) were examined by transient transfection assay. The level of expression was determined by Western blot assay and transcriptional activity was measured by luciferase assay. As shown in Figure 2C, none of the deletion mutants could stimulate transcription in the presence of hGABPα, even though these truncated molecules were expressed at levels comparable with that of full-length hGABPβ1 (data not shown). Previously, a gel shift assay showed that the N-terminal deletion mutant β1N133 could not bind hGABPα and that the C-terminal deletion mutants β1C248 and β1C332 could bind hGABPα but could not form heterotetrameric complexes functional in transcription activation *in vitro*. Note that the internal deletion mutant β1I153/267 could not stimulate transcription in this assay, although it showed a slight capacity to stimulate transcription activation *in vitro* (7).

Co-expression of hGABPα with β1I243/NLS/330, which has a NLS derived from the SV40 large T antigen inserted into the deletion region of β1I243/330 (Fig. 1B), resulted in a ~10-fold increase in luciferase activity compared with the expression vector containing no hGABPβ or β1I243/330 construct cDNA (Fig. 4, lanes 1 and 4). This NLS-fused mutant was observed only in the nucleus by immunofluorescence assay (Fig. 2r). These results suggest that the C-terminal sequence (amino acids 330–353) of hGABPβ1 is required for hGABP-induced transcription activation *in vivo* and that transcription activation is relatively independent of amino acids 243–330, which are necessary for nuclear localization of the hGABP complex.

Site-directed mutagenic analysis of the C-terminal region of hGABPβ1

The results above indicate that the C-terminal region of hGABPβ1 (amino acids 330–353) is important for transcription activation *in vivo*, which is consistent with previous *in vitro* data (7). This leads to the conclusion that this region is important for both heterotetrameric complex formation and transcription activation by hGABP. To further dissect the functional domains in this region, we carried out site-directed mutagenesis. *In vitro* analysis revealed that this region has homodimerization activity (7,13). Besides, it is free of α-helix destabilizing residues and has a hydrophobic phase composed of leucine and alanine residues when displayed on an idealized α-helix projection. Therefore, it may function as a leucine-zipper structure for homodimerization (28,29). We systematically constructed a series of vectors expressing hGABPβ1 mutants having two sequential amino acids substituted by the α-helix destabilizing residues glycine and threonine within this region (Fig. 4). These substitution mutants were expressed in *E.coli* and purified as described in Materials

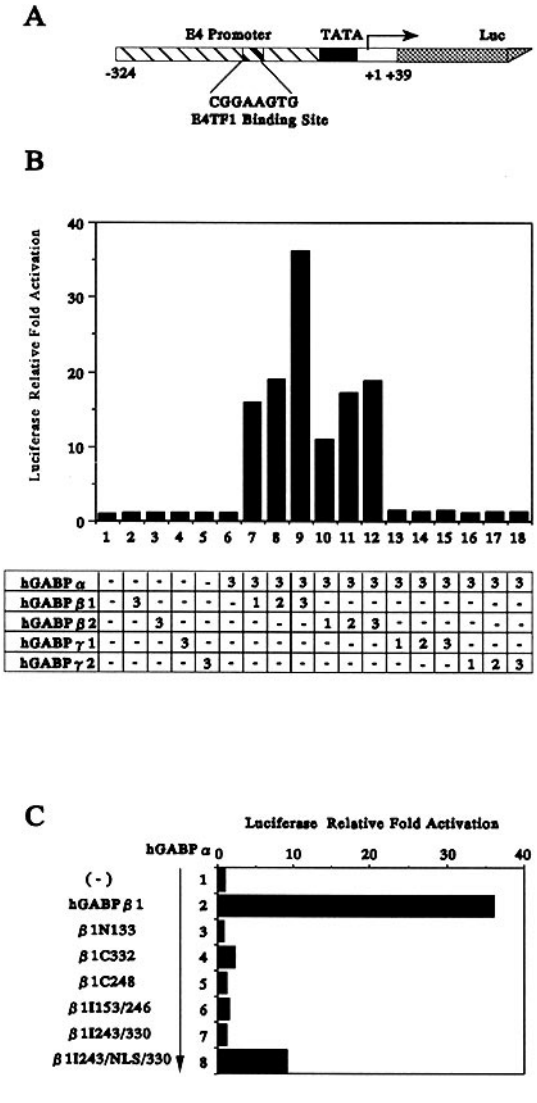

Figure 3. hGABPβ1 and hGABPβ2 activate transcription from the adenovirus E4 promoter with hGABPα in SL2 cells. (**A**) A schematic diagram of the adenovirus E4 promoter (from –324 to +39) of the reporter plasmid pE4-luciferase. This promoter was linked to the luciferase gene in the reporter plasmid. (**B**) SL2 cells were transfected with 2 μg pE4-luciferase, 1 μg β-galactosidase vector and various amounts (μg) of expression vector as indicated below. The cells were harvested 40 h after transfection. Luciferase activities were determined and standardized for β-galactosidase activities. (**C**) Aliquots of 3 μg of various hGABPβ1 mutants were used to transfect SL2 cells along with 2 μg pE4-luciferase and 3 μg hGABPα expression vector. Forty hours later, the cells were lysed and luciferase activity was determined as described in Materials and Methods.

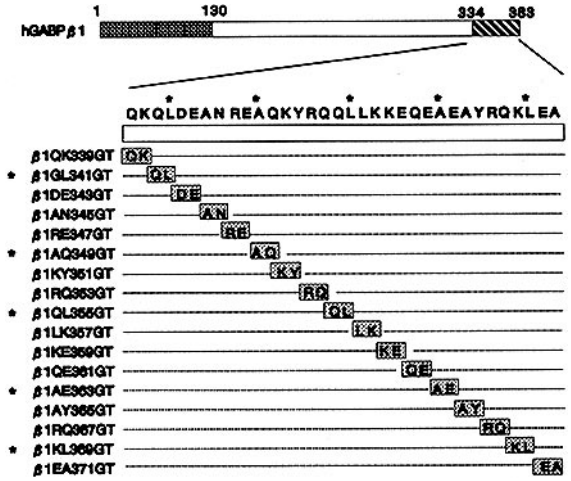

Figure 4. Schematic structures of hGABPβ1 mutants with substitutions of two sequential amino acids within the hGABPβ-specific region. The names and the substitution sites of hGABPβ1 mutants are illustrated. The detailed amino acid sequence in the hypothetical leucine zipper structure of wild-type hGABPβ1 is represented at the top. The amino acid sequence glycine–threonine replaces the substitution sites indicated by open squares. Leucines and alanines within the hydrophobic phase in the hypothetical leucine zipper structure and the mutants containing substitutions for the amino acids are shown by an asterisk (*).

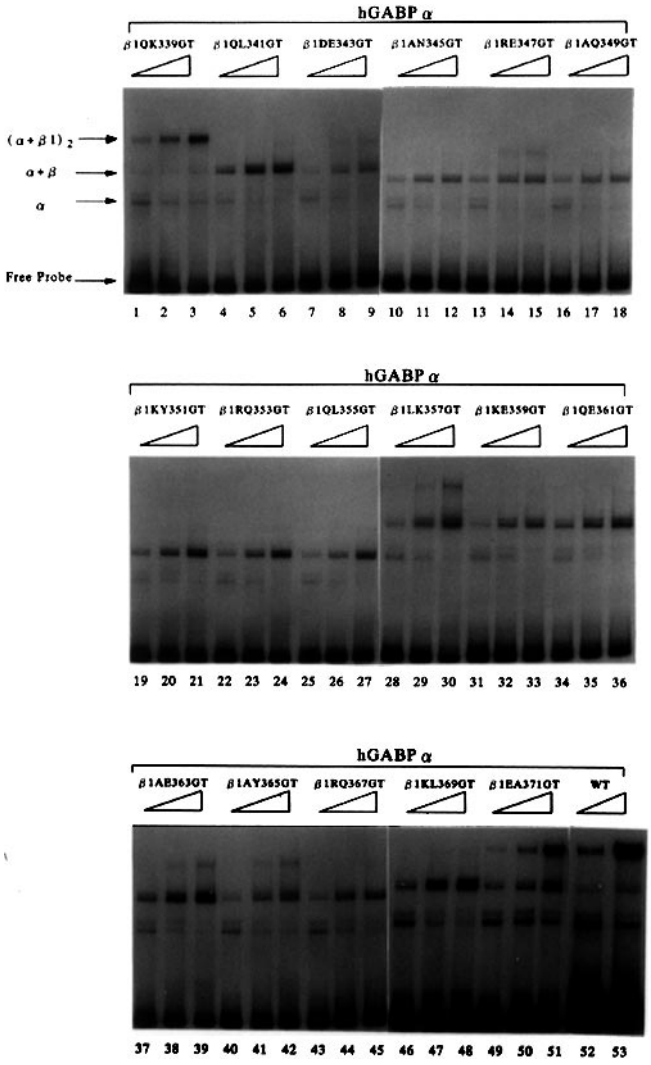

Figure 5. hGABP tetramerization activity of the substitution mutants of hGABPβ1 in the presence of hGABPα. The gel shift assay was performed using various hGABPβ1 mutants as indicated above. 10 ng hGABPα and 1 ng radiolabeled DNA probe containing the E4 promoter (–29 to –182) were incubated with 3-fold incremental additions (3, 10 and 30 ng) of the mutants β1QK339GT (lanes 1–3), β1QL341GT (lanes 4–6), β1DE343GT (lanes 7–9), β1AN345GT (lanes 10–12), β1RE347GT (lanes 13–15), β1AQ349GT (lanes 16–18), β1KY351GT (lanes 19–21), β1RQ353GT (lanes 22–24), β1QL355GT (lanes 25–27), β1LK357GT (lanes 28–30), β1KE359GT (lanes 31–33), β1QE361GT (lanes 34–36), β1AE363GT (lanes 37–39), β1AY365GT (lanes 40–42), β1RQ367GT (lanes 43–45), β1KL369GT (lanes 46–48) or β1EA371GT (lanes 49–51) and 2-fold incremental additions (3 and 10 ng) of wild-type hGABPβ1 (lanes 52–53).

and Methods. Their capacity to form heterotetramers with hGABPα was examined by gel shift assay, as shown in Figure 5. Although β1QK339GT (lanes 1–3) and β1EA371GT (lanes 49–51) could form a heterotetramer with hGABPα as efficiently as wild-type hGABPβ1 (lanes 52 and 53), other mutants and especially those that contained a mutations within the hydrophobic phase of the hypothetical α-helix structure, such as β1AN345GT (lanes 10–12), β1KY351GT (lanes 19–21) and β1KE359GT (lanes 31–33), could not form a heterotetrameric complex with hGABPα efficiently. Three leucine-defective mutants, β1GL341GT (lanes 4–6), β1QL355GT (lanes 26–27) and β1KL369GT (lanes 46–48), all failed to form the heterotetrameric complex, but two alanine-defective mutants, β1AQ349GT (lanes 16–18) and β1AE363GT (lanes 37–39), could form the complex, albeit less efficiently than wild-type hGABPβ1. These results suggest that amino acids 341–369 are important for formation of the hGABP heterotetrameric complex. Also, it seems possible that this region forms an α-helix structure and that the three leucine residues are part of a leucine zipper structure necessary for homodimerization.

Next, in order to study the relationship between hGABP tetrameric complex formation and transcription activation by hGABPα and hGABPβ1, we examined some of these mutants for their activity in the transient transfection assay. The results are shown in Figure 6. An ~20-fold activation of transcription was observed when β1QK339GT (lane 3) or β1EA371GT (lane 10) was co-expressed with hGABPα. These two mutants could form a tetramer as efficiently as wild-type hGABPβ1, as measured by gel shift assay. Mutants with a reduced capacity to tetramerize with hGABPα, such as β1AQ349GT (lane 5), β1LK357GT (lane 7) and β1AE363GT (lane 8), were severely impaired in their ability to stimulate transcription. These results indicate that it is difficult to functionally separate the regions responsible for tetrameric complex formation and transcription activation and that *in vivo* the activities of these two regions are intimately linked.

DISCUSSION

The studies reported here further extend our understanding of the relationship between structure and function in hGABPβ1 (summarized in Fig. 7). The identification of two functional regions in hGABPβ1 was previously indicated by *in vitro* analysis (7). The N-terminal region containing four tandem repeats with homology to Notch/ankyrin was shown to be required for binding to

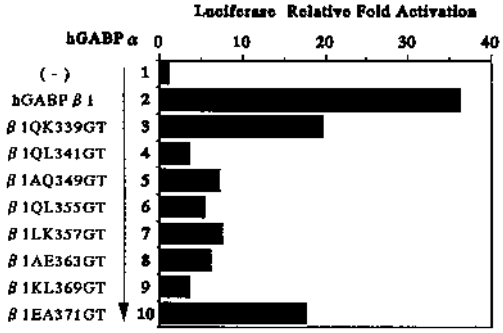

Figure 6. Comparison of the potential transcriptional activities of the wild-type and substitution mutants of hGABPβ1. Aliquots of 3 µg expression vectors carrying the different substitution mutants of hGABPβ1 were transfected into SL2 cells, along with 2 µg reporter plasmid pE4-luciferase and 3 µg hGABPα expression vector. Forty hours later, the cells were lysed and the luciferase activities were determined as described in Materials and Methods.

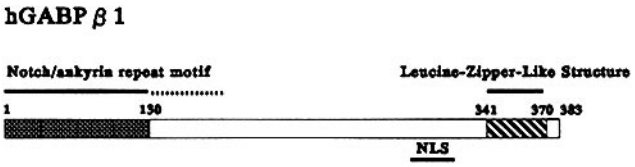

Figure 7. Summary of the functional domains of hGABPβ1. Stippled region represents four tamdem repeats of a Notch/ankyrin motif required for heterodimer formation with hGABPα. The hatched region represents a leucine zipper-like structure. This domain is necessary for homodimerization, which activity is critical for transcription activation. The underlined region (amino acids 243–317) is expected to play an important role in nuclear localization.

hGABPα. The C-terminal region containing the leucine zipper-like motif was found to be responsible for transcription activation and homodimerization. Here, we identify a new functional region necessary for nuclear localization of hGABPβ1 and show that this region is also necessary for efficient nuclear localization of hGABPα. Also, we have extended our study of the C-terminal region of hGABPβ1 by identifying key amino acids necessary for both stimulation of transcription and homodimerization. This further underlines the coincidental nature of these two activities within the C-terminal region of hGABPβ1.

A nuclear factor hGABP

Transcription factors must be able to enter and become localized in the nucleus. This ability has been shown to depend on short regions termed NLS (30,31). In this report, hGABPβ1 and hGABPγ1 were shown to be localized predominantly in the nucleus regardless of the presence of hGABPα. Mutational studies revealed that hGABPβ1 and hGABPγ1 contain an NLS in the amino acid sequence 243–330. This region is not necessary for the transcriptional activity of hGABP. Further analyses will be necessary to further delimit the minimal sequence necessary and sufficient for nuclear localization. We speculate that the PPAKR sequence (amino acids 313–317) and the neighboring region may play an important role, because NLSs have been reported to be rich in proline and basic amino acids (31).

hGABPα alone was not able to direct nuclear localization efficiently. This absolutely required the concomitant expression of either hGABPβ or hGABPγ. It is possible that hGABPβ or hGABPγ bind hGABPα in the cytoplasm to assist hGABPα transport into the nucleus. This is different from the case of other ets-related proteins, ETS-1 and ER81, whose ETS domains alone are sufficient for nuclear localization (32,33). There may be a mechanism that inhibits hGABPα entry into the nucleus. hGABPα may only succeed in so doing on forming a complex with its partner protein hGABPβ or hGABPγ.

The homodimerization domain of hGABPβ1

hGABPβ1 has homodimerization activity in its C-terminal region, as shown by an *in vitro* analysis (7). Here, we have shown that this region is located between amino acids 341 and 371 of hGABPβ1 and that this region probably adopts an α-helix structure in which three leucine residues play an important role in homodimerization. Consistent with our observations, de la Brousse reported that this dimerization domain could functionally replace the leucine zipper of the b-ZIP transcription factor C/EBP and that it exists predominantly as an α-helical structure, as shown by CD spectroscopic studies (13). Therefore, this region is suggested to mediate homodimerization via the three leucine residues, which probably adopt a leucine zipper structure.

The transcription activation domain within hGABPβ1

We have demonstrated that each of the hGABP subunits alone fails to stimulate transcription *in vivo*, consistent with our previous report obtained by *in vitro* analysis (2,6). However, hGABPβ1 stimulates transcription from the adenovirus E4 promoter in the presence of hGABPα. Furthermore, hGABPβ1 and hGABPβ2 were found to differ by ~2-fold in their capacity to activate transcription. It seems that the inserted amino acid sequence present in hGABPβ2 plays a role in inhibiting transcription activity in this assay. As this region is rich in serine residues, there exists the possibility that phosphorylation of at least some of these serine residues may regulate transcription activation activity. hGABPγ was found to have no influence on basal transcription in the presence hGABPα. As this is inconsistent with the results obtained in the *in vitro* assay (6), some unknown cellular factors could function cooperatively with the complex composed of hGABPα and hGABPγ.

Our deletion mutational studies of hGABPβ1 demonstrated that its C-terminal region was necessary for transcription activation *in vivo*, consistent with our previous report obtained by *in vitro* analysis (7). This region coincides with the homodimerization domain. Furthermore, substitution mutational studies of the C-terminal region of hGABPβ1 revealed that the amino acids important for homodimerization were also required for full transcription activation. This indicates that the dimerization structure of this region is critical for transcription activation.

Gugneja *et al.* reported that the β and γ subunits of NFR-2, which are identical to hGABPβ and hGABPγ respectively, have the same transcriptional activity when fused with the GAL4 DNA binding domain as measured using the transient transfection assay. They also reported that the transactivation domains are located in the repeated glutamine-containing hydrophobic clusters (19). However, we have observed that hGABPγ does not mediate transcription activation in the presence of hGABPα. Also, the repeated glutamine-containing hydrophobic clusters

would not appear to always be essential for transcription activation, as the hGABPβ mutant β1I243/NLS/330 lacks this region. One explanation for this discrepancy is that dimerization is required because both the GAL4 DNA binding domain and hGABPβ exist as homodimers on their respective promoter DNAs in these assays. Also, it is possible that the region necessary for transcription activation extends from the repeated glutamine-containing hydrophobic clusters to the C-terminal region. This would explain why the mutant β1I243/NLS/330 possesses only one third of the transcription stimulatory activity of the wild-type. The expanded region may interact with the transcriptional apparatus, some general transcription factors and/or a bridging factor between the pre-initiation complex and the hGABP complex.

ACKNOWLEDGEMENTS

The contribution of M.Ikeda and K.Tamai to the production of monoclonal antibodies against hGABPα and β is gratefully acknowledged. We thank Dr J.-i.Inoue for the gift of SL2 cells and SL2 expression vectors. We also thank Dr S.-i.Hisanaga for technical advice on DAPI staining. We are grateful to Drs T.Wada and T.Imai for helpful discussions and M.Usui for reviewing the manuscript. This work was supported by Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists.

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