Specificities of protein–protein and protein–DNA interaction of GABPα and two newly defined ets-related proteins

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The ets-related protein GABPα interacts with the four ankyrin-type (ANK) repeats of GABPB to form a high-affinity DNA-binding complex that recognizes a site important for herpes simplex virus type I immediate early gene activation. To investigate the selectivity and specificity of the GABP complex, we have isolated two new ETS family members, termed ER81 and ER71. ER81 and GABPα were present in most tissues of adult mice, whereas ER71 was restricted to testis. We have compared the DNA-binding specificities of these proteins by binding site selection. GABPα, ER71, and ER81 recognized the common pentanucleotide DNA sequence 5'-CGGAA/T-3'. Although subtle differences were observed for nucleotide preferences flanking this pentanucleotide core, the overall similarity of the selected sequences was most striking. Given the observation that GABPα interaction with GABPB requires its intact ETS domain, we further compared the ability of GABPB to interact with other ETS proteins. GABPB did not augment the DNA-binding activity of the highly similar ETS domains of ER81, ER71, or Ets-1. Moreover, probing of total tissue extracts with radiolabeled GABPB demonstrated its exceedingly stringent specificity for GABPα. Given that the DNA-binding specificities of these ETS proteins are similar and that the protein–protein interactions between GABPB and GABPα are highly specific, we conclude that the protein interactions determine the target site selection by GABPα.

[Key Words: ETS domain; transcription factor; Notch; ets; DNA-binding protein; testis; ankyrin repeats]

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The heteromeric DNA-binding complex termed GABP was isolated previously on the basis of its ability to bind to a cis-regulatory DNA sequence important for herpes simplex virus type 1 (HSV-1) immediate early (IE) gene activation [Triezenberg et al. 1988; LaMarco and McKnight 1989; LaMarco et al. 1991]. Characterization of GABP revealed that two distinct subunits, GABPα and GABPβ, associate to form an α2β2 tetramer [Thompson et al. 1991]. Subunit association was shown to increase the affinity of binding to two directly repeated GA-rich cis-regulatory elements present in the promoters of HSV-1 IE genes. Four 32- to 33-amino-acid ankyrin-type (ANK) repeats, similar to those found in a wide variety of cellular proteins [Michaely and Bennett 1992], mediate the ability of GABPB to interact with GABPα [Thompson et al. 1991]. The portion of GABPα sufficient for subunit association includes an ETS domain [Thompson et al. 1991].

Members of the ETS family of DNA-binding proteins contain an ~85-amino-acid region of similarity called the ETS domain [Karim et al. 1990]. This domain, characterized by a hydrophobic region containing 3 tryptophan residues spaced 17–18 residues apart, followed by a segment rich in basic amino acids, is sufficient for sequence-specific binding to DNA at a purine-rich core sequence, GGAA/T [Klemsz et al. 1990; Thompson et al. 1991; Nye et al. 1992]. In several cases, including Ets-1, PEA3, SAP-1, and PU.1, these proteins have been shown to function as transcriptional activators [Klemsz et al. 1990; Wasylyk et al. 1991; Dalton and Treisman 1992; Xin et al. 1992].

ETS family members have been implicated in a variety of biologically important processes. Ets-1 was originally identified as the progenitor of v-ets, a portion of an oncogene associated with transformation of erythroblasts by avian retrovirus E26 [Leprince et al. 1983; Nunn et al. 1983]. Since that time, PU.1/Spi-1 and Fli-1 have also been implicated in the induction of erythroleukemia [Moreau-Gachelin et al. 1988; Goebb 1990; Klemsz et al. 1990; Ben-David et al. 1991]. Other ETS proteins have been isolated by virtue of their ability to bind to viral gene promoters. PEA3, for example, binds to polyoma virus enhancer [Xin et al. 1992], GABP binds to HSV-1 IE gene promoters [LaMarco et al. 1991], and a GABP-related factor binds to adenovirus promoters [Watanaba et
al. 1990]. It is now apparent that many members of this family exist in multicellular organisms, including ets-1, ets-2, erg, elk-1, PU.1, E74, Flt-1, elf-1, SAP-1, PEA-3, and GABPα (see Fig. 2, below, and references therein). Given that each of these proteins appears to bind similar DNA sequences, the question arises as to how these factors might undertake biologically distinct functions. As part of our efforts in understanding the action of the GABP family and other ETS family members, we have isolated two new members of this gene family, termed ER71 and ER81. We have compared their tissue distribution, DNA-binding specificity, and ability to interact with GABPβ. The results of our studies dramatically emphasize the importance of protein–protein interaction in the functional specificity of ETS proteins.

Results

Isolation of the ets-related genes ER71 and ER81

An 8.5-day mouse embryo cDNA library was screened using degenerate oligonucleotides designed to recognize predicted to be 335 amino acids in length corresponding to a molecular mass of 37,047 daltons. The cDNA contained an open reading frame (ORF) beginning at its 5’ terminus and ending at a stop codon just before a contiguous segment of adenine residues. The amino acid numbering of the ORF begins at the first methionine residue downstream from the 5’ terminus of the cDNA. An in-frame stop codon was not identified upstream of the first methionine residue. Therefore, we cannot be certain that this clone represents the entire coding portion of the mRNA. However, Northern blot analysis showed the embryo mRNA to be ~1.2 kb in length (Fig. 3, below). Given that the native poly(A) region should add ~100–200 nucleotides not present in the cDNA, it is likely that this cDNA clone represents a nearly full-length copy of the mRNA expressed in mouse embryos. The encoded ER71 protein is predicted to be 335 amino acids in length corresponding to a molecular mass of 37,047 daltons.

The ER81 cDNA consisted of 2514 nucleotides and contained a single long ORF. The ER81 protein product was predicted to be 477 amino acids corresponding to a molecular mass of 55,040 daltons. Both ER71 and ER81 encode an ETS domain located near their respective carboxyl terminals. Sequence comparisons with the ETS domains of other related proteins and a percent identity to GABPα are shown in Figure 2. Comparison of this 85-amino-acid region with known proteins present in the GenBank data base revealed that ER71 was most similar in amino acid sequence to ets-1 (68% identical) and ER81 was most similar to PEA3 (95% identical). ER71 shared no significant amino acid sequence similarity to other ets-related proteins outside of the ETS domain. ER81 was, however, 54% identical to murine PEA3 throughout the entire ORF of the protein.

Expression patterns of ER71 and ER81

The sizes and distributions of the ER81 and ER71 mRNAs were determined by Northern blot analysis of mRNA prepared from various mouse tissues (Fig. 3). ER81 was found in a wide variety of tissues. In most tissues, two mRNA species of ~6.5 and ~3.2 kb were observed. These two ER81 mRNAs were most abundant in kidney; moderately abundant in heart, brain, lung, and embryo; low in spleen, intestine, testis, and thymus; and undetectable in liver. In addition, testis contained moderately abundant levels of an ~2.5-kb ER81 mRNA. This probe did not detect the related PEA3 mRNA under these conditions because the sizes and distribution of ER81 mRNAs are different from those reported for PEA3 [Xin et al. 1992]. In the adult mouse, ER71 mRNA was detected only in testis. In testis, the ER71 mRNA was ~1.7 kb, whereas embryos expressed a smaller mRNA of ~1.2 kb.

The identical filter used for analysis of ER81 and ER71 mRNA was also probed for GABPα mRNA (Fig. 3). With several exceptions, Northern blot analysis of GABPα mRNA expression was similar to that published previously [LaMarco et al. 1991]. Three predominant species of GABPα (~5.0, ~2.8, and ~2.6 kb) were most abundant in preparations of thymus and embryo mRNA yet were also present in a wide variety of tissues. In addition to these previously recognized species, the current analysis revealed an additional ~1.7-kb GABPα mRNA present in spleen, testis, embryo, and thymus. In our previously published report, GABPα was clearly expressed in the brain [LaMarco et al. 1991]. However, in two separate experiments we have been unable to detect GABPα mRNA in the brains of 18-month-old mice. The mRNA isolated from these brains was shown in both cases to be intact by two criteria. First, methylene blue staining showed the RNA to be intact (not shown). Moreover, when the identical filter containing the brain mRNA was probed with ER81 cDNA, its corresponding mRNA was clearly detected (see Fig. 3). The absence of detectable GABPα mRNA in RNA samples that clearly contained intact mRNA may be indicative of physiologically important regulation of GABPα in the brain (see Discussion).

Comparison of optimal DNA-binding site for three ETS family members

Given the widespread expression of GABPα and ER81 mRNA, it is likely that multiple ETS family members are expressed in the same cell at the same time. The question arises, therefore, as to what mechanisms determine the selective targeting of different genes by different ETS family members. One possible explanation is that different ets-related proteins, despite sharing highly related DNA-binding domains, recognize different DNA sequences. To test this possibility, we used the polymerase chain reaction (PCR)-based binding site selection method of Blackwell and Weintraub (1990) to compare...
Figure 1. Nucleotide and deduced amino acid sequences of ER71 (left) and ER81 (right). The deduced amino acid sequences are shown in single-letter code beneath the corresponding codons of the nucleotide sequence. Nucleotide and amino acid are shown in single-letter code before the first methionine residue in the cDNA.

The optimal binding sites for GABPa, ER71, and ER81 (Fig. 4). Polypeptides containing the entire ETS domain of GABPa, ER71, and ER81 were used to select binding sites from an oligonucleotide population prepared with 4 random nucleotides flanking both sides of a central GA dinucleotide. Following three iterations of gel-shift and...
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Figure 2. Comparison of the ETS domain of ER71, ER81, and other known ETS gene family members with GABPα. Conserved amino acids are shaded, and the percent identity with GABPα is indicated. The consensus sequence is shown at the bottom. Sources of gene family members are as follows: M-GABPα (LaMarco et al. 1991), M-ets-1 (Gunther et al. 1990), M-ets-2 (Watson et al. 1988), H-erg (Reddy et al. 1987), H-elk-1 (Rao et al. 1989), M-PU.1 (Klemsz et al. 1990), D-E74 (Burtis et al. 1990), M-fli-1 (Ben-David et al. 1991), H-elf-1 (Thompson et al. 1992), H-SAP-1 (Dalton and Triesman 1992), M-PEA-3 (Xin et al. 1992), D-elg-1 (Pribyl et al. 1991, Chen et al. 1992).

PCR amplification, the selected populations were sequenced. For the purpose of discussion we refer to the following system: 5'-NNNNGANNNN-3' is numbered -4, -3, -2, -1, 0, 0, 1, 1, 2, +3, +4 (see Table 1).

The binding sites selected by GABPα, ER71, and ER81 contained the core sequence GGAA/T. Subtle differences in binding specificity were detected at positions outside of this core. The most noticeable difference was that ER71 selected a C residue at position +4, whereas neither GABPα nor ER81 exhibited any preference at this position. At position -4, GABPα selected G or A equally, whereas ER81 selected G most strongly with modest selectivity for A. At this same -4 site, ER71 selected a G or C equally. Slight differences were also detected in the ratio of C versus G at position -3 and the ratio of A versus T at position +1. At position -3, GABPα favored C over G, ER81 favored G over C, and ER71 tolerated G or C equally. Finally, GABPα favored A over T at position +1, whereas ER71 and ER81 tolerated an A or T equally.

Perhaps more striking than the slight differences noted in the aforementioned paragraph was the overall similarity of the selected sequences. The sequences selected by these three ets-related proteins were almost identical for 8 nucleotides: SMGGAWRY (DNA degenerate alphabet: N = A, C, G, or T; S = G or C; R = A or G; M = A or C; W = A or T; Y = C or T). Their are several reasons to believe that the subtle differences in binding site selectivity may be insufficient to account for selectivity in target gene activation by the members of this family of transcription factors. For example, the GABP-binding site in the promoter of the ICP4 gene (AACGGAAGCG-GAAACC) contains two sites that very closely resemble the optimal sequences of these three ets-related proteins. Based on the binding site selection alone, you would expect the second site to be preferentially recognized by ER81 because of the G residue present at position -3. However, oligonucleotide affinity chromatography of rat tissue extracts resulted in the purification of only GABP and not ER81 or other ETS proteins (LaMarco and Mc-Knight 1989, LaMarco et al. 1991). It would appear, therefore, that selectivity for this site is governed by other determinants, such as post-translational modification or protein–protein interactions. Consistent with this hypothesis, we have demonstrated previously that complex formation of GABPα at the duplicate GA-rich binding sites in the HSV-1 ICP4 gene is dramatically influenced by a second protein termed GABPβ.

Figure 3. Northern blot analysis of GABPα, ER71, and ER81 mRNAs from various mouse tissues. Ten micrograms of poly(A)+ RNA was loaded in each lane. Multiple proings of a single filter are represented. The probe used is indicated above each blot, and the tissue source of RNA is indicated above each lane. The positions of RNA standards are indicated at left.

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Figure 4. Nucleotide sequence of the binding site selected by GABPα, ER81, and ER71. Autoradiography of DNA sequencing after three rounds of gel-shift and PCR amplification by the protein indicated above each panel. The sequence of the starting oligonucleotide is shown in the left panel (Free). The selected nucleotides are designated by letters at the right as the complementary nucleotide determined from the sequencing gel.

GABPβ does not change the specificity of GABPα binding at a single site

We have demonstrated previously the dramatic quantitative effect of GABPβ on the DNA-binding affinity of GABPα. We have also demonstrated that GABPβ can be cross-linked to DNA by UV irradiation, indicating its close proximity to DNA [Thompson et al. 1991]. Here, we have tested whether the proximity of GABPβ to DNA has a qualitative effect on the DNA-binding specificity of GABPα (Fig. 5). Binding site selection in the presence and absence of GABPβ revealed that no sequence differences were detected in the populations of oligonucleotides selected by GABPα alone or the GABPα/GABPβ complex. Therefore, GABPβ somehow affects the affinity of DNA interaction without altering the specificity of sequence recognition.

GABPβ does not associate with or abet the DNA-binding activity of ER71, ER81, or Ets-1

If the functional selectivity of ets-related proteins is significantly influenced by protein–protein interactions, one might anticipate that such interactions would be highly specific. We have tested the functional specificity of GABPβ by assessing its ability to interact with several ets-related proteins. Portions of GABPα, ER71, ER81, and Ets-1 containing the ETS domain were expressed in bacteria and purified [see Material and methods]. Each of these ets-related proteins was analyzed for its ability to interact with DNA and GABPβ in a gel-shift assay. All proteins tested, GABPα, ER71, ER81, and Ets-1, effectively bound an oligonucleotide containing a single CG-GAA site (Fig. 6). Upon addition of GABPβ, GABPα was quantitatively shifted to a slower migrating complex. Similarly supershifted complexes were not observed with ER71, ER81, or Ets-1. Given that Ets-1 is more similar to GABPα than any murine ETS family member yet cloned, these observations provide the initial indication that GABPβ may be dedicated to only one of the ETS family members (Fig. 2).

GABPβ is highly specific for GABPα

Although GABPβ did not functionally abet the DNA-binding activity of ER71, ER81, or Ets-1, it is possible...
that it recognizes an as yet unidentified ets-related protein or possibly an unrelated protein. To test this possibility, we used radiolabeled GABPβ to probe total tissue extracts. Various mouse tissues were homogenized directly in boiling SDS/β-mercaptoethanol containing buffer. Approximately equal amounts of protein, as judged by Coomassie blue staining (Fig. 7A), were subjected to SDS-PAGE and transferred to Immobilon membranes. Following denaturation and renaturation, the membrane was probed with 32P-labeled GABPβ [see Material and methods]. Predominantly one protein band of ~58 kD was recognized by 32P-labeled GABPβ (Fig. 7B). This band was identified as GABPα by two criteria. First, it comigrated with *Escherichia coli* expressed GABPα. Second, immunochemical staining with antibodies directed against GABPα detected this 58-kD species in the same relative amounts that were detected by 32P-labeled GABPβ (Fig. 7C). These results indicated that GABPβ forms a very specific high-affinity complex with GABPα. Even in tissues that express numerous ETS family members, such as thymus, GABPα was the only major protein species detected by 32P-labeled GABPβ. Two caveats are that GABPβ may target a protein that does not renature under these conditions or possibly a very rare protein below the limit of detection.

**Residues within and immediately carboxy-terminal to the ETS domain of GABPα are required for high-affinity interaction with GABPβ**

In earlier experiments it was demonstrated that GABPβ is capable of recognizing a truncated derivative of GABPα containing the ETS domain and the remaining carboxy-terminal residues (Thompson et al. 1991). A construct containing only the ETS domain, termed GABPαN313/C407 [Fig. 8], was able to bind DNA but could no longer interact with GABPβ [Thompson et al.]

**Figure 5.** The nucleotide sequence of the binding site selected by GABPα in the presence and absence of GABPβ. Autoradiography of DNA sequencing after three rounds of gel-shift and PCR amplification. The sequence of the starting oligonucleotide is shown in the left panel [Free]. The selected nucleotides (complement of those determined by the sequencing) are designated by the letters at right.

**Figure 6.** Gel-shift assay of DNA-binding of GABPα, ER71, ER81, and Ets-1 in the presence and absence of GABPβ. E. coli expressed proteins were assayed for binding to an oligonucleotide containing a single ets-binding site. The proteins added to each binding reaction are indicated above each lane. The ER71-shifted species is shown as the intense band that migrates near the free oligonucleotide.

**Figure 7.** Protein interaction blotting of mouse tissue extracts with 32P-labeled GABPβ. Various mouse tissues were homogenized in boiling SDS–sample buffer and subjected to SDS-PAGE. (A) Coomassie blue-stained gel; (B) autoradiography of tissue extracts on Immobilon membranes probed with 32P-labeled GABPβ; (C) Western blot with rabbit anti-GABPα antibodies.
Figure 8. Gel-shift assay of GABPα and ER71 chimeric proteins. (A) Chimeric proteins were constructed as shown using PCR. Junctions were designed between two conserved amino acids with no added or deleted amino acids. (B) Gel-shift assay of chimeric proteins in the presence and absence of GABPβ. The oligonucleotide probe contained two ets-binding sites as found in the HSV-1 ICP4 gene.

From these constructs, it was concluded that the region of GABPα required for interaction with GABPβ occurs within or near the conserved ETS domain. However, because truncations into the ETS domain of GABPα produced small proteins of limited solubility, it was not rigorously established as to whether the ETS domain of GABPα contributed to the interaction with GABPβ. In addition, these ETS domain-deleted proteins lacked DNA-binding activity, thereby preventing detection of GABPα/GABPβ interaction in a gel-shift assay.

To overcome the technical limitations in determining whether GABPβ recognizes determinants within the ETS domain of GABPα, we designed fusion proteins composed of ER71 and GABPα. The chimeric proteins were engineered such that junctions occurred between 2 conserved residues and no extra amino acids were inserted [Fig. 8A]. All constructs were expressed in E. coli and analyzed by gel-shift assay with an oligonucleotide containing two ets-binding sites as they occur in the HSV-1 ICP4 gene [Fig. 8B]. All chimeric proteins bound to this oligonucleotide. Addition of GABPβ to the binding reaction quantitatively supershifted the DNA-bound GABPαN313 but, as expected, did not affect ER71. Replacement of the amino-terminal approximately one-third of the ETS domain of GABPα with that of ER71 (chimera A) markedly decreased the ability of the chimera to complex with GABPβ. Replacement of about two-thirds of the ETS domain of GABPα with that of ER71 (chimera B) further decreased the amount of GABPβ complex detected. Finally, addition of the carboxy-terminal region of GABPβ outside of the ETS domain to the end of the ER71-coding sequence [chimera C] afforded no detectable interaction with GABPβ. Chimeric proteins A, B, and C were also immobilized onto filters, denatured/renatured, and assayed for interaction with 32P-labeled GABPβ as described in the preceding section. Under these conditions, none of the chimeric proteins demonstrated detectable interaction with GABPβ [T. Brown, unpubl.]. Therefore, both assays indicated that portions of the ETS domain of GABPα were important for interaction with GABPβ.

In preparing chimeric proteins composed of two different ETS domains, we have made the assumption that the overall structural framework generated by this motif is similar. The ability of these chimeric proteins to specifically recognize an ets-binding site provides evidence that the protein can fold and bind DNA in a sequence-specific manner. The simplest interpretation of these studies is that sequences throughout the ETS domain and extending 37 amino acids toward the carboxyl terminus of GABPα are required for interaction with GABPβ. Despite sharing a similar structural framework with other ETS proteins, GABPα is somehow highly qualified for interaction with GABPβ.

Discussion

In this report we have analyzed the DNA-binding and protein–protein interaction specificity of several ETS proteins. As part of this study, we report the molecular cloning and characterization of two new members of the ETS family, termed ER81 and ER71. Given their amino acid sequence similarity to other ETS family members and their capacity to bind DNA in a sequence-specific manner, we assume that ER71 and ER81 function as transcriptional regulatory proteins.

Certain ets-related genes are expressed with strict tissue specificity, whereas others are expressed in a wide variety of tissues. Ets-1 and Spi-1 are found only in hematopoietic cells [Bhat et al. 1987; Klemsz et al. 1990].
Ets-2 and Fli-1 are expressed in a variety of tissues including thymus and spleen [Bhat et al. 1987; Ben-David et al. 1991]; PEA3 is only found in brain, epididymis, and mammary glands [Xin et al. 1992]; Elk-1 is limited to testes, lung, liver, and brain [Rao et al. 1989]. ER71 represents a new example of the tissue-specific class of ETS proteins. In adult mice, ER71 mRNA was found only in testis. ER71 may regulate the transcription of genes important in testis specific processes such as spermatogenesis. For example, ER71 might represent the TIN-1 activity thought to repress X chromosomal genes during spermatogenesis [Goto et al. 1991].

ER81, like GABPα, has a widespread tissue distribution. ER81 mRNAs were found in all tissues tested except liver. Previously, GABPα mRNA was found in all tissues tested [LaMarco et al. 1991]. In this report, however, we were unable to detect GABPα mRNA in the brain. The complete absence of a hybridization signal indicates that this message is tightly regulated in the brain under some, as yet undefined, physiological conditions. Such observations may be relevant to the pattern of infectivity of HSV-1. As we have shown, GABP binds to a cis-regulatory element important for HSV-1 IE gene activation [Trizzenberg et al. 1988; LaMarco and McKnight 1989]. Perhaps the absence of GABPα in the brain of adult mammals influences the growth cycle of HSV-1, favoring latency in neuronal cells over the lytic cycle common to other tissues and cell types.

Several recent reports indicate that different ETS family members have different DNA-binding specificities [Table 1]. Two independent groups concur on the binding specificity of Ets-1: 5'-RCMGGAWRY-3' [Nye et al. 1992; Woods et al. 1992]. As Nye et al. [1992] noted, the specificity of Ets-1 for residues in the minor groove differs slightly from that of E74: 5'-AAYCMGGAACT-3' [Urness and Thummel 1990]. The major difference is that E74 prefers a pyrimidine residue, 4 nucleotides 5' of the central GA pair, whereas Ets-1 prefers a purine at this position. Also, all of the oligonucleotides recognized by E74 had an A at position +1, whereas selection by Ets-1 gave an A/T ratio of 1.4 : 1 [Urness and Thummel 1990; Woods et al. 1992]. It was suggested that differences in the flanking sequences in the recognition site mediate the specificity of the binding of different ETS proteins [Nye et al. 1992]. In this study we have compared the binding site specificities of GABPα, ER71, and ER81. This analysis confirms that the ETS domain is capable of highly specific interactions with DNA and can discriminate between sequence variations spanning at least 10 nucleotides. Also, slight differences in binding site specificity exist between these ETS family members [Table 1]. In some instances, the DNA-binding specificity detected in vitro may account for specificity of promoter action in vivo. For example, two ets-type DNA-binding sites in the interleukin-2 enhancer are binding sites for elf-1 but are not recognized by either ets-1 or ets-2 [Thompson et al. 1992]. However, there are numerous members of the ETS family, and this motif recognizes a core DNA sequence that is nearly identical. Most striking, in our opinion, is the similarity of the optimal binding sites of GABPα, ER81, ER71, and Ets-1. It seems likely that discrimination of different promoter elements by the ETS family members will be governed by more compelling forces than these subtle differences in DNA-binding specificity alone. We believe that protein–protein interactions, such as those we have characterized in the GABPα/GABPβ complex, could provide the selectivity necessary for the discrimination of different promoters by different members of the ets family.

We have demonstrated, by gel-shift assay with known ETS proteins and by blotting of total tissue extracts, that GABPβ specifically recognizes only GABPα. We predict that specific protein–protein interactions, such as those between GABPα and GABPβ, facilitate target selection by ETS family members. Target site recognition would be directed by the assembly ETS proteins with interacting factors, much like a jigsaw puzzle [Johnson and McKnight 1989; Thompson and McKnight 1992]. For example, as diagramed in Figure 9, the ability of GABPβ to homodimerize brings two molecules of GABPα together in an α2β2 tetramer. Tetramer formation drives GABP to bind with high affinity to the two tandem ets DNA-binding sites of the HSV-1 IE gene promoter. Similarly, it has been demonstrated by Dalton and Treisman [1992] that protein–protein interactions between SAP-1 and the serum response factor [SRF] are required for the activity of SAP-1 adjacent to the serum response element [SRE] of the c-fos promoter (Fig. 9). Other examples include ets-1 action adjacent to jun/fos at the API site of the stromelysin promoter [Wasylyk et al. 1990, 1991] and PU.1 interaction with a protein at the immunoglobulin κ3 enhancer [Pongubala et al. 1992]. We conclude that an
important mechanism for providing specificity of ETS proteins is specific protein–protein interactions occurring at promoters. Protein–protein interactions direct GABP to preferentially act at promoters containing multiple ets sites, Ets-1 to act at sites adjacent to an AP-1 site, and SAP-1 to act adjacent to a SRE.

Material and methods

cDNA cloning of ER71 and ER81

The following four degenerate oligonucleotide probes were designed to hybridize to the conserved regions of the ETS domain: 5′-ATCCAGCAGTGCAGAAGTTTCGCT-3′, 5′-ATCCAGTGTGCAGAAGTTTCGCT-3′, 5′-ATCCAGCAGTGCAGAAGTTTCGCT-3′, and 5′-ATCCAGTGTGCAGAAGTTTCGCT-3′. These oligonucleotides were used to screen an 8.5-day mouse embryo library (S.-J. Lee 1990).

E. coli expression

Full-length GABPa, GABPaN313, GABPaN313/C407, and full-length GABPb were expressed in E. coli and purified as described previously (Thompson et al. 1991). E. coli expression vectors of chimeric proteins were constructed by PCR such that chimera A contained amino acids 207–261 of ER71 fused to amino acids 348–454 of GABPa; chimera B contained amino acids 207–290 of ER71 fused to amino acids 376–454 of GABPa; and chimera C contained amino acids 207–335 of ER71 fused to amino acids 410–454 of GABPc. For chimera A, PC primers were designed to produce an amplified DNA fragment coding for amino acids 207–261 of ER71 with a 5′ BamHI site and a 3′ EcoRI site, and PC primers were used to produce an amplified DNA fragment coding for amino acids 348–454 of GABPa with a 5′ EcoRI site and a 3′ HindIII site. These two fragment were ligated in a three-fragment ligation with BamHI/HindIII-cut E. coli expression vector pT5 (Thompson et al. 1991). The EcoRI site regenerates the Glu-Phe residues that are conserved between GABPs and ER71. Chimera B was constructed by similar methods except that the junction was an XbaI site between conserved Ser-Arg residues. In chimera C, the junction was constructed by the addition of an EcoRI site. In this case, the cloning led to the addition of a Glu-Phe dipeptide at the junction. The ETS domains of ER71 (amino acids 208–335), ER81 (amino acids 333–477), Ets-1 (amino acids 170–440, as defined in Nye et al. 1992) and full-length GABPb were cloned by PCR into the E. coli expression vector pAR(AR1) that has been described by Blanar and Rutter (1992). This vector produced proteins under the conditions described above. After three rounds of selection and amplification, the final oligonucleotide population was sequenced using primer A.

Gel-shift assay

DNA-binding reactions were done with E. coli-expressed proteins and the indicated 32P-labeled DNA probe in 25 mM Tris-HCl (pH 8.0), 0.3 mM MgCl₂, 0.5 mM EDTA, 50 mM KCl, 2 mM DTT, 10% glycerol, and 50 μg/ml of bovine serum albumin with 1 μg of poly[dI–dC] per 20 μl reaction. Reactions were incubated at 4°C for 30 min and subjected to electrophoresis on 5% polyacrylamide gels in recirculating 0.25× TBE at 4°C. The double-stranded oligonucleotide containing two ets-binding sites was created by annealing 5′-AGCTTGCGGAACCGGGATCG-3′ and 5′-GATCCGATTCCGCCGTTCCTCGGTCGCG-3′. The double-stranded oligonucleotide containing a single ets-binding site was created by annealing 5′-AACCAAGCTTGAATCCGGAAGAGTAGA-CAACG-3′ and 5′-AACCAAGCTTGAATCCGGAAGAGTAGACACGC-3′. Probing with GABPb Various mouse tissues were homogenized in boiling 0.625 M Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol using a Poltron homogenizer (Brinkmann). Approximately equal amounts of protein were subjected to SDS-PAGE and electrophoretically transferred to Immobilon membranes (Millipore). Filters were denatured and renatured as described (Vinson et al. 1988). Filters were then treated for 60 min in blocking buffer [20 mM HEPES-KOH (pH 7.7), 5% Carnation nonfat dry milk, 0.05% Tween 20, 1 mM DTT], followed by 30 min in HYB buffer [20 mM HEPES-KOH (pH 7.7), 75 mM KCl, 1% Carnation nonfat dry milk, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Tween 20, 1 mM DTT]. HMK–GABPb was radiolabeled with [γ-32P]ATP using HMK (Sigma) and hybridized at 4°C for 4 hr (250,000 cpm/ml in HYB buffer). After hybridization, the filter was washed three times at 4°C with HYB buffer for 10 min each time and exposed to X-ray film.

Binding site selection

Binding site selection [SAAB] was performed essentially as described by Blackwell and Weintraub (1990), with the following exceptions. The sequences of the oligonucleotides were as follows: Template, 5′-AGAGCCTACATTGCAACCCCCN-NNNNGNNNCTGATCTGTAGCAATTCGGA-3′; primer A, 5′-TCCGAATTGCTACAG-3′; and primer B, 5′-AGACCG-TACATTGG-3′. Binding reactions were done with E. coli-expressed proteins under the conditions described above. After three rounds of selection and amplification, the final oligonucleotide population was sequenced using primer A.

Northern blotting

Poly(A)+ RNA was isolated from 18-month-old mice (CD-1, Charles River Laboratory) by standard methods. Ten micrograms of once-selected poly(A)+ RNA was subjected to electrophoresis through denaturing formaldehyde–agarose gels, transferred to GeneScreen membranes (New England Nuclear), and probed with randomly primed 32P-labeled DNA probes corresponding to ER71, ER81, and GABPa. Between successive probes the filter was stripped by boiling in water for 20 min. Molecular mass estimates were obtained by comparison with RNA standards (BRL).

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Note added in proof

The nucleotide sequences of ER81 and ER71 have been submitted to the GenBank data library.

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