Specificities of protein–protein and protein–DNA interaction of GABP α and two newly defined *ets*-related proteins

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The *ets*-related protein GABP α interacts with the four ankyrin-type (ANK) repeats of GABP β 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 GABP α 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. GABP α , 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 GABP α interaction with GABP β requires its intact ETS domain, we further compared the ability of GABP β to interact with other ETS proteins. GABP β 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 GABP β demonstrated its exceedingly stringent specificity for GABP α . Given that the DNA-binding specificities of these ETS proteins are similar and that the protein–protein interactions between GABP β and GABP α are highly specific, we conclude that the protein interactions determine the target site selection by GABP α .

[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, GABP α and GABP β , associate to form an $\alpha_2\beta_2$ 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 GABP β to interact with GABP α (Thompson et al. 1991). The portion of GABP α 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, char-

acterized 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; Goebel 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 (Watanabe et

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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*, *Fli-1*, *elf-1*, *SAP-1*, *PEA-3*, and *GABP α* (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 *GABP* 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 *GABP β* . 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 two conserved regions within the DNA-binding domain of *ets*-related proteins (see Materials and methods). Two different cDNA clones, termed *ets*-related 71 (ER71) and *ets*-related 81 (ER81), were isolated and sequenced (Fig. 1). The largest ER71 clone obtained was 1066 bp in length. 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 termini. Sequence comparisons with the ETS domains of other related proteins and percent identity to *GABP α* 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 *GABP α* mRNA (Fig. 3). With several exceptions, Northern blot analysis of *GABP α* mRNA expression was similar to that published previously (LaMarco et al. 1991). Three predominant species of *GABP α* (~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 *GABP α* mRNA present in spleen, testis, embryo, and thymus. In our previously published report, *GABP α* was clearly expressed in the brain (LaMarco et al. 1991). However, in two separate experiments we have been unable to detect *GABP α* 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 *GABP α* mRNA in RNA samples that clearly contained intact mRNA may be indicative of physiologically important regulation of *GABP α* in the brain (see Discussion).

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

Given the widespread expression of *GABP α* 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

ER71

AGAACCGTCAGAACAGCATCCATGGACCTGTGGAAGTGGGATGAGCGTCACCTGCAGGA 60
 e p s e q a s M D L W N W D E A S L Q E 13
 AGTGCCCTCTGGGGACAAGCTGACAGGACTGGGAGCGGAATTTGGTTTCTATTTCCTCTGA 120
 V P P G D K L T G L G A E F G F Y F P E 33
 AGTGCTCTACAAGAGGACACCCGATCACACCAATGAACGTAGAAGGCTGCTGGAAAGG 180
 V A L Q E D T P I T P M N V E G C W K G 53
 GTTCCAGAGCTGGACTGGAACCCCGCTTTACCTCAGGAAGACGTACCTTTCCAGGCGGA 240
 F P E L D W N P A L P H E D V P F Q A E 73
 GCCCGTTGCTACCCCTTCCGTGGTGGGAGACTGGACAGACCTGGGATGCAACACCTC 300
 P V A H P L P W S R D W T D L G C N T S 93
 GGACCCGTGGAGCTGTGCTTACAGAGCCGAGGCGCTGCCCTCTGGCAGGAGCCCTC 360
 D P W S C A S Q T P G P A P P G T S P S 113
 CCCCTTCGTGGCTTTGAAGGGGCGACCGGCGCAGAACTCTGCCACCTGGGAGGAGGGGT 420
 P F V G F E G A T G Q N P A T S A G G V 133
 CCCCTGTGGTGGACCCCTCCAGCTGCCTGGAGACTACAGCTGGGACTGTTCTGTGGG 480
 P S W A H P L P W S R D W T D L G C N T S 153
 CCCAGTGGCGCCACCTACTGGGACAATGGCTGGGCGGGGAAGCGCATGAGGACTATAA 540
 P S G A T Y W D N G L G G E A H E D Y K 173
 AATGTCATGGGGGGGTCTGCGGTTTCGACTACACACCGTGAATACCTGGGCTGCA 600
 M S W G G S A G S D Y T T T W N T G L Q 193
 GGACTGCAGCATCCCTTTGAGGGGCGACAGAGTCCAGCATTCACACCGCTCCAAATC 660
 D C S I P F E G H Q S P A F T T P S K S 213
 GAACAAGCAGTCTGATAGAGCCACATTGACTCGCTACTCCAAAATAACACCGAGGTCC 720
 N K Q S D R A T L T R Y S K T N H R G P 233
 CATTCAGCTGTGGCAATTCCTCCTGGAGCTGCTCAAGACGGGGCTCGCAGCAGCTGCAT 780
 I Q L W Q F L L E L L Q D G A R S S C I 253
 CCGCTGGACGGGCAATAGCCGCGAGTTCCAGCTGTGCGACCCCAAGAGGTGGCCCGGCT 840
 R W T G N S R E F Q L C D P K E V A R L 273
 GTGGGGCGAGCGCAAGAGGAAGCGGGAATGAATTATGAGAACTGAGTCGAGGTCTACG 900
 W G E R K R K P G M N Y E K L S R G L R 293
 TTATTATTACCGCGCGACATCGTGTCTCAAGAGTGGTGGCGCAAGTACACATACCGCTT 960
 Y Y Y R R D I V L K S G G R K Y T Y R F 313
 CGGGGACGTGTGCTGTCTCGCTATCAGGATGATATGGGGCATCTGCCAGGTGSCAGA 1020
 G G R V P V L A Y Q D D M G H L P G A E 333
 AGGCCAATAAAACAAAAACAAAAACAAAAACAAAAACAAAAACAAAAACAAAAACAAAA 1066
 G Q 335

ER81

CAGCTCTGTCTAGAGGCGCTTTCCGGCTTCCAAGGGGAAGTGTGGGCAATAATTAATGTTT 60
 TTAATTAAATTTGGAGGGAAATTTTTCAGCGCCGTTCGCTAGCGTGGCCCTCAGGTTGATA 120
 GAAGTCCAGATCCCGAGGAAATCTCCAGCTAAATGCTCAAAATATATAAACACAGTGAAGTAA 180
 TATTTCGGAAGAGGAGCAGAAATGGATGGATTTTATGACCCAGCAAGTGCCTTACGTGGTCA 240
 M D G F Y D Q Q V P Y V V T 14
 CCAACAGTCAGCTGGGAGAACTGTACCGAGAAACCAACAAATGTGAGGAAAAGAAAAT 300
 N S Q R G R N C T E K P T N V R K R K F 34
 TCATTAAACAGAGATCTGGCTCATGATTGAGAAAGTCTTTCAAGATCTGAGTCAGTTAC 360
 I N R D L A H D S E E L F Q D L S Q L Q 54
 AAGAAACATGGCTGTCTGAGAGCTCAGGTACCTGACAATGATGAGCAGTTGTGTCCAGACT 420
 E T W L A E A Q V P D N D E Q F V T P D Y 74
 ATCAGGCTGAAAGTTTGGCTTTTCATGGCTCCCACTGAAATCAAGAAAGAACCCACACA 480
 Q A E S L A F H G L P L K I K K E P H S 94
 GCCCATGTTTCAAGCTGGGTCTGCTTTCAGTCAAGAGCAGCCCTTTAAATTCAGCTATG 540
 P C S E L G S A C S Q E Q P F K F S Y G 114
 GAGAAAAGTGCCTGTCAATGTGAGTGCCTATGATCAGAAGCCACAAGTGGGAATGAGG 600
 E K C L Y N V S A Y D D E Q F V M R P 134
 CCTCAATCCCGCAGACCATCCAGCACTCCAGTGTCCCACTACACCATGCATCTCCAA 660
 S N P P T P S S T P V S P L H H A S P N 154
 ACACAGCTCACTCCGAAACCTGACGGGGCTTCCAGCTCAGCTCCCTCTCTCAGT 720
 T A H T P K P D R A F A P A H L P S Q S 174
 CCATACACAGCAGCCTACCCCATGGACACAGATTTTCGCCGCGAGCTTTCTGAACCT 780
 I P D S T Y P M D H R F R R Q L S E P C 194
 GTAATTCTTTTCTCTCTTTCGCGACAATGCCAAGGGAAGGCGCTCTATGTACCAACGCC 840
 N S F P P L P T M P R E G R P M Y Q R Q 214
 AGATGTCTGAGCCAAACATCCCTTCCCAACCAAGGCTTTAAGCAGGATACACGACC 900
 M S E P N I P F P P Q G F K Q G E Y H D P 234
 CAGTCTATGAACATACACCATGGTGGCGGTGAGCGAGCCAAAGCTTCCCGCTCTT 960
 V Y E H T T M V G G A A S Q S F P P P L 254
 TGATGATCAACAGGAACCCAGAGATTTTGCATATGACTCAGAAGTGCCTAGCTGCCACT 1020
 M I K Q E P R D F A Y D S E V P S C H S 274
 CCATTTATATGAGGCAAGAGGCTTCTGCTCATCCCAAGCAGAACAGGCGTCATGT 1080
 I Y M R Q E G F L A H P S R T E G C M F 294
 TTGAGAAGGGTCCAGGAGTCTATGATGACACCTGTGTGGTCCCGGAGAAAGTTCGATG 1140
 E K G P R Q F Y D D T C V V P E K F D G 314
 GGGACATTAAAGCAAGAGCCTGGAATGTACCGGGAAGGACCCAGTACAGAGGCGAGGAT 1200
 D I K Q E P G M Y R E G P T Y Q R R G S 334
 CCCTCCAGCTCTGGCAGTTTTCGTTAGCTCTTCTGGATGACCTTCCAAATCTCAHTTCA 1260
 L Q L W Q F L V A L L D D P S N S H F I 354
 TTGCTGGACTGGACGAGGATGGAATTTAACTGATTGAGCCGCAAGAGGTGGCCCGGC 1320
 A W T G R G M E F K L I E P E E V A R R 374
 GTTGGGCGATTGAGAAGACAGCGCGGCGATGAATATGACAACTTAGTCTGTTCTCTCC 1380
 W G I Q K N R P A M N Y D K L S R S L R 394
 GCTATTATGAGAAGGGAATCATGCAAAAGTGGCTGGAGAAAGATACGTGTACAAAT 1440
 Y Y Y E K G I M Q K V A G E R Y V Y K F 414
 TTGTGTGTGACCCGGAAGCCCTTTTCTCTATGGCTTTCCGGATAACAGCGGCCACTGC 1500
 V C D P E A L F S M A F P D N Q R P L L 434
 TGAAGACGGACATGGAACGTCACATCAACGAAGGACACAGTGCCTCTGTCTCACTTTG 1560
 K T D M E R H I N E E D T V P L S H F D 454
 ATGAGAGCATGACCTACATGCCGGAAGGGGCTGTGCAACCTTCACCCCTACAACGAAG 1620
 E S M T Y M P E G G C C N P H P Y N E G 474
 GATACGTGTACTAATCAGTAAACCCGTCAAGCAAGGCCCGCTGTTCCGCTCTTTT 1680
 Y V Y 477
 TTTCAAGATGCGAGAGAATACCGAATTCCTCTGATGTTTGTGTTTATTCTGTTGTTG 1740
 TACTTTATTTTAAATGATAATACAAAAGGGGCTTTTCCTGTTGCATTATCTATGTT 1800
 CTGCCATGGACTGCGCACTTTATTTGCTGTGGGCGGAGTAATCTAGACATTCATTCTT 1860
 TGTAACAGGAAGATGGCGGATGAGTGGGCGAAGGAGCTGAGGGAATCTTTTGTCTTAG 1920
 GCTTGGAAAGGAGTCTACAGGTTTCTGATGATGATGCTATATCATATTTGTTCTGATTT 1980
 CATATAAGCTAAGATAATTTTCCTCGGTATCTACGGTACAGTTGATTTACAGTTGTGT 2040
 AAATATCTCTTGGAGACCATTTGCTTGGGCAATTTCCCGCATCTACTGAGTCTCTGC 2100
 AGGTGTACAAAAATCTACTGTAAATGGCAGTTTAAATGTTAGAAATTTACTGTTTGCACCT 2160
 CTGTGAAAAAAAATATTTAGCAATGCAATTTGTGTTCTTTCTGTTTCAATAGCTTT 2220
 ACAGATGACTTGTAGAGAAAAACCTTAATGTGGGCAATTTCTCTGAAAGTTGAGTAAT 2280
 CACCATGACTGTAAATGCGGGCCACCGTTTGGAGCTTGGCTCCAACTGAGTCAACAGGCC 2340
 AGTAGCATTTTATGTATCTGGTGCCATCTGCTGTTTGTGTTACTACAAATCTGCTCTTT 2400
 AAATATGTTGATGCCCATCTCACTTAAATAATGACATGTCATGCTCTTCGGAATCTTCA 2460
 TTTAAGTGTAAAGCTGGGATCACAATGAAGCAAAAAATAAAAACAAAAACAAAAAC 2514

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 numbers are shown at the *right*. The lowercase letters in ER71 depict amino acid sequence corresponding to the predicted ORF before the first methionine residue in the cDNA.

the optimal binding sites for GABP α , ER71, and ER81 (Fig. 4). Polypeptides containing the entire ETS domain of GABP α , 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

GABP α	100	NNGQ-IQLWQFLLLELLTDKD-ARDCTSWV-GDEGEFKLNQPELVA--QKWGQRK-NKPTMNYEKLRSALRYYYDGDIMCKVQGRFVYKFD
D-ELG1	74	NNGQ-VQLWQFLLLELLTDCE-HTVIEVWV-GTEGEFKLTDDEVA--RLWGGRK-NKPAMNYEKLRSALRYYYDGMISKVSGKRFAYKFD
M-ETS-1	67	GSQP-IQLWQFLLLELLTDKS-CQSFISWT-GDGWEFKLSDPEVA--RRWGGRK-NKPMNYEKLRSGLRYYYDKNIHKTAGKRYVYRFV
M-ETS-2	66	GSQP-IQLWQFLLLELLSDKS-CQSFISWT-GDGWEFKLADPEVA--RRWGGRK-NKPMNYEKLRSGLRYYYDKNIHKTSGKRYVYRFV
H-ERG	62	GSQP-IQLWQFLLLELLSDSS-NSSCITWE-GTNGEFKMTDPEVA--RRWGGRK-SKPMNYDKLSRALRYYYDKNIMTKVHGKRYAYKFD
M-FLI1	62	GSQP-IQLWQFLLLELLSDSA-NASCITWE-GTNGEFKMTDPEVA--RRWGGRK-SKPMNYDKLSRALRYYYDKNIMTKVHGKRYAYKFD
H-SAP1	58	--MDSATLWQFLLQLLQKQP-NKHMICWTSMD-GQFKLLQAEVA--RLWGGRK-NKPMNYDKLSRALRYYYVKNIIKKVNGQRFVYKFD
H-ELK1	58	FS---VTLWQFLLQLLEQQ-NGHITSWTSRDGEFKLVDAEVA--RLWGGRK-NKPMNYDKLSRALRYYYDKNIIRKVSQGRFVYKFD
M-PEA3	55	RRGA-LQLWQFLLVALLDDPT-NAHFIAMT-GRGMEFKLIEPEVA--RLWGGRK-NRPAMNYDKLSRLRYYYEGKIMQKVGKRYVYKFD
M-ER81	55	RRGS-LQLWQFLLVALLDDPS-NSHFIAMT-GRGMEFKLIEPEVA--RRWGGRK-NRPAMNYDKLSRLRYYYEGKIMQKVGKRYVYKFD
M-ER71	55	HRGP-IQLWQFLLLELLQDGA-RSSCIRWT-GNSREFQLCDPEVA--RLWGGRK-NKPMNYEKLRSGLRYYYRDIIVLKSQGRKYTYRFQ
H-ELF-1	49	-KGNTIYLWFLALLQDKATCPKYIKWTQREKICFKLVDSKAVS--RLWGGRK-NKPMNYETMGRALRYYYQKILAKVGGKRLVYQF-
D-E74A	47	GST--TYLWFLKLLQDREYCPRFIKWTNREKGVFKLVDSKAVS--RLWGGRK-NKPMNYETMGRALRYYYQKILAKVGGKRLVYQF-
M-PU.1	40	GSKKIRIYQFLLQLLRSQD-MKDSIWWYDKDKGTQFQFSKHKEALAHRWGQKGNRKKMTYQKMARALRYYYGKTGEVKKV-KKKLTYQES
consensus		L..FL..LL.....I.W.....F.....WG..K.....M.Y....R.LR.Y..K....K.....Y.F.

Figure 2. Comparison of the ETS domain of ER71, ER81, and other known ETS gene family members with GABP α . Conserved amino acids are shaded, and the percent identity with GABP α is indicated. The consensus sequence is shown at the bottom. Sources of gene family members are as follows: M-GABP α (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, +2, +3, +4 (see Table 1).

The binding sites selected by GABP α , 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 GABP α nor ER81 exhibited any preference at this position. At position -4, GABP α 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, GABP α favored C over G, ER81 favored G over C, and ER71 tolerated G or C equally. Finally, GABP α 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 McKnight 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 GABP α at the duplicate GA-rich binding sites in the HSV-1 ICP4 gene is dramatically influenced by a second protein termed GABP β .

Figure 3. Northern blot analysis of GABP α , ER71, and ER81 mRNAs from various mouse tissues. Ten micrograms of poly(A)⁺ RNA was loaded in each lane. Multiple probes 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.

Figure 4. Nucleotide sequence of the binding site selected by GABP α , 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.

GABP β does not change the specificity of GABP α binding at a single site

We have demonstrated previously the dramatic quantitative effect of GABP β on the DNA-binding affinity of GABP α . We have also demonstrated that GABP β 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 GABP β to DNA has a qualitative effect on the DNA-binding specificity of GABP α (Fig. 5). Binding site selection in the presence and absence of GABP β revealed that no sequence differences were detected in the populations of oligonucleotides selected by GABP α alone or the GABP α /GABP β complex. Therefore, GABP β somehow affects the affinity of DNA interaction without altering the specificity of sequence recognition.

GABP β 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 GABP β by assessing its ability to interact with several *ets*-related proteins. Portions of GABP α , 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 GABP β in a gel-shift assay. All proteins tested, GABP α , ER71, ER81, and *Ets*-1, effectively bound an oligonucleotide containing a single CG-GAA site (Fig. 6). Upon addition of GABP β , GABP α 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 GABP α than any murine ETS family member yet cloned, these observations provide the initial indication that GABP β may be dedicated to only one of the ETS family members (Fig. 2).

GABP β is highly specific for GABP α

Although GABP β did not functionally abet the DNA-binding activity of ER71, ER81, or *Ets*-1, it is possible

Table 1. Nucleotide sequence determined by binding site selection

	-4	-3	-2	-1	0	0	+1	+2	+3	+4
	N	N	N	N	G	A	N	N	N	N
GABP α	G	C	C	G	G	A	A	G	T	N
	A	g	a				t	a	c	
ER81	G	G	C	G	G	A	A	G	T	N
	a	c	a				T	a	c	
ER71	G	C	C	G	G	A	T	G	T	C
	C	G	a				A	a	c	
<i>Ets</i> -1	A	C	C	G	G	A	A	G	C	N
	g		a				T	a	T	
E74	C	C	C	G	G	A	A	G	T	N
	T		a					a		

GABP α -, ER81-, and ER71-binding site selection was determined with an oligonucleotide population prepared with GA dinucleotide flanked by 4 random nucleotides on each side (see Fig. 4). Two uppercase letters at a position indicate approximately equal selection; a lowercase letter indicates a less-favored nucleotide. Also shown are the optimal sequences for *Ets*-1 (Nye et al. 1992; Woods et al. 1992) and E74 (Urness and Thummel 1990).

Figure 5. The nucleotide sequence of the binding site selected by GABP α in the presence and absence of GABP β . 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.

that it recognizes an as yet unidentified *ets*-related protein or possibly an unrelated protein. To test this possibility, we used radiolabeled GABP β 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 32 P-labeled GABP β (see Materials and methods). Predominantly one protein band of ~58 kD was recognized by 32 P-labeled GABP β (Fig. 7B). This band was identified as GABP α by two criteria. First, it comigrated with *Escherichia coli* expressed GABP α . Second, immunochemical staining with antibodies directed against GABP α detected this 58-kD species in the same relative amounts that were detected by 32 P-labeled GABP β (Fig. 7C). These results indicated that GABP β forms a very specific high-affinity complex with GABP α . Even in tissues that express numerous ETS family members, such as thymus, GABP α was the only major protein species detected by 32 P-labeled GABP β . Two caveats are that GABP β 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 GABP α are required for high-affinity interaction with GABP β

In earlier experiments it was demonstrated that GABP β is capable of recognizing a truncated derivative of GABP α containing the ETS domain and the remaining

Figure 6. Gel-shift assay of DNA-binding of GABP α , ER71, ER81, and *Ets*-1 in the presence and absence of GABP β . *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.

carboxy-terminal residues (Thompson et al. 1991). A construct containing only the ETS domain, termed GABP α N313/C407 (Fig. 8), was able to bind DNA but could no longer interact with GABP β (Thompson et al.

Figure 7. Protein interaction blotting of mouse tissue extracts with 32 P-labeled GABP β . 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 32 P-labeled GABP β ; (C) Western blot with rabbit anti-GABP α 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.

1991). 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 GABPα 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 GABP α , has a widespread tissue distribution. ER81 mRNAs were found in all tissues tested except liver. Previously, GABP α mRNA was found in all tissues tested (LaMarco et al. 1991). In this report, however, we were unable to detect GABP α 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 undetermined, 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 (Triezenberg et al. 1988; LaMarco and McKnight 1989). Perhaps the absence of GABP α 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'-AAYCMGGAAGT-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 GABP α , 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 bind-

ing sites of GABP α , 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 GABP α /GABP β 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 GABP β specifically recognizes only GABP α . We predict that specific protein-protein interactions, such as those between GABP α and GABP β , 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 GABP β to homodimerize brings two molecules of GABP α together in an $\alpha_2\beta_2$ 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 AP1 site of the stromelysin promoter (Wasylyk et al. 1990, 1991) and PU.1 interaction with a protein at the immunoglobulin κ 3' enhancer (Pongubala et al. 1992). We conclude that an

Figure 9. Protein-protein interactions contribute to the selection of DNA-binding sites by ETS proteins. (A) GABP α binds only weakly to the promoters of HSV-1 IE genes. The presence of GABP β promotes the association of GABP α into an $\alpha_2\beta_2$ tetramer, resulting in high-affinity binding to two tandem *ets* sites (Thompson et al. 1991). (B) SAP-1 requires protein-protein interaction with SRF to act adjacent to SREs important in the induction of *c-fos* transcription (Dalton and Treisman 1992).

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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'-ATCCAGCTITGGCAGTTCCTICTIGAGCTICT-3'; 5'-ATCTCITGGA / GT / CIGGIGACGGA / GIIIGAGTTCAAGCT-3'; 5'-ATGAAGTATGAGAAGCTITCICGIGG / CICTICGITATT-3'; and 5'-GTIGCICA / GIIITGGGGIA / CAGCGIAAGA-ACAA-3'. These oligonucleotides were used to screen an 8.5-day mouse embryo library (S.-J. Lee 1990).

E. coli expression

Full-length GABP α , GABP α N313, GABP α N313/C407, and full-length GABP β 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 GABP α ; chimera B contained amino acids 207–290 of ER71 fused to amino acids 376–454 of GABP α ; and chimera C contained amino acids 207–335 of ER71 fused to amino acids 410–454 of GABP α . For chimera A, PCR primers were designed to produce an amplified DNA fragment coding for amino acids 207–261 of ER71 with a 5' *Bam*HI site and a 3' *Eco*RI site, and PCR primers were used to produce an amplified DNA fragment coding for amino acids 348–454 of GABP α with a 5' *Eco*RI site and a 3' *Hind*III site. These two fragments were ligated in a three-fragment ligation with *Bam*HI/*Hind*III-cut *E. coli* expression vector pT5 (Thompson et al. 1991). The *Eco*RI site regenerated the Glu-Phe residues that are conserved between GABP α and ER71. Chimera B was constructed by similar methods except that the junction was an *Xba*I site between conserved Ser-Arg residues. In chimera C, the junction was constructed by the addition of an *Eco*RI 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 GABP β were cloned by PCR into the *E. coli* expression vector pAR(Δ R1) that has been described by Blonar and Rutter (1992). This vector produced proteins that were tagged with an antibody recognition site (FLAG) and a substrate site for heart muscle protein kinase (HMK). The expressed proteins were isolated and partially purified by chromatography on a mono-Q FPLC column (Pharmacia).

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), 3.0 mM MgCl₂, 0.5 mM EDTA, 50 mM KCl, 2 mM DTT, 10% glycerol, and 50 μ g/ml of bovine serum albumin with 1 μ g of poly[d(I-C)] per 20 μ l reaction. Reactions were incubated at 4°C for 30 min and subjected to electrophoresis on 5% polyacrylamide gels in recirculating 0.25 $\times$ TBE at 4°C. The

double-stranded oligonucleotide containing two *ets*-binding sites was created by annealing 5'-AGCTTGCGGAACGGAA-GCGGAAACCGCCGGATCG-3' and 5'-GATCCGATCCG-GCGGTTTCCGCTTCCGTTCGCG-3'. The double-stranded oligonucleotide containing a single *ets*-binding site was created by annealing 5'-AACCAAGCTTGAGTACCGGAAGAGTA-CACCG-3' and 5'-AACCAAGCTTCCGGTGACTCTTCCGG-TACTC-3'.

Probing with GABP β

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-GABP β 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'-AGACCGTACCATTGCAACCCCN-NNNGANNNNCTGATCTGTAGCAATTTCGGA-3'; primer A, 5'-TCCGAATTGCTACAG-3'; and primer B, 5'-AGACCG-TACCATTGCA-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 GABP α . 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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Specificities of protein-protein and protein-DNA interaction of GABP alpha and two newly defined ets-related proteins.

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