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# Ets Proteins: New Factors That Regulate Immunoglobulin Heavy-Chain Gene Expression

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**We used a DNA-protein interaction screening method to isolate a cDNA, Erg-3, whose product binds to a site, designated π, present in the immunoglobulin (Ig) heavy-chain gene enhancer. Erg-3 is an alternatively spliced product of the *erg* gene and contains an Ets DNA-binding domain. Fli-1 and PU.1, related Ets proteins, also bind to the same site. In addition, PU.1 binds to a second site, designated μB, in the Ig heavy-chain enhancer. We demonstrate that the π binding site is crucial for Ig heavy-chain gene enhancer function. In addition, we show that Erg-3 and Fli.1, but not PU.1, can activate a reporter construct containing a multimer of protein-binding sites, synergistically with helix-loop-helix protein E12. We discuss how combinatorial interactions between members of the helix-loop-helix and Ets families may account for the tissue specificity of these proteins.**

Developmental regulation of immunoglobulin (Ig) gene expression is dependent on various sequence motifs present in the Ig heavy- and light-chain gene enhancers. One class of such sequences are the E boxes, first identified by in vivo methylation protection studies (5, 9). Five E-box sites are present in the Ig heavy-chain gene enhancer, designated μE1, μE2, μE3, μE4, and μE5. Three E-box sequences are present in the Ig kappa-chain gene enhancer, designated κE1, κE2, and κE3 (17). The E-box sequences were shown to be important for B-cell-specific Ig gene regulation by mutational analysis (15, 17). Most important are sequence elements that contain an E2 box (GCAGNTG), present in both the Ig heavy (μE2 and μE5)- and light-chain (κE2) gene enhancers (17).

Recently, a family of proteins that bind the E2 box was identified. They share a common sequence element designated the helix-loop-helix (HLH) motif (12, 22). The HLH domain has the potential to form two amphipathic helices separated by a loop (22). The HLH domain mediates both homo- and heterodimerization with other HLH proteins (6, 8, 21). Adjacent to the HLH domain is a basic region that is responsible for DNA binding (8). Two members of the HLH proteins, E12 and E47, are involved in various developmental pathways. In B cells, two tissue-specific complexes, designated BCF1 and BCF2, that contain homo-oligomers of E12 and/or E47 are present (1, 2).

In addition to the highly conserved E-box motifs, several other sequence elements that are crucial for Ig gene regulation have been identified. One such element, designated μB, binds a lymphoid-specific protein and plays an important role in the control of Ig heavy-chain gene expression (19, 23). In addition, DNase I protection analysis showed that a lymphoid-specific protein binds a site, designated π, located adjacent to the μE2 site (19). Here, we demonstrate that the π site is crucial for Ig heavy-chain enhancer function and identify two Ets proteins, Erg-3 and Fli.1, that bind with high affinity to the π site. We also show that PU.1, a related Ets protein, binds to both the π site and a second site, μB. We show that Erg-3 and Fli.1, but not PU.1, can activate a reporter construct containing a multimer of μE2-π binding sites, synergistically with HLH protein E12.

## MATERIALS AND METHODS

**Construction and purification of GST fusion proteins.** pGEX-Erg-3 was constructed by first introducing the Erg-3 cDNA into the *Eco*RI site of pBSK and then subcloning it into the *Eco*RI site of pGEX-1. Full-length cDNAs of PU.1 and Fli-1 (a kind gift from R. Maki) were used to construct the GST fusion proteins pGex-Fli-1 and pGex-PU.1. Full-length PU.1 was excised by *Eco*RI digestion and ligated, in frame, into the *Eco*RI site of pGex-1 by standard techniques. Fli-1 cDNA was digested with *Xho*I, blunt-ended with Klenow fragment, and then excised with *Bam*HI. The nearly full-length cDNA, lacking the first 74 amino acids, was ligated into the *Bam*HI-*Sma*I sites of pGex-2TK by using standard techniques. Expression of glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* (BL21 DE3) and purification on glutathione-Sepharose was performed as described previously (14). The purity, size, and concentration of eluted proteins were examined by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels.

**Hybridization of filters.** Phages from the λgt11 pre-B-cell library 22D6 (a kind gift from Astar Winoto) were grown for 3 hours at 42°C; β-galactosidase fusion protein expression was then induced by placing nitrocellulose filters, prewet in 10 mM isopropyl-β-D-thiogalactopyranoside, over the plates and growing the phages for an additional 5 hours at 37°C. Filters were blocked overnight in 1× HBB (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.7], 25 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40) and 5% nonfat powdered milk. Filters were washed once in TNE 50 (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). Filters were next placed in hybridization solution (TNE 50, 100 μl of 1-mg/ml sonicated salmon sperm DNA per ml of hybridization solution). To the hybridization solution, $1 \times 10^6$ to $2 \times 10^6$ cpm of [γ-$^{32}$P]ATP-end-labeled probe per ml of hybridization solution was added. The probe was prepared by end labeling a trimer of μE2-π sites digested from pBSK. The probes were end labeled with [γ-$^{32}$P]ATP by using T4 polynucleotide

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kinase and purified in Elutip columns (Schleicher & Schuell). Filters were hybridized for 1 h at room temperature. Following hybridization, the filters were washed three times (10 min per wash) in TNE 50. The filters were dried briefly, covered with Saran Wrap, and exposed to film at −70°C with an intensifying screen.

**Cell culture.** The human fibroblast cell line HeLa was grown in DME-regular glucose containing 10% fetal calf serum. The mouse pre-B-cell line 300-19 and the mouse hybridoma cell line LK were grown in RPMI 1640 supplemented with 10% fetal calf serum. Cell lines were grown at 37°C in a humidified 5% $CO_2$-containing atmosphere.

**Transient transfection.** Fli-1, PU.1, and Erg-3 cDNAs were cloned into the expression vector pJ3Ω. Full-length Fli-1 cDNA was excised from the parental plasmid by *Eco*RI-*Xho*I digestion, blunt-ended with Klenow fragment, and subcloned into the *Sma*I site of pJ3Ω. Full-length PU.1 cDNA was removed from the parental plasmid p25.1 by *Eco*RI digestion and was subcloned into the *Eco*RI site of pJ3Ω. Erg-3 was first subcloned into the *Sma*I site of pBS-ATG (22). pBS-ATG/Erg-3 was then digested with *Hin*dIII-*Eco*RI and ligated into the *Hin*dIII-*Eco*RI sites of pJ3Ω. Transfections were performed by using DEAE-dextran in HeLa cells. To determine the ability of Fli-1, Erg-3, and PU.1 to activate transcription, 5 μg of each construct was transfected alone or cotransfected with 5 μg of pJ3ΩE12 in duplicate with 10 μg of J-21 (μE2-π) chloramphenicol acetyltransferase (CAT), a reporter containing a trimer of either wild-type or mutant μE2-π sequences. Cells were harvested 48 h later and lysed by freeze-thawing in 0.25 M Tris (pH 7.8), and CAT activity was determined as described previously (29).

Wild-type and mutant J-21 (E-μB) CAT reporter plasmids were constructed by subcloning bp 267 to 459 (9) of the IgH enhancer into J-21 (11), which contains a minimal c-*fos* promoter fused to the bacterial CAT gene. Inserts were excised from pBSK by *Eco*RI-*Xba*I digestion, blunt-ended with Klenow fragment, and subcloned into the filled-in *Sal*I and *Eco*RV sites of J-21 to place the enhancer 5′ and 3′ of the CAT gene, respectively. Clustered mutations of Ig enhancer-binding sites were introduced by polymerase chain reaction (PCR). To determine the effects of mutations in μE5, ME2, and π sites on transcription, 10 μg of each reporter was transfected into 300-19 and LK cells by using DEAE-dextran. Cells were harvested 48 h later and lysed by freeze-thawing in 0.25 M Tris (pH 7.8), and CAT activity was determined as described above.

**DNase I footprinting.** bp 267 to 459 (9) of the μ enhancer were cloned out by PCR, and the *Eco*RI and *Xba*I sites were introduced. The μ enhancer fragment was then cloned into pBSK *Eco*RI-*Xba*I. The μ enhancer-containing plasmid pBSKμ267-459 was then cut with *Eco*RI, treated with calf intestinal phosphatase, and end labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase. The μ enhancer probe was then cut out with *Xba*I and purified from a 5% polyacrylamide gel. Footprinting reactions were carried out in 50-μl binding reaction mixtures with 10,000 cpm of probe DNA with increasing amounts of GST fusion proteins for 30 min at room temperature. Binding reactions were carried out in a buffer containing 25 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM dithiotheritol, 1 mM EDTA, 10 μg of bovine serum albumin, 0.01% Nonidet P-40, and 0.5 μg of poly(dIC) as a nonspecific competitor DNA. Breakdown reactions were allowed to take place for 1.5 min in a final concentration of 3 to 5 mM $MgCl_2$ with 0.2 U of DNase I. After 1.5 min, the reaction was quenched with a solution containing 2% sodium dodecyl sulfate (SDS), 200 mM EDTA, and 1 μg of salmon sperm DNA. After extraction with phenol-chloroform, reaction mixtures were analyzed by electrophoresis on an 8% polyacrylamide gel containing 8 M urea.

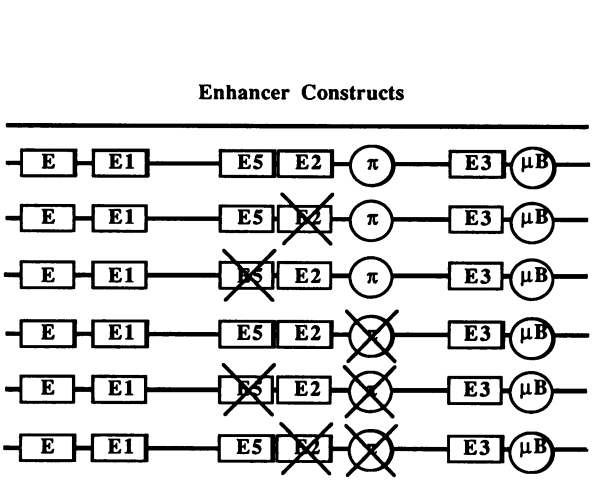
**Northern (RNA) blotting.** RNA was isolated from cell lines as described by Sambrook et al. (29). RNA was fractionated on a 1% agarose gel containing formaldehyde and transferred to nitrocellulose. cDNA probes 25.1, Fli-1, and Erg-3 were random primed by using the Prime-It II kit (Stratagene). Hybridizations were performed at 68°C overnight in Quikhyb (Stratagene). Filters were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 15 min and once for 30 min in 0.1× SSC–0.1% SDS at 60°C. Filters were then dried briefly and exposed to film at −70°C with an intensifying screen.

**DNA sequencing.** Double-stranded DNA sequencing was performed by using a Sequenase 2.0 kit (U.S. Biochemical) and the protocol provided by the manufacturer.

## RESULTS

**The π binding site is crucial for Ig heavy-chain gene enhancer function in B cells.** Recent DNase I protection analysis showed that a lymphoid-specific protein binds a site, designated π, located in the Ig heavy-chain gene enhancer (19). To define a functional role of the π binding site, we made a cluster of mutations in the π, μE2, and μE5 sites. In addition, we introduced double mutations in both the π and E-box sites. In these studies a fragment of the Ig heavy-chain gene enhancer, containing the E, μE1, μE5, μE2, π, μE3, and μB sites, was used (Fig. 1). The activity of wild-type and mutant enhancers was measured when positioned either upstream or downstream of a chloramphenicol acetyltransferase gene placed under the control of a minimal *fos* promoter. Wild-type and mutant enhancers were transfected into both a pre-B-cell line, 300-19, and a hybridoma cell line, LK (Fig. 1). Mutations in the μE5 and μE2 sites reduce transcriptional activation in hybridoma cells as described previously (Fig. 1) (15). However, the most striking effect was caused by mutations in the π binding site, reducing transactivation to approximately 25% of wild-type levels when the enhancer was positioned immediately proximal to the promoter and to 16% of wild-type levels when positioned distal to the promoter (Fig. 1). A double mutation in either the μE2 or μE5 site and the π site did not further reduce the transactivation ability of the reporter construct, suggesting that both sites are required for full activation (Fig. 1). Thus, occupancy of the π binding site is crucial for Ig heavy-chain enhancer function.

**Screening for π-binding proteins in B cells.** In order to isolate and characterize cDNA clones that encode for proteins that bind the π site, we screened an expression library derived from a pre-B-cell line, 22D6, with a radiolabeled π fragment. Two cDNA clones that encoded interacting proteins were identified. Clone 2 was characterized extensively (Fig. 2). An 816-bp open reading frame started at the fusion between the β-galactosidase sequence and clone 2. DNA sequence analysis of clone 2 showed that it was identical to Erg-1 and Erg-2, except for a 63-bp insertion (27) (Fig. 2). Analysis of several cDNAs that were subsequently isolated revealed that many of them contained this insertion, suggesting that the insertion is likely the result of alternative splicing, rather than a cloning artifact (Fig. 2). Erg-1 and Erg-2 are Ets proteins, a recently identified new family of DNA-binding proteins (26). Thus, we isolated a cDNA




| % Relative Activation | | | |
|---|---|---|---|
| LK | | 300-19 | |
| Enhancer Position | | | |
| 5' | 3' | 5' | 3' |
| 100 | 100 | 100 | 100 |
| 66 | 33 | 19 | 18 |
| 30 | 30 | 26 | 48 |
| 25 | 16 | 5 | 8 |
| 18 | 18 | 5 | 14 |
| ND | 17 | ND | ND |

FIG. 1. Mutations in the Ets-binding site reduce transcriptional activity of the Ig heavy-chain gene enhancer in B cell lines. LK and 300-19 cells were transfected with IgH enhancer CAT reporter constructs containing wild-type and mutant protein-binding sites. Reporters contained bp 267 to 459 (9) of the IgH enhancer upstream or downstream of the CAT gene of J-21. Shown are the results of a representative experiment done in duplicate; the values for duplicate experiments were averaged, and the CAT activity of mutants is shown relative to wild-type levels. ND, not done.

encoding an Ets protein, designated Erg-3, that binds to the π site.

**Erg-3 recognizes the π site present in the Ig heavy-chain gene enhancer.** To determine whether Erg-3 could bind to the

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  1  GAT GAC TTC CAG CGG CTC ACG CCG AGC TAC AAT GCC GAC ATT CTT CTC   48
(1)  D   D   F   Q   R   L   T   P   S   Y   N   A   D   I   L   L  (16)

 49  TCA CAT CTC CAC TAC CTC AGA GAG ACT CCC CTT CCA CAT CTG ACT TCC   96
(17)  S   H   L   H   Y   L   R   E   T   P   L   P   H   L   T   S  (32)

 97  GAT GAC GTT GAT AAG GCT TTA CAA AAC TCT CCA CGG TTA ATG CAT GCC  144
(33)  D   D   V   D   K   A   L   Q   N   S   P   R   L   M   H   A  (48)

145  AGA AAC ACA GGG GGT GCA GCT TTT ATT TTC CCA AAT ACT TCA GTA TAT  192
(49)  R   N   T   G   G   A   A   F   I   F   P   N   T   S   V   Y  (64)
     **************************************************
193  CCC GAA GCT ACG CAA AGA ATT ACA ACT AGG CCA GAT TTA CCT TAT GAG  240
(65)  P   E   A   T   Q   R   I   T   T   R   P   D   L   P   Y   E  (80)
     ******************************************
241  CCT CCC AGG AGA TCA GCC TGG ACC GGC CAC AGC CAC CTC ACC CCT CAG  288
(81)  P   P   R   R   S   A   W   T   G   H   S   H   L   T   P   Q  (96)

289  TCC AAA GCT GCT CAG CCA TCT CCC TCT GCA GTG CCC AAA ACT GAA GAC  336
(97)  S   K   A   A   Q   P   S   P   S   A   V   P   K   T   E   D (112)

337  CAG CGT CCT CAG TTA GAT CCT TAC CAG ATC CTG GGA CCG ACC AGT AGC  384
(113)  Q   R   P   Q   L   D   P   Y   Q   I   L   G   P   T   S   S (128)

385  CGC CTT GCT AAT CCA GGT AGT GGC CAG ATC CAG CTG TGG CAG TTC CTG  432
(129)  R   L   A   N   P   G   S   G   Q   I   Q   L   W   Q   F   L (144)

433  CTC GAA CTC CTG TCA GAC AGC TCC AAC TCC AAC TGC ATC ACC TGG GAA  480
(145)  L   E   L   L   S   D   S   S   N   S   N   C   I   T   W   E (160)

481  GGC ACC AAC GGG GAG TTC AAG ATG ACA GAC CCG GAC GAG GTG GCT CGG  528
(161)  G   T   N   G   E   F   K   M   T   D   P   D   E   V   A   R (176)

529  CGC TGG GGG GAG AGG AAG AGC AAG CCC AAC ATG AAC TAT GAC AAG CTC  576
(177)  R   W   G   E   R   K   S   K   P   N   M   N   Y   D   K   L (192)

577  AGC CGC GCC CTC CGC TAC TAC TAC GAC AAA AAC ATC ATG ACC AAG GTG  624
(193)  S   R   A   L   R   Y   Y   Y   D   K   N   I   M   T   K   V (208)

625  CAC GGG AAG CGC TAC GCC TAC AAG TTT GAC TTC CAC GGG ATT GCC CAG  672
(209)  H   G   K   R   Y   A   Y   K   F   D   F   H   G   I   A   Q (224)

673  GCC CTG CAG CCC CAC CCT CCT GAG TCG TCC CTG TAC AAG TAC CCC TCC  720
(225)  A   L   Q   P   H   P   P   E   S   S   L   Y   K   Y   P   S (240)

721  GAC CTG CCA TAC ATG GGC TCC TAT CAC GCC CAC CCC CAG AAG ATG AAC  768
(241)  D   L   P   Y   M   G   S   Y   H   A   H   P   Q   K   M   N (256)

769  TTT GTG TCT CCC CAC CCT CCC GCT CTC CCA GTC ACA TCT TCC AGT TTC  816
(257)  F   V   S   P   H   P   P   A   L   P   V   T   S   S   S   F (272)
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FIG. 2. Nucleotide sequence of the Erg-3 cDNA clone and its predicted amino acid sequence. Amino acid numbering is indicated in parentheses. The Ets domain is underlined. A 24-amino-acid insertion unique to Erg-3 is indicated by asterisks.

π site in solution, we mapped the contacts that Erg-3 makes with the Ig heavy-chain gene enhancer, using DNase I protection analysis. Erg-3 protects a region of approximately 12 nucleotides centered over the π site (Fig. 3A). No protection was found over the μB element (Fig. 3A). As expected, a series of clustered mutations in the GAA motif in the π binding site prevented Erg-3 from binding to this site (Fig. 3A). Interestingly, exactly the same pattern of protection over the π site was previously observed in a DNase I protection study of nuclear extracts derived from B cells but not from other cell types (19). Thus, Erg-3 binds the π site, a site located in the Ig heavy-chain gene enhancer.

**Fli.1 and PU.1 bind to sites present in the Ig heavy-chain gene enhancer.** The finding that Erg-3 binds the Ig heavy-chain gene enhancer led us to examine whether additional Ets-DNA-binding proteins interact with the π element. The Ets protein Fli.1 was a good candidate. Fli.1 and Erg-2 are closely related (67% identity) over the entire coding sequence, except between residues 200 and 260 of Fli.1 (3). However, in the Ets DNA-binding domain, comprising approximately 85 residues, Fli.1 and Erg-3 are 98% identical (3). Therefore, we first tested whether Fli.1 also binds the π element, using DNaseI protection analysis (Fig. 3B). Predictably, Fli.1 gives a pattern of protection similar to that of Erg-3, suggesting that Fli.1 contacts DNA in a similar manner (Fig. 3B).

Less closely related to Erg-3 is PU.1. PU.1 and Erg share only 23% identity over the whole coding region and 38% identity in the Ets DNA-binding domain (3, 16). To test whether PU.1 also recognizes sites present in the Ig heavy-chain gene enhancer, we tested the binding of PU.1 by DNase I protection analysis. Surprisingly, PU.1 gives protection over two different sites (Fig. 3C). Protection from cleavage was observed over the μB element. Some DNase I-hypersensitive bands, flanking this protected area, were observed. The μB site binds a B-cell-specific protein in nuclear extracts, and functional analysis has shown that this element is crucial for B-cell-specific gene expression (19, 23). In addition to that of μB, protection was observed over the π site (Fig. 3C). However, we note that the affinity of PU.1 to the π site is lower than that to the μB element (Fig.

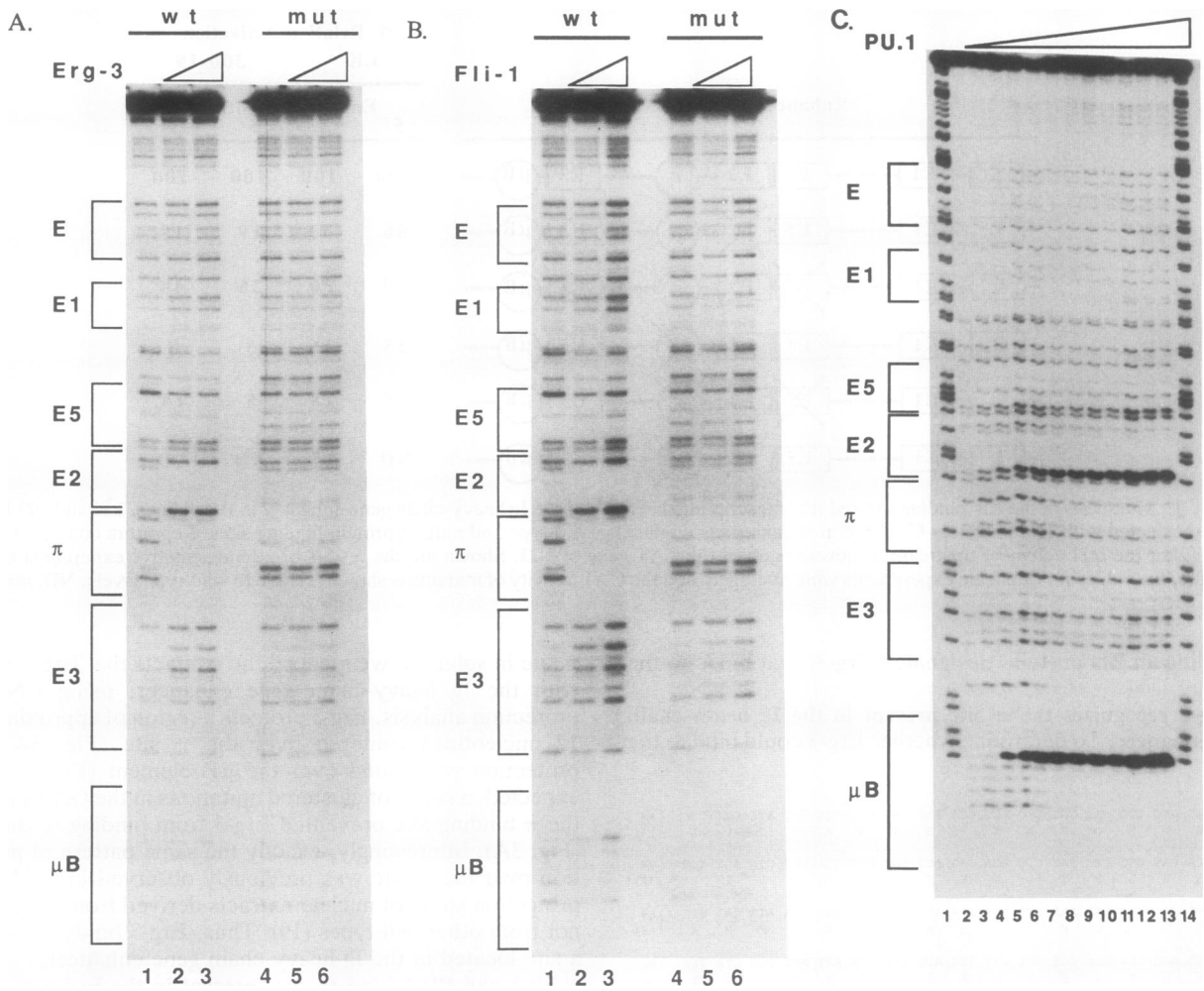


FIG. 3. DNase I protection analysis of Erg-3 (A), Fli.1 (B), and PU.1 (C). DNase I protection analysis of Ets proteins was done with the Ig heavy-chain gene enhancer as a probe. Mutant probes contained mutant π sites in which four nucleotides of the core consensus, GGAA, had been changed to TCGT. Ets proteins were produced as GST fusion proteins. Boxes indicate known binding sites present on this probe. For DNase I footprint analysis of purified GST-Erg-3 (A) and GST-Fli.1 (B), binding reactions were done with wild-type (wt) and mutant (mut) probes in the absence (lanes 1 and 4) or presence (lanes 2 and 5) of 125 ng of GST-Erg3 or GST-Fli.1 and with 250 ng (lanes 3 and 6) of GST-Erg-3 or GST-Fli-1. (C) For DNase I footprint analysis of GST-PU.1, binding reactions were done with the wild-type enhancer probe in the absence (lane 2) or presence of increasing amounts of GST-PU.1. Lanes 3 to 13 contain 16, 32, 48, 64, 80, 96, 112, 128, 144, 160, and 176 ng of GST-PU.1, respectively. G+A reference ladders are present in lanes 1 and 14.

3C). Thus, Erg-3, Fli.1, and PU.1 bind to various sites present in the Ig heavy-chain gene enhancer.

**Ets and HLH proteins activate transcription synergistically.** In addition to the cloning of Ets proteins using the π site as a probe, we identified identical cDNAs, including Erg-3, in a screen, using radiolabeled E12 protein. We attempted to demonstrate interaction between E12 and Erg-3 by various assays, but we were unable to convincingly demonstrate either E12-Erg interaction in solution or cooperativity in DNA binding (27a). Thus, E12 and Erg-3 interact on a filter, but this interaction is too weak to be demonstrated in solution in either the absence or presence of DNA. However, because E12 and Erg-3 bind to sites adjacent to each other, we tested whether HLH and Ets proteins could activate transcription in a synergistic fashion. E12, Erg-3, PU.1, and Fli.1 were expressed in HeLa cells in the presence of a reporter construct containing a multimer of μE2 and π sites. Neither E12 nor any of the Ets proteins could activate a reporter construct containing a multimer of μE2 and π sites (Fig. 4). In contrast, coexpression of E12 and Erg-3 led to a dramatic increase in transcriptional activation (Fig. 4). Similarly, while overexpression of Fli.1 did not result in transactivation, cotransfection of E12 and Fli.1 resulted in a dramatic increase in transactivation (Fig. 4). By using a reporter construct containing multiple mutant π sites, no activation was observed (27a). Interestingly, PU.1 did not synergize with E12 in activating transcription (27b). We note, however, that we have been unable to detect synergistic activation using the complete Ig heavy-chain gene enhancer as a reporter construct in non-B cells. Perhaps, negative regulation in non-B cells prevents the HLH and Ets proteins from activating the Ig enhancer, or, alternatively, additional factors are required.

**Northern (RNA) analysis of Erg-3, Fli.1, and PU.1.** We showed that the π site is important for regulation of the Ig heavy-chain gene enhancer. To determine whether the Fli.1,

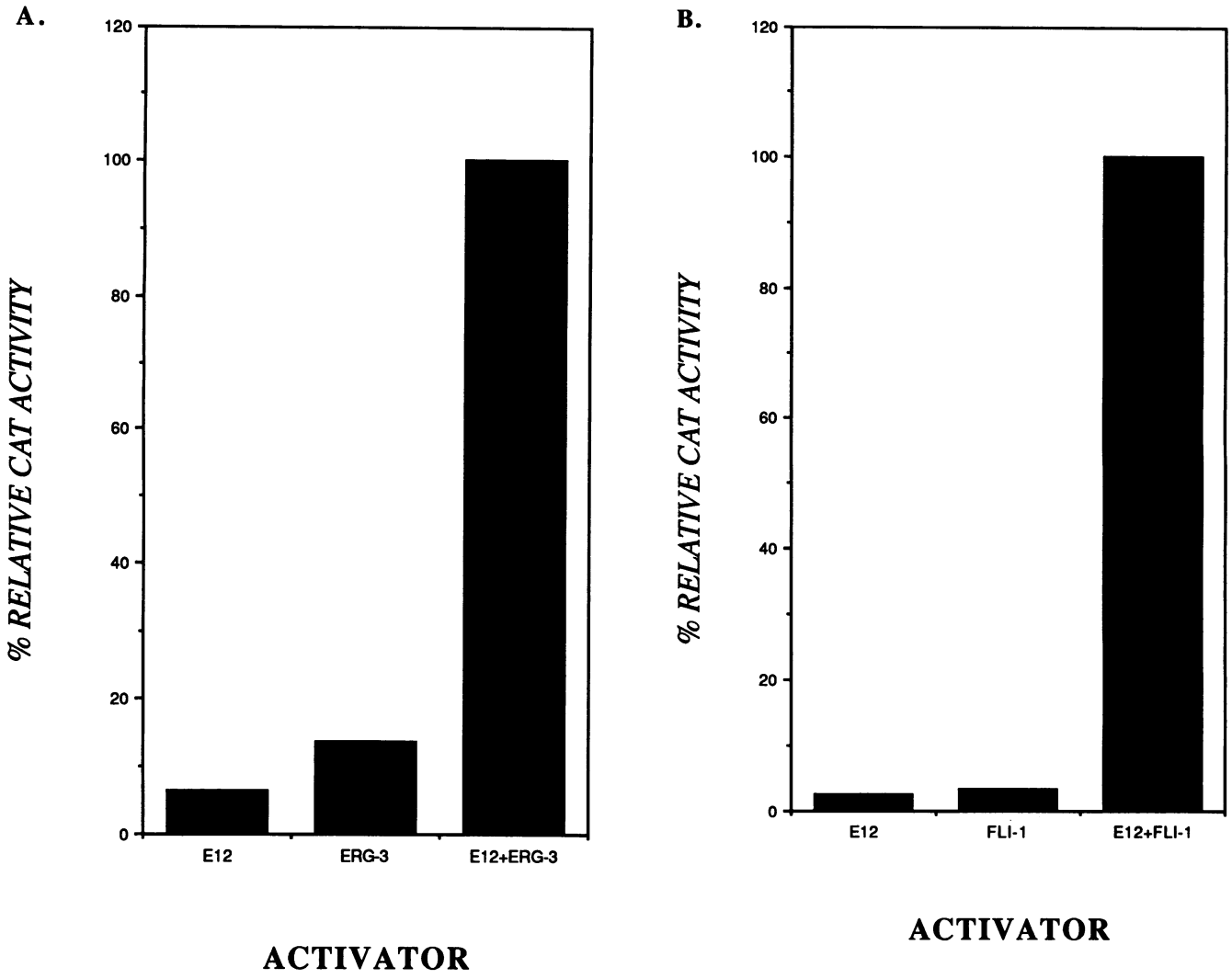


FIG. 4. Fli-1 and Erg-3 synergistically activate transcription with E12. HeLa cells were transfected with a reporter gene containing three copies of the μE2-π sequence upstream of the CAT gene and expression vectors containing E12, Erg-3, or Fli.1. All transfections were repeated at least twice in duplicate. Shown are data from a representative experiment done in duplicate; values from the duplicates were averaged, and CAT activity is shown relative to E12 plus Erg-3 (A) or E12 plus Fli.1 (B).

Erg-3, and PU.1 genes are expressed in B cells and to determine their relative levels during B-cell development, we probed a Northern blot containing RNA derived from fibroblast, pre-T, early pre-B-, pre-B-, and mature-B-cell lines. Previous reports showed that Erg-1 was expressed in a colon carcinoma cell line, COLO 320, and a T-cell line, Molt-4 (27). Here, we show that Erg-3 is expressed at relatively high levels in early pre-B cells and at low levels in pre-B and mature B cells (Fig. 5A). However, little or no Erg-3 RNA can be detected in 10T1/2, 3T3, and 2017, a pre-T cell line. Fli.1 RNA is present at equal levels in early pre-B (HAFTL), pre-B (PD31), and mature B (WEHI231) cells and in a pre-T-cell line (2017) (Fig. 5B). No expression was found in 3T3 cells (Fig. 5B). While lower levels of Fli.1 expression were found in 10T1/2 cells, no Fli.1 RNA could be detected in myoblasts and myotubes (27b). As previously reported, PU.1 is expressed in B cells but not in T cells and fibroblast-like cells (16). We confirmed those results and found in addition that PU.1 RNA levels increase during B-cell differentiation (Fig. 5C). The highest levels of PU.1 are found in mature B cells (Fig. 5C). Thus, Erg-3, Fli.1, and PU.1 are each expressed in B cells, and both Erg-3 and PU.1 RNA levels change during B-cell differentiation. In summary, each Ets protein studied here is expressed in cell lines derived from the B-cell lineage, but their RNA levels change during differentiation.

## DISCUSSION

A paradox in understanding the function of Ets and HLH proteins is that their recognition sequences are present in a variety of enhancers that regulate tissue-specific gene expression (26, 32). For example, the DNA-binding specificities of many HLH proteins are nearly indistinguishable in vitro (4, 30); however, they act with great biological specificity during development. For example, in B cells, homodimers of E12 and/or E47 bind to the E-box sites in the Ig gene enhancers (2). However, when tested, neither the muscle creatine kinase (MCK) gene enhancer nor the insulin gene enhancer is activated in B cells (2a). Thus, the HLH

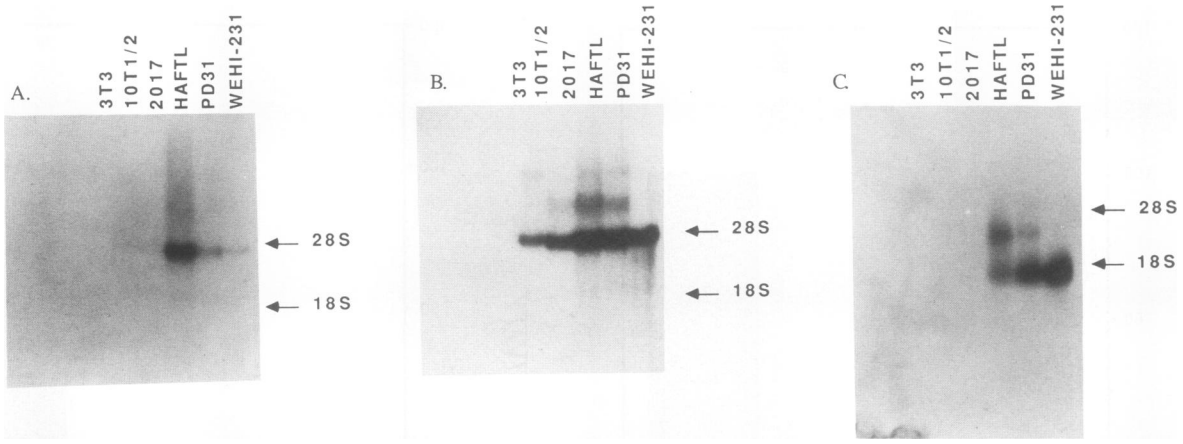


FIG. 5. Northern blot analysis of Erg-3, Fli.1, and PU.1 expression. Total RNA (15 μg) from cell lines representing mature B (WEHI-231), pre-B (PD31 and HAFTL), pre-T (2017), 10T1/2, and fibroblast (3T3) cells was probed with a labeled Erg-3 (A), Fli.1 (B), or PU.1 (25.1) (C) cDNA insert. The positions of 28S and 18S rRNAs are indicated on the right.

proteins act with a high degree of biological specificity that cannot simply be explained by differences in DNA-binding specificity and affinity. We and others previously postulated that sets of activators and repressors together determine the tissue specificity of Ig gene expression (18, 21). We show here that an Ets protein and an HLH protein bind to adjacent sites present in the Ig heavy-chain gene enhancer and activate transcription synergistically. In contrast, muscle- and pancreas-specific enhancers lack Ets consensus sites directly adjacent to their E2-box elements (7, 8, 20, 25). The lack of Ets-like sequences adjacent to the E-box elements may prevent efficient occupancy of the MCK or insulin gene enhancer and thus prevent the inappropriate activation of the MCK gene in B cells. A similar scenario may occur in pancreas-specific gene activation. It recently has been shown that β-cell-specific gene expression of the insulin gene is controlled by synergistic activation of an HLH (pan-1, E47) and a homeobox-containing protein, designated 1mx-1 (10). Likewise, it is conceivable that in myotubes a MyoD-E12 heterodimer interacts with yet another class of DNA-binding proteins, providing specificity to the muscle-specific HLH regulators.

In addition to the Ets-HLH synergistic activation, it has recently been demonstrated that E47 and TFE3 are involved in cooperative activation of the Ig heavy-chain gene enhancer (28). Similarly, we attempted to demonstrate E12-Ets synergistic activation, using the complete Ig heavy-chain gene enhancer, but were unsuccessful. Likely, additional factors are required to activate the Ig heavy-chain gene enhancer in non-B cells. Alternatively, negative regulatory elements may prevent the activation of the Ig heavy-chain enhancer in HeLa cells and may be dominant in E12-Ets activation.

After this paper was submitted for publication, a paper with very similar results reported the binding of proto-oncogene ets-1 to the π site (24). These data increase the number of factors that potentially can interact with this element, and it remains to be determined which of the Ets domain proteins are functional in B cells. From our Northern analysis and other previous studies, at least five Ets proteins are present in B cells, including Erg-3, Fli.1, PU.1, ets-1, and ets-2 (13, 31). We and Nelsen and collaborators (24) show that the levels of these proteins change during B-cell differentiation. The various Ets domain proteins might compete for the same binding sites. Perhaps each of them has a function in a certain stage of B-cell differentiation. Regardless of which Ets subunit(s) binds the π site, we provide evidence that the π site is crucial for Ig heavy-chain gene enhancer function.

In summary, we demonstrate that various Ets proteins bind to the Ig heavy-chain gene enhancer and show that their binding sites are crucial for Ig heavy-chain enhancer function. To account for the biological specificity of Ets and HLH proteins we propose that Ets and/or HLH proteins act in concert to activate the Ig heavy-chain gene only in B cells.

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