A Three-protein-DNA Complex on a B Cell-specific Domain of the Immunoglobulin μ Heavy Chain Gene Enhancer

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The lymphoid-specific immunoglobulin μ heavy chain gene intron enhancer (μE) contains multiple binding sites for trans-acting nuclear factors. We have used a combination of in vitro and in vivo assays to reconstruct protein-DNA interactions on a minimal B cell-specific μ enhancer that contains three motifs, μA, μB, and μE3. Using ETS-domain proteins that transactivate the minimal enhancer in non-lymphoid cells, we show that (i) PU.1 binds coordinately to both μA and μB sites in vitro and (ii) in the presence of Ets-1, this factor binds to the μA site and PU.1 to the μB site. Two factors, TFE3 and USF, bind to the μE3 element. When the ETS proteins are present together with μE3 binding proteins, a three-protein-DNA complex is generated. Furthermore, we provide evidence for protein-protein interactions between Ets-1 and PU.1 proteins that bind to μA and μB sites, and between Ets-1 and TFE3 bound to the μA and μE3 sites. We propose that this domain of the μ enhancer is assembled into a nucleoprotein complex that contains two tissue-restricted ETS domain proteins that recognize DNA from the same side of the helix and one ubiquitously expressed bHLH-leucine zipper protein that binds between them, recognizing its site from a different side of the helix.

The immunoglobulin μ heavy chain (IgH) gene intron enhancer is a tissue-specific regulatory element that is necessary for expression of the IgH gene (1, 2). Since the proposal of Alt and colleagues of the correlation between transcription and immunoglobulin gene rearrangements (3–5), the μ enhancer has been considered a likely candidate to be the sequence that regulates rearrangements at this locus. Recently, this hypothesis has been directly verified in mice containing disrupted μ enhancer alleles (6, 7). Thus, the enhancer is activated at very early stages of B cell differentiation, prior to the onset of DμH to Jμ rearrangements. In addition, experiments in transgenic mice have shown that this enhancer is sufficient to direct expression of heterologous genes in B and T lymphoid cells (8–11).

The μ enhancer contains binding sites for multiple DNA binding proteins, and tissue-specific activity has been proposed to be the consequence of both positive and negative regulatory proteins. Positive regulation is effected by lymphoid-specific transcription activators, such as those that bind to the μA, μB (12–15), and octamer elements (16–18). Negative regulation is effected by proteins found in non-lymphoid cells that may suppress enhancer activity (19–22), such as those that bind to the μE5 site within the enhancer (23, 24). In addition, there are several sites within the enhancer referred to as E motifs. These elements, μE1–μE5, fit the consensus CANNTG and bind proteins belonging to the basic helix-loop-helix family of transcription factors (25) that are expressed in both lymphoid and non-lymphoid cells. We have previously shown that mutation of either μA or μB elements abrogates B cell-specific activity of a 170 nucleotide enhancer (μ170) that contains both these elements as well as μE1, μE3, and μE5. Mutation of individual E motifs in the enhancer reduces, but does not abolish, enhancer activity (2). For example, a mutation in μE3 results in a 2-fold reduction in the activity of μ170 (14). Such results indicate functional redundancy among the several E motifs present in the μ enhancer. To simplify the enhancer for more detailed characterization, we have previously defined a 70-nucleotide minimal μ enhancer (μ70). This fragment contains only the μA, μB, and μE3 elements and activates transcription from a heterologous promoter in S194 plasma cells, when assayed either as a monomer or a dimer. Mutation of the μE3 element in μ70 results in a more drastic reduction of activity compared to the corresponding mutation in μ170. Based on these results we have proposed that the μA and μB elements define a minimal enhancer whose activity is raised by the presence of proximal E motifs (14).

The μA and μB sites bind members of the ETS domain protein family (14, 26, 27). Binding of the B cell and macrophage-specific factor PU.1 to the wild type μB element and a panel of three nucleotide substitution mutants within the element correlates with the ability of the mutants to activate transcription. These results suggested that PU.1 was a likely candidate for a functionally relevant μB-binding protein. The μA site binds the lymphoid-restricted Ets-1 protein, and mutations in μA, but not μB, affect Ets-1 binding to the μ enhancer in vitro. Furthermore, the minimal enhancer can be transactivated by co-expressing PU.1 and Ets-1 in COS non-lymphoid cells, thereby strengthening the idea that these factors can complement the lack of lymphoid-specific factors required for μ enhancer activity (14).

In this study we report the detailed interactions of ETS domain proteins and bHLH proteins with the minimal μ enhancer in vitro. Methylation and ethylation interference assays show that the contacts made by PU.1 protein on the μB element are very similar to those made by Ets-1 protein bound to its cognate DNA site. These results indicate that these two most distantly related members of the ETS family recognize DNA similarly. Furthermore, electrophoretic mobility shift and DNase I footprint assays demonstrate that PU.1 protein binds coordinately to both μA and μB elements, although the μA site has a significantly lower affinity for PU.1 compared to μB.
contrast, full length Ets-1 protein binds only to the μA site, within the range of concentrations used in these experiments. When both proteins are present together, Ets-1 and PU.1 bind to the μA and μB sites respectively to generate a tri-molecular complex. The third cis component of the minimal μ enhancer is the μE3 element. Two μE3 binding proteins, TFE3 and USF, can bind to μ enhancer DNA in the presence of ETS domain proteins to generate a three protein-DNA complex in which all three sites, μA, μB, and μE3, are occupied. Furthermore, we provide evidence for protein-protein interaction between PU.1 and Ets-1, and Ets-1 and TFE3. We propose that the μ70 enhancer is activated by a nucleoprotein complex that contains two lymphoid-specific ETS-domain proteins that recognize DNA from one face of the helix and one ubiquitous bHLH-leucine zipper protein that binds between them, probably recognizing the DNA from a different side.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins

**GST-Pu**—The coding region of PU.1 was amplified using polymerase chain reaction and cloned into SmaI-digested pGEX-3X. 5′ primer, 5′-GGT AGG CTT ATG TTA CAG GGC TGG AAA ATG-3′; and 3′ primer, 5′-CTG CAG GCT CTT GGT G-3′. The 5′ primer introduced three additional amino acids between the coding regions for glutathione S-transferase and PU.1. GST fusion proteins were purified as described previously (28). An overnight culture of HB101 cells transformed with the appropriate plasmid was diluted 1:10 in LB/ampicillin medium and grown for an additional hour. Expression of the recombinant protein was induced by the addition of isopropyl-1-thio-

5-

9-

GGTAGGCCTATGTTACAGGCGTGCAAAATG-3

pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Extracts were prepared by sonication (three pulses for 10 s each) at 0 °C. The insoluble debris was removed by centrifugation, and the fusion protein was purified from the supernatant by adsorption to glutathione-agarose. Briefly, sonicated supernatant was mixed with the affinity matrix at a ratio of 1 ml of 50% slurry of beads for 1 liter of bacterial culture, with shaking for 1–2 h at 4 °C. The slurry was poured into a 10-ml column and washed with 50 column volumes of cold NETN three times. Ad- sorbed proteins were eluted by the addition of reduced glutathione at a concentration of 5 mM. One column volume eluates were collected and analyzed by SDS-PAGE, and the most concentrated fractions were pooled and dialyzed against buffer D. Aliquots were frozen in liquid nitrogen and stored at −70 °C.

**His.Pu**—A 1.2-kilobase pair EcoRI fragment containing murine PU.1 DNA was treated with the Klenow fragment of DNA polymerase plus dNTPs and cloned into XhoI-digested, Klenow-treated pET14b plasmid (Novagen). For protein expression, the plasmids were transformed into BL21 bacterial strain, and proteins were purified as described by the manufacturer (Novagen Inc.).

**His.Ets**—The coding region of murine Ets-1 was isoisoisoamerod as a BamHI fragment from the previously described expression vector pEVRFP0-Ets (14). After filling in the ends, this fragment was cloned into pET-14b cut with BamHI and treated with Klenow to create blunt ends. His.Ets was expressed in Escherichia coli strain BL21 and purified by affinity chromatography.

**Methylation and Ethylation Interference Assays**

DNA fragments (residues 359–433) (29) from the wild-type, μA, μB, and M103 mutant enhancers (14) were isolated as Sau3A1-BamHI fragments (note that the BamHI site was introduced by site-specific mutagenesis of the first core site, and the mutation has no effect in transient transfection assays) and cloned into pSP72 cut with BamHI. For labeling each strand, these plasmids were linearized either with EcoRI or XhoI, treated with calf intestinal phosphatase (Boehringer Mannheim), phosphorylated with polynucleotide kinase and [γ-32P]ATP, recut with XhoI or EcoRI, respectively, and a 102-base pair DNA probe purified by preparation of poly(dI-dC), 4% polyvinyl alcohol) containing 50 μg of bovine serum albumin in Buffer D. After incubation for 10 min on ice 50 μl of 10 mM MgCl₂ and 5 mM CaCl₂ were added, followed by DNase I for 1 min to a final concentration of 17 μg/ml, determined empirically. DNase I digestions were quenched by the addition of 90 μl of stop solution (20 mM EDTA, pH 8.0, 1% SDS, 0.2 mM NaCl, 250 μg/ml tRNA), and the DNA was purified after one extraction with phenol/chloroform (1:1) and precipitated with ethanol. Samples were analyzed by electrophoresis through denaturing 6% polyacrylamide/urea gels. Gels were dried onto Whatman 3MM paper and visualized by autoradiography.

**Partial Proteolysis Assays**

His.Ets-1 alone, or together with His.TFE3, His-p50, or bovine serum albumin, was incubated in a final volume of 12 μl (containing 20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 20% glycerol) for 10 min at 0°C. Proteolysis was initiated by the addition of 1 μl of 150 ng/μl trypsin and carried out for 15 min at room temperature. Reactions were quenched by the addition of 2 × SDS sample buffer and heating to 100 °C for 3 min, and proteins were separated by 12% SDS-PAGE. The proteins were transferred to nitrocellulose membranes and probed using an anti-Ets-1 antibody (Santa Cruz SC350, 1:1000). Chemiluminescence detection was carried out using the ECL immuno-detection protocol according to the manufacturer’s specifications (Amersham Corp.). His.Ets-1 and His.TFE3 were used at 400 ng/reaction, bovine serum albumin and p50 were used at 2 μg/reaction, and DNA was 20 ng of a 51-base pair double-stranded synthetic oligonucleotide containing the μA, μB, and μE3 motifs.

**Transient Transfection Assays**

CO5 and NIH3T3 cells were transfected with calcium phosphate using reporter plasmids containing wild-type, or mutated, μ70 dimers as described previously (14). Transfections contained 2 μg of reporter
RESULTS

**PU.1 and Ets-1 Make Similar DNA Contacts**—To identify nucleotides involved in PU.1 recognition of the μE element, we used methylation and ethylation interference assays. Preparative binding reactions were carried out with partially methylated μ enhancer DNA followed by separation of the protein bound DNA and free DNA by non-denaturing gel electrophoresis. Bound and free DNA were electroeluted, treated with piperidine to effect strand cleavage, and analyzed by electrophoresis through urea-containing sequencing gels. On the coding strand, methylation of four contiguous guanines affected PU.1 binding as shown by the significant depletion of the bands corresponding to these residues in the bound DNA (Fig. 1A, compare lanes 4–6). On the noncoding strand, bands corresponding to three contiguous adenines were diminished in the bound DNA (Fig. 1A, compare lanes 1–3) identifying these residues as being important for nucleoprotein complex formation.

Phosphate contacts were similarly assayed using ethylated probes in *in vitro* binding assays. On the noncoding strand, ethylation of four phosphates at the 3' end of the μE site affected protein binding as shown by the absence of these bands in the bound DNA lanes (Fig. 1B, lanes 1–3). Four additional phosphate contacts were identified on the coding strand at the 5' end of the μE element (Fig. 1B, lanes 4–6). These results are summarized in the helix diagram and sequence shown in Fig. 1C. The A and G residues identified by methylation interference studies are marked with asterisks and include the two guanines that are part of the core GGA recognition site of ETS domain proteins. Methylation at the N3 position of adenines lies in the minor groove of the DNA and can affect protein binding by direct interference or by altering DNA conformation. PU.1 binding was diminished by methylation of 3 As on the noncoding strand lying up to five nucleotides upstream of the core GGA. Phosphates identified by ethylation interference are indicated by vertical lines in the sequence and black circles in the helix diagram. Nye *et al.* (34) have previously mapped at high resolution the DNA contacts made by Ets-1 protein. Methylation and ethylation interference assays described here, as well as induction of a DNase I hypersensitive site on the noncoding strand (see Fig. 6), indicate that the contacts made by PU.1 are similar to those described for Ets-1. We conclude that these two most divergent ETS domains recognize DNA by similar mechanisms.

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**Fig. 1. Interference assays to identify contacts of PU.1 with the μ enhancer μE element.** A, methylation interference assay. A DNA fragment encompassing residues 359–433 of the murine μ heavy chain gene enhancer was labeled with γ32P-ATP and polynucleotide kinase on either the coding or the noncoding strand, as described under *Experimental Procedures.* Probes were partially methylated using dimethyl sulfate and used in *in vitro* binding assays with purified GST.PU.1 fusion protein. The protein-bound DNA and the unbound DNA were separated by electrophoresis through nondenaturing polyacrylamide gels and the DNA was isolated by electroelution as described. Bound and free DNAs were cleaved by piperidine treatment and analyzed by electrophoresis through 6% polyacrylamide gels containing 8 M urea. Free (lanes 1, 3, 4, and 6), represents unbound probe DNA isolated from a preparative mobility shift assay gel, and complex (lanes 2 and 5) represents probe DNA present in the nucleoprotein complex. Positions and sequences of the μB and μA sites are indicated. On the noncoding strand, the missing bands in the complex lane correspond to the three A residues and on the coding strand to the four G residues within the μE element. B, backbone phosphate contacts identified by ethylation interference assays. Radioactive probes were as described in A, except that chemical modification was induced by treatment with ethyl-nitrosourea as described. Free and Complex labels above the lanes are the same as described above, and the positions of the μB and μA sites are indicated. C, summary of interference assay results. Helical representation of 12 nucleotides containing the μB and 2 μg of each transactivator. In those cases where only one, or no, transactivator was used, total DNA was made up to 6 μg using an empty expression vector. 48 h after transfection whole cell extracts were prepared and CAT enzyme assayed using 75 μg of heat-treated extracts for 1 h. S194 cells were transfected by the DEAE-dextran procedure using 1 μg of reporter plasmids and 2 μg of TFE3 expression vector, or the empty expression vector. Extracts prepared 48 h later were assayed for CAT enzyme expression by ELISA (CAT ELISA, Boehringer Mannheim).
Analysis of Mutation M103—We have previously analyzed a panel of mutations within the μB sequence element by transfection into S194 plasma cells (12). Nucleotides modified by the three mutations M102–104 are indicated by horizontal lines within the sequence in Fig. 1C. In functional assays, M102 and M104 significantly decreased enhancer activity, whereas M103 had little or no effect. The effects of mutations M102 and M104 are readily explained by the strong adenine interferences that coincide with nucleotides changed in M102 and alteration of the GGAA core, including the fourth G identified by interference assays in M104. However, wild-type activity of M103 is harder to explain since this mutation changed three guanine residues that score strongly in the interference assay. In M103 the three guanines indicated were changed to three adenines, so one interpretation is that purine substitutions at these positions are tolerated by the PU.1 protein. Alternatively, it was possible that PU.1 bound to a different GGAA sequence located 3’ to the identified μB site in M103 resulting in the observed enhancer activity. To distinguish between these possibilities, we studied PU.1 binding to M103 in greater detail.

Methylation interference analysis showed that modification of the three G residues located 3’ of the M104 mutation did not interfere with PU.1 binding (Fig. 2A, lanes 6–8) indicating that the putative downstream GGAA was not an alternate PU.1 binding site. Methylation of the remaining guanine (Fig. 1C, position 10) in the μB element, that was unaltered by the M103 mutation, partially inhibited PU.1 binding. On the noncoding strand, methylation of adenines at positions 4–6 (numbering as in Fig. 1C) affected PU.1 binding as observed with the wild-type site, showing that the protein recognized the M103 mutant μB element. Interestingly, we detected strong interferences with G and A residues that make up the adjacent μA element which we have shown binds the Ets-1 protein (Fig. 2A, lanes 6–8 marked μA). No residues within the μA site on the noncoding strand were identified by this assay (Fig. 2A, lanes 2–4).

Involvment of the μA site in the binding of PU.1 to M103 was further confirmed by ethylation interference assays. Ethylation of coding strand phosphates within the mutated μB sequence resulted in partial interference with PU.1 protein binding (Fig. 2B, lanes 7–9), whereas phosphate residues within the μA sequence interfered more strongly. On the noncoding strand as well, ethylation within the μA motif interfered strongly and those within the μB motif interfered weakly with PU.1 binding (Fig. 2B, lanes 2–4). First, these results show that PU.1 does not bind an alternate flanking GGAA site, downstream of the μB element, in the M103 mutation. DNase I footprinting experiments described below further confirm this observation. Second, significant interference caused by nucleotide or phosphate modifications within the μA site indicates that optimal binding of PU.1 to the M103 mutant requires dual recognition of both μA site and the mutated μB site. A lesser role of the μA element in the wild-type context suggests that case most of the binding energy comes from PU.1/μB interaction.

DNase I Footprint Analysis of PU.1/μ Enhancer Interactions—Occupancy of both the μA and μB sites in the WT and mutant enhancers was directly visualized by DNase I footprinting assays. On the coding strand there was a conspicuous absence of bands corresponding to DNase I cleavage sites within the μB sequence with no added protein (Fig. 3). We
observed protection in two regions that correspond to the 5' end of the μB element and the 3' flank of the μB element. In addition, two weak DNase I hypersensitive sites that fell between the regions of protection described above, were also induced in response to PU.1 binding (Fig. 3). Similarly, protection in the region of the μA element was also detectable, though weak. Surprisingly, a strong DNase I footprint was observed in a region 3' of the μB element where several bands were protected and one prominent hypersensitive band induced (Fig. 3). The μ enhancer contains three elements homologous to the core element identified by mutational analysis of the SV40 enhancer, and this footprint maps to the region of the most downstream core site. Previous mutational analysis concluded that these core sites were not required for B cell-specific activity of the μ enhancer suggesting that PU.1 binding to this site may represent a nonfunctional interaction (35).

PU.1 binding to all three sites on the enhancer was more clearly discernible by analysis of the noncoding strand due to the induction of a strong DNase I hypersensitive site within each element (Fig. 4A, lanes 2–6). Addition of increasing amounts of GST-PU.1 fusion protein resulted in a dose-dependent increase of a band within the μB motif that was barely detectable in the absence of protein. A similar band was induced within the μA element (labeled μA) and the third core site (at the bottom of the gel). The location of the hypersensitive site within the μB sequence is shown in Fig. 1C and corresponds to the position where a similar band is induced in response to Ets-1 binding to its cognate sequence element. Using DNase I footprinting, Rivera et al. (26) have previously shown that PU.1 binds to both μA (π, in their nomenclature) and μB sequence elements.

Analysis of the μB− and μA− (inactive) enhancer mutations showed that in each case only one of these two sites bound PU.1. With the μB− DNA probe, no hypersensitive band was observed at the μB site in the range of protein concentrations used (Fig. 4B, lanes 2–6), whereas the hypersensitive site at μA was still induced although higher concentrations of PU.1 were required. This is consistent with competition analysis that showed that the μA site bound PU.1 approximately 8-fold more weakly (data not shown). Conversely, only the hypersensitive band corresponding to μB site occupancy was seen when the μA− enhancer was analyzed (Fig. 4C, lanes 2–6). Both mutated enhancers still showed PU.1 binding to the third core site as evidenced by the DNase I hypersensitive site in this region (Fig. 4A–C, lower part of the gel). In contrast, the functional M103 mutation bound PU.1 efficiently to both the mutated μB and the wild-type μA elements as shown by the generation of the characteristic DNase I hypersensitive bands at both positions with increasing amounts of PU.1 protein (Fig. 4D, lanes 2–6). These experiments directly demonstrate that mutations in μA or μB elements that inactivate the enhancer allow PU.1 binding to only one site, whereas both sites can be filled in the functional M103 mutation despite the lower affinity of the M103 μB site for PU.1. These observations provide an explanation for the observed activity of the M103 enhancer in transient transfection assays. We propose that (i) interaction with the proximal μA site and (ii) the pool of available PU.1 protein in S194 cells compensate for the decreased affinity of the M103 μB site.

A GST-PU.1 fusion protein was used in these studies of PU.1 binding to wild-type and mutated enhancers. To rule out a possible contribution of the GST portion of the fusion protein, we analyzed the binding of two other forms of PU.1 to the wild-type μ enhancer. First, the GST part of the GST-PU.1 fusion protein was proteolytically removed by digestion with Factor X. Alternatively, the PU.1 cDNA was cloned in the vector pET14b to generate a hexahistidine tagged fusion protein (His.PU) in bacteria. Both new PU.1 derivatives showed similar patterns of binding to the μA and μB sites of the μ enhancer (data not shown).

Binding of PU.1 and Ets-1 to Minimal μ Enhancer—Pu.1 binding to both μA and μB sites of the wild-type and M103 mutant μ enhancers, or to only one or the other site in the μA− and μB− mutated enhancers, correlates well with the functional characteristics of the mutations in S194 B cells. That is, those enhancers where both sites are bound by PU.1 are active in transient transfection assays, whereas those enhancers
where only one site is bound are inactive. However, in COS cell co-transfection assays, both PU.1 and Ets-1 are required for optimal enhancer activity, indicating that PU.1 binding to both sites is not sufficient for transcriptional activation (14). To analyze binding of both ETS-domain proteins, we expressed polyhistidine tagged full length Ets-1 (His-Ets-1) in bacteria and used the purified protein in DNase I footprint assays.

DNase I footprint analysis of His-Ets-1 resulted in a protection pattern over the μA site that was substantially different from that observed with PU.1. On the noncoding strand (Fig. 5A), in addition to the hypersensitive site within μA that was induced with either PU.1 or Ets-1 (marked with an asterisk), several bands were prominently protected against DNase I digestion only with Ets-1 (indicated by the open triangles), but not with PU.1. Second, in the range of protein concentrations used, Ets-1 did not bind over the μB site as shown by (i) the absence of the strong hypersensitive band within μB that is induced upon PU.1 binding and (ii) no protection of the weak bands indicated by the black triangles that are protected by PU.1 (Fig. 5A, lanes 10–13). Addition of Ets-1 protein, in the presence of PU.1, showed a pattern of protection and hypersensitive sites that indicated binding of Ets-1 and PU.1 to the μA and μB sites respectively (Fig. 5A, lanes 7–9). Specifically, Ets-1 binding to the μA site was shown by the protections over the μA site, whereas PU.1 binding to the μB site was shown by induction of the strong hypersensitive site within this sequence. Because the constant amount of PU.1 used in these experiments was sufficient to fill both sites (Fig. 5A, lane 6), these results indicate that Ets-1 bind preferentially to the μA site.

A similar pattern was observed on the coding strand (Fig. 5B). PU.1 alone protected two sets of bands within the μB element indicated by the black triangles and induced three DNase I hypersensitive sites indicated by asterisks. PU.1 protection within the μA element was quite weak. Ets-1 alone (Fig. 5B, lane 5) strongly protected several bands within the μA site, indicated by the open triangles, but did not protect any bands or induce DNase I hypersensitivity within the μB site. Thus, PU.1-dependent protection and hypersensitivity within the μB site and Ets-1-dependent protection of the μA sequence characterize binding of each protein to these sites. Incubation of both proteins together showed a composite protection pattern (Fig. 5B, lanes 6 and 7), indicating that both sites were simultaneously occupied by the ETS-domain proteins. We conclude that although PU.1 alone can bind both μA and μB sites, in the presence of Ets-1 these sites are occupied by the two different ETS proteins. Taken together with the COS cell transfection data, we propose that the trimolecular PU.1-Ets-1-μA enhancer complex is the functional ETS domain protein complex.

Further Functional Analysis of the μ70 Enhancer—The minimal μ enhancer contains three elements, μA, μB, and μE3. A monomer, or a dimer, of this enhancer activates a heterologous promoter in S194 plasma cells, and we have previously shown that all three sites are necessary for enhancer activity in these cells. The μA and μB sites bind tissue-restricted ETS domain proteins, whereas the μE3 site binds several factors that contain a basic helix-loop-helix plus a leucine zipper domain (bHLH-zip), such as TFE3 and USF. Unlike the ETS domain proteins, μE3 binding proteins are ubiquitously expressed in a variety of cell types. However, in the earliest in vivo footprinting experiments, Ephrussi et al. (29) had shown that the μE3 site was bound by a factor in B cells only. To resolve the apparent inconsistency between the observed expression and in vivo DNA binding by μE3 proteins we proposed that the μ enhancer was inaccessible to μE3 binding proteins in non-B cells (36). Alternatively, it is possible that there exist B cell-specific forms of μE3 binding proteins which are necessary for μ enhancer function in B cells. Because the μ enhancer activates transcription in B cells only, it has been difficult to ascertain whether E binding proteins expressed in non-B cells can activate this enhancer or not.

Co-expression of PU.1 and Ets-1 transactivates the minimal μ enhancer in COS non-lymphoid cells (14). To determine whether the μE3 sequence contributed to enhancer activity in COS cells, we used a reporter plasmid containing a μE3 mutated enhancer fragment. This mutation decreases activity of the minimal enhancer by approximately 80% in S194 cells (14). In COS cell co-transfections, the combination of PU.1 and Ets-1 transactivated the minimal enhancer strongly, and mutation of either the μA, μB, or μE3 elements reduced activity approximately 10-fold (Fig. 6A). Therefore, the site usage in COS cells in the presence of transfected PU.1 and Ets-1 closely paralleled that seen in S194 cells in the absence of additional transfected trans-activators. Although we cannot rule out the possibility that the transfected PU.1 and (or) Ets-1 induced the synthesis of a B cell-specific form of a μE3 binding protein, we favor the interpretation that COS cell μE3 binding proteins can activate
the \( \mu \) enhancer in the presence of a co-transfected PU.1 and Ets-1.

To extend these results we assayed \( \mu \) enhancer activity in a second non-lymphoid cell line. In NIH3T3 cells as well, co-expression of PU.1 and Ets-1 transactivated a minimal \( \mu \) enhancer containing reporter plasmid (Fig. 6B), and mutation of either \( \mu A \), \( \mu B \), or \( \mu E3 \) sites significantly reduced enhancer function. We conclude that \( \mu E3 \) binding proteins present in these non-lymphoid cells can activate the \( \mu \) enhancer only in the presence of transfected ETS domain genes. Furthermore, the combined transactivation and mutational analysis suggests that a two-protein DNA complex is not sufficient to reconstitute a functional enhancer. For example, since PU.1, or Ets-1, alone do not activate the enhancer despite the presence of endogenous \( \mu E3 \)-binding proteins, it suggests that any one ETS protein plus a \( \mu E3 \)-binding protein is not sufficient for enhancer function. Conversely, lack of activity of the \( \mu E3 \) mutation suggests that binding of both ETS proteins is also insufficient for activity. We therefore propose that the three-protein-DNA complex consisting of two ETS domain proteins and a \( \mu E3 \)-binding protein is required for \( \mu 70 \) enhancer activity.

In the studies described above, we observed a requirement for endogenous \( \mu E3 \)-binding proteins present in non-lymphoid cells to activate the minimal \( \mu \) enhancer in the presence of transfected ETS proteins. We further examined the effects of expressing exogenous \( \mu E3 \)-binding proteins in the presence of endogenous ETS proteins that activate the enhancer in B cells. As shown earlier, in S194 cells the \( \mu 70 \) dimer is a functional enhancer whose activity requires both the \( \mu E3 \) site and the \( \mu B \) site (Fig. 7, bars marked with a minus sign, indicating the absence of co-transfected TFE3 protein). Co-transfection of a murine TFE3 expression vector increased the activity of the \( \mu 70 \) enhancer approximately 2-fold. Increased activity was dependent on all three sites being intact as shown by the significantly reduced activity of the \( \mu E3^- \) or \( \mu B^- \) reporter plasmids (Fig. 7, bars marked with a plus sign). These results further strengthen the proposal that occupancy of all three sites in the
ETS and bHLH Proteins form a Multiprotein Complex on the RU Enhancer—Because transfection analyses suggested that μE3-binding proteins present in non-lymphoid cells can activate the μ enhancer, we used cloned, ubiquitously expressed bHLH zip proteins to determine whether a three-protein-DNA complex could form on the μ enhancer. Full-length TFE3 (37, 38), a truncated TFE3 containing the DNA binding domain (39) and full-length USF (40) were expressed in bacteria as GST fusion proteins. DNase I footprint analysis showed that each protein bound to the μE3 element generating a protected region that extended toward the μA site (data not shown).

Co-incubation of μE3 binding proteins and PU.1, followed by DNase I footprinting resulted in a pattern consistent with the simultaneous occupancy of all three sites, μA, μB, and μE3. As described in the preceding sections, addition of increasing amounts of PU.1 alone generates two strong DNase I hypersensitive sites indicating occupancy of both μA and μB sites (Fig. 8A, lanes 2–6). In addition to these, several weaker bands in both sites were protected against DNase I digestion. No protection was observed in the core of the μE3 element, although three bands in the 3’ end of the μA bracket were protected by either μE3-binding proteins alone (data not shown) or by PU.1 binding to the μA site (Fig. 8A, lanes 2–6) indicating a region of partial overlap between the binding proteins. For the co-incubation experiments we used a fixed amount of μE3 binding proteins that results in complete protection over the μE3 element and added increasing amounts of GST.PU corresponding to lanes 2–6 of Fig. 8A. Under these conditions, we observed protection of the μE3 sequences indicating occupancy of this site, as well as the strong induction of DNase I hypersensitive sites within the μA and μB motifs, indicating that both of these sites were filled as well (Fig. 8A, lanes 7–21). We conclude that all three elements of the minimal μ enhancer can be simultaneously occupied by factors: ETS domain proteins, such as PU.1, binding to the μA and μB elements, and bHLH proteins, such as TFE3 and USF, binding to the μE3 element. Analysis of the coding DNA strand was consistent with this conclusion (data not shown).

Because enhancer activity requires Ets-1 in addition to PU.1, we also analyzed the binding of PU.1, Ets-1 and USF to the μ enhancer (Fig. 8B). On the coding strand, the footprints generated by Ets-1 and USF overlap considerably. Inclusion of all three proteins together resulted in a composite pattern (Fig. 8B, lane 6), indicating that all three proteins bound simultaneously to the enhancer.

Protein-DNA and Protein-Protein Interactions on the μ Enhancer—Visualization of the minimal enhancer sequence in double-helical form shows that the GGAA cores of the μA and μB sites lie on the same side of the double helix. Thus, ETS domain proteins bound to these sites are well positioned to interact with each other. We further investigated whether μA, μB, and μE3 binding proteins interacted directly with each other. Full-length PU.1, or an N-terminal deletion mutant, were cloned in frame 3’ of a LexA DNA binding domain in the expression vector pEG202 (Fig. 9A) (32). PU.1 and Ets-1 sequences were also cloned into the pJG vector containing the strong B42 transcription activation domain (Fig. 9A, lines 4–6), and as negative controls we used two other pJG derivatives shown in Fig. 9A (lines 7 and 8). Yeast strains containing different combinations of the Lex and pJG derivatives and a lac Z reporter plasmid were selected and assayed for lac Z expression. Co-expression of PU.1 or Ets-1, containing pJG derivatives together with PU.1 containing pEG derivatives consistently resulted in lac Z expression as assayed by development of blue colonies in the presence of X-gal. In contrast, pJG-Crk or pJG-Per co-expression with pEG-Pu(1–272) yielded only white colored colonies. To quantify the results, three colonies were randomly selected from yeast strains summarized in Fig. 9B, and β-galactosidase
activity was quantified in extracts prepared from cultures. The average β-galactosidase activity obtained is shown in Fig. 9B. We conclude that PU.1 can interact with itself or with Ets-1. Furthermore, this interaction requires only the C-terminal 171 amino acids of PU.1 that contain the ETS domain.

We assayed possible interactions between Ets-1 and TFE3 by yeast two-hybrid analysis. ETS-domain-protein interactions on m Enhancer. We have previously shown that the m70 domain of the Ig m enhancer, that encompasses μA, μB, and μE3 elements, is a B cell-specific transcription activator. Transcription activity can be reconstituted in non-lymphoid cells by co-expressing the ETS domain proteins PU.1 and Ets-1 that bind to the μB and μA sites, respectively. Here we show that m70 activity in non-lymphoid cells requires an intact μE3 element, suggesting that an endogenous μE3-binding protein is functionally recruited.

**DISCUSSION**

Fig. 10. Assay for Ets-1/TFE3 interactions. A, Ets-1 alone, or in the presence of other proteins and m enhancer DNA as described above the figure, was incubated for 15 min at room temperature followed by treatment with 150 ng of trypsin for 10 min. The proteolysis was quenched by the addition of 2 × SDS-PAGE sample buffer followed by electrophoresis of the reaction mixture through 8% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes and probed with an anti-Ets-1 antiserum directed against a peptide epitope at the C terminus of Ets-1. Detection was carried out using enhanced chemiluminescence procedure (Amersham). Full-length Ets-1 is shown in lane 1. The faster migrating band in lanes 2–9 represents a tryptic fragment of Ets-1. B, schematic of the Ets-1 protein showing the approximate location of the trypsin sensitive site that is masked by TFE3. The dark shaded portion represents the DNA binding ETS domain, the hatched portion at the N terminus represents a previously identified transcriptional activation domain, and the stippled region on either side of the ETS domain represent inhibitory domains that reduce DNA binding by the full-length protein. The C-terminal peptide epitope, which is recognized by the antiserum used in these studies, is shown as the bold line below the Ets-1 protein and the vertical arrow marks the location of the trypsin-sensitive site as approximated from the mobility of the shorter protein fragment in SDS-PAGE.
during the transactivation assay. Because transfection of PU.1 or Ets-1, individually, did not activate the \( \mu \)70 enhancer in non-lymphoid cells, these observations suggest that neither ETS protein alone works efficiently together with the endogenous \( \mu \)E3 binding protein. Further evidence for the importance of all three sites was obtained by assessing the effects of TFE3 expression on \( \mu \)70 activity in B cells that contain endogenous \( \mu \)A and \( \mu \)B binding proteins. Optimal transactivation of the \( \mu \)70 enhancer by TFE3 also required intact \( \mu \)A and \( \mu \)B sites. Taken together, our analyses in lymphoid and non-lymphoid cell lines indicate that a three-protein-DNA complex, consisting of factors bound to the \( \mu \)A, \( \mu \)B, and \( \mu \)E3 sites, is required to activate the \( \mu \)70 domain of the Ig enhancer.

We next examined the \textit{in vitro} interactions of proteins that reconstituted minimal enhancer activity. We found that, despite a lower affinity for the \( \mu \)A site compared to the \( \mu \)B site, PU.1 bound both sites \textit{in vitro}. These results provide a partial explanation for our previous observation that the M103 \( \mu \)B mutation that changes the core GGA in the PU.1 recognition site retains wild-type enhancer activity in B cells. Although the M103 mutation resulted in an approximately 4-fold weaker PU.1 site, the mutated enhancer bound PU.1 efficiently at both \( \mu \)A and \( \mu \)B sites. Furthermore, the contacts identified by interference assays for PU.1 recognition of the \( \mu \)B site were very similar to those described previously for Ets-1, indicating that these two most divergent ETS domain proteins recognize DNA similarly.

While this work was in progress, structural studies of the PU.1 and Ets-1 ETS domains were reported (41–43). Our biochemical analysis of PU.1/DNA interactions correlate closely with the x-ray crystal structure of the PU.1 ETS domain in several aspects, but also point to other interesting properties that are not easily evident from the crystal structure. The most obvious similarity between the interference and high resolution structures are the close correspondence of the phosphodiester backbone contacts on both coding and noncoding strands, and direct contact of PU.1 with nucleotides forming the core GGA recognition site. Although no direct protein/DNA contacts were noted 5' of the GGAA sequence in the crystal structure, we found that three adenosine residues on the noncoding strand located several nucleotides upstream of the core scored strongly in methylation interference assays (see Fig. 1C), suggesting possible involvement of these residues in PU.1/\( \mu \)B interactions. Furthermore, the M102 mutation that changes these nucleotides also reduced PU.1 binding and \( \mu \) enhancer activity significantly, suggesting that these residues contribute to PU.1/DNA interactions. These observations indicate that the PU.1 recognition site extends at least five nucleotides upstream of the GGAA sequence. Recognition of the upstream sequences may be mediated by direct base-specific contacts by the protein, which were not detected in the crystal structure because of the particular sequence used in that study. Alternatively, it is possible that sequence specificity at these positions is determined by a combination of stereochemical and electronic properties of the DNA helix (dependent on the sequence) without direct base recognition by the polypeptide. Because the methyl group of methyl adenosines lies in the minor groove, these results also indicate that the protein is located in the minor groove at this position.

Our results differ most significantly from those of the Kondandapani et al. (41) by the identification of a strong DNase I hypersensitive site on the noncoding strand between the A and C residues (Fig. 1C). The crystal structure of the PU.1 ETS domain did not reveal any structural distortions in the DNA helix that could easily account for the generation of this hypersensitive site. We suggest that this site is caused by a PU.1-induced distortion of the helix. Furthermore, this distortion is probably not binding site-specific because a similar hypersensitive site was also induced within the \( \mu \)A element, when PU.1 was bound at that site.

In mobility shift assays full-length Ets-1 bound poorly to the \( \mu \) enhancer. This was most likely due to the previously characterized inhibitory domain in Ets-1 (44–46) that lies just before the DNA binding ETS domain. Using DNase I footprint assays, we observed Ets-1 binding to the \( \mu \)A, but not the \( \mu \)B, site of the enhancer. When both ETS proteins were present together, Ets-1 bound to the \( \mu \)A site and PU.1 bound to the \( \mu \)B site to generate a ternary complex. Our observation that PU.1 and Ets-1 associate in the yeast two-hybrid assay supports the idea that the two proteins may interact when bound to the \( \mu \) enhancer. We have recently shown that the ETS domain of PU.1 is sufficient to transactivate the enhancer together with Ets-1 (47), and it is interesting to note that the same domain of PU.1 is sufficient for interaction with Ets-1 in the two-hybrid assay. Our working model is that PU.1/Ets-1 interactions mediated by the ETS domain of PU.1 may provide a combined transactivation domain, either by jointly “touching” the basal transcription machinery directly or by jointly recruiting additional co-activators. Despite the correlation between the domains of PU.1 required for transcriptional activity and interaction with Ets-1, we note that the yeast two-hybrid assay does not closely resemble the B cell nucleus, and additional studies are required to prove the physiological relevance of this interaction.

As noted earlier, the two-protein-DNA complex discussed above is necessary, but not sufficient, for transcriptional activity of the \( \mu \)70 enhancer. The third factor required to activate this enhancer is the \( \mu \)E3 binding protein(s). We show here that \( \mu \)E3 binding proteins can bind to the \( \mu \) enhancer in the presence of ETS proteins to generate a three-protein-DNA complex, which we propose to be a functional unit of this B cell-specific enhancer. Crystal structures of bHLH zipper family of transcription factors, of which TFE3 is one, have recently been reported (48, 49). These structures reveal that the basic residues take on an induced helical conformation and make contacts in the major groove of DNA. Contacts in the \( \mu \)A, \( \mu \)B, and \( \mu \)E3 regions suggest that the ETS-domain proteins recognize the \( \mu \)A and \( \mu \)B sites from the same side of the DNA helix, leaving the opposite side accessible to \( \mu \)E3 binding bHLH-zip proteins. The proposed structure has the interesting feature that the enhancer binding proteins appear to “coat” or “surround” the DNA. Perhaps this results in a weakening of the interaction of DNA with nucleosomal proteins that is manifested as altered chromatin structures in regions of the genome that contain active enhancers.

In addition we provide evidence that Ets-1 and TFE3 may interact directly. The trypsin sensitive site in Ets-1 that is masked in the presence of TFE3 is located in the N-terminal domain of Ets-1 that has no similarity to PU.1. The simplest interpretation of this observation is that the N-terminal domain of Ets-1 directly contacts TFE3. This is particularly interesting because we have shown that an N-terminal domain in Ets-1 is necessary for activation of the \( \mu \)70 enhancer together with PU.1 (47). It is possible that this domain functions by recruiting TFE3 to the PU.1/Ets-1 bound \( \mu \) enhancer, thus completing the functional quaternary nucleoprotein complex. Our studies suggest the following model of the \( \mu \)70 enhancer. PU.1 and Ets-1 bind to the \( \mu \)B and \( \mu \)A sites of the enhancer, and interact via the PU.1 ETS domain. This interaction may be facilitated by DNA bending induced by PU.1 (50). TFE3 interacts with an N-terminal domain of Ets-1, while binding between the two ETS proteins on the other side of the DNA helix.
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