An IgH Enhancer That Drives Transcription through Basic Helix-Loop-Helix and Oct Transcription Factor Binding Motifs

FUNCTIONAL ANALYSIS OF THE Eμ3' ENHANCER OF THE CATFISH* 

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The transcriptional enhancer (Eμ3') of the IgH locus of the channel catfish, Ictalurus punctatus, shows strong B cell-specific activity and differs from the mammalian Eμ enhancer in both location and structure. It occurs between the μ and δ genes and contains numerous transcription factor binding sites, predominantly octamer and μE5 motifs of consensus and variant sequences. It lacks the classical μA-μE3(CBF)μB core array of binding motifs seen within mammalian IgH Eμ enhancers. To determine the functionally important motifs, a series of mutant enhancers was created using sequence-targeted polymerase chain reaction. Whereas the mutation of consensus and variant octamer motifs (individually or in multiples) decreased enhancer function, mutation of a single consensus μE5 motif destroyed the function of this enhancer in mammalian plasmacytomas. Mutation of this consensus μE5 site, combined with mutations of certain octamer sites, destroyed function in catfish B cells. Experiments using artificial enhancers containing multimers of motifs or short regions of the native enhancer suggested that the minimal Eμ3' enhancer (α) contains a consensus μE5 site and two octamer sites, (b) is B cell-specific, and (c) is active across species. The dependence of an Ig enhancer on sites that bind basic helix-loop-helix and Oct transcription factors has not previously been observed and confirms large differences in structure and function between fish and mammalian IgH enhancers.

Teleost (bony) fishes represent the evolutionary lineage that is most divergent from mammals yet still shares with them the organization of the IgH1 locus. In both mammals and bony fish the Vμ⁴, D, Jμ, and C gene segments are in tandem arrays, also known as the translocon arrangement (1). Within the IgH locus of mammals, there are six described enhancers, required for the processes of V-D-J recombination, class switching, somatic hypermutation, and expression of the functionally rearranged locus (2–4). These enhancers are both internal to and 3′ of the locus and are tightly regulated in function at different stages of B cell development (5, 6). The Eμ enhancer, located in the JH–Cμ intron, is arguably the most important; not only is it required for expression of the rearranged IgH locus, but it also plays an important role in the processes of VH–D–JH recombination and somatic hypermutation and is not deleted by any of the recombination processes that occur during B cell development. The IgH locus of the channel catfish, the best-established model for the teleost immune system, is less complex than that of mammals; only two classes of immunoglobulin (IgM and IgD) are produced through alternative processing of the primary transcript from the IgH locus (7, 8). Class switching by chromosomal recombination does not occur in teleost fish. The single identified enhancer (Eμ3') driving IgH transcription is located in the μ–δ intergenic region (9). The structure and organization of Eμ and Eμ3' differ greatly. Mammalian Eμ is ~500 bp in length and contains several DNA binding motifs (μE1–5, E-box, μA, μB, CBF, Oct) each present only once (10–12). The Eμ3' enhancer region of the channel catfish is considerably larger, 1.5 kilobases in length, and it contains many repeats of octamer and μE motifs of both consensus and variant sequences (9). Despite these differences Eμ and Eμ3' are both strong B cell-specific transcriptional activators that function in both mouse and catfish B cells (9, 13–15). Here we report the results of an analysis of Eμ3', defining the functional motifs essential for its activity and identifying a minimal enhancer.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids for Mutagenesis and Transfection—CAT reporter constructs to test enhancer function at a distal site were based upon those previously described (9, 13). Briefly, mutated enhancers were inserted at the KpnI site of the pFVH/CAT/modified vector that contains the full promoter region of a goldfish Vμ⁴ gene (9). To test DNA binding motifs and enhancer fragments at a proximal (promoter) position, the pUC56/CAT construct was used (16). This vector contains the minimal c-fos promoter, from −56 to +109 bp relative to the transcription start site, and includes a minimal TATA box (16). All PCR-generated enhancer or promoter fragments were made using Taq DNA polymerase (Life Technologies, Inc.), with restriction sites incorporated in the primer sequences to facilitate cloning. Mutagenesis of the full enhancer was performed in a modified pUC19 vector. The pUC19ΔHinDIII site of pUC19 was destroyed by digestion with HinDIII and Klenow fill-in to permit the screening of clones for mutations by the introduction of a HinDIII site. The 1782-bp enhancer fragment that contains full activity (ELF11), hereafter referred to as the full enhancer, was ligated into pUC19ΔHinDIII via the HindIII site. 

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§ The abbreviations used are: Vμ⁴ variable region of immunoglobulin heavy chain genes; D, diversity region of immunoglobulin heavy chain genes; Jμ, joining region of immunoglobulin heavy chain genes; C, constant region of immunoglobulin heavy chain genes; bp, base pair(s); CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction.

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KpnI site of the polylinker and subjected to mutagenesis. After successful mutagenesis was confirmed by sequencing, the mutant enhancer was recloned into the KpnI site of pEVH/CAT (modified). Mutagenic primers were designed to replace the DNA binding motif of interest with a sequence of identical length containing a HinDIII site. The only exception was the mutated motif of the pA56 c-fos CAT construct. Clones were sequenced to confirm the presence or absence of HinDIII sites in the 230 bp of the full enhancer. Primers designed to amplify the full enhancer (Eμ3’) with the addition of HinDIII sites were as follows: 5’-TTTAAGCTTATCACATGCAATGCGTACTTATACATCAGGTA-3’. Primers designed to amplify region 1 (see Fig. 1) of the full enhancer, which contains the mammalian consensus octamer 5, variant μE5-1 motif, and variant octamer 6, with the addition of HinDIII ends were as follows: 5’-TTTAAGCTTTGATTTACAATCTCATGCTCTATGGAATACATCAGGTA-3’. After annealing, the double-stranded DNA fragment was purified by electrophoresis on a 12% non-denaturing polyacrylamide gel followed by electroelution. Purified fragments were cloned into the HinDIII site in the same orientation as they appear in the full enhancer. Primers designed to amplify the Eμ3’ with the addition of HinDIII ends were as follows: 5’-TTTAAGCTTATCACATGCAATGCGTACTTATACATCAGGTA-3’. Primers designed to amplify region 3 (see Fig. 1) of the full enhancer, which contains the mammalian consensus octamer 5, variant μE5-1 motif, and variant octamer 10, variant octamer 11, and the mammalian consensus μE5-2 motif, with the addition of HinDIII ends were as follows: 5’-TTTAAGCTTTGATTTACAATCTCATGCTCTATGGAATACATCAGGTA-3’ and 5’-TTTAAGCTGTATGCAATGCGTACTTATACATCAGGTA-3’. Primers designed to amplify region 3 (see Fig. 1) of the full enhancer, which contains the mammalian consensus octamer 5, variant μE5-1 motif, and variant octamer 14, with the addition of HinDIII ends were as follows: 5’-TTTAAGCTTTGATTTACAATCTCATGCTCTATGGAATACATCAGGTA-3’ and 5’-TTTAAGCTGTATGCAATGCGTACTTATACATCAGGTA-3’.

PCR-amplified regions from the full enhancer were purified by electrophoresis on a 0.8% agarose gel and extracted using the NucleoTrap gel extraction kit (Clontech). Clones were sequenced to confirm enhancer fragment presence, sequence, and copy number. Plasmids for mutagenesis and transformation were prepared by transfection of chemically competent XL1 Blue MRF’ cells (Stratagene), grown under ampicillin selection. Subsequent purification of DNA was accomplished using QiaFilter Plasmid maxi and mega kits (Qiagen).

**Transfection of Cell Lines Using Reporter Constructs—**

The catfish B lymphoblastoid cell line 1B10 and the catfish T cell line G14D, derived from the peripheral blood lymphocytes, have been useful for further transfection analysis (13). These cell lines were maintained in AL-5 medium (50% A5M V (Life Technologies, Inc.) and 50% Leibovitz-L15 medium (Life Technologies, Inc.) adjusted to adjust cell isotypic by culturing 91 with water). AL-5 was supplemented with heat-inactivated catfish serum to a final concentration of 5% (17), 100 μg/ml streptomycin (ICN), 100 units/ml penicillin (ICN), 50 μg/ml dexamethasone (Sigma), 10 μm sodium bicarbonate (Sigma), and 0.1% sodium bicarbonate (Sigma). 1B10 and G14D cells were cultured at 27 °C in a 5% CO2 atmosphere. The murine plasmacytoma cell lines J558L and S194 were maintained in RPMI 1640 medium (Life Technologies, Inc.) with a final concentration of 5% fetal calf serum (Life Technologies, Inc.). Mouse cell lines were grown at 37 °C in a 5% CO2 atmosphere. All transfections were done using a BTX600 electrotransporator and 2-mm gap cuvettes (Biotechnologies & Experimental Research, Inc.). Cells were harvested during logarithmic growth, washed in serum-free RPMI 1640 (at the appropriate titer), and resuspended in serum-free RPMI 1640 for storage. Directly prior to transfection, 180 μl of cells (8 × 10^6) cells of B10, 5 × 10^6 cells of G14D, or 4 × 10^6 cells of J558L and S194) were mixed with 20 μl of a DNA mixture. DNA solutions contained 2.5–8.0 ng/ml (2–5 μg/ml) of DNA depending on the cell line transfected. Three to nine μg of a second DNA construct, pRSVLuc (18), expressing firefly luciferase driven by the Rosa sarcoma virus promoter, were cotransfected to permit assessment of transfection efficiencies. Optimal electroporation values were determined as previously described (9). Briefly, 1B10 cells harvested at a density of 3.5–4.0 × 10^8 cells/ml were electroporated at 210 V, 1100 microfarads, and 48 ohms. G14D cells were harvested at a density of 2.5–3.0 × 10^8 cells/ml were electroporated at 190 V, 1100 microfarads, and 48 ohms. J558L and S194 cells, harvested at a density of 8.0 × 10^5 cells/ml, were electroporated at 130 or 168 V, respectively, 1100 microfarads, and 48 ohms.

**Reporter Assays—**

Expression of reporter constructs was measured 30–48 h (depending upon the cell line) after electroporation. CAT and luciferase activities were measured using cell extracts prepared with

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*Note: The document is a scientific research paper that describes the analysis of a specific DNA binding motif in the context of catfish genomic sequences.*

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*To further explore the context and implications of this research, consider the following points:*

1. **DNA Binding Motif Analysis:** The paper focuses on the analysis of a specific DNA binding motif (Eμ3’ Igh Enhancer) in the context of catfish genomic sequences. The authors use PCR amplification, mutagenesis, and reporter assays to study the motif’s functional properties.

2. **Transfection Studies:** The research involves transfecting various cell lines to evaluate the expression of the DNA binding motif. This includes the use of BTX600 electrotransporators and BTX842 electrotransporators for transfection.

3. **Functional Analysis:** The study analyzes the motif’s functional properties, including expression levels and reporter assays, under different conditions.

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*By detailing these aspects, one gains a deeper understanding of the experimental design and outcomes presented in the paper.*
of the catfish IgH locus is driven by the E

Though it has previously been demonstrated that transcription of the E

(top) and the location of the Eμ3‘ enhancer (bottom). The catfish IgH enhancer overlaps the second transmembrane exon (ExM 2) of IgM. O, octamer; B, μ; A, α; 5, μE5; and 3, μE3. A repeating substructure within the Eμ3‘ enhancer is shown as three regions, each containing a single μE5 and two octamer motifs (regions 1–3). DNA binding motifs are numbered based upon their order of occurrence in the recombinant genomic λ-phage clone 12C, EMBL accession number X79482 (9).

Promega reporter lysis buffer (Promega). Cells were harvested by centrifugation, washed in phosphate-buffered saline, and resuspended in 100 μl of 1× reporter lysis buffer (Promega). After a 15-min incubation at room temperature, cell lysis was cleared by centrifugation and aliquoted for immediate use. Luciferase was measured using the Promega luciferase assay system (Promega) and a TD-20/20 luminometer (Turner Instruments) set to standard sensitivity. Values were corrected to exclude background activity. CAT activity was measured using CAT assay grade L-threo-(dichloroacetyl-1,2,4,5-tetrachlorophenyl) and n-butyryl coenzyme A (Sigma) (9). CAT values were corrected for background activity and for transfection efficiency by normalizing with luciferase values.

Sequence and Analysis—Sequencing of PCR-mutated enhancers, Eμ3‘ fragment multimers, and multimers of DNA binding was accomplished using the Circumvent DNA sequencing kit (New England Biolabs) or the sequencing facility of the Medical University of South Carolina.

RESULTS

Functional Significance of a Consensus μE5 Motif—Although it has previously been demonstrated that transcription of the catfish IgH locus is driven by the Eμ3‘ enhancer (9), the motifs and transcription factors through which it functions are unknown. Indeed, the Eμ3‘ enhancer contains significant numbers of motifs that have the potential to contribute to function. As illustrated in Fig. 1, these include 10 consensus and variant octamer motifs, of which eight have been shown to be transcriptionally active (9), a single variant μE3 site, and four μE5 sites of consensus or variant sequence. To determine the degree to which each of these sites might contribute to enhancer function, targeted mutations were made in the enhancer. In an initial experiment each μE5 site and the μE3 site were mutated individually, whereas the consensus octamer 5 (ATGC/3AAAT) was mutated in conjunction with the μE5-1 site that it overlaps, and the closely spaced dimer of octamers (octamers 10 and 11) of identical sequence (ATGtAAAT) were mutated to-gether. The function of the mutated enhancers was tested in catfish B cells (Fig. 2). From these results (Figs. 2 and 3) it appears that factors bound to the μE5-2 motif and as many as five octamer sites act synergistically in the Eμ3‘ enhancer in catfish B cells.

Effect of Mutating Multiple Octamer Motifs—With the consensus μE5-2 motif left intact, mutation of three octamer sites (the variant octamer dimer composed of octamer motifs 10 and 11, along with the consensus octamer 5, which also overlaps the μE5-1 site) reduced function in catfish B cells by only around 35% (Fig. 3A), a similar effect to that seen with mutation of the octamer pair (octamers 10 and 11) alone. However, mutation of this particular combination of three octamer motifs reduced enhancer function by >70% in murine J558L plasmacytoma cells (Fig. 3C), indicating further differences in the function of this enhancer in fish and mouse B cells.

Function of Arrays of Individual Motifs—Because mutations of several octamer motifs and the consensus μE5-2 motif strongly affect native enhancer function, the ability of these individual motifs to drive transcription of a reporter construct was tested. Trimers of μE5 and μE3 failed to function as promoters in both catfish and murine cell lines (Fig. 4, A and B). In contrast, trimers of octamer sequences, either consensus 5 or variant 10 and 11, increased the rate of transcription in both catfish and mouse B cells 14–17-fold. These results indicate that in catfish B cells, the function of μE motif is dependent upon the presence of additional transcription factors bound to other sites, whereas octamer motifs are functional by themselves when associated with a minimal promoter.

A Minimal Enhancer within Eμ3‘—Inspection of the organization of the Eμ3‘ enhancer (Fig. 1) reveals that it contains a
repeating substructure: a μE5 motif associated with two octamer motifs. This occurs three times (regions 1–3; Fig. 1). The results of the motif mutation experiments suggest that the second of these regions (containing the μE5-2 consensus and octamers 10 and 11) may be a minimal functional unit within the enhancer, and experiments were undertaken to test this and to determine whether the other two regions (1 and 3; Fig. 1) were transcriptionally active. Accordingly, fragments; 300 bp in length were PCR-amplified from the three regions of the native enhancer identified in Fig. 1 and ligated into reporter constructs. In catfish B cells the full enhancer increased reporter activity 6-fold, whereas enhancer region 2 (containing the μE5-2 and octamer 10 and 11 motifs) increased reporter gene expression almost 5-fold. Other regions failed to activate transcription above control levels (Fig. 5A). To test the contribution of the consensus μE5-2 site to the function of this fragment, this motif was mutated. This mutation reduced the activity of the enhancer fragment, as was also observed upon mutation of this motif within the full enhancer (Fig. 2C). When tested in murine J558L cells (Fig. 5B), the full enhancer increased reporter activity 6-fold, whereas enhancer region 2 (containing the μE5-2 and octamer 10 and 11 motifs) increased reporter gene expression almost 5-fold. Other regions failed to activate transcription above control levels (Fig. 5A). To test the contribution of the consensus μE5-2 site to the function of this fragment, this motif was mutated. This mutation reduced the activity of the enhancer fragment, as was also observed upon mutation of this motif within the full enhancer (Fig. 2C). When tested in murine J558L cells (Fig. 5B), the full enhancer increased reporter activity almost 15-fold, whereas region 2 alone increased reporter gene expression almost 10-fold. Other regions failed to activate transcription above control levels. Mutation of the mammalian consensus μE5-2 site from region 2 reduced activity by more than 60% in J558L cells (Fig. 5B). To determine whether the activity of region 2 was B cell-specific, its ability to activate transcription in catfish T cells was evaluated (Fig. 5C). Both the full enhancer and all three regions of the enhancer failed to drive transcription of the reporter gene in these T cells. Thus, region 2 of the enhancer shows the same B cell specificity as the full enhancer (9, 13–15).
The vector alone or vector containing three fos transcription factor binding motifs were cloned into the HinDIII site of the vector pα56 c-fos/CAT. The vector alone or vector containing three copies of the motif was transfected into 1B10 cells (A) and J558L cells (B). Expression was assayed after 30–48 h depending on the cell line tested. M, mock transfection; V, vector alone (set to 1); E5–1, μE5–1; E5–2, μE5–2; E5–3, μE5–3; E5–4, μE5–4; E3, μE3; O5, octamer 5; O10, octamer 10 (see Fig. 1). Results are the averages of two transfections carried out in triplicate. Normalized values of CAT activity are given as the mean ± S.E., relative to the transcriptional activity of the vector alone.

**DISCUSSION**

The results of this study demonstrate that the function of Eμ3′, the enhancer driving expression of the IgH locus of the catfish, depends on transcription factors binding to a single consensus μE5 motif and to a number of octamer motifs, only one of which is of consensus sequence. This contrasts with the situation in the mammalian IgH locus, where, although μE5 and octamer sites play contributory roles in the enhancers, a number of other motifs play major, indeed essential, roles (19, 20). This is particularly clear in the case of the Eμ enhancer, found in the mammalian Jμ-Cμ intron, where the core of this enhancer has been shown to consist of a small region containing three motifs: a μA site, a μE3 or a CBF site, and a μB site (21). The factors binding to these sites (Ets-1 binding to μA, PU.1 binding to μB, and TFE3 or core binding factor binding to the μE3/CFB site) interact in a manner that requires precise spacing and orientation of the binding sites (22, 23). Although the involvement of additional sites in the catfish Eμ3′ enhancer cannot yet be completely discounted, there is no evidence that it utilizes ETS family transcription factors; nor does it appear to require TFE3 binding sites (9, 13).

Of the multiple μE5 sites identified in the catfish enhancer, only the site of exact mammalian consensus sequence was shown to exert a major positive influence on transcription. In fact, the μE5–1 site, which differs by one base from consensus and which overlaps the consensus octamer site, appeared to have a negative influence, by depressing the contribution to function made by the overlapping octamer motif (Fig. 2). In contrast, the octamer sites within Eμ3′ that differed from consensus sequence by single base changes in the 5′ half of the site (i.e. ATGC, the binding site for the POU-specific domain of Oct transcription factors) retained function. This is consistent with the previously described ability of these variant octamer sequences both to bind catfish Oct2 and to drive transcription in B cells when placed at the promoter site of a reporter construct (13–15). One unusual aspect of the catfish Eμ3′ enhancer...
revealed in the present study is the potential of multiple octamer sites to act cooperatively with a single consensus μE5 site. Although two of the octamers (octamers 10 and 11) are spaced two full turns of the DNA helix apart, which might facilitate the interaction of bound transcription factors, other octamers that are shown to have function in the enhancer are distributed over 1.4 kilobases. The consensus μE5 site is only 24 bp away from the closest functional octamer, whereas the other octamers that function are as far away from the μE5 site as 892 bp. This cooperative action of transcription factors bound to widely separated sites is consistent with the involvement of coactivators, the nature of which is not known. A coactivator, OCA-B2, has been postulated to explain the action of Oct2 bound to octamer sites in the mammalian Eμ enhancer (24–26). Octamer-binding transcription factors (in particular two isoforms of Oct2) have been cloned in the catfish and show clear similarity with their mammalian homologues, including strong sequence conservation of the DNA binding site, the presence of N- and C-terminal domains that are probably transcriptional activators, and the ability to act synergistically with the OCA-B (BOB-1) coactivator (14, 15). They have a restricted tissue distribution similar to that described for mammalian Oct2 (14). The factor(s) binding to the catfish μE5 site are not yet defined. In mammals the USF proteins, the E2A-encoded basic helix-loop-helix motif proteins E12 and E47, and the zinc finger protein ZEB bind to both μE5 and μE2 sites and are implicated in regulation of the Eμ enhancer in B cells (27–29). Enhancer function dependent upon Oct transcription factors and factors bound to a μE5 site has not been described in mammalian systems. The definition of a B cell-specific functional subunit of the catfish Eμ3’ enhancer, which contains the consensus μE5 motif and two functional octamer motifs, in which both μE5 and octamer sites are functional (Fig. 1), will permit the study of potential interactions between octamer- and μE5-binding transcription factors in a simpler system than is afforded by the full, native enhancer.

The comparison of enhancers between the IgH loci of catfish and mammals reveals substantial differences that contrast with the overall conservation of exon structure observed in vertebrate Ig genes. The enhancers differ in position, size, composition, and mechanism of action. This probably reflects the complexity of the mammalian locus, which undergoes class switching by chromosomal recombination (which does not occur in fish), and a very complex program of B cell development (6). In light of their substantial differences, it is surprising that the catfish Eμ3’ and mouse Eμ enhancers both function effectively and B cell-specifically when transfected across species (9, 13). This observation would be explicable if B lineage cells of all vertebrates possessed a large complement of transcription factors, of both tissue-specific and ubiquitous expression. Different Ig enhancers would then be able to use different combinations of these transcription factors to drive B cell-specific transcription, with the details of the particular mechanisms reflecting specific needs for fine control of B cell development and differentiation.

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