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Interaction between E-protein and Oct transcription factors in the function of the catfish *IGH* enhancer

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Abstract

Transcriptional control of the immunoglobulin heavy chain (*IGH*) locus in the channel catfish, *Ictalurus punctatus*, is incompletely understood. It is however known that 2 variant octamer motifs and a μ E5 motif in the core region of the enhancer ($E\mu 3'$) are important in driving transcription, and it has been suggested that interaction between transcription factors (Oct factors and E-proteins) bound to these sites contributes to enhancer function. In this study the functional relationships between the μ E5 motif, the proximal octamer motif, and the factors that bind them have been examined. The results of mutational analysis of these motifs showed that their interaction is important to driving transcription from the enhancer. Furthermore, the catfish Oct transcription factors were capable of a physical interaction with the catfish E-proteins. These results support a role for interaction between transcription factors bound to the octamer and μ E5 motifs in the function of the $E\mu 3'$ enhancer.

Keywords

E-protein; Oct1 Oct2; transcription factor; *IGH*; $E\mu 3'$ enhancer; channel catfish

1. Introduction

The picture that has emerged from studies of transcriptional control of the *IGH* locus in teleost fish [1-6] is that there is a single enhancer ($E\mu 3'$) that resides between the μ and δ genes. Whilst E-protein binding sites (e.g. μ E5 motifs) are clearly of major importance in the function of the $E\mu 3'$ enhancer, octamer motifs can also play a role (as shown in the channel catfish *Ictalurus punctatus*) in driving transcription [2,4]. The core region of the catfish $E\mu 3'$ enhancer differs in position, structure and function from the mammalian intronic *IGH* enhancer, $E\mu$ [1,2,7,8]. It contains 2 variant octamer motifs and a consensus μ E5 site [4] that are capable of binding to Oct transcription factors and E-box binding proteins (E-proteins) respectively [9-13]. In

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contrast the transcription factors that bind to the minimal E μ enhancer in the mouse *IGH* locus are Ets family members and the TFE3 (bHLH-zip) transcription factor [7,8].

The role of the 2 octamer motifs and the μ E5 site in the function of the E μ 3' enhancer has previously been investigated [4,5]. In summary, 1) the mutational inactivation of the 2 octamer motifs results in the loss of approximately 30% of the activity in the enhancer, 2) mutation of the μ E5 site results in a loss of 50% activity, indicating that the μ E5 site in this enhancer has an important function, 3) an increased distance between the 2 octamer motifs resulted in increased activity, 4) the binding of Oct transcription factors to the octamer motif induced bending of the DNA (as is also the case with mammalian Oct factors, [14]) and 5) the octamer factors have been suggested to interact with other factors bound to sites within E μ 3'. These interactions may include: Oct factors with E-protein; Oct factors with a coactivator; and Oct factors with an unknown protein binding to a recently described motif present between the 2 octamer sites [4,5,15]. In this study, the contribution to enhancer function of interactions between factors bound to the μ E5 site and to the proximal octamer site have been investigated.

2. Materials and Methods

2.1. DNA constructs

Schematics showing the octamer and μ E5 motifs present within the core region of E μ 3', and the mutations made to them are presented in Figs 1A and 2B. Eight reporter constructs for luciferase expression were used in this study, 1) the minimal *c-fos* promoter (Δ 56-Empty; pGL3/ Δ 56), 2) the minimal *c-fos* promoter with the core region of the catfish enhancer (R#2-WT; pGL3/ Δ 56/R#2), 3) the core region with double-mutated octamer#11 and μ E5 motifs (R#2- Δ Oct#11& Δ μ E5; pGL3/ Δ 56/R#2- Δ Oct#11& Δ μ E5), 4) the core region with mutated octamer#11 motif (R#2- Δ Oct#11; pGL3/ Δ 56/R#2- Δ Oct#11), 5) the core region with mutated μ E5 site (R#2- Δ μ E5; pGL3/ Δ 56/R#2- Δ μ E5#2), 6) the core region with 20bp addition in the position between octamer#11 and μ E5 (O/E+20bp; pGL3/ Δ 56/R#2-O/E+20bp), 7) the core region with 14bp addition in the position between octamer#11 and μ E5 (O/E+14bp; pGL3/ Δ 56/R#2-O/E+14bp), 8) the core region with a 5bp deletion between octamer#11 and μ E5 (O/E-5bp; pGL3/ Δ 56/R#2-O/E-5bp). The reporter constructs #1, 2, and 5 were described in previous study [11].

The reporter constructs pGL3/ Δ 56/R#2- Δ Oct#11& Δ μ E5#2, pGL3/ Δ 56/R#2- Δ Oct#1, and pGL3/ Δ 56/R#2-O/E-5bp were constructed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the following primers. The primer set for pGL3/ Δ 56/R#2- Δ Oct#11 and pGL3/ Δ 56/R#2- Δ Oct#11& Δ μ E5#2 was: 5'-GTC TAA TAA TGG ACT GCA GTG TAG CAC TTC TTC C (sense: G-2093, 34-mer), and 5'-GGA AGA AGT GCT ACA CTG CAG TCC ATT ATT AGA C-3' (antisense: G-2094, 34-mer). The primer set for pGL3/ Δ 56/R#2-O/E-5bp was: 5'-TGG ATG TAA ATG TAG CAC CCT GTG CAG GTG TGT TTC-3' (sense: G-1883, 36-mer), and 5'-GAA ACA CAC CTG CAC AGG GTG CTA CAT TTA CAT CCA-3' (antisense: G-1884, 36-mer). PCR was performed for 18 cycles, with the following profile: 30 s at 95 °C, 1 min at 55 °C and 11 min at 68 °C. The plasmids of pGL3/ Δ 56/R#2- Δ μ E5#2 (for the #3 construct) or pGL3/ Δ 56/R#2 (for the #4 and 8 constructs) were used as template DNA for these reactions. The DNA fragments used to construct reporters, pGL3/ Δ 56/R#2-O/E+20bp and pGL3/ Δ 56/R#2-O/E+14bp, were PCR-amplified by Ex-Taq polymerase (Takara Bio Inc., Shiga, Japan) using the following primer sets containing the cloning site (underlined). The primer sets for pGL3/ Δ 56/R#2-O/E+20bp were: 5'-GGA ATT CTA CAA AGT CCT GTG CAG GTG TGT TTC AC-3' (sense: G-2097, 35-mer), and 5'-GGA ATT CAT AAT GCA GAA GTG CTA CAT TTA CAT CC-3' (antisense: G-2098, 35-mer). The primer sets for pGL3/ Δ 56/R#2-O/E+14bp were: 5'-TTT AAG CTT ATG TCT TCC TGT GCA GGT GTG TTT CAC TCC-3' (sense, G-1885, 39-mer), and 5'-TTT AAG CTT CAC AAA GTG CTA CAT TTA CAT CCA TTA TTA GAC-3' (antisense: G-1886, 42-mer).

Using pGL3/Δ56/R#2 as a template DNA, PCR was performed for 35 cycles with the following profile: 15 s at 95 °C and 12 min at 68 °C. PCR-products were digested with *Eco*RI or *Hind*III, self-ligated to circle, and transformed into *Escherichia coli*. Purified and mutated DNA fragments were re-cloned in the original pGL3/Δ56 (construct #1) described above. Plasmids for transfection were purified using Nucleobond AX mega prep kits (BD Biosciences Clontech, Palo Alto, CA) and dissolved in dH₂O.

The catfish recombinant E-proteins (CFEB1, CFEB2, and E2A1) fused into S-peptide tag (S-tag) using *in vitro* transcription and translation (TNT) system (Promega) with expression constructs cloned in pCITE+4b vector (pCITE-CFEB1+S, pCITE-CFEB2+S, and pCITE-E2A1+S), have been described previously [11,12]. Recombinant proteins without S-tag of catfish Oct transcription factors (Oct2α, Oct2β, and Oct1) have been also produced from pCITE-Oct2α, pCITE-Oct2β, and pCITE-Oct1 in our previous studies [6,10].

2.2. Cell lines, DNA transfection, and Luciferase Reporter Assay

The catfish B lymphoblastoid cell line, 1G8, which is a subclone of the 1B10 cell line [16] was used for transfections as described previously [4]. Equimolar amounts of construct were transfected in each experiment. Each group contained the same amount of reporter, 3.05 pM (corresponding to 10 μg for pGL/Δ56, 10.24 μg for pGL/Δ56/R#2, pGL3/Δ56/R#2-ΔμE5#2, pGL3/Δ56/R#2-ΔOct#11, and pGL3/Δ56/R#2-ΔOct#11&ΔE5#2, 10.28 μg for pGL3/Δ56/R#2-O/E+20bp, 10.27 μg for pGL3/Δ56/R#2-O/E+14bp, and 10.23 μg for pGL3/Δ56/R#2-O/E-5bp), and the same amount of *Renilla* luciferase construct pRL/CMV, 0.371 pM (1.0 μg) as an internal control for transfection efficiency. Optimal electroporation conditions for transfection were determined as previously described [4].

Transfected cells were harvested 36-40 hours after electroporation and the luciferase activity was measured using the Dual-luciferase reporter assay system (Promega) in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Normalization for transfection activity was performed using the activity of the *Renilla* luciferase, and values were calculated as mean ± SE [11].

2.3. Assay of E-protein Interactions

The catfish E-proteins (E2A1, CFEB1, and CFEB2) and Oct transcription factors (Oct1, Oct2α, and Oct2β) were produced by *in vitro* transcription and translation using the TNT T7 Quick Coupled Transcription/Translation system (Promega). Two forms of the proteins were synthesized: ³⁵S-labeled proteins (Oct1, Oct2α, and Oct2β) without an S-tag, and non-radioactive proteins (E2A1, CFEB1, and CFEB2) with an S-tag. Fifty μl of the *in vitro* synthesized non-radioactive S-tagged catfish E2A1, CFEB1 and CFEB2 was bound to 25 μl of S-protein agarose resin for 5 hours at 4°C in 250 μl of binding/washing (B/W) buffer (25mM Tris-HCl [pH 7.5], 100mM NaCl, 2mM EDTA, 0.5% Triton X-100, 1mM phenylmethylsulfonyl fluoride), and then washed twice with 500 μl of the B/W buffer. Fifty μl of ³⁵S-labeled *in vitro* synthesized Oct1, Oct2α, and Oct2β proteins (without S-tag) were then incubated with the resin bound E2A1, CFEB1, and CFEB2 proteins for 5 hours at 4°C in 250 μl of the B/W buffer. The samples were then washed four times in 500 μl of the B/W buffer, boiled in 25 μl of 2× sample buffer and analyzed on a 7% SDS-PAGE gel. The gel was dried completely, exposed to a phosphorimager screen for 16-20 hours, and analyzed using a Typhoon phosphorimager and the ImageQuant program (Amersham Biosciences).

3. Results and Discussion

3.1. Contributions of the μ E5 and proximal octamer motifs to transcriptional activity

To understand the individual and interactive contributions of transcription factors bound to the Oct#11 and μ E5 motifs in the core region of E μ 3' enhancer (Fig 1A), reporter constructs containing motifs inactivated by mutation were transfected into catfish B cells and their activity was measured. Motifs (Δ Oct#11 or $\Delta\mu$ E5) were inactivated individually or in combination. The effects of these mutations on transcriptional activity were analyzed within the functional framework illustrated in Fig 1B. In summary, the contribution of the μ E5 site to transcriptional activity was considered to consist of two components; its independent contribution (**a** in Fig 1A); and its contribution through interaction with (factors bound at) the octamer site (**d** in Fig 1A). The contribution of the octamer site was similarly considered to consist of three components; its independent contribution (**b** in Fig 1A); (c) its interaction with the second octamer site (#10, **c** in Fig 1A); and its interaction with the μ E5 site (**d** in Fig 1A). Analysis of the data indicated that the contribution to the total activity of the E μ 3' enhancer by the μ E5 site was 77%, of which 28% was derived from interaction with the proximal octamer site. The total contribution to the activity of the E μ 3' enhancer from the proximal octamer site was 44%. Of this, 28% was contributed by interaction with the μ E5 site, and the remaining 16% contributed by the site alone plus its interaction with the second octamer site (#10, Fig 1A), which could not be distinguished by the design of this experiment. The potential interaction of factors bound to the octamer #10 site with those at the μ E5 site could also not be addressed directly through the design of this experiment.

If the assumption is correct that there are interactions between the μ E5 and proximal octamer sites (or rather, between the transcription factors bound to them) then two predictions can be made. The first is that increasing the distance between the proximal octamer site and the μ E5 site should alter the transcriptional activity of the construct, most likely by reducing it. The second prediction is that there should be demonstrable physical interaction between the factors that bind the octamer site (the Oct transcription factors) and those that bind the μ E5 site (E-proteins). These predictions were both tested, as described below.

3.2. Influence of distance between the octamer and μ E5 sites on transcriptional activity

Analysis of the relative contributions of the Oct#11 and μ E5 motifs to transcriptional activation driven by the core region of the catfish E μ 3' enhancer, as inferred from mutational inactivation of the sites, suggested interaction of factors bound to these sites. To understand the importance of physical distance between the Oct#11 and μ E5 motifs for this interaction, the distance between these two motifs was altered in a series of reporter constructs that were then transfected into catfish B cells. This series of constructs contained the following changes: 1) an insertion of 20bp that moved the Oct#11 and μ E5 motifs apart while maintaining their orientation on the same face of the DNA helix; 2) an insertion of 14bp that moved the motifs apart while placing them on opposite faces of the DNA helix; and 3) a deletion of 5bp that moved the motifs closer and oriented them on opposite faces of the DNA helix (Fig. 2A). Their transcriptional activity was compared to that of the wild-type unmutated enhancer (R#2-WT). An insertion of 20bp between the motifs significantly reduced the activity of the enhancer, while moving the motifs apart by 14bp had little effect on the wild type activity and moving them closer by 5bp significantly increased activity (Fig. 2B, C). These results suggest that the transcriptional activity resulting from the functional interaction of these motifs was primarily dependent upon (and inversely proportional to) the distance by which they were separated. The orientation of the motifs (in terms of the face of the DNA helix) did not affect transcriptional activity, a conclusion also reached previously with respect to the activities of the octamer motifs #10 and #11 [5]. The decrease in activity seen as the octamer and μ E5 motifs were moved apart

supports the suggestion that there is a physical interaction between the transcription factors bound at these sites. The occurrence of such an interaction was thus investigated directly.

3.3. Physical interaction between Oct transcription factors and E-proteins

The possible interactions between catfish octamer-binding transcription factors (Oct1, Oct2 α , and Oct2 β [9,10]) and catfish E-proteins (CFEB1, CFEB2, and E2A1 [11,12]) was directly tested in pull-down experiments using *in vitro* transcribed/translated proteins (Fig. 2D). The results of these experiments clearly demonstrated interactions between Oct transcription factors and E-proteins. All 3 of the Oct transcription factors tested were capable of binding to all 3 of the E-proteins cloned in the catfish (Fig. 2D). The specificity of this interaction was demonstrated by the failure of ³⁵S-labeled proteins to bind to the S-peptide beads in the absence of the S-tagged, unlabeled protein. Figure 2E shows the positive control, demonstrating the binding of ³⁵S-labeled S-tagged proteins to the S-protein agarose beads. The interaction between the Oct2 transcription factor and CFEB1 appears to be stronger than the interaction with Oct1, although a more quantitative assay of the affinity of this interaction would be needed to confirm these results. Evidence of a physical interaction between the Oct transcription factors and E-proteins has not previously been reported in any vertebrate. Based on the results of the present and prior studies [2,4,5] we propose that the integrated function of the catfish E μ 3' enhancer includes interaction between a catfish E-protein bound to the μ E5 site and an Oct transcription factor bound to the proximal (#11) octamer site. While potentially important aspects of the function of the E μ 3' enhancer, such as the role of BOB.1 [17] and of the transcription factor binding to a site between the Oct motifs [6] remain to be elucidated, it is clear that transcriptional control of the *IGH* locus in a teleost fish is dependent upon interactions between Oct transcription factors and E-proteins, constrained by the spatial arrangement of the enhancer [2,4,5].

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Abbreviations

IGH	immunoglobulin heavy chain
bHLH	basic helix-loop-helix
CFEB	catfish E-box binding protein
O/E	the octamer#11 and μ E5#2 motifs
R#2	Region#2 (core region of μ E3' enhancer)
WT	wild type
EMSA	electrophoretic mobility shift assay

SE	standard error
EDTA	ethlenediamine tetraacetic acid
TBE	Tris-Borate-EDTA
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis

Figure 1. Effect of mutational inactivation of octamer and μE5 motifs in the Eμ3' enhancer
(A) The Oct#11 and μE5 motifs were inactivated singly or together by site-directed mutation. A schematic illustrating the motifs mutated in the core region (R#2) of the Eμ3' enhancer is shown on the left. The oval, rectangle, and diamond in the schematic illustrations indicate the motifs to be bound with transcription factors: Oct, E-protein, and an unknown protein (X; Hikima et al, 2006), respectively. Covered black crosses indicate a mutated motif. Transcriptional activity of the mutated reporter constructs, shown as percent activity relative to the wild type (WT) R#2, upon transient transfection into catfish 1G8 B cells. The standard error (SE) is denoted by the error bars, and the asterisk “*” indicates the significant difference between each of these constructs at $p < 0.01$, as determined by two-tailed Student's t -test ($n=9$).
(B) Schematic showing potential interactions between transcription factors and calculations for the contributions of the Oct and μE5 motifs derived from the results in (A). Abbreviations: O#10, octamer#10; O#11, octamer#11.

Figure 2. Interaction between Oct transcription factors and E-proteins in the function of the E μ 3' enhancer

(A) Transcriptional activation of the E μ 3' enhancer: effect of changing the distance between the octamer and μ E5 motifs. The distance between Oct#11 and μ E5 site was increased or decreased as indicated in the sequences of O/E+20bp, O/E+14bp, and O/E-5bp. (B) Schematic illustrating the orientation of the motifs on the same or opposite faces of the DNA helix and the structures of luciferase (LUC) reporter vectors are shown on the left side of graph. Transcriptional activity of mutated reporter constructs, shown as fold activation, upon transient transfection into catfish 1G8 cells. The orientation (direction) of box at μ E5 site indicates the actual face of DNA motif to bind with an E-protein. The standard error (SE) is indicated with the error bars, and the number of replicates is indicated in parentheses to the right. The asterisk “*” indicates the significant difference between each of experimental results at $p > 0.01$, as determined by two-tailed Student's *t*-test. (C) The results from B plotted as a function of the separation (bp) between the Oct#11 and μ E5 motifs. Abbreviations: WT; wild type, O/E; octamer #11 and μ E5. (D) Interaction was assayed in pull-down experiments by affinity

chromatography on S-protein agarose. *In vitro* synthesized ^{35}S -labeled catfish Oct transcription factors (Oct2 α , Oct2 β , or Oct1) were mixed with *in vitro* synthesized non-labeled S-tagged catfish E-proteins (CFEB1, CFEB2, or E2A1) pre-bound to S-protein agarose beads. Lanes labeled "Input (1/50)" show 2% of the starting material (^{35}S -labeled Oct transcription factors) entered into the reaction. That the interactions detected in this assay are attributable to the catfish E-proteins and not to the epitope tag is shown by the lack of interaction when the S-tag peptide alone was used (lanes labeled "S-peptide"). (**E**) Control confirming S-tag function using the *in vitro* synthesized ^{35}S -labeled and S-tagged proteins (CFEB1, CFEB2, E2A1), captured by S-protein chromatography and separated on 7% SDS-PAGE.