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Evolution of antibody class switching: Identification and transcriptional control of an I ν exon in the Duck (*Anas platyrhynchos*)^{*}

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Abstract

Immunoglobulin class switching is characteristic to the tetrapod lineage, but the nature of this process has been elucidated only in mammals, where I-exon transcription initiates and directs the recombination in the IgH locus. Here, it is shown that an I ν exon occurs 5' of the ν (IgY constant region) gene of the duck (*Anas platyrhynchos*): it is longer than mammalian I-exons and comprised primarily of tandem repeats. The I ν promoter was identified and shown to be responsive to stimulation with IL-4 but not LPS. It contains Oct, LYF-1, ATF, and C/EBP motifs. Site directed mutagenesis indicates that 2 C/EBP motifs are uniquely necessary for the response of the promoter to IL-4, as tested in the mouse pre-B cell line, 70Z/3. These results support the conclusion that the signal transduction pathways controlling I-exon promoter responses to cytokines have been highly conserved in vertebrate evolution.

Keywords

Class switch; Evolution; Avian; Interleukin 4

Abbreviations

H, heavy; L, light; V, variable; C, constant; bp, basepair; S, switch; VNTR, variable number tandem repeats; INR, initiation region

1. Introduction

In the vertebrate antibody response, diversity of ligand recognition is achieved through sequence variation in the variable (V) domains of the heavy (H) and light (L) chains. In addition, diversity of secondary biological function is achieved through the process of class switching, in which a B lymphocyte switches the isotype of expressed H chain. Whereas IgM and IgD are produced by alternative pathways of processing of a primary transcript that includes both μ and δ sequences [1], switching from IgM to other antibody classes (in mammals IgG, IgA

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and IgE) is achieved by chromosomal recombination [2]. While the mechanism of class switching has been described only in mammals, the evolutionary history of this phenomenon is of great interest, particularly since it is now recognized that both class switch recombination and the generation of high affinity binding sites in the Ig-V regions are mechanistically linked [3]; they both require the expression of activation-induced cytidine deaminase [4]. In mammalian B-cells, the switch from expression of IgM to the production of other antibody classes (except, typically, for IgD) is dependent on the expression of germ-line transcripts of the constant (C) region gene targeted for switching. These germ-line transcripts lack V-encoding sequences, contain the C region exons, and originate with *I*-exons 5' of the switch (S) regions [5]. An *I*-exon is located 5' of the S region for each C region gene, is typically 100–500 bp in length and contains multiple stop codons in all three reading frames. The function of the *I*-exon is not fully understood as it can readily be exchanged with an unrelated sequence provided that splicing to the C region exons is maintained [6]. This has led to the conclusion that it is not the *I*-exons per se, but rather the sterile transcription and RNA processing, especially stabilized R-loop structures of the sterile transcripts [7], that are important for the class switch to occur. The germ-line transcripts are driven from the TATA-less promoters of the *I*-exons. While elements within the $E\mu$ enhancer have been shown to promote the constitutive production of μ germ-line transcripts [8], the *I*-exon promoters of the other heavy chain genes, (γ , α , and ϵ), are induced by cytokines following B-cell activation. In vitro treatment of mitogen activated mouse B-cells with combinations of cytokines has led to the conclusion that class switching can be directed to specific isotypes by specific sets of stimuli. For example, IL-4 induces switching to IgE and IgG1 [9–11], TGF- β promotes the production of IgA [12–14] and IgG2b [15]; while IFN- γ targets the IgG2a [16] and IgG3 genes [17]. There are recent reports suggesting that the target of the class switch machinery also involves, besides the cytokine-induced transcription factors, DNA binding factors that distinguish between the different S regions [18]. The C region genes are typically all in the same transcriptional orientation. Thus, class switch recombination involves a looping-out mechanism in which the intervening DNA is deleted as a circle [19]. Although class switch recombination is believed to occur not only in mammals but also in amphibia, reptiles and birds [20,21], the mechanisms by which this process is initiated, directed and controlled are not known for any non-mammalian species. The organization of the IgH locus in the Pekin duck [22,23], and chicken [24], presents interesting questions regarding the control and mechanism of class switching in birds. This is because the IgH locus is organized with the C genes in the order μ – α – ν , with the α gene in inverse transcriptional orientation. Thus, while the switch from μ (IgM production) to ν (IgY production) follows a looping-out/deletion mechanism, the switch to α (IgA production) must be dependent on the inversion of the chromosomal segment containing the μ and α genes [22,24]. In this study, the genetic structures that are predicted to underlie class switching to IgY in the duck (*Anas platyrhynchos*) have been addressed, to determine whether a cytokine-responsive *I*-exon homologue lies upstream of the ν gene.

2. Materials and methods

2.1. Animals and cell lines

American commercial ducks (MLF, Milford, IN) were the source of materials used in this study. Chicken cell lines, DT40 (a B-cell, [25,26]) and 132B (a T-cell, [27]), were maintained in Iscove's Modified Dulbecco's Medium with GlutaMAX (Gibco, Grand Island, NY) and supplemented to 1% with chicken serum (Sigma, St Louis, MO), to 5% with fetal bovine serum (Hyclone, Logan, UT), and with 50 U/50 μ g per ml each of penicillin and streptomycin (Gibco, Grand Island, NY), respectively. The mouse pre-B cell line 70Z/3 [28] was generously provided by Dr Lucille London and grown in RPMI medium 1640 with GlutaMAX (Gibco, Grand Island, NY), supplemented to 10% with fetal bovine serum, 50 μ M 2-mercaptoethanol and 100 U/100

µg per ml each of penicillin and streptomycin. All cell lines were maintained at 37 °C in a humidified 5% carbon dioxide atmosphere.

2.2. cDNA library and screening

Spleen RNA from an 8-week-old female duck was extracted using the RNeasy extraction kit (Qiagen, Valencia, CA). Approximately 5 µg of total RNA was reverse-transcribed and used in the construction of a λZAPII cDNA library (Stratagene, La Jolla, CA). Differential screening for V_H negative/Cv1 positive recombinants was performed to isolate a total of seven clones. These were converted to plasmids prior to sequencing on both strands using vector and gene specific primers at the Biotechnology Resource Facility (Medical University of South Carolina). Sequences were analyzed in the DNASTar software (DNASTar Inc., Madison, WI). A novel exon was identified by comparing cDNA sequences with the previously published genomic sequence of the duck IgH locus (22) (Acc #AJ314754). Putative transcription factor-binding motifs were identified using the transfac database (www.gene-regulation.com) with matrix and core identities set to 75%.

2.3. RT-PCR analysis

Messenger RNA was obtained from the spleens of an 8-week-old unimmunized female (the individual used for library construction) and an immunized 14-week-old male duck. Three weeks prior to the sampling the male duck had been administered an intramuscular injection of 100 µl containing an emulsion of *Limulus polyphemus* hemocyanin (1 mg/ml in phosphate-buffered saline) with Freund's complete adjuvant. The mRNA was reverse-transcribed with an oligo dT primer (G-413; 5'-TCTGAATTCTCGAGTCGACATC-T₍₁₇₎-3') using the PowerScript enzyme (Clontech, Palo Alto CA) and used as template in a PCR reaction. The forward primer (G-1992; 5'-AAATGGGGGAGCCTGG/GCAGC-3') overlapped the I- ν /Cv1 splice site by five nucleotides, while the two reverse primers used were specific for either the 3' untranslated region of $\nu(\Delta Fc)$ (G-1993; 5'-CGGTGGTTCATGGCCAGCAGGG-3') or the Cv3 exon (G-1994; 5'-GAGGCTACCGGGGCTTGGTGG-3'). The amplifications were performed in a two-step PCR with 30 cycles of 95 °C for 30 s and an annealing/elongation step of 68 °C for 3 min, using the Advantage-2 PCR kit (Clontech, Palo Alto CA). The amplified products were separated on a 1.2% agarose gel, purified, cloned and sequenced.

2.4. Promoter region constructs

Two overlapping fragments, 475 bp including the Oct motif and 310 bp (excluding the Oct motif) in length (positions 40985–41460 and 40985–41295 in Acc #AJ314754) were used in the initial characterization for promoter activity. These fragments were PCR-amplified from the duck genomic clone λ2.1 (22) using a common forward primer (G-2153; 5'-CGAGATCTCACAGCGACCCCGGTGGC) and two reverse primers (G-2154; 5'-TTAGATCTCAGAGTTTTGGAGCCCATATTG and G-2155; 5'-TTAGATCTCGATTTTGGGCACCGAGCTCT-3'). Both fragments were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and verified by sequencing. A *Bgl*/II site (*italics*), introduced during PCR on either side of the amplicons, allowed cloning into the pGL3-enhancer vector (Promega, Madison WI), a luciferase reporter vector lacking endogenous promoter but containing the SV40 enhancer. All constructs were then again verified by sequencing for orientation and sequence identity prior to transfection.

2.5. Site-directed mutagenesis of the I ν promoter

The longer of the two promoter fragments, cloned into pCR2.1, was subjected to PCR based site directed mutagenesis to generate a set of single, double and triple mutated promoter regions. The mutation of the third C/EBP site, in the single (Δ C/EBP#3) and triple (Δ C/EBP#1,2,3) mutated constructs, was generated using overlapping primers that introduced a *Cl*a I site

(underlined) in the consensus motif of C/EBP#3 (forward primer G-2690 5'-GGGATCGATCCTTTTGGGTTATTTCTGTTATTGC, reverse primer G-2691 5'-GGGATCGATAACAACCCCATTTGGCTGCTTCC). All the other putative transcription factor binding sites were mutated using a complementary primer set and the Site directed mutagenesis kit (Stratagene, LaJolla, CA); Δ C/EBP#1 (G-2573 and G2573 5'-CGGTGGCTGTGGTTTGGCGAAATTCA); Δ C/EBP#2 (G-2574 and G-2575 5'-GCCGATTCGGCATAGGTTACCCCA); Δ C/EBP #1,2 (G-2612 and G-2613 5'-CCACGGTGGCTGTGGTTGGCGTAGGTTACCCAAAT); Δ LYF-1 (G-2570 and G-2571 5'-CTGCAGAAATTCAATTGGATGCGTGGGAG); Δ ATF#1 (G-2580 and G-2581 5'-GAGTGGTGAGTACGTCATCAGCC); Δ ATF#2 (G-2582 and G-2583 5'-GAGTGGTGATGACGTACTCAGCCCG); Δ ATF#1,2 (G-2584 and G-2585 5'-GAGTGGTGATGATATCATCAGCC); Δ Oct (G- 2212 and G-2213 5'-GACCTCAAAATGTTTATCCGG GCTC). Following mutagenesis and sequence verification, the mutated promoter regions were up cloned into the pGL3-enhancer vector and again sequenced to verify the orientation and sequence of the constructs to be transfected.

2.6. Transfections

The mouse pre B- (70Z/3) and chicken B- (DT40) and T- (132B) cell lines were transiently transfected by electroporation. $1-2 \times 10^6$ cells in 180 μ l of serum-free medium were mixed with 20 μ l of DNA in TE buffer. Electroporation conditions were, 155 V, 1100 μ F for 70Z/3 and 170 V, 1100 μ F for the two chicken cell lines. Experimental constructs, positive control (pGL3-control) and negative control (pGL3- enhancer) were all transfected in equimolar ratios, standardized to 8 μ g per transfection for the positive control. As a transfection efficiency control, the *Renilla* luciferase-expressing pRL construct (Promega, Madison, WI) was co-transfected at 2 or 0.5 μ g for mouse and chicken cells, respectively. The experiments also included a mock transfection (TE buffer, no DNA) to determine auto-luminescence. Induction of the putative promoter region was investigated by, 30 min post transfection, supplementing the culture media with *E. coli* lipopolysaccharide (LPS, Calbiochem, La Jolla, CA) and IL-4 (Sigma, St Louis, MO), separately and in combination to a final concentration of 5 μ g and 10 ng per ml, respectively. Transfected cells were cultured for 24 h at 37 °C before being harvested and assayed using the Dual- Luciferase Reporter Assay System (Promega, Madison, WI). The samples were read in a luminometer (20/20 instrument, Turner Designs, Sunnyvale, CA), and the light units were adjusted for auto-luminescence, normalized for transfection efficiency and calculated as fold-increase compared to the negative control.

2.7. Statistical analysis

To test for the significance of differences in response to IL-4 between the native promoter and promoters bearing mutations, the following approach was used. For each promoter construct, the individual data points from untreated cells (i.e. expression in the absence of IL-4) were subtracted from the individual data points from IL-4 treated cells. Three replicate experiments were carried out with IL-4 treatment for each promoter construct. Each experiment utilized triplicate transfections for each promoter construct/ IL-4 treatment. This approach resulted in a total of 81 (9×9) new data points reflecting the IL-4 responsiveness of each promoter. These data points were then pruned of outliers, defined as values greater than two standard deviations from the mean. Means and standard deviations were then calculated, and the IL-4 responsiveness of each mutated promoter was compared to that of the native promoter, using twotailed *T*-tests and assuming unequal variances.

3. Results

3.1. Identification of a putative *I_u*-exon

The initial screening of the cDNA library yielded seven clones that were *C_u* positive and *V_H* negative. Further characterization of these clones revealed that most were truncated in the *C_uI* exon or in FR4 of the *V_H* domain. However, a single clone, 01-113 (Acc #AJ517505), was found to contain at its 5' end, sequence that was derived neither from *V_H* nor from the intron immediately 5' of the *C_uI* exon. This sequence was 958 bp in length and constituted the 5' part of a message that also contained exons *C_u1* through *C_u4* and a poly(A) tail. Comparisons of the obtained cDNA sequence with the genomic sequence of the duck IgH locus (Fig. 1, [22]) (Acc #AJ314754) revealed a novel exon that maps 5' of the putative *S_u* region (Fig. 1). It was thus identifiable as the putative duck *I_u*-exon. Within this *I*-exon a tandem repeat, of consensus sequence (TCTCCATTATGGACCCAAAA), made up nearly 95% of the length of the exon. The *I_u* exon sequences derived from genomic sequencing (Acc #AJ314754) and cDNA cloning differ in length by one repeat unit, and show in addition 7 single nucleotide substitutions. Based on the observations of repeat length polymorphism and the degree of allelic variation in the Ig exons [22], these differences are interpreted as another example of allelic polymorphisms in the duck IgH locus. Although it is generally accepted that the function of *I*-exons in class switching is independent of the expression of an encoded peptide, some reports indicate that *I*-exon spliced messages are expressible as a protein product [29]. Analyzing the sequence of the genomic duck *I_u* exon, and the *I_u*-containing cDNA resulted in two conceptual translations of different lengths. These translations were made in the single reading frame that continues through the constant region of the *u* gene. The most 5' ATG-sites compatible with an open reading frame are shown (Fig. 1B). In neither case was the start codon associated with a consensus ribosomal attachment (Kozak) site [30].

3.2. Two *I_u*-containing transcripts

Duck serum IgY is expressed as two isoforms: the full-length IgY molecule and a truncated IgY(ΔFc). Production of the full-length IgY precedes, in an immune response, the production of the IgY(ΔFc), which predominates later in the response [31–33]. Thus, it would be predicted that the class switch to IgY would be preceded by the production of *I_u* transcripts spliced to the full-length *u*, rather than to *u*(ΔFc). In order to test this hypothesis, an initial northern blot analysis of *u* transcripts was carried out on spleen mRNA from two ducks. The results obtained with northern blotting (data not shown) were suggestive of *I_u* splicing to both full-length and truncated *u* transcripts, thereby indicating that the pathways of alternative splicing of the constant exons are active prior to the switch to IgY production. Thus, a more definitive understanding of the transcripts that contained both *I_u* and *C_u* sequences was sought using RT-PCR analyses combined with cloning and sequencing (Fig. 2). Products were amplified using a forward primer spanning the *I_u*–*C_u1* splice site and the reverse primers used were specific for either the *C_u3* exon or the 3'UT region of the *u*(ΔFc). Each primer combination amplified a product of the predicted size, and these were cloned and sequenced. The sequences (Acc #AJ534873 and AJ534872) clearly confirmed splicing of *I_u* to both the full-length *u* and to the *u*(ΔFc) coding sequences, respectively.

3.3. A promoter region immediately upstream of the *I_u* exon

The genomic sequence immediately upstream of, and overlapping, the *I_u* exon was analyzed for transcription factor binding sites, for TATA boxes and for transcription initiation regions (INR). Similarly to mammalian *I*-exon promoter regions, the putative duck *I_u* promoter region lacks a TATA box and thus must utilize an INR as the origin of transcription. Potential transcription factor binding motifs identified in the duck *I_u* promoter were C/EBP (3 sites), LYF-1 (1 site), ATF (2 sites) and Oct (1 site) (www.gene-regulation.com) as shown in Fig. 3. The promoter region also contained a potential INR that could direct transcription through the

v gene (Fig. 3). To investigate the function of the putative promoter, two fragments (denoted long and short) were cloned into the pGL3-enhancer vector (Fig. 4). Constructs with the fragments in the orientation that would drive expression of the *v* gene were assessed for baseline promoter activity by transfection into mouse pre-B cells (70Z/3) and chicken B- (DT40) and T cells (132B) (Fig. 4). The constructs containing both long and short fragments of the putative *I_v* promoter all showed higher transcriptional activity than the promoterless control in the three cell lines. Also, the promoter activity of the shorter construct is higher than that of the longer in the 3 cell lines. Bearing in mind the positions of the identified transcription factor binding sites, only the Oct site is missing in the short construct. From our transfections with mutagenized binding sites in the mouse pre-B cell line (Fig. 6), we can see that deleting the Oct site in the long construct results in a similar increase of promoter activity, about 2-fold above that of the unmutated long construct. This suggests that the Oct motif may function as a negative regulator of the *I_v* promoter activity.

3.4. Induction of transcription from the *I_v* promoter

The transcriptional activity of mammalian *I*-exon promoters is selectively upregulated by the exposure of B cells to stimuli such as cytokines and LPS. Cytokine-responsive bird cell lines have not been described, and it was therefore decided to test the duck *I_v* promoter using the well-established mouse 70Z/3 pre-B cell model of cytokine-responsiveness. This utilization of mouse cells involves testing duck promoter function across a substantial phylogenetic distance; the advantage of this system is that, if responsiveness is observed, it permits inferences concerning the evolutionary conservation of signal transduction pathways. Accordingly, the construct with the longer promoter fragment (Fig. 4) was transfected into 70Z/3 cells and its transcriptional response to stimulation with LPS and IL-4 (alone or in combination) was tested. Treatment of transfected 70Z/3 cells with IL-4 resulted in a strong induction of transcription driven by the *I_v* promoter (Fig. 5), whereas LPS treatment alone did not stimulate the activity of the *I_v* promoter. Consistent with this lack of induction by LPS, when LPS treatment was combined with IL-4 it reduced the level of stimulation from that seen with IL-4 alone (Fig. 5). This effect of LPS was not due to a general toxic effect on the 70Z/3 cells, as shown by the LPS-induced stimulation of transcription in constructs driven by the SV40 promoter (Fig. 5).

3.5. C/EBP sites control the IL-4 responsiveness of the *I_v* promoter

The duck *I_v* promoter contains seven readily-identifiable transcription factor binding motifs (Fig. 3). In order to determine which of these sites might be involved in the response to IL-4, a series of site-directed mutations was introduced into the promoter. These mutations selectively inactivated the motifs either individually or in combinations (Fig. 6). The responsiveness of the mutated promoter constructs to IL-4 induction in 70Z/3 cells was assessed. The results (Fig. 6) showed that the mutation of any of the LYF-1, ATF or Oct sites did not significantly reduce the responsiveness of the promoter to IL-4 stimulation. Whereas an enhancement of IL-4 responsiveness was seen with mutation of the C/EBP #3 site (Fig. 6), in contrast, mutation of either of the other 2 C/EBP sites (#1 and #2) markedly reduced the transcriptional response to IL-4 stimulation. While the unmutated promoter showed a > 10-fold increase in transcription in response to IL-4 treatment, the enhancement seen in the case of the Δ C/EBP #1 and Δ C/EBP #2 constructs was only approximately 2.5-fold. When the mutations of the C/EBP motifs were combined (all 3 sites, or just #1 plus #2), the response of the promoter to IL-4 was completely eliminated. Statistical analysis to verify the significance of the observed changes in promoter responsiveness to IL-4 was performed as described in Section 2 and the results are listed in Table 1.

4. Discussion

This is the first report of an *I*-exon homologue in a non-mammalian species. The evidence that an *I_ν* homologue has been identified is three-fold: (1) The *I_ν* exon lies upstream of the exons encoding *C_ν*, from which it is separated by the putative switch (*S_ν*) region; (2) The *I_ν* exon splices directly to the *C_νI* exon; and (3) The *I_ν* exon is under the control of a promoter that is transcriptionally responsive to IL-4 stimulation. In comparison with the relatively loose definitions of an *I*-exon in mammals, the duck *I_ν* exon was found to exhibit some remarkable differences compared to previously-described *I*-exons. Its length is nearly twice that of the longest mammalian *I*-exons [2] and it consists primarily of tandem repeat sequences. In addition to the differences from other *I*-exons, the duck *I_ν* exon displays substantial allelic polymorphism, manifested in both the number of VNTR units as well as in sequence variations. This is consistent with previous analyses indicating a very high prevalence of VNTR-like sequences and allelic variation within the duck IgH locus [22].

The duck *ν* gene encodes two secreted isoforms, a full-length *ν* and a truncated *ν*(ΔFc) that lacks the 2 C-terminal domains. These isoforms are produced through an alternative processing of a common primary transcript [34,35]. While the full-length IgY is produced early in an antibody response, the IgY(ΔFc) is the predominant isoform characterizing the late antibody response [31–33]. Thus, a question arises concerning the stage of duck B cell development at which the RNA processing pathway for synthesis of the IgY(ΔFc) is active: is it utilized only late in the IgY response? Examination of *I_ν* transcripts by RT-PCR/sequencing analyses clearly showed the presence of transcripts containing *I_ν* sequences spliced to both the full-length *ν* and the truncated *ν*(ΔFc) sequences. Thus, it can be concluded that the RNA processing pathways that generate the full-length *ν* and the truncated *ν*(ΔFc) are both active prior to the switch to IgY production.

The evolutionary position of IgY as the precursor to both IgG and IgE has been deduced from the sequence and organization of the protein-encoding exons (reviewed in Ref. [36]). The identification of the *I_ν* exon offers the further opportunity to study the transcriptional control of Ig class switching in an evolutionary context. The analysis of the cytokine responsiveness of the duck *I_ν* promoter was carried out using a cytokine-responsive mouse cell line, because cytokine-responsive avian lymphocyte cell lines have not been developed. However, this was not entirely a disadvantage, because the use of mouse cells allowed testing of the hypothesis that cytokine-responsive signal transduction pathways involved in *I*-promoter activation have been evolutionarily conserved. Such conservation is strongly supported by the observation that the duck *I_ν* promoter is IL-4 responsive. These results suggest that the essential molecular machinery mediating Ig class switching evolved over 300 million years ago, prior to the split between the lineages from which birds and mammals would evolve [37]. While the duck *I_ν* promoter is similar to mammalian *I*-exon promoters in being TATA-less, it also lacks many of the sites described to be important in the regulation of mammalian *I*-exon promoters, such as the cytokine responsive elements of INF- γ , IL-4 and TGF- β , and binding sites for NFkB and STAT6 [38–41]. Nor was any sequence similarity observed with the evolutionarily conserved element that has been reported in mammalian *I γ* promoter regions [42–44]. The cytokine responsiveness of mammalian *I*-exon promoters is both complex and subtle. For example, in the mouse the IL-4 responsive *I ϵ* and *I γ 1* promoters bind STAT6 and NFkB, whose interactions and outcome in class switching are also influenced by interactions with additional promoter-binding factors, such as (as in the case of *I γ 1*) ATF2 and AP-1 [45] and in the case of *I ϵ* , BCL-6, Pax5/BSAP and PU-1 [46]. The influence of C/EBP sites is context-dependent: in the *I γ 1* promoter C/EBP enhances transcription [38], whereas in the *I γ I* promoter, the binding of C/EBP β is inhibitory [45]. Mutational analysis of the duck *I_ν* promoter to determine the basis of IL-4 responsiveness gave a very simple result: 2 adjacent C/EBP sites are uniquely necessary for this response. Mutational inactivation of Oct, ATF and LYF-1 motifs, as well as

of a third C/EBP site, were without effect on the IL-4 responsiveness of the promoter. Thus, it is clear that functional aspects rather than the *I_H* promoter sequence itself must be considered, in order to draw conclusions of the exact evolutionary relationship of the *I*-exons, their promoters and the highly diverse C region genes of the mammalian IgH locus. This is not unexpected, since it has been observed before that in the vertebrate IgH locus the transcriptional control elements have undergone far more rapid evolutionary divergence than have the protein-encoding elements [47,48].

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References

1. Moore KW, Rogers J, Hunkapiller T, Early P, Nottenburg C, Weissman I, et al. Expression of IgD may use both DNA rearrangement and RNA splicing mechanisms. *Proc Natl Acad Sci USA* 1981;78(3):1800–4. [PubMed: 6262826]
2. Stavnezer J, Kinoshita K, Muramatsu M, Honjo T. Molecular mechanism of class switch recombination. In: Honjo T, Alt FW, Neuberger M, editors. *Molecular Biology of B Cells*. London: Elsevier Academic Press; 2004. p. 307–26.
3. Kinoshita K, Lee CG, Tashiro J, Muramatsu M, Chen XC, Yoshikawa K, et al. Molecular mechanism of immunoglobulin class switch recombination. *Cold Spring Harb Symp Quant Biol* 1999;64:217–26. [PubMed: 11232289]
4. Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* 1999;274(26):18,470–18,476.
5. Kemp DJ, Harris AW, Cory S, Adams JM. Expression of the immunoglobulin C mu gene in mouse T and B lymphoid and myeloid cell lines. *Proc Natl Acad Sci USA* 1980;77(12):2876–80. [PubMed: 6771761]
6. Hein K, Lorenz MG, Siebenkotten G, Petry K, Christine R, Radbruch A. Processing of switch transcripts is required for targeting of antibody class switch recombination. *J Exp Med* 1998;188(12):2369–74. [PubMed: 9858523]
7. Yu K, Chedin F, Hsieh CL, Wilson TE, Lieber MR. R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. *Nat Immunol* 2003;4(5):442–51. [PubMed: 12679812]
8. Lennon GG, Perry RP. C mu-containing transcripts initiate heterogeneously within the IgH enhancer region and contain a novel 5'-nontranslatable exon. *Nature* 1985;318(6045):475–8. [PubMed: 3934561]
9. Snapper CM, Finkelman FD, Paul WE. Regulation of IgG1 and IgE production by interleukin 4. *Immunol Rev* 1988;102:51–75. [PubMed: 3284816]
10. Snapper CM, Finkelman FD, Paul WE. Differential regulation of IgG1 and IgE synthesis by interleukin 4. *J Exp Med* 1988;167(1):183–96. [PubMed: 3257252]
11. Snapper CM, Finkelman FD, Stefany D, Conrad DH, Paul WE. IL-4 induces co-expression of intrinsic membrane IgG1 and IgE by murine B cells stimulated with lipopolysaccharide. *J Immunol* 1988;141(2):489–98. [PubMed: 3260253]
12. Sonoda E, Matsumoto R, Hitoshi Y, Ishii T, Sugimoto M, Araki S, et al. Transforming growth factor beta induces IgA production and acts additively with interleukin 5 for IgA production. *J Exp Med* 1989;170(4):1415–20. [PubMed: 2677210]
13. Coffman RL, Lebman DA, Shrader B. Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J Exp Med* 1989;170(3):1039–44. [PubMed: 2788703]
14. McIntyre TM, Kehry MR, Snapper CM. Novel in vitro model for high-rate IgA class switching. *J Immunol* 1995;154(7):3156–61. [PubMed: 7897205]

15. McIntyre TM, Klinman DR, Rothman P, Lugo M, Dasch JR, Mond JJ, et al. Transforming growth factor beta 1 selectivity stimulates immunoglobulin G2b secretion by lipopolysaccharide- activated murine B cells. *J Exp Med* 1993;177(4):1031–7. [PubMed: 8459202]
16. Snapper CM, Peschel C, Paul WE. IFN-gamma stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. *J Immunol* 1988;140(7):2121–7. [PubMed: 3127461]
17. Snapper CM, McIntyre TM, Mandler R, Pecanha LM, Finkelman FD, Lees A, et al. Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J Exp Med* 1992;175(5):1367–71. [PubMed: 1373759]
18. Shanmugam A, Shi MJ, Yauch L, Stavnezer J, Kenter AL. Evidence for class-specific factors in immunoglobulin isotype switching. *J Exp Med* 2000;191(8):1365–80. [PubMed: 10770803]
19. Jack HM, McDowell M, Steinberg CM, Wabl M. Looping out and deletion mechanism for the immunoglobulin heavy-chain class switch. *Proc Natl Acad Sci USA* 1988;85(5):1581–5. [PubMed: 2830623]
20. Bengten E, Wilson M, Miller N, Clem LW, Pilstrom L, Warr GW. Immunoglobulin isotypes: structure, function, and genetics. *Curr Top Microbiol Immunol* 2000;248:189–219. [PubMed: 10793479]
21. Mussmann R, Courtet M, Schwager J, Du Pasquier L. Microsites for immunoglobulin switch recombination breakpoints from *Xenopus* to mammals. *Eur J Immunol* 1997;27(10):2610–9. [PubMed: 9368617]
22. Lundqvist ML, Middleton DL, Hazard S, Warr GW. The immunoglobulin heavy chain locus of the duck. Genomic organization and expression of D, J, and C region genes. *J Biol Chem* 2001;276(50):46,729–46,736.
23. Magor KE, Higgins DA, Middleton DL, Warr GW. Opposite orientation of the alpha- and epsilon-chain constant region genes in the immunoglobulin heavy chain locus of the duck. *Immunogenetics* 1999;49(7–8):692–5. [PubMed: 10369928]
24. Zhao Y, Rabbani H, Shimizu A, Hammarstrom L. Mapping of the chicken immunoglobulin heavy-chain constant region gene locus reveals an inverted alpha gene upstream of a condensed epsilon gene. *Immunology* 2000;101(3):348–53. [PubMed: 11106938]
25. Baba TW, Giroir BP, Humphries EH. Cell lines derived from avian lymphomas exhibit two distinct phenotypes. *Virology* 1985;144(1):139–51. [PubMed: 2998040]
26. Baba TW, Humphries EH. Differential response to avian leukosis virus infection exhibited by two chicken lines. *Virology* 1984;135(1):181–8. [PubMed: 6328748]
27. Calnek BW, Lucioi B, Schat KA. Pathogenesis of Marek's disease virus-induced lesions. 2. Influence of virus strain and host genotype. In: Kato S, Huriuchi T, Mikami T, Hirari K, editors. *Advances in Marek's disease research*. Osaka, Japan: Japanese Association on Marek's Disease; 1988. p. 324–30.
28. Paige CJ, Kincade PW, Ralph P. Murine B cell leukemia line with inducible surface immunoglobulin expression. *J Immunol* 1978;121(2):641–7. [PubMed: 308074]
29. Bachl J, Turck CW, Wabl M. Translatable immunoglobulin germ-line transcript. *Eur J Immunol* 1996;26(4):870–4. [PubMed: 8625981]
30. Kozak M. Regulation of translation in eukaryotic systems. *Annu Rev Cell Biol* 1992;8:197–225. [PubMed: 1335743]
31. Grey HM. Duck immunoglobulins. II. Biologic and immunochemical studies. *J Immunol* 1967;98(4):820–6. [PubMed: 4164559]
32. Grey HM. Duck immunoglobulins. I. Structural studies on a 5.7S and 7.8S gamma-globulin. *J Immunol* 1967;98(4):811–9. [PubMed: 4164558]
33. Zimmerman B, Shalatin N, Grey HM. Structural studies on the duck 5.7S and 7.8S immunoglobulins. *Biochemistry* 1971;10(3):482–8. [PubMed: 5543979]
34. Magor KE, Warr GW, Middleton D, Wilson MR, Higgins DA. Structural relationship between the two IgY of the duck, *Anas platyrhynchos*: molecular genetic evidence. *J Immunol* 1992;149(8):2627–33. [PubMed: 1401901]
35. Magor KE, Higgins DA, Middleton DL, Warr GW. One gene encodes the heavy chains for three different forms of IgY in the duck. *J Immunol* 1994;153(12):5549–55. [PubMed: 7989756]

36. Warr GW, Magor KE, Higgins DA. IgY: clues to the origins of modern antibodies. *Immunol Today* 1995;16(8):392–8. [PubMed: 7546196]
37. Kumar S, Hedges SB. A molecular timescale for vertebrate evolution. *Nature* 1998;392(6679):917–20. [PubMed: 9582070]
38. Delphin S, Stavnezer J. Characterization of an interleukin 4 (IL-4) responsive region in the immunoglobulin heavy chain germline epsilon promoter: regulation by NF-IL-4, a C/EBP family member and NF-kappa B/p50. *J Exp Med* 1995;181(1):181–92. [PubMed: 7807002]
39. Berton MT, Linehan LA. IL-4 activates a latent DNA-binding factor that binds a shared IFN-gamma and IL-4 response element present in the germ-line gamma 1 Ig promoter. *J Immunol* 1995;154(9):4513–25. [PubMed: 7722306]
40. Lin SC, Stavnezer J. Activation of NF-kappaB/Rel by CD40 engagement induces the mouse germ line immunoglobulin Cgamma1 promoter. *Mol Cell Biol* 1996;16(9):4591–603. [PubMed: 8756615]
41. Iciek LA, Delphin SA, Stavnezer J. CD40 cross-linking induces Ig epsilon germline transcripts in B cells via activation of NF-kappa B: synergy with IL-4 induction. *J Immunol* 1997;158(10):4769–79. [PubMed: 9144491]
42. Schaffer A, Cerutti A, Shah S, Zan H, Casali P. The evolutionarily conserved sequence upstream of the human Ig heavy chain S gamma 3 region is an inducible promoter: synergistic activation by CD40 ligand and IL-4 via cooperative NF-kappa B and STAT-6 binding sites. *J Immunol* 1999;162(9):5327–36. [PubMed: 10228008]
43. Mills FC, Mitchell MP, Harindranath N, Max EE. Human Ig S gamma regions and their participation in sequential switching to IgE. *J Immunol* 1995;155(6):3021–36. [PubMed: 7673720]
44. Sideras P, Mizuta TR, Kanamori H, Suzuki N, Okamoto M, Kuze K, et al. Production of sterile transcripts of C gamma genes in an IgM-producing human neoplastic B cell line that switches to IgG-producing cells. *Int Immunol* 1989;1(6):631–42. [PubMed: 2518730]
45. Mao CS, Stavnezer J. Differential regulation of mouse germline Ig gamma 1 and epsilon promoters by IL-4 and CD40. *J Immunol* 2001;167(3):1522–34. [PubMed: 11466373]
46. Oettgen HC. Regulation of the IgE isotype switch: new insights on cytokine signals and the functions of epsilon germline transcripts. *Curr Opin Immunol* 2000;12(6):618–23.
47. Magor BG, Ross DA, Pilstrom L, Warr GW. Transcriptional enhancers and the evolution of the IgH locus. *Immunol Today* 1999;20(1):13–17. [PubMed: 10081224]
48. Cioffi CC, Middleton DL, Wilson MR, Miller NW, Clem LW, Warr GW. An IgH enhancer that drives transcription through basic helix-loop-helix and Oct transcription factor binding motifs. Functional analysis of the E(mu)3' enhancer of the catfish. *J Biol Chem* 2001;276(30):27,825–27,830.

Fig. 1.

The *Iv* exon of the duck: (A) Schematic showing the duck *IgH* locus and the relationship between the *v* gene and the germ-line transcript, as cDNA. The position of the *Iv* exon, the switch (*Sv*) region, the constant (*C*) domain exons, the short terminal (*T*) exon and the transmembrane (*TM*) exons are indicated. The splicing event resulting in the characterized cDNA clone is shown. Figure is not drawn to scale. The corresponding positions in the duck genomic sequence (AJ314754) are for the *I* exon 41241–42218, and for the *Cv1* exon 45500–45784. The donor and acceptor sites for the *Iv*–*Cv* splice are TGG/GTATGG and CCACAG/GCA, respectively with the above mentioned positions in the genomic sequence; (B) Conceptual translation of the 3' end of the *Iv* exon examined as cDNA (this study) and from a genomic clone (21). The conceptual translations of the *Iv* reading frame (defined by the *Cv1* to which the *Iv* exon is spliced) were aligned from the 5'-most methionine codon (ATG) that excluded downstream stop codons. Amino acid identities are indicated as apostrophes ('). The differences in length and amino acid residues between the two sequences are inferred to reflect allelic polymorphism between the cDNA and genomic sequences examined. The Met codon for the genomic *Iv* exon corresponds to position 42092–42094 in AJ314754.

Fig. 2.

Analysis (by agarose gel electrophoresis and ethidium bromide staining) of RT-PCR products of the I_ν germ-line transcripts (I_ν(ΔFc) and I_ν). Spleen mRNA from an immunized male duck (see Section 2) was used as template in the RT-PCR reaction. The amplicons were generated using a forward primer covering the I_ν/C_ν1 splice site, and reverse primers specific for the 3' UT region of ν(ΔFc) (lane 2 and upper right image) and C_ν3 (lane 3 and lower right image). Lane 1 is a 100 bp ladder. The position of primers and the size of the predicted products (bp) are indicated on the schematic to the right of the gel, with the ν(ΔFc) form on top and the full length form below.

Fig. 3.

Schematic showing the relative positions of the Iv promoter, Iv exon and Sv region (top). The sequence of the putative promoter region is shown below (pos. 40976–41470 in Acc no AJ314754). The identified transcription factor-binding sites are indicated above the sequence. Potential initiation regions (INRs, of consensus sequence YYANWYY, where N is the first nt transcribed) are shown in bold and arrow (→). PCR primers used to amplify the promoter region are underlined and lower case letters above the sequence indicates the *Bgl*II sites introduced to allow site specific cloning. The start of the cDNA clone (Acc. no. AJ517505) is shown with a (-[*graphic not found*]-).

Fig. 4.

Transcriptional activity of the *Iv* promoter region: (A) Constructs of two different lengths (long and short), were generated by PCR and cloned in forward orientation into pGL3/Enh, a promoter-less luciferase reporter vector containing the SV40 enhancer; (B) The constructs were transiently transfected into the mouse pre-B cell line (70Z/3), and chicken B (DT40) and T (132B) cell lines. The pGL3 control vector, driven by the SV40 promoter and enhancer, was used as the positive control. The negative control was the pGL3/Enh vector. Each construct was transfected six times, and the luciferase activity of the constructs was normalized for transfection efficiency and expressed (mean $\pm$ standard deviation) as fold-induction compared to the negative control.

Fig. 5.

Induction of the $I\gamma$ promoter. The constructs (negative and positive controls, and the 'long' $I\gamma$ promoter in forward orientation) were transfected into the 70Z/3 mouse pre-B cell line. Post transfection, the cells were stimulated with LPS and IL-4 (indicated by + or - signs below the graph) either alone or in combination. Two experiments were conducted, in which each transfection/treatment was carried out three times. Both experiments showed the same effects, and the results of one experiment are shown. The activity of the constructs was normalized for transfection efficiency and expressed (mean $\pm$ SE) as fold-induction compared to the negative control.

Fig. 6.

C/EBP sites are necessary for the response of the I α promoter to IL-4: (A) Schematic representation of the native promoter and the sites mutated in each construct; (B) and (C) Transcriptional responses of the native and mutated I α promoter constructs. Transfections with no IL-4 stimulation (white bars) and with IL-4 stimulation (black bars) are shown as means $\pm$ standard deviation from one representative set of transfections in triplicate. The effects of the mutations on the response to IL-4 were calculated for three independent sets of triplicate transfections of each promoter construct and are displayed in Table 1.

Table 1Statistical analysis of the influence of site-directed mutagenesis on the IL-4 responsiveness of the I ν promoter

Construct	Mean activity ^a	Standard deviation	<i>p</i> -value
I ν promoter	20.06	6.29	—
Δ C/EBP#1	6.11	4.71	8.65×10^{-28}
Δ C/EBP#2	2.09	1.84	1.03×10^{-33}
Δ C/EBP#3	23.37	14.18	0.09
Δ C/EBP#1,2	0.29	4.5	3.20×10^{-38}
Δ C/EBP#1,2,3	-0.15	3.59	6.62×10^{-38}
Δ LYF-1	20.45	13.62	0.83
Δ ATF#1	23.56	19.66	0.14
Δ ATF#2	17.87	11.67	0.15
Δ ATF#1,2	17.86	10.66	0.12
Δ oct	23.73	13.28	0.03

^aIL-4 stimulation calculated as described in Section 2.