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Yin Yang 1, Oct1, and NFAT-4 Form Repeating, Cyclosporin-Sensitive Regulatory Modules Within the Murine CD21 Intrinsic Control Region

Mark D. Zabel, Wells Wheeler, Janis J. Weis, and John H. Weis

The murine complement receptor type 2 gene (Cr2/CD21) is expressed by murine B and follicular dendritic cells, but not murine T cells. We have previously shown that appropriate transcriptional control of the CD21 gene requires the CD21 promoter as well as intrinsic sequences. We have also demonstrated that altering chromatin structure by inhibiting histone deacetylases induces CD21 expression in murine T cells by increasing the accessibility of promoter and intrinsic regulatory elements. In this report, we identify seven distinct regulatory areas within the first intron of the murine CD21 gene that are conserved between mouse and human CD21 intronic sequences. EMSA competition and supershift analyses reveal the formation of multiple DNA-protein complexes at these sites that include Yin Yang 1, Oct1, and NFAT-4. NFAT-containing complexes were altered in B cells treated with the NFAT inhibitor cyclosporin A and correlated with a repression of CD21 gene transcription implicating NFAT transcriptional control. Functional data revealed that no single region conferred cell-specific reporter gene expression, but rather the entire CD21 regulatory element was required to confer cell-specific gene expression. Taken together, these data demonstrate the formation of repeating, overlapping regulatory modules, all of which are required to coordinately control the cell-specific expression of the murine CD21 gene. We propose a model in which Yin Yang 1 and Oct1 may recruit histone deacetylase to multiple sites in the CD21 intrinsic regulatory element in nonexpressing cells and NFAT either displaces this histone deacetylase or recruits a histone acetylase to allow the formation of a functional transcriptional complex in expressing cells. The Journal of Immunology, 2002, 168: 3341–3350.

The murine complement receptor type 2 (CD21) is an important receptor for the innate and acquired immune response (1). CD21 facilitates internalization of immune complexes by B cells to enhance Ag presentation. CD21, in association with CD19/CD81, also serves as a coaccessory activation complex with the B cell Ag receptor, permitting a lower Ag concentration to achieve maximal B cell activation (2). Murine B cells and follicular dendritic cells of the spleen are the only two cell types that express CD21 (3). Murine T cells do not express CD21 (4).

Significant progress has been made in elucidating the mechanisms of cell-specific expression of the murine CD21 gene. Appropriate transcriptional control of the CD21 gene requires both the CD21 promoter as well as intrinsic sequences which possess enhancer and suppressor functions (5). A minimal promoter consisting of 2 kb of sequence 5′ to the initiating ATG site of the CD21 gene facilitates reporter gene expression, but does so equally well in both expressing B cells and nonexpressing T cells. Addition of the first 1.6 kb of CD21 intron 1 restores cell specificity (B>T). This intronic sequence has been shown to require the endogenous CD21 promoter to maintain cell-specific expression of such reporter constructs (6).

Chromatin remodeling via histone acetylation is now a well-established regulatory mechanism of gene expression (7, 8). We have implicated chromatin structure as a regulatory mechanism for the coordination of CD21 promoter and intrinsic control sequences by limiting access by transcription factors. We have demonstrated by DNase I hypersensitivity assays that differential accessibility to CD21 regulatory elements exists in B cells and T cells (6). However, when chromatin structure is altered by using deacetylase inhibitors to create a hyperacetylated state in T cells, the accessibility of DNase I to CD21 regulatory elements in T cells increases dramatically, closely resembling the B cell profile. Increased accessibility to CD21 regulatory elements directly correlates to induced CD21 expression in hyperacetylated T cells (9).

In this report, we extend previous studies on the intrinsic control elements to elucidate sites that are important for the cell-specific transcriptional control of the murine CD21 gene. We analyzed the entire first intron of the mouse and human CD21 genes, and identified seven areas that exhibit significant sequence homology between the mouse and human CD21 genes. Interestingly, all seven of these homologous regions were found in or near the previously identified regulatory elements for both genes. These regions were found to share spatial as well as sequence homology.

The exact mechanism for controlling accessibility to CD21 regulatory elements has remained elusive. Hypothesizing that these seven mouse/human homology regions (termed mouse human homology intron regions 1–7 (MHi1–7)3) are functionally important

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for cell-specific CD21 gene expression, we analyzed them for transcription factor binding sites. EMSA analyses revealed that five of these seven sites, MH1, MH2, MH3, MH4, and MH5, formed complexes with B and T cell nuclear extracts. Competition and supershift EMSA analyses revealed that each of these complexes contained many of the same transcription factors, including Yin Yang, Oct1, and NFAT-4. Interestingly, inhibiting NFAT translocation and DNA binding by treating B cells with cyclosporin A (CsA) resulted in the transcriptional repression of the CD21 gene in a time- and dose-dependent manner, implicating this factor as key in controlling CD21 transcription in the permissive B cell.

Materials and Methods

Cell lines and culture conditions

The murine CD21-expressing B cell lymphoma cell 2PK3 and the CD21-negative T cell lymphoma cell EL4 were obtained from American Type Culture Collection (Manassas, VA). 2PK3 and A20 cells (a CD21-expressing pre-B cell line) were maintained at 37°C/5% CO2 in DMEM (Life Technologies, Bethesda, Maryland) with 10% FCS. (HyClone Laboratories, Logan, UT) and 1% penicillin-streptomycin (Pen-Strep). Cells were isolated from adult NIH outbred mice and maintained in single-cell suspensions with DMEM with 10% FCS and Pen-Strep. Cells were treated with indicated doses of CsA (Sigma-Aldrich, St. Louis, MO) or an equal volume of ethanol (EtOH) vehicle in media for the indicated times.

Luciferase reporter constructs

PCD21Luc, pCD21LucIntron 1, pCD21LucA, pCD21LucAa, and pCD21LucAb luciferase reporter plasmids were generated as previously described (5). CD21 intron 1 segments were generated by PCR using the following primers. A

Described (5). CD21 intron 1 segments were generated by PCR using the following primers. A

Plasmids containing CD21 intrinsic deletions were generated by either of two PCR methods; PCR, using divergent primers that added 5'-GCG CGC GGT CAC TCA AAT ACG GAA TTC TCA CAC C-3' and 1533 (5'-GCC CGT CGA CCC ACT TTG TGA AAC CCT ACA AGA G-3') into pCD21Luc 3'. These PCR products were digested with EcoRI and BamHI restriction enzymes, gel purified, and sequenced in both directions using the chain termination method (10). These PCR products were inserted by restriction digest and sequencing in both directions using the chain termination method (10).

RNA preparation and cDNA synthesis

Total RNA from cells was isolated using the RNeasy kit (Qiagen, Chantilly, VA). Serial injection of substrates and monitoring of light emission for 10 s was performed for both firefly and Renilla luciferase. Computer software (Dynex) automatically subtracted background and normalized raw data by calculating the ratio of firefly:Renilla light emission values. Normalized data were relativized to luciferase activity seen in pCD21Luc.

EMSA

The protocol was performed as previously described (11) with minor modifications (6). Nuclear extracts were prepared from 2PK3 and EL4 cells and quantified by the Bradford assay (12). DNA fragments were prepared either by excision from pTVECTOR plasmids by BamHI/SalI digestion or by PCR using primers specific for each segment of CD21 intronic fragments as described above. Such PCR products were digested with EcoRI, purified by 4% PAGE and radioactively labeled via a fill-in reaction with [α-32P]dCTP, d(A,G,T)TPs, and Klenow enzyme. All binding reactions were performed at room temperature for 30 min in a 30-μl volume containing 0–5 μg nuclear extract, 10,000–30,000 cpm fragments, and 2 μg poly(dIdC) in binding buffer (3 mM Tris (pH 7.9), 5 mM MgCl2, 0.5 mM EDTA, and 3% glycerol). Reactions were subjected to 4% PAGE for 2 h at 250 V and resolved by autoradiography.

The oligonucleotide competition assay is a variation on the above protocol in which 1000-fold excess of unlabelled double-stranded competitor oligonucleotides were added to the binding reactions. Oligonucleotides used to compete specific binding sites are shown in each of the EMSA figures within the sequence illustration.

RNA preparation and cDNA synthesis

The supershift assay is a variation of the EMSA protocol in which 2 μg of anti-Yin Yang 1 (YY1), anti-NFAT-4, anti-Oct-1, or a nonspecific Ab (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the binding reactions.

Continuous monitoring PCR

PCR using fluorescence detection was performed as previously described (9). Murine CD21 transcripts were amplified using primers 45 (5'-AGT GAC TCT TGG GGT CAC TCA AAT ATC TCA CCT GGA TCC GCC GCT GGT CAT TAT CCA GCA GAT ATT GAT TAT CCA CAC G-3') and 1565 (5'-GGA GTG AAA AAT ACC TTA ACT TTT GCA GAG GCT CAG AAG-3') for i12Δ. These PCR products were inserted into pCD21Luc 3' of the firefly luciferase gene to generate pCD21Luc i12Δ and i12Δ. All plasmid constructs were confirmed by restriction digest and sequencing in both directions using the chain termination method (10).

Transient transfection and assay of luciferase reporter constructs

Transfections were performed as previously described (5) utilizing 5×104 2PK3 or EL4 cells. Equimolar amounts of plasmid were added relative to 10 μg of pGL3Basic luciferase reporter construct (Promega, Madison, WI). One microgram of pRl-TK Renilla luciferase reporter plasmid (Promega) was added to control for transfection efficiency. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Ten microliters of the transfected cell lysate was loaded into the well of a white opaque microtiter plate and the dual luciferase assay was performed automatically by the MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA). Serial injection of substrates and monitoring of light emission for 10 s was performed for both firefly and Renilla luciferase.

Computer software (Dynex) automatically subtracted background and normalized raw data by calculating the ratio of firefly:Renilla light emission values. Normalized data were relativized to luciferase activity seen in pCD21Luc.
and CD21 transcript levels were reported relative to levels found in EtOH vehicle-treated splenocytes. Melting curve profiles were used to confirm amplification of specific transcripts. Statistical analyses was performed using Microsoft Excel (Microsoft, Seattle, WA).

DNA sequence analysis

The first intron of the mouse and human CD21 genes were aligned for sequence homology using MacVector software (IBI, New Haven, CT). CD21 intronic sequences were analyzed for putative transcription factor binding sites using Transcription Element Search software at http://www.cbil.upenn.edu/tess, and by visual inspection.

**Results**

Mouse and human CD21 intronic regulatory elements share significant sequence homology

It has been previously shown that the first ~2.4 kb of the first intron of the human CD21 gene silences expression of CD21-driven reporter constructs in nonexpressing cell lines (13). We have demonstrated similar intronic control for the murine CD21 gene (5). To determine whether the mouse and human intronic regulatory elements share sequences that may be important to

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** A, Schematic of murine CD21 promoter and intron 1 regulatory elements. The promoter is shown arbitrarily divided into six overlapping segments of ~200 bp each (p6–p1). The first half of intron 1 is shown arbitrarily divided into 12 overlapping segments of ~200 bp each (i1–i12). The gray shaded areas depict the relative size and location of each of the regions homologous to the human CD21 promoter (MHp1 and MHp2) and intron 1 (MHi1–7). B, Intronic sequence comparison of the mouse (top line) and human (bottom lines) CD21 genes. The base pairs of the sequences are noted. Exact matches between the human and mouse sequences are denoted by the ∧ while differences are noted by the v. Exact matches are also noted by the upper case letters in the human sequence.
control cell-specific expression of the CD21 gene in both species, we performed a homology search between the entire first intron of mouse and human CD21 genes. We identified seven areas with a high degree of homology between the first introns of the mouse and human CD21 genes and two such regions within the promoter region (Fig. 1A). All seven of these homologous regions were found in or near the previously identified regulatory elements for both genes. These regions were found to share spatial as well as sequence homology (Fig. 1B). Thus, MHi1, the first homology region, matched the murine sequence beginning at nt 1206 relative to the first nucleotide in the intron, to the human sequence beginning at 1225. MHi1 conserves 81 of 96 nt between mouse and human sequence. MHi2 conserves 21 of 30 nt beginning at mouse nt 1856 to the human sequence beginning at nt 1782. MHi3 conserves 57 of 67 nt beginning at mouse nt 1950 to the human sequence beginning at nt 1890. MHi4 conserves 25 of 31 nt beginning at mouse nt 2110 to the human sequence beginning at 2052, and 17 of 19 nt beginning at mouse nt 2157 to the human sequence beginning at 2098. MHi5 conserves 38 of 54 nt beginning at mouse nt 2201 to the human sequence beginning at nt 2154. MHi6 conserves 24 of 31 nt beginning at mouse nt 2326 to the human sequence beginning at nt 2293. MHi7 conserves 23 of 28 nt beginning at mouse nt 2456 to the human sequence beginning at nt 2429. Sequence conservation between noncoding regions of genes from two different species strongly suggests a conserved functional role for these sequences. This observation, along with previous results showing regulatory functions for these sequences in the human CD21 gene, prompted a further investigation into the roles that these sequences play in the regulation of the murine CD21 gene. We approached this analysis from two perspectives. The first was to use such sequences to find putative transcriptional control factors by DNA-binding assays, and the second was to use transfection reporter assays to assay the function(s) of specific regions of the intronic sequence.

Differential DNA-protein complexes formed with B and T cell nuclear extracts that include YY1, Oct1, and NFAT-4 occur at or near regions MHi1, i2, and i3

We have previously demonstrated that the first third of murine CD21 intron 1, which was designated fragment A, conferred cell-specific expression of CD21-driven reporter constructs (5). We have also shown by EMSA that fragment A contains many nuclear protein binding sites, some of which form complexes only with proteins from B cell nuclear extracts (6). These complexes are interesting because they may confer tissue-specific expression of the murine CD21 gene. We approached this analysis from two perspectives. The first was to use such sequences to find putative transcriptional control factors by DNA-binding assays, and the second was to use transfection reporter assays to assay the function(s) of specific regions of the intronic sequence.

EMSA analysis of i7 produced only one major band in extracts obtained from 2PK3 B cells and EL4 T cells (Fig. 2A) which could be competed with the binding site (BS) 1 oligonucleotide (Fig. 2B). Analysis of the sequence for putative transcription factor binding sites identified many candidate proteins, including YY1, NFAT, and Oct1, that may be responsible for forming these complexes. Addition of Abs to i7-binding reactions revealed that YY1 formed a complex in B and T cells resulting in an apparent loss of the band. An Ab against NFAT-4 also appeared to slightly alter the migration of a fraction of this complex in the B cell extracts. Oct1 was not part of this complex.

The i8 intronic sequence which also contains part of the MHi1 homology region was similarly analyzed. Three distinct bands were generated with the B cell nuclear extract, while only two were evident with the T cell extract. Supershift analysis suggested the lowest band was due to YY1 binding, the middle due to Oct1, and the top band, only evident in the B cell extract, was the result of NFAT-4 binding (Fig. 3A). Competition analysis for this fragment with specific oligonucleotides indicated that this fragment possesses two distinct binding sites. Both of these sites bind YY1 which thus requires the full i8 fragment (or mixtures of both competition oligonucleotides; data not shown) to extinguish YY1 binding (Fig. 3B). However, these sites do show specificity for either Oct1 (BS2) or NFAT-4 (BS3). Thus, the B cell extract provides for a YY1-NFAT-4 complex to form on the BS3 site while the T cell extract does not (Fig. 3C).

The remaining MHi regions were arbitrarily segmented into three overlapping segments, termed i10, i11, and i12 (see Fig. 1 for map). These segments were analyzed by competition EMSA with 20-bp oligonucleotides to map binding sites. These sites were then analyzed for putative transcription factor binding sites to derive a small set of candidate proteins and tested for their involvement in complex formation by Ab supershift EMSA.

EMSA analysis revealed that segment i10, containing MHi2 and 3, formed complexes resulting in three distinct bands in extracts obtained from B cells and two such bands generated from T
cell extracts (Fig. 4A). Competition EMSA analysis revealed that two binding sites, BS4 and BS5, contribute to complex formation. BS4 and BS5 are located in MHi2 and MHi3, respectively (Fig. 4B). Ab supershift analysis indicated that the lowest migrating band in both cell types was due to YY1 binding while the next band was supershifted with the anti-Oct1 antisera. The sequence of this region contains a consensus NFAT site; however, the identification of a clear NFAT-DNA complex was difficult to ascertain. (NFAT binding data to this site are also addressed below; see Fig. 9.) Oligonucleotide competition studies demonstrated that YY1 preferentially binds to the BS4 site while Oct1 appears to bind to both BS4 and BS5 sites (data not shown). The data obtained in the EMSA analyses of the Mhi1, 2, and 3 regions essentially provide for two conclusions. The first is the correlation of sequence conservation in the MHi regions to the binding of nuclear factors. The second is that the central difference in the B and T cell EMSA shifts for these regions appears to be due to the binding of a NFAT protein (at least NFAT-4) present from the B cell extracts but absent from the analogous T cell extracts.

DNA-protein complexes formed with B and T cell nuclear extracts occur at MHi4, MHi5, and adjacent to MHi7 and involve YY1 and Oct1

Formation of a DNA-protein complex with segment i11, containing MHi4 and MHi5, resulted in a single bandshift (Fig. 5A). Competition EMSA analysis revealed two distinct binding sites, BS6 and BS7, located in MHi4 and MHi5, respectively (Fig. 5B). Ab supershift EMSA indicated that a single protein, YY1, bound to both of these sites.

Formation of a DNA-protein complex with segment i12, containing MHi6 and 7, resulted in two primary bandshifts identified

FIGURE 3. i8 EMSA with competition and supershift analyses. Binding reactions were performed as described above. A, i8 fragment incubated alone (first lane) or with 2PK3 or EL4 extracts in the absence (−) or presence of factor-specific antisera. The migration of the pertinent YY1, Oct1, and NFAT-4 complexes is marked on the left. B, Competition EMSA using either 2PK3 or EL4 nuclear extracts in the absence or presence of the BS2 or BS3 oligonucleotides, or the whole unlabeled i8 fragment. C, Partial sequence of the i8 sequence denoting the sequences of the BS2 and BS3 oligonucleotides (underlined) and the YY1, Oct, and NFAT consensus sequences marked in bold type. Gray shaded sequence denotes that within the mouse/human conserved regions.

FIGURE 4. i10 EMSA with competition and supershift analyses. A, Binding reactions were performed as described above using the 2PK3 B cell and EL4 T cell nuclear extracts. Arrows, Pertinent protein-DNA complexes. B, Partial sequence of i10 denoting the two defined binding sites, BS4 and BS5. Nomenclature is as described above. Gray shaded sequence denotes that within the mouse/human conserved regions.
as either an Oct1- or YY1-dependent shift (Fig. 6A). Competition EMSA revealed that four continuous overlapping oligonucleotides competed for binding of the complexes, identifying three apparent binding sites BS8 (for Oct1), BS9 (for YY1), and BS10 (for Oct1). Unlike most of the complexes identified in the MHi regions, all of the i12 complexes were formed with DNA sequences located outside of both MHi6 and 7. In addition, the complexes formed with i11 and i12 were identical to both B and T cell nuclear extracts. These data suggest that complexes formed on these sequences may serve to be less significant to cell-specific control of CD21 gene expression than those described for the MHi1, 2, and 3 regions.

The modular nature of CD21 intronic regulatory elements

We have previously shown that the first intron of CD21 directs cell-specific expression of luciferase constructs transiently transfected into 2PK3 B and EL4 T cells (5). The preceding EMSA experiments implicated many DNA-protein complexes within intron 1 that may facilitate cell-specific expression of the murine CD21 gene. To determine which, if any, of these MHi sequences are necessary and/or sufficient to confer cell specificity to CD21 gene expression, we performed luciferase reporter assays using different CD21 intronic fragments inserted 3’ of a luciferase gene that was driven by the CD21 promoter.

Cell-specific expression of the CD21 gene can be maintained if only the first 1.6 kb of intron 1, termed fragment A, is present (Fig. 7). However, suppression of luciferase activity in T cells by intron 1 was never fully recapitulated by fragment A. To determine whether the additional sequences 3’ of fragment A that contain MHi3–7 contribute to cell-specific CD21 gene expression, we sequentially added these regions back onto fragment A. Thus, i1–10 possesses all of fragment A plus the i10 sequence; i1–11 possesses the A fragment, i10, and i11 sequences; and A+ contains the A fragment plus the i10, i11, and i12 sequences. As these sequences were added back to fragment A and the constructs assayed, full cell-specific gene expression was restored such that pCD21LucA+ conferred the same cell specificity as pCD21LucIntron1 (Fig. 7). Thus, ~2.6 kb of intron 1 is required for full cell-specific control of CD21 gene expression exhibited by the full-length intron 1. Interestingly, the size of this murine CD21 intronic regulatory element closely matches that of the human CD21 intronic regulatory element and includes all of the MHi regions.

We have shown previously that the i7 and i8 fragments inserted into the reporter plasmid alone significantly suppressed reporter gene expression, but not in a cell-specific manner (again as shown in Fig. 7). However, insertion of either the i10, i11, or i12 sequences into a luciferase construct had minimal effect upon the expression of the construct in T or B cells (Fig. 7). When the protein binding sites present on the i10, i11, and i12 sequences were deleted (denoted by the i10Δ, i11Δ, and i12Δ symbols), no significant difference in expression was noted between the different cell types or with the nondeleted construct. Similar constructs were prepared for the i7 and i8 fragments in which the protein binding sites defined earlier in this manuscript were deleted and analyzed. These deletion constructs (denoted i7Δ and i8Δ) demonstrated a loss of transcriptional suppression following transfection, confirming that these sites were integral in the general suppressive effect of the fragments. To test for the effect of a whole intron 1 containing construct lacking just the i7 or i8 protein binding sites, such plasmids were prepared and analyzed (Fig. 7). As shown, the intron 1 construct lacking the i8 binding sites (Intron1 i8Δ) and the analogous construct lacking the i7 binding sites (Intron1 i7Δ) demonstrated virtually the same reporter activity as did the construct possessing the entire intron (pCD21Luc Intron1). These data in
total demonstrate that no single DNA sequence can recapitulate the effect of the A+ sequence (or the entire intron) in allowing for B+T cell expression and that, on their own, such sequences can have contrasting and unexpected effects upon transcriptional control. Thus, the murine CD21 gene possesses multiple regulatory modules in the first intron whose cooperation is required to coordinately control cell-specific gene expression.

Cyclosporin A treatment inhibits CD21 gene expression

The EMSA analysis of the binding sites within the MHi1, 2, and 3 regions shown above suggested that B cells possess a member(s) of the NFAT family (at least NFAT-4) capable of generating stable DNA-protein complexes in the CD21 intronic regions. This was in contrast to the T cell extracts which did not readily demonstrate this complex formation. The NFAT family possesses at least four members that are known to regulate the transcription of a variety of genes (14). NFAT proteins are usually sequestered in the cytoplasm requiring nuclear translocation for function. This activation pathway is accomplished by the dephosphorylation of the proteins in the cytoplasm by calcineurin which can be specifically inhibited by CsA and FK506. A variety of studies have shown that transcriptional suppression by these agents is through the inhibition of calcineurin activity blocking NFAT nuclear transport.

To determine whether our EMSA analyses were correct in implicating NFAT-4 as key for the expression of CD21 in B cells, we treated single-cell suspensions of splenocytes with 1 μM CsA for 16 h and performed semiquantitative real-time RT-PCR analyses to investigate CD21 gene expression. CsA-treated murine splenocytes exhibited a nearly 5-fold decrease in CD21 gene expression compared with EtOH vehicle-treated cells (Fig. 8A). CsA-treated splenocytes demonstrated a similar decrease in expression of the CD5 gene, previously shown to be activated by NFAT (14). Expression of the CD19 gene was unaffected by CsA treatment. CD21 transcriptional repression by CsA was both time and dose dependent (Fig. 8, B and C). CsA-treated 2PK3 cells and the A20 pre-B cell line (both of which normally express CD21) exhibited an ~2- to 3-fold decrease in CD21 gene expression (data not shown). Taken together, these data suggest that CsA treatment inhibits NFAT-mediated activation of the CD21 gene.

Cyclosporin treatment alters protein-DNA complexes formed at MH regions i1 and i3 that contain NFAT binding sites

To determine whether decreased CD21 gene transcription correlated with an alteration in NFAT-containing complex formation, we performed EMSA analyses using nuclear extracts prepared from CsA- and EtOH vehicle-treated B cell line 2PK3 cells. As shown in Fig. 9, the EMSA pattern of an intronic fragment possessing a NFAT binding site (i10, A) was dramatically altered with CsA treatment while the EMSA pattern of an intronic fragment without a NFAT site (i11, B) was unaffected. Interestingly, the CsA-treated extracts with the i10 fragment demonstrated a loss of the Oct1-possessing band as well as the NFAT band, suggesting that NFAT is required for Oct1 binding to that site. CsA treatment does not result in a generalized loss of YY1 or Oct1 within the extract since the i12 patterns comparing vehicle- or CsA-treated extracts were identical. These data thus strongly implicate a NFAT family member playing a role in protein complex formation within the CD21 intron.
Discussion

We have previously shown that sequences within the first intron control the cell-specific expression of the murine CD21 gene (5) and that the acetylation status of the chromatin encompassing these elements regulate their accessibility by transcription factors (6). Intronic control of cell-specific human CD21 gene expression has also been demonstrated (13). Comparative analysis of mouse and human intron 1 identified seven areas of significant sequence homology (Mhi1–7) which are located at or near sites previously identified as important for cell-specific CD21 gene expression in both mouse and human. Identification of key components of complexes formed at these mouse/human intronic homology regions offers valuable insight into the possible mechanism of cell-specific transcriptional regulation of the CD21 gene. To define the mechanism of B cell expression and T cell suppression of CD21 transcription, we analyzed the mouse/human homology regions to define the protein complexes that are formed.

Three primary observations were disclosed in this investigation. First, that the B cell-specific complexes included at least one member of the NFAT family and such complexes primarily mapped to the human/mouse conserved sequences. Indeed, the association of the NFAT complexes with the CD21 sequences was directly linked to the increased expression of the gene. Second, no single intronic unit was capable of defining B/T cell-specific transcription such that deletion of any single binding site had minimal effect upon the cell-specific transcription pattern. Third, that the treatment of CD21-expressing cells with CsA (which has potent and specific inhibitory properties regulating NFAT functionality) resulted in the specific loss of CD21 transcripts supporting our EMSA observations that NFAT was a member of the observed B cell-specific complexes.

Sequence competition and Ab supershift analysis utilizing nuclear extracts from T and B cells has identified the primary constituents as Oct1, YY1, NFAT-4, and potentially other NFAT members as well (summarized in Fig. 10). YY1 (δ, NF-E1, UCRBP, CF1) is a ubiquitously expressed 65-kDa protein that is highly conserved among human, mouse, Xenopus, and Drosophila (15). A member of the GL-Kruppel family of zinc finger transcription factors, YY1 was originally isolated as a repressor of the Ig3 enhancer (16). At the same time, YY1 was reported to be an activator of c-myc (17). These seminal reports illustrate the reason for naming this protein YY1: depending on the cellular context, YY1 can act as a repressor or activator, often performing these disparate functions on different genes within the same cell at the same time (18). YY1 has been reported to be involved in regulating a myriad of different genes, including c-myc, c-fos, IgH, Igκ, and IFN-γ (19–21).

YY1 depends on cofactors to define its role as a suppressor or inducer of gene activation. The most significant YY1 corepressors identified so far are the mammalian histone deacetylases.

FIGURE 8. Effect of CsA treatment on gene expression in primary cells. A, Splenocytes were treated with 1 μM CsA or EtOH vehicle for 16 h and assayed for CD5, CD19, and CD21 gene transcripts by RT-PCR. B, Splenocytes were treated with 1 μM CsA or EtOH vehicle for the indicated time points and assayed for CD21 gene transcript. C, Splenocytes were treated with the indicated doses of CsA or EtOH vehicle for 16 h and assayed for CD21 gene transcript. Data are averages of at least six experiments.

FIGURE 9. EMSA analyses using nuclear extract from 2PK3 cells treated for 16 h with 1 μM CsA or EtOH vehicle. Binding reactions were performed as previously described. Arrows, Protein-DNA complexes. A, i10 EMSA with supershift analysis. B, i12 EMSA with supershift analysis. ex, Extract; Rx, treatment.
FIGURE 10. Model for the cell-specific expression of the murine CD21 gene. Diagram is of nonexpressing T cell CD21 gene on top and a CD21-expressing B cell CD21 gene on the bottom, focusing solely upon the intronic region. HAT, Histone acetyltransferase.

(HDACs). Three HDACs have been cloned that belong to the RPD3 family, all of which have been shown to interact with YY1 in vitro (22-24). The repressor mechanism involving these two proteins would require YY1 binding to DNA and subsequent recruitment of HDAC, or interaction with HDAC first, then recruitment to DNA. Overexpression of HDAC2 has been shown to enhance YY1-mediated repression of transcription in reporter constructs containing YY1 binding sites (25).

YY1 has been reported to complex with Oct1 at an IL-5 promoter element to down-regulate IL-5 transcription in human T cells (26). Oct1 was originally identified as a transcriptional factor that regulates Ig promoter function (27). Oct1 is a ubiquitously expressed factor that, with the B cell-specific coactivator OCA-B, activates B cell-specific gene transcription (28, 29). More recently, Oct1 has also been shown to act as a repressor in concert with other transcription factors, including C/EBP and Pbx (30-32). We propose that YY1 and the Oct1 complex at murine CD21 regulatory elements repress gene transcription in nonexpressing cells by recruiting HDAC to the regulatory elements (see Fig. 10).

If a YY1-Oct1-HDAC complex acts as a repressor of murine CD21 gene transcription, what is the repressor mechanism and how does it affect gene transcription? One potential candidate is NFAT. NFAT, as the name implies, was first identified in T cells as an inducible transcription factor that bound the human IL-2 promoter upon T cell activation (33). However, several NFAT family members have been detected in many other hemopoietic cells, including mast cells (34), macrophages (35), NK cells (36), as well as neuronal cells (37). Three different NFAT family members, NFAT-1, NFAT-2, and NFAT-3, are expressed in B cells (38-40). NFAT function in human B cells seems to mirror its function in T cells (14). T and B cell activation via cross-linking of Ag receptors leads to calcium influx that activates calcineurin to dephosphorylate cytoplasmic NFAT. Dephosphorylated NFAT translocates to the nucleus, where it binds to NFAT sequences on promoters and activates gene transcription of many different cytokines and cell surface molecules, including IL-2, IL-4, CD40 ligand, CD25 and IFN-γ on T cells, and CD5 and Igα on B cells. Fos-Jun binding at AP-1 sites stabilizes NFAT-DNA complexes and facilitates transcription. In addition, lower levels of NFAT have been detected in the nuclei of unstimulated B and T cells (41) that may serve to activate constitutive expression of certain genes. We propose that CD21 may be one of those genes. NFAT binds to CD21 intronic regulatory elements specifically in B cells. YY1, Oct1, or both may stabilize this NFAT-DNA complex instead of, or in addition to, the Fos-Jun complex (Fig. 10). Recently, NFAT has been shown to bind with YY1 at the human IL-5 promoter (42), albeit to repress transcription of the IL-5 gene. Given the dichotomous character of YY1, this complex may function as an activating complex in the CD21 gene.

A potentially alternative method of transcriptional control of the CD21 gene expression by a YY1-NFAT-Oct1 complex might involve the active recruitment of a HDAC (see Fig. 10). As an inducer, YY1 has been shown to interact with CBP and p300 (43) (20), both of which contain HDAC activity (44). It has also been suggested that NFAT may actively recruit a HDAC (45). It will be interesting to see whether histone acetyltransferases can be identified in B cell-specific complexes at intronic or promoter regulatory elements of the murine CD21 gene.

A very intriguing question remains: how do CD21 regulatory modules coordinate with each other to perform their cell-specific regulatory functions? Certain modules may act to recruit HDACs or histone acetyltransferases to sites, acting as molecular sentinels at other protein binding sites, which then perform more traditional activation roles by recruiting transcription factors or RNA PolII subunits. It has been demonstrated in yeast systems that HDACs affect only a few hundred base pairs of chromatin per binding site (46, 47). The CD21 intronic regulatory element is ∼2.6 kb, suggesting that multiple binding sites are required to open and close chromatin encompassing this region. Indeed, our transient transfection data suggest no single site in the CD21 intron is responsible for the type of control observed. Deciphering the exact mechanism of CD21 regulatory module coordination will provide insight into

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the transcriptional control of other genes involved in similarly intricate regulatory mechanisms.

References


