Myocyte Enhancer Factor-related B-MEF2 Is Developmentally Expressed in B Cells and Regulates the Immunoglobulin J Chain Promoter*

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Immunoglobulin J chain gene expression is induced by the delivery of a lymphokine signal to antigen-activated B cells in a primary immune response. A major interleukin 2 (IL-2)-responsive region that contains two adjacent control elements (JA and JB) exists within the J chain promoter. Transcription factor PU.1 positively regulates J chain gene expression by binding to one of the control elements (JB) in the J chain promoter. In the present study we have determined that a myocyte enhancer factor 2 (MEF2)-related nuclear factor, named B-MEF2, positively regulates the J chain gene promoter activity via the second control element (JA). An in vitro translated MEF2 family member, MEF2C, was found to bind the JA site with identical properties as endogenously expressed B-MEF2 in B cell lines. Moreover, in vivo experiments showed that a dominant negative mutant of MEF2C blocked B-MEF2 regulation of the J chain promoter. Consistent with its role as positive regulator of J chain gene expression, B-MEF2 levels were enhanced in highly differentiated B cells. In addition, induction of an IL-2-responsive presecretor cell line BCL1 with IL-2 or IL-5 (which up-regulates J chain gene expression) resulted in an increased expression of B-MEF2. We conclude that a MEF2-related transcriptional factor, B-MEF2, acts as a stage-specific positive regulator of J chain gene expression in the B cell lineage.

The differentiation of B lymphocytes from committed precursor cells to antibody-secreting plasma cells is distinguished by changes in the expression of genes encoding immunoglobulins and accessory molecules. On activation of naive mature B cells by antigens, cytokines stimulate these cells to express the J chain gene, resulting in the assembly and secretion of the pentamer IgM antibody (1). The J chain gene is expressed only in activated B cells in response to IL-21 and IL-5, and its synthesis is tightly regulated at the transcriptional level. A single nuclease-hypersensitive site is co-induced with J chain gene expression either in normal B lymphocytes stimulated with mitogens or in BCL1 cells stimulated with recombinant IL-2 (2). The IL-2 signal therefore alters the 5’ structure of the J chain gene, allowing access to the transcriptional machinery. Previous studies have shown the presence of an IL-2-responsive region of the J chain promoter that contains adjacent elements: a positive T-rich sequence (JA) and an A + G-rich sequence (JB) with overlapping positive and negative regulatory motifs (3). In a previous study, transcription factor PU.1 was identified as one of the elements that binds to the JB site of the J chain promoter (4). In the present work we have identified a second positive regulator, B-MEF2, which through binding to the JA region, activates J chain gene expression.

Myocyte Enhancer Factor 2 (MEF2) proteins belong to the MADS box family of transcription factors named MCM1 that controls mating type-specific genes in yeast, Agamous and Dicicenis, that function as homeotic genes in plants and serum response factor, which regulates muscle specific and serum-inducible gene expression (5). Members of the MADS family share homology within a 56-amino acid domain termed the MADS box, which mediates DNA binding and dimerization (6). An additional 29-amino acid sequence immediately adjacent to the MADS box called the MEF2 domain is also highly conserved among MEF2 proteins (A, B, C, and D) but is absent from other MADS box proteins. MEF2 factors form homo- and heterodimers that bind to the consensus site (C/T)TA(A/G) originally identified in the promoters of several muscle-specific genes (7–9). The MADS and the MEF2 domains have been shown to be necessary and sufficient for dimerization and DNA binding (10).

All of the MEF2 proteins characterized so far are subject to complex forms of regulation at many levels. Alternative mRNA splicing of MEF2A gives rise to muscle-specific isoforms, and their role in muscle development has been extensively studied (11, 12). The expression of MEF2C mRNA is primarily restricted to skeletal muscle (13), brain (14, 15), and spleen (16), whereas transcripts of MEF2A, B, and D are widely expressed. Although MEF2 proteins have been shown to be present in muscle and non-muscle cells (17, 18), the role of these factors in non-muscle cells is less well understood. Recent studies have shown that MEF2C is activated by MAP kinase P38 during inflammation, resulting in an increase in c-Jun gene transcription (19). These studies indicate that MEF2C activation may play a role in host defense and inflammatory responses during an infection.

In the present report we have identified the recognition site for a MEF2 transcription factor family member within the IL-2-responsive region upstream of the J chain gene. The protein was identified as a MEF2 family member by several criteria, including binding of the J chain promoter by the product of a MEF2 family member cDNA clone, specific binding competition with mutant oligonucleotides, and reactivity of DNA-protein complexes with a MEF2 antibody. Finally, we show that B-MEF2 is
responsive to IL-2 or IL-5, and its expression correlates with J chain gene expression during B cell differentiation.

MATERIALS AND METHODS

Recombinant Interleukins—Recombinant human IL-2 (10⁵ units/ml) was obtained from Takeda Chemical Industries, Osaka, and recombinant IL-5 (10⁵ units/mg) was obtained from DNAX. The lymphokine doses were as follows: IL-2, 100 units/ml and recombinant IL-5, 25 units/ml for a 3-day incubation.

Cell Lines—The myeloma cell line S194 was grown at 37 °C under 8% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 50 mM β-mercaptoethanol, 100 units/ml penicillin, and 100 µg/ml streptomycin. The pre-B cell line PD36, immature B cell line WEHI 231, pre-secretor BCL 1, and mature B-cell line K46R were grown in RPMI 1640 medium supplemented as above.

Plasmids—Progressive 5’ deletions were made in the J chain promoter and cloned into the reference pCAT3M plasmid as described previously (3). The 4-bp mutation in the JA site was introduced in the 1.2-kb J chain promoter by site-directed mutagenesis (Stratagene). For linked promoter analysis, the oligonucleotides representing the J chain promoter region were transiently transfected into the plasmacytoma S194 cell line and compared for CAT activity with the reference construct. Asterisks represent mutations introduced into the JA site. Mean percentages were calculated from four independent duplicate determinations. B, schematic representation of the J chain promoter, indicating the JA and JB sites. The mutated residues are underlined.

were washed three times for 10 min each in 1× phosphate-buffered saline. The filters were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech) for 1 h at room temperature. The filters were washed again three times with 1× phosphate-buffered saline and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS

Identification of a Positive Regulator, NFJA in the J Chain Promoter—In a previous study, a series of 5’ deletions within the J chain promoter led to the identification of a major IL-2-responsive control region containing both positive and negative elements (3). In the present study, a more precise deletion analysis of this region was performed to characterize the positive regulatory element within the J chain promoter. The reporter plasmid, pCAT3M, containing the 1.24-kb 5’-flanking J chain sequence upstream of the CAT gene was used to assess the activity of various deletion mutants of the J chain promoter region as described previously (3). These constructs of the J chain promoter region were transiently transfected using DEAE-dextran into the J chain-positive plasmacytoma cell line S194 to determine their relative abilities to activate transcription of the CAT gene. As shown in Fig. 1, deleting base pairs from −70 to −61 or −70 to −60 resulted in a 10-fold decrease in CAT activity. Moreover, site-specific mutations introduced between nucleotides 60 and 63 within the context of the J chain promoter resulted in a similar loss in CAT activity. These results indicate the presence of a major positive control element within the region upstream of the J chain gene. This positive element located within a previously identified hypersensitive site was designated JA, and the factor binding this element was termed nuclear factor JA (NFJA).

A computer search of previously known DNA binding sites performed to identify the NFJA factor revealed that the consensus recognition sequence for MEF2 family showed a strong homology to the JA sequence. The putative MEF2 element in the J chain promoter extending from nucleotide −56 to −65 (5’-TTATTTTAAG-3’) is the reverse complement of the consensus sequence (5’-(T/C)(T/A)(T/A)AAATA(A/G)-3’) recognized
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**Characterization of NFJA as a MEF2 Family Member**—The results above suggested that the region of the J chain promoter from −70 to −56 might contain a binding site for a MEF2-related transcription factor. We therefore performed gel shift assays with nuclear extracts from S194 using the B8(−70/45) probe, a 24-bp fragment containing the JA and JB sites, and detected the presence of several major DNA-protein complexes (Fig. 3A). The specificity of the complexes was determined by competition for binding with a 50-fold excess of unlabeled oligonucleotides. Competition of the B8(−70/45)32P-labeled probe with excess unlabeled double-stranded oligonucleotide B10, an 18-bp (−74/−56) J chain promoter sequence containing the MEF2 site, resulted in specific displacement of complex 1 and 2 binding. In contrast, unlabeled sequence PU.1(−63/−45) corresponding to the PU.1 site competed successfully for the binding of complex 3. To correlate specific binding of the putative MEF2 core sequence with the functional expression shown in Fig. 2, we examined the ability of the mutant oligonucleotide (−72/−57; mutant JA, 5′-CTGGGTTGTCGGGTAAG-3′) not containing the PU.1 site but with a 4-bp substitution within the MEF2 site (which abolished reporter specific expression) to compete for binding of the MEF2 site. As shown in Fig. 3A, the mutant oligonucleotide was unable to effectively compete for binding.

To initially assess whether the binding complex might reflect binding of a MEF2 family member, binding experiments were carried out with in vitro translated MEF2C (which binds the common consensus site of the MEF2 family) and the B8 probe (−70 to −45), which contains the putative MEF2 and PU.1 binding sites. The MEF2C cDNA was transcribed and translated in vitro, yielding a protein of apparent molecular mass of 50 kDa on SDS/polyacrylamide gel, in agreement with the predicted size based on the published cDNA sequence (11). Western blot analysis (using a MEF2 antibody cross-reactive with MEF2A, C, and D) of the translated product along with nuclear extracts from B cell lines revealed the presence of in vitro translated MEF2C and a band in nuclear extracts with similar size and mobility (Fig. 3B). Gel mobility shift assays showed that in vitro translated MEF2C bound the B8 probe strongly (Fig. 3C, 5th lane). Moreover, in vitro translated MEF2C protein and endogenous protein from the myeloma cell line S194 had similar DNA binding properties in terms of mobility of the complex. To directly demonstrate that the binding was in fact due to a

**Fig. 2.** Linked promoter analysis. Constructs containing single or four tandem repeats of the JA oligomer were assayed for their effects on a heterologous promoter-driven CAT expression system in J chain-positive S194 cell line. Hatched boxes indicate the 4-bp mutations introduced into the JA site.

**Fig. 3.** A, specificity of factor binding to the JA element in nuclear extracts from S194. Oligonucleotides were used as competitors to determine the specificity of factors binding to complexes 1, 2, and 3. B8 represents an oligonucleotide that contains binding sites for PU.1 and NFJA, B10 contains the binding site for NFJA, PU.1 contains the binding site for PU.1, and MJA represents the mutant oligonucleotide with a 4-bp mutation within the MEF2 site. B, Western blot comparing the mobility of in vitro translated MEF2C (lane 3) with endogenous B-MEF2 from nuclear extracts (lane 1, S194; lane 2, K46). C, effect of antibodies from PU.1 and MEF2 on the mobility shift assays of nuclear extracts from the S194 cell line and in vitro translated MEF2C.
MEF2 family member, we performed antibody blocking gel mobility shift assay. Incubation of the S194 extracts with a MEF2 antibody resulted in inhibition of the formation of complex 2 (Fig. 3C, 3rd lane), confirming the presence of a MEF2-specific DNA binding complex. We observed a similar inhibition of complex formation in the case of the in vitro translated protein (Fig. 3C, 7th lane). In contrast, control antiserum (anti-PU.1) did not result in any inhibition of complex 2 formation (Fig. 3C, 2nd and 6th lanes) but instead inhibited the formation of complex 3 (PU.1 specific DNA complex). The MEF2 antibody used in this study recognizes and cross-reacts with mouse MEF2A, MEF2C, and MEF2D proteins. Antibodies specific to MEF2B and MEF2D were used to further characterize the MEF2 family member present in the complex. Since neither antibody was able to inhibit formation of the complex (data not shown), these studies indicate that the MEF2 family member binding the MEF2 site is either MEF2A, MEF2C, or a previously unidentified family member. The antibodies available cannot distinguish between these possible family members. These results indicate that NFJA is a MEF2 family member (referred to as B-MEF2) with similar in vitro binding properties and an identical consensus binding site.

Functional Role of B-MEF2 in the J Chain Promoter—The MEF2 family of transcription factors shows a high degree of homology in the MADS box and the adjacent MEF2 domain, which together constitute the DNA binding domain of these proteins (12). Indeed, previous studies have shown that MEF2 proteins with mutations within this DNA binding region act as dominant negative mutants, which can inhibit activation of MEF2-dependent genes by wild type MEF2C (10). To investigate the functional in vivo role of B-MEF2 in J chain gene expression, therefore, a construct containing the DNA binding domain of MEF2C was overexpressed. If this mutant protein could function as a dominant negative mutant, it should suppress the activity of endogenously expressed B-MEF2. The DNA binding domain of MEF2C was cloned into the vector pcDNA1 (containing the cytomegalovirus promoter and enhancer) and co-transfected into S194 cells along with a CAT reporter gene construct containing 1.24 kb of the J chain promoter sequence. Control transfections included expression vectors containing no insert or a reporter construct in which the J chain promoter sequence. These results indicate that the dominant negative effect of the MEF2 mutant was specific to the J chain promoter MEF2 site.

To determine whether MEF2C could transactivate via the MEF2 site within the J chain promoter in B cells, cotransfection experiments were performed using MEF2C cDNA cloned into pcDNA1 as the expression plasmid and various reporter constructs containing the J chain promoter MEF2 site. The reporter constructs contained a heterologous minimal promoter (γ-fibrinogen) and four copies of the MEF2 binding site (JA) driving the bacterial CAT gene. The reporter and effector plasmids were then introduced into the S194 cell line. Transactivation of the CAT reporter gene was observed when MEF2C was expressed simultaneously. In S194 there was a 10-fold increase in CAT expression when both the reporter and MEF2C cDNA were cotransfected together. As shown in Fig. 4B, activation of the reporter constructs was dependent solely on the presence of the intact MEF2 binding sites and the presence of MEF2C. No increase in CAT activity was observed in the absence of JA sites. These results indicate that MEF2C can function as a transcriptional activator, specifically via the target JA site on the J chain promoter in B cells.

Correlation of B-MEF2 Binding with B Cell Differentiation—The expression of the J chain gene has been shown to correlate with the different stages of B cell differentiation. The J chain gene remains silent during the early antigen-independent stages of differentiation but becomes activated when the mature B cell makes contact with antigen and T cell lymphokines (22). The binding properties of B-MEF2 were therefore analyzed over a range of B lymphoid cell lines to determine whether a similar expression pattern during B cell differentiation was displayed. Binding properties of B-MEF2 were examined by gel mobility shift assays using the B8 probe (which includes the PU.1 site) and nuclear extracts from cell lines representing different stages in B cell development. As shown in Fig. 5A, the B-MEF2 complex appeared in the highly differentiated S194 cell line. Low or almost undetectable amounts were observed in the pre-B cell line PD96, immature B cell line WEHI 231, and the mature B cell line K46R, representing the early stages of B cell differentiation. To determine whether the amounts of bound B-MEF2 correlated with the levels of MEF2 family member proteins present in these cell lines, we performed Western blot analysis. Nuclear extracts from B cell lines representing different stages in B cell differentiation were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting using antibodies rec-
recognizing MEF2 family members. The same blots were also probed with antibodies specific for actin to confirm equivalent loading of sample protein. As shown in Fig. 5B, detectable levels of MEF2 proteins were observed in cell lines representing the early stages of B cell differentiation. These results indicate that the presence of MEF2 transcription family members in early B cells does not correlate with increased binding to the MEF2 site. However, the presence of high levels of expressed and bound MEF2 complex in the highly differentiated plasmacytoma S194 cell line is consistent with the role of B-MEF2 as a positive regulator of the J chain gene.

Regulation of B-MEF2 by IL-2 and IL-5—In a primary immune response J chain, gene expression is induced by cytokines such as IL-2 or IL-5, leading to the secretion of the pentameric (IgM)_5J antibody. This J chain response has been duplicated in vitro in the cloned murine B cell lines with physiological doses of recombinant IL-2 and/or IL-5. The effect of addition of these cytokines was therefore analyzed to determine whether there were any changes in the levels of MEF2 family member proteins. We utilized the BCL1 cell line as a model for normal B cell activation, because it can be induced to express high levels of J chain protein after IL-2 or IL-5 stimulation. Nuclear proteins were extracted after 3 days of IL-2 or IL-5 stimulation and assayed for MEF2 proteins by Western blot analysis. We found that the addition of IL-2 or IL-5 resulted in increased expression of MEF2 proteins (Fig. 6A). This is consistent with the levels of expression of the J chain gene in BCL1 cells on induction by IL-2 or IL-5 (Fig. 6A). We also performed mobility shift experiments with nuclear extracts that showed an increased binding of B-MEF2 protein in activated BCL1 cells in comparison with unactivated BCL1 cells (Fig. 6B, 1st and 2nd lanes). In contrast, as we reported earlier, the levels of PU.1 were unchanged after the addition of IL-2 (4). Oligonucleotides specific for PU.1, MEF2, and mutant MEF2 were used to show specificity of the bound complexes (Fig. 6B, 3rd–5th lanes). These results suggest that the amounts of B-MEF2 protein are induced by IL-2 and IL-5, similar to the induction observed with J chain.

**DISCUSSION**

In the present study we have demonstrated that a member of the transcription factor MEF2 family, B-MEF2, specifically binds the JA element of the J chain promoter. By deletion analysis, the binding site for MEF2 was identified between 274 and 256 nucleotides within the J chain promoter. Mutations in the JA site resulted in an almost complete loss of CAT activity when the JA site was placed under the control of the J chain promoter or with a heterologous minimal promoter. By several criteria we show that the DNA-binding protein that interacts with the JA site is a MEF2-related protein. First, in vitro translated MEF2C recognizes and binds the JA site with strong and comparable affinity as endogenously expressed B-MEF2. Second, oligonucleotides corresponding to the JA site competed specifically with the B8 probe for binding to the B-MEF2 protein in nuclear extracts, whereas a mutant oligonucleotide failed to compete with the B8 probe in similar binding experiments. Also, antibodies recognizing MEF2 family members were able to inhibit complex formation in both nuclear and in vitro translated extracts. Finally, we show that MEF2C trans-activates reporter constructs containing the JA site, and a dominant negative mutant of MEF2C suppresses the transcriptional activity of the J chain reporter construct.

The presence of a MEF2 binding site within the J chain promoter was unexpected since MEF2 factors have previously
been described to be muscle-specific factors (23, 24). Indeed, all the MEF2 factors form hetero- and homodimers that bind to an AT rich consensus sequence found in the control regions of numerous muscle-specific genes. More recently however, transcripts of MEF2 family members have been shown to be more widely expressed. The potential function of MEF2 proteins in these various non-muscle tissues has yet to be elucidated. In this study we provide evidence for a novel role for the MEF2 family of transcription factors in B cell development. The role of B-MEF2 in immune cell development described here is specifically important, since this expands the regulatory potential of these proteins to regulate gene transcription during development in cell types not restricted to muscle lineage. Moreover, these proteins along with their isofoms could recruit other transcription factors into combinatorial interactions forming regulatory complexes, providing a network of transcription control.

The pattern of MEF2 binding and expression in B cell lines representing different stages of differentiation suggests that low levels of B-MEF2 are expressed through the early stages of development. However, in activated B cell lines, the amounts of bound B-MEF2 protein increase, correlating with the expression of J chain during B cell differentiation. A similar correlation of MEF2 expression with differentiation of myocytes is also known to exist. For example, similar to the pattern observed in B cell differentiation during embryogenesis, MEF2 transcripts appear initially in precursors of the cardiac and skeletal muscle lineage and are subsequently expressed at high levels in these differentiated muscle cell types (25, 26). Also, mutations of the D-mef2 gene in Drosophila melanogaster suggest that MEF2 is an essential cofactor for differentiation of skeletal, cardiac, and visceral muscle cells (27). In the absence of D-MEF2, myoblasts fail to differentiate despite being correctly positioned. These observations along with our results in B cell differentiation suggest a more wide-ranging role for MEF2 in differentiation processes that has previously been envisaged.

Previous studies have shown that either IL-2 or IL-5 stimulate antigen-activated B cells to express the J chain gene necessary for the secretion and assembly of pentameric (IgM) J antibody (28, 29). Further analyses of the IL-2 signal revealed that transcription of the J chain gene correlates with chromatin changes in the 5′ region. We show here that the levels of B-MEF2 are also induced by treating BCL1 cells with IL-2 or IL-5. The induction of B-MEF2 by cytokines is significant because it demonstrates a direct link between B-MEF2 activation and the induction of the J chain promoter by cytokines. These studies also indicate that MEF2 family members may regulate gene expression via IL-2 and IL-5 signaling pathways. Earlier studies have shown that in monocytic cells, activation by LPS increases the transactivation activity of MEF2C through MAP kinase p38-catalyzed phosphorylation, resulting in an increased transcription of the c-jun gene (19). Also, serum treatment has been shown to regulate the activation of MEF2C (30) and MEF-2D (31), thereby increasing expression of the c-jun gene. The serum-induced MEF-2C activation requires the MEK5/BMK1 mitogen-activated protein kinase-signaling pathway. Since mitogen-activated protein kinases regulate several cellular processes including differentiation, growth, and apoptosis (32, 33), these studies indicate that MEF2 proteins may have a broader role in regulating gene expression. These findings also indicate that members of the MEF2 family can mediate responses from various external agents including cytokines by using different signaling pathways.

We were also interested in determining whether adjacently bound PU.1 synergizes with B-MEF2 to activate the transcription of the J chain gene. We found that B-MEF2 and PU.1 have transactivating capabilities that are likely to function independent of each other (data not shown). Furthermore, when they are present together, they bind the J chain promoter, resulting in an additive rather than a synergistic effect. Moreover, we found no evidence of any in vitro interaction between B-MEF2 and PU.1 using co-immunoprecipitation experiments (data not shown). These results are consistent with our earlier observations, which indicated that PU.1 does not require the cooperation of a second nuclear factor for its transactivation function (4).

The mechanism by which B-MEF2 serves to activate transcription of the J chain gene is not known and is currently under investigation. Among the possibilities is the interaction with the recently discovered enhancer upstream of the J chain promoter (34). This enhancer element, which also responds to IL-2, has several binding sites for transcription factors including CBF (core binding protein) and bHLH protein (E protein), which could interact with B-MEF2 to promote transcription of the J chain gene. Similar mechanisms have been proposed for the role of MEF2 in myogenesis (35). For instance, it is known that though MEF2 factors lack myogenic activity, they have been shown to potentiate the activity of myogenic bHLH factors. This potentiation appears to be mediated by direct protein-protein interactions between MEF2 and heterodimers formed between bHLH factors and E proteins (36, 37). Therefore, we propose that B-MEF2 may function as an accessory factor for another transcription factor in the activation of J chain gene transcription, similar to the role of other members of the MADS box factor family.

The results of the present study demonstrate that J chain gene expression is regulated by B-MEF2. Further investigation is necessary to determine whether this factor is MEF2A, MEF2C, or a novel related protein. The selective expression and function of B-MEF2 in the non-muscle system of B lymphocyte differentiation suggests a wide-ranging role of this factor in developmental processes.

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