Characterization of a Cis-Acting Regulatory Element which Silences Expression of the Class II-A β Gene in Epithelium

By Shelley E. Albert,* Frank Strutz,* Kathleen Shelton,* Thomas Haverty,* Mae Jane Sun,* Shao-ran Li,* Amy Denham,* Richard A. Maki,$ and Eric G. Neilson*†

From the *Penn Center for Molecular Studies of Kidney Diseases, Renal-Electrolyte Division of the Department of Medicine, †the Graduate Groups in Immunology and Cell Biology at the University of Pennsylvania, Philadelphia, Pennsylvania 19104; and the ‡La Jolla Cancer Research Institute, La Jolla, California 92161

Summary

Class II major histocompatibility complex (MHC) genes encode for α/β chain pairs that are constitutively expressed principally on mature B cells and dendritic cells in mice. These gene products are easily induced on macrophages with cytokines, and may also aberrantly appear on the surface of epithelium during immune injury. The appearance of class II determinants in parenchymal tissue potentially renders these somatic cells capable of antigen presentation to circulating CD4+ T lymphocytes, and their absence may be protective for normal tissues expressing self-antigens. The low surface class II expression observed on parenchymal cells generally correlates with low levels of mRNA, suggesting that transcription rate is a major element in class II regulation. To understand the transcriptional mechanism maintaining low basal surface expression of class II in somatic cells, we transiently transfected mini-gene reporter constructs to study the regulation of the murine Aβ promoter in a cultured renal epithelial cell line. We describe here a negative cis-acting regulatory region located between -552 and -489 bp upstream of the Aβ cap site that silences the transcriptional activity of the Aβ promoter in epithelial cells in an orientation-dependent manner, and is also able to silence a heterologous promoter. This region is not active in class II-expressing B cells (BAL-17) in culture, but is functional in two other murine class II-negative cell lines, fibroblasts and thymoma T cells. Using competition electrophoretic mobility shift assays, we have localized the core protein binding site within this region to an 8-10-bp response element, designated AβNRE, at -543 to -534 bp. A nuclear extract from BAL-17 cells does not bind to this element. Mutation of this site abrogates the transcriptional silencing activity of the region. We conclude that the transcription of class II-Aβ in parenchymal cells, and some lymphocytes, can be actively repressed by an upstream silencing element.

The gene products of the class II region of the MHC are required for the recognition of antigen by CD4+ T lymphocytes (1). Class II molecules are normally expressed constitutively in mature B lymphocytes (2) and dendritic cells (3), and are easily induced in antigen-presenting macrophages with IFN-γ or TNF-α (4). Other somatic cells, such as organ epithelium, normally express little or no class II on their cell surface, but can increase their expression during episodes of local inflammation. This upregulation has been noted in such settings as type I diabetes mellitus (5), GVHD (6), and various forms of tubulointerstitial nephropathy (7, 8). Again, IFN-γ has been implicated as the primary mediator (for reviews see references 9 and 10).

Under circumstances of increased class II expression, some nonlymphoid cells (11-13) including renal tubular cells (14-17), are capable of presenting both self and foreign antigen to CD4+ T lymphocytes or hybridomas. The surface expression of class II as a gene product correlates well with levels of mRNA transcript, and a variety of studies suggest that levels of mRNA encoding AαAβ/EαEβ are regulated by transcriptional controls rather than by modulation of stability of transcript levels (9). Transcript levels are generally abundant in mature B cells, and very low in somatic parenchymal cells. Thus, an insight into how class II determinants are normally repressed in somatic cells, particularly at the transcriptional level, may be important in understanding the antigen-presenting function of parenchymal cells in contributing to the maintenance of a local immune response against self (8, 18, 19).

The cis-acting response elements and associated trans-acting
nuclear proteins involved in the positive enhancement of class II transcription in murine and human lymphoid cells have been well characterized, with most of the key elements clustering in a proximal promoter region in the first 200 bp upstream of the transcription start site (9). Two lines of evidence suggest that the absence of class II expression on other cell types is at least partly a function of active silencing by negative-acting nuclear factors, as opposed to simply a lack of transcriptional enhancers. First, fusion of class II–positive B cells with either class II–negative plasma cells (20) or fibrosarcoma cells (21) extinguishes both basal and inducible mRNA expression of class II genes in the hybrid. Second, many of the trans-acting proteins that bind to the conserved X and Y box promoter elements upstream of all class II genes can be detected in both class II–positive and –negative cell lines (22, 23), although it has been suggested that these do not occupy their binding sites in class II–negative cells in vivo (22). Here we describe a novel cis-acting element upstream of the murine Aβ gene which acts to silence transcription of that gene in low class II–expressing renal tubular epithelium, fibroblasts, and T cells, but not in class II–positive B cells.

Materials and Methods

Cell Lines. MCT epithelium is a low class II–expressing (AαAβ+/EαEβ−) murine proximal renal tubular cell line (14) that has been maintained in culture in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. BAL-17 is an H-2d B lymphoma cell line that expresses high levels of class II (kindly provided by Dr. John Monroe, University of Pennsylvania; 24), and was maintained in RPMI 1640 medium with 10% FCS, penicillin, and streptomycin, and 0.001% ZnSO4, but was otherwise unchanged. BAIA7 nuclei were extracted in 20 mM Hepes, 15% glycerol, 1.5 mM MgCl2, 5 mM dithiothreitol (DTT), and 0.4 M KCl, then prehybridized, and hybridized with a 0.79-kb fragment from cDNA clone p71sa encoding murine Aβ (26). Selected filters were stripped and rehybridized with a cDNA probe encoding the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to account for small loading and transfer variations.

Transfections and CAT Assay. Transient transfections of MCT, BAL-17, and 3T3 cells were carried out using calcium phosphate precipitation (28); replicate experiments were performed as indicated in the figure legends. 5 × 10^5 MCT cells were plated in 60-mm dishes and transfected 24 h later with 10 μg of plasmid DNA (or equimolar equivalents), incubated in 5% CO2 at 37°C for 24 h, after which time the precipitate was removed and the cells were incubated for a further 24 h in complete medium. Cells were lysed by repeated freeze–thaw cycles. Transfections of BAL-17 cells were carried out using DEAE-dextran (29). 10^7 BAL-17 cells were transfected with 20 μg plasmid DNA in 500 μg/ml DEAE-dextran for 30 min followed by a 3-min shock in 10% DMSO. Cells were harvested 64 h after transfection. CAT assays were performed with all cell lysates heat inactivated at 65°C for 10 min, and incubated for 2 h in the case of MCT cells, or 16 h in the case of BAL-17 cells (28). Loading of cell lysates for incubation was normalized for protein content.

Nuclear Extract Preparation and Gel Shift Assay. MCT nuclear extracts were prepared according to the method of Dignam et al. (30) with the following modifications: three additional protease inhibitors, leupeptin, aprotinin, and pepstatin A (Boehringer Mannheim Biochemicals, Indianapolis, IN) were added to all buffers. Nuclei were extracted in 20 mM Hepes, 15% glycerol, 1.5 M MgCl2, 5 mM dithiothreitol (DTT), 0.4 M KCl, 1 mM EDTA, and 0.5% NP-40. Buffer D contained 15% glycerol and 0.5 M ZnSO4, but was otherwise unchanged. BAL-17 nuclei were extracted in the presence of 0.4 M NaCl rather than KCl, and in the absence of NP-40. The probe S89A was created by amplification of an 88-bp fragment from −605 to −517 of plasmid 12.19 using the flanking oligomers 5'-gtcaacGAGGGCTGAGACACACATTA-3' and 5'-gtcaacAGAGCAGTGTGATGTGGTT-3'. The product was cloned into the vector pCR II and digested with Accl to produce 5' overhangs which were filled in with [32P]dCTP and [32P]dATP (Amersham Life Sciences, Arlington Heights, IL) using Klenow fragment polymerase. 80,000 cpm of probe was incubated with 15 μg of nuclear extract in the presence of 2 μg poly d(IC), other competitor DNA as indicated, 5% glycerol, 50 mM NaCl, 10 mM Tris (pH 7.9), 1 mM EDTA, 5 mM MgCl2, 1 mM DTT, and 3 μg BSA in a 15-μl volume for 15 min at 21°C. Reaction products were electrophoresed through a 5% polyacrylamide gel in 0.5× TBE (Tris-borate/EDTA) at 10 V/cm for 2 h. The gels were dried and exposed to x-ray film without enhancement overnight.
Results

MCT Epithelial Cells Express Low but Detectable Levels of Aβ mRNA. We sought to determine the transcriptional mechanism whereby parenchymal epithelium maintains immunologic neutrality by means of low MHC class II expression, and made use of a renal tubular cell line to do so. MCT epithelial cells normally express little to no Aα/Aβ on their cell surface, but this expression can be upregulated somewhat with IFN-γ (8). Unlike mature B cells which constitutively express class II determinants, MCT cells would be predicted to have very low levels of mRNA encoding class II. Fig. 1 presents a 10-d exposure of a Northern blot of the Aβ mRNA message level in MCT cells compared with the class II-expressing BAL-17 B cell line. Basal class II mRNA message levels in MCT cells are clearly much lower than in B cells. We have also previously isolated full-length cDNA clones encoding Aβ from an MCT library, sequence analysis of which indicated that there are no mutations in the coding region of Aβ which would explain its low surface abundance by aborted translation (26).

A DNA Segment Upstream of the Aβ Gene Silences the Homologous and a Heterologous Promoter in MCT Cells. In Fig. 2, a representative transfection of MCT cells with Aβ gene deletion constructs demonstrates strong CAT activity with the shorter promoter segments which drops off in activity between −669 and −489 bp, indicating the possible presence of a negative cis-acting element in this region. The position independence of this region was established by creating plasmid Δ669/489.8, in which region −669 to −489 was ligated directly to the homologous proximal promoter for Aβ contained in a 322-bp segment upstream of the gene. As can be observed in Fig. 3 A, this region was still able to silence the strong positive CAT activity of the downstream region in MCT cells, but only when placed in the forward orientation (F); similar results were observed with 3T3 fibroblasts (Fig. 3 B) and 2B4 thymoma T cells (Fig. 3 C). Transfection of BAL-17 cells with these constructs (Fig. 4), however, revealed no silencing of proximal CAT activity by Δ669/489.8, suggesting the absence of a relevant trans-acting factor in these class II-expressing B cells.

To functionally map the silencing region more closely, further constructs were created in which portions of this region were linked to the proximal homologous promoter (31), as diagrammed in Fig. 5. Transfection of these constructs into MCT cells further localized the silencing element to a region between −552 and −489. Again, the orientation dependence of this silencing effect is evident. This same region was also able to silence the heterologous SV40 enhancerless promoter, as shown in Fig. 6. This region in Aβ was of identical sequence to that found in the genomic DNA used to assemble the original AβCAT constructs used above (25), with the exception of a single base pair substitution at position −547.

Mapping the Core Binding Site of the Putative Silencing Factor. Gel shift analysis was performed using an 88-bp probe from −605 to −517, and revealed one major shifted band of interest (Fig. 7, lane 1). Competition analysis using 100-fold molar excess of the unlabeled double-stranded oligomers diagrammed in Fig. 7 revealed that the S12 oligonucleotide (lane 3), but not S11 or S14 (lanes 2 and 4), was able to com-
Figure 3. Effect of -669 to -489 region on homologous promoter in MCT cells (A), 3T3 fibroblasts (B), and 2B4 thymoma T cells (C). The -669 to -489 region was linked directly to the proximal promoter region (contained in a 322-bp segment upstream of the cap site) in the forward (F) and reverse (R) orientation. Percent acetylation (average of three independent transfections) is expressed relative to that of 8.54, which was set at 100%.

Figure 4. Effect of -669 to -489 region on homologous promoter in BAL-17 cells. Results expressed as described in legend to Fig. 3.

Figure 5. Localization of silencer region to -552 to -489 bp. Diagrammed constructs were transfected into MCT cells with results expressed as described in legend to Fig. 3.

Discussion

The immunologic implications of aberrant expression of class II MHC gene products on nonlymphoid cells, as seen...
in a wide array of settings and likely mediated primarily by IFN-γ, have been the subject of considerable interest in recent years. Although several groups have been able to demonstrate a T cell proliferative response to intact (15, 33) or processed (11) antigen presented by class II-expressing parenchymal cells, implying that such cells contribute, under certain pathologic conditions, to the local immune response, others have suggested that such "nontraditional" antigen presentation may in fact lead to T cell anergy, and thus contribute to peripheral self-tolerance (34). In either event, studying the normal mechanisms maintaining low epithelial class II expression (and thus immunologic silence) will contribute to an understanding of issues pertaining to peripheral tolerance and organ-specific autoimmunity.

It has become increasingly appreciated that transcription of eukaryotic genes may be modulated by negative-acting elements in addition to the transcriptional enhancers that have long been recognized. Since the first description of silencing elements in yeast (35), numerous studies have demonstrated factors that exert a negative effect on the transcription of a wide range of genes (31, 32, 36, 37) including MHC class II (38). That such a silencing factor might be operative in suppressing the transcription of class II genes in nonexpressing cells is suggested by fusion studies that have demonstrated extinction of class II expression in hybrids of class II-negative and -positive cells (20, 21). In one of these (21), multiple clones of four independent fusions of L929 fibrosarcoma cells with splenic B cells were studied for both basal and inducible class II message and gene product. Although the hybrids retained expression of class I genes derived from both fusion partners and retained the Eβ gene at the genomic level, none of the clones had any detectable class II expression. Similar findings were noted using transient fusion techniques, so that chromosomal loss appeared not to have been the mechanism of class II extinction. A more recent study found reactivation of low levels of class II transcript in plasmacytoma cells and human T cells on transient fusion with B lymphoblastoid cells and to a much lesser extent with splenocytes (39). Whereas these opposing results are difficult to reconcile, the study by Stuart et al. (21) is highly suggestive of the presence of a negative trans-acting factor operating in at least some cell types. In addition, two studies have previously demonstrated functional evidence of a class II silencer element in the region we report. Boss and Strominger (40), using stable transfection of DQβ constructs into fibroblasts, found a threefold increase in transcription on deletion of upstream DNA from −2,500 to −590 bp, and a further 3.3-fold increase on deletion to −159 bp. Thanos et al. (41), using transient transfection of Eα constructs into a xeroderma cell line, found an increase in basal expression on deletion from −873 to −353 bp.

**Figure 6.** Silencer region can repress a heterologous promoter. The −552 to −489 silencer region was linked to the enhancerless SV40 promoter transfected into MCT cells. Percent acetylation is expressed relative to that for the SV40 promoter \(Spr\) alone.

**Figure 7.** Gel shift analysis of silencer region. Labeled probe S89A (sequence shown) was incubated with MCT nuclear extract without (lane 1), or with 100-fold molar excess of an unlabeled double-stranded oligonucleotide as indicated (lanes 2–9). (−) Nucleotide identity with the S89A probe. (Arrow) Position of major shifted band. Base pairs found to be essential for protein binding by competition analysis are underlined.
bp. To date, the region in the range of -1,200 to -400 bp upstream of the transcription start site for class II genes has not been examined in further detail.

We have mapped a novel silencing region to a segment between -552 and -489 bp upstream of the murine Aβ cap site. This region, which acts in a position-independent, but orientation-dependent manner, is active in a low class II–expressing renal tubular epithelial cell line (MCT), but not in a class II–positive B lymphoma cell line (BAL-17). Gel shift competition analysis using a series of mutated oligonucleotides localized the core protein binding site, termed AβNRE, to an 8–10-bp AT-rich element at position -543. Mutation of this binding site abolished the ability of the region to silence the transcriptional activity of the downstream positive-acting elements of the Aβ gene when linked directly upstream of them. Our studies of this region in two other murine class II–negative cell lines (one a fibroblast and the other a T cell) suggest that this upstream silencer may represent a relatively ubiquitous mechanism of class II transcriptional repression.

A search of the GenBank™ database revealed no homology between AβNRE and other known transcriptional elements. However, a novel member of the bZIP family of DNA-binding proteins, E4BP4, has recently been isolated from a human placental cDNA library (42), and its optimum binding site, (G/A)TTATGTAAC(C/T), bears a marked degree of homology of AβNRE, (CC)ATTATTAA(C). Cotransfection of an E4BP4 expression plasmid with a reporter bearing its binding site upstream of a heterologous promoter revealed it to have transcriptional repressing activity. The function and distribution of this binding protein is currently unknown. Although AβNRE is not found in the first 1,296 bp 5' of the translation start site in the Aα gene, the first 8 bp of the element, CCAATTATT, are found 480 bp upstream of the transcriptional start site of Aβ's analogous human gene, DQβ. In fact, an examination of the proximal promoter region reveals a greater degree of identity of conserved elements between Aβ and DQβ than between Aβ and Aα, with at least one element (a nuclear factor kB binding site) which is present in both Aα and DQα but not in the corresponding β chain promoters (10). Whether Aα is coordinately silenced by a poorly conserved version of AβNRE or by an entirely different mechanism remains to be determined.

Transcriptional repression of eukaryotic promoters is mediated by DNA-binding proteins which act either by competing for DNA-binding sites, by quenching or sequestering of activator proteins, or by direct repression of the basal transcription complex (43, 44). The mechanism of action of the putative silencing protein that binds to AβNRE can only be speculated upon, but any model must take into account two key findings. First, Liou et al. (45) have found that class II–expressing cells possess three DNase I–hypersensitive sites in their Aα genes, at -1,400 bp, in the first intron, and in the proximal promoter region. The latter of these, D2, was absent in class II–negative cell lines, and on fusion of T and B cells (in which class II expression was extinguished), the authors demonstrated the absence of the D2 site derived from the Aα gene of either fusion partner in the hybrid. Second, it has been shown that the class II promoter elements are not occupied in vivo in class II–negative cell lines, although their binding factors can be detected in in vitro gel shift assays (22). Thus, the AβNRE binding protein might be expected to act in a manner analogous to the α2 repressor of the yeast S. cerevisiae, which is able to place a nucleosome near a promoter site (46). This would have the effect of rendering the promoter both inaccessible to activating factors and insensitive to DNase I. However, silencers that function in this manner tend to demonstrate both position and orientation independence (43), and so the marked orientation dependence of AβNRE is somewhat puzzling. A lesser degree of orientation dependence was noted when a 274-bp segment from the first intron of the α1(I) collagen gene was placed upstream of the promoter of that gene (36), so our finding is not without precedent. IFN-γ (or factors induced by it), which exerts its class II upregulating effect through the proximal promoter region (47), could disrupt this nucleosome structure, as has been shown in the case of the glucocorticoid–glucocorticoid receptor complex and the murine mammary tumor...
virus promoter (48). Alternatively, both constitutive class II-expressing and IFN-γ induced cells might produce a factor that is able to sequester the silencing protein and prevent its effects on promoter accessibility. A fuller understanding of the mechanism of action of the nuclear factor recognizing A3NRE must await isolation of this protein.

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Address correspondence to Dr. Eric G. Neilson, 700 Clinical Research Building, University of Pennsylvania, 422 Curie Building, Philadelphia, PA 19104-6144. S. Albert's current address is the Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ont., Canada, N6G 1X8.

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