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The locus control region of the MHC class II promoter acts as a repressor element, the activity of which is inhibited by CIITA

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ABSTRACT

The closest region of the promoter of *MHC II* genes and particularly three conserved boxes (X, Y and S) are fundamental for the transcriptional regulation. A second set of conserved sequences is present approximately 1200–1500 bp upstream in opposite orientation. In transient transfection experiments in IFN-γ-treated macrophages and in B lymphocytes, we determined the expression of a fragment of 2035 bp of the *I-Aβ* gene, which contains the upstream boxes. Mutation of the distal boxes increased induction, thereby suggesting a repressive effect on transcription. *In vitro*, the proximal and distal ends of I-Aβ promoter were ligated in the presence of nuclear extracts from untreated macrophages but not when the extracts were obtained from IFN-γ-stimulated cells. The mutation of distal or proximal boxes resulted in a decrease in the ligation assay. The addition of recombinant CIITA to untreated nuclear extracts decreased the capacity of the promoter to be ligated. Finally, we observed increased capacity to ligate the promoter in extracts from B cells lacking CIITA, but not from B cells lacking RFXANK. These results allow us to postulate a model where the proteins in the proximal and distal conserved sequences interact. When CIITA is induced, these proteins make an enhanceosome, allowing chromatin to open and initiate transcription.

1. Introduction

MHC class II (MHC II) proteins play a key role in immune response (LeGuern, 2003; Watts, 2004). They participate in the generation of the T cell repertoire in the thymus and are required for antigen presentation to T lymphocytes. MHC II proteins are normally expressed on a limited number of cell types, including B, thymic epithelial, dendritic and glial cells, as well as activated macrophages (Glimcher and Kara, 1992). The aberrant expression of MHC II proteins has been implicated in immune dysfunction. The absence of class II expression in humans (Reith and Mach, 2001) and in experimental models (Grusby and Glimcher, 1995) leads to a severe combined immunodeficiency. Abnormal expression of MHC II molecules may be linked to the development of autoimmune diseases (Bottazzo et al., 1983; Foulis and Farquharson, 1986).

The expression of MHC II molecules is constitutive in some cells such as B lymphocytes and dendritic cells, while in others, such as macrophages, *MHC II* genes can be induced by the stimulation with IFN-γ. This expression pattern is controlled mainly by transcriptional regulation (Benoist and Mathis, 1990; Mach et al., 1996; Reith and Mach, 2001; Ting and Trowsdale, 2002). However, IFN-γ also regulates the expression of MHC II molecules that act at post-transcriptional level (Cullell-Young et al., 2001; Gonalons et al., 1998).

Within 150–300 bp upstream of the core promoter of all *MHC II* genes, there is a characteristic module of three elements (S, X and Y boxes), which is tightly conserved in sequence, orientation and spacing (Benoist and Mathis, 1990; Mach et al., 1996). *In vitro* reporter gene experiments have shown that these three cis-acting elements are essential for the transcriptional regulation of *MHC II* genes. Nuclear factors bind each element. The S–Y module is recognized by an MHC II-specific transcription factor called regulatory factor X (RFX) (Reith and Mach, 2001; Ting and Trowsdale, 2002), which comprises three subunits RFX5, RFXANK and RFXAP. The NFY complex (composed by NFYA, B and C) binds to the Y box. Finally, CREB binds to the X box (Ting and Trowsdale, 2002). The binding of RFX, NFY and CREB to DNA creates a large multiprotein enhanceosome complex that is required for the recruitment of CIITA, the expression of which provides the tissue specificity of *MHC II* genes (Masternak et al., 2000; Reith and Mach, 2001; Ting and Trowsdale, 2002). Both the enhanceosome assembly and the recruitment of CIITA are essential for transcription of *MHC II* genes.

Although the proximal promoter can direct cell type-specific and IFN-γ-induced expression of reporter gene constructs transfected transiently into a variety of cell lines (Ting and Trowsdale,

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2002), this promoter is not sufficient to reproduce the normal pattern of MHC II expression *in vivo* in the context of chromatin. For instance, expression of *H2-Eα* transgene in B cells requires not only the proximal promoter but also a distal region situated between 1.3 and 20 kb upstream (Carson and Wiles, 1993; Dorn et al., 1988). This distal region functions as a regulatory element known as a locus control region (LCR) (Dean, 2006). This LCR is bound by RFX and CIITA, thereby inducing long-range histone acetylation, RNA polymerase II recruitment and the synthesis of extragenic transcripts within the LCR (Masternak et al., 2003).

Here we show that in the absence of CIITA, the LCR acts as a repressor element. The proteins that bind to the proximal and distal elements interact, thereby allowing the formation of a loop. When CIITA is present, the loop is open, which allows transcription. Here we report an unexpected regulatory mechanism of *MHC II* genes.

2. Materials and methods

2.1. Cells and reagents

The following murine cell lines were used: WEHI 3B, RAW 264.7 and WR19M.1 (myelomonocytic), D2SC/1 (dendritic cells), 70Z/3 (pro-B cell), A20 (B cell lymphoma), NS1 (plasma cell), EL-4 (T cell) and L929 (fibroblast). These lines were maintained in DMEM medium (BioWhitaker, Verviers, Belgium) containing 2 mM glutamine, 50 μM 2β-mercaptoethanol, 10% heat-inactivated FCS (PAA Laboratories, Pasching, Austria), 100 U/ml penicillin, and 100 μg/ml streptomycin. The human B lymphocyte cell lines Raji, RJ2.25 (cells lacking CIITA) and BLS-1 (RFANK-deficient) were also used and cultured as described (Reith et al., 1988). In some experiments bone marrow-derived macrophages were used and obtained as described (Celada et al., 1984). Animal use was approved by the Animal Research Committee of the University of Barcelona (# 2523). Recombinant cytokines were obtained from R&D Systems Inc. (Minneapolis, MN). All other chemicals were of the highest purity grade available and were purchased from Sigma.

2.2. Plasmids

The H2 I-Aβ promoter (from −1960 to 65 bp) (Celada et al., 1988) was cloned directionally into the luciferase reporter plasmid pGL3-Basic (Promega). The constructs carrying distinct deletions of the 5′ region were made either by PCR amplification and cloning or by endonuclease digestion from the complete promoter. The construct lacking the region from −1960 to −216 bp was created by PCR DNA amplification with the forward-(GGGAAAGAGACTTATTGT) and reverse-(CTCTAAGCTTCAGGTAATGGCAG) primers and sequential digestion with PvuII and PstI enzymes. The distal-less pGL3-I-Aβ module was obtained by EcoRV and StuI digestion and religation starting from the complete plasmid pGL3-I-Aβ. A similar strategy was used to obtain deletions from −1391 to −389 bp or from −1483 to −136 bp in the region comprised between distal and proximal modules and to introduce the distal module in the opposite orientation. To mutate the S′ X′ and Y′ boxes, a new restriction site was introduced. Briefly, two PCR were performed, one using the original 5′ oligonucleotide and a mutated 3′ primer which contained the mutation, and the other using the original 3′ primer and a new 5′ oligonucleotide introducing the mutation. Both fragments were then cloned separately into the pCR2.1 vector and restricted, thus obtaining two fragments, one corresponding to the new mutation of the 5′ end of the promoter and the other from the new mutation to the 3′ end. The ligation of these two fragments into the pGL3-Basic vector rendered the full-length promoter with the mutation. All the mutations were checked by sequencing. A fragment of 2678 bp corresponding to the upstream region of the human *HLA-DRA* gene was cloned and inserted into a pcDNA3 vector (Invitrogen) by EcoRI and XhoI restriction sites. The CIITA vector was kindly provided by Dr. Richard A. Flavell (Yale University School of Medicine, New Haven, CT) (Chang and Flavell, 1995).

2.3. Transient transfections and luciferase assays

WEHI 3B cells were transfected using Fugene 6 reagent (Roche). 2×10^5 cells were transfected with 1 μg of plasmid following the manufacturer's instructions. Twenty-four hours after the transfection, cells were stimulated with IFN-γ (300 U/ml) for another 24 h, then lysed, and luciferase activity was measured with a Luciferase Dual System Assay (Promega) on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) (Marques et al., 2004). Luciferase activity was normalized to protein concentration.

2.4. Nuclear extracts and ligation assay

Nuclear extracts from all the cells were prepared as described (Herrero et al., 2001). To perform the ligation assay, the incubation was performed for 20 min at 16 °C. The mixture included 20 ng of the I-Aβ promoter, lineal with blunted ends, 20 pg of pGL3 vector, which was used as internal control, and 20 μg of nuclear extracts. Then 1 U of T4 DNA ligase (Roche) and the appropriate buffer was added and the ligations were performed in the preliminary assays overnight at 16 °C. For quantification of DNA using PCR, the incubation was for 20 min. In control experiments, DNA was digested with BglII and the resulting samples were run in an agarose gel. DNA was purified by chloropan extraction and ethanol precipitation and digested for 1 h at 37 °C with 50 U of Exonuclease III (Takara), to prevent the formation of concatenamers, and proteins were later removed with proteinase K (Sigma). The ligation products were measured by real-time PCR using SYBR Green PCR Core Reagents (Applied Biosystems) in an AB1 Prism 7900 Sequence Detection System (Applied Biosystems) (Marques et al., 2004). The primers (5′-GTGACTGCCATTACCTG-3′ and 5′-AATCAGTGCTTCAGTGG-3′) were designed in such way that they amplified only circularized DNA, and as an internal control we amplified a random fragment of the pGL3-Basic vector (5′-GGTAACTATGCTCTTGACTCC-3′ and 5′-CTGTTCTTCTAGTGTAGCCG-3′). CIITA protein was synthesized by *in vitro* transcription-translation kit (TNT Quick Master Mix, Promega), following the manufacturer's protocol.

2.5. Quantitative RT-PCR analysis

Cells were washed twice with cold PBS, and total RNA was extracted with the EZ-RNA kit (Biological Industries, Israel), following the manufacturer's instructions. For quantitative RT-PCR analysis, RNA was treated with DNAse (Roche, Basel, Switzerland) to eliminate contaminating DNA (Marques et al., 2004). For cDNA synthesis, 1 μg RNA and M-MLV Reverse transcriptase RNase H Minus, Point Mutant, oligo$(dT)_{15}$ primer and PCR Nucleotide mix were used, following the manufacturer's instructions (Promega Corporation, Madison, WI). The primers and probes used to amplify mouse were: *IA-β*: 5′-ACCCAGCCAAGATCAAAGTGC-3′ and 5′-TGCTCCACGTGACAGGTGTAGA-3′ (accession number NM_207105); *CIITA* (detects all isotypes): 5′-CACCCCCAGATGTGTATGTGCT-3′ and 5′-ACGAGGTTTCCCAGTCCAGAA-3′ (NM_007575). Real-time monitoring of the PCR amplification of cDNAs was performed using SYBR Green PCR Core Reagents (Applied Biosystems) in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The relative quantification of gene expression was performed as described in the SYBR Green user's manual using β-actin (5′-ACTATTGGCAACGAGCGGTTC-3′ and 5′-AAGGAAGGCTGGAAAAGAGCC-3′). The threshold cycle (C_T) is