Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA

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The highly complex pattern of expression of major histocompatibility complex class II (MHC-II) molecules determines both the immune repertoire during development and subsequently the triggering and the control of immune responses. These distinct functions result from cell type-restricted expression, developmental control and either constitutive or inducible expression of MHC-II genes. Yet, in these various situations, MHC-II gene expression is always under the control of a unique transactivator, CIITA. Here we show that the CIITA gene is controlled by several distinct promoters, two of which direct specific constitutive expression in dendritic cells and B lymphocytes respectively, while another mediates γ -interferon-induced expression. Thus the cellular, temporal and functional diversity of MHC-II expression is ultimately controlled by differential activation of different promoters of a single transactivator gene. This provides novel experimental tools to dissect compartment-specific gain or loss of MHC-II function *in vivo*.

Keywords: CIITA/MHC class II/promoter/transactivator

Introduction

The level of major histocompatibility class II (MHC-II) expression directly influences T-lymphocyte activation. The highly complex regulation of MHC-II expression thus controls both the generation of the immune repertoire and the triggering and maintenance of an immune response (Benacerraf, 1981; Janeway *et al.*, 1984). It also represents an interesting example of a tight and complex control of a multi-gene family. Two main modes of MHC-II expression can be distinguished, constitutive or inducible. MHC-II genes are expressed constitutively in only a very restricted number of cell types, specialized in antigen presentation, such as dendritic cells and B lymphocytes. Within these cell lineages, MHC-II expression is also subject to strict developmental control. For example, in the murine B-lymphocyte compartment, early pro-B cells are MHC-II negative, expression in pre-B cells is inducible by interleukin-4 (IL-4), mature B-cells show constitutive MHC-II expression, while terminal differentiation into plasma cells is accompanied by the extinction of MHC-II

expression (Glimcher and Kara, 1992). MHC-II expression can also be induced in a large variety of other cell types, in particular by γ -interferon (IFN- γ). In addition, modulation of MHC-II gene expression under a variety of other physiological and pathological conditions has been described (Glimcher and Kara, 1992; Mach *et al.*, 1996). This complex cellular and temporal control of MHC-II genes has important functional consequences in terms of both normal and aberrant T lymphocyte activation.

Our current understanding of the mechanisms of MHC-II gene expression results to a large extent from the study of various regulatory mutant cell lines, affected in certain essential MHC-II regulatory factors (Griscelli *et al.*, 1989; Mach *et al.*, 1994). In particular, analysis of cell lines from patients suffering from hereditary MHC-II deficiency (also called bare lymphocyte syndrome; BLS), a genetically heterogeneous disease of gene regulation, has led us to the discovery of three distinct, MHC-II-specific, transacting factors: RFX-5, RFX-AP and CIITA. Each of these factors is essential for the expression of all MHC-II genes and, in all three cases, mutations in the relevant regulatory genes could be identified in BLS patients. RFX-5 (Steimle *et al.*, 1995) and RFX-AP (RFX-associated protein) (Durand *et al.*, 1997) are components of the multi-subunit RFX complex (Reith *et al.*, 1988) that binds specifically to the X box of all MHC-II promoters.

The MHC-II transactivator CIITA (Steimle *et al.*, 1993) has been found to act as a master-regulator of MHC-II expression (Reith *et al.*, 1995; Mach *et al.*, 1996). Whereas the different protein subunits of the RFX complex are expressed ubiquitously and are also present in MHC-II-negative cells, CIITA expression can only be detected in MHC-II-positive cell types and tissues (Steimle *et al.*, 1993, 1994). CIITA and MHC-II expression are not only qualitatively but also quantitatively correlated (L.Otten, V.Steimle, S.Bontron and B.Mach, in preparation). In addition to its essential role in constitutive MHC-II expression, CIITA is also the obligatory mediator of IFN- γ -inducible MHC-II expression, since IFN- γ induces CIITA expression which in turn activates MHC-II transcription (Steimle *et al.*, 1994). Furthermore, in plasmacytes, which are MHC-II negative, it was shown that CIITA expression is abolished and that re-expression of CIITA is sufficient to reactivate MHC-II expression in these cells (Silacci *et al.*, 1994). In most cell types, introduction and expression of the CIITA gene is sufficient to activate expression of all MHC-II genes (Mach *et al.*, 1996).

These examples demonstrate that control of MHC-II expression is ultimately dependent on the control of expression of the CIITA gene itself. It was therefore an obvious challenge to explore the mechanisms that regulate CIITA expression in various cell types and biological situations and thus to understand how a single rate-limiting

Fig. 1. The four different 5' ends of the human CIITA mRNAs. Coding regions are represented by wide boxes, 5'-untranslated regions by narrow boxes. The non-homologous regions are hatched differently. The positions of the two primers (P₁, P₂) used for RACE-PCR analysis are indicated by small arrows. The 3' boundary of exon 2 has not been determined.

transactivator controls the highly complex pattern of regulation of MHC-II genes, including constitutive and inducible expression. We show here that expression of CIITA is controlled, in an unexpected manner, by four independent CIITA promoters leading to CIITA transcripts with four distinct first exons. The individual CIITA promoters are used in an alternative and tissue-specific manner. Distinct CIITA promoters control either MHC-II constitutive expression in dendritic cells (promoter I, PI) and in B lymphocytes (PIII), or IFN- γ -inducible expression (PIV) in a variety of MHC-II-negative cell types. We conclude that the cellular, temporal and functional diversity in MHC-II expression is not regulated at the level of the MHC-II genes themselves, but is ultimately under the control of several promoter sequences that activate differentially the same transactivator gene.

Results

Multiple 5' ends of CIITA mRNA

When the murine homologue of human CIITA (Steimle *et al.*, 1993) was isolated from a mouse cDNA library by low stringency hybridization (L.Otten, V.Steimle, S.Bontron and B.Mach, in preparation), sequence comparison between human and mouse CIITA cDNAs showed excellent identity downstream of position +167 of the human CIITA cDNA, but totally divergent 5' ends. To establish the structure of the 5' region of CIITA mRNA, a systematic analysis of CIITA 5' ends was performed by RACE-PCR both on murine and human RNAs isolated from different tissues, using primers from the homologous region (Figure 1). These assays led to the isolation of four different CIITA cDNA 5' sequences in human and three in mouse. Sequence analysis revealed that all these different cDNA clones had identical 3' ends but different 5' ends. In each case, the boundary between the diverging 5' ends and the common 3' ends was located, in human and mouse, 21 nucleotides upstream of the second ATG codon of the CIITA cDNA sequence described previously (Steimle *et al.*, 1993) (Figure 1). The different CIITA 5'

Fig. 2. Genomic organization of the 5'-flanking regions of the human and murine CIITA gene. The black boxes represent the different first exons, the small open boxes correspond to introns. The restriction sites for *EcoRI* (E), *KpnI* (K), *NheI* (N), *SacI* (S), *XbaI* (X) and *XhoI* (Xh) are indicated. The arrows represent the major initiation site of each first exon.

ends were numbered I–IV according to their relative position in the genomic map of the 5'-flanking region of the CIITA gene (see below). CIITA type I, III and IV 5' sequences were identified in human and mouse genomic DNA, while the CIITA type II 5' end was found only in human DNA.

Of the four different forms of CIITA transcripts which were thus identified, human CIITA type III corresponds to the previously described form of CIITA cDNA (Steimle *et al.*, 1993). The four transcripts share a common open reading frame starting from the AUG which is located 21 bp downstream of the 5' end of the common nucleotide sequence (Figure 1). For CIITA forms II and IV, this AUG, located in the context of a perfect Kozak consensus (Kozak, 1989), is the first initiation codon (Figures 1 and 3B and D). CIITA forms I and III display, in addition, upstream AUGs in favourable translation initiation contexts, which potentially lead to CIITA proteins with an additional 101 or 24 N-terminal amino acids respectively (Figures 1 and 3A and C). These features are shared between human and murine CIITA 5' ends I, III and IV. The respective nucleotide conservation within these different CIITA 5' ends is 75, 68 and 66%. For the sake of coherence and clarity, we propose to maintain the numbering of the amino acid sequence of the body of the CIITA sequence as published originally for CIITA type III (Steimle *et al.*, 1993).

Since the nucleotide sequences of the 5' region of all four different CIITA mRNAs differ, they could be generated either by alternative splicing of different 5' exons in the CIITA gene or by a differential usage of four different promoters. Complete lack of sequence homology at the very 5' ends of the divergent sequences favoured the second hypothesis.

Genomic organization of the 5'-flanking region of the CIITA gene

A human and a murine genomic λ phage library were screened using DNA fragments from the human type III and the murine type I and III 5' ends of CIITA cDNAs, respectively. Three overlapping human genomic clones and five overlapping murine genomic clones covering 24 and 35 kb were obtained, and a detailed restriction map was established (Figure 2). Fragments hybridizing to the

four different 5' ends were identified, subcloned and sequenced. In all cases, the genomic sequence corresponded exactly to the sequences of the different 5' ends of CIITA cDNA clones and thus represented distinct exons. Furthermore, the nucleotide sequences of exon-intron boundaries agreed with consensus splice donor sequences (Breathnach and Chambon, 1981). Screening of the murine genomic clones with a human CIITA type II 5' end cDNA probe did not yield a positive signal. The four different 5' exons (i.e. exon 1 of CIITA mRNA I, II, III and IV respectively) were mapped within the 5'-flanking region of the CIITA gene. The distance between exon 1 of CIITA type I and exon 1 of CIITA type IV is ~13 kb in human and 10 kb in the mouse. The relative order and, in part, the genomic distances between homologous promoters are maintained between the two species, indicating a strongly conserved genomic organization (Figure 2).

Multiple transcription initiation sites reveal four distinct CIITA promoters

The transcription initiation sites of the different human CIITA mRNAs were mapped by RNase protection assays with fragments covering the different CIITA exons 1 and their respective 5'-flanking regions. For CIITA type I, three protected fragments were detected using mRNA from liver (data not shown). The major protected fragment corresponded to a major transcription initiation site located 380 bp upstream of the 3' end of exon 1. It has been defined as nucleotide +1 of CIITA type I mRNA (Figure 3A). The two minor fragments indicate initiation start sites at positions -14 and +8 of CIITA type I. These transcription initiation sites fit well with those obtained by RACE-PCR in mouse.

For CIITA type III, several protected fragments were obtained using mRNA from a B-lymphocyte cell line (data not shown). The major transcription initiation site located 183 bp upstream of the 3' end of exon 1 defines position +1 of CIITA type III mRNA (Figure 3C). Two other main initiation start sites are located at positions -8 and -4, and several minor sites from positions -23 to +34. The longest CIITA type III cDNAs obtained by RACE-PCR fit with these initiation start sites. The CIITA cDNA sequence initially described (Steimle *et al.*, 1993) begins at position +17.

For CIITA type IV, multiple fragments were protected, using mRNA from an IFN- γ -induced melanoma cell line, with the major transcription initiation site located 75 bp upstream of the 3' end of exon 1 (data not shown). This defines nucleotide +1 of CIITA type IV mRNA (Figure 3D). A second major and six minor transcription initiation sites were identified at position +17 and between positions -54 and +69 of exon 1 of CIITA type IV, respectively. The 5' ends of many CIITA cDNAs obtained by RACE-PCR coincide with these initiation sites. No tissue or cell line could be identified so far in which the CIITA type II 5' end is expressed at a significant level; the exact initiation site of this form of CIITA transcript was, therefore, not analysed further.

Since all types of CIITA mRNAs were transcribed from multiple transcription initiation sites, we can conclude that these sites represent bona fide 5' ends of transcription products from four distinct CIITA promoters (PI, PII, PIII

and PIV) located upstream of four different CIITA exons 1 (of type I, II, III and IV respectively) (Figure 2).

Analysis of promoter sequences

Comparison of the sequences of the four different CIITA promoters showed no significant homology to each other, both in human and mouse. None of the regions upstream of the different CIITA first exons contains a consensus TATA box element or GC box. The absence of a TATA box is consistent with the finding that there are multiple initiation start sites (Breathnach and Chambon, 1981). In contrast, comparison of the homologous CIITA promoters between human and mouse showed significant sequence homology. For CIITA promoters I, III and IV, this homology extends over 120, 800 and 350 nucleotides respectively (Figure 3). More interestingly, within these promoter regions, small segments that are very well conserved within the two species could be identified (Figure 3). This sequence conservation suggests functional relevance and potential *cis*-acting elements. On the basis of this strong human-mouse conservation, as well as on the basis of sequence homology with known *cis*-acting elements, the following potential *cis*-acting elements of the different CIITA promoters can be pointed out.

CIITA promoter I contains an NF-GMb site (Shannon *et al.*, 1988), an NF-IL6 site (Akira and Kishimoto, 1992), two NF-IL6 inverted sites, a PEA3 site (Wasylyk *et al.*, 1989), a PEA3 inverted site and an E2A site (Murre *et al.*, 1989) in human and mouse respectively. In addition, human CIITA promoter I includes an AP1 site (Pollock and Treisman, 1990) and a CCAAT box (Dorn *et al.*, 1987) (Figure 3A).

In CIITA promoter III, one observes an E2A inverted box (Murre *et al.*, 1989), an IRF1/2 site (Tanaka *et al.*, 1993), an MYC inverted site (Agira *et al.*, 1989) and an OCT inverted site (Rosales *et al.*, 1987) in human and mouse, respectively, as well as two CCAAT boxes in mouse (Figure 3C).

CIITA promoter IV, the IFN- γ -responsive promoter (see below), contains a NF-GMa site (Shannon *et al.*, 1988), a GAS box (Pellegrini and Schindler, 1993), an adjacent E box (Blackwell *et al.*, 1990) and an IRF1/2 site, both in human and mouse (Figure 3D). In addition, there is an NFkB site (Sen and Baltimore, 1986) in human and two AP1 sites in mouse. Although it is beyond the scope of this report, a systematic functional analysis of the role of these various potential *cis*-acting motifs is obviously required.

Differential usage of the multiple CIITA promoters

To study the pattern of expression of the different forms of CIITA mRNAs, and thus of the different CIITA promoters, RNase protection analyses using probes specific for each type of CIITA mRNA were performed (Figure 4A). A panel of different tissues and cell lines expressing the CIITA gene either constitutively or following IFN- γ induction were analysed. The results were quantified by PhosphorImager. Interestingly, there is a striking difference in the specific usage of each of the different CIITA promoters. CIITA type I mRNA, resulting from the use of promoter I, is expressed at a remarkably high level in dendritic cells (Figures 4B and 5). It is barely detectable

The presence of CIITA type III mRNA is detected at a high level in different B-lymphocyte cell lines, as well as in tissues rich in B lymphocytes such as spleen and tonsils, and also in the thymus (Figure 4C, upper panel, and Figure 5). In contrast, type III mRNA was detected only at a low level in dendritic cells and even less so in a variety of IFN- γ -induced cell lines (Figures 4C and 5).

C

D

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hIII CTGCAGAGGTTGGCAGATATTGGCAGCTGGCCACCACT - 286
mIII CTGCAGSAG-----AAT - 244

hIII GCGGTTCATTGTGATCATCATTTCTGAACGTCAGACTGTTGAAGGTTCCCCAACAGAC - 226
mIII GTGTGTCAGAAATGCAATATATCATTTTCAAG----TGACTG-----AGCTGAG - 204

hIII TTTCTCTGCAACTTTTCTGTCTTCACCAAAATTCAGTCCACAGTAGGAAGTGAAAATAATT - 166
mIII ----GTCCAGCTTC-TGTGGTCACTAAATTGGGCCACAAGAGGAATGAAATTAACA - 150

hIII TCAGAGG-TGTAGGGAGGGCTTAAGGGAGTTGGTGTAAAAATTAGAGGGTGTTCAAGAAACAG - 107
mIII TAAGAAGCTGTGGT---GGC-TAAGGGAGCATGGGCCAAATTAGAGGGTATCCAATACAG - 94

hIII AAATCTGACCGCTTGGGGCCACCTTGCAGGGAGAGTTTTTTTTTGATGATCCCTCACT-TGT - 48
mIII GA-TATGAACATTT-----TGTCGAGA-----ATTTCAGGTGGTCCCTTGCTCTGT - 48

hIII TTCTTTGCATCTTTGGCTAGCTTGGCTGGGCTCCCAACTGGTGACTGGTT-AGTGATG-AG + 11
mIII TTCTTTGCATCTTAGCTGAGCTTGGTAGGTTCTGGGCTCTCTAAGTATAGACAGCAT + 13

hIII GCTAGTAGTGAGGCTGTGTGCTTCTGAGCTGGGCATCCGAAGGCATCTTGGGGAAGCTG + 71
mIII GTAGGGGATGAGGCTGCTGCTGCCACCTGGGCATCTGAGGACCITTTTGGAGA-CCT + 71

hIII AGGGCACG---AGGAGGGGCTGCCAGACTCCGGAGTCTGCTGCTGGCTGGGATCTCTACA + 129
mIII CCGGCACGCCAGGAGGGGCGCTGAGCTACAGACGTTACTGCATCACTCTGCTCTCTAAA + 131

hIII M R C L A P R P A G S Y L S E P Q |
CAATGCGTGGCTGGCTCCAGCCCTGCTGGGTCTACCTGTGCAGAGCCCAAGgtaaga
mIII TCTGCTGCTGCTGCTTCTGGCCCTCTGGGCTTACCTGCCGAGGCTGTCAAGgtaagt
M R C L V P G P S G S Y L P E L Q

D

hIV GGTGGGACTG - 345
mIV GGTGGGCTG - 363

hIV AGTTGGAGAGAAACAGAGACCCACCCAGGGTGGGGACAAGCTCCCTG-CAACTCAGGAC - 286
mIV AGATAGAGTGAATATAGAGAGCCACC-AGGGGTGTAGATGAGGTGGCTTACAAACCA----- - 308

hIV TTGCAGATCACTTGGCCCAAGTGGC-TCCCTAGCTCTCTGGCTCTTGAGCCCGGGGCGCTGGGA - 227
mIV -----AACACCTGGCT---TGAAATTCAGTTCTACCTGGCTCAATCTGTCTGCTCT--- - 261

hIV CTCTCCCGGAAGTGGGGCTGGCCACTGTGAGGAACCGACTGGAGGCAGGGA-CCTCTTGG - 168
mIV CTCCTCTGTAGCAGGAGCTGTGCTAAGAGTGCAG-ATCTGCCCTGTAGGAGGGGCTCTCTGGA - 202

hIV ATGCCCCCAGT-----AGTTGGATGCCAC-----TCTGTAFAAGGACAGCTGTGG - 122
mIV TAGACTCTGTCCAAGCAAACTGGGTTGCAATGTGGCAGCTTCTGGAAGGACAGCTGTGG - 142

hIV CCA-----CAGTAGGTGCTTGGTTGCTCTACAGCC-----TGSCCGGA-GCTCA - 78
mIV ATATCACTTTTCAGGGGAGGAGGGGTTCTCTGCTAGCTCTGCTTTGGSCCAAAGCTGAA - 82

hIV GCCTGCAGAAAATAAGTGAAGGGA-AAAAGACATCTCGGGGAGGCG-----GGAGGTA - 25
mIV -GGTGATAGACAAAGTGAAGGGGGGAAAGGCCACAGATATCCCTATTATTGTGAGATA - 23

hIV GGATGACCAGCGCAGAGCTGCCACAGACTTGGCCGCCCTCAGAGCTTGGCGGAGGGAG + 36
mIV GC-TG-CAGGAGACTGCCCGCCCAAGCTCTTAGGAGCCAGGAGCTGGCGGAGGGAG + 36

hIV AGGCCACCAAGCAGC-----GGCGCGGGGAGCCCGGGGAACA-----GCgttaggt
mIV A--CTGCATGCAGGCAGCACTCAGAAGACCGGGGCAGCCACAGCCGCGgttaggt
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D

hIV AGTTGGAGAGAAACAGAGACCCACCCAGGGGTGGGGACAAGCTCCCTG-CAACTCAGGAC- 286
mIV AGATAGAGTGAARTAGAGAGCCACC-AGGGGTGTAGATAGAGTGGCTTCAAAACCA---- 308

hIV TTGCAGATCACTTGTCCCAAGTGCC-TCCCTAGCTCCTGGCTCCTGGCCCCGGGGCCTGGGA 227
mIV -----AACACCTGCTCT--TGGAACTCAGTTCTCACTGGCTCTAATCTGCTGCTCT- 261

hIV **NF- κ B** CTCTCTCCGAAGTGGGGTGGCCACTGTGAGGAACCGACTGGAGCGAGGGA-CCTCTTGG 168
mIV CTCTCCTTGAGCAGGGAAGTGGTCTAAGTGTAG-ATCTGCCCTTAGGGAGGGGGTCTCTTGGA 202

hIV ATGCCCCAGGC-----AGTTGGGATGCCAC-----TTCTGTATAAACACAGTGTGG 122
mIV **NF κ B** **GAS** **E box** AGACTCTGTCCAAAGCAACTTGGGTTCGATCTGTGCAGCTTCTGAGAAACACGTGTGG 142

hIV CCA-----CAGTAGGTGCTTGGTGTCTCCACAGCC-----TGSCCCGA-GCTCAG 78
mIV ATATCACTTTTTCAGGGGAGGAGGGGTTCCTGCTAGCTCTGCCTTTTGCCCAAGAGCTGAA 82

hIV CGCTGCAGAAAGAAAGTGAAGGGA-AAAAGAACTGCGGGGAGGCG-----GGGAGGTA 25
mIV -GGTGTAGACAGAAAGTGAAGGSGGGAAGAGCCACAGATACTCCCTATTGTGTAGATA 23

hIV GGATGACCACGCGACGAGCTGCCACAGACTGCCCGGCCCGACAGCTGGCGGGAGGGAG + 36
mIV GC-TG-CCAGGAGACTGCCCGCCCAAGCTCTTAGAGGCCAGGAGCTGGCGGGAGGGAG + 36

hIV AGGCCACCAAGCAGC-GCGCGGGGAGCCCGGGGAACA-----GCGgtaggt
mIV A--CTGCATCGAGGCAGCACTCAGAAGACGGGGCAGCCACAGCCGCGgtaggt

Fig. 3. Sequence comparison of the first exons of CIITA with their respective 5'-flanking regions in human and mouse. **(A)** CIITA type I. **(B)** CIITA type II. **(C)** CIITA type III. **(D)** CIITA type IV. For CIITA types I, III and IV, the upstream regions showing significant sequence homology between human and mouse are shown. Major mRNA initiation sites are indicated by horizontal arrows. The +1 positions of the murine sequences were defined as the homologous positions of the human +1 sites. For human CIITA type II, the +1 site is defined by RACE-PCR. Exonic regions are shaded. For CIITA types I and III, the predicted amino acid sequences starting from the upstream AUGs (white boxes) are shown in the one-letter code. The 3' exon-intron boundaries of the different exons I are indicated by a vertical bar and the splice donor sites are shown in lower case letters. The different binding sites for known transcription factors are indicated. The white circle represents an AP1 site, the grey box a CCAAT box. Box1 is composed of either two NF-IL6 sites or two PEA3 sites in palindromic orientation.

CIITA type IV mRNA is the major form expressed following induction by IFN- γ . This was observed in different IFN- γ -inducible cell lines, such as melanoma (Me67.1), monocytes (THP1), endothelial cells (HUVEC) and fibroblasts (PP2) (Figure 4C, lower panel, and Figure 5). In contrast, only a low level of type IV CIITA transcript was detectable in B lymphocytes or in dendritic cells (Figure 4C, upper panel, and Figure 5). No cell line or tissue could be identified to date in which a significant

Fig. 4. Differential expression of CIITA types I, III and IV mRNAs. (A) Schematic representation of the probes used for analysis of expression pattern of the multiple CIITA mRNAs by RNase protection assays. The different specific probes are indicated with their sizes before and after RNase digestion. Each probe covers a part of exon 1 and 226 nucleotides. For example, the CIITA type I-specific probe protects a 333 nucleotide type I-specific fragment and protection of the 226 nucleotide fragment is due to hybridization with all other types of CIITA mRNAs (non-type I). The same applies to probes specific for CIITA type III and type IV. The RNAs were hybridized with the specific CIITA probe, an internal CIITA probe ('internal') and a probe for TBP ('control'). (B) Analysis of CIITA type I expression. (C) Analysis of CIITA type III (upper panel) and CIITA type IV (lower panel). The positions of the protected fragments are indicated.

amount of CIITA type II mRNA is detectable by RNase protection assay (data not shown). The quantification of the differential expression of the different CIITA transcripts, from their respective endogenous promoters, is represented schematically in Figure 5, and the percentages of relative promoter usage are indicated in Table I.

Differential CIITA expression is under transcriptional control

To determine if the expression of the CIITA gene is controlled at the level of transcription, an alternative method to the classical run-on assay was carried out for the analysis of the transcription rate of CIITA RNA. Nascent RNA chains were isolated from nuclei by precipitation of a chromatin pellet containing DNA, histones and ternary transcription complexes (Wuarin and Schibler, 1994). Nascent RNA chains were then isolated from the chromatin pellet and analysed by RNase protection assays.

The transcription rate of the CIITA gene was analysed in the monocyte cell line THP1 before and after induction by IFN- γ . Before induction, no CIITA nascent RNA could be detected (Figure 6, lane 3). After induction by IFN- γ , CIITA nascent RNA was detected at a high level (Figure

6, lane 5). As a control, nascent RNA from a B-lymphocyte cell line (Raji), which expresses CIITA constitutively, was analysed (Figure 6, lane 1). The level of nascent RNA was similar in the B-cell line and in IFN- γ -induced THP1 cells. These results show that the induction of the CIITA gene by IFN- γ is controlled mainly at the level of transcription. Moreover, no nascent RNA could be detected in a T-lymphoma cell line (CEM) constitutively negative for CIITA expression (data not shown). The constitutive expression of CIITA RNA is thus also controlled at the level of transcription. Comparison of lanes 5 and 6 shows that the relative amount of CIITA RNA versus TATA box-binding protein (TBP) RNA is completely different between nascent RNA and nuclear RNA. This excludes any contamination of the nascent RNA chains by nuclear RNA.

The promoter-proximal regions control alternative usage of different CIITA promoters

The functional activity and tissue specificity of CIITA promoters III (PIII) and IV (PIV) were analysed by transient transfection assays of promoter-reporter gene constructs. Since CIITA type III mRNA is the major form expressed in B lymphocytes and CIITA type IV mRNA

Fig. 5. Schematic representation of the differential expression of the four types of CIITA transcripts. The amount of the different types of CIITA mRNAs is given as a percentage of the total amount of CIITA mRNA expression as measured by the internal CIITA probe after quantification by PhosphorImager of the protected fragments obtained by RNase protection analysis.

is expressed preferentially in IFN- γ -induced cells, the B-lymphocyte cell line Raji and the melanoma cell line Me67.8 were chosen for these functional assays. Because of the very low level of expression of the CIITA gene (Steimle *et al.*, 1993), it was necessary to use a highly sensitive reporter gene assay, such as quantitative RT-PCR transcription assay (Sperisen *et al.*, 1992). For both CIITA promoters pIII and pIV, two different fragment sizes were analysed and compared with the promoterless plasmid pG β G(+) as a negative control. Transfections of pIII-974 and pIII-322 in B lymphocytes revealed a high activity of CIITA promoter pIII, whereas in Me67.8 cells, the same promoter pIII was inactive, before and after induction by IFN- γ (Figure 7A). In B lymphocytes, the activity of the short pIII-322 promoter was higher than that of the longer promoter pIII-974 (signal ratios of 8.2 and 2.6 respectively). The control pG β G(+) was negative in both cell types.

In contrast, the CIITA promoter IV (pIV-950 and pIV-461) showed only a baseline activity in B lymphocytes (Raji) (signal ratios of 0.4 and 1.1 respectively), whereas

Table I. Percentage of the different types of CIITA mRNAs observed in different tissues and cell lines (see Figures 4 and 5)

	Type I	Type III	Type IV
Spleen	3.5	67	33
Tonsil	0	96	17
Thymus	6	60	33
Raji	0	86	2.5
Mann	0	72	17
Dendritic	74	39	2.7
Me67.1 + IFN- γ	0	2	88
THP1 + IFN- γ	0	14	62
HUVEC + IFN- γ	ND	10	68
PP2 + IFN- γ	ND	16	66

Fig. 6. Analysis of transcription from CIITA promoters III and IV. Nascent RNAs (lanes 1, 3 and 5) and free nuclear transcripts (lanes 2, 4 and 6) were analysed by RNase protection assays in B lymphocytes (Raji), and in monocytic cells (THP1) before (–) and after (+) induction by IFN- γ . The RNAs were hybridized with the internal CIITA probe, the TBP probe and the CIITA type III or type IV probe for Raji and THP1, respectively.

the same CIITA promoter IV was highly induced by IFN- γ in Me67.8 cells (Figure 7B), as well as in other IFN- γ -inducible cell lines (HeLa, 2FTGH, data not shown). Signal ratios of promoters pIV-950 and pIV-461 rose from 0.13 and 0.18 before induction to 7.9 and 29.6, respectively, after IFN- γ induction. These results provide functional evidence for the existence of distinct CIITA promoter regions, located immediately upstream of the alternative 5' exons type III and IV. They also showed that the use of these two distinct CIITA promoters is indeed controlled in a tissue-specific manner, either constitutively or following induction, as observed earlier for the transcription of the endogenous CIITA gene into distinct CIITA mRNA.

Discussion

The very complex and tight regulation of expression of the MHC-II gene family has direct implications for T-lymphocyte activation and thus for the control of the immune response. In addition, significant pathophysiological consequences such as immunodeficiency or aberrant T-cell activation may arise from the dysregulation of MHC-II gene expression. The unsuspected findings reported here allow us to attribute the tissue-specific and temporal control of expression of MHC-II molecules, and hence of T-cell activation, to the selective usage of distinct alternative promoters of the regulatory gene CIITA. This indicates that the complex pattern of regulation that

Fig. 7. Functional analysis of CIITA promoters III and IV. (A) Transient transfections of Raji and Me67.8 cells with the plasmids pIII-974, pIII-322 or the promoterless plasmid pGβG(+) and the reference plasmid. The input ratios between CIITA constructs and the reference plasmid were 4:1 for Raji and 9:1 for Me67.8. c, RT-PCR signals from CIITA 5'-flanking region-β-globin reporter gene construct; r, RT-PCR signals from reference plasmid. The signal ratio is determined by normalizing the mRNA signal derived from the CIITA construct to that obtained with the reference plasmid (see Materials and methods). (B) Transient transfections of Raji and Me67.8 with the plasmids pIV-950, pIV-461 or pGβG(+) and the reference plasmid, with the same ratio as in (A).

determines the diverse functions of MHC-II molecules, in positive and negative selection for the generation of the immune repertoire, as well as in the induction of an immune response by antigen-presenting cells (APCs), is controlled entirely by the differential activation of multiple promoters of a single transactivator gene.

Expression of MHC-II molecules can be modulated by a number of different stimuli including immune, neuroendocrine and even viral mediators (Glimcher and Kara, 1992; Mach *et al.*, 1996). On a restricted number of specific cell types, one observes up- or down-regulation of MHC-II expression, occasionally with opposite effects of certain mediators depending on the target cell. Within this highly complex pattern, MHC-II regulation is characterized by constitutive expression in professional APCs, such as dendritic cells and B lymphocytes, and inducible expression in non-professional APCs, in particular by IFN-γ. Yet, in all these cases, the control of MHC-II expression is directly dependent on the expression of the same transactivator, CIITA. We have shown here that tissue-specific expression of this transactivator is controlled by distinct promoters and that individual CIITA promoters control MHC-II expression in professional versus non-professional APCs. It follows that the controlled expression of a family of target genes, with its cellular and developmental specificity and with its different functional consequences, ultimately is exercised by alternative activation of individual promoters of the CIITA gene. We suggest that this multi-promoter system has been developed to allow the fine regulation of CIITA expression by different transcription factors operating on alternative

Fig. 8. Schematic representation of the complexity of promoter-specific control of CIITA and MHC-II expression.

promoters, in different cell types and after different signals (Figure 8). Additional levels of complexity might be introduced through differential post-transcriptional control operating on distinct CIITA transcripts.

A systematic analysis of CIITA mRNA expression in different tissues by RACE-PCR led to the discovery of four different 5' exons (Figures 1 and 3). Each of the four alternative first exons of CIITA is spliced to a common exon 2. The first exon of CIITA mRNA type I and III each encodes an additional upstream AUG, in-frame with

the common AUG located in exon 2, which is the first AUG for CIITA type II and IV. Therefore, the alternative expression of CIITA mRNA can lead to three different forms of CIITA protein. Interestingly, comparison of human and mouse nucleotide and amino acid sequences of the first exon of CIITA type I indicates a relative excess of mutations at the third base position of the codon, a situation that favours conservation at the amino acid level. This suggests evolutionary pressure for conservation of this sequence and thus translation of this segment. We have not observed differences in the biological activities of CIITA initiated at either the common AUG or the first AUG of the type III transcript in transfection experiments (V.Steimle *et al.*, unpublished data), but this possibility needs to be explored further, especially for CIITA type I, since it may give rise to an additional level of complexity in the fine tuning of CIITA function.

The pattern of CIITA promoter usage was analysed by RNase protection assays on the specific transcripts of the endogenous CIITA gene. Surprisingly, this analysis revealed a strong bias in the selective use of each of the different CIITA promoters, with two different kinds of specificities. First, there is a tissue-specific use of two distinct constitutive promoters (PI and PIII), with a preferential use of promoter I in dendritic cells and of promoter III in B lymphocytes. Second, distinct CIITA promoters are used for constitutive (PI and PIII) versus IFN- γ -inducible (PIV) expression of the regulatory gene, with the preferred utilization of promoter IV in cells activated by IFN- γ (Figures 4 and 5, Table I). Direct transcription assays confirmed that the control of both constitutive and inducible expression of CIITA is indeed largely executed at the level of transcription (Figure 6). This reinforces the importance of multiple promoters in the control of CIITA gene expression.

Functional analysis of CIITA promoters III and IV showed that the region immediately upstream of the respective first exons does confer tissue-specific expression of a reporter gene construct (Figure 7). Indeed, PIII-322 shows a specific expression in B lymphocytes, while PIV-461 is functional only in IFN- γ -induced cells. It is remarkable that, in both cases, a small upstream region proximal to the initiation start site is sufficient to confer tissue specificity or inducibility by IFN- γ , as was observed for the expression of the endogenous CIITA gene. In view of the short genomic distance between CIITA promoters III and IV, it will be interesting to explore whether, in certain cases, they can act on each other as enhancers. An effect over that distance might explain the low level of CIITA type IV transcript found in B lymphocytes and that of CIITA type III mRNA observed in induced cells (Figures 4 and 5, Table I).

Whereas no common features were observed between the different CIITA promoters within a given species, there is a striking sequence conservation between homologous promoters in human and mouse (Figure 3). Detailed sequence comparison of promoters I, III and IV in both species points to highly conserved sequence elements, suggesting functional relevance and pointing to possible targets for DNA-binding proteins. A systematic study, including functional assays, will be required to dissect the relevant *cis*-acting elements of each of the different CIITA promoters. It is worth pointing out that the IFN- γ -inducible

CIITA promoter contains a conserved consensus GAS box. This is compatible with the known involvement of STAT1 in the induction of MHC-II genes by IFN- γ (Meraz *et al.*, 1996). It also contains a conserved IRF1-binding site, which is a factor involved in the regulation of several genes induced by IFN- γ (Briken *et al.*, 1995). The study of mutant cell lines specifically mutated in the responsiveness of the CIITA gene to IFN- γ will be of great interest in the search for *trans*-acting factors acting selectively on CIITA promoter IV. One can also anticipate an interest in possible polymorphisms within the CIITA promoters. For instance, small differences in the responsiveness to IFN- γ in terms of CIITA and MHC-II induction, and thus ultimately in terms of the magnitude of the T-cell response, would be expected to have important functional implications.

Alternative promoters have been identified in several eukaryotic genes. Their differential usage can afford an extra level of flexibility in the regulation of these genes (Schibler and Sierra, 1987; Ayoubi and Van De Ven, 1996). One promoter, for example, can be functional in a given tissue or during a particular developmental stage. Gene transcription from multiple promoters allows differential post-transcriptional control, since RNA transcripts with different 5'-untranslated regions could exhibit different stability or different translational efficiency (Kozak, 1991). The unique features of the system described here concern the hierarchy in the control of a family of target genes, with its highly complex pattern of regulation and diverse functional consequences, by a single regulator gene, itself controlled by selective activation of alternative promoters.

Finally, it is worth pointing out that the unexpected finding of alternative usage of different CIITA promoters for constitutive versus inducible expression suggests novel strategies of immunomodulation. First, it is remarkable that CIITA promoter I is expressed selectively in dendritic cells. This may provide an experimental model for controlled expression of genes in dendritic cells *in vivo*, a point of interest in the study of the immune system, as well as for DNA vaccination, given the scarcity of known dendritic cell-specific promoters. Second, it will allow us selectively to modulate the expression of CIITA, and thus of MHC-II molecules, *in vivo* in a conditional fashion by inducing selective recombination within specific CIITA promoters. For instance, while maintaining normal expression of MHC-II molecules on professional APCs, it will be possible to repress selectively inducible MHC-II expression, with both spatial and temporal control. The potential selectively to prevent or trigger MHC-II expression in dendritic cells alone, or in B lymphocytes, also offers interesting possibilities of immunomodulation *in vivo*. Such novel forms of controlled experimental modulation of MHC-II genes are relevant to studies of the development of the immune repertoire as well as to studies on the pathogenesis of several inflammatory or autoimmune syndromes. Third, the distinct role of different CIITA promoters should eventually make possible the identification of agents capable of selectively inhibiting MHC-II expression on certain cell types, such as non-professional APCs. Selective immunosuppression of this type could be of clinical interest.

Materials and methods

Cell lines and culture

The cell lines Raji [Epstein-Barr virus (EBV)-positive Burkitt lymphoma cell line], Mann (EBV-transformed human B-lymphocyte cell line), CEM (T-lymphoblastoid cell line), THP1 (monocytic cell line) and PP2 (foreskin primary fibroblasts, provided by J.-M.Dayer), Me67 (melanoma cell line, provided by S.Carrell) and HUVEC (human umbilical cord vein endothelial cells, a gift from J.-L.Magnenat), were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 U/ml penicillin, 10 mg/ml streptomycin and 2 mM L-glutamine and incubated at 37°C in 5% CO₂. IFN- γ (500 U/ml for Me67, 1000 U/ml for THP1, PP2 and HUVEC) was added for induction experiments during the time specified in the figure legends. BC1 dendritic cells were kindly provided as a frozen pellet by A.Lanzavecchia (Sallusto and Lanzavecchia, 1994).

RNAs

Total RNA from BC1 was prepared with Trizol reagent (Gibco-BRL). Cytoplasmic RNAs from Raji, Mann, Me67.1, THP1, HUVEC and PP2 were extracted as described (Wilkinson, 1988). RNA from human spleen, thymus, tonsil and kidney were a gift from P.Sapino.

RACE-PCR

RACE-PCR (Frohman *et al.*, 1988) was carried out on total RNA from Raji, IFN- γ -induced Me67 and kidney with a RACE-PCR-kit (Gibco-BRL) according to the manufacturer's instructions with the following modifications. Tailing was done using dATP. For PCR amplification, 5 μ l of dA-tailed cDNA was added to 40 μ l of amplification mix containing 4.5 μ l of 10 $\times$ synthesis buffer, 200 μ M each dNTP, 25 pmol of gene-specific primer P₂, 25 pmol of adapter primer ADXSC and 10 pmol of adapter primer XSCT17. The sample was incubated for 5 min at 95°C, then maintained at 80°C. Five μ l of diluted *Taq* polymerase (2 U) was added, and the sample was amplified by 30 cycles of 94°C for 45 s, 54°C for 25 s and 72°C for 2 min; the last extension was for 10 min at 72°C. The sequences of the primers are: P₁, 5'-GTCCAGTTCGCGATATTGG-3', P₂, 5'-TCCCTGGTCTCTTCA-TCA-3'; ADXSC, 5'-GACTCGAGTCGACATCG-3'; XSCT17, 5'-GACTCGAGTCGACATCGAT17-3'. Conditions for RACE-PCR on mouse RNA will be described elsewhere (L.Otten, V.Steimle, S.Bontron and B.Mach, in preparation).

Isolation of genomic clones

A mouse genomic library in phage λ gem11 (kindly provided by M.Aguet) and a human genomic library in phage EMBL3 were screened with random primed labelled CIITA cDNA probes using standard protocols (Sambrook *et al.*, 1989). Restriction maps were determined by complete or partial digestions of overlapping clones and hybridization with specific cDNA probes. Overlapping restriction fragments were cloned into pBluescript KS vector and the exons and their flanking regions were sequenced by the deoxy chain termination method using the T₇ G/A-Deaza sequencing kit of Pharmacia.

RNase protection assays

Three different probes were constructed to determine transcription initiation sites of CIITA mRNAs. For CIITA type I mRNA, the probe covers position -91, relative to the cap site, to the end of exon 1. For CIITA type III mRNA, the probe spans position -322 (*Pst*I) to +101 (*Hpa*II) around the cap site. The probe used for CIITA type IV mRNA covers 176 bp upstream of the cap site, the 75 bp of the exon 1 and 226 bp downstream of the 3' end of exon 1.

For analysis of differential CIITA promoter usage, four CIITA cDNA fragments were prepared as RNase protection probes, specific for each type of CIITA mRNAs. The four probes share in their 3' ends the region of 226 nucleotides downstream of the end of exon 1, but have distinct 5' ends, which are specific for the type of CIITA mRNA they protect (Figure 4A). As an internal control for total CIITA expression, a fragment was prepared that covers nucleotides 1152 (*Pst*I) to 1344 (*Nco*I), protecting 193 bp of CIITA mRNA in the common region. As an RNA loading control, a fragment was constructed that protects 161 bp (*Ssp*I-*Apa*LI) of TBP mRNA (Kao *et al.*, 1990).

RNase protection assays with 25 μ g of RNA per reaction were carried out as previously described (Steimle *et al.*, 1993). The results were quantified by PhosphorImager. Promoter usage was quantified as the ratio of the expression of the type-specific CIITA mRNA to the expression of total CIITA mRNAs as measured by the internal CIITA probe.

Isolation of nascent RNAs

Nascent RNAs and free nuclear RNAs were isolated from 200 $\times$ 10⁶ cells as described (Wuarin and Schibler, 1994). Isolation of nuclei was performed as reported earlier (Steimle *et al.*, 1995). Nascent RNA and free nuclear RNA were treated with RNase-free DNase I (Boehringer). Nascent RNA and free nuclear RNA isolated from 5 $\times$ 10⁶ and 1.25 $\times$ 10⁶ cells, respectively, were analysed by RNase protection assay.

Reporter gene assay

Four CIITA promoter-reporter gene plasmids were constructed by subcloning CIITA 5'-flanking regions upstream of the rabbit β -globin gene of plasmid pG β G(+) (Sperisen *et al.*, 1992). Plasmids pIII-974 and pIII-322 contain the -974 (*Nhe*I)/+101 (*Hpa*II) and the -322 (*Pst*I)/+101 (*Hpa*II) fragments, respectively, of the CIITA 5'-flanking region of exon 1 type III. Plasmids pIV-950 and pIV-461 contain the -950 (*Xho*I)/+75 and the -461 (*Kpn*I)/+75 fragment, respectively, of the 5'-flanking region of exon 1 type IV.

Reporter gene expression was measured by quantitative RT-PCR as previously described (Sperisen *et al.*, 1992) with the following modifications. Raji (5 $\times$ 10⁶) and Me67.8 (2.5 $\times$ 10⁶) cells were transiently transfected by electroporation at 250 V, 960 μ F (Genepulser, Bio-Rad), with 20 μ g of plasmid DNA and 400 μ g of *Escherichia coli* tRNA as carrier in 750 μ l of RPMI medium. Plasmid DNA is composed of a defined ratio of a CIITA promoter-rabbit β -globin gene construct and of reference plasmid. The latter contains a rabbit β -globin gene with a 40 bp deletion in the second exon and is transcribed under the control of the constitutive chicken β -actin promoter (pG β Ac β GID) (Sperisen *et al.*, 1992). For IFN- γ inductions, cultures were split into halves after transfection and cultured in the absence or presence of IFN- γ (500 U/ml). The cells were cultured at 37°C for 48 h; total RNA was extracted with Trizol reagent (Gibco-BRL) and treated with RNase-free DNase I (Boehringer). One μ g of total RNA was used for the first strand cDNA synthesis from a (dT)₂₀ primer with Superscript RNase H⁻ reverse transcriptase (50 U, Gibco-BRL) and 10 U of RNase inhibitor. β -Globin cDNA was amplified by PCR: 1/10 of the reverse transcription product was adjusted to 50 μ l containing 5 μ l of Vent 10 $\times$ buffer [100 mM KCl, 100 mM (NH₄)₂SO₄, 200 mM Tris-HCl pH 8.8, 20 mM MgSO₄, 1% Triton X-100], 300 μ M dNTP, 40 pmol of primer β GP5' (5'-TCCCCCAAACAGACAGAAATGG-3'), 40 pmol of primer β GP3' (5'-GTCACAGTGCAGTTCACTCAG-3'), 5% LB and 2 μ Ci of [α -³²P]dCTP (Amersham). After 3 min at 95°C, 1 μ l of Vent DNA polymerase (2 U, NEB) was added and the samples were amplified by 30 cycles of 94°C for 40 s, 59°C for 30 s, 72°C for 60 s. PCR products were denatured and separated by electrophoresis on a 6% polyacrylamide-8 M urea gel. mRNA-derived signals were quantified by PhosphorImager. The input ratios between the CIITA promoter plasmids and the reference plasmid were determined experimentally so as to yield comparable signals for the test (c) and reference (r) RT-PCR bands. In order to permit comparison of signal ratios between different experiments, a correction factor was applied for the quantification, bringing all promoter to reference plasmid ratios to 10:1.

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