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Site α Is Crucial for Two Routes of IFNγ-Induced MHC Class I Transactivation: The ISRE-Mediated Route and a Novel Pathway Involving CIITA

Sam J. P. Gobin, Ad Peijnenburg, Vivian Keijsers, and Peter J. van den Elsen
Department of Immunohaematology and Blood Bank
University Hospital Leiden
2333 ZA Leiden
The Netherlands

Summary

The constitutive and cytokine-induced levels of major histocompatibility (MHC) class I expression are tightly controlled at the transcriptional level. In this study, it is shown that the *cis*-acting regulatory element site α of the MHC class I promoter is essential for the IFNγ-induced transactivation of MHC class I gene expression through the ISRE. Moreover, it was discovered that the class II transactivator (CIITA), which is itself under the control of the IFNγ induction pathway, strongly transactivates MHC class I gene expression and exerts its activity through site α. Therefore, site α is a crucial regulatory element, mediating the classic route of IFNγ induction via the ISRE as well as a novel route of MHC class I transactivation involving CIITA.

Introduction

The expression of major histocompatibility (MHC) class I molecules is essential in the immune response because they present antigen-derived peptides to cytotoxic T lymphocytes (Germain, 1994). In accordance with this key role in antigen presentation, MHC class I molecules are ubiquitously expressed and their basal level of expression can be induced by a number of cytokines and viral factors (reviewed by Singer and Maguire, 1990; Ting and Baldwin, 1993; Le Bouteiller, 1994). Expression of MHC class I molecules is tightly regulated at the transcriptional level during development and also in fully differentiated cells.

The level of MHC class I gene expression is controlled by transcription factors binding to *cis*-acting regulatory elements within the MHC class I promoter. The main control elements include enhancer A, the interferon-stimulated response element (ISRE), and site α (reviewed by Ting and Baldwin, 1993; Le Bouteiller, 1994). Enhancer A is bound by transcription factors of the NF-κB/rel family and is thought to be essential for constitutive and cytokine-induced expression (reviewed by Baeuerle and Henkel, 1994; Le Bouteiller, 1994). The ISRE is the target DNA-binding site for factors of the interferon regulatory factor (IRF) family, and it mediates the induction of MHC class I expression by type I and type II interferons (reviewed by Schindler and Darnell, 1995).

In particular, interferon-γ (IFNγ) is a potent inducer of MHC class I membrane expression: in addition to the MHC class I heavy chain, it also enhances the expression of β_2m, TAP1, TAP2, LMP2, and LMP7 (Gussow et al., 1987; Yang et al., 1992; Wright et al., 1995; Min et al., 1996), all of which play an important role in MHC class I–mediated antigen presentation (reviewed by Lehner and Cresswell, 1996; Marusina and Monaco, 1996). The IFNγ induction pathway that leads to MHC class I transactivation is initiated by the binding of IFNγ to its receptor, which then leads to the activation of the tyrosine kinases JAK1 and JAK2 (Schindler and Darnell, 1995). These tyrosine kinases phosphorylate the signal transducer and activator of transcription-1α (STAT1α; also termed p91, STAT91, or GAF). STAT1α binds the γ-activated site of genes, such as interferon regulatory factor-1 (IRF-1; Fujita et al., 1989; Harada et al., 1989, 1994), as a homodimer (Bluyssens et al., 1995; Schindler and Darnell, 1995), inducing transcription of these IFNγ-inducible genes. IRF-1 is the principal transcription factor binding the ISRE in the MHC class I promoter (Chang et al., 1992; Girdlestone et al., 1993; Johnson and Pober, 1994).

The regulatory element site α is homologous to the cAMP-response element (CRE) and is constitutively occupied in vivo, as demonstrated by footprinting experiments (Korber et al., 1988; Israël et al., 1989; Dey et al., 1992). Its role in MHC class I transactivation has not been well characterized, but some studies have suggested that site α may have a role in constitutive MHC class I expression in lymphoid cells (Dey et al., 1992).

The recently identified class II transactivator (CIITA) has been shown to be essential for transcriptional activation of MHC class II genes (Steimle et al., 1993, 1994; Mach et al., 1996). MHC class II gene promoters contain a set of conserved regulatory elements, known as the S (W or Z), X1, X2, and Y boxes. These regulatory elements are bound by a number of DNA-binding proteins. These include the RFX protein complex, which binds the X1 box; X2BP and other Fos/Jun- and ATF/CREB-related proteins, which bind the X2 box; and NF-Y, which binds the Y box (reviewed by Benoist and Mathis, 1990; Glimcher and Kara, 1992; Ting and Baldwin, 1993; Mach et al., 1996). CIITA is thought to act as a coactivator and, since no DNA-binding motif has been found (Steimle et al., 1993), CIITA is believed to exert its activity through these DNA-binding proteins (Riley et al., 1995; Zhou and Glimcher, 1995; Mach et al., 1996). In particular, the S and X2 boxes and their DNA-binding proteins appear to be crucial for mediating transactivation by CIITA (Zhou and Glimcher, 1995). CIITA contains several domains involved in MHC class II transactivation: an N-terminal acidic domain with transactivation properties; a proline-, serine-, and threonine-rich domain; and a C-terminal domain (Riley et al., 1995; Zhou and Glimcher, 1995). CIITA is constitutively expressed in MHC class II–positive cells such as B cells (Steimle et al., 1993). In other cell types, the expression of CIITA can be induced by IFNγ and is under the control of the IFNγ-mediated signal transduction pathway (Chang et al., 1994; Chin et al., 1994; Steimle et al., 1994; Lee and Benveniste, 1996; Meraz et al., 1996). Therefore, MHC

class II genes are inducible by IFNγ, despite a lack of ISRE and γ-activated site elements in their promoter region.

The importance of CIITA in the regulation of MHC class II expression is illustrated in patients with MHC class II deficiency, also referred to as bare lymphocyte syndrome (BLS). In B-LCL and fibroblast cell lines from patients belonging to BLS complementation group A, the lack of MHC class II expression is caused by mutations in the CIITA gene (reviewed by Mach et al., 1996). As a result, these cells are thought to express mutated or truncated forms of CIITA, which are not functional in MHC class II transactivation. Transfection experiments have shown that CIITA under the control of a constitutive promoter was sufficient to induce MHC class II expression in these cell lines (Steimle et al., 1993, 1994). Therefore, it was concluded that CIITA governs both constitutive and IFNγ-inducible expression of MHC class II genes (Chang et al., 1994; Steimle et al., 1994; Lee and Benveniste, 1996).

CIITA is also important in the regulation of expression of invariant chain (Ii), HLA-DMA, and HLA-DMB (Chin et al., 1994; Chang and Flavell, 1995; Kern et al., 1995), which, like MHC class II genes, contain X and Y boxes within the promoter region (O'Sullivan et al., 1986; Kelly et al., 1991a). Coordinate expression of these proteins is essential for MHC class II complex assembly and translocation to the cell surface membrane (Germain, 1994; Fling et al., 1994; Sloan et al., 1995).

This study shows that site α is essential for the IFNγ-induced transactivation of MHC class I through the ISRE. Furthermore, a second route of IFNγ-induced transactivation of MHC class I is revealed, and this route, which is mediated by CIITA, is also dependent on site α. Therefore, site α is crucial for both the classic route of IFNγ induction via the ISRE and a novel route of activation mediated by CIITA. Furthermore, the identification of CIITA as mediator of this route reveals a direct link between the cytokine-induced activation of MHC class I and MHC class II gene expression.

Results

Constitutive MHC Class I Expression Requires the Promoter Element Site α

Several regulatory elements in the MHC class I promoter have been implicated in constitutive expression. The importance of site α in constitutive expression was evaluated by transient transfection experiments in Epstein-Barr virus (EBV)–transformed B cells, which have a constitutively high expression of MHC class I. Transfection of luciferase reporter constructs containing promoter fragments of the human MHC class I gene HLA-B7 linked to the firefly luciferase gene showed that transactivation was highest in constructs containing the regulatory elements enhancer A, ISRE, and site α (pGL3-B250). Absence of both enhancer A and the ISRE in the promoter constructs (pGL3-B140) resulted in a reduced level of transactivation when compared to the full-length promoter constructs pGL3-B250 (Figure 1). Reporter constructs containing all upstream regulatory elements (enhancer A and ISRE) of the HLA-B7 promoter region but in which the 6 bp core sequence of site α is deleted (pGL3-B250Δα) did not result in any significant transactivation (Figure 1). This strongly suggests that site α fulfills a crucial role in cell lines that constitutively express MHC class I.

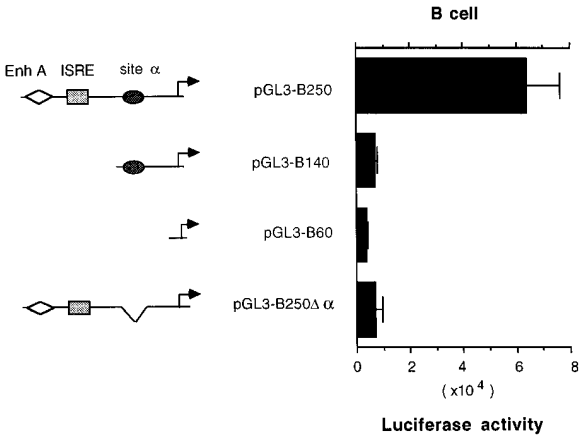

Figure 1. The Role of Site α in Constitutive HLA Class I Expression in B Cells

Transient transfection of the HLA-B7 promoter–luciferase reporter plasmids (pGL3-B250, pGL3-B140, pGL3-B60, and pGL3-B250Δα) in EBV-transformed B cells. Luciferase activity is shown as mean ± SD (n = 3).

Site α Is Crucial for IFNγ-Induced Transactivation via the ISRE

Transient transfection experiments were conducted in the cervix carcinoma cell line HeLa to determine the contributions of the different regulatory elements to IFNγ-induced MHC class I transactivation. IFNγ treatment of cells transfected with pGL3-B250 gave rise to a 4-fold induction of transcription (Figure 2). The importance of site α in IFNγ-induced MHC class I transcription was demonstrated using the site α deletion construct pGL3-B250Δα, which could not confer IFNγ-induced MHC class I transactivation. As expected, lack of the ISRE in the HLA-B7 promoter construct pGL3-B140 strongly impaired IFNγ transcription (Figure 2). Comparable results were obtained with a similar set of reporter constructs driven by the HLA-A2 promoter (data not shown). On the basis of these results, it can be concluded that site α is crucial for the functioning of the ISRE, which mediates IFNγ-induced HLA class I transactivation.

ATF/CREB Family Factors Bind Site α Constitutively

To determine what proteins interact with site α, electromobility shift assays using nuclear extracts from cell lines of different cell type origins were performed. These assays revealed the binding of several protein complexes to the site α probe in both untreated and IFNγ-treated HeLa cells (Figure 3). A similar pattern of bands was found using nuclear extracts from untreated and IFNγ-treated teratocarcinoma 2102Ep cells and B cells (Figure 3). In competition experiments, the complex could be competed for only with the consensus HLA-B7 site α probe and not with the mutant oligonucleotides

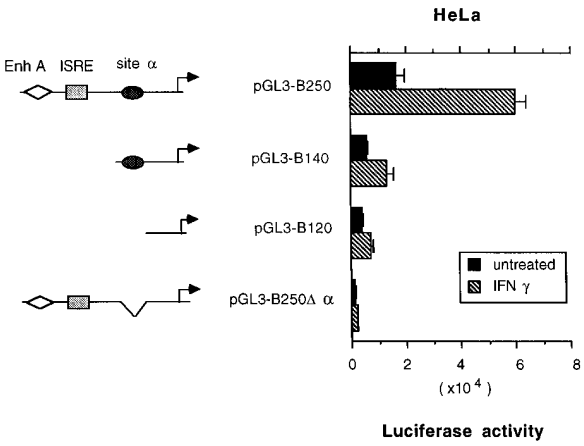

Figure 2. Site α Is Crucial for IFNγ-Induced Transactivation of HLA Class I via the ISRE

Transient transfection of reporter constructs, containing HLA-B promoter fragments of decreasing length or mutated in their site α core sequence (pGL3-B250, pGL3-B140, pGL3-B120, and pGL3-B250Δα) in HeLa cells. Cells were treated with IFNγ (500 U/ml) for 24 hr. Luciferase activity, corrected for transfection efficiency with β-galactosidase values, is shown as mean ± SD (n = 4).

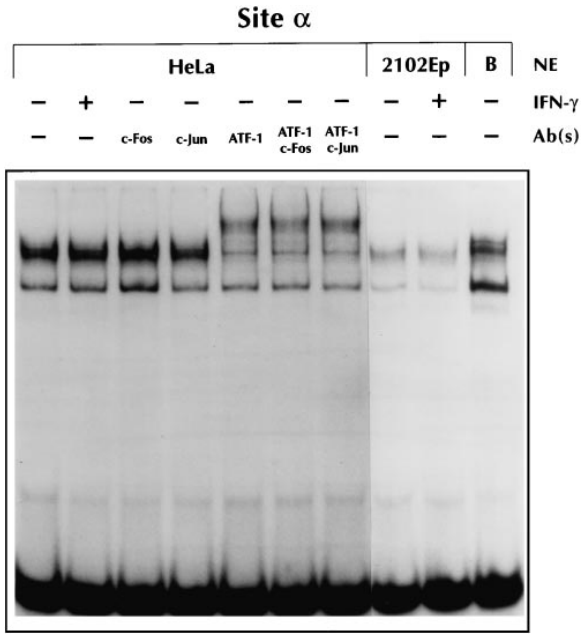

Figure 3. Site α Is Constitutively Bound by Factors of the ATF/CREB Family of Transcription Factors

Electromobility gel shift assay using a probe of the region containing site α of HLA-B7 (see Experimental Procedures) with nuclear extracts (NE) of HeLa, 2102Ep, and EBV-transformed B cells either untreated or treated with IFNγ (2 hr, 500 U/ml). For supershift assays, an anti-c-Fos antibody (Ab), an anti-c-Jun/AP-1 Ab, and an anti-ATF-1 Ab (see Experimental Procedures) were used as indicated.

(see Experimental Procedures) deleted in the site α core sequence (TGACGC) or containing a mutated site α core sequence (GGACGC) (data not shown), demonstrating the specificity of the complex. The nature of the transcription factors that bind to the site α probe was determined by supershift assays. Since the core sequence of site α (TGACGC) is homologous to AP-1– and CRE-binding sites, antisera directed against the Fos/Jun and ATF/CREB families of transcription factors were employed. As is shown in Figure 3, with the use of the anti-ATF-1 antibody (reactive with ATF-1, CREB-1, and CREM-1), the protein(s) contained in the complexes binding site α could be identified as members of the ATF/CREB family of transcription factors. There was no apparent binding of proteins of the Fos/Jun family of transcription factors to site α under these conditions, not even when the complex was first supershifted with the anti-ATF-1 antibody, revealing another band migrating at similar height (Figure 3). Similar results were found in supershift assays using nuclear extracts from 2102Ep and B cells (data not shown).

CIITA Is a Transactivator of MHC Class I

Although site α was shown to be involved in the IFNγ-induced MHC class I transactivation through the ISRE, no additional complex that binds site α could be detected after IFNγ induction. This led us to the hypothesis that an additional, putative non–DNA-binding protein was involved in the IFNγ-induced transactivation of MHC class I. For MHC class II transactivation, such a role had already been assigned to the coactivator CIITA, which is itself under the control of the IFNγ induction pathway (Steimle et al., 1993; Steimle et al., 1994). Furthermore, the X2 box in MHC class II promoters, which has been shown to be important in CIITA-mediated transactivation of MHC class II genes (Zhou and Glimcher, 1995), also has binding affinity for Fos/Jun- and ATF/CREB-like proteins (Anderson and Peterlin, 1990; Liou et al., 1990; Ono et al., 1991a, 1991b; Cox and Goding, 1992; Moreno et al., 1995).

To test whether CIITA could transactivate MHC class I gene expression, transient transfection experiments were performed in several IFNγ-sensitive cell lines. Cotransfection of the episomal expression vector pREP4-CIITA with pGL3-B250, resulted in a markedly induced HLA-B7–driven transcription in HeLa and 2102Ep cells (Figure 4). Similarly, HLA-A2–driven transcription was induced by cotransfection of pREP4-CIITA. As expected, HLA-DRA–driven gene transcription was strongly induced by cotransfection of pREP4-CIITA. HLA-G–driven reporter constructs served as a negative control in these experiments, since HLA-G lacks most of the MHC class I promoter regulatory elements and is insensitive to IFNγ induction (data not shown; Le Bouteiller, 1994). Similar results were found in several other cell lines, such as the teratocarcinoma cell line Tera-2, the melanoma IGR39D, and SV40-transformed dermal fibroblast cells (data not shown). On the basis of these results, it can be concluded that CIITA is capable of transactivating classic MHC class I genes.

Transactivation by CIITA Is Mediated through Site α

To determine whether CIITA would be acting via site α, the HLA-B7 promoter constructs were tested in HeLa and 2102Ep cells. As shown in Figure 5, lack of the enhancer A and ISRE in pGL3-B140 did not influence

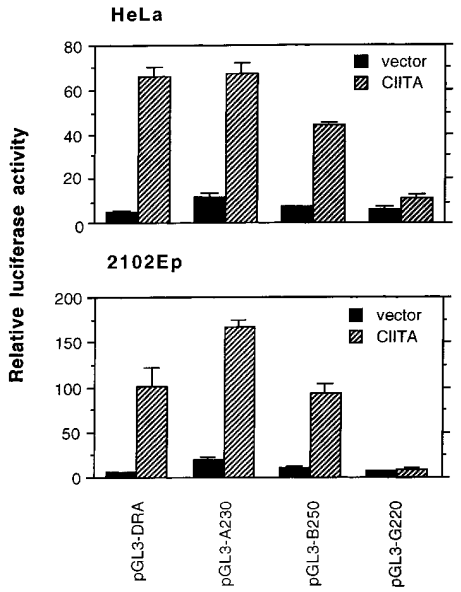

Figure 4. HLA-A and HLA-B Transcription Is Induced by CIITA

Transient cotransfection of the HLA-DRA, HLA-A2, or HLA-B7 promoter–luciferase reporter plasmids (pGL3-DRA, pGL3-A230, and pGL3-B250, respectively) with pREP4-CIITA in HeLa and 2102Ep. HLA-G promoter–luciferase reporter plasmid (pGL3-G220) served as a negative control. Relative luciferase activity ($\times 10^3$) is shown as mean ± SD (n = 4) and was calculated as the luciferase activity of the test constructs divided by the luciferase activity values of pGL3-Basic. Luciferase values were corrected for transfection efficiency with β-galactosidase activity.

the ability of CIITA to transactivate HLA-B7–driven transcription. However, absence of the promoter region between −140 and −120 bp upstream of the transcription start (containing site α) resulted in a complete loss of CIITA-induced expression (pGL3-B120; Figure 5). In addition, the site α mutant construct pGL3-B250Δα could not be transactivated by CIITA (Figure 5). Comparable results were obtained with a similar set of reporter constructs driven by the HLA-A2 promoter (data not shown). From these experiments it can be concluded that site α is crucial for MHC class I gene transactivation by CIITA.

Mutated Forms of CIITA That Are Not Functional in MHC Class II Transactivation Are Unable to Transactivate MHC Class I

Earlier studies have shown that CIITA contains three domains involved in transactivation of MHC class II gene expression (Riley et al., 1995; Zhou and Glimcher, 1995). These include an N-terminal acidic domain with transactivation properties; a proline-, serine-, and threonine-rich domain; and a C-terminal domain. To evaluate which domains of CIITA are essential for MHC class I transactivation, several constructs of CIITA expressing truncated forms or forms mutated in the C-terminal domain were tested in cotransfection experiments. The CIITA form found in cell line RJ2.2.5 (pREP4-CTIIA-RJ), belonging to the BLS complementation group A, did not manifest transactivating activity of MHC class I, and neither did cotransfection of the truncated forms of CIITA, pREP4-CIITA-N110 and pREP4-CIITA-N410 (Figure 6). This indicates that the N-terminal and the proline-, serine-, and threonine-rich domains are not sufficient and require in addition the C-terminal domain for MHC class I transactivation.

Only the MHC Class I Heavy Chain Is Induced by CIITA

To evaluate the potential transcriptional induction of the antigen- processing genes by CIITA, cotransfections were performed with pREP4-CIITA and reporter constructs containing the TAP1 or LMP2 promoter region. These transient transfection experiments showed that CIITA was not able to induce either TAP1 or LMP2 transcription in HeLa cells (Figure 7A). To assess the capacity of CIITA to transactivate endogenous MHC class I, TAP, and LMP genes, stably CIITA-transfected HeLa and 2102Ep cells were generated. Pools of hygromycin-resistant colonies were analyzed by immunofluorescence for HLA-DR cell surface expression, and the MHC class II–positive cells among these primary pools were enriched for by cell sorting and then used for further analysis. A semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay was used to determine the level of CIITA transcripts in these stably transfected cells. In the CIITA-transfected cells, a high level of CIITA transcripts could be detected, and this

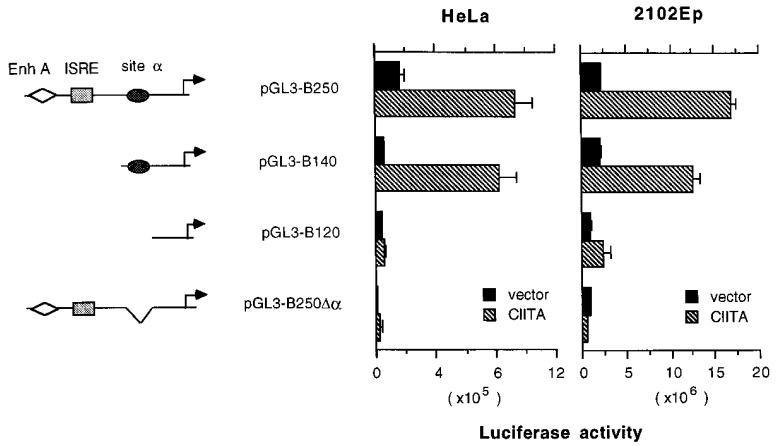

Figure 5. Site α Is Crucial in CIITA-Mediated Transactivation of HLA Class I

Transient cotransfection in HeLa and 2102Ep cells of pREP4-CIITA with reporter constructs, containing HLA-B7 promoter fragments of decreasing length or mutated in the site α core sequence. Luciferase activity, corrected for transfection efficiency with β-galactosidase values, is shown as mean ± SD (n = 4).

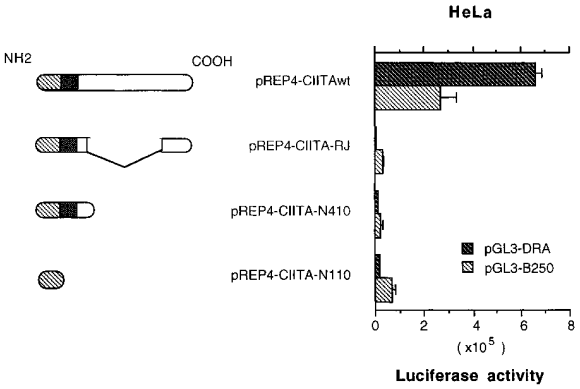

Figure 6. Mutated Forms of CIITA That Are Not Functional in HLA Class II Transactivation Are Unable to Transactivate HLA Class I

The capacity of the deleted CIITA form of RJ2.2.5 or truncated CIITA forms to transactivate HLA class I was tested by transient cotransfection with the HLA-B7 promoter–driven luciferase reporter plasmids. HLA-DRA promoter constructs served as a control. Luciferase activity, corrected for transfection efficiency with β-galactosidase values, is shown as mean ± SD (n = 4).

level increased only slightly upon IFNγ treatment; in the mock-transfected cells, CIITA transcripts were difficult to detect in noninduced cells but were markedly increased after IFNγ treatment (Figure 7B). Northern blot analysis revealed that the CIITA-transfected cells displayed an induced level of endogenous MHC class I and class II transcripts (Figure 7C and Table 1). In contrast, β_2m, TAP1, TAP2, LMP2, and LMP7 genes displayed no significant increase in the level of transcription in CIITA-transfected cells (Figure 7C and Table 1). The increased level of MHC class I transcription was also reflected in an increased expression of MHC class I heavy chain protein, as determined by Western blot analysis (Figure 7D).

CIITA Can Enhance the Level of MHC Class I Cell Surface Expression

Since only MHC class I heavy chain was induced by CIITA and since the genes that encode molecules involved in antigen processing and assembly of MHC class I molecules were not, we evaluated possible changes in cell surface expression in an extended panel of CIITA-transfected cells. The level of MHC class I and class II cell surface expression was determined by fluorescence-activated cell sorter analysis (Figure 8). Expression of the exogenous CIITA gene resulted in a negligible increase in the level of MHC class I cell surface expression on HeLa, 2102Ep, and WSI (fibroblast) cells, whereas cell surface expression of MHC class II was clearly induced (Figure 8). In contrast, CIITA had a marked effect on the level of MHC class I cell surface expression in several other cell lines (the chronic myelogenous leukemia cell line K562, the fibroblast cell line ATU, and the fibrosarcoma cell line G3A; see Experimental Procedures). A significant increase in the level of MHC class I molecules expressed at the cell surface was observed in CIITA- transfected K562, ATU, and G3A cells (Figure 8). The population of CIITA-transfected G3A cells was heterogeneous: about 50% of the cells were MHC class II positive, and the same heterogeneity was seen for the elevated level of MHC class I expression (Figure 8).

Table 1. Quantification of Transcription Levels in CIITA-Transfected Cells

	Fold Induction	
	HeLa	2102Ep
HLA-DRA	24.19	7.70
HLA class I	1.56	1.46
β_2m	0.79	0.98
TAP1	0.94	1.22
TAP2	0.78	1.06
LMP2	0.95	1.11
LMP7	0.88	1.28

Values are calculated as the ratio of the normalized densitometric values of CIITA-transfected cells over those of mock-transfected cells. Densitometric values were normalized to the corresponding GAPDH values and were derived from the Northern blots shown in Figure 7C.

Discussion

The expression of MHC class I genes is tightly regulated both during development and also in fully differentiated cells. Control at the transcriptional level is determined by various regulatory elements in the MHC class I promoter. Site α, positioned −130 bp upstream of the transcription start site, is highly conserved among the various MHC class I loci, with the exception of HLA-G (Dey et al., 1992; Ting and Baldwin, 1993; Le Bouteiller, 1994). The exact role of site α in MHC class I transcriptional control has hitherto been poorly defined. It has been shown that site α, together with other regulatory elements, contributes to the constitutive expression of MHC class I in lymphoid cells (Dey et al., 1992; Baeuerle and Henkel, 1994; Le Bouteiller, 1994; this study). The core sequence of site α (TGACGC) present in HLA-B, HLA-C, HLA-E, and HLA-F (C/GGACGC in HLA-A) is homologous to CRE- or AP-1– binding sites, and it has been speculated that proteins of the Fos/Jun or ATF/CREB family of transcription factors bind to this site (Korber et al., 1988; Israël et al., 1989; Dey et al., 1992). In this study, it could be demonstrated that in both lymphoid and nonlymphoid cells, members of the ATF/CREB family of transcription factors do indeed bind site α and do so in a constitutive manner. Binding by proteins of the Fos/Jun family of transcription factors could not be detected under these conditions. In view of these findings, it is noteworthy that the X2 box in MHC class II promoters, which has been shown to be important in CIITA-mediated transactivation of MHC class II genes (Zhou and Glimcher, 1995), also has binding affinity for ATF/CREB-like proteins (Anderson and Peterlin, 1990; Liou et al., 1990; Ono et al., 1991a; Cox and Goding, 1992; Moreno et al., 1995).

The IFNγ induction of MHC class I genes is known to be mediated through binding of the transactivator IRF-1 to the ISRE (Chang et al., 1992; Girdlestone et al., 1993; Johnson and Pober, 1994; unpublished data). Here, it is shown that site α is crucial for this classic route of IFNγ-induced MHC class I gene expression. However,

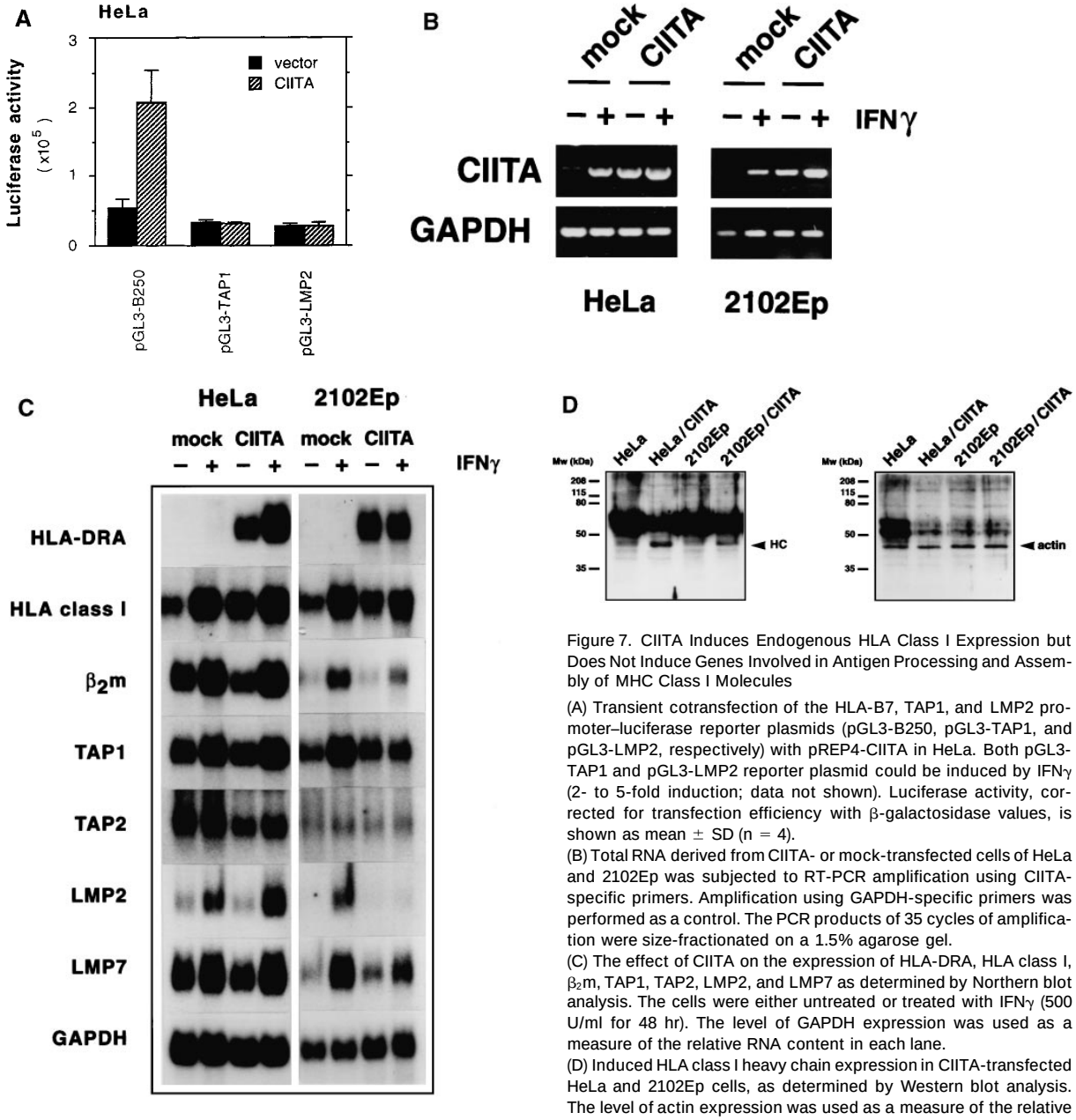

Figure 7. CIITA Induces Endogenous HLA Class I Expression but Does Not Induce Genes Involved in Antigen Processing and Assembly of MHC Class I Molecules

(A) Transient cotransfection of the HLA-B7, TAP1, and LMP2 promoter–luciferase reporter plasmids (pGL3-B250, pGL3-TAP1, and pGL3-LMP2, respectively) with pREP4-CIITA in HeLa. Both pGL3-TAP1 and pGL3-LMP2 reporter plasmid could be induced by IFNγ (2- to 5-fold induction; data not shown). Luciferase activity, corrected for transfection efficiency with β-galactosidase values, is shown as mean ± SD (n = 4).

(B) Total RNA derived from CIITA- or mock-transfected cells of HeLa and 2102Ep was subjected to RT-PCR amplification using CIITA-specific primers. Amplification using GAPDH-specific primers was performed as a control. The PCR products of 35 cycles of amplification were size-fractionated on a 1.5% agarose gel.

(C) The effect of CIITA on the expression of HLA-DRA, HLA class I, β_2m, TAP1, TAP2, LMP2, and LMP7 as determined by Northern blot analysis. The cells were either untreated or treated with IFNγ (500 U/ml for 48 hr). The level of GAPDH expression was used as a measure of the relative RNA content in each lane.

(D) Induced HLA class I heavy chain expression in CIITA-transfected HeLa and 2102Ep cells, as determined by Western blot analysis. The level of actin expression was used as a measure of the relative protein content in each lane.

no new DNA-binding complex could be detected binding site α after IFNγ treatment. Site α, which is shown to be constitutively occupied, may allow interaction between proteins of the IRF family binding the ISRE and proteins of the ATF/CREB family binding site α. The protein complex binding site α may also facilitate interactions with a coactivator. In this study, it is shown that CIITA, which originally was described as a transcriptional coactivator specific for genes involved in MHC class II–mediated antigen presentation, is also capable of up-regulating MHC class I expression in human (tumor) cell lines of various tissue origin. Therefore, CIITA represents a direct link between IFNγ-induced MHC class I and class II gene regulation. In mutational analysis of the MHC class I promoter region, it was found that site α mediates transactivation by CIITA and is therefore also crucial for this pathway of transactivation. Similar results are found for the mouse MHC class I promoter (Martin et al., 1997 [*Immunity*, this issue]).

CIITA contains three domains essential for transactivation of MHC class II: an N-terminal acidic domain with transactivation properties; a proline-, serine-, and threonine-rich domain; and a C-terminal domain (Riley et al., 1995; Zhou and Glimcher, 1995). Mutation analysis has shown that the N-terminal acidic domain is essential for general transcription activation of MHC class II; the proline-, serine-, and threonine-rich domain is required for optimal transcriptional activity; and the C-terminal domain is essential for specific interactions with MHC class II transcription regulators. Because the truncated

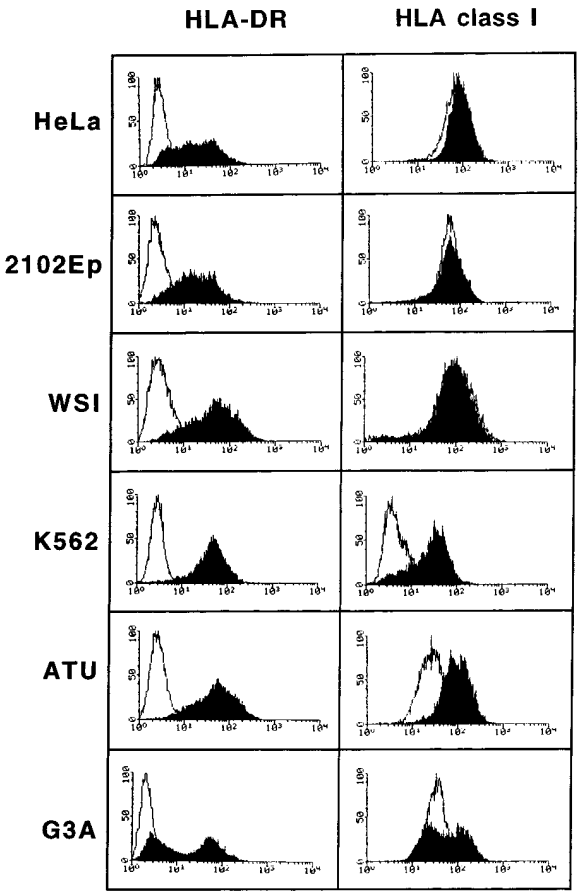

Figure 8. CIITA Can Enhance the Level of MHC Class I Cell Surface Expression

The level of HLA class II and class I cell surface expression on CIITA-transfected HeLa, 2102Ep, WSI, K562, ATU, and G3A cells was determined by fluorescence-activated cell sorter analysis. Filled and open profiles represent CIITA- and mock-transfected cells, respectively.

forms of CIITA tested were unable to transactivate MHC class I, it is very possible that all three domains are required and carry out a similar function in the transcriptional regulation of both MHC class I and class II. This is supported by evidence from a detailed structural domain analysis by Martin et al. (1997).

The results presented here demonstrate that CIITA induces MHC class I gene transcription, leading to increased levels of MHC class I heavy chain protein. However, it was shown that CIITA had no apparent regulatory effect on the expression of β_2m and the antigen-processing genes important for MHC class I–mediated antigen presentation at the cell surface, which is in agreement with findings by Martin et al. (1997). This may explain why the observed induced level of transcription of the MHC class I heavy chain gene in the CIITA-transfected cells did not result in a significant increase in cell surface expression in HeLa, 2102Ep, and fibroblast cells. It can be envisaged that in these cells the CIITA-induced MHC class I heavy chain expression is not sufficient to induce class I cell surface expression because the levels of expression of the β_2m, TAP, or LMP genes remain unchanged and could therefore become limiting. To induce the level of MHC class I cell surface expression in these cells, other components of the IFNγ induction pathway would be needed for a concommitant induction of the β_2m, TAP, or LMP genes. In contrast, in cell lines in which the cell surface expression is limited by low levels of MHC class I heavy chain expression, CIITA is able to enhance the level of MHC class I cell surface expression. This is best illustrated in the MHC class I heavy chain–deficient K562 (Sutherland et al., 1985; Chen et al., 1986; Blanchet et al., 1991), in which CIITA was able to restore MHC class I cell surface expression. In addition, cell lines such as G3A (Chin et al., 1994) and ATU (BLS fibroblast cell line; A. P. et al., unpublished data), in which the level of MHC class I heavy chain is reduced, also displayed a marked increase in MHC class I cell surface expression upon CIITA transfection.

Transcriptional gene expression is generally regulated by a number of transactivating factors acting in concert. Therefore, lack of one transcription factor may be of influence but could be compensated by others. For the recently generated CIITA$^{-/-}$ knockout mice (Chang et al., 1996), it was not reported whether the constitutive and IFNγ-induced level of MHC class I expression in the different cell types tested was similar to that in wild-type mice, but if so, this could be an example of such compensation by other (related) transcription factors. In this respect, the complete dependency of MHC class II gene expression on one transactivator is more likely an exception than a rule.

Taking all of these findings into account, we propose a new model for IFNγ-induced transactivation of MHC class I, in which site α plays a central role (Figure 9). This pathway is initiated by activation of factors of the JAK/STAT pathway by the binding of IFNγ to its receptor (Darnell et al., 1994; Schindler and Darnell, 1995). This results in the induction or activation of transcription factors of the IRF family (such as IRF-1), which bind the ISRE and transactivate MHC class I genes (Ting and Baldwin, 1993; Le Bouteiller, 1994). Although this pathway is dependent on site α, it is not clear at this time whether this is the result of interactions between the transcription factors occupying both regulatory elements. The observed induction of endogenous CIITA expression by IFNγ and the enhanced level of MHC class I expression in CIITA-transfected cells strongly suggest a parallel activation route for IFNγ-induced MHC class I gene expression. In this second route, the activation of the JAK/STAT pathway leads to the induced expression of CIITA (Steimle et al., 1994; Lee and Benveniste, 1996; Meraz et al., 1996). CIITA could actuate an increase in MHC class I heavy chain expression by acting as a coactivator, presumably interacting with the ATF/CREB transcription factors occupying site α. This hypothesis is supported by the observation that deletion of the site α core sequence abrogates transactivation by CIITA. Under these experimental conditions, site α is apparently not dependent on the ISRE and its binding proteins to mediate transactivation by CIITA, because promoter constructs lacking elements upstream from site α are still activated by CIITA. Whether CIITA itself can transactivate the basal transcription complex or is

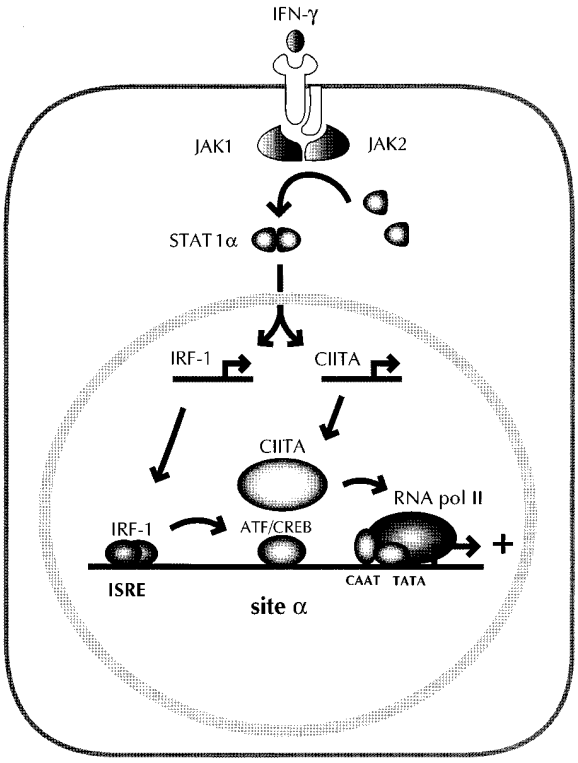

Figure 9. Model for IFNγ-Induced MHC Class I Gene Transcription Regulation

In the proposed model, the classic route of IFNγ induction via the ISRE and the new route of activation mediated by CIITA are merged by a mutual dependency on site α. The classic route by which the induced or activated transcription factors of the interferon regulatory factor family (such as IRF-1) bind the ISRE and transactivate MHC class I genes is paralleled by a second route by which the IFNγ-induced expression of CIITA actuates an increase of MHC class I heavy chain expression by acting as a coactivator possibly interacting with the ATF/CREB transcription factors that occupy site α.

merely an activator of site α–binding factors in MHC class I transactivation remains to be determined.

In conclusion, it is shown that site α is essential for the IFNγ-induced transactivation of MHC class I through the ISRE. In addition, this report provides evidence for a second site α–dependent pathway of IFNγ-induced activation of MHC class I genes. This new route of transactivation mediated by CIITA is the first direct link between MHC class I and MHC class II gene activation. Site α is therefore crucial for both the classic route of IFNγ induction via the ISRE and the novel route of activation mediated by CIITA.

Experimental Procedures

Cell Culture

Cell lines used in this study were the human cervical carcinoma cell line HeLa, the teratocarcinoma cell line 2102Ep (Andrews et al., 1987), the teratocarcinoma cell line Tera-2, the melanoma cell line IGR39D (a kind gift from Dr. P. I. Schrier; Versteeg et al., 1988), an EBV-transformed B cell line (MBW), the SV40-transformed fibroblast cell line WSI, the chronic myelogenous leukemia cell line K562, the in vitro generated mutant fibrosarcoma cell line G3A (a kind gift from Dr. J. P.-Y. Ting; Chin et al., 1994), and the SV40-transformed fibroblast cell line ATU (a kind gift of Dr. Ö. Sanal). The cell line ATU is derived from a BLS patient and is characterized by a lack of (IFNγ-induced) MHC class II cell surface expression, which can be restored upon CIITA transfection (A. P. et al., unpublished data). All cells were grown in Iscove's modified Dulbecco's modified minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco, Paisley, Scotland), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Recombinant human IFNγ (Boehringer-Ingelheim, Ingelheim, Germany) was used at a concentration of 500 U/ml.

Plasmids

The episomal expression vector construct pREP4-CIITA contains the 4.5 kb CIITA cDNA fragment (Steimle et al., 1993) cloned in the XhoI site of pREP4 (Invitrogen, San Diego, CA). The expression of CIITA is driven by promoter sequences from the Rous sarcoma virus long terminal repeat. The truncated CIITA constructs pREP4-CIITA-N110 and pREP4-CIITA-N410 contain a 445 bp NotI–PvuII and 1345 bp NotI–NotI fragment of pREP4-CIITA, respectively. A mutated CIITA form present in the cell line RJ2.2.5 of BLS complementation group A was generated by PCR amplification (forward primer ATG GCTAGCGATTCCTACACAATGCGTTGCCTGGCTC, reverse primer ATGCTCGAGCAAGGTCCAGCGTGGTTAGTGTCCTCAG; Riley et al., 1995). The PCR–amplified cDNA fragments were cloned into pREP4/NheI–XhoI to give rise to pREP4-CIITA-RJ.

Reporter constructs were all based on the luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI) and contained either a 294 bp XbaI–SacI HLA-DRA promoter fragment (pGL3-DRA), a 230 bp BglI–AhaII HLA-A2.1 promoter fragment (pGL3-A230), a 140 bp PpuMI–AhaII HLA-A2.1 promoter fragment (pGL3-A140), a 120 bp AvaII–AhaII HLA-A2.1 promoter fragment (pGL3-A120), a 268 bp AspI–AhaII HLA-B7 promoter fragment (pGL3-B250), a 140 bp PpuMI–AhaII HLA-B7 promoter fragment (pGL3-B140), a 120 bp MluI–AhaII HLA-B7 promoter fragment (pGL3-B120), a 60 bp XbaI–AhaII HLA-B7 promoter fragment (pGL3-B60) or a 220 bp HLA-G promoter PCR fragment (pGL3-G).

Site α mutant constructs, deleted in the 6 bp core sequence, were generated by site-directed mutagenesis using the overlapping extension method (Ho et al., 1989). Primers used for amplification of HLA-A2 promoter fragments were A_{A2}: 5′-GATAGATCTCAGGCC CCGAAGGC; B_{A2}: 5′-ACTGGGTCCTGAGTATCCAGGAAGAAGGA; C_{A2}: 5′-TGGATACTCAGGACCCAGTTCTCACTCC; and D_{A2}: 5′-GAT AAGCTTCGGCGTCTGGGGAGAAT. Primers used for amplification of HLA-B7 promoter fragments were A_{B7}: 5′-GATAGATCTCAGGAC GAAGTCCCAGG; B_{B7}: 5′-AAGTGGGGACGAGTATCCTAGAAGAA GGA; C_{B7}:5′- AGGATACTCGTCCCCACTTCCCACTCC; and D_{B7}: 5′-GATAAGCTTCGGCGTCTGAGGAGACT. First, two separate PCR fragments were generated (25 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min) with primer sets A/B and C/D. Next, the two products were annealed by virtue of overlapping sequences in primers B and C, and a second PCR amplification was performed on the elongated template (35 cycles at 95°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min) using primer set A/D. The PCR fragments were digested at their introduced BglII and HindIII sites and ligated into the BglII and HindIII sites of pGL3-Basic (Promega). All plasmids were verified by sequence analysis (T7-polymerase sequence kit; Amersham, Buckinghamshire, England).

Reporter constructs containing the TAP1 or LMP2 promoter used were pGL3-Enhancer vector-based plasmids (a kind gift from Dr. B. Seliger, III Medizinische Klinik, J. Gutenberg Universität Mainz, Germany) as well as the CAT reporter plasmids (a kind gift from Dr. J. P.-Y. Ting; Wright et al., 1995).

Transient Transfection

The EBV-transformed B-cells (5×10^6) were transfected by electroporation (300 V, 960 μF; Genepulser, Bio-Rad Laboratories, CA) with 20 μg pGL3 reporter plasmid and harvested 40 hr after transfection. Adherent cells were transfected by the calcium phosphate coprecipitation method (Chen and Okayama, 1987). In each of four wells in a six-well plate, 0.25×10^6 cells were transfected with 2.5 μg pGL3 reporter plasmid, 6.25 μg of either pREP4 or pREP4-CIITA, and 2.5 μg pRSVLacZ plasmid (Promega) and harvested 40 hr after transfection. For dose-response experiments, 0.5 μg pGL3 reporter plasmid was cotransfected with various concentrations of pREP4-CIITA (2.44 ng to 20 μg) supplemented with pREP4 to a final amount

of 20 μg, and 2.5 μg pRSVLacZ control plasmid. The pGL3-based reporter constructs were linearized prior to transfection. Luciferase activity was determined using a luminometer (Tropix, Bedford, MA) and corrected for β-galactosidase activity and is shown as mean ± standard deviation (SD). In some experiments, the luciferase values are expressed as relative luciferase activity, which is the luciferase activity of the promoter-containing constructs divided by the luciferase activity value of pGL3-Basic and which is shown as mean ± SD.

Preparation of Nuclear Extracts

Nuclear extracts were prepared from 10×10^6 cells. The cells were harvested, washed with phosphate-buffered saline and taken up in 300 μl (three cell volumes) of hypotonic solution (20 mM HEPES [pH 8.0], 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 1 mM dithiothreitol [DTT], 0.5 mM AEBSF) and were left on ice for 15 min. The cells were lysed with NP-40 (final concentration of 0.1% for B cells, 0.2% for 2102Ep, and 0.4% for HeLa) for 3–5 min. Eighty microliters (80% of the cell volume) of a sucrose solution (50 mM HEPES [pH 8.0], 10 mM KCl, 0.25 mM EDTA, 1 mM DTT, 0.5 mM AEBSF, 70% w/v sucrose) was added, and the nuclei were centrifuged cold at 5000 rpm for 5 min. The supernatant was discarded and the pellet was gently taken up in 300 μl (three cell volumes) of solution B (10 mM HEPES [pH 8.0], 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM AEBSF, 25% v/v glycerol) and centrifuged at 4°C at 5000 rpm for 5 min. After the supernatant was discarded, the cell pellet was taken up in 200 μl (two to three cell volumes) of extraction solution (10 mM HEPES [pH 8.0], 400 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM AEBSF, 25% glycerol) and left on ice for 30 min with intermittent vortexing. The extracted nuclei were centrifuged at 4°C at 14,000 rpm for 5 min, and the supernatant was aliquoted and stored at −80°C.

Electromobility Gel Shift Assay

Nuclear extracts (about 5 μg) were incubated in binding buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 10% v/v glycerol, 0.5 mM DTT, 0.1 mM EDTA, 1 μg poly(dI-dC), and 1 μg sonicated single-stranded herring sperm DNA) with 1 ng [^{32}P]-radiolabeled probe for 15 min at 4°C. The samples were run on a 6% polyacrylamide gel in 0.25× TBE buffer at 200 V for 2 hr. The gels were fixed and dried onto Whatman paper and exposed to X-ray film. The double-stranded oligonucleotide representing HLA-B7 site α (GGATACTCGTGACGC GTC) was used as a probe.

Competition experiments were performed with the HLA-B7 site α probe and two different mutant oligonucleotides: B7Δα, which has a 6 bp mutation of the site α core sequence (AGGATACTCGTCCCCA CTTCCCACTCC), or B7αA3, which contains a variant of the site α core sequence (TACTCGCGGACGCGTCC) present in HLA-A3. Each (cold) double-stranded oligonucleotide was added at 50×, 100×, and 200× excess before incubation with radiolabeled HLA-B7 site α probe.

In supershift assays, 1 μg of each antibody directed against member of the Fos/Jun or ATF/CREB family of transcription factors were added to nuclear extracts and probe mixture and incubated overnight at 4°C. The anti-c-Fos antibody (sc-413) is broadly reactive with c-Fos, Fos B, Fra-1, and Fra-2; the anti-c-Jun/AP-1 antibody (sc-44) is broadly reactive with c-Jun, Jun B, and Jun D; and the anti-ATF-1 antibody (sc-270) is reactive with ATF-1, CREB-1, and CREM-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Generation of Stable Cell Lines Transfected with pREP4-CIITA

Adherent cell lines were transfected according to the calcium phosphate coprecipitation method (Chen and Okayama, 1987), and the nonadherent cell line K562 was transfected with the lipofectin Tfx-50 (Promega) according to the manufacturer's recommendations. The cells were transfected with either pREP4-CIITA or control plasmid and were grown on selection medium. After selection on 100 μg/ml (HeLa) or 50 μg/ml (2102Ep) Hygromycin B (Boehringer Mannheim, Mannheim, Germany) for approximately 2 weeks, hygromycin-resistant colonies were pooled.

Flow Cytometric Analysis and Cell Sorting

The MHC class II positive cells in the CIITA-transfected pool (5%–10%) were enriched for by cell sorting using a FACSTAR flow cytometer (Becton-Dickinson, Mountain View, CA). Pools of the cell lines transfected with pREP4 (mock-transfected cells) were used as a negative control. Subconfluent cultures of the CIITA- or mock-transfected cells were stained by indirect immunofluorescence, with an anti-HLA-DR monoclonal antibody or the anti-MHC class I monoclonal antibody W6/32 as first antibody and anti-mouse IgG as second antibody (Becton-Dickinson). The analysis was performed on a FACScan flow cytometer (Becton-Dickinson) using FACScan and LYSIS software.

The MHC class II–positive cells in the CIITA-transfected pool were enriched for by cell sorting using either a FACSTAR flow cytometer (Becton-Dickinson) or Dynabeads coated with sheep anti-mouse IgG as second antibody (Dynal AS, Oslo, Norway). Pools of the cell lines transfected with pREP4 (mock-transfected cells) served as a negative control.

RT-PCR Assay

For semiquantitative RT-PCR analysis, total RNA was isolated with RNAzol (Cinna/Biotecx Laboratories, Houston, TX) following the manufacturer's instructions. First strand cDNA was synthesized in the presence of random hexamer (Pharmacia) using the Riboclone cDNA Synthesis System (Promega) according to the manufacturer's instructions. Semiquantitative PCR was performed in the linear phase of amplification by testing PCR products after varying numbers of cycles. For the PCR, 1 μl of cDNA sample was used in a total reaction volume of 100 μl containing 10 mM Tris-HCl [pH 8.4], 50 mM KCl, 4 mM $MgCl_2$, 0.6 μg/ml BSA, 0.5 mM of each dNTP, 20 pmol of 5′ primer, and 20 pmol of 3′ primer. The following primer pairs were used: CIITA, sense 5′-GCTTGTGGCCGGCTTCCCCAGT and anti-sense 5′-TGGAAGATCAGCCCAGCCAGAAAGC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5′-GGTCGG AGTCAACGGATTTG and anti-sense 5′-ATGAGCCCCAGCCTTCTC CAT. The samples were heated to 95°C for 2 min and cooled to 85°C, at which point 2.5 U AmpliTaq DNA polymerase was added (Perkin-Elmer Cetus, Emeryville, CA), and the PCR amplification was run for 10, 20, 30, or 40 cycles (1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C). The PCR products, 15 μl for CIITA samples and 5 μl for GAPDH samples, were size fractionated on an 1.5% agarose gel, transferred to a Hybond N^+ nylon membrane (Amersham), and subsequently hybridized with ^{32}P-labeled human CIITA (Steimle et al., 1993) or GAPDH probes (Versteeg et al., 1988), to verify the amplified products. Half of the total RNA of each sample was subjected to the same protocol but without reverse transcriptase in the reaction mixture, to serve as a control for DNA contamination.

Northern Blot Analysis

Total RNA was isolated from subconfluent cultures by the RNAzol extraction method (Cinna/Biotecx Laboratories). RNA samples (20 μg/lane) were run on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to a Hybond N membrane (Amersham), and hybridized with ^{32}P-labeled probes (random priming; DuPont–NEN, Boston, MA) for HLA-DRA (Marcadet et al., 1987), HLA class I (Sood et al., 1981; Marcadet et al., 1987), β_2m (Gussow et al., 1987), TAP1 (Spies et al., 1990), TAP2 (Bahram et al., 1991), LMP2 (Kelly et al., 1991b), LMP7 (Glynne et al., 1991), and β-actin (Cleveland et al., 1980). For quantitation, the autoradiograms were scanned using an Eagle Eye II apparatus and Eagle Sight 3.0 image-capture and analyzing software (Stratagene, La Jolla, CA).

Western Blot Analysis

Cells (5×10^6) were harvested in lysis buffer (0.5% [v/v] NP-40, 50 mM Tris-HCl [pH 7.9], 150 mM NaCl, 5 mM EDTA, 2 mM PMSF), and the content of solubilized protein in the lysates was determined (BCA protein assay kit; Pierce, Rockford, IL). Protein extracts (500 ng/lane) were separated on a 10% SDS polyacrylamide gel, transferred to a PVDF transfer membrane (Polyscreen; NEN, Boston, MA). The proteins were immunostained with anti-MHC class I heavy chain antibody (HC, 1:1000; Neefjes and Ploegh, 1988) or anti-actin antibody (1:1000; Sigma Chemical, St. Louis, MO) followed by horseradish peroxidase–conjugated anti-IgG antibodies. The blots were developed using an enhanced chemoluminescence Western blotting detection system (Amersham) according to the manufacturer's recommendations.

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