Suppression of MHC class II expression by human class II trans-activator constructs lacking the N-terminal domain

Sheng Yun, Kenth Gustafsson and John W. Fabre

Division of Cell and Molecular Biology, Institute of Child Health, University College London Medical School, 30 Guilford Street, London WC1N 1EH, UK

Keywords: class II trans-activator, MHC class II, N-terminal domain

Abstract

The class II trans-activator (CIITA) is a bi- or multi-functional domain protein which plays a critical role in the expression of MHC class II genes. We report that removal of the N-terminal 151 amino acids, encompassing all of the acidic domain but leaving intact the proline-serine/threonine-rich domain, results in a mutant protein with potent suppressive properties for MHC class II expression. HeLa cells stably or transiently transfected with mutant CIITA constructs showed up to 99% suppression of MHC class II antigen induction by IFN-γ and marked suppression of HLA-DRA mRNA expression. Transient transfection of a B lymphoma line resulted in up to 89% reduction of constitutive MHC class II expression within 5 days and suppression of HLA-DRA mRNA synthesis.

Introduction

MHC-encoded class II molecules are heterodimeric cell surface molecules, the function of which is to present peptides to CD4+ thymocytes and peripheral T lymphocytes. They play a critical role in both thymic T cell ontogeny and the activation of peripheral T cell immune responses (1). Not surprisingly, genetic deficiency of MHC class II results in severe immunodeficiency, which has been called the 'bare lymphocyte syndrome' (2,3).

Complementation studies with cells from bare lymphocyte syndrome patients have demonstrated a minimum of four recessive loci, all outside the MHC, and therefore presumably coding for trans-acting regulatory factors (4). One of these regulatory factors has been identified and designated the class II trans-activator (CIITA) (5). Since the original identification, the CIITA molecule has been shown to play a critical role in IFN-γ-induced (6) and developmentally regulated (7) class II expression. In many situations, CIITA induction alone is sufficient to induce class II expression (8). Moreover, CIITA induces the expression of both the invariant chain and HLA-DM locus class II molecules (9), both of which play crucial roles in the intracellular trafficking, peptide loading and cell surface expression of MHC class II molecules (10).

The promoter region of class II MHC genes in CIITA-deficient patients is fully occupied by DNA binding proteins (11). This, together with the fact that CIITA shows no sequence homology to DNA binding proteins, suggests that it might bind to the proteins in the promoter region (5). The N-terminus has an acidic region, with 30% glutamate or aspartate residues between amino acids 26 and 137. This is followed by three regions rich in proline, serine and threonine (amino acids 163–195, 209–237 and 261–322) (5). Transcriptional activators have been shown to have acidic, proline-rich or glutamine-rich regions (for review see 12), and it was therefore suggested that CIITA might consist of an acidic N-terminal activation domain and a C-terminal domain for binding to MHC class II region promoter proteins (5).

Model studies using fusion proteins of different regions of CIITA with the DNA binding domain of a transcriptional activator have established that the acidic domain is indeed a potent transcriptional activator. All or most transcription activation activity could be ascribed to amino acids 1–125 (13) or 1–114 (14). The first two proline-serine/threonine-rich regions by themselves (amino acids 163–195, 209–237 and 261–322) had no transcription activation activity (13). Moreover, the C-terminal 813 amino acids, when fused to a general transcriptional activator, could specifically direct transcription from the DRA promoters, although substantially less potently than the native CIITA molecule (14).

Distinct functional domains in several transcriptional activators have enabled the construction of dominant negative mutants by deletion of the activation domain, while retaining the DNA binding domain (15–18). Whether or not this is possible...
with CIITA depends on the structural independence of the transcrip-
tion activation region and the promoter protein binding
region. The reduced ability of the 813 amino acid C-terminal
region to direct transcription from the DRA promoter suggests
that the conformation or other characteristics of this fragment
might not be optimal. Indeed, this fragment did not inhibit class
II expression in the Raji B lymphoblastoid cell line (14). As the
acidic domain has strong transcription activation activity and
the isolated proline-rich domain has none, we have studied
mutant molecules lacking the acidic regions only, to leave the
longest possible C-terminal fragment.

We report that deleting 151 amino acids from the N-terminal
region of human CIITA results in a 979 amino acid molecule
which strongly suppresses both constitutive and IFN-γ-
induced MHC class II expression. Such a construct has
clear potential for regulating MHC class II expression for
experimental and clinical purposes.

Methods

Cell culture

The HeLa cell line was maintained in DMEM and the
DoHH2 human B lymphoma cell line in RPMI 1640 (both
media from Gibco/BRL, Paisley, UK). In each case the
medium was supplemented with 10% FCS, 2 mM glutamine,
100 units/ml penicillin and 100 µg/ml streptomycin. For
passaging and all other manipulations, the HeLa cells were
recovered by scraping. All cultures were at 37°C in 95%
air/5% CO2.

Induction of MHC class II antigens

Human recombinant IFN-γ (a kind gift of Dr N. Klein,
Institute of Child Health, London) was added at 500 units/
ml to HeLa cells at 50% confluence. The cells were
harvested 24 and 48 h later. From each cell culture, 5
ml to HeLa cells at 50% confluence. The cells were
Institute of Child Health, London) was added at 500 units/
air/5% CO2.

We report that deleting 151 amino acids from the N-terminal
region of human CIITA results in a 979 amino acid molecule
which strongly suppresses both constitutive and IFN-γ-
induced MHC class II expression. Such a construct has
clear potential for regulating MHC class II expression for
experimental and clinical purposes.

Suppression of MHC class II by mutant CIITA

We report that deleting 151 amino acids from the N-terminal
region of human CIITA results in a 979 amino acid molecule
which strongly suppresses both constitutive and IFN-γ-
induced MHC class II expression. Such a construct has
clear potential for regulating MHC class II expression for
experimental and clinical purposes.

Construction of CIITA deletion mutants

The full sequence of the CIITA cDNA present in pBlueScript
(5) (kindly provided by Dr V. Steimle, Geneva) was moved
to the pcDNA3 expression vector using EcoRI and Xhol.

pcDNA3mutCIITA2 (Fig. 1A). This was constructed in two
steps. First, the pcDNA3 CIITA construct was cut with NotI
and Xhol, and the resulting fragment (representing position
1340 to the 3' end of the CIITA) was subcloned into NotI
and Xhol sites of pcDNA3. Next, PCR was used to
synthesize the fragment from amino acid 152 to the
NotI site. The upstream primer, 5'-ACTCGATATCATCAGGCA-
GAGCTGAAACAT-3', contained an introduced EcoRV site
(underlined) at position 567 of the original sequence, while
the downstream primer, 5'-GCTCAGTGTCCAGGCGCAATA-
3', was complementary to the CIITA sequence immediately
downstream of the NotI site. The amplified product was
cut with EcoRV and NotI, purified on a Chromaspin+TE-
100 column (Clontech, Palo Alto, CA), and inserted at the
EcoRV and NotI sites, resulting in pcDNA3mutCIITA2. Following
insertion into pcDNA3, the PCR product was
sequenced between the EcoRI and NotI sites, and confirmed
to correspond exactly to the published sequence (5). This
construct was used as a control since it lacked an initiation
codon and would not give rise to CIITA protein.

pcDNA3mutCIITA3 (Fig. 1B). In order to support translation
of the mutated cDNA, an oligonucleotide containing an
initiation codon, followed by five codons corresponding to
amino acids 146–150 of native CIITA was incorporated at
the 5' end of the pcDNA3mutCIITA2 construct. Two
complementary oligonucleotides were synthesized contain-
ing the ATG initiation codon (underlined) on the sense
strand, an EcoRI site at the 5' end of the sense
strand and a blunt end at the other as follows: 5' AATGTACACAAATGGGTGCTGCCGCA-TCA-3' and 5'TGGA-
GCGAGCCACGGCATGTTAG-3'. These were annealed to
each other by heating at 95°C followed by cooling to room

Flow cytometry

All experiments were performed on cells grown in tissue
culture, using the FACScalibur flow cytometer (Becton
Dickinson, NJ). Cells were initially analysed using forward
and side scatter. With both HeLa and DoHH2 cells, the
large majority of cells formed a tight cluster which was
gated for the fluorescence studies.

The percentage suppression of MHC class II expression
was calculated as: 100

\[ \frac{(\text{test value} - \text{negative control})}{(\text{positive control} - \text{negative control})} \times 100\%
\]

For example, the percentage suppression of MHC class II expression
was 2.6 and 4.9 respectively. The corresponding figures for the
control HeLa clone were 2.7 and 88.1. The percentage
suppression was calculated as: 100×[1 – ((4.9–2.6)/(88.1–
2.7))] = 97.3%.

All flow cytometry was performed in the Department of
Immunology, St Mary's Hospital, London. A FACScalibur
flow cytometer (Becton Dickinson, NJ) was used for
the analysis. HeLa cells were washed twice in 0.5% BSA in PBS and resuspended to 2
×10^6 cells/ml in 0.5% BSA/PBS. The cells were
washed twice as above. The pellet of the second wash
was resuspended in 1 ml of 2% formalin in PBS and 5000
cells were analysed at room temperature in a FACScalibur
flow cytometer (Becton Dickinson, NJ).

The data were analysed on CellQuest software (Becton
Dickinson). Cells were initially analysed using forward and
right angle scatter. With both HeLa and DoHH2 cells, the
large majority of cells formed a tight cluster which was
gated for the fluorescence studies.

The percentage suppression of MHC class II expression
was calculated as: 100

\[ \frac{(\text{test value} - \text{negative control})}{(\text{positive control} - \text{negative control})} \times 100\%
\]

For example, the percentage suppression of MHC class II expression
was 2.6 and 4.9 respectively. The corresponding figures for the
control HeLa clone were 2.7 and 88.1. The percentage
suppression was calculated as: 100×[1 – ((4.9–2.6)/(88.1–
2.7))] = 97.3%.

Construction of CIITA deletion mutants

The full sequence of the CIITA cDNA present in pBlueScript
(5) (kindly provided by Dr V. Steimle, Geneva) was moved
to the pcDNA3 expression vector using EcoRI and Xhol.

pcDNA3mutCIITA2 (Fig. 1A). This was constructed in two
steps. First, the pcDNA3 CIITA construct was cut with NotI
and Xhol, and the resulting fragment (representing position
1340 to the 3' end of the CIITA) was subcloned into NotI
and Xhol sites of pcDNA3. Next, PCR was used to
synthesize the fragment from amino acid 152 to the
NotI site. The upstream primer, 5'-ACTCGATATCATCAGGCA-
GAGCTGAAACAT-3', contained an introduced EcoRV site
(underlined) at position 567 of the original sequence, while
the downstream primer, 5'-GCTCAGTGTCCAGGCGCAATA-
3', was complementary to the CIITA sequence immediately
downstream of the NotI site. The amplified product was
cut with EcoRV and NotI, purified on a Chromaspin+TE-
100 column (Clontech, Palo Alto, CA), and inserted at the
EcoRV and NotI sites, resulting in pcDNA3mutCIITA2. Following
insertion into pcDNA3, the PCR product was
sequenced between the EcoRI and NotI sites, and confirmed
to correspond exactly to the published sequence (5). This
construct was used as a control since it lacked an initiation
codon and would not give rise to CIITA protein.
Suppression of MHC class II by mutant CIITA

Fig. 1. Mutated CIITA constructs. (A) The pcDNA3mutCIITA2 construct was made by inserting a subclone from the original full-length cDNA, between a NotI site at position 1340 and a XhoI site at position 4543, followed by the insertion of a PCR product incorporating an introduced EcoRV site at position 567, and the NotI site at position 1340. This is a control construct, without an initiation codon, and would not yield CIITA protein. (B) The pcDNA3mutCIITA3 and pcDNA3mutCIITA4 constructs were made by inserting synthetic double-stranded oligonucleotides between the EcoRI and EcoRV sites of pcDNA3mutCIITA2. ATG denotes initiation codon. (C) pCEP4mutCIITA2 was made by subcloning the fragment from EcoRI to XhoI from pcDNA3mutCIITA2 into pCEP4. Similarly, pCEP4mutCIITA3 and pCEP4mutCIITA4 were made by transferring the EcoRI–XhoI fragment from pcDNA3mutCIITA3 and pcDNA3mutCIITA4 respectively.

DNA sequence analysis

The pcDNA3mutCIITA2, 3 and 4 constructs were sequenced between the EcoRI and NotI sites, using four oligonucleotide primers. One primer was complementary to the sequence of the pcDNA3 CMV promoter, 5'-ATACGACTCACTATAGG-3'. The other three primers were complementary to the original CIITA cDNA sequence (5) at positions 819–838, 1002–1026 and 1180–1208. Then, 10 µl plasmid DNA (1.5 µg/µl) was subjected to sequencing reactions and run on an ALF System (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions.

Transfections

HeLa and DoHH2 cells were washed twice in OPTIMEM-1 serum-free medium (Gibco/BRL) and 10⁶ cells in 0.8 ml of OPTIMEM-1 were seeded into each well of six-well tissue culture plates (Becton Dickinson). For the transfections, 3 µg of DNA construct and 10 µg of lipofectamine (Gibco/BRL) were mixed in 200 µl of OPTIMEM-1 and incubated for 30 min at room temperature to allow DNA–lipofectamine complexes to form. This was added to the appropriate well on the tissue culture plate, mixed gently to ensure uniform distribution and incubated for 5 h at 37°C in 95% air/5% CO₂. At the end of this incubation, 4 ml of the appropriate medium with 12.5% FCS was added to each well.

At 48 h after transfection, the HeLa cells were passaged into medium containing 500 µg/ml G418 (Gibco/BRL). The medium was changed every 2 days. Colonies were picked after 2–4 weeks in the selective medium and were maintained in flasks in selective medium.

The DoHH2 cells (which grow as a suspension culture) were taken 48 h after transfection and maintained with 500 µg/ml of G418 in the medium.

pcDNA3mutCIITA4 (Fig. 1B). In order to ensure transport of the mutated CIITA molecule to the nucleus, another synthetic oligonucleotide construct was made. This was similar to that for pcDNA3mutCIITA3, but contained in addition 21 nucleotides encoding a nuclear localization signal (NLS). The complementary oligonucleotides were: 5'-AATTCTACACATGGCGTTGGCTGCCAAAGAAGCGCAAGGTGCTC3' and 5'-GA-CCTTGCGGTTCCTTCTTCAGAGCCAGCAAGCGATTGTGTA-3'. This pair of oligonucleotides contained (in addition to the ATG initiation codon, the EcoRI site and the blunt end) the NLS of the SV40 large T antigen (Lys/Lys/Lys/Arg/Arg/Lys/Val) (22,23). These were ligated into pcDNA3mutCIITA2 at the EcoRI and EcoRV sites, resulting in pcDNA3mutCIITA4.

pCEP4 vectors (Fig. 1C). The pcDNA3mutCIITA2, 3 and 4 vectors were cut by EcoRI, treated with Klenow large fragment DNA polymerase to create blunt ends, and subsequently cut with XhoI. These fragments were purified using QiaexII Gel Extraction (Qiagen, Hilden, Germany) and cloned into the expression vector pCEP4 in the PvuII and XhoI sites, to create respectively pCEP4mutCIITA2, 3 and 4.

DNA sequence analysis

The pcDNA3mutCIITA2, 3 and 4 constructs were sequenced between the EcoRI and NotI sites, using four oligonucleotide primers. One primer was complementary to the sequence of the pcDNA3 CMV promoter, 5'-ATACGACTCACTATAGG-3'. The other three primers were complementary to the original CIITA cDNA sequence (5) at positions 819–838, 1002–1026 and 1180–1208. Then, 10 µl plasmid DNA (1.5 µg/µl) was subjected to sequencing reactions and run on an ALF System (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions.

Transfections

HeLa and DoHH2 cells were washed twice in OPTIMEM-1 serum-free medium (Gibco/BRL) and 10⁶ cells in 0.8 ml of OPTIMEM-1 were seeded into each well of six-well tissue culture plates (Becton Dickinson). For the transfections, 3 µg of DNA construct and 10 µg of lipofectamine (Gibco/BRL) were mixed in 200 µl of OPTIMEM-1 and incubated for 30 min at room temperature to allow DNA–lipofectamine complexes to form. This was added to the appropriate well on the tissue culture plate, mixed gently to ensure uniform distribution and incubated for 5 h at 37°C in 95% air/5% CO₂. At the end of this incubation, 4 ml of the appropriate medium with 12.5% FCS was added to each well.

At 48 h after transfection, the HeLa cells were passaged into medium containing 500 µg/ml G418 (Gibco/BRL). The medium was changed every 2 days. Colonies were picked after 2–4 weeks in the selective medium and were maintained in flasks in selective medium.

The DoHH2 cells (which grow as a suspension culture) were taken 48 h after transfection and maintained with 500 µg/ml of G418 in the medium.
Suppression of MHC class II by mutant CIITA

A.

Fig. 2. Base-line studies on HeLa and DoHH2 cells. The HeLa and DoHH2 cell lines were studied by RT-PCR (A) and flow cytometry (B) before and 24 h after IFN-γ treatment. The cells used for the RT-PCR studies in (A) were from the same cultures used for flow cytometry studies in (B). (A) HeLa cells before (lane 1) and after (lane 2) IFN-γ treatment and DoHH2 cells before (lane 3) and after (lane 4) IFN-γ treatment. mRNA (80 ng) from each culture was used for the RT-PCR studies. (B) Flow cytometry profiles with the control antibody (dashed line) are given for HeLa cells in panel 2 and for DoHH2 cells in panel 3. HeLa cells were examined for MHC class II antigens (panel 1) and MHC class I antigens (panel 2), both before IFN-γ treatment (open profiles) and after IFN-γ treatment (shaded profiles). DoHH2 cells (panel 3) were studied for MHC class II and MHC class I antigens, as indicated.

B.

Semi-quantitative RT-PCR analysis

Messenger RNA was prepared from HeLa and DoHH2 cells using an mRNA Purification System (Pharmacia Biotech), and the amount of mRNA recovered was established spectrophotometrically using a GeneQuant (Pharmacia Biotech). Complementary DNA was synthesized using 320 ng mRNA and the First-Strand cDNA Synthesis Kit (Pharmacia Biotech) according to the manufacturer’s instructions. The solution was denatured at 95°C for 5 min and different amounts of template (equivalent to 200, 50, 10, 2 and 0.4 ng mRNA) were amplified in the presence of primers specific for actin, endogenous CIITA, HLA-DRA and mutated CIITA as follows:

**HLA-DRA chain:**
- upstream primer: 5’-CGAGTTCTCTATCTGAATCCTG-3’ (from exon 1)
- downstream primer: 5’-GTTCTGCTGCATTGCTTTTGC-3’ (from exon 2)

**Endogenous CIITA:**
- upstream primer: 5’-ACTCCGGGAGCTGCTGCCCTGGC-3’
- downstream primer: 5’-CCTGGAAGACATACTGGTCC-3’

**Transfected CIITA:**
- upstream primer: 5’-AATTCTACACAAATGGCGCGTGCCTGCTCCA-3’
- downstream primer: 5’-GTTGGGAGGCCGTGGAGTG-3’

**Actin:**
- upstream primer: 5’-GGGCATGGGTCAGAAGGATT-3’ (from exon 3)
- downstream primer: 5’-TACATGGCTGGGGTGTTGAA-3’ (from exon 4)

The upstream primer for endogenous CIITA is from the regions deleted in all mutant constructs. It therefore cannot recognize the mutated CIITAs, but is specific for endogenous CIITA.

Results

Construction of deletion mutants of the human CIITA molecule

Our objective was to remove the smallest portion of the N-terminal region which would completely abolish activation of transcription, in order to give the optimal chance for the remainder of the molecule to retain its native conformation. We chose to remove the first 151 amino acids (bases 1–566) in the first instance.

All three mutated CIITA constructs were placed in the expression vectors pcDNA3 and pCEP4, in both of which transcription is controlled by the CMV promoter (Fig. 1C). The pCEP4 vector has the potential to support episomal growth in human cells, via an Epsetin–Barr virus origin of replication.

Base-line studies

RT-PCR experiments demonstrated that neither CIITA nor HLA-DRA mRNA could be detected in HeLa cells prior to stimulation with IFN-γ. However, these were readily detectable within 24 h of stimulation (Fig. 2A, lanes 1 and 2). By contrast, the DoHH2 cell line had readily detectable mRNA for both...
Suppression of MHC class II by mutant CIITA

Fig. 3. Suppression of MHC class II induction in stably transfected HeLa cell clones. HeLa cell clones stably transfected with pcDNA3mutCIITA2 (clone 1), pcDNA3mutCIITA3 (clone 2) and pcDNA3mutCIITA4 (clone 3) were studied by semi-quantitative RT-PCR (A) and flow cytometry (B). The cells used for the RT-PCR studies in (A) were from the same cultures used for flow cytometry studies in (B). (A) RT-PCR studies on HeLa cells 24 h after treatment with IFN-γ. The amount of mRNA used for RT-PCR is shown above the wells. The 200 ng gel was run on a separate occasion to the remainder of the gel. (B) HeLa cells were analysed for MHC class II antigens (left column) and MHC class I antigens (right column), with the control antibody (dashed line) shown in the right column. Open profiles correspond to cultures before IFN-γ treatment and shaded profiles to cultures after IFN-γ treatment.

CIITA and HLA-DRA, which was not obviously influenced by exposure to IFN-γ (Fig. 2A, lanes 3 and 4).

In agreement with the mRNA data, HLA-DR protein was readily detectable by flow cytometry after, but not before, IFN-γ stimulation of HeLa cells (Fig. 2B, panel 1). HeLa cells strongly express MHC class I molecules, which are further up-regulated by IFN-γ (Fig. 2B, panel 2). This provides an excellent control for the class II expression studies. As expected, the DoHH2 cell line strongly and constitutively expressed both HLA-DR and class I molecules (Fig. 2B, panel 3), and these were not up-regulated by IFN-γ (data not shown).

Suppression of HLA-DR induction in HeLa cells by mutated CIITA

pcDNA3 expression vector: Initial studies involved transfection of HeLa cells with each of the three mutant CIITA constructs in the pcDNA3 vector and the establishment of stable, transfected clones. Between 27 and 95 stable HeLa clones were established for each mutant construct, and each clone was screened for class II MHC expression before and after treatment with IFN-γ, using flow cytometry. A minimum of 10 clones for each construct was examined for HLA-DRA and mutant CIITA mRNA expression by semi-quantitative RT-PCR.

All 28 clones transfected with the empty pcDNA3 vector and all 27 clones transfected with the control CIITA construct without the initiation codon (pcDNA3mutCIITA2) had normal class II MHC induction by flow cytometry. In excess of 10 clones with each construct were also tested by semi-quantitative RT-PCR and all gave essentially the same result.

A representative clone transfected with pcDNA3mutCIITA2 and analysed by flow cytometry (Fig. 3B, clone 1) and by semi-quantitative RT-PCR (Fig. 3A, clone 1) showed normal class II and class I MHC antigen induction, as well as normal induction of mRNA for CIITA and HLA-DRA.

Thirty-six of 62 HeLa clones transfected with pcDNA3mutCIITA3 (with initiation codon) and 54 of 95 HeLa clones transfected with pcDNA3mutCIITA4 (with initiation codon and NLS)
Suppression of MHC class II by mutant CIITA

Fig. 4. Suppression of MHC class II induction in transiently transfected HeLa cells. HeLa cell cultures transiently transfected with pCEP4mutCIITA2 (culture 1) and pCEP4mutCIITA4 (culture 2) were studied by semi-quantitative RT-PCR (A) and flow cytometry (b). Other details are precisely as in the legend to Fig. 3.

showed clear (>35%) suppression of class II MHC induction. Nineteen of the 36 and 22 of the 54 clones showed very strong suppression of MHC class II induction and were chosen for further study. Flow cytometry profiles for representative clones are shown in Fig. 3(B, clones 2 and 3), with semi-quantitative RT-PCR analysis on these same cultures in Fig. 3(A, clones 2 and 3). The flow cytometry demonstrated a down-regulation in mean fluorescence for class II of 97% with pcDNA3mutCIITA3 and 98% with pcDNA3mutCIITA4, without any reduction in class I expression. The RT-PCR studies showed normal induction of endogenous CIITA mRNA in clones 2 and 3 but, in spite of this, a substantial (~20-fold) suppression of HLA-DRA mRNA expression. There was no consistent advantage of pcDNA3mutCIITA4 (with initiation codon and NLS) over pcDNA3mutCIITA3 (with initiation codon only). The variability in effectiveness of the mutated constructs in different clones was presumably a consequence of the fact that the site of integration into the host cell genome can influence the level of gene expression.

None of the HeLa clones expressing the pcDNA3mutCIITA3 or 4 constructs showed any expression of class II antigens prior to IFN-γ stimulation (data not shown). Thus, removal of the N-terminal 151 amino acids abolished transcription activation by CIITA even though the truncated CIITA molecules could presumably bind well to the promoter proteins to compete effectively with native CIITA.

Expression of mutant CIITA mRNA was clearly visible (Fig. 3A, clones 2 and 3, 200 ng gel). Omission of the RT step resulted, in no PCR products (data not shown), formally demonstrating that the mutated CIITA PCR product was derived from expressed mRNA and not genomic DNA or surviving plasmid DNA.

pCEP4 expression vector. Because of the possibility of higher levels of expression of mutated CIITA from multiple episomal copies, the vector pCEP4 was also used. Transiently transfected HeLa cell cultures were used, but hygromycin selection was applied to remove non-transfected cells. HeLa cells were transfected with the control mutCIITA2 and the mutCIITA4 constructs in pCEP4. Five to 11 days after selection with hygromycin, the bulk cultures were stimulated with IFN-γ and subjected to flow cytometry and semi-quantit-
Fig. 5. Suppression of constitutive MHC class II expression in DoHH2 cells. The DoHH2 B lymphoblastoid cell line was transiently transfected with empty pcDNA3 vector (1), pcDNA3mutCIITA2 containing a non-functional CIITA gene (2), pcDNA3mutCIITA3 (3) and pcDNA3mutCIITA4 (4). Semi-quantitative RT-PCR (A) and flow cytometry studies (B) were performed 5 days following transfection. The cells used for the RT-PCR studies were from the same cultures used for flow cytometry. The amount of mRNA used for RT-PCR is shown above the wells. The 200 ng gel was run on a separate occasion to the remainder of the gel. In (B), the cultures were stained for MHC class II antigens. The profile for the control antibody (dashed line) for cells transfected with the empty pcDNA3 vector is shown. Profiles for MHC class I expression were the same for all cultures (not shown).

ive RT-PCR analysis. The experiment was repeated on 11 occasions. The control cultures transfected with the empty pCEP4 vector (data not shown) or the control pCEP4mutCIITA2 construct (Fig. 4A and B, culture 1) showed the expected normal pattern of MHC class I and class II protein expression, and CIITA and HLA-DRA mRNA expression. However, on seven of the 11 occasions, the cultures transfected with pCEP4mutCIITA4 constructs showed >60% suppression of class II MHC expression, the suppression being >93% in four of these seven cultures. The results for one of the transfections with the mutCIITA4 construct are given in Fig. 4(A and B). The down-regulation in mean fluorescence for class II was 99% for this culture and HLA-DRA mRNA was substantially reduced (~20-fold). Expression of the mutant CIITA construct was readily visible in the 200 ng gel.

Suppression of constitutive HLA-DR expression in a B lymphoblastoid cell line by mutated CIITA

The DoHH2 cell line was transiently transfected with each of the pcDNA3mutCIITA constructs, but G418 selection was applied to remove non-transfected cells. At days 5 and 8 following selection with G418, the cultures were examined by flow cytometry and semi-quantitative RT-PCR. The experiment was repeated on 20 occasions. For any particular experiment, the results at days 5 and 8 were very similar.

The flow cytometry studies showed no suppression of class I MHC expression at any stage in any of the cultures (data not shown). Flow cytometry studies for MHC class II expression are given in Fig. 5(B). Cultures transfected with pcDNA3 alone (culture 1) or pcDNA3mutCIITA2 (culture 2) (data not shown) showed no reduction in class II expression. However, 11 of 20 cultures transfected with pcDNA3mutCIITA3 showed >35% suppression of MHC class II expression, with four of these showing >75% suppression. One of these latter cultures, showing 87% suppression, is illustrated in Fig. 5(B, panel 1, culture 3). Eight of the 20 cultures transfected with pcDNA3mutCIITA4 showed >35% suppression of MHC class II suppression, with four of these showing >80% suppression. One of these latter cultures, showing 89% suppression, is illustrated in Fig. 5(B, panel 2, culture 4). There was no suppression of endogenous CIITA mRNA expression in any of the cultures (Fig. 5A). However, in the pcDNA3mutCIITA3 (Fig. 5A, culture 3) and pcDNA3mutCIITA4 (Fig. 5A, culture 4) transfected B cells, HLA-DRA mRNA was substantially (~10- to 20-fold) reduced.

It is difficult to relate the level of expression of cell surface
MHC class II antigens to levels of suppression of CIITA mRNA in the DoHH2 cells. The expression of MHC class II antigens will depend (among other things) on their half-life on the cell surface, antigen dilution by cell division, the levels of CIITA protein required for MHC class II mRNA transcription and the half-life of CIITA protein.

Discussion
The original description of the cDNA sequence of CIITA by Steimle et al. (5) suggested that the N-terminal region of this molecule might be involved in the activation of transcription of MHC class II genes. Subsequent studies by Riley et al. (13) and Zhou and Glimcher (14) established that this was indeed the case. Zhou and Glimcher (14) also demonstrated that the C-terminal 830 amino acids could specifically direct transcription from the HLA-DRA promoter, although less efficiently than the full-length 1130 amino acid CIITA molecule. However, this 830 amino acid fragment was unable to suppress constitutive MHC class II expression. Here we demonstrate that removal of the N-terminal 151 amino acid acidic domain leaves a truncated protein of 979 amino acids which inhibits both constitutive and IFN-γ-induced MHC class II expression. The three proline/serine/threonine-rich domains, which are found at amino acid positions 163–322 in the native CIITA protein, are intact in our construct, but missing from that of Zhou and Glimcher (14). It would therefore seem likely that this region of CIITA either plays a critical role in the conformation of the remainder of the protein or is itself directly involved in critical protein–protein interactions.

Our mutated CIITA proteins might function as dominant negative suppressors by competing effectively with endogenously produced CIITA for binding to proteins in the promoter region of MHC class II genes. However, the relative expression of endogenous CIITA mRNA and mutant CIITA mRNA in the transfected cell lines cannot be assessed from our RT-PCR studies, as the efficiency of the primers for PCR is likely to vary for the two molecules. Whereas a long strand of sequence was available to select the optimal upstream primer specific for endogenous CIITA, that for mutant CIITA was restricted to the inserted oligonucleotide containing the initiation codon. Nevertheless, it is worth noting that transcription activators frequently are active as dimers or higher multimers (e.g. 16,24,25). If this is the case for CIITA, it is possible that in addition to competing for binding to proteins in the promoter region, the mutated proteins either are unable to form multimers, or that dimers or multimers incorporating a mutant CIITA molecule are functionally compromised. In these circumstances the suppressive effect of mutated CIITA molecules would be greater than would be expected from the relative concentrations of endogenous and mutated forms.

Although the region of the CIITA protein important for nuclear localization is not known, our studies suggest that it is not present in the N-terminal 151 amino acids. This can be inferred from the equal effectiveness of mutant constructs, irrespective of whether or not a known NLS is added. As NLS tend to be basic (26), one would in fact not expect this signal to be in the acidic region of the molecule.

The suppression of MHC class II expression by these mutant CIITA proteins has potentially important applications for regulating clinically relevant immune responses, especially in autoimmunity and transplantation. For example, it has been suggested that class II MHC expression on vascular endothelial cells plays a critical role in the long-term immunogenicity of transplanted human organs (27). The suppression of this expression might substantially reduce the incidence of chronic rejection and the requirement for long-term immunosuppression. In the pig-to-human xenograft system, it is now well established that human T cells can respond directly to porcine MHC class II molecules (28) and these molecules therefore present potentially important targets for immune regulation. It is important to note, from studies down-regulating MHC expression in B cells by antisense oligonucleotides, that partial suppression of MHC expression on antigen-presenting cells can have major effects on the efficacy of antigen presentation (29).

For experimental studies, the in vivo down-regulation of class II expression has been possible by the use of the gene knock-out approach, in particular for CIITA (30). These approaches give rise to global suppression of class II MHC genes. Our construct would allow tissue-specific down-regulation, by the generation of transgenic mice carrying the mutant CIITA genes under tissue specific promoters.

Acknowledgements
This work was supported by the British Heart Foundation.

Abbreviations
CIITA class II trans-activator
NLS nuclear localization signal

References
10 Roche, P. A. 1995. HLA-DM: an in vivo facilitator of MHC class II peptide loading. *Immunology* 3:259
14 Zhou, H. and Glimcher, L. H. 1995. Human MHC class II gene transcription directed by the carboxyl terminus of CIITA, one of the defective genes in type II MHC combined immune deficiency. *Immunity* 2:545.
27 Fabre, J. 1982. The rat kidney allograft model: was it all too good to be true? *Transplantation.* 34:223
30 Chang, C.-H., Hong, S.-C., van Ewijk, W. and Flavell, R. A. 1996. Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression. *Immunity* 4:167