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Regulation and Specificity of MHC2TA Promoter Usage in Human Primary T Lymphocytes and Cell Line

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Although activated human T cells express MHC class II antigens, the regulation of these antigens in T cells is poorly understood. This study focuses on the control of the MHC2TA gene in these cells. MHC2TA encodes the transcriptional master regulator of MHC class II, the class II trans-activator (CIITA). It has at least three distinct promoters (PI, PIII, and PIV), each active in an overlapping subset of cell types and directing a slightly different product. This report used highly purified blood T cells prepared by negative immunoselection to analyze CIITA. Real-time PCR analysis indicates that resting T cells do not express detectable CIITA transcript, while activated T cells express the PIII CIITA form. Transient transfection of activated blood T cells using wild-type and mutant PIII promoter-reporter constructs shows that two promoter elements, activation response element-1 (ARE-1) and ARE-2, are important for PIII function. cAMP response element binding protein, a known activator of gene expression in activated T cells, activates PIII in primary T cells. However, an intact ARE-2 site is not required for this activation, indicating that cAMP response element binding protein does not activate via this site. EMSAs indicate that an activating transcription factor/cAMP response element binding protein/cAMP response element modulator family member, but not phosphorylated cAMP response element binding protein-1, binds to ARE-2. ARE-2 also forms a complex with an unidentified protein. The ARE-2 binding protein is constitutively expressed in a DR⁺ T cell line, reflecting differences between the DR⁺ cell line and primary blood lymphocytes. These results show that MHC2TA PIII is induced in activated T lymphocytes, and that the induced binding of ARE-2 is a crucial step in this process. The Journal of Immunology, 2002, 169: 3112–3119.
Previous studies have shown that MHC class II gene expression in a human transformed T cell line is correlated with the presence of CIITA. Transfection of the MHCIIC gene into HLA-DR T cell lines can cause MHC class II expression (22). Analysis of MHC class II gene control in primary T cells has been limited to the delineation of DR promoter usage (23). Beyond these observations, the molecular pathway by which MHC class II expression is achieved in T cell lines, much less primary T cells, is unclear. This study focuses on CIITA gene control in primary human blood T cells and a human T cell line. We show here that the PIII CIITA isoform, but not PI or PIV, is expressed in activated human T lymphocytes purified by immunoselection. Transfection of a promoter-reporter construct for PIII shows the utilization of this promoter in both activated T cells and a DR+ T cell line. PIII usage is enhanced by molecular activators of T cells, such as the cAMP response element (CRE) binding protein (CREB) (24). Additionally, two elements within PIPI, activation response elements 1 (ARE-1) and ARE-2, are required for PIII activation. While the HLA-DR T cell line constitutively expresses ARE-2 DNA-binding activity, primary T cells exhibit a major departure, in that ARE-2 binding is undetectable in resting T cells, but is induced upon mitogenic stimulation. This indicates that primary T cells require activation signals to cause ARE-2 binding activity, leading to CIITA expression and the subsequent induction of MHC class II. In contrast, the rare DR+ transformed T cell line, H9, is frozen in its activation state, where ARE-2 is persistently expressed, as is MHC class II. This strict correlation between ARE-2 binding activity and MHC class II gene expression in normal and transformed T cells indicates that the recognition of ARE-2 by transcription factor(s) is crucial for PIII activation in these cells.

Materials and Methods

Constructs

Constructs containing sequential deletions of PIII were cloned as previously described (18). The site-specific PIPI mutants were generated as previously described (19). Rous sarcoma virus (RSV)-CREB was provided by S. Kenney (University of North Carolina Lineberger Cancer Center, Chapel Hill, NC). RSV-AF-1 contains the activating transcription factor (ATF)-1 cDNA in the RSV-PECE vector (gift from M. Green) (25). All plasmids were purified using a Qiagen column (Qiagen, Chatsworth, CA) before transfection.

Cell lines and growth conditions

The H9 HLA-DR+ human T cell line, the Raji human B cell line, and THP-I human monocyte cell lines were maintained in RPMI (Life Technologies, Gaithersburg, MD) supplemented with 2 mM t-glutamine, 10% FBS (Sigma-Aldrich, St. Louis, MO), and 100 U/ml penicillin and streptomycin (Life Technologies). DCs were prepared from PBMCs as previously described (26). THP-I cells were treated with 500 U/ml IFNγ (PeproTech, Rocky Hill, NJ) 24 hr before RNA isolation.

Generation of primary human T cells

PBMCs were isolated by Ficoll-Hypaque density-gradient centrifugation (ICN Biomedicals, Aurora, OH) from anti-coagulated venous blood. T cells were isolated by stringent immunomagnetic negative selection using the Pan T cell isolation kit (Miltenyi Biotec, Auburn, CA). This kit includes Apheragen (27) and, additionally, CD11b, CD16, CD19, CD36, and CD56 to remove monocytes, DCs, NK cells, B cells, early erythrocyte cells, platelets, and basophils. After purification, cells were cultured for 3 days in RPMI supplemented with 2 mM t-glutamine, 10% FBS, 100 U/ml penicillin, and 2 μg/ml PHA (Sigma) for stimulation. The lack of B cell and macrophage contamination was verified by RT-PCR for CD19 and CD14, respectively (see below).

Flow cytometry

Cells were pelleted, washed twice with 15 ml PBS, resuspended in 1 ml PBS, and then incubated with 70 μl of a 3 mg/ml solution of mouse Ig (IgG fraction) on ice for 10 min. Cell suspension (50 μl) was added to tubes (USA Scientific, Ocala, FL) containing panels of fluoresceinated or phycoerythrin-labeled mAbs to CD19, CD14, CD3, or HLA-DR (BD Biosciences, San Jose, CA; CalTAG Laboratories, South San Francisco, CA) as well as to tubes containing either isotype controls or no Abs. Cells were incubated on ice for 15 min. All samples were washed with 0.5 ml PBS with 2% BSA (Sigma-Aldrich) and fixed in 0.5 ml 1% Ultrapure formaldehyde (Polysciences, Warrington, PA). Forward and side scatter and two colors of fluorescence were measured for each sample on a FACScan flow cytometer (BD Biosciences). Ten thousand events were analyzed in each sample. Data were analyzed using FlowJo data analysis software (TreeStar, Stanford, CA).

Transfection and luciferase assays

H9 cells were electroporated with 10 μg of a luciferase reporter construct in a 300-μl final volume of serum-free RPMI containing 3 × 106 cells. Primary human T cells were electroporated with 20 μg of a luciferase reporter construct in a 300-μl final volume of serum-free RPMI containing 3 × 106 cells (23). Cells were harvested 24 hr post-transfection. Luciferase assays were performed using an LB 953 AutoLumat (EG&G, Berthold, Germany), as previously described (27).

RT-PCR

RNA was prepared using the SV Total RNA Isolation System (Promega, Madison, WI). RT-PCR was performed using the Access RT-PCR System (Promega) with the following oligonucleotides: PI sense, 5′-TACCAGCAGCTCTGTCCTCGAGG-3′; PIII sense, 5′-CCTGCTCCACCCGCCTGCTG-3′; PIV sense, 5′-GAGCTGCGGCGAGGAGG-3′; PI, PIII, and PIV antisense, 5′-GAACCTGTGCGGATGTAGG-3′; CD19 sense, 5′-CAGTCCGCCCAACATCACAC-3′; CD19 antisense, 5′-AGGATACAAAGGAGGTCTG-3′; GAPDH sense, 5′-CCATGGAGGAGCTCTG-3′, and GAPDH antisense, 5′-CAAAATGTTTCATGAGTACC-3′. Reactions were run for 35 cycles with an annealing temperature of 60°C. Amplification products were analyzed by gel electrophoresis through 2% agarose gels.

Real-time PCR

cDNA was synthesized from total RNA using random hexamers, Moloney murine leukemia virus reverse transcriptase (Life Technologies), and RNASin RNase inhibitor (Promega). Real-time PCR was performed using the ABI PRISM 7900 sequence detection system (PerkinElmer, Foster City, CA). Primers and probes (Oligo Facility, University of North Carolina) were designed to span exon-intron junctions. CIITA probes were labeled at the 5′ end with the reporter dye FAM and at the 3′ end with the quencher dye TAMRA. The 18S rRNA probe was labeled at the 5′ end with the reporter dye TET and at the 3′ end with the quencher dye TAMRA. Primer and probe sequences are as follows: PI probe, 5′-CAGGCCCTTCTGACAACTGCTT-3′; sense primer, 5′-GCCATCTCATGCAGTACACTTCAGGAGA-3′; antisense primer, 5′-CATGAGAGCAGAGTGCTAGTGATTAT-3′; PIII probe, 5′-CCACGCCCTGCTGGTCTTCT-3′; PIV probe, 5′-GACTCCAGGCGGACAGTTCGTTTCCC-3′; sense primer, 5′-GGGAGGACCGACACGAGAGCTAGTTTCCC-3′; antisense primer, 5′-GCTCCAGGTTGACCCACTTCT-3′; and 18S rRNA probe, 5′-CAAAATTCCACTCCACGGCC-3′; sense primer, 5′-GCTCCAGGTTGACCCACTTCT-3′; antisense primer, 5′-CGCTGACCCAGAATTCC-3′; and 18S rRNA probe, 5′-CGCTGACCCAGAATTCC-3′. PCR of cDNA specimens and standards were conducted in a total volume of 15 μL with 2× Platinum Quantitative Supermix-UDG (Invitrogen, San Diego, CA). Thermal cycle parameters included 2 min at 50°C, 2 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Values were calculated based on standard curves generated for each gene. Normalization of samples was determined by dividing copies of CIITA by copies of 18S rRNA.

Nuclear extract preparation and EMSA

Nuclear extracts were prepared as described previously (28). The blood T cells were prepared by negative selection using immunomagnetic negative selection with the Pan T cell beads (Miltenyi Biotec) described above. Gel-shift analysis was performed as described previously (29) using synthetic oligonucleotides and 700 ng of the nonspecific competitor poly(dI:dC). The ARE-2 oligonucleotide spans from −66 to −51 bp of the promoter relative to the transcription start site and is 5′-GATCCCTTGA TGATCCCTCACTAGATC-3′. Sequences of the competitor probes are as follows: mouse ARE-2 oligonucleotide, 5′-GATCCCTTGA TGATCCCTCACTAGATC-3′; consensus PU.1 oligonucleotide, 5′-GGCTGCTGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGA-3′; and consensus B cell-specific activator protein (BSAP)-CD19, 5′-CCGCAGACACCCATGTTGATGCCTCGAC-3′.

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cytes use PI, PIII, and also PIV (16, 30). If contaminating B cells because B cells use predominantly PIII, while macrophage/monocyte contamination is free of macrophage and B cell contamination, respectively (Fig. 1A). As expected, these T cells do not express abundant class II MHC, since they have not been activated (Fig. 1B). Staining for CD14 and CD19 indicates that the T cells are free from macrophage/monocyte and B cell contamination, two primary sources of contaminating MHC class II Ags (Fig. 1, C and D). To further assess the purity of the T cell population with higher sensitivity, we used RT-PCR analysis. RT-PCR analysis performed on RNA isolated from a purified population of primary human T cells using primers for CD14 and CD19 indicates that the population of T cells is free of macrophage and B cell contamination, respectively (Fig. 2B, top and middle panels, lanes 4 and 5). RT-PCR analysis was also used to determine the CIITA isoform that is expressed in human T cells. The four promoters and primer sets are depicted in Fig. 2A. It was important to confirm that the T cell population was pure, because B cells use predominantly PIII, while macrophage/monocytes use PI, PIII, and also PIV (16, 30). If contaminating B cells and monocytes were present in the preparation, they would greatly alter the integrity of the data. RT-PCR results of the purified T cell population show that the transcript driven by PIII, but not by PI or PIV, is the primary one expressed in these purified, activated primary human T cells (Fig. 2B, middle panel, lanes 1–3). Resting T cells do not express any detectable CIITA isoforms (Fig. 2B, top panel, lanes 1–3). RT-PCR analysis of RNA harvested from positive controls for each primer set includes primary human DCs for PI, Raji B cell line for PIII, and THP-1 (monocyte cell line) induced with IFN-γ for PIV. All the positive controls produced the expected products, indicating that these primer pairs can amplify

![Figure 1](https://via.placeholder.com/150)

**FIGURE 1.** The magnetically selected T cell population is free of B cell or macrophage contamination. Anti-CD14, -CD19, -CD3, and -HLA-DR flow cytometric evaluation of primary human T cells isolated by negative immunoselection with a magnetic cell separator shows the purity of the T cell population.

**FIGURE 2.** PIII of CIITA is expressed in activated primary human T cells. A, Schematic representation of the four promoters that control expression of the CIITA gene. B, RT-PCR analysis of CIITA promoter expression. Primary human T cells were immunoselected using a magnetic cell separator. RNA was harvested from the T cells after 3 days of stimulation with PHA. RT-PCR was performed on RNA harvested from resting T cells (top panel) and activated T cells (middle panel). T cells using primers for PI, PIII, and PIV CIITA. CD14 and CD19 primers were used to detect any macrophage or B cell contamination. Positive control RNA from the primary human dendritic cells (PI, PIII), THP-1 human monocyte cell line (PIV, CD14), and Raji human B cell line (CD19, GAPDH) were included in the RT-PCR analysis (bottom panel). Bands shown are from different gel lanes that were cut and aligned artificially for presentation, although they are of different sizes. PCR products sizes are as follows: PI, 346 bp; PIII, 225 bp; PIV, 240 bp; CD14, 212 bp; CD19, 174 bp; and GAPDH, 228 bp. C, Real-time PCR analysis of CIITA promoter expression. cDNA made from the same RNA samples used for the RT-PCR analysis was used for the real-time PCR. Values are normalized with respect to 18S rRNA levels and expressed as fold activation over resting T cells. All reactions were performed in triplicate.
the proper product (Fig. 2B, bottom panel). RT-PCR analysis of CIITA expression in the H9 class II-positive T cell line indicated usage of PIII as well as minor PIV (data not shown). As H9s are a transformed cell line, it is possible that expression differs slightly from primary T cells. For more quantitative results and higher sensitivity, we used real-time PCR analysis to confirm the RT-PCR findings (Fig. 2C). As detected by RT-PCR, PIII CIITA is the predominant form expressed in activated T cells, although a hint of PI is also seen. DCs express both PI and PIII and a small amount of PIV, Raji express primarily PIII and a slight amount of PIV, while IFN-γ-treated THP-1 monocytes express predominantly PIV, but also a significant amount of PIII. The same RNAs used for RT-PCR were used here.

The ARE-1 and ARE-2 sites within PIII are required for PIII CIITA expression in PHA-activated T cells and H9 cells

To delineate the promoter region of PII that is required for expression in T cells, luciferase reporter constructs containing sequential deletions of PIII were transiently transfected in H9 or primary T cells (Fig. 3A). Cell lysates were assayed by luciferase assay to assess expression levels of these constructs. The results of the transfection and luciferase assay analysis in H9, an MHC class II-expressing T cell line, indicate PIII expression and show that PIII promoter activity increases slightly from the 113-bp to the 195-bp construct (Fig. 3B). Transfection of activated primary human T cells also shows that PIII activity is modestly increased when the promoter is lengthened from 113 to 195 bp. Activity does not increase significantly with the transfection of constructs containing more upstream PIII sequences (Fig. 3C).

In a previous study of B cell lines, the functional regions of PIII were subjected to in vivo footprinting, and five sites of protein/DNA interaction were identified within the first 195 bp of PIII. These are referred to as site A, site B, ARE-2, ARE-1, and site C (19) (Fig. 4A). Luciferase reporter constructs containing mutations

![Diagram of the PIII sequence](http://www.jimmunol.org/)

**FIGURE 3.** PIII CIITA is active in H9, a class II-expressing T cell and activated primary human T cells. A. Deletions of the PIII region of CIITA were cloned into luciferase reporter constructs as previously described (18). B. PIII CIITA is active in H9, a human T cell line that expresses class II MHC. Cells (3 x 10^6) were electroporated with the specific PIII deletion constructs. Cells were harvested 24 h post-transfection and were assayed for luciferase activity. pGL2-Basic is the empty vector control. Data represent three independent experiments in which each data point was determined in triplicate. RLU values were normalized against the amount of protein in extract. C. PIII activity in primary human T cells. Primary human T cells were purified away from B cells and macrophages using a magnetic cell separator. Cells (3 x 10^6) were electroporated with the specific PIII deletion constructs. Cells were harvested 24 h post-transfection and assayed for luciferase activity. Data represent three independent experiments in which each data point was determined in triplicate. RLU values were normalized against the amount of protein in extract.

![Diagram of the PIII sequence](http://www.jimmunol.org/)

**FIGURE 4.** ARE-1 and ARE-2 are important for PIII activity in both H9 and activated primary human T cells. A. Diagram of the PIII sequence showing the elements at which site-specific mutations were generated. Mutated base pairs are indicated in lower case in the sequence directly below each site. PIII luciferase reporter constructs containing site-specific mutations were transfected into both H9 and activated primary human T cells. B. ARE-1 and ARE-2 are critical for PIII activity in an HLA-DR^+ T cell line. H9 cells were electroporated with luciferase reporter constructs containing PIII with site-specific mutations. Cells were harvested for luciferase reporter assays 24 h post-transfection. C. ARE-1 and ARE-2 are important for PIII activity in primary human T cells. Primary human T cells were electroporated with luciferase reporter constructs containing PIII site-specific mutations. Cells were harvested for luciferase reporter assays 24 h post-transfection.

![Diagram of the PIII sequence](http://www.jimmunol.org/)
at each of these sites were generated based on the PIII.545 construct, as previously described (19). Transfection of the site-specific mutant constructs into H9 and activated primary human T cells followed by luciferase assay analysis shows that mutations in site A, B, or C had little effect on PIII activity, but mutations of ARE-1 or ARE-2 significantly reduced activity (Fig. 4, B and C). An ARE-1 or ARE-2 mutation obliterated reporter expression in H9 cells in the composite of three experiments shown in this figure, although in other experiments some residual expression remained. Residual expression is also seen in blood T cells with the ARE-1 and ARE-2 single mutation. In blood T cells, mutation of site A, B, or C actually resulted in a slight enhancement of reporter activity, which may be due to the possibility that the sites are repressor elements. Nonetheless, these results taken together indicate that, like B cells, the expression of PIII CIITA in a T cell line and blood T cells depends on intact ARE-1 and ARE-2 sites.

**PIII activity in H9 and primary T cells can be enhanced by CREB independently of an intact ARE-2 site**

Recent studies have found an important role for the CREB in T cell activation. Low levels of CREB have been observed in resting, naive CD4+ T cells and were elevated following TCR stimulation (31). CREB is also shown to be a critical activator of the IL-2 promoter (32), is induced by the CD28 costimulator (33), and has a role in T cell differentiation by down-regulating IL-4 (3, 34). To determine whether CREB may enhance PIII activity in primary T cells, the PIII.545 luciferase reporter construct and an expression vector containing CREB were cotransfected into these cells (Fig. 5). The inclusion of CREB caused a 3- to 4-fold enhancement of PIII activity (wild-type PIII.545 activation levels reported as relative light units (RLU) per micrograms differ from those in Fig. 4 due to the use of a different luminometer). Since we showed that ARE-2 is important for PIII activity and the ARE-2 site is the only one of the five known sites with any homology to a CREB binding site (CRE), we decided to test whether CREB activated PIII by binding ARE-2. The enhancement of PIII by CREB is not dependent on an intact ARE-2 site, as mutation of the ARE-2 site did not affect this enhancement, although this mutation did decrease the overall level of expression as expected (Fig. 5). We further explored whether a CRE-like site in the −254 to −248 bp region (relative to the transcription start site) of PIII could be mediating the effects of CREB. However, mutating both this site and ARE-2 did not affect the activation of PIII by CREB (data not shown).

**Binding to ARE-2 is observed in DR+ T cell line and primary T cells, but not DR-negative resting T cells**

EMSA was performed on nuclear extracts from H9 and primary human T cells to determine whether the ARE-2 element interacts with DNA-binding factors from cells of the T cell lineage. Nuclear extracts from blood T cells were prepared by immunomagnetic negative selection using Pan T cell Miltenyi magnetic beads, and each preparation from one blood bag gives at most 4 × 10^7 cells and usually much less than that. For this reason, each nuclear extract preparation is only sufficient for one experiment, and each experiment required a separate donor and cell preparation, attributing to some variations in the appearance of the EMSA. Nonetheless, EMSA was used to determine 1) differences or similarities between transformed and primary blood T cells, and 2) differences between resting and activated blood T cells. EMSAs using nuclear extract from Raji cells and competitive probes were included as controls (Fig. 6, lanes 1–4). The mouse ARE-2 oligo included as

**FIGURE 5.** CREB activates PIII CIITA, but not by binding the ARE-2 site. H9 cells (3 × 10^6; A) or activated T cells (B) were cotransfected via electroporation with PIII reporter constructs containing either a wild-type or mutant ARE-2 site in the presence or absence of CREB. Cells were harvested 24 h post-transfection and assayed for luciferase activity. Data represent three independent experiments in which each data point was determined in triplicate. RLU values were normalized against amount of protein in extract.

**FIGURE 6.** ARE-2 site interacts with DNA binding factors present in activated human T cells and a T cell line. EMSAs were performed by incubating an oligonucleotide containing the ARE-2 sequence element with nuclear extract from H9 or activated and resting primary human T cells. Extracts that have been incubated with an oligonucleotide containing the ARE-2 sequence element (CRE), we decided to test whether CREB activated PIII by binding ARE-2. The enhancement of PIII by CREB is not dependent on an intact ARE-2 site, as mutation of the ARE-2 site did not affect this enhancement, although this mutation did decrease the overall level of expression as expected (Fig. 5). We further explored whether a CRE-like site in the −254 to −248 bp region (relative to the transcription start site) of PIII could be mediating the effects of CREB. However, mutating both this site and ARE-2 did not affect the activation of PIII by CREB (data not shown).

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The ARE-2 probe detects two ARE-2 binding protein complexes in activated, primary T cells. The top band is specific for the ATF-1 Ab that recognizes ATF-1, CREM, and CREB-1. The middle band is specific for an unidentified ARE-2 binding protein. The lower band is nonspecific. In lanes 2–4, activated T cell extract was incubated with a competitor oligonucleotide, as indicated above each lane. In lanes 6–9, activated T cell extract was incubated with Ab, as indicated above each lane.

**FIGURE 7.** The ARE-2 probe detects two ARE-2 binding protein complexes in activated, primary T cells. The upper band is specific for the ATF-1 Ab that recognizes ATF-1, CREM, and CREB-1. The middle band is specific for an unidentified ARE-2 binding protein. The lower band is nonspecific. In lanes 2–4, activated T cell extract was incubated with a competitor oligonucleotide, as indicated above each lane. In lanes 6–9, activated T cell extract was incubated with Ab, as indicated above each lane.

A negative control did not eliminate the ARE-2 band, as it is substantially different from the human ARE-2 site in three of 11 bases (Fig. 6, lane 3). The top band is a specific band that was competed with the ARE-2 competitor, while the bottom band is nonspecific. Incubation of H9 nuclear extracts with an oligonucleotide containing the ARE-2 element resulted in a similar complex formation (Fig. 6, lane 5). Addition of ARE-2 competitor oligonucleotide eliminated this complex (Fig. 6, lane 7), but the addition of an unrelated oligonucleotide did not (Fig. 6, lane 6). EMSA analysis of extracts prepared from primary blood T cells showed two complex formations only in extracts from activated T cells, but not resting T cells (Fig. 6, lanes 8 and 9). This is the first circumstance where ARE-2 binding activity is inducible. A probe for NF-Y was used on both resting and activated T cell extracts to indicate that the absence of an ARE-2 complex in resting T cells was not due to decreased levels of resting T cell extract (Fig. 6, lanes 10 and 11). EMSA analysis of T cell extracts for ARE-1 is not shown because the gel-shift band is not clearly visible despite a variety of optimization protocols. This is similar to previous findings that the in vitro ARE-1 complex in B cells is very weak (19). Due to limitation in the amount of nuclear extract, another T cell preparation was prepared to show competition of gel-shift complex formation with an ARE-2 oligonucleotide, but not an identical oligonucleotide with mutations in the ARE-2 site, a CREB sequence or a B cell-specific activator protein sequence.

**Analysis of the ARE-2 binding activity in primary T cells.** A further analysis of the ARE-2 site in nuclear extracts isolated from activated T cells was performed in an attempt to identify the protein that interacts with this site. Three protein-DNA complexes observed are shown in Fig. 6 (lanes 8 and 9), but only two bands are specific for ARE-2, because addition of the ARE-2 competitor oligonucleotide removes both upper bands but not the lower band (Fig. 7, compare lanes 1 and 2). A CREB-1 oligonucleotide eliminated the formation of the top band, but not the second one. A nonspecific oligonucleotide encoding the PU.1 sequence is shown in lane 4, and the intensity of the second band is comparable in lanes 3 and 4. A supershift analysis using an array of Abs was then performed. The top band is specific for the ATF-1 Ab, which also recognizes CREB-1, CREM-1, and ATF-1 family members. Incubation with this Ab significantly reduced the intensity of the top band and caused the formation of a shifted band. The lower band was not altered by this Ab (compare lane 6 to the nonspecific serum control, lane 9). This suggests that an ATF-1/CREB-1/CREM-1 family member constitutes the upper band formed with ARE-2. The lower band is specific for an unidentified protein that binds ARE-2. An anti-CREB-2 Ab did not significantly alter the two bands. Phosphorylation of the serine 133 residue of CREB is required for its ability to activate transcription (24). Therefore, we analyzed another CREB-1 Ab (lane 8), which is specific for the form that contains the phosphorylated serine 133. This Ab also did not supershift either band, suggesting that phosphorylated CREB-1 is not associated with the ARE-2 binding complex (Fig. 7, lane 4). These results suggest that an ATF-1/CREB-1/CREM family member may be associated with the ARE-2 binding complex, while another unknown protein is also associated with this sequence. However, the luciferase assay shown in Fig. 5 indicates that CREB does not require ARE-2 for trans-activation of the PIII reporter, and phosphorylated, transcriptionally active CREB-1 was not detected by supershift assay (Fig. 7). ATF-1 was originally identified as a factor that bound to CRE-like elements present in adenovirus promoters and is 65% identical with CREB in primary structure. Like CREB family members, it is a transcription factor that binds to CREs (24). Since the supershift assay (Fig. 7) identified an ATF-1/CREB-1/CREM-1 family member binding to ARE-2, it is important to functionally assess whether ATF-1 activates PIII through ARE-2 by a luciferase assay. Both H9 (Fig. 8A) and activated primary T cells (Fig. 8B) were cotransfected with a vector containing the ATF-1 gene or its empty vector control together with wild-type or mutant ARE-2 PIII reporter. Our data show that ATF-1 activates PIII CIITA 3- to 4-fold (Fig. 8), an activation level achieved with the
cotransfection of CREB and PIII reporter (Fig. 5). Similar to the results from the CREB activation assay (Fig. 5), ATF-1 activation of PIII is not mediated through the ARE-2 sequence because activation remained unchanged when the ARE-2 site was mutated. Thus, both CREB and ATF-1 appear to activate PIII CIITA, but not through binding of the ARE-2 site. Additional experiments are necessary to identify the other ARE-2 binding proteins.

**Discussion**

Although the majority of circulating human T cells lack class II expression, they can express HLA-DR, -DP, and -DQ after activation (10). The expression of class II on T cells indicates that they have the potential to present Ag to T cells; and in vitro have been shown to present soluble Ag such as HIV-gp120 (35) or hepatitis B envelope Ag (36). Class II expression on activated human T cells could function as an additional mechanism to deliver costimulatory signal to inactivated T cells, since it has been suggested that activated T cells stimulate Ag-specific primary responses by inducing unprimed peripheral blood T cells to respond (37). CIITA is crucial in regulating class II expression in professional APCs; thus, it is important whether it plays a role in activating class II expression in activated T cells.

Results from this report show congruent findings in transformed and primary human T cells, in that the PIII isoform of CIITA is expressed, and this expression is dependent on the ARE-1 and ARE-2 elements within this promoter. ARE-1 and ARE-2 were previously identified as two sites that are most important for PIII expression in B cell lines (16). ARE-1 is a transcription enhancer factor-2-like sequence that belongs to the Kruppel family of transcription factors, which includes proteins that bind to CTCCC motifs and are ubiquitously expressed. The identity of the ARE-2 binding protein has remained elusive, and at least one component may represent a novel protein. ARE-1 and ARE-2 binding sites are also occupied in both immature and mature DCs, implying that the proteins that recognize these two sequences are constitutively expressed at different stages of DC maturation (15). These results underscore the importance of ARE-1 and ARE-2, which have now been broadly observed in three prominent DR+ cell types; B cells, activated T cells, and DCs.

Although our findings of the T cell line, H9, agree with the analysis of PIII in B cell lines (19), primary T cells show a major departure, in that PIII is only expressed in activated human T cells. Correlatively, ARE-2 binding activity is only found in activated human T cells. This indicates that ARE-2 binding activity is induced upon T cell activation and strongly implies that ARE-2 binding is a critical step for the control of PIII CIITA in primary T cells. The identification of ARE-2 binding protein is crucial in further understanding how PIII is regulated in a variety of cell types.

One candidate protein that we pursued as a possible factor that recognizes ARE-2 is CREB. This is particularly relevant, as a recent report published during the review of this manuscript concludes that ARE-2 is a CREB/ATF binding site (38). CREB is a protein known to be involved in T cell activation, and it was initially included in this study based on its role in T cell activation (32, 33). Data from our gel shifts indicate that CREB-1 or other family members, ATF-1 and CREM (Fig. 7, lane 6), can bind ARE-2 or associate with the ARE-2 binding complex. However, it is unlikely that CREB activates through association with the ARE-2 site because 1) the phosphorylated form of CREB, which is the form required for transcriptional activation, was not detected by gel supershift; and 2) CREB does not activate a PIII reporter through the ARE-2 site. Based on data from the gel shift (Fig. 7), we also used a luciferase reporter assay to assess whether ATF-1 activates PIII by its binding to ARE-2. We show that similar to CREB, ATF-1 activated PIII, but this is independent of an intact ARE-2 site (Fig. 8). Therefore, it is unlikely that ATF-1 is the protein that binds ARE-2. It is crucial to exhaustively test other CREB, ATF, and CREM family members in a functional assay to assess their role in the activation of PIII in primary T cells.

We also attempted to further identify the CREB recognition site in this promoter. A potential CREB binding site is present in the −252 to −248 region of PIII. Mutating this sequence had no effect on the activation of PIII by CREB. One possible explanation for these findings is that the activation of PIII by CREB occurs via an indirect pathway. For example, the binding of CREB to promoter elements of the IFN-γ gene was observed in distinct T cell populations, suggesting that CREB may play a role in regulating IFN-γ gene transcription (31). CREB may enhance PIII CIITA by inducing IFN-γ expression, which then causes the up-regulation of PIII CIITA expression.

A comparison of the recently published report shows data that are in agreement with our findings (38). They found a predominance of PIII usage and genomic footprints over the PIII promoters in blood T cells. They additionally found that AML-2 can bind to the ARE-1 site. Both papers show the functional importance of ARE-1 and ARE-2 in PIII using T cell lines, and this paper extended the analysis to activated blood T cells. Our work shows RT-PCR and real-time PCR analyses of different promoter usage and indicates a prominent PIII usage, although a slight amount of PI usage is also observed. In DCs we detected usage of all three (PI, PIII, and PIV); however, the last is a minor population. The usage of PI, PIII, and PIV in DCs has been recently reported by Landmann et al. (15). Finally, the prominent use of PIII in Raji has been reported (17, 18), although we saw slight PIV usage as well. In THP-1 cells both PII- and PIV-induced transcripts were found, confirming previous findings that both promoters are IFN-γ responsive (16, 18, 39).

In summary, this report examines the molecular control of CIITA in primary and transformed T cells to further our understanding of MHC class II gene activation/expression in these cells. Our results show a crucial role for both ARE-1 and ARE-2 in this activation. It further shows the induction of an ARE-2 binding complex in activated primary T cells, which is correlated with the importance of ARE-2 in PIII expression in these cells. This provides the first evidence of a system where ARE-2 binding is inducible and provides strong impetus to identify the ARE-2 binding protein. It also points out a notable difference between transformed and nontransformed T cells and underscores the significance of studying the latter to obtain the most physiologically relevant data.

**References**


6. Ko, H. S., S. M. Fu, R. J. Winchester, D. T. Yu, and H. G. Kunkel. 1979. Inducible and nontransformed T cells and underscores the significance of studying the latter to obtain the most physiologically relevant data.