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To the Graduate Council:

I am submitting herewith a dissertation written by Noel Elyssa Day entitled "Expression and Characterization of the MHC Class II Transactivator in K-562 Cells." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Albert T. Ichiki, Major Professor

We have read this dissertation and recommend its acceptance:

Karla Matteson, Robert N. Moore, Carmen Lozzio

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Anne Mayhew
Vice Provost and Dean of
Graduate Studies

(Original Signatures are on file in the Graduate Student Services Office.)

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EXPRESSION AND CHARACTERIZATION OF THE MHC CLASS II TRANSACTIVATOR IN K-562 CELLS

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Noel Elyssa Day

December 2001

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For Brad

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Acknowledgment

I would like to express my gratitude to my major professor, Dr. Albert Ichiki, for his guidance and friendship. His understanding and encouragement have made it possible for me to complete this first step in my training as a scientist.

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Abstract

The K-562 cell line was developed from the pleural effusion of a patient with chronic myelogenous leukemia (CML) in blast crisis and has served as an excellent model to study the properties of CML cells. The highly undifferentiated cells can be induced by a variety of chemicals to follow multiple pathways of differentiation. When K-562 cells were treated with phorbol myristate acetate (PMA), they exhibited accessory cell functions by replacing monocytes in the activation of resting Th2 lymphocytes. However, K-562 cells are negative for the expression of class II major histocompatibility complex (MHC) antigens HLA-DR, -DP, and -DQ. The focus of this study was to determine the functional capacity of the class II transactivator (CIITA), a crucial regulatory factor of MHC class II genes, in K-562 cells. CIITA is a co-activator, which is regulated in a tissue-specific manner by four alternative promoters. The transcription factor is recruited to the MHC class II promoter by factors that associate with the promoter, such as regulatory factor X (RFX). The CIITA mRNA is present in K-562 cells and, due to alternative splicing, there is an insertion of an additional genomic sequence not present in the wild-type. The insertion occurs at the carboxy terminus of the CIITA gene, introducing a stop codon at nucleotide 2796, which results in a truncated protein of 932 rather than 1130 amino acids. Although a truncation of this type does not interfere with the ability of CIITA to associate with the MHC class II promoter or with the RFX complex *in vivo*, it does lead to an inactive protein, which might explain the absence of MHC class II molecules on K-562 cells. The most active CIITA promoter in these cells is promoter III, which is generally specific to B cells. Although IFN-γ

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treatment of K-562 cells does not result in MHC class II expression, the IFN-γ inducible CIITA promoter IV does exhibit low levels of basal activity, and the activity of promoter IV is greatly enhanced upon treatment with IFN-γ. These results provide insight into the role of CIITA in malignant cells, as well as supporting the hypothesis that the negative expression of MHC class II molecules is caused by the alternatively spliced CIITA transcript identified in K-562 cells.

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CHAPTER 1

Literature Review

Chronic Myelogenous Leukemia

Etiology

Chronic myelogenous leukemia (CML) is a myeloproliferative disease affecting about 1 in every 100,000 people. The disease, generally considered to be triphasic, begins with a chronic phase and later progresses to the blast phase (Figure 1). In most cases, there is an intermediate phase referred to as the accelerated phase. Once the patient has reached the blast phase, the disease is nearly always fatal. CML accounts for 20% of all leukemias and is slightly more common in men (Figure 2.A). Because the diagnosis of CML in children is very rare, it is widely considered to be an adult disease (Figure 2.B) (Gunz, 1977). Furthermore, the probability of developing the disease increases with age, with a median age of 53. Common symptoms of the disease include weight loss, increased sweating, bone pain, a persistent, unexplained fever, and abdominal discomfort due to splenomegaly.

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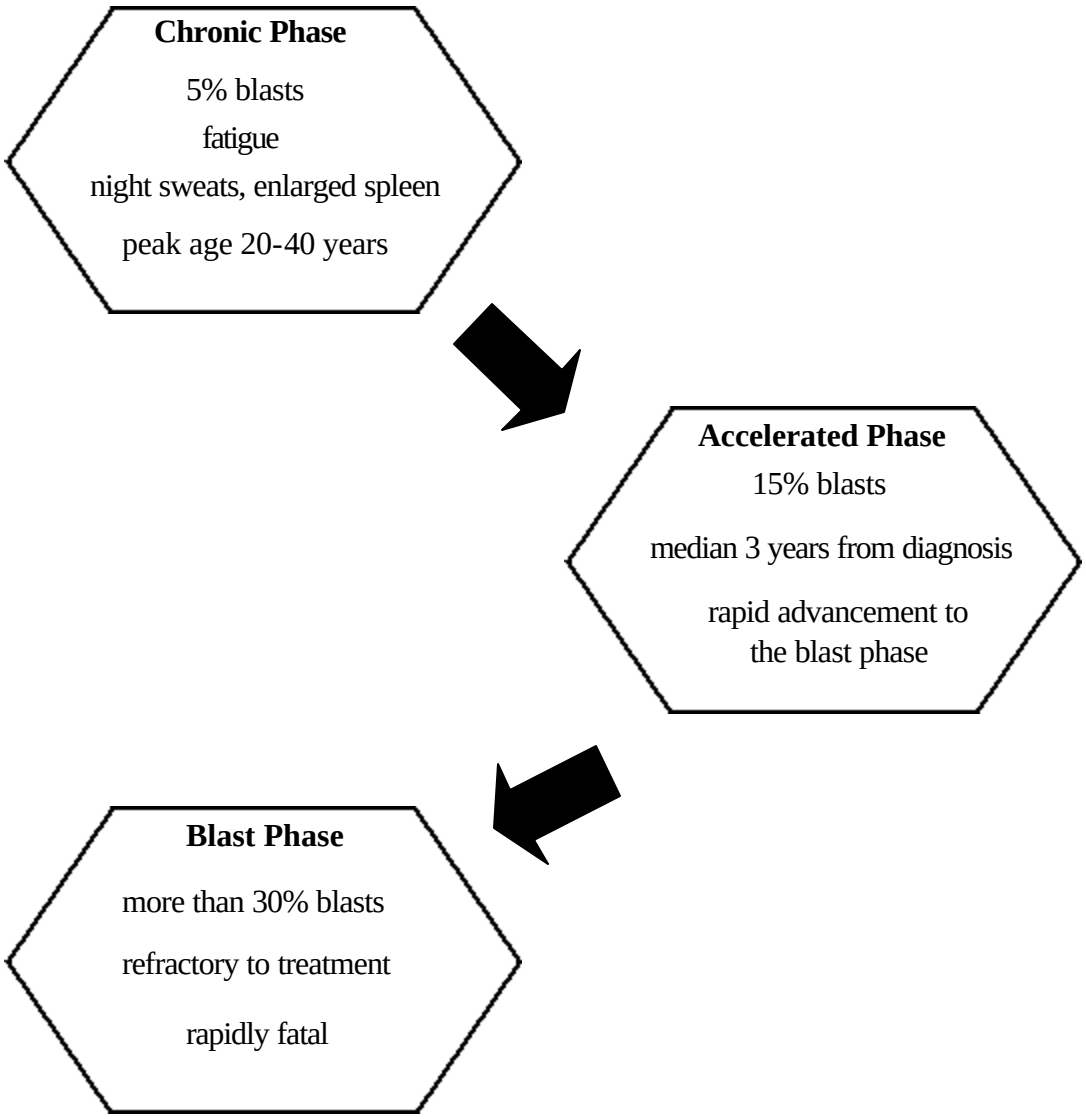

Figure 1. A diagrammatic representation of the three phases of chronic myelogenous leukemia.

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A.

Year	Total		Male		Female	
	Count	Rate	Count	Rate	Count	Rate
1992	68	1.5	41	2.1	27	1.0
1993	64	1.4	36	1.8	28	1.1
1994	71	1.5	39	1.9	32	1.3
1995	67	1.4	39	1.8	28	1.0
1996	80	1.7	41	2.0	39	1.5
1997	65	1.3	41	1.9	24	0.8
1998	55	1.1	24	1.1	31	1.2

B.

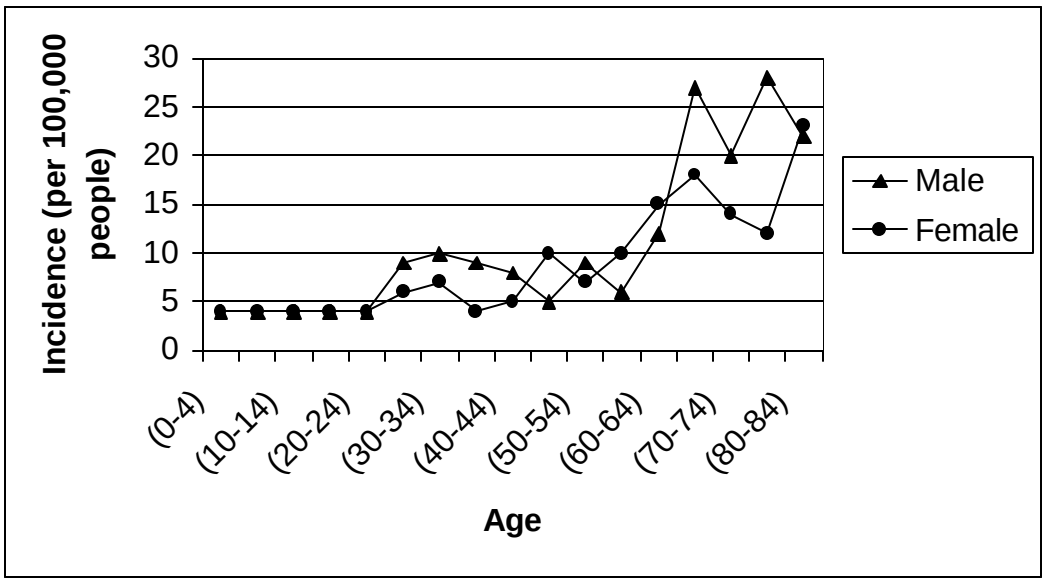

Figure 2. Incidence of CML. **A.** Annual case counts and age-adjusted rates of chronic myelogenous leukemia per 100,000 population. **B.** Incidence of chronic myelogenous leukemia in male and female populations.

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Treatment and Diagnosis

CML can be diagnosed from a peripheral blood smear; the blood count typically shows leukocytosis and the marrow becomes hypercellular. When the disease is present, the platelet count may be high, the differential count will show peaks involving neutrophils and myelocytes, there will be absolute basophilia, and monocyte counts will be low. The presence of these features, combined with the Philadelphia chromosome (Ph), the cytogenetic marker of CML, provides physicians with a very good diagnostic tool (de Klein et al., 1982).

Many different treatments for CML have been found which act by prolonging the chronic phase. At this time, the treatments are not curative because they do not prevent the blast phase. One treatment, which has been used to treat CML patients, is the administration of interferon-α (IFN-α). In the majority of patients, hematologic remission is accomplished and, in some patients, normal hematopoiesis returns. IFN-α, however, does not prevent transformation to the blast phase (Niederle et al., 1987; Talpaz et al., 1987). Until recently, the most promising treatment for CML patients was an allogeneic bone marrow transplant; this procedure has been found to be curative in many cases. However, the effectiveness of the treatment depends on the stage of the disease and the prior treatments the patient has received. Studies have shown disease free survival rates as high as 60%. Treatment usually consists of extreme doses of chemotherapy or radiation in order to kill the CML cells and cause immunosuppression. The suppression of the immune system is essential for the acceptance of the donor allograft. The donor graft will contain cells that can restore normal hematopoiesis and

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cells that will kill residual and newly formed CML cells. Although allogeneic bone marrow transplantation is the most successful treatment for CML, the treatment has a 20% morbidity rate, due to immunosuppression, graft rejection and graft versus host disease (GvH) (Carella et al., 1991; Haines et al., 1984). Recently, considerably progress has been made by using the molecular source of the disease as a target for the treatment of CML, and very promising results have been obtained with the drug STI571, commercially known as Gleevec (Druker et al., 2001; Mauro, 2001). One of the many benefits of the drug is that it does not involve suppression of the immune system, or serious side effects if administered in the right dose (300 to 1000 mg/day). In recent clinical trials of patients in either the chronic phase or the blast phase of CML, the results of treatment with STI571 were evaluated in terms of a hematologic response and a cytogenetic response (Figure 3). A hematologic response was characterized by the return to normal blood counts, and a cytogenetic response involved the loss of the Ph chromosome. As one would expect, the results were better for the patients in the chronic phase, 91% complete hematological response (CHR) and 55% major cytogenetic response (MCR), than for the patients in the blast phase, 4% CHR and 14% MCR. These responses have been maintained in 70% of the clinical subjects for more than one year. Clinical trials for patients in the accelerated phase have just been initiated, and as such, no results are available at this time. In previous trials, minor side effects included skin rash, fatigue, diarrhea and cramps; however, these results were unprecedented, given that the patients in the trial group had all received prior treatment with no response. For the

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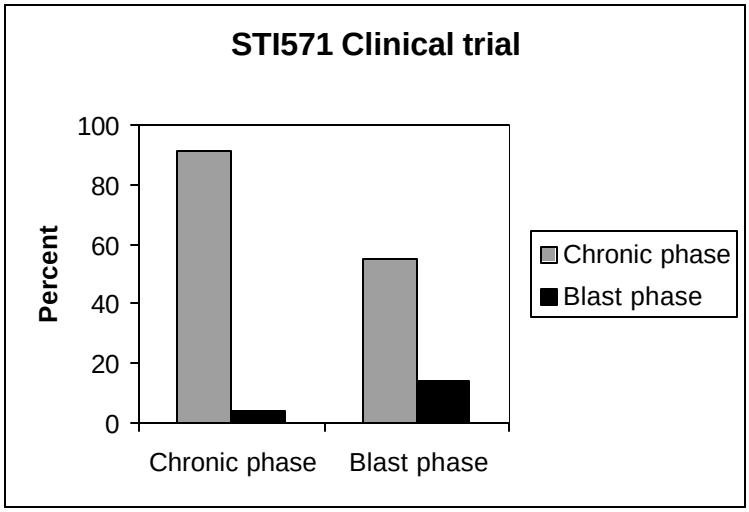

Figure 3. A graph of the results of clinical trials for patients in the chronic and blast phases of chronic myelogenous leukemia treated with STI571.

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first time, it seems possible that rather than just a treatment for CML, there may be a cure.

The Philadelphia Chromosome

The hallmark of CML is the Philadelphia chromosome, which is present in greater than 90% of CML patients. In 1960 the process of identifying the Ph chromosome began (Nowell, 1960). The first step in the process was the identification of an unusually short autosome, chromosome 22, found in both the blood and marrow cells of CML patients. More than a decade later the cause of this shortened autosome was discovered (Rowley, 1973). Using more advanced chromosomal banding techniques, researchers were able to demonstrate that there was a reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] (Figure 4). This abnormality became known as the Philadelphia chromosome. Further studies revealed that the Ph chromosome was the result of a rearrangement of the *c-abl* gene, the human equivalent of the *Abelson* viral oncogene in mice, from chromosome 9, and the ubiquitously expressed *bcr* (breakpoint cluster region) gene on chromosome 22. The product of this translocation is the *bcr-abl* fusion gene (Heisterkamp et al., 1983). Studies involving transgenic mice have shown that the gene product can cause leukemia *in vitro* and *in vivo* (Heisterkamp et al., 1990; McLaughlin et al., 1987). A duplication of the Ph chromosome is one of the secondary mutations that may occur in the blast phase of the disease.

Due to the consistently increasing number of cells in K-562 cultures, researchers believed that the cells were proliferating more rapidly than non-malignant cells.

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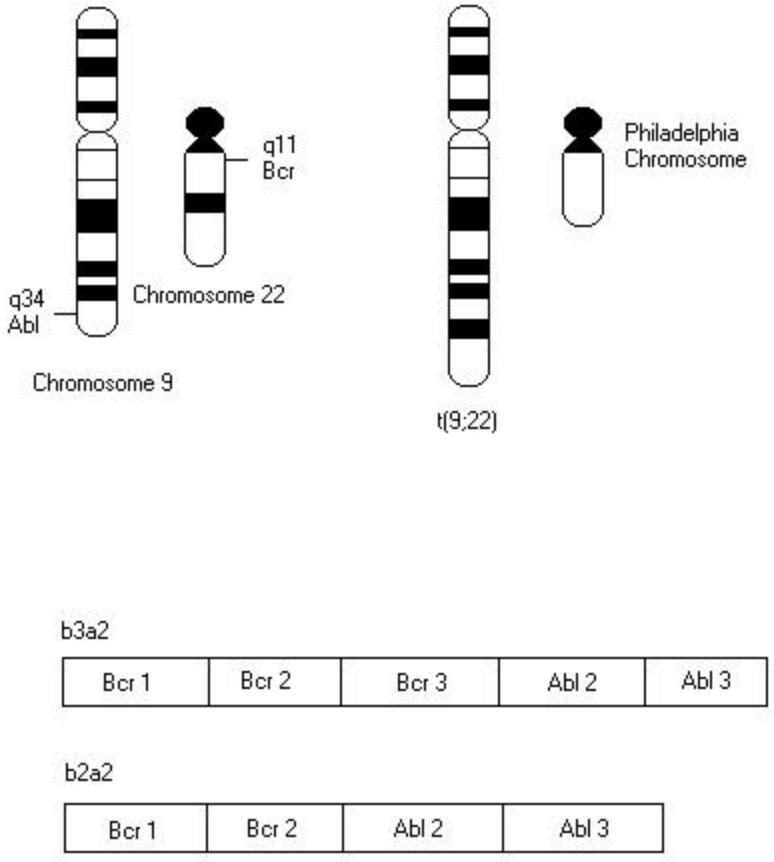

Figure 4. A diagrammatic representation of the *bcr-abl* translocation resulting in the Philadelphia chromosome. The predominant hybrid transcripts in chronic myelogenous leukemia are depicted.

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Konopka *et al*. (1984) discovered that the junction of the *c-abl* gene with the *bcr* gene resulted in an altered Bcr-Abl protein, p210. The resulting protein was larger than its normal C-Abl counterpart, p145, and unlike p145, the tyrosine residues are phosphorylated *in vitro* and *in vivo*. It was hypothesized that this altered protein may have an effect on cellular proliferation. Subsequently, Kashige *et al*. (2000) showed that p210 leads to activation of the Ras pathway by inhibiting a down-regulator of the signal transducer. K-562 cells are notably resistant to apoptosis, the process of programmed cell death, which serves to regulate the number of a particular cell type at any given time (Diomede, 1993; Martin et al., 1990; McGahon, 1997). Their resistance to apoptosis leads to high numbers of K-562 cells in culture, which results in the appearance of excessive proliferation. Although they are notoriously resistant, the cells can be induced to undergo apoptosis. When this occurs, they typically do not develop the DNA ladder pattern that is the hallmark of apoptosis. While this means that internucleosomal DNA fragmentation may not occur, it has been shown that large molecular weight fragments are produced. McGahon *et al*. (1994), attributed the resistance of K-562 cells to apoptosis to the presence of the *bcr-abl* fusion gene and the resulting increase in tyrosine kinase activity.

This up-regulated tyrosine kinase activity was the target for the researchers who designed the previously mentioned STI571 drug for the treatment of CML. The drug functions by binding to the active site of the enzyme produced by the p210 protein, thereby blocking access to ATP (Figure 5). ATP is an energy source that is needed by

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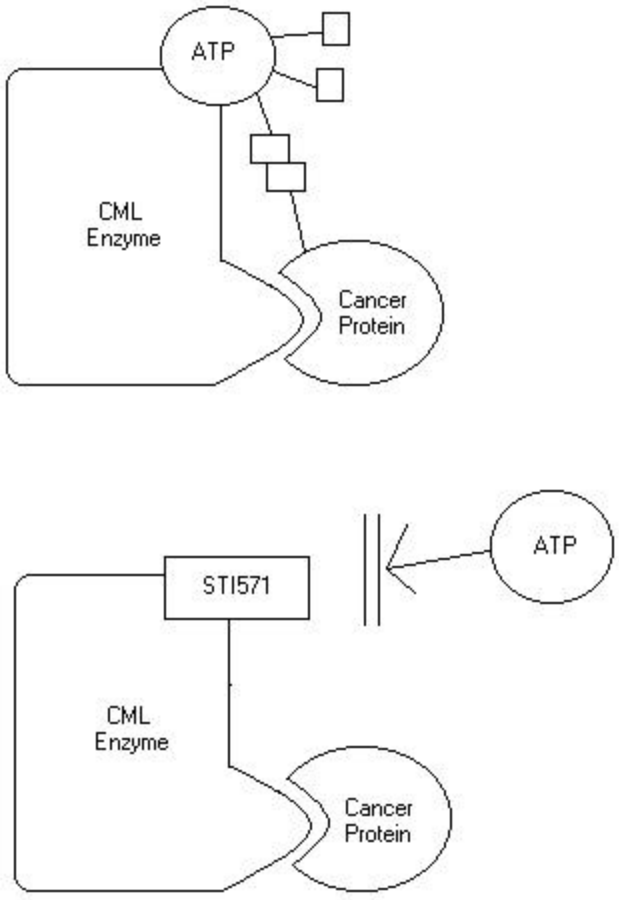

Figure 5. A schematic diagram of the mechanism of action of the *bcr-abl* inhibitor, STI571.

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the cancer protein to function. This essentially starves the cancer cells, resulting in cell death (Druker et al., 2001; le Coutre et al., 1999).

The History of K-562 Cells

In an effort to learn more about the aspects of the disease, many attempts to culture CML cells have been made. The majority of these attempts have failed for various reasons, but the most common problem was the loss of the Ph chromosome after the cells had been sub-cultured. There was, however, a successful attempt at The University of Tennessee Medical Center, Knoxville, Tennessee (Lozzio, 1975). In 1970, the first successful long-term culture of malignant cells from a patient with CML began. The K-562 cell line is a pluripotent line derived from the pleural effusion of a patient with chronic myelogenous leukemia in blast crisis. The cells began actively proliferating as soon as they were placed in culture media. After several years of culturing the cells through serial passages, the Ph chromosome remains present in the cell line. K-562 cells are commonly used to study natural killer (NK) cells because of their sensitivity to NK cell mediated lysis (Gronberg et al., 1985; Ichiki et al., 1988). Although the cells typically grow in suspension, they can be induced by various methods to undergo adherence (Jarvinen et al., 1993; Lundell et al., 1996) or homotypic aggregation (Fitter et al., 1999; Hickstein et al., 1993). The cell line has come to be known as the universal target of NK cell activity in vitro. The establishment of this cell line provided leukemia researchers with a means of performing not only *in vitro*, but also *in vivo* studies.

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The origin of these primitive cells, which vary greatly in size and shape, was not immediately determined. It was undisputed that the cells were highly undifferentiated, however, there was a great deal of controversy as to what paths of differentiation the cells could be induced to follow. Depending on the inducing agent used, the cells can display the characteristics of granulocytic, erythroid or megakaryocytic differentiation (Sutherland et al., 1986). For this reason, K-562 cells serve as an important tool for studying hematopoiesis. K-562 cells were originally thought to be of myeloid origin due to their lack of T and B cell markers, and the presence of the Ph chromosome (Lozzio et al., 1976; McGahon, 1997; Tetteroo et al., 1984a). Morphologically, the cells display surface characteristics similar to those of granulocytic leukemia cells. Furthermore, the cells react to granulocytotoxic-positive antisera, suggesting the presence of granulocyte antigens (Drew, 1977; Martin et al., 1990). However, the granulocytic lineage of the cells came into question when controversial studies showed that K-562 cells synthesize and express glycophorin A on their surface.

Glycophorin A is the erythrocyte lineage antigen, and it has been found that the production of glycophorin A significantly increases when the K-562 cells are stimulated with sodium butyrate (Andersson et al., 1979; Sutherland et al., 1986). Adding to the confusion was the publication in which Andersson *et al.* (1979) claimed that the cells, when treated with sodium butyrate, synthesized hemoglobin, demonstrated by benzidine staining and radioimmunoassays. Soon after this finding was published, Rutherford *et al.* (1979) claimed that culturing the cells with hemin led to large amounts of fetal hemoglobin production. Lozzio *et al.* (1979) responded to these claims by publishing a

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paper stating the characteristics of the original K-562 cell line. Among these characteristics they noted that the cells did not produce fetal hemoglobin when treated with hemin and that sodium butyrate was toxic to the cells. These discrepancies were attributed to the differences of various K-562 sublines, which have been well established (Ichiki, 1986). Subsequently, it is accepted that both sodium butyrate and hemin result in the production of hemoglobin in a subline of K-562 cells (Sutherland et al., 1986). In 1981, Vainchenker *et al*. observed that sodium butyrate stimulation of K-562 cells also leads to the formation of megakaryoblasts. This conclusion was based on staining for platelet peroxidase (PA), which was localized to the nuclear envelope and the endoplasmic reticulum (ER). PA is not known to be present in the ER of erythroid cells . Further evidence for megakaryocytic differentiation came when it was discovered that treatment with phorbol 12-myristate 13-acetate (PMA) results in up-regulation of the platelet glycoprotein complex, gpIIb/IIIa (CD41/61) (Alitalo, 1990; Tetteroo et al., 1984a). Because PMA significantly alters cell proliferation, it is thought to induce terminal differentiation in K-562 cells (Sutherland et al., 1986; Tetteroo et al., 1984a). In 1981, Lozzio *et al*. concluded that the cell line was in fact derived from a pluripotential hematopoietic stem cell.

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Major Histocompatibility Antigens

Characteristics

The major histocompatibility complex (MHC), located on the short arm of chromosome 6, encompasses approximately 4 million base pairs, and is comprised of several genes that encode MHC molecules (Figure 6). MHC molecules are also referred to as human leukocyte antigens (HLA), because the antibodies that recognize them react to leukocytes, but not erythrocytes. These molecules, essential to antigen presentation to T cells, are divided into two major categories, MHC class I and II. They are highly polymorphic, and as such, are the major reason for graft rejection and GvH in transplantation. Each HLA molecule is composed of a heavy and light chain, that come together to form the binding groove for foreign peptides. While over-expression of MHC molecules is associated with autoimmune diseases, impaired expression leads to immunodeficiency. In either case, the result will likely be the death of the patient.

MHC class I molecules (HLA-A, B, C) bind foreign peptides in the cytosol and present them to the cytotoxic $CD8^+$ T cells, which act by killing the infected cell. They are expressed on most hemopoietic stem cells and their progeny and can be induced in most cell types by Interferon-γ (IFN-γ). The light chain of the class I molecule is referred to as β_2-microglobulin (β_2m), and unlike the class I heavy chain and class II heavy and light chains, it is located on chromosome 15. The binding groove of the class I molecule is closed, meaning that it is connected at both sides, limiting the size of the peptide to be

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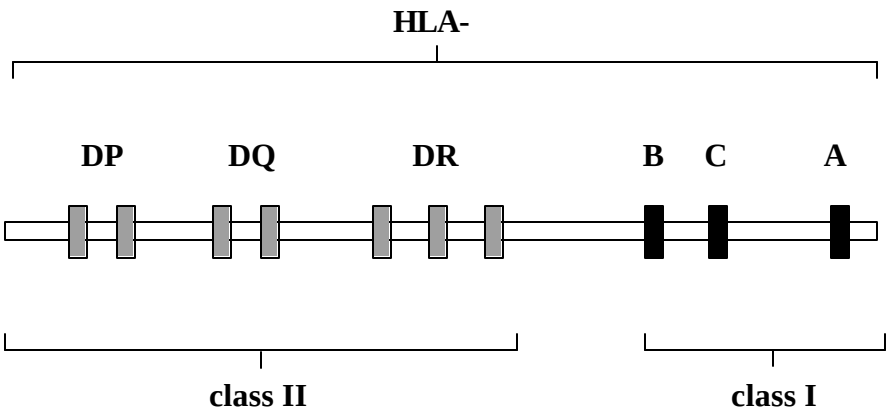

Figure 6. The major histocompatibility complex class I and II genes are located on chromosome 6.

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bound to 8-10 amino acids. The minimum and maximum number of class I alleles an individual can express are 3 (homozygous) and 6, respectively.

The MHC class II molecules (HLA-DP, -DQ, -DR) are found primarily in professional antigen presenting cells (APC), namely B cells, dendritic cells and macrophages (Shipp et al., 1983), and can be induced by IFN-γ in many cell types (Basta et al., 1987; Collins et al., 1984; Glimcher and Kara, 1992; Mach et al., 1996; Rohn et al., 1996; Ting and Baldwin, 1993). This class of molecules acts by degrading antigens in intracellular vesicles and presenting them to T cells. The class II molecule, complexed with the antigen, is then recognized by $CD4^+$ T helper cells. While the function of $CD8^+$ cells is to kill infected cells, $CD4^+$ cells function by activating other cells, such as macrophages (cell mediated immunity) or B cells (humoral immunity). The heavy and light chains of class II molecules are called the α and β chains, respectively. Interestingly, the α chain of HLA-DR is monomorphic. The class II binding groove is open, meaning that it is not attached at the sides, such that peptides of 13-25 amino acids can be bound efficiently. An individual can express as few as 3 (homozygote), or as many as 8 different class II alleles.

In addition to the classical MHC class II genes, HLA-DM and the invariant chain (Ii) play important roles in the class II response. Both classes of MHC molecules are assembled in the ER; however, they are processed in different manners. The class I molecule cannot leave the ER until a peptide is bound. On the other hand, the class II molecule will not leave the ER until Ii covers the binding groove, preventing the class II molecules from binding the wrong type of peptide (Figure 7). In the ER, Ii binds the

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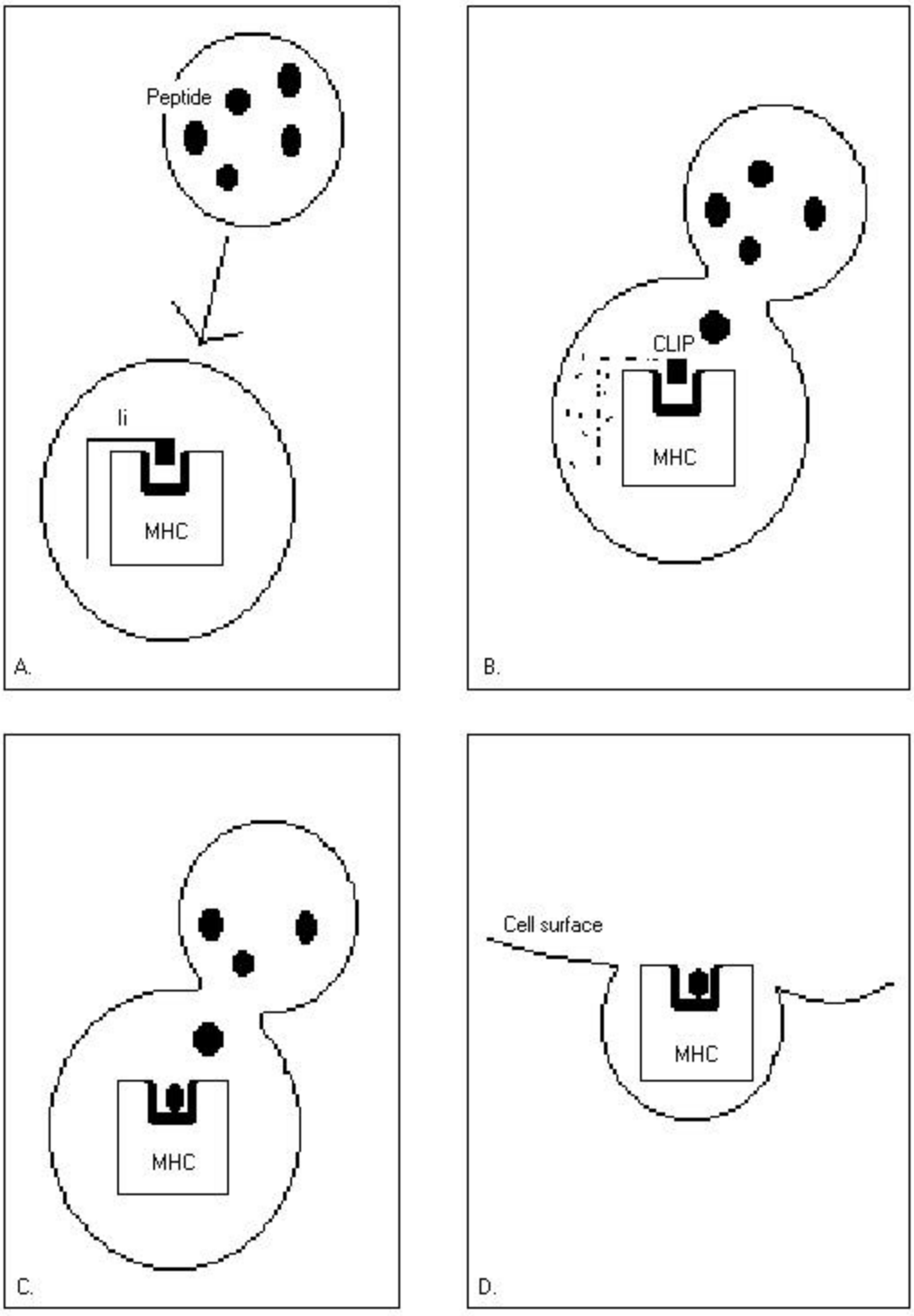

Figure 7. Ii mode of action. A. Ii covers the binding groove. B. Ii is degraded, CLIP remains. C. The peptide is loaded. D. Translocation of the MHC complex to the surface.

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MHC class II molecule and remains associated with it until peptide loading occurs in the endosomal compartments. Here, the majority of Ii is degraded, leaving only the class II-associated Ii polypeptide (CLIP) attached to the class II molecule. Once the peptide has been loaded, CLIP is removed by HLA-DM (Bertolino and Rabourdin-Combe, 1996).

Located on chromosome 5, Ii, also known as CD74, is called the invariant chain because it is a nonpolymorphic chain with very little variation across species. However, there are different isoforms of Ii in both humans and mice. Alternative splicing at the seventh exon results in both a major 33 kDa isoform (31 kDa in mice) and a minor 41 kDa isoform (Strubin et al., 1986). Ii expression is constitutive in some cell lines, namely, professional APC, and IFN-γ-inducible in others (Barr and Saunders, 1991). Studies of Ii knockout mice have helped researchers to uncover the many functions of the non-traditional class II gene (Bikoff et al., 1993; Matza et al., 2001; Viville et al., 1993). These mice not only have very low levels of MHC class II expression (Viville et al., 1993), they also have reduced numbers of $CD4^+$ T cells (Bikoff et al., 1993). This suggests that Ii is involved in the maturation of the $CD4^+$ T cell repertoire. Furthermore, the B cells of these mice are arrested at the immature stage. Transfection of the $Ii^{-/-}$ mice with the p31 protein results in the maturation of the B cells, as determined by the surface expression of IgD (Matza et al., 2001).

Another nontraditional class II molecule, HLA-DM, is responsible for assisting the loading of the foreign peptide onto the class II molecule, and for removing CLIP once the peptide has been loaded. Although crystallography of HLA-DM has not revealed the site of interaction with the class II molecule, a recent study shed some light on this topic

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(Doebele et al., 2000). When HLA-DM is unable to interact with HLA-DR, the result is the accumulation of HLA-DR/CLIP complexes at the cell surface (Fling et al., 1994; Riberdy et al., 1992). The study took advantage of this fact to determine whether HLA-DM was able to interact with HLA-DR after a series of point mutations in the DR α and β chains. The results indicated that there was a site surrounding the antigen-binding groove of HLA-DR in which mutations stabilized the DR/CLIP complex, and an additional site on the lateral face of the DR molecule where the N terminus of CLIP is located. Mutations in these sites led to the accumulation of HLA-DR/CLIP complexes on the cell surface.

Once the MHC-peptide complex reaches the cell surface and is recognized by the T cell receptor (TCR) of the appropriate T cell, a series of reactions begins. The first step is the activation of the T cell; however, in addition to the recognition of the MHC-peptide complex by the TCR, there must be an interaction of co-stimulatory molecules from both the T cell and the APC. If this does not occur, rather than becoming activated, the T cell will die (Medema and Borst, 1999). B7-1 and B7-2 are two accessory molecules present on APC that interact with CD28 on the T cell (Sperling and Bluestone, 1996; Van Gool et al., 1996). This interaction signals a pathway involving the release of a cascade of cytokines that are key elements of the immune response.

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MHC expression in K-562 Cells

It has been demonstrated that, under various conditions, K-562 cells can replace monocytes in stimulating a mitogen-induced T cell response (Wakasugi et al., 1983; Wakasugi et al., 1984). It was further demonstrated by Baker *et al*. (2000), that this response was, specifically, an expansion of T helper type 2 (Th2) cells, indicating a shift towards a humoral immune response (Baker et al., 2000). In light of these discoveries, it is a puzzling, albeit well documented, observation that K-562 cells do not express HLA-A, B, C, β_2m or HLA-DR on their surface (Benz, 1980; Drew, 1977; Garson, 1985; Klein, 1980; Lozzio, 1983; Maziarz, 1990). Although it has not been detected on the cell surface, K-562 cells do possess intracellular β_2m (Klein, 1980; Ziegler et al., 1981). As the result of a binding assay with the W6/32.HLK hybrid and K-562 cells, Ziegler *et al*. (1981) proposed that the cells did express HLA antigens, but at levels too low to be detected by cytotoxic assays. Chen *et al*. (1987), found that the absence of class I surface antigens on K-562 cells was due to both positive and negative transcriptional regulators. When MHC class II molecules are absent from cells, as the result of inefficient antigen presentation, T cell proliferation is significantly impaired (Gilfillan, 1991). HLA class I molecules can be induced on K-562 cells using various agents. IFN-γ is a cytokine released by T cells and NK cells to combat viral infection. Among the many effects it has on target cells is the up-regulation of MHC class I molecules including β_2m and, in some cases, MHC class II molecules (Sutherland, 1985; Wallach, 1982). Sutherland *et*

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al. (1985), found that K-562 cells cultured with IFN-γ for 4 days showed detectable class I antigens; however, HLA-DR was still not expressed on the cells. Ichiki *et al*. (1987), found that as many as 90% of cells cultured in IFN-γ showed a reversible expression of MHC class I antigens. It was then discovered that these IFN-γ-treated, class I expressing cells became resistant to lysis by NK cells. It was originally thought that this resistance was due to the class I molecules expressed on the cell surface, but several years later it was shown that the resistance was actually a result of the cellular effects of IFN-γ treatment (Gronberg et al., 1985). When the cells were transfected to express class I molecules without IFN-γ treatment, they did not become resistant to the NK cell-mediated lysis. However, when the transfected cells were treated with IFN-γ, they did become resistant to NK cell lysis. Surprisingly, it has been demonstrated that K-562 cells transfected to express HLA-DR become resistant to NK cell lysis (Jiang et al., 1996).

HLA-DR is the best-characterized class II molecule. It is expressed on the surface of B cells, dendritic cells, macrophages and activated T cells (Shipp et al., 1983). The molecule orchestrates the interactions of these cells to produce an immune response. Of these responses, perhaps the most important is the production of antibodies by B cells. K-562 cells are notoriously negative for the expression of HLA-DR (Drew, 1977; Klein et al., 1976; Ress et al., 1991). It was, at one time, thought that the expression of HLA-DR was determined by the amount of methylation of the gene (Gambari et al., 1986). Theoretically, there would be a negative correlation between the methylation of DNA and HLA-DR transcription. However, this hypothesis was abandoned when it was discovered that the sites examined in K-562 cells were entirely unmethylated, while the sites in Colo

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38 cells were highly methylated. This discredited the theory because the K-562 cells do not express the HLA-DR molecule, but it is actively transcribed in the Colo 38 cells. In the experiments performed, it was found that the amount of HLA-DR mRNA in the K-562 cells was significantly lower than the Colo 38 cells but, nevertheless, it was present. In 1991, Ress *et al*. observed that K-562 cells cultured in T cell conditioned media (CM) were induced to express HLA-DR. After two weeks of culture, HLA-DR was expressed, dependent on the K-562 subline used, on as many as 90% of the cells.

The Class II Transactivator

Discovery

The class II transactivator (CIITA) is a co-activator of MHC class II genes. It is often referred to as the master switch of MHC class II expression, because it is absolutely required for the transcription of class II genes. In fact, the correlation between MHC class II and CIITA expression is so precise that cells constitutively expressing one will also constitutively express the other. CIITA knockout mice are also found to be MHC class II negative (Itoh-Lindstrom et al., 1999). Furthermore, MHC class II expression is quantitatively correlated with CIITA expression (Otten et al., 1998). Interestingly, although Ii expression is significantly reduced in these mice, it is not destroyed (Chang et al., 1996; Lee et al., 1997). Steimle et al. (1993) originally identified CIITA through complementation cloning of a bare lymphocyte syndrome (BLS) cell line. BLS cells lack expression of both MHC class I and II molecules. For one group of BLS cells,

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designated group A, introduction of the wild type CIITA, through a complementation assay, was found to restore MHC class II expression. It was then found that the introduction of CIITA into normal cells leads to the expression of MHC class II genes (Bradley et al., 1997; Chang et al., 1994; Chin et al., 1994; Steimle et al., 1994). Regulation of MHC class II expression by CIITA is so precise, that single amino acid substitutions (F961S in the carboxy terminal portion of the gene or L469F in the midregion of the gene) in the CIITA gene can lead to MHC class II deficiency (Quan et al., 1999; Wiszniewski et al., 2001). Once this transcription factor was identified as an important regulatory factor for MHC class II expression, many investigators began to focus their efforts on the characterization of CIITA.

Characterization

Located on chromosome 16, the gene that encodes CIITA was found to produce a 4.5Kb cDNA, encoding a protein of 1130 amino acids. Although the predicted molecular mass is 123.5 kDa, the apparent mass is 135 kDa (Steimle et al., 1993), suggesting some post-translation modifications. Although the intron-exon structure of the human CIITA gene has not been determined, the mouse counterpart spans 42 kb of genomic DNA and consists of 19 exons (Reith, 2001). It was soon discovered that the protein does not

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bind the MHC promoter directly; rather, it associates with several of the proteins that occupy the MHC promoter, forming a type of molecular scaffold referred to as the enhancesome (Figure 8). Regulatory Factor-X (RFX) and Nuclear Factor-Y (NF-Y) are two such proteins. These factors are known to bind to consensus sequences of the MHC class II promoter, referred to as the X and Y boxes. The X box can be subdivided into the X1 and X2 boxes. The X1 box is bound by RFX-5, a subunit of the multimeric RFX protein. The X2 box is bound by the X2 box-binding protein (X2BP), which was later identified to be the cyclic AMP response element-binding protein (CREB) (Moreno et al., 1999). Similar to RFX, NF-Y is multimeric and can be divided into different subunits. In addition to the X and Y boxes, CIITA interacts with the W box. The W box is essential to IFN-γ-induced MHC class II expression and is also necessary for maximal B cell expression (Brown et al., 1998). The interaction of CIITA with these elements requires strict spatial-helical arrangements, such that the introduction of a partial helical turn would destroy the ability of CIITA to associate with the MHC class II promoter complex, resulting in the null expression of MHC class II molecules (Zhu et al., 2000). It is thought that the assembly of these factors on the class II promoter signals the recruitment of CIITA to the complex. Although these factors can assemble in the absence of CIITA, transactivation does not occur and, subsequently, the class II gene is not transcribed (Steimle et al., 1993). In the absence of RFX, the promoter-associating complex cannot form (Kara and Glimcher, 1991; Kara and Glimcher, 1993). Mutations in the various subunits of RFX, which is expressed ubiquitously in all tissues, have been identified as the cause of BLS types B, C and D (Durand et al., 1997; Masternak et al.,

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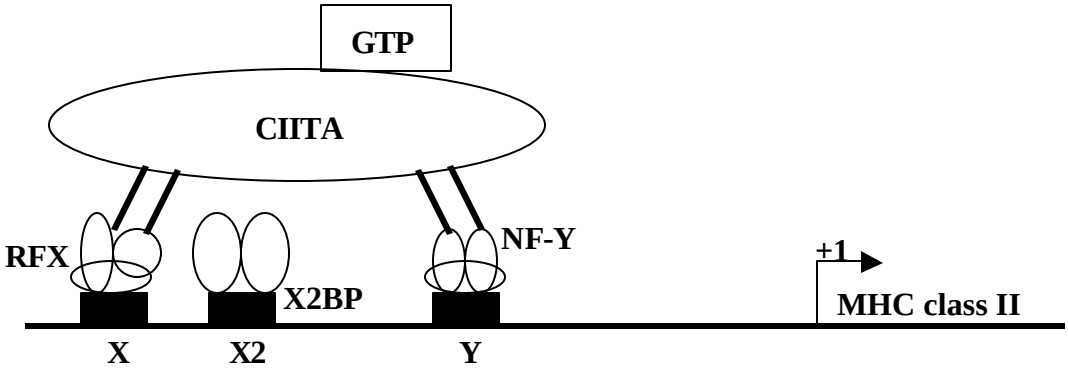

Figure 8. The class II transactivator associates with multiple factors bound to the MHC class II promoter to form the enhancesome.

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1998; Steimle et al., 1995). In a BLS patient of complementation group C, a premature stop codon in RFX5 was found to be the cause of the lack of MHC class II expression and the diminished class I expression. Surprisingly, transfection of the cells with exogenous CIITA was able to partially overcome this defect, resulting in the expression of HLA-DR, but not the other class II molecules, namely HLA-DP and DQ (Peijnenburg et al., 1999). However, the transfected cells remained negative for antigen-presenting functions, providing yet another example of the tight control and exact parameters required for proper immune expression.

The CREB binding protein (CBP/p300) and the associated factor PCAF activate CIITA by acetylating it at lysine residues that are located within a nuclear localization residue (Spilianakis et al., 2000). The effect of this acetylation is the accumulation of CIITA in the nucleus. Likewise, mutation of the acetylation targets of CIITA leads to the reduced transactivation of MHC class II genes. There is evidence that CIITA also acts by chromatin remodeling (Rigaud et al., 1996; Ting et al., 1997; Wright et al., 1998). Cells that lack CIITA have a closed chromatin structure at the class II promoters (Rigaud et al., 1996; Wright et al., 1998). However, when CIITA is introduced, the class II promoters, as well as HLA-DM and Ii promoters, that were previously closed, open and become occupied (Ting et al., 1997; Wright et al., 1998). A recent study showed that the binding of CIITA to the complex, which assembles at the MHC class II promoter, results in acetylation and deacetylation of histones H3 and H4 in the HLA-DR promoter (Beresford and Boss, 2001). Furthermore, the acetylation modifications can be seen following IFN-γ

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treatment of IFN-γ-inducible cells. The acetyl transferase activity of CIITA was mapped to the N-terminal region between amino acids 36 and 132 (Raval et al., 2001).

In addition to MHC class II genes, CIITA is also involved in the regulation of MHC class I genes (Gobin et al., 1997; Martin et al., 1997) and IL-4 production (Gourley et al., 1999; Sisk et al., 2000), and is itself regulated by IL-1β (Rohn et al., 1999). The regulation of class I genes by CIITA has been attributed to a synergistic relationship between CIITA and RelA (Girdlestone, 2000), a transcription factor that binds the ENH-A region upstream of the class I genes (Girdlestone et al., 1993). However, CIITA represses the transcription of IL-4 (Gourley et al., 1999), a cytokine produced by Th2 cells that is involved in the regulation of antibody production (Mosmann and Coffman, 1989; Paul, 1991). When CIITA deficient mice were stimulated with IL-12, the Th1 cells produced an abundance of IL-4, indicating that CIITA negatively regulates IL-4 production by Th1 cells. Interactions between NF-AT and CBP/p300 enhance the IL-4 promoter activity (Avots et al., 1999; Kubo et al., 1997). The mechanism of this transcriptional repression by CIITA was found to be the competition with NF-AT to bind CBP/p300 (Sisk et al., 2000). Lastly, it has been shown that IL-1β inhibits the IFN-γ-induced transcription of CIITA (Rohn et al., 1999). However, it is not known how this process takes place, since the binding of STAT1 and IRF-1 to their respective targets was not affected.

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Protein Domains

Several protein domains of CIITA have been identified and are essential to the function of the transcription factor (Figure 9) (Bourne et al., 1991; Chin et al., 1997; Steimle et al., 1994). Beginning at the amino end, there is a domain that is rich in acidic amino acids. Following the acidic domain is a proline-serine-threonine-rich region. Next is the nucleotide binding domain, which encompasses three GTP-binding motifs (nucleotides 420-427, 461-464 and 558-561). In the carboxy end of CIITA there are several leucine-rich repeats (LRR). The importance of these domains is evident from mutation experiments. Many transcription factors contain acidic domains, which have been shown to be important for activation. CIITA contains an acidic domain (amino acids 30-160), followed by domains rich in proline (amino acids 163-195), serine (amino acids 209-237) and threonine (amino acids 260-322). When the acidic domain of CIITA was replaced with the acidic domain of another transcription factor (HSV1), there was a reduction in the function of the CIITA protein (Chin et al., 1997). A deletion of the proline-serine-threonine-rich portion of CIITA led to a complete loss of transcriptional activation. However, when the proline- and serine-threonine-rich regions were deleted individually, there was no affect on the function of the protein. These experiments indicate that the acidic domain is necessary, but not sufficient for the activation of CIITA. Furthermore, the proline- and serine-threonine-rich domains may serve overlapping functions in the activation of CIITA.

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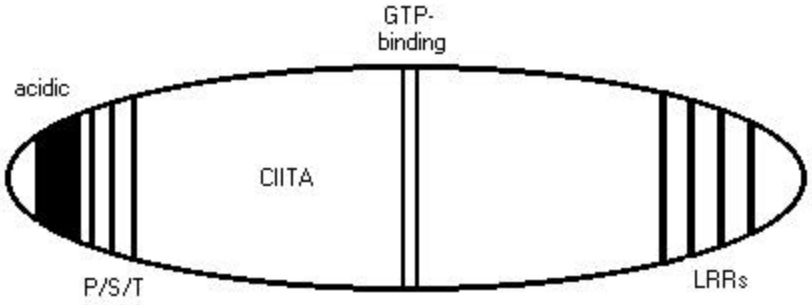

Figure 9. A schematic representation of the important protein domains of the class II transactivator. The diagram represents the acidic domain, the proline-serine-threonine-rich region (P/S/T), the GTP-binding domain and the leucine-rich repeats (LRR).

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In 1999 several important discoveries were made about the GTP-binding domain of CIITA (Harton, 1999; Itoh-Lindstrom et al., 1999). It had previously been established that CIITA has no GTPase activity, but, nonetheless, the GTP-binding domain is crucial to the function of the transcription factor. Studies with knockout mice expressing a deletion of this domain revealed that the mice had no detectable CIITA mRNA, and that they represent mice with a deletion of the CIITA gene, rather than a deletion of a specific domain (Itoh-Lindstrom et al., 1999). The animals had almost no expression of MHC class II molecules, based on RT-PCR analysis, and induction of MHC class II expression by lipopolysaccharide (LPS), IL-4 and IFN-γ was not detectable. Furthermore, expression of the invariant chain was greatly reduced but not destroyed. In a second study, where CIITA was mutated to induce GTPase activity, surprisingly, the result was reduced transcriptional activation (Harton, 1999). It was subsequently discovered that the binding of GTP to CIITA is required for nuclear import of the complex, supported by the finding that immunostaining of cells expressing the mutated CIITA revealed that the protein was present only in the cytoplasm and not the nucleus of the cells. Clearly, the transcription factor cannot function if it cannot enter the nucleus to associate with the MHC class II promoter.

Studies in which the leucine-rich repeats of CIITA are mutated by substituting an alanine for one or more amino acid residues revealed that although the transcriptional capacity of CIITA was abolished, the protein was still able to associate with RFX and NF-Y. However, it was demonstrated that in vivo binding of CIITA to the MHC class II promoter is dependent on the LRR (Hake et al., 2000).

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Recently, it was discovered that CIITA contains two distinct domains that mediate self-association. The latter of the two domains includes amino acids 939-1130, and mutations in this region lead to abrogation of the transactivation ability of CIITA (Linhoff et al., 2001). A second report showed that the central region of CIITA, including the GTP-binding region, is sufficient for self-association and that this region reacts with both the carboxy-terminal LRR and the amino-terminal acidic domain (Sisk et al., 2001). Regardless of the location of the essential elements, mutational analysis indicates that self-association is important for CIITA to function.

Using RT-PCR, Riley et al. (1995), identified the wild-type CIITA from Raji cells as a major band in an agarose gel. Unexpectedly, they also noted several minor bands. They determined that Raji cells expressed multiple isoforms of the CIITA gene; however, only the wild-type transcript produced a functional protein. MHC CIITA clone 10 is an alternatively spliced CIITA transcript that contains an insertion of genomic sequence. The insertion introduces a premature stop codon, resulting in a protein of 884 amino acids, rather than 1130. As the result of the loss of several crucial protein domains, the clone 10 protein is inactive. This discovery further emphasized the importance of the carboxy-terminal protein domains of CIITA.

Regulation of the CIITA Gene

In 1997, using RACE-PCR, Muhlethaler-Mottet et al. found that the transcription of CIITA is regulated by 4 distinct promoters, designated P- I, II, III and IV (Figure 10).

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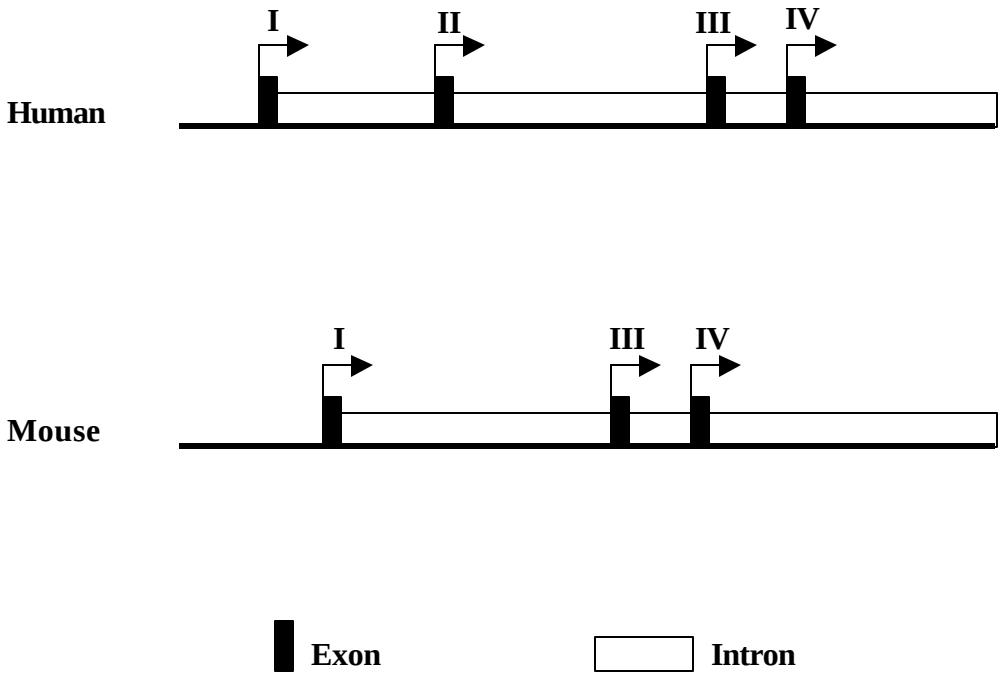

Figure 10. Alternative CIITA promoters in both mice and humans. The promoters are expressed in a tissue-specific manner. Promoter I is found in dendritic cells, promoter III in B cells and promoter IV in IFN-γ-inducible cells. Promoter II has not yet been identified in any particular tissue.

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Each of the 4 promoters has a distinct first exon, which is spliced to a common second exon. Three of the 4 promoters are found in specific cell types, and are responsible for the activation and control of the transcription factor. Two of these cell types, dendritic cells and B cells, express CIITA constitutively, whereas one is interferon-γ-inducible. The type II promoter has not yet been identified in any specific cell type and, as such, there is far less information available that pertains to PII. Due to the fact that CIITA was originally isolated from Raji cells, a B cell leukemia line, the published wild type sequence is that of PIII (Steimle et al., 1993). Promoters I, III and IV, but not promoter II, have been identified in mice, and show significant sequence homology to the human promoters (Muhlethaler-Mottet et al., 1997).

For each of the different CIITA promoters, which lie within 13 kb of one another, binding elements have been identified in the sequences (Muhlethaler-Mottet et al., 1997). For PI (Figure 11), in both the human and the mouse promoter, there is an NF-GMb site (Shannon et al., 1988), an NF-IL6 site (Akira and Kishimoto, 1992), two NF-IL6 inverted sites, a PEA3 site (Wasylyk et al., 1989), an inverted PEA3 site, and an E2 site (Murre et al., 1989). Box 1 is composed of either two NF-IL6 or two PEA3 sites in palindromic orientation (Muhlethaler-Mottet et al., 1997). In addition to these sites, the human PI has an AP1 site (Pollock and Treisman, 1990) and a CCAAT box (Dorn et al., 1987). In both the human and the mouse PIII sequence (Figure 12), there is an interferon regulatory factor binding element (IRF) (Tanaka et al., 1993), an inverted OCT site (Rosales et al., 1987), an inverted MYC site (Agira, 1989), and a C site (Ghosh et al., 1999). Additionally, the mouse PIII has two CCAAT boxes. For both the human and the mouse

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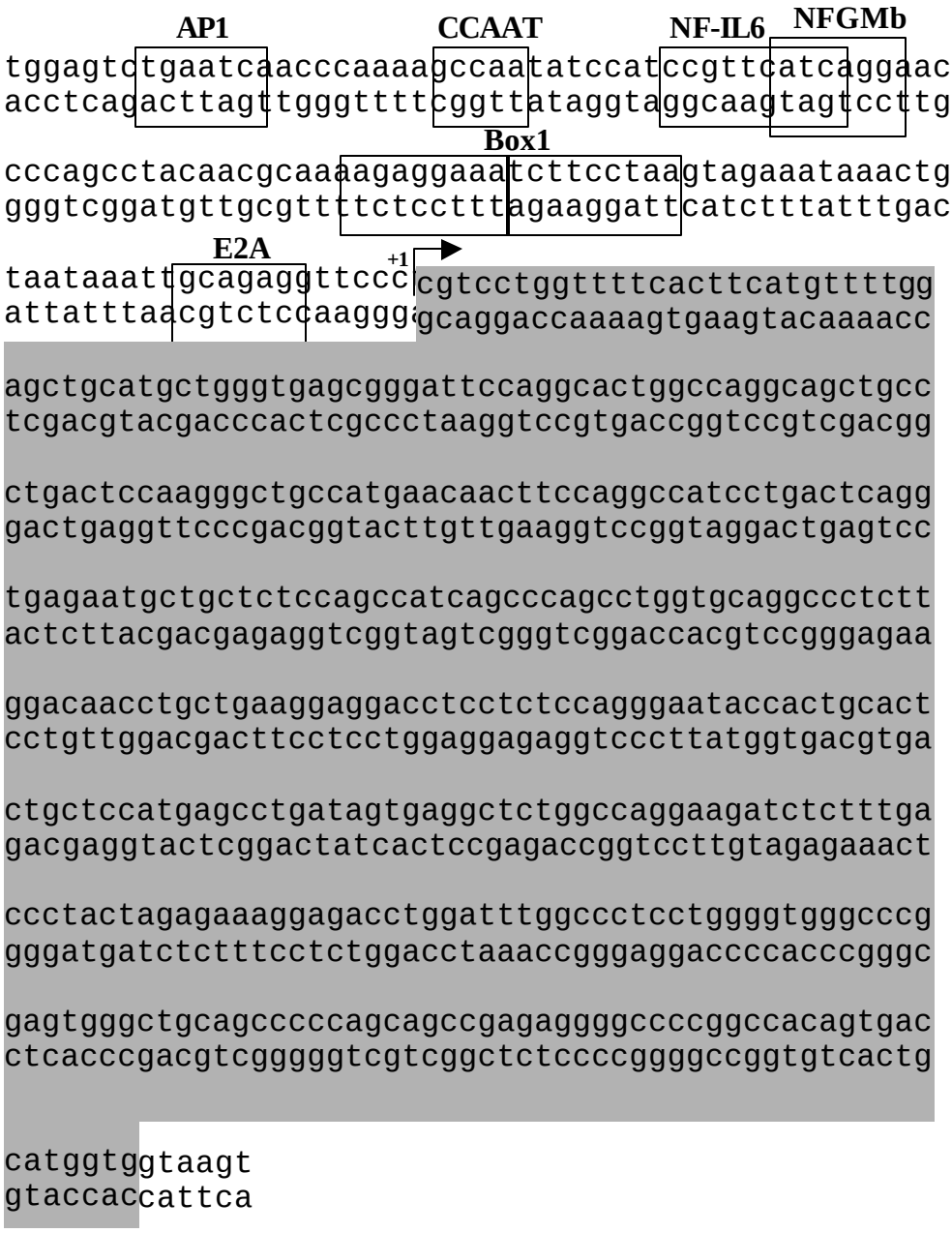

Figure 11. The binding elements identified in the human CIITA promoter I.

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Figure 12. The important binding elements identified in the human CIITA promoter III.

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PIV (Figure 13), there is an IFN-γ activation sequence (GAS) (Pellegrini and Schindler, 1993) and adjacent E box (Blackwell et al., 1990), and an IRF. Additionally, the human promoter contains an NFêB site, and the mouse promoter has two AP1 sites.

Enhancement of the CIITA promoter is thought to occur only in dendritic cells. Dendritic cells are professional APC that have a high level of MHC class II surface expression, and this high expression level was shown to be the result of enhanced transactivation activity of the dendritic cell-specific CIITA (Nickerson et al., 2001). The first exon of CIITA, unique to PI, encodes an extended N-terminal region of CIITA. This region has been shown to contain a domain with high homology to a caspase-recruitment domain (CARD). Although CARDs are known to interact with caspases and are involved in the regulations of apoptosis, this is not the case of the dendritic cell CIITA CARD. However, when the CARD of the dendritic cell CIITA is altered, there is no longer enhanced transactivation activity. It has been proposed, but not yet verified, that there is a CARD-CARD interaction that occurs in dendritic cells, resulting in this increased activity of CIITA.

Although the expression of PIII is largely constitutive, it can also be induced by IFN-γ and inhibited by transforming growth factor-β (TGF-β) (Piskurich et al., 1998). TGF-β knockout mice develop severe autoimmune disease as a result of the over-expression of MHC class II genes (Geiser et al., 1993; Shull et al., 1992). A study of the DNA sequence of the region flanking PIII led to the discovery of a segment located at nucleotides -545 to -113 (relative to the transcription start site) that was important for the constitutive expression of PIII. Further analysis revealed that there was an additional

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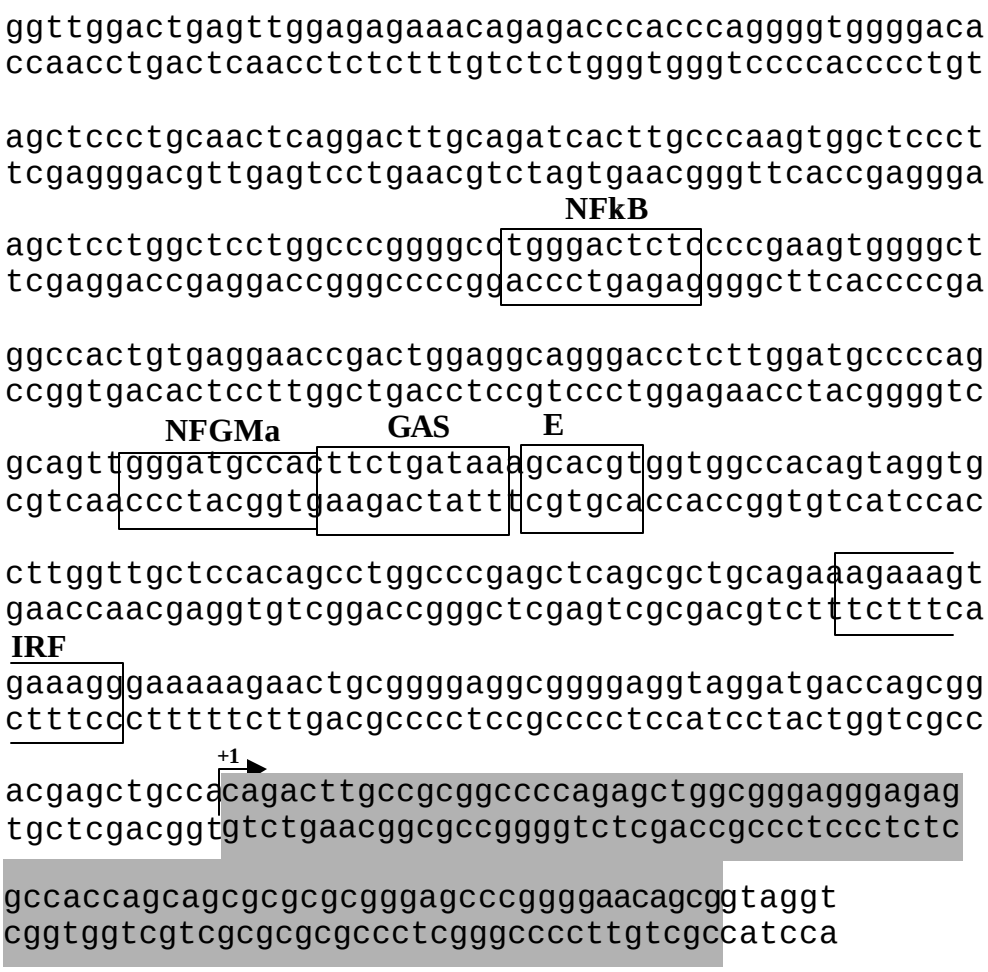

Figure 13. The important binding elements identified in the human CIITA promoter IV.

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region approximately 4 kb upstream of the promoter that was necessary for IFN-γ-inducible activation of PIII. This 1 kb region was termed the IFN-γ response sequence. A later study found that the elements that are sufficient for the constitutive expression of PIII are located within 319 bases of the start site (Ghosh et al., 1999). In vivo genomic footprinting showed that within this region there were several sites of protected DNA. The activation response elements (ARE) were sites of strong protection, and proved to be essential for the constitutive expression of PIII. Sites A, B and C showed weaker protection, and proved to be weak enhancers of PIII expression. ARE-1 is located between –133 and –142, and is a TEF-2-like element (Davidson et al., 1988; Mercurio and Karin, 1989). ARE-2 is located between -56 and –64, and binds a novel transcription activator. Site A is located between -18 and -27, and corresponds to a sequence that has homology to an NF-1 transcription factor-binding site (Leegwater et al., 1985). Site B is located between -45 and –38, and corresponds to an octamer-binding site (Muhlethaler-Mottet et al., 1997). Site C is located at position –181. In the myeloma cell line, NCI-H929, PIII is completely unoccupied (Ghosh et al., 1999). Subsequently, there is no MHC class II expression.

For PIV, the essential elements of the promoter sequence have been identified. Three cis-acting elements within a 154 bp region of the promoter were found to be critical for IFN-γ-inducible expression of PIV (Dong et al., 1999). The first is the GAS element, which is adjacent to the E box, followed by the IRF element. These elements are bound by the signal transducers and activators of transcription (STAT1), the upstream stimulating factor (USF-1) and IRF-1/IRF-2, respectively (Muhlethaler-Mottet et al.,

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1998). IRF-1 and STAT1 are IFN-γ-inducible transcription factors, while USF-1 is ubiquitously expressed. It is believed that the binding of IFNγ to its receptor on the cell surface signals a cascade of events in which STAT1 is phosphorylated by the protein tyrosine janus activated kinases, JAK1 and JAK2 (Figure 14). STAT1 is then translocated to the nucleus where it binds the GAS element (Darnell, 1997; Darnell et al., 1994; Schindler and Darnell, 1995). However, STAT1 will only bind the GAS site in the presence of USF-1 (Muhlethaler-Mottet et al., 1998). Thus, the binding of USF-1 and STAT1 to their respective sites is cooperative and essential for IFN-γ-inducible MHC class II expression. The macrophages of STAT1 knockout mice cannot be induced to express CIITA mRNA (Meraz et al., 1996). Similarly, in IRF-1 knockout mice, there is a significant reduction of IFN-γ-induced CIITA mRNA found in the kidneys, suggesting that IRF-1 is one of the crucial elements of IFN-γ-inducible MHC class II expression (Hobart et al., 1997). Later experiments revealed that IRF-1 and IRF- 2 simultaneously occupy the promoter via the IRF element, and synergistically activate PIV (Xi et al., 1999). IRF-2 generally acts as a repressor of transcription by competing with IRF-1 to bind the IRF element (Harada et al., 1989; Harada et al., 1990; Tanaka et al., 1996; Taniguchi et al., 1995; Taniguchi et al., 1997); however, IRF-2 knockout mice have impaired MHC CIITA expression (Xi et al., 2001). In macrophages, the suppressors of cytokine signaling (SOCS-1) protein inhibits the expression and binding of STAT1 and IRF-1 to their respective elements, thereby inhibiting the IFN-γ-inducible expression of CIITA (O'Keefe et al., 2001).

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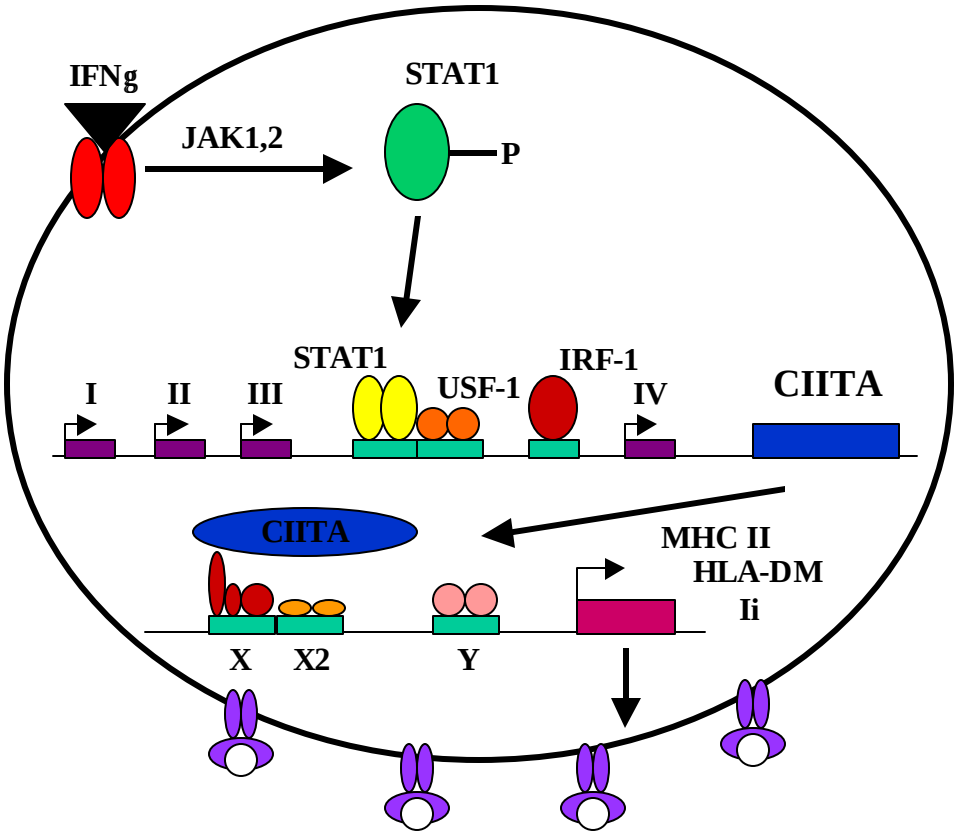

Figure 14. CIITA promoter IV is activated via the JAK/STAT signal transduction pathway by the binding of IFN-γ to its receptor on the cell surface.

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It appears that there is not only differential usage of the CIITA promoters in different cell types, but also under different physiological conditions. When experimental autoimmune encephalitis (EAE), an autoimmune disease that is caused by the introduction of various myelin proteins (Wekerle et al., 1994), was induced in the central nervous system (CNS) of mice, the CIITA types I and IV were found in the brain and spinal cord. Neither form of CIITA was observed in the brain or spinal cord of control mice (Suter et al., 2000). Additionally, the active promoter can vary depending on the different stages of development of a particular cell line. For example, early pro-B cells have IFN-γ-inducible MHC class II expression, but mature B-cells exhibit constitutive expression. In addition to the differential usage of promoters, various mechanisms of silencing or enhancing the CIITA promoters have been identified. Although it is not known how the promoter to be used is chosen, it has been proposed that alternative splicing plays a key role in the process (Muhlethaler-Mottet et al., 1997).

One cell type where promoter silencing occurs is fetal trophoblasts. Fetal trophoblasts lack both constitutive and IFN-γ-inducible expression of MHC class II molecules. It was subsequently discovered that they also lack both constitutive and IFN-γ-inducible expression of CIITA. It is thought that inhibition of CIITA and, therefore, MHC class II genes is critical for maternal-fetal tolerance. It was demonstrated that, in these cells, the regulatory factors of the CIITA gene (STAT1 and IRF-1) were unable to assemble at PIV. Furthermore, it was shown that these factors were present, and were able to activate a reporter gene in an expression assay. The reason for the failure to assemble was determined to be the methylation of PIV (Morris et al., 2000).

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Another example of CIITA promoter silencing involves the terminal differentiation of B cells into plasma cells. During this process there is a loss of MHC class II expression that was found to correlate with a loss of CIITA expression (Silacci et al., 1994). In mice, B lymphocyte-induced maturation protein I (BLIMP-I) is a transcriptional repressor that is involved in the differentiation of B cells to plasma cells. BLIMP-I was found to be a repressor of CIITA that acts by binding to CIITA PIII. It was also found that the induction of the expression of BLIMP-I leads to the repression of mRNA for not only CIITA, HLA-DR, -DP and -DQ, but also the non-traditional MHC class II genes, Ii and HLA-DM (Piskurich et al., 2000). The human equivalent of BLIMP-I is the positive regulatory domain I binding factor 1 (PRDI-BF1), which has been implicated in the loss of CIITA expression in plasma cell tumors (Ghosh et al., 2001).

In some cases of melanoma, rather than silencing of the CIITA promoters, there is constitutive expression, which is usually limited to professional APCs. Constitutive expression of MHC class II molecules on melanoma cells is associated with a poorer prognosis, and is considered to be a marker of the disease progression (Barnhill, 1993; Ostmeier et al., 1999). Recent studies have demonstrated that the unusual expression pattern of the class II molecules in melanoma cells is the result of the constitutive expression of CIITA, specifically, CIITA types III and IV (Deffrennes et al., 2001; Goodwin et al., 2001). The constant activity of PIII was attributed to factors acting on the upstream enhancer, known as the IFN-γ response sequence. The constitutive

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activation of PIV was unprecedented in any cell type, and was thought to be activated by IRF-2, as IRF-1 was not constitutively expressed in the melanoma cells.

Rationale for the Present Work

The K-562 cell line is a pluripotent cell line that has been shown to differentiate along several different pathways. Although MHC class I expression can be induced by IFN-γ on the cells, a method for inducing HLA-DR remains to be identified. This observation, combined with studies demonstrating the ability of K-562 cells to function as accessory cells in T cell activation assays, raises the question of the role of the cell line in the immune response. Compromised MHC class II expression has been shown to result in severe immunodeficiency, caused by inefficient T cell activation and a diminished T cell repertoire. In the case of CML, the malignant cells slowly replace the hematopoietic stem cells, leading to diminished immunity in the blast phase, and ultimately, the death of the patient. For this reason it is important to identify the cause of the negative expression of MHC class II molecules on K-562 cells.

In 1999, Liu et al. observed that CIITA mRNA was not expressed in K-562 cells. The study then showed that a complementation assay of the wild type CIITA in K-562 cells led to the recovery of MHC class II expression. The conclusion was that the lack of MHC class II expression is caused by the absence of CIITA expression in K-562 cells. Although CIITA mRNA has been identified in K-562 cells in our laboratory, the results of the complementation assay by Liu et al. suggested that there was some malfunction of

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the CIITA gene. The work presented here addresses the nature of this malfunction and its effect on the MHC class II expression of K-562 cells.

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CHAPTER 2

Materials and Methods

Chemicals

Trizol, reverse transcriptase MEM, RPMI 1640 and all other cell culture reagents, with the exception of gentamicin, which was purchased from Sigma Chemical Company (St. Louis, MO), were obtained from Invitrogen life technologies (Carlsbad, CA). The CIITA full-length cDNA was a gift from Dr. Matija Peterlin (The University of California, San Francisco, Cancer Center, San Francisco, California). The phytohemagglutinin (PHA) crude extract was purchased from ICN (Costa Mesa, CA). With the exception of the CIITA and RFX-B antibodies, which were purchased from Santa Cruz (Santa Cruz, CA), all monoclonal antibodies were purchased from BD Pharmingen (San Jose, CA). Most enzymes, nucleic acid purification kits and the dual luciferase assay were purchased from Promega (Madison, WI). All other chemicals were purchased from Sigma Chemical Company.

Culture Media, Growth Conditions, and Proliferation Assays

Bacteria

All transformations were performed in JM109 *Escherichia coli* high competency cells (Promega). Vectors used are listed in Table 1. Bacteria was grown and maintained

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Table 1. Plasmid vectors used in this study.

Name	Function	Resistance
pGEM-T Easy	Cloning of PCR products	ampicillin
pGL3-basic	Firefly Luciferase reporter	ampicillin
pRL-TK	*Renilla* (Sea Pansy) luciferase reporter	ampicillin
pBABE	Expression vector	ampicillin, puromycin

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on Luria broth (LB) medium (Sambrook *et al.*, 1989) supplemented with 50 µg/ml ampicillin, at 37°C. Liquid cultures were allowed to shake overnight at 37°C.

Mammalian cell culture

A continuous culture of the K-562 cell line was kindly provided by Dr. Carmen Lozzio (The University of Tennessee Medical Center, Knoxville, TN) and maintained in Eagle's minimal essential medium (MEM) supplemented with gentamicin, 15% fetal bovine serum (FBS), 2 mM L-glutamine and 1% nonessential amino acids. Aliquots of cells were periodically frozen in Eagle's MEM with 15% FBS and 5% glycerol and stored in liquid nitrogen. Once a week, a new passage was made by seeding $1x10^5$ cells/ml in RPMI 1640 medium supplemented with gentamicin, 2 mM L-glutamine and 10% FBS. The MHC class II-positive Raji cell line was purchased from the Human Science Research Resource Bank (Osaka, Japan) and cultured in RPMI 1640 medium with gentamicin, 2 mM L-glutamine and 10% FBS. All cultures were grown in a humidified atmosphere at 37°C with 5% CO_2.

T cell growth-conditioned medium

For the production of T cell-growth-conditioned medium (CM), whole blood extracts were obtained from various donors. Peripheral blood mononuclear cells (PBMC) were then isolated using a ficol-hypaque gradient (Sigma). The PBMC were cultured at a concentration of $5x10^5$ cells/ml in RPMI 1640 medium with 10% FBS in the presence of a crude extract of PHA (5 µg/ml). After 96 hours, the PBMC were removed by

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centrifugation at 800 rpm using a TS-7 rotor (Tomy Seiko Ltd., Tokyo, Japan). The medium was then further purified by vacuum filtration with a .22 µm cellulose acetate filter (Corning Inc., Corning, NY) and stored at -80°C. For the flow cytometry and microscopy experiments, K-562 cells were incubated with 50% CM for 24 hours.

Scintillation counting

K-562 cells were labeled with 1 µCi/ml [^{3}H]thymidine (Amersham Pharmacia Biotech, Bucks, UK) and harvested at various time points. The counts per minute (CPM) were recorded in 5 ml Scintisol EX-H solution (Dotite, Tokyo, Japan) using a Beckman LS5000CE counter (Beckman Coulter, Fullerton, CA). For the chromatin immunoprecipitation experiment, aliquots of the various chromatin fractions were assayed to determine which fractions contained cross-linked chromatin.

Proliferation assay

In a 96-well plate, [^{3}H]thymidine was added to CM alone, 1x10^5 K-562 cells in RPMI medium and 1x10^5 K-562 cells in 50% CM. The samples were harvested at various time points and analyzed by scintillation counting. The results of the proliferation assay were graphed in Excel.

T cell activation assay

For the T cell activation assay, PBMC were isolated using a ficol-hypaque gradient and then further purified by incubation in a plastic flask at 37°C for 1.5 hours to

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remove the adherent monocytes. T cells were isolated from the PBMC using Human $CD3^+$ T cell Enrichment Columns (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The K-562 cells ($1x10^6$ cells/ml) were incubated in the dark with mitocycin C (Sigma Chemical Company) at 45°C for 1 hour, washed twice with media and transferred to a 96-well plate, where they were incubated with media alone or media and T cells. At various time points, the cells were harvested and analyzed by scintillation counting.

Caspase assay

In order to detect caspase-3 activity in K-562 cells treated with 0.2 nM PMA, the caspase-3 substrate, Ac-DEVD-pNA (Calbiochem, San Diego, CA), was used according to the manufacturer's instructions. The cells were incubated for various times before the addition of the colorimetric substrate. Once the cells had been lysed and the substrate had been added, the results were read on a Wallac Arvo SX Multilabel Counter (Amersham). The data was then graphed in Excel.

Nucleic Acid Manipulation

Isolation of nucleic acids

For Northern blotting, reverse transcription-polymerase chain reactions (RT-PCR) and real-time PCR, total RNA was isolated from $6x10^6$ Raji or K-562 cells using Trizol Reagent (Invitrogen life technologies) according to the manufacturer's instructions. Prior

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to reverse transcription, RNA was treated with 1 U/µg RNase free DNase for 30 min at 37°C (Promega).

For the isolation of genomic DNA, $2x10^7$ Raji or K-562 cells were lysed in high molecular weight buffer (10 mM Tris-HCl, 0.1 M EDTA, 0.5% SDS, and 100 µg/ml proteinase K) for 1.5 hour at 55°C. Genomic DNA was then extracted three times with phenol-chloroform (1:1) and three times with ether, followed by ethanol precipitation. Finally, the pelleted DNA was resuspended in Tris-EDTA (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) containing RNase A (20 µg/ml).

Plasmid DNA was isolated from *E. coli*, according to the alkaline lysis method described by Sambrook *et al.* (1989), or by using the Wizard Plus Miniprep DNA Purification system (Promega). For all nucleic acid isolations, the quantity and quality of the DNA or RNA was assessed by monitoring the optical density (OD) at 260 nm and by gel electrophoresis.

Northern blotting

Northern blotting was performed according to the glyoxal method described by Sambrook *et al.* (1989). After treatment with glyoxal, dimethyl sulfoxide (DMSO) and 100 mM Na_2PO_4, 10 µg of total RNA was separated on a 1% agarose gel containing 10 mM Na_2PO_4. The gel was washed briefly in dH_20 and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech). The membranes were UV-cross-linked prior to hybridization with the probe. The hybridization solution used was Rapid-hyb buffer (Amersham Pharmacia Biotech).

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The [^{32}P]CIITA probe was made from the full length CIITA cDNA (4.5 kb) provided by Dr. Matija Peterlin (The University of California, San Francisco, Cancer Center, San Francisco, California) using the Random Primed DNA Labeling Kit from Roche Molecular Biochemicals (Mannheim, Germany), followed by purification with a MicroSpin G-25 column (Amersham). Hybridizations and washing were performed according to the manufacturer's instructions. The results were visualized with a Bas 2500 Phospho-imager (Fuji Film, Tokyo, Japan).

Reverse transcription

Reverse transcription was performed using 5 µg of total RNA (final concentration 250 ng/µl) with 0.5 µg Oligo $(dT)_{12-18}$ as the primer. In most cases, Superscript II Moloney's Murine Leukemia Virus (M-MLV) reverse transcriptase (RT) was used and the reaction was carried out at 42-50°C for 1 hour. In the case of the CIITA transcript, Thermoscript Avian Myeloblastosis Virus (AMV) RT was used. Prior to the addition of the enzyme, the samples were heated to 70°C for 10 minutes to disrupt any secondary structure. The enzyme mixture was pre-heated to 60°C before adding it to the RNA/oligo-dT mixture. The reaction was allowed to proceed for 1 hour at 60°C. The reverse transcribed cDNA was then further treated with RNase H for 20 min at 37°C.

Polymerase chain reaction

Amplification by PCR was performed with *Taq* DNA Polymerase according to the manufacturer's specifications (Promega). Primers used for PCR and sequencing were

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purchased from either Invitrogen or Hokkaido Systems Science (Hokkaido, Japan) and are listed in Tables 2, 3 and 4. The CIITA primers correspond to overlapping regions within the CIITA cDNA (Figure 15). Prior to cycling, the samples were heated to 94°C for 2 minutes. PCR conditions were as follows: 94°C, 1 min; annealing temperature, 1 min; 72°C, 3 min. The reaction was allowed to proceed for 30-35 cycles, with a final extension of 10 min at 72°C.

Real-time PCR

Quantitative PCR was carried out using an ABI Prism 7700 sequence detector and the Platinum Quantitative PCR system (Invitrogen life technologies). The probe and spanned the insertion site and the mutant probe and primer set corresponded to a region primer sequences are found in Table 5. The wild type Taqman probe and primer set within the insert of the alternative transcript (Figure 16). The template used was the cDNA produced with Thermoscript reverse transcriptase (Invitrogen life technologies) at 60°C with dH_20 as the negative control. The samples were annealed at 64°C for 37 cycles.

Cloning of PCR products

Fresh (<1 day old) PCR products were ligated into the pGEM-T Easy vector using the pGEM-T Easy vector system (Promega) according to the manufacturer's protocol. Ligated products were transformed into JM109 competent cells and grown on LB/ampicillin plates. Blue/white visual screening was used to detect the colonies containing the insert according to the method described by Sambrook *et al*. (1989).

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Table 2. CIITA RT-PCR primers used in this study.

Name	Primer Sequence	Location (nt)	T_m °C
CIITA A Forward 1	5'-TCCTACACAATGCGTTGCCTG-3'	107-127	71
CIITA A Reverse 1	5'-GGCCGGTCTGGAGATGTTGGG-3'	966-946	77
CIITA A Forward 2	5'-TGAAGTGATGGGTGAGAGTATGGA-3'	489-513	72
CIITA A Reverse 2	5'-GCTTCCAGTGCTTCAGGTCT-3'	593-574	70
CIITA B Forward 1	5'-GGCAAATCTCTGAGGCTGGAAC-3'	837-858	73
CIITA B Reverse 1	5'-GTGCTGTGCAGGAAGCCATCT-3'	1656-1636	73
CIITA B Forward 2	5'-AAGAGCCTGGAGCGGGAACT-3'	1241-1260	72
CIITA B Reverse 2	5'-GCCCAGCACAGCAATCACTCG-3'	1376-1355	75
CIITA C Forward 1	5'-AGGTTTTCAGCCACATCTTGA-3'	1570-1590	67
CIITA C Reverse 1	5'-CCTCCAGCCAGTTGTCATAGG-3'	2372-2352	73
CIITA C Forward 2	5'-GTCACAGCCACAGCCCTACTT-3'	1932-1952	73
CIITA C Reverse 2	5'-CCCAGCAGGCCGACATAGAGT-3'	2052-2032	75
CIITA D Forward 1	5'-TGTGGCTGGCTCTGAGTGGCG-3'	2274-2294	77
CIITA D Reverse 1	5'-CAGGTCTTCCACATCCTTCAG-3'	2908-2888	71
CIITA D Forward 2	5'-CTCGGTGGACAGGAAGCAGAAGG-3'	2455-2477	77
CIITA D Reverse 2	5'-GCTCCTGTACCACGTGCTGCCAA-3'	2600-2578	77
CIITA E Forward 1	5'-GGCAGCAGAGGAGAAGTTCAC-3'	2842-2862	73
CIITA E Reverse 1	5'-GAGAACATGCCTGTCCAGAGC-3'	3541-3521	73
CIITA E Forward 2	5'-ACTGACCTGGGTGCCTACAAAC-3'	3197-3218	73
CIITA E Reverse 2	5'-CACGTCGCAGATGCAGTTATTGTA-3'	3292-3269	72
CIITA F Forward	5'-GCCTCTACCACTTCTATGACC-3'	252-273	71
CIITA F Reverse	5'-GACTTTTCTGCCCAACTTCTG-3'	522-501	69

Table 3. Additional RT-PCR primers used in this study.

Name	Primer Sequence	T_m °C
β-actin Forward	5'-ATCTGGCACACCTTCTACAATGAGCTGCG-3'	82
β-actin Reverse	5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'	82
HLA-DR Forward	5'-CGACAAGTTCACCCCACCAGT-3'	73
HLA-DR Reverse	5'-CAGGAAAAGGCAATAGACAGG-3'	69
Ii Forward	5'-GAGGAGAAGCAGGAGCTGTCG-3'	61
Ii Reverse	5'-TCTTAAGGTGTCTCAGGTTCTCCG-3'	59

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Table 4. Promoter primers used for PCR in this study.

Name	Primer Sequence	T_m °C
CIITA PI Forward	5'-CAAAAGCCAATATCCATCCGTTC-3'	57
CIITA PI Reverse	5'-CCAGGTCTCCTTTCTCTAGTAGGGTC-3'	64
CIITA PII Forward	5'-CTCAGGACTTGCAGATCACTTGC-3'	61
CIITA PII Reverse	5'-ACTTTCTTTCTGCAGCGCTGAG-3'	59
CIITA PIII Forward	5'-ATTGTGATCATCATTTCTGAACGTCA-3'	56
CIITA PIII Reverse	5'-CCTCATCACTAGCCTCATCACTAACC-3'	62
CIITA PIV Forward	5'-GCATCAGAGGAGTGAATAGCTCAGTT-3'	61
CIITA PIV Reverse	5'-GGCAAGTCTGTGGCAGCTC-3'	59
PI Luc. Forward	5'-GCGCTAGCTGGAGTCTGAATCAACCCAAAAGC-3'	67
PI Luc. Reverse	5'-GCCTCGAGCCAGGTCTCCTTTCTCTAGTAGGGTC-3'	70
PIII Luc. Forward	5'-GCGCTAGCCTGCAGAAGGTGGCAGATATTG-3'	67
PIII Luc. Reverse	5'-GCCTCGAGCCTCATCACTAGCCTCATCACTAACC-3'	69
PIV Luc. Forward	5'-GCGCTAGCGCATCAGAGGAGTGAATAGCTCAGTT-3'	68
PIV Luc. Reverse	5'-GCCTCGAGGCAAGTCTGTGGCAGCTC-3'	68
DR Forward	5'-GCGCTAGCAACTGGTTCAAACCTTTCAAGTAC-3'	65
DR Reverse	5'-GCCTCGAGTCTCACTCAGGGAGAACTATGAAC-3'	68
Ii Forward	5'-GGTGTCTTCTGTTTCAAAGTGCTTTC-3'	73
Ii Reverse	5'-CACTCCGCCCACTTGGTAGAT-3'	73
HLA-DM Forward	5'-CTACGGAAATCTACTGGTTGTTCTG-3'	73
HLA-DM Reverse	5'-ATTAAATCTGTTCCTTCCAGCTCAC-3'	71
HLA-DP Forward	5'-ATGAGTATCACTGTCTTTCCTCCG-3'	72
HLA-DP Reverse	5'-GAGACCATGAACCCAAGTAGTCTTC-3'	74
HLA-DR Forward	5'-CAACTGGTTCAAACCTTTCAAGTAC-3'	71
HLA-DR Reverse	5'-AGTCTCACTCAGGGAGAACTATGAAC-3'	74

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Wild-type

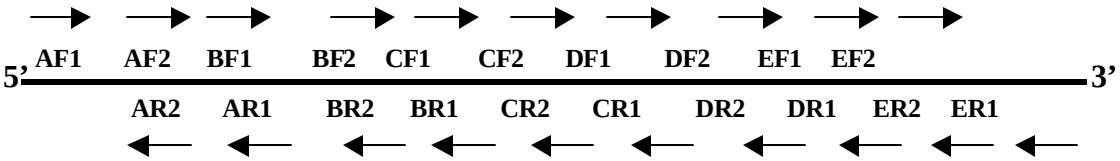

Figure 15. Location of the CIITA primers in the wild-type sequence.

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Table 5. Probes and Primers used for Real-Time PCR in this study.

Function	Name	Sequence	T_m °C
Probe	Wild-type	5'-Vic-CCCGTTTCAGGGCTGCCTTGAG-Tamra-3'	68
Primer	WT Forward	5'-TCCGCAGCACTGGCATTT-3'	60
Primer	WT Reverse	5'-CCTCTGCCTGAAGTAGCTT-3'	59
Probe	Alternative	5'-Fam-TCAATATTTGAAGGCCCGCCATGTG-Tamra-3'	69
Primer	Alt Forward	5'-CAAGTGGAGAGGCAATGGCATT-3'	59
Primer	Alt Reverse	5'-AAGAAAACGGTGGCGTGCT-3'	59

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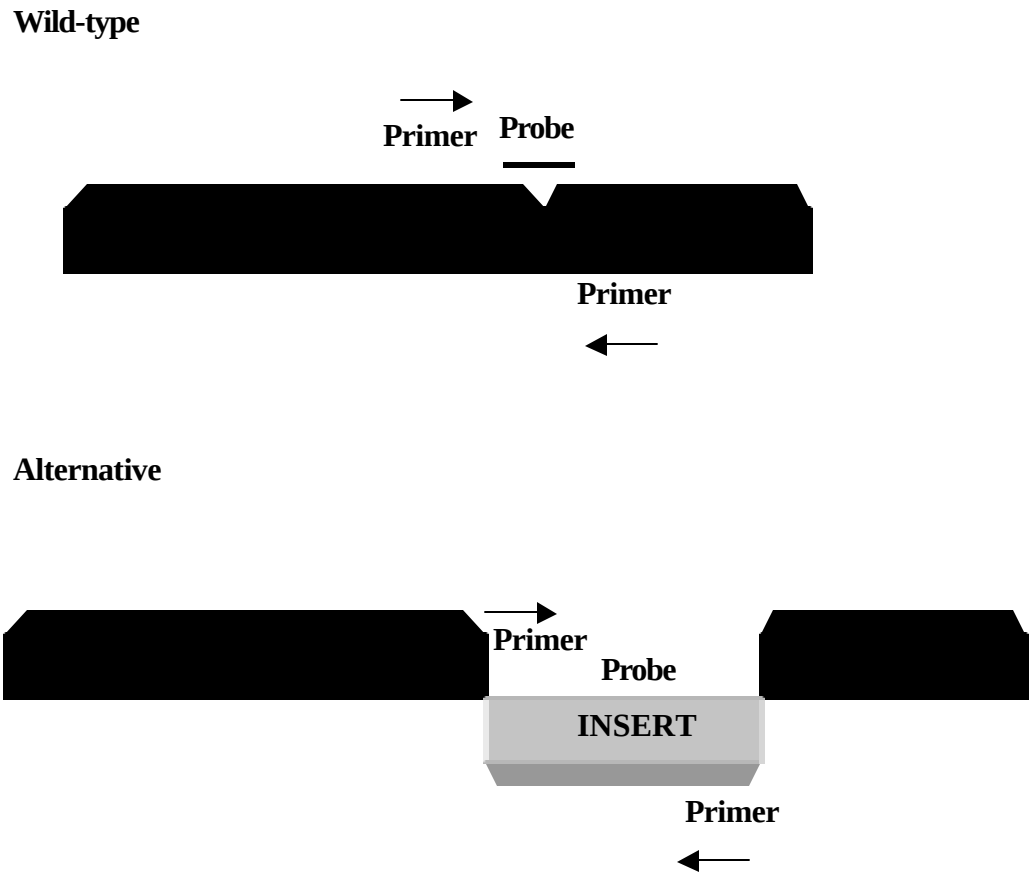

Figure 16. Location of the primers and probes for real time PCR in the wild-type and alternative CIITA transcripts.

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Restriction digestion

The restriction enzymes used in this study, *Eco RI, Nhe I, Sal I* and *Xho I,* were purchased from New England Biolabs (Beverly, MA). The digestions were carried out at 37°C, according to the manufacturer's protocol, using the buffers provided. When the *Eco RI* and *Sal I* buffers were used in combination, the *Sal I* buffer was used. In the case of the screening of colonies for the appropriate insert, 5 µl of the digestion reaction was electrophoresed on an agarose gel and visualized by ethidium bromide staining. When the excised insert was to be used for the production of DNA constructs, the entire digestion reaction was electrophoresed on an agarose gel and the appropriate band was excised from the gel. The DNA was then purified from the gel piece using GenElute Minus EtBr Spin columns (Sigma), according to the manufacturer's instructions.

Sequencing

Sequencing was carried out by the dideoxy chain termination method on an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA) using a Big Dye dGTP sequencing kit (Applied Biosystems). Sequencing primers were M13 forward and M13 reverse and gene-specific primers listed in Table 6. PCR conditions were as follows: 96°C, 30 sec; 55°C, 15 sec; 65°C, 4 min, and the reaction was allowed to proceed for 30 cycles.

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Table 6. Sequencing primers used in this study.

Name	Primer Sequence	T_m °C
3'UTR 1 Forward	5'-CCTCACCTTCTCCTCTCTTACC-3'	60
3'UTR 2 Forward	5'-CTGATTGTATCCCATCCCTGC-3'	59
3'UTR Reverse	5'-GGAGTGGAAGAGGAGAGAATGG-3'	60
CIITA D3 Forward	5'-TTGGCAGCACGTGGTACAGGAGC-3'	64

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Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed according to the method of Orlando *et al*. (1997). Briefly, $1x10^9$ cells were labeled with 1 µCi/ml ^{3}H-thymidine for 48 hours and fixed by adding one-tenth volume fixation buffer containing 11% formaldehyde. After 1 hour at 4°C, the reaction was stopped with 0.125 M glycine. Cells were collected by centrifugation and washed for 10 min each in 15 ml of Buffer 1 (0.25% Triton X-100, 10 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM Tris-HCl, pH 8.0), and in 15 ml of Buffer 2 (0.2 M NaCl, 1 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM Tris-HCl, pH 8.0). The final pellets were resuspended in 10 ml of Buffer 2, and stored on ice. The samples were sonicated twice for 30 seconds each time, adjusted to 0.5% sodium lauryl sarcosine, and then applied to a cesium chloride gradient (1.42 g/cm^3) for chromatin isolation. After ultra-centrifugation at 40,000 rpm for 72 hours in a Beckman SW55Ti rotor (Beckman Coulter), the fractions were collected and analyzed by scintillation counting. The fractions containing cross-linked chromatin were dialyzed overnight at 4°C against 5% glycerol, 1 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM Tris-HCl, pH 8.0. The samples were then adjusted to RIPA buffer (1% Triton-X, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF) by adding each component sequentially, followed by the addition of the CIITA or RFX-B antibody, and incubated overnight at 4°C. As a negative control, a sample was performed in tandem, in which no antibody was added. Protein-A Sepharose beads (Sigma) were used to recover immuno-complexes. The beads were washed under stringent conditions for 10 minutes each, five times in RIPA buffer (Wash 1), once in Wash 2 (0.25 M LiCl, 0.5% NP-40,

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0.5% sodium deoxycholate, 1 mM Na-EDTA, 10 mM Tris-HCl, pH 8.0), and twice in Wash 3 (1 mM Na-EDTA, 10 mM Tris-HCl, pH 8.0), and resuspended in the 100 µl of Wash 3. They were then treated with 50 µg/ml RNase A at 37°C for 30 minutes, followed by 0.5% SDS, 50 µg/ml proteinase K overnight. After the cross-links were reversed at 65°C for 6 hours, the DNA was extracted with phenol-chloroform (1:1). The organic phase back-extracted with 0.5 ml Extraction Buffer (50 mM Tris-HCl, pH 8.0), followed by a final extraction of the aqueous phase with chloroform-isoamyl alcohol (1:1). The DNA was then ethanol precipitated overnight in the presence of 3 M sodium acetate, using 20 µg glycogen as a carrier, and dissolved in DEPC-treated H_2O. Following precipitation of the DNA, PCR was performed on the samples using primers for the HLA-DR promoter (Table 4) and CIITA primer pair F (Table 2). The amplified products were then electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Transfections and Luciferase Assay

Luciferase Constructs

For the production of the luciferase constructs (Figure 17), the CIITA promoters were amplified from the genomic DNA of Raji and K-562 cells by PCR, using primers containing the appropriate restriction site for directional ligation into the pGL3 luciferase reporter (forward primers, *Nhe I*; reverse primers, *Xho I*). The PCR products were first cloned in the pGEM-T Easy vector system and selected by blue/white screening on

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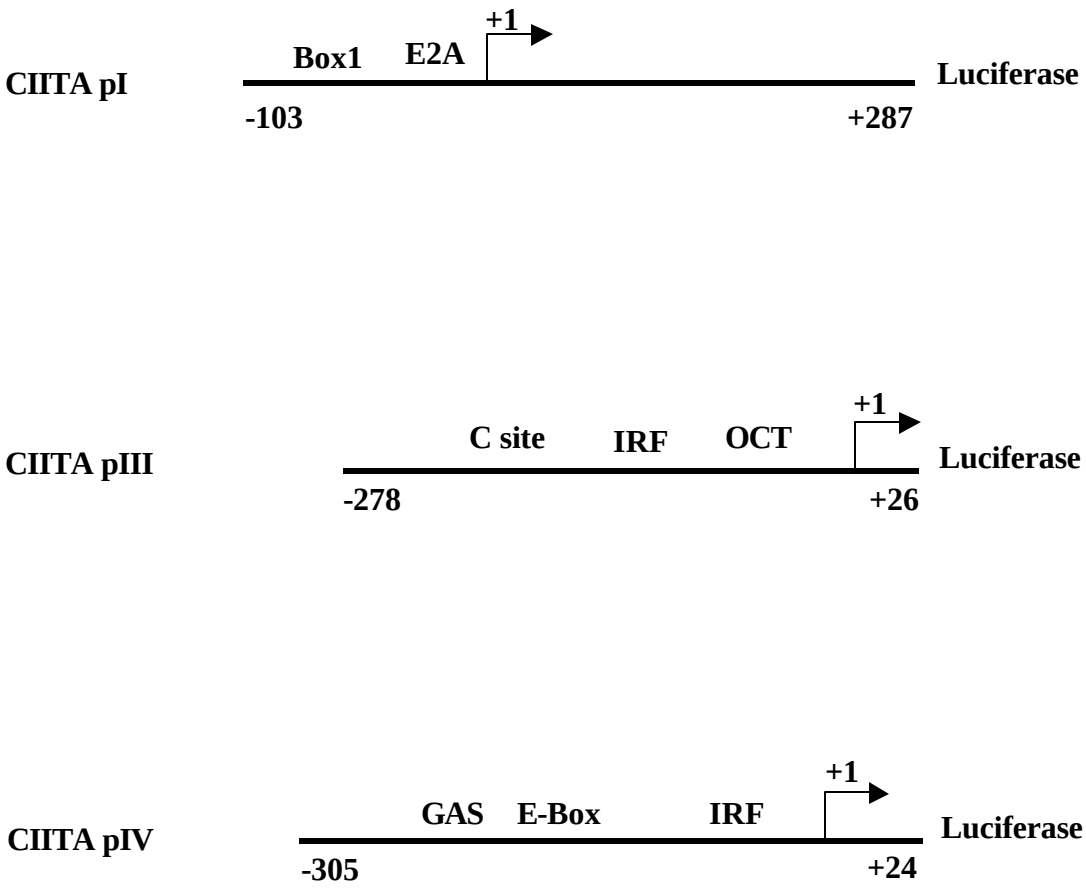

Figure 17. Luciferase constructs of the CIITA promoters.

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LB/ampicillin plates. The plasmid was isolated and the insert was excised and ligated to the *Nhe I* and *Xho I* digested, CIAP-treated pGL3 luciferase reporter vector. JM109 cells were transformed and selected on LB/ampicillin plates, followed by plasmid purification of the positive colonies.

Transfection

Stable and transient transfection of mammalian cells was performed using Lipofectamine 2000 Reagent (Invitrogen life technologies) according to the manufacturer's protocol. For the luciferase assay, 0.6 μg of the pRL-TK vector (Promega) was co-transfected with 0.6 μg of the appropriate CIITA promoter construct, and the cells were harvested after 24 hours. For the IFN-γ-treated cells, four hours after transfection 500 U/ml IFN-γ was added and incubated for 24 hours. For stable transfections, the cells were transfected with 1.2 μg p31-BABE for 36 hours. After the transfection, the cells were transferred to RPMI medium containing 2 μg/ml puromycin for negative selection.

Luciferase assay

For the luciferase assay, the Promega Dual Luciferase System was used according to the manufacturer's protocol. Samples were lysed with passive lysis buffer (PLB) and read on a Wallac Arvo SX Multilabel Counter (Amersham). The results were normalized by dividing the firefly luciferase counts per second (CPS), by the sea pansy luciferase CPS, and graphed in Excel.

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Flow Cytometry and Microscopy

Flow Cytometry

Flow cytometric analysis was performed on a FACScan (BD Pharmingen). $1x10^6$ cells were collected and incubated with saturating concentrations of monoclonal antibodies (mAb) listed in Table 7. Control cells and cells according to the experimental protocol were stained with the mAb for 30 minutes in the dark at room temperature. The cells were then washed with PBS before being resuspended in 1% para-formaldehyde. Ten thousand cells were counted for each sample and analyzed using Cell Quest software (BD Pharmingen). For the detection of apoptosis, an Apo-direct assay (BD Pharmingen) was performed according to the manufacturer's specifications.

Light Microscopy

For light microscopy, cells were observed in suspension with an Olympus IX70 microscope and photographed with an Olympus PM-C35DX 35 mm camera (Olympus, Tokyo, Japan). The photographs were taken with a blue filter using Fuji Sensia II color reversal film, ISO 100.

Electron Microscopy

For electron microscopy, K-562 cell suspensions from various treatments were fixed with an equal volume of Carson's fixative for 30 minutes, centrifuged into a pellet, and then suspended in fresh Carson's fixative for 2-3 hours. The cells were then washed

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Table 7. Summary of the antibodies used in this study.

Antibody	Conjugate	Characteristics
Anti-human IgG γ1/γ2a	PE/FITC	Control for nonspecific binding
Anti-human IgM	FITC	B cell marker
Anti-human HLA-DR	PE	MHC class II antigen
Anti-human CD15s	FITC	Adhesion molecule of the sialomucin family

PE, phycoerythrin; FITC, fluorine isothiocyanate.

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in sterile water and post-fixed with 2% aqueous osmium tetraoxide for 1 hour. The samples were then washed again with sterile water and dehydrated through a graded series of acetone and a final wash with 100% propylene oxide. The cells were pelleted and embedded in Spurr's plastic. Sixty to ninety nanometer sections were cut from the blocks with a diamond knife on a Richert ultramicrotome. The sections were post-stained with uranyl acetate and lead citrate. The cells were examined and photographed in a Hitachi 800 intermediate voltage transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

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CHAPTER 3

Results

Identification of an alternatively spliced CIITA gene in K-562 cells

Understanding the mechanisms of the transcription of immune regulatory genes is essential to understanding the underlying causes of immunodeficiency. To that end, a defect, possibly contributing to the negative MHC class II expression of K-562 human leukemia cells, was identified. Previous studies have demonstrated the ability of mutations of the C terminal portion of the CIITA protein to abolish MHC class II expression (Chin et al., 1997; Hake et al., 2000; Linhoff et al., 2001; Quan et al., 1999). The data presented here shows that K-562 cells lack 198 amino acids of the carboxy terminal region.

K-562 cells do not exhibit surface expression or mRNA expression of HLA-DR

To verify that HLA-DR is not expressed on the cell surface, flow cytometry was performed on K-562 cells. Untreated K-562 cells were stained with either the γ1/γ2a antibody as a control, or with an HLA-DR monoclonal antibody (Chapter 2, Table 7). The MHC class II-positive Raji cell line was used as a positive control. The results were consistent with previous studies, which have reported that there is no surface expression

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of HLA-DR on K-562 cells, and that there is a high level of expression for Raji cells (Figure 18 A and B).

Additionally, to demonstrate that HLA-DR mRNA is not expressed in K-562 cells, RT-PCR was performed using primers specific for HLA-DR (Chapter 2, Table 3, HLA-DR Forward and Reverse). As a positive control, the MHC class II-positive Raji cell line was used, as were primers for the constitutively expressed β-actin gene (Chapter 2, Table 3, β-actin Forward and Reverse). The results show that HLA-DR mRNA is not expressed in K-562 cells, and that the β-actin gene was amplified, indicating that the cDNA was viable (Figure 18 C). Furthermore, both β-actin and HLA-DR were amplified in Raji cells.

Northern blot analysis reveals the presence of CIITA mRNA in K-562 cells

In order to identify the CIITA gene, RT-PCR was performed on RNA from Raji and K-562 cells using primers specific for CIITA. Initial attempts at amplifying the CIITA gene in K-562 cells under normal conditions failed. The results were not entirely conclusive, however, because bands of the expected size were not seen when the PCR products were visualized by ethidium bromide staining following agarose gel electrophoresis (Figure 19 A). In order to determine whether or not the CIITA mRNA was present in K-562 cells, Northern blotting was performed (Figure 19 B). The results of the Northern blot were positive, suggesting that the problem with the RT-PCR could be the reverse transcription. To further investigate the K-562 CIITA transcript, RT-PCR

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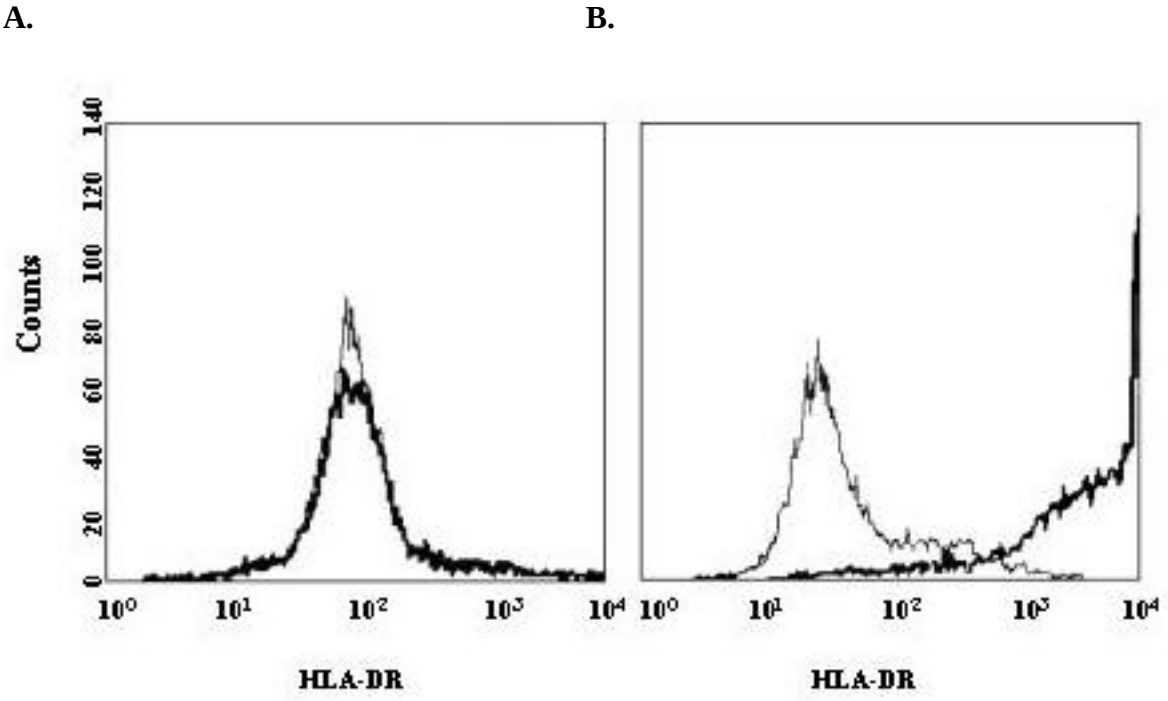

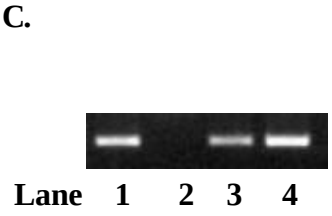

Figure 18. HLA-DR expression in Raji and K-562 cells. **A.** Flow cytometry analysis of the surface expression of HLA-DR in K-562 cells. **B.** Flow cytometry analysis of the surface expression of HLA-DR in Raji cells. **C.** RT-PCR of HLA-DR; lane 1, Raji cells and lane 2, K-562 cells, and β-actin; lane 3, Raji cells and lane 4, K-562 cells.

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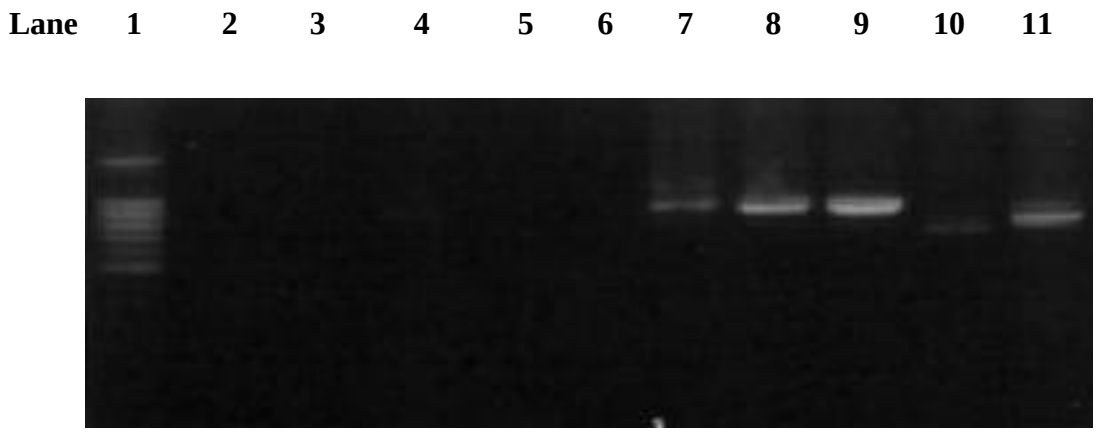
A.



B.

Figure 19. CIITA expression in Raji and K-562 cells. **A.** RT-PCR of CIITA in K-562 cells (lanes 2-6) and Raji cells (lanes 7-11). Lane 1 is the marker. **B.** Northern blot analysis of CIITA mRNA expression in Raji and K-562 cells.

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was performed under varying conditions. In order to amplify the desired product, it was necessary to perform the reverse transcription at an unusually high temperature (60°C) using a relatively high concentration of mRNA (250ng/µl).

K-562 CIITA contains an insert of genomic sequence that is alternatively spliced in Raji cells

Once the conditions for RT-PCR were optimized, the CIITA transcript was amplified from K-562 cells. For the primer combination designated CF2/DR1 (Chapter 2, Table 2 and Figure 15), the expected product size of the amplified region was 976 bp. While RT-PCR of Raji cells resulted in a product of this size, K-562 cDNA did not produce the expected results (Figure 20). For this particular primer combination, the K-562 fragment appeared to be approximately 870 bp larger (1.8 Kb) than the Raji fragment.

To demonstrate that the genomic sequence of K-562 CIITA was different than the wild type sequence, PCR was performed using K-562 and Raji genomic DNA with the same primer combination (CF2/DR1) as in Figure 20. Based on their migration in a 1% agarose gel, the genomic products of both cell lines appeared to be approximately the same size as the K-562 RT-PCR product (Figure 21). This data suggested that the additional bases in the K-562 CIITA cDNA originated from the CIITA genomic sequence, as a result of alternative splicing, and that the insert is a complete intron.

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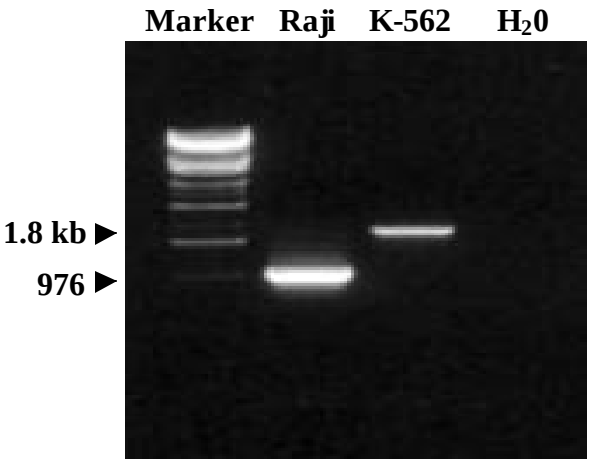

Figure 20. RT-PCR amplification of CIITA (primers CF2 and DR1) in Raji and K-562 cells.

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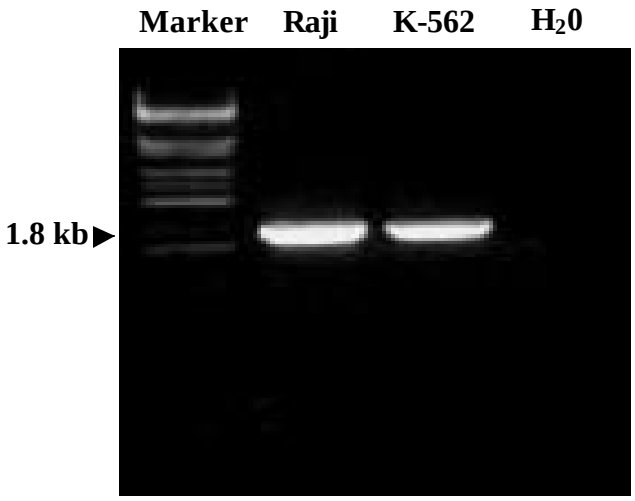

Figure 21. Genomic PCR amplification of CIITA (primers CF2 and DR1) in Raji and K-562 cells.

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To verify that the additional bases of the K-562 CIITA cDNA were in fact part of the genomic sequence, automated sequencing was performed on the cloned products of the RT-PCR reaction. A basic local alignment search tool (BLAST) (Altschul, 1990) search of the cloned PCR products revealed that, while part of the gene corresponded to the wild type CIITA, part of the gene corresponded to MHC CIITA clone 10 (Riley et al., 1995). However, there was an additional 140 bp of sequence that did not correspond to any identifiable sequences in the database. MHC CIITA clone 10 was identified by Riley *et al.* (1995) as an alternatively spliced form of CIITA, which is present in at low levels Raji cells. The clone has an insertion of CIITA genomic sequence at nucleotide 2761 (nt 2773 of the wild-type cDNA). Although Riley *et al.* found four different alternatively spliced isoforms of CIITA in these MHC class II positive cells, only one splice site combination was able to restore class II expression to mutant cells.

Alternative splicing of the K-562 CIITA is not caused by a mutation at the splice site

Although there were 140 bases of sequence that were not present in the wild type or MHC CIITA clone 10 sequences, it was now known that a large portion of the additional 870 bases originated from the genomic sequence of CIITA. In order to determine the origin of the additional 140 bases of K-562 CIITA, sequencing was performed on genomic DNA from Raji and K-562 cells. Comparison of the unknown sequence to the sequence of the Raji and K-562 cells revealed that the additional bases were in fact part of the CIITA genomic sequence (Figures 22 and 23). Additionally, the genomic sequences were used to determine if there was a mutation at the splice site of the

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Figure 22. K-562 CIITA cDNA sequence; GenBank accession number AF410154.

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   1 tgatgaggct gtgtgcttct gagctgggca tccgaaggca tccttgggga
  51 agctgagggc acgaggaggg gctgccagac tccgggagct gctgcctggc
 101 tgggattcct acacaatgcg ttgcctggct ccacgccctg ctgggtccta
 151 cctgtcagag ccccaaggca gctcacagtg tgccaccatg gagttggggc
 201 ccctagaagg tggctacctg gagcttctta acagcgatgc tgacccccctg
 251 tgcctctacc acttctatga ccagatggac ctggctggag aagaagagat
 301 tgagctctac tcagaacccg acacagacac catcaactgc gaccagttca
 351 gcaggctgtt gtgtgacatg gaaggtgatg agagaccagg gaggcttatg
 401 ccaatatcgc ggaactggac cagtatgtct tccaggactc ccagctggag
 451 gcctgagcaa ggacatttтc aagcacatag gaccagatga agtgatcggt
 501 gagagtatgg agatgccagc agaagttggg cagaaaagtc agaaaagacc
 551 cttcccagag gagcttccgg cagacctgaa gcactggaag ccagctgagc
 601 cccccactgt ggtgactggc agtctcctag tgggaccagt gagcgactgc
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 701 ctccggccag atgcgcctgg agaaaaccga ccagattccc atgcctttct
 751 ccagttcctc gttgagctgc ctgaatctcc ctgagggacc catccagttt
 801 gtccccacca tctccactct gccccatggg ctctggcaaa tctctgaggc
 851 tggaacaggg gtctccagta tattcatcta ccatggtgag gtgccccagg
 901 ccagccaagt acccccтccc agtggattca ctgtccacgg cctcccaaca
 951 tctccagacc ggccaggctc caccagcccc ttcgctccat cagccactga
1001 cctgcccagc atgcctgaac ctgccctgac ctcccgagca aacatgacag
1051 agcacaagac gtcccccacc caatgcccgg cagctggaga ggtctccaac
1101 aagcttccaa aatggcctga gccggtggag cagttctacc gctcactgag
1151 gacacgtatg gtgccgagcc cgcaggcccg gatggcatcc tagtggaggt
1201 ggatctggtg caggccaggc tggagaggag cagcagcaag agcctggagc
1251 gggaactggc caccccggac tgggcagaac ggcagctggc ccaaggaggc
1301 ctggctgagg tgctgttggc tgccaaggag caccggcggc cgcgtgagac
1351 acgagtgatt gctgtgctgg gcaaagctgg tcagggcaag agctattggg
1401 ctggggcagt gagccgggcc tgggcttgtg gccggcttcc ccagtacgac
1451 tttgtcttct ctgtcccctg ccattgcttg aaccgtccgg gggatgccta
1501 tggcctgcag gatctgctct tctccctggg cccacagcca ctcgtggcgg
1551 ccgatgaggt tttcagccac atcttgaaga gacctgaccg cgttctgctc
1601 atcctagacg ccttcgagga gctggaagcg caagatggct tcctgcacag
1651 cacgtgcgga ccggcacggc ggagccctgc tccctccggg ggctgctggc
1701 cggccttttc cagaagaagc tgctccgagg ttgcaccctc ctcctcacag
1751 cccggccccg gggccgcctg gtccagagcc tgagcaaggc cgacgcccta
1801 tttgagctgt ccggcttctc catggagcag gcccaggcat acgtgatgcg
1851 ctactttgag agctcaggga tgacagagca ccaagacaga gccctgacgc
1901 tcctccggga ccggccactt cttctcagtc acagccacag ccctactttg
1951 tgccgggcag tgtgccagct ctcagaggcc ctgctggagc ttggggagga
2001 cgccaagctg ccctccacgc tcacgggact ctatgtcggc ctgctgggcc
2051 gtgcagccct cgacagcccc cccggggccc tggcagagct ggccaagctg
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2101 cccatccgca gacgtgagga cctgggcgat ggccaaaggc ttagtccaac
2151 acccaccgcg ggccgcagag tccgagctgg ccttccccag cttcctcctg
2201 caatgcttcc tgggggccct gtggctggct ctgagtggcg aaatcaagga
2251 caaggagctc ccgcagtacc tagcattgac cccaaggaag aagaggccct
2301 atgacaactg gctggagggc gtgccacgct ttctggctgg gctgatcttc
2351 cagcctcccg cccgctgcct gggagcccta ctcgggccat cggcggctgc
2401 ctcggtggac aggaagcaga aggtgcttgc gaggtacctg aagcggctgc
2451 agccggggac actgcgggcg cggcagctgc ttgagctgct gcactgcgcc
2501 cacgaggccg aggaggctgg aatttggcag cacgtggtac aggagctccc
2551 cggccgcctc tcttttctgg gcacccgcct cacgcctcct gatgcacatg
2601 tactgggcaa ggccttggag gcggcgggcc aagacttctc cctggacctc
2651 cgcagcactg gcatttgccc ctctggattg gggagcctcg tgggactcag
2701 ctgtgtcacc cgtttcaggt ggggtgaggg gcttggaaga gacatccttg
2751 tgttgggcat taactgcggt cttggtgcca agcccagtgc tctgtggggt
2801 cctttttagta tgcagagcag ccgggtgggg cagaatggat tctctccatt
2851 tttaagatga ggatgttgag gctcagagag gggcagccac ttgccacaca
2901 gcaagtgaga ggcaatggca ttctcccagt caatatttga aggcccgcca
2951 tgtgccagtc actggggtat gtctagaatc tgagactgac ctgggctcaa
3001 atttgtttta ttctttccac cccctgagca cgccaccgtt ttcttatgct
3051 aagagtaaag ccatggcctc cccttggact ctctgcctcc attctctcct
3101 cttccactcc attttgtatt cagcaaccag accaatcttc tcagaacttg
3151 aatctgattg tatcccatcc ctgcttacaa tccttcaggg acactccacc
3201 actgtcagga tgaaggctaa atttcttaat ttggtttcat taagtcggtc
3251 tgcaatctgc ttgagcattt cagcttaatc gccagaggat tgcttccata
3301 tttcccccta aacatacttt acccaagctg taaggtccta cataattgtg
3351 ccaataattt agcagtgagc ttcctggtag ccgaagcaaa aagggaaaga
3401 aaaccactgt gtgagttgtg agaaagtagg aatcaataaa ggctggagtg
3451 gtcatggaag gctttctggg aaaggtagag gttgagctaa ggaaagaaag
3501 tattttaata ggtaggagga cccttcatgg agctgccctt ccattaaggt
3551 ctagcctggt caccgtgcct gggtctgagg ccctccctcc acagggctgc
3601 cttgagcgac acggtggcgc tgtgggagtc cctgcggcag catggggaga
3651 ccaagctact tcaggcagca gaggagaagt tcaccatcga gcctttcaaa
3701 gccaagtccc tgaaggatgt ggaagacctg ggaaagcttg tgcagactca
3751 gaggacgaga agttcctcgg aagacacagc tggggagctc cctgctgttc
3801 gggacctaaa gaaactggag tttgcgctgg gccctgtctc aggcccccag
3851 gctttcccca aactggtgcg gatcctcacg gccttttcct ccctgcagca
3901 tctggacctg gatgcgctga gtgagaacaa gatcggggac gagggtgtct
3951 cgcagctctc agccaccttc ccccagctga agtccttgga aaccctcaat
4001 ctgtcccaga acaacatcac tgacctgggt gcctacaaac tcgccgaggc
4051 cctgccttcg ctcgctgcat ccctgctcag gctaagcttg tacaataact
4101 gcatctgcga cgtgggagcc gagagcttgg ctcgtgtgct tccggacatg
4151 gtgtccctcc gggtgatgga cgtccagtac aacaagttca cggctgccgg
4201 ggcccagcag ctcgctgcca gccttcggag gtgtcctcat gtggagacgc

Figure 22, continued

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```
4251 tggcgatgtg gacgcccacc atcccattca gtgtccagga acacctgcaa
4301 caacaggatt cacggatcag cctgagatga tcccagctgt gctctggaca
4351 ggcatgttct ctgaggacac taaccacgct ggaccttgaa ctgggtactt
4401 gtggacacag ctcttctcca ggctgtatcc catgaggcct cagcatcctg
4451 gcacccggcc cctgctggtt cagggttggc ccctgcccgg ctgcggaatg
4501 aaccacatct tgctctgctg acagacacag gcccggctcc aggctccttt
4551 agcgcccagt tgggtggatg cctggtggca gctgcggtcc acccaggagc
4601 cccgaggcct tctctgaagg acattgcgga caccacggcc aggccagagg
4651 gagtgacaga ggcagcccca ttctgcctgc ccaggcccct gccaccctgg
4701 gaagaaagta cttctttttt tttatttta gacagagtct cactgttgcc
4751 caggctggcg tgcagtggtg cgatctgggt tcactgcaac ctccgcctct
4801 tgggttcaag cgattcttct gcttcagcct cccgagtagc tgggactaca
4851 ggcacccacc atcatgtctg gctaattttt catttttagt agagacaggg
4901 ttttgccatg ttggccaggc tggtctcaaa ctcttgacct caggtgatcc
4951 acccacctca gcctcccaaa gtgctgggga ttacaagcgt gagccactgc
5001 accgggccac aagaaagtac ttctccaccc tgctctccga ccagacacct
5051 tgacagggca caccgggcac tcagaagaca ctgatgggca acccccagcc
5101 tgctaattcc ccagattgca acaggctggg cttcagtggc aggctgcttt
5151 tgtctatggg actcaatgca ctgacattgt tggccaaagc caaagctagg
5201 cctggccaga tgcaccaggc ccttagcagg aaacagctaa tgggacacta
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5301 tctttt
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Figure 22, continued

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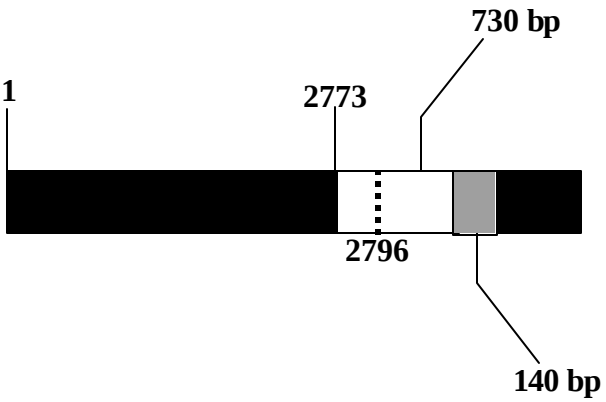

Figure 23. Schematic diagram of the K-562 CIITA cDNA. The closed box represents the wild-type sequence, the open box represents the additional sequence of MHC CIITA clone 10, the hatched box represents the additional CIITA genomic sequence, and the dashed line indicates the location of the stop codon.

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K-562 CIITA. Sequence analysis of the PCR products revealed that there was no difference in the sequence immediately flanking the splice site [5'-ACTCA GCTGTGTCACCCGTTCAGG/GCTGCCTTGAGCGACACGGTGGCGC-3'] (Figure 24). This finding is further evidence that the genomic insertion is the result of alternative splicing and not a mutation of the splice site.

Alternative splicing of the K-562 CIITA introduces a stop codon, resulting in an inactive protein

The genomic insert of MHC CIITA done 10 contains a stop codon, which results in a protein that is unable to revert MHC class II-negative cells. To ascertain whether this was the case with K-562 CIITA, the predicted amino acid sequence was determined. The results indicated that a stop codon was introduced in all reading frames, resulting in a truncated protein. The result was a protein of 932 amino acids rather than 1130 (Figure 25). Although the function of the carboxy-terminal region of CIITA has not been fully elucidated, previous reports have shown that it is essential for the transactivating ability of CIITA. When the integrity of one or more crucial elements within this region is compromised, ultimately the result is immunodeficient cells, further supporting the hypothesis that the alternatively spliced transcript of CIITA encodes an inactive protein that results in the null expression of MHC class II molecules by K-562 cells.

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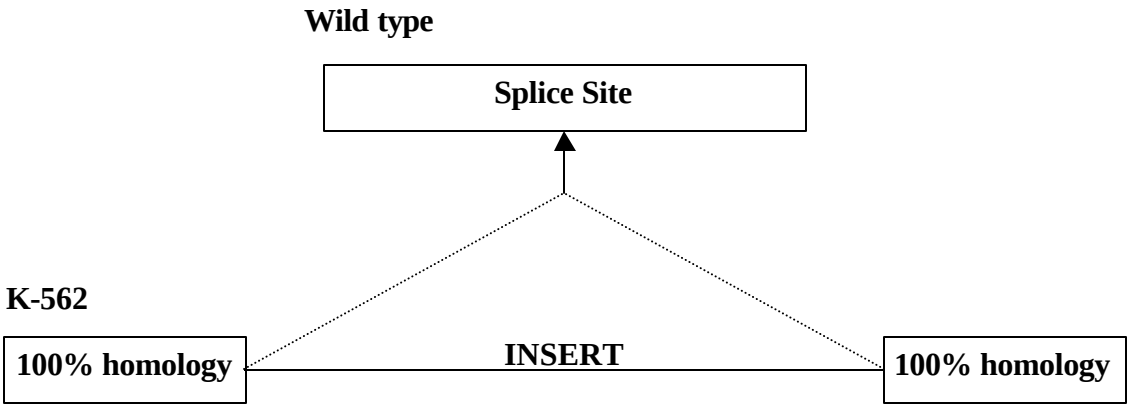

Figure 24. A schematic diagram of the homology of the sequence flanking the insertion site in Raji and K-562 cells.

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Figure 25. Comparison of the putative amino acid sequence in K-562 and Raji cells. The K-562 sequence is on the top line and the Raji sequence is on the bottom line.

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  1 MRCLAPRPAGSYLSEPQGSSQCATMELGPLEGGYLELLNSDADPLCLYHFYDQMDLAGEE   60
  1 MRCLAPRPAGSYLSEPQGSSQCATMELGPLEGGYLELLNSDADPLCLYHFYDQMDLAGEE   60

 61 EIELYSEPDTDTINCDQFSRLLCDMEGDEETREAYANIAELDQYVFQDSQLEGLSKDIFK  120
 61 EIELYSEPDTDTINCDQFSRLLCDMEGDEETREAYANIAELDQYVFQDSQLEGLSKDIFK  120

121 HIGPDEVIGESMEMPAEVGQKSQKRPFPEELPADLKHWKPAEPPTVVTGSLLVGPVSDCS 180
121 HIGPDEVIGESMEMPAEVGQKSQKRPFPEELPADLKHWKPAEPPTVVTGSLLVGPVSDCS 180

181 TLPCLPLPALFNQEPASGQMRLEKTDQIPMPFSSSSLSCLNLPEGPIQFVPTISTLPHGL 240
181 TLPCLPLPALFNQEPASGQMRLEKTDQIPMPFSSSSLSCLNLPEGPIQFVPTISTLPHGL 240

241 WQISEAGTGVSSIFIYHGEVPQASQVPPPSGFTVHGLPTSPDRPGSTSPFAPSATDLPSM 300
241 WQISEAGTGVSSIFIYHGEVPQASQVPPPSGFTVHGLPTSPDRPGSTSPFAPSATDLPSM 300

301 PEPALTSRANMTEHKTSPTQCPAAGEVSNKLPKWPEPVEQFYRSLQDTYGAEPAGPDGIL 360
301 PEPALTSRANMTEHKTSPTQCPAAGEVSNKLPKWPEPVEQFYRSLQDTYGAEPAGPDGIL 360

361 VEVDLVQARLERSSSKSLERELATPDWAERQLAQGGLAEVLLAAKEHRRPRETRVIAVLG 420
361 VEVDLVQARLERSSSKSLERELATPDWAERQLAQGGLAEVLLAAKEHRRPRETRVIAVLG 420

421 KAGQGKSYWAGAVSRAWACGRLPQYDFVFSVPCHCLNRPGDAYGLQDLLFSLGPQPLVAA 480
421 KAGQGKSYWAGAVSRAWACGRLPQYDFVFSVPCHCLNRPGDAYGLQDLLFSLGPQPLVAA 480

481 DEVFSHILKRPDRVLLILDAFEELEAQDGFLHSTCGPAPAEPCSLRGLLAGLFQKKLLRG 540
481 DEVFSHILKRPDRVLLILDAFEELEAQDGFLHSTCGPAPAEPCSLRGLLAGLFQKKLLRG 540

541 CTLLLTARPRGRLVQSLSKADALFELSGFSMEQAQAYVMRYFESSGMTEHQDRALTLLRD 600
541 CTLLLTARPRGRLVQSLSKADALFELSGFSMEQAQAYVMRYFESSGMTEHQDRALTLLRD 600

601 RPLLLSHSHSPTLCRAVCQLSEALLELGEDAKLPSTLTGLYVGLLGRAALDSPPGALAEL 660
601 RPLLLSHSHSPTLCRAVCQLSEALLELGEDAKLPSTLTGLYVGLLGRAALDSPPGALAEL 660

661 AKLAWELGRRHQSTLQEDQFPSADVRTWAMAKGLVQHPPRAAESELAFPSFLLQCFLGAL 720
661 AKLAWELGRRHQSTLQEDQFPSADVRTWAMAKGLVQHPPRAAESELAFPSFLLQCFLGAL 720

721 WLALSGEIKDKELPQYLALTPRKKRPYDNWLEGVPRFLAGLIFQPPARCLGALLGPSAAA 780
721 WLALSGEIKDKELPQYLALTPRKKRPYDNWLEGVPRFLAGLIFQPPARCLGALLGPSAAA 780

781 SVDRKQKVLARYLKRLQPGTLRARQLLELLHCAHEAEEAGIWQHVVQELPGRLSFLGTRL 840
781 SVDRKQKVLARYLKRLQPGTLRARQLLELLHCAHEAEEAGIWQHVVQELPGRLSFLGTRL 840

841 TPPDAHVLGKALEAAGQDFSLDLRSTGICPSGLGSLVGLSCVTRFRWGEGLGRDILVLGI 900
841 TPPDAHVLGKALEAAGQDFSLDLRSTGICPSGLGSLVGLSCVTRFRAALSDTVALWESLR 900

901 NCGLGAKPSALWGPFSMQSSRVGQNGFSPFLR---------------------------- 932
901 QHGETKLLQAAEEKFTIEPFKAKSLKDVEDLGKLVQTQRTRSSSEDTAGELPAVRDLKKL 960
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 933 ------------------------------------------------------------  933
 961 EFALGPVSGPQAFPKLVRILTAFSSLQHLDLDALSENKIGDEGVSQLSATFPQLKSLETL 1020

 933 ------------------------------------------------------------  933
1021 NLSQNNITDLGAYKLAEALPSLAASLLRLSLYNNCICDVGAESLARVLPDMVSLRVMDVQ 1080

 933 ------------------------------------------------           933
1081 YNKFTAAGAQQLAASLRRCPHVETLAMWTPTIPFSVQEHLQQQDSRISLR          1130
```

Figure 25, continued

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The alternative CIITA transcript is present in both Raji and K-562 cells

Since previous reports have shown that MHC class II-positive Raji cells express multiple isoforms of CIITA, the possibility arose that there might be more than one isoform present in K-562 cells. Real-Time PCR was used to determine the relative levels of the alternative transcript in Raji and K-562 cells, and to assess the possibility of the presence of more than one CIITA isoform in K-562 cells (Chapter 2, Table 5 and Figure 16). The results showed that the alternative transcript, but not the wild type, was present in K-562 cells (Figure 26 A). The alternative transcript was present in both Raji and K-562 cells, yet at a higher level in the latter (Figure 26 B). This is consistent with the observation that MHC CIITA clone 10 is present at low levels in Raji cells (Riley et al., 1995). Presumably, the alternative transcript is not easily detectable in Raji cells by RT-PCR due to the competition created by the more abundant wild type transcript. The lack of the wild type transcript in K-562 cells provides further evidence for CIITA as the cause of the null expression of MHC class II molecules.

The MHC class II promoters are present in the K-562 genome

In an effort to verify the presence of the MHC class II promoters in the K-562 genome, PCR was performed using genomic DNA from Raji and K-562 cells as the template, with primers specific for the MHC class II promoters, HLA-DR, -DP, -DQ and Ii (Chapter 2, Table 4). The results showed that all four promoter sequences were present in the K-562 genome (Figure 27), and sequence analysis revealed that there were no significant differences as compared to the Raji promoters (data not shown). This data

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Figure 26. Real-time PCR of the two CIITA transcripts. **A.** Quantitation by real-time PCR of the wild-type CIITA transcript in Raji and K-562 cells. The wild-type transcript is only present in Raji cells. **B.** Quantitation by real-time PCR of the alternative CIITA transcript in Raji and K-562 cells. The alternative transcript is present in both cell lines, but at a higher level in K-562 cells.

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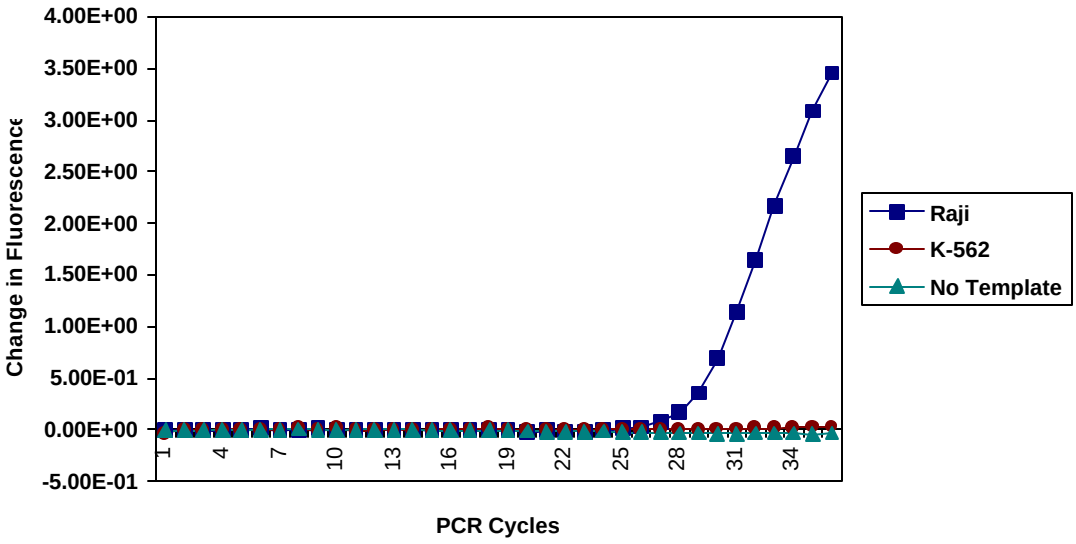
A.



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B.

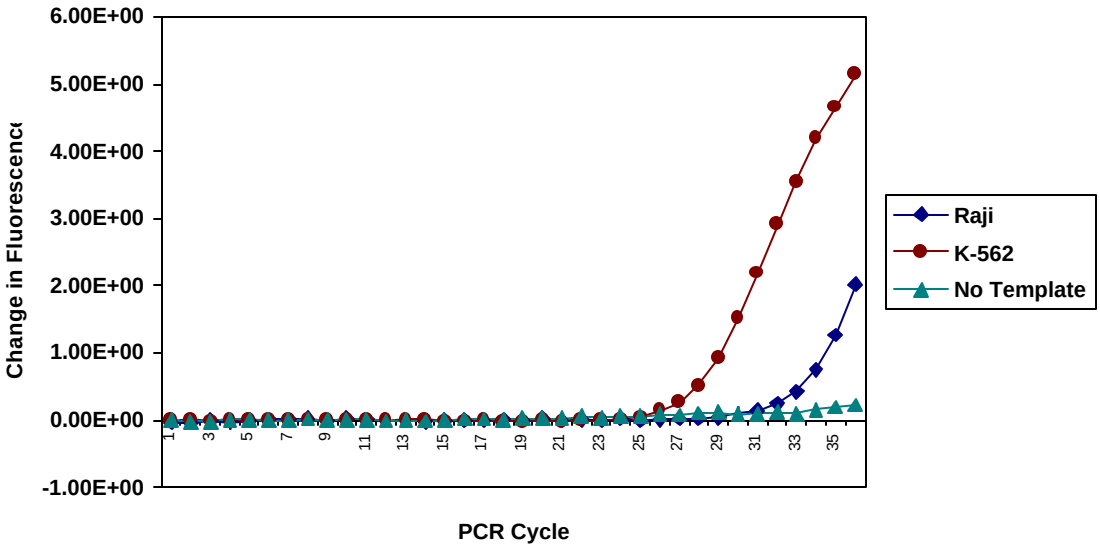

Figure 26, continued

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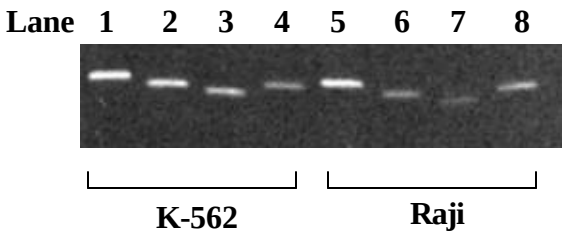

Figure 27. Expression of the MHC class II promoters in Raji and K-562 cells. PCR was performed on genomic DNA from Raji and K-562 cells using primers for the MHC class II promoters, HLA-DR (lanes 1 and 5), HLA-DP (lanes 2 and 6), HLA-DM (lanes 3 and 7) and Ii (lanes 4 and 8).

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supported the hypothesis that the lack of MHC class II expression in K-562 cells is caused by a defect in one or more regulatory factors, and not in the class II gene itself.

The alternatively spliced CIITA protein associates with both RFX-B and the HLA-DR promoter in vivo

To determine if the truncation of the alternatively spliced CIITA protein interferes with the ability of CIITA to associate with the MHC class II promoter or the RFX complex, chromatin immunoprecipitation assays were performed in viable K-562 cells. Cross-linked chromatin-protein complexes were immunoprecipitated with the CIITA antibody, and the associated DNA was subsequently analyzed by PCR with primers specific for the HLA-DR promoter (Chapter 2, Table 4). Because CIITA does not bind the class II promoter directly but associates with the RFX complex, which is bound to the class II promoter, precipitation of the class II promoter with the CIITA antibody would mean that the CIITA protein was also associating with the RFX complex. As shown in Figure 28, lane 1, gel electrophoresis revealed that the HLA-DR promoter was amplified by PCR, which indicated that the alternatively spliced CIITA did associate with both the MHC class II promoter and the RFX complex. This data showed that the missing portion at the carboxy terminus of CIITA is not required for association of CIITA with the class II promoter complex, although the region is essential to the transactivating ability of CIITA. Moreover, the primer pair specific for CIITA, F (Chapter 2, Table 2, CIITA F Forward and Reverse), amplified a fragment of the CIITA DNA (Figure 28, lane 3). Following cloning of the PCR product, sequencing analysis revealed that the CIITA PCR

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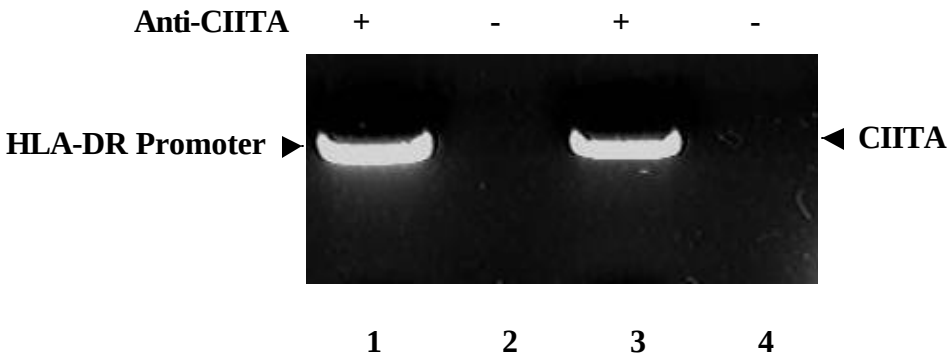

Figure 28. PCR of K-562 chromatin immunoprecipitation samples. The samples were precipitated with or without CIITA antibody and the DNA was amplified with primers for the HLA-DR promoter (lanes 1 and 2) and a small fragment of CIITA (lanes 3 and 4).

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product was 100% homologous to the wild-type CIITA (Figure 29). This data suggested that there was a self-association of CIITA, and that the region amplified was involved in this association, which was verified by later reports (Linhoff et al., 2001; Sisk et al., 2001).

CIITA promoter activity in K-562 cells

In order to assess the role of CIITA in MHC class II negative K-562 cells, it is essential to characterize the gene. An essential element to this characterization is the identification of the promoter involved in the transcription of the K-562 CIITA gene. The pluripotential lineage of the cell line and the tissue-specific activation of the CIITA promoters make this a particularly important factor in understanding the immune expression of these human leukemia cells. The data presented here shows that there is a previously undocumented switch of the active CIITA promoter in K-562 cells.

The four CIITA promoters are present in the K-562 genomic sequence

To verify the presence of the CIITA promoters in the genomic sequence of K-562 cells, PCR was performed using genomic DNA from K-562 cells and primers specific for each of the CIITA promoters I, II, III and IV (Chapter 2, Table 4). As a positive control, genomic DNA from MHC class II-positive Raji cells was also used (Figure 30). Agarose gel electrophoresis revealed that all four promoters were present in K-562 genome.

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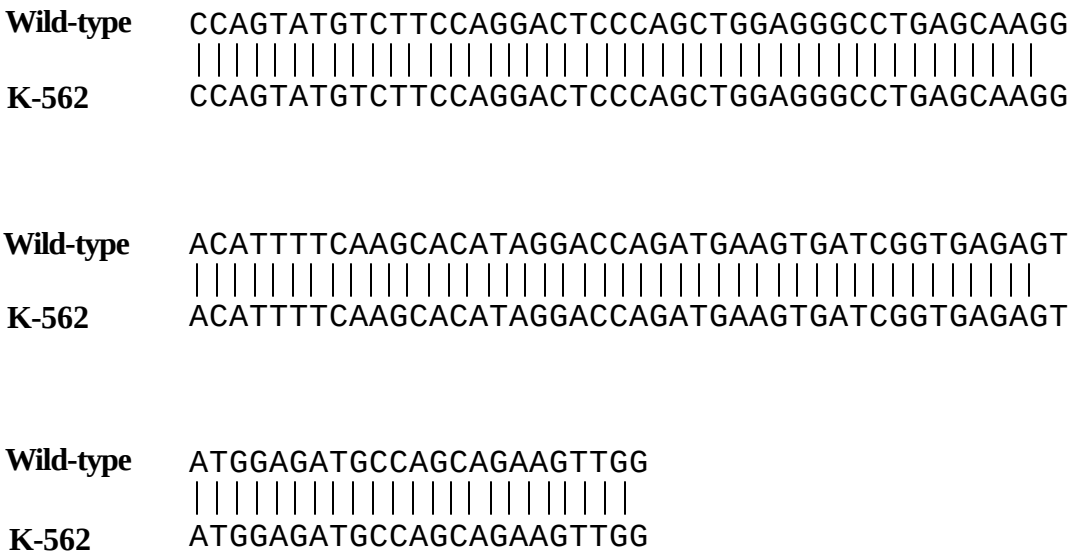

Figure 29. Alignment of the wild-type CIITA K-562 CIITA fragment isolated by chromatin immunoprecipitation.

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Figure 30. PCR amplification of the CIITA promoters from Raji and K-562 genomic DNA. Raji cells are shown in lane 1 and K-562 cells are shown in lane 2.

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Furthermore, the migration of the K-562 samples appeared to be the same as that of the Raji cell samples.

CIITA Promoter III is the active promoter in K-562 cells

To ascertain which of the CIITA promoters is active in K-562 cells, a luciferase assay was performed using DNA constructs made from the different K-562 CIITA promoters (Chapter 2, Figure 17). The results showed that the relative luciferase activity of the B cell-specific promoter, PIII, was considerably higher than the dendritic cell-specific promoter, PI (Figure 31). Although the activity of PIII was as much as 85% higher than PI, it was only 40% higher than the IFN-γ-inducible promoter, PIV. In previous reports, only one of the 4 possible promoters has been shown to be active in a particular cell type. This data suggested that K-562 cells were an exception to this common observation.

IFN-g treatment of K-562 cells leads to a change in the active CIITA promoter

The observation that the IFN-γ-inducible CIITA promoter, PIV, produced 60% as much luciferase activity as the more active PIII, lead to the question of what effect IFN-γ treatment would have on the cells. Again, the K-562 cells were transfected with the luciferase reporter constructs made from the different CIITA promoters of K-562 cells, but this time 500 U/ml IFN-γ was added four hours after transfection. Surprisingly, the results revealed a change in the most active promoter from PIII to PIV (Figure 32 A).

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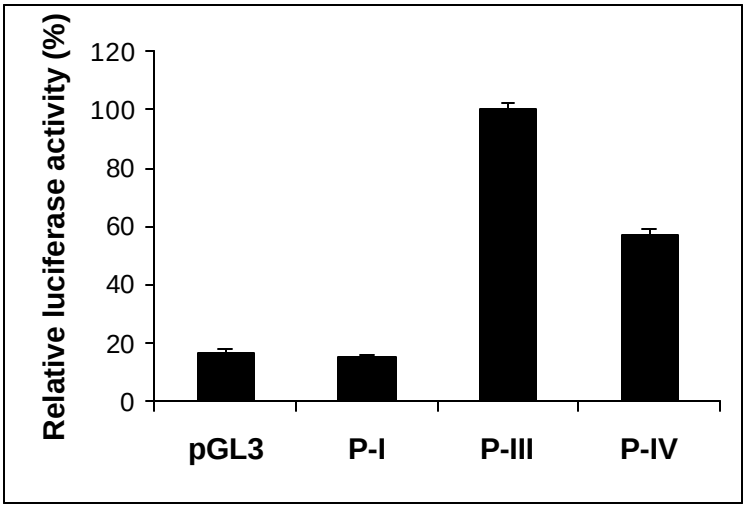

Figure 31. Relative luciferase activity of K-562 cells transfected with K-562 CIITA promoter constructs PI, PIII and PIV or the pGL3 reporter vector alone.

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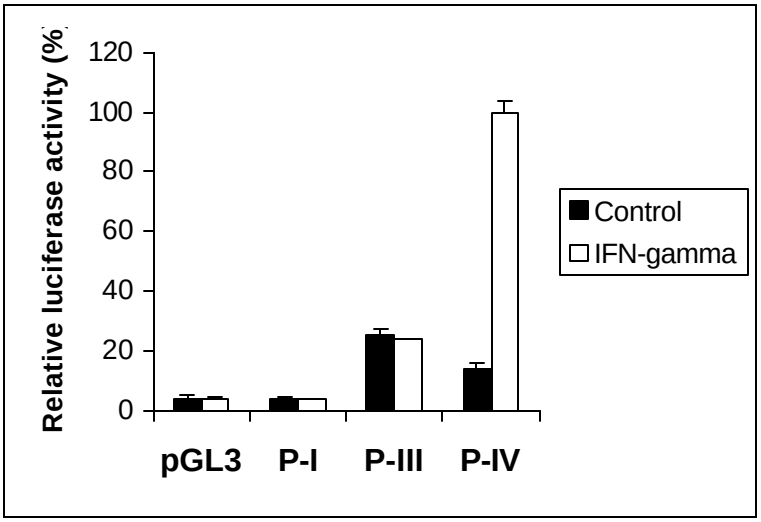
A.



B.

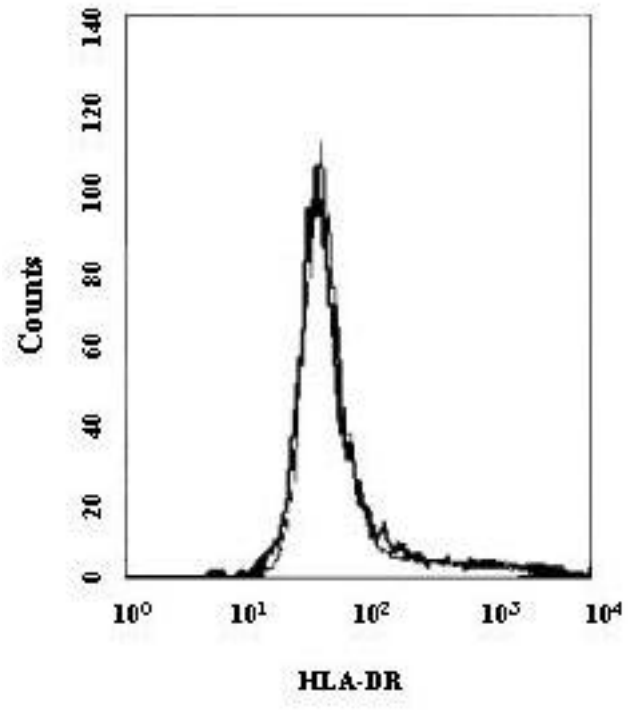


Figure 32. Promoter activity of the K-562 CIITA promoters after treatment with IFN-γ. **A.** Relative luciferase activity of untreated and IFN-γ-treated K-562 cells transfected with K-562 CIITA promoter constructs PI, PIII and PIV or the pGL3 reporter vector alone. **B.** Flow cytometry analysis of the HLA-DR expression of PIV transfected K-562 cells (thick histogram), as compared to control cells (thin histogram).

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After the IFN-γ treatment, PIV was 95% and 72% more active than PI and PIII, respectively. Currently, there are no known reports in which the active CIITA promoter changes upon treatment with IFN-γ.

Because it has been documented that IFN-γ treatment does not induce MHC class II expression in K-562 cells, flow cytometry was performed on the transfected cells to verify that this is still the case (Figure 32 B). The results indicated that although the IFN-γ-inducible CIITA promoter was up-regulated in the treated cells, there was no surface expression of the MHC class II molecule, HLA-DR. This result is consistent with previous reports (Ichiki, 1987; Sutherland, 1985).

The K-562 PIV sequence is identical to the Raji cell sequence

To determine if the change in the active promoter was the result of a novel element present in the K-562 CIITA promoter sequence, the genomic sequence was determined for both the Raji and K-562 cell PIV (Figure 33). The sequence was identical for the two cell lines, including an intact GAS element, E-Box and IRF. To test this further, luciferase constructs were made using the CIITA promoters from Raji cell genomic DNA. K-562 cells were then transfected with the Raji cell constructs. If the promoter change was the result of the PIV sequence, the Raji cell constructs should produce different results than the K-562 constructs did. In both cases, untreated or 500 U/ml IFN-γ, the results of the luciferase assay are very similar to those obtained with the K-562 constructs (Figures 34 and 35 A). Furthermore, flow cytometry analysis revealed

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Figure 33. Alignment of the CIITA promoter IV sequence from Raji and K-562 cells. The Raji cell sequence is on the top line and the K-562 sequence is on the bottom line. The open boxes represent the GAS, E box and IRF.

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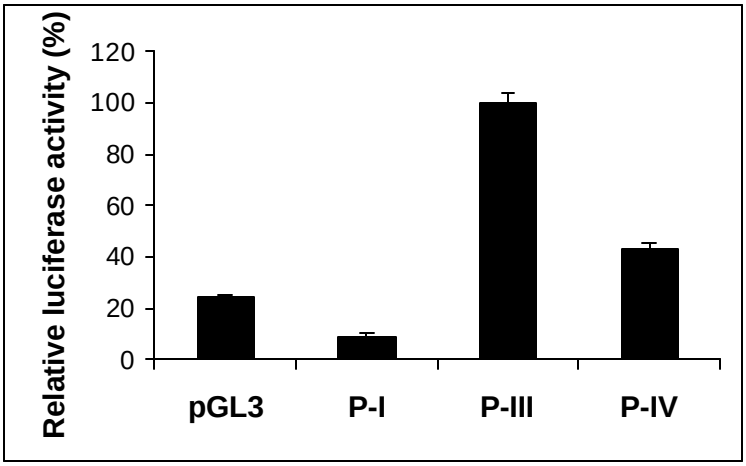

Figure 34. Relative luciferase activity of K-562 cells transfected with Raji CIITA promoter constructs PI, PIII and PIV or the pGL3 reporter vector alone.

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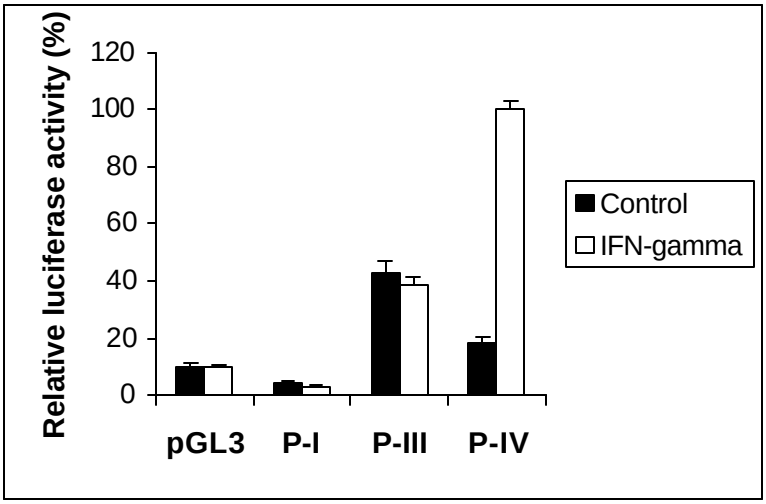
A.



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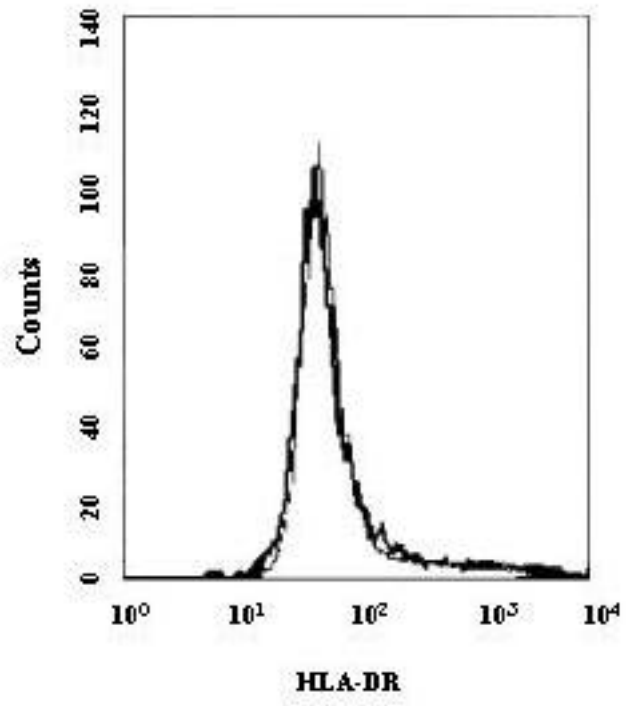

Figure 35. Promoter activity of the Raji cell CIITA promoters after treatment with IFN-γ. **A.** Relative luciferase activity of untreated and IFN-γ-treated K-562 cells transfected with Raji CIITA promoter constructs PI, PIII and PIV or the pGL3 reporter vector alone. **B.** Flow cytometry analysis of the HLA-DR expression of PIV transfected K-562 cells (thick histogram), as compared to control cells (thin histogram).

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that the Raji cell CIITA promoter constructs were not able to induce MHC class II surface expression in K-562 cells (Figure 35 B). This suggested that the change in the active promoter was not caused by the promoter sequence, but rather an additional element of the K-562 cell chemistry.

Calcium ionophore and STI571 have no effect on the activity of the CIITA promoters in K-562 cells

Since IFN-γ stimulated a change in the activity of promoter IV in K-562 cells, the effects of other chemical agents on the CIITA promoters were examined. Once again, K-562 cells were transfected with the luciferase constructs of the CIITA promoters I, III and IV. Reportedly, calcium ionophore induces CML cells to acquire the characteristics of mature dendritic cells via the mobilization of calcium (Engels et al., 2000). For this reason, it was hypothesized that there might be an increase in the activity of the dendritic cell-specific promoter, PI, upon treatment with calcium ionophore. The transfected K-562 cells were treated with 375 ng/ml calcium ionophore, 24 hours prior to assaying for luciferase activity. The results, however, did not support the hypothesis, as the calcium ionophore-treated cells produced the same results as untreated cells (Figure 36). Additionally, transfected K-562 cells were treated with the bcr-abl inhibitor, STI571 (1.0 μM) (le Coutre et al., 1999). As was the case with calcium ionophore, the STI571 treated cells produced the same results as the untreated cells (Figure 36).

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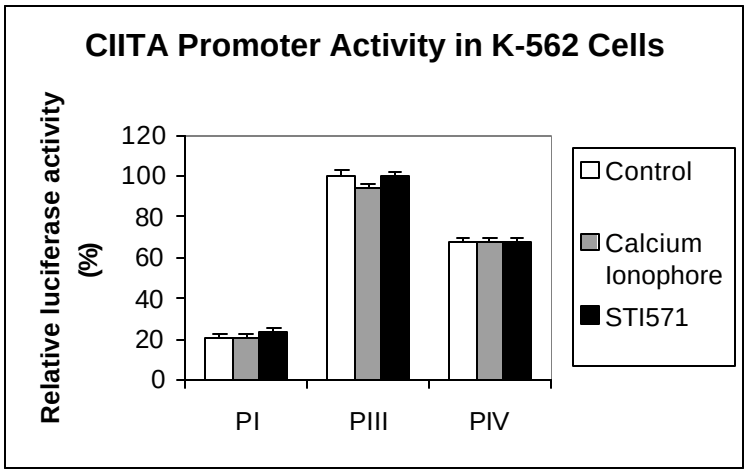

Figure 36. Relative luciferase activity of untreated, calcium ionophore-treated and STI571-treated K-562 cells transfected with K-562 CIITA promoter constructs PI, PIII and PIV.

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The invariant chain does not induce B cell maturation in K-562 cells

The observation that CIITA PIII, the B cell specific-promoter, was active in K-562 cells was unexpected, given that K-562 cells do not express immunoglobulins on their surface (Lozzio et al., 1976). A recent report showing that the transfection of Ii-deficient mice with wild-type Ii, leads to B cell maturation (Matza et al., 2001) prompted investigation into the expression of Ii in K-562 cells. Semi-quantitative RT-PCR was performed on Raji and K-562 cells using primers specific for Ii (Chapter 2, Table 3). The results showed that Ii mRNA is expressed in both Raji and K-562 cells, although the amplification product was significantly more intense for the Raji cell sample (Figure 37). This result is consistent with previous reports which show that Ii expression is reduced in CIITA-deficient cells (Chang et al., 1996; Lee et al., 1997). To determine if the seemingly diminished expression of CIITA could be the cause of the negative immunoglobulin expression in K-562 cells, the cells were stably transfected with an Ii expression vector generously provided by Dr. Idit Shachar (The Weizmann Institute of Science, Rehovot, Israel). Following selection with puromycin, flow cytometry was performed with anti-IgM. The results indicated that despite the transfection of Ii, the K-562 cells still did not express IgM on the cell surface (Figure 38).

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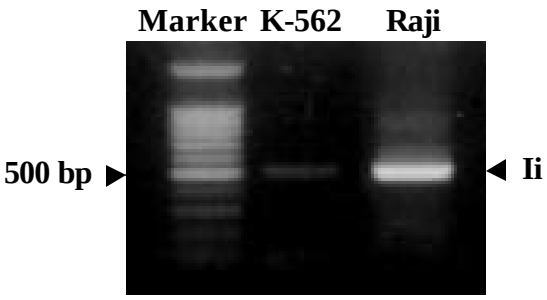

Figure 37. RT-PCR of the invariant chain (Ii) cDNA in Raji and K-562 cells.

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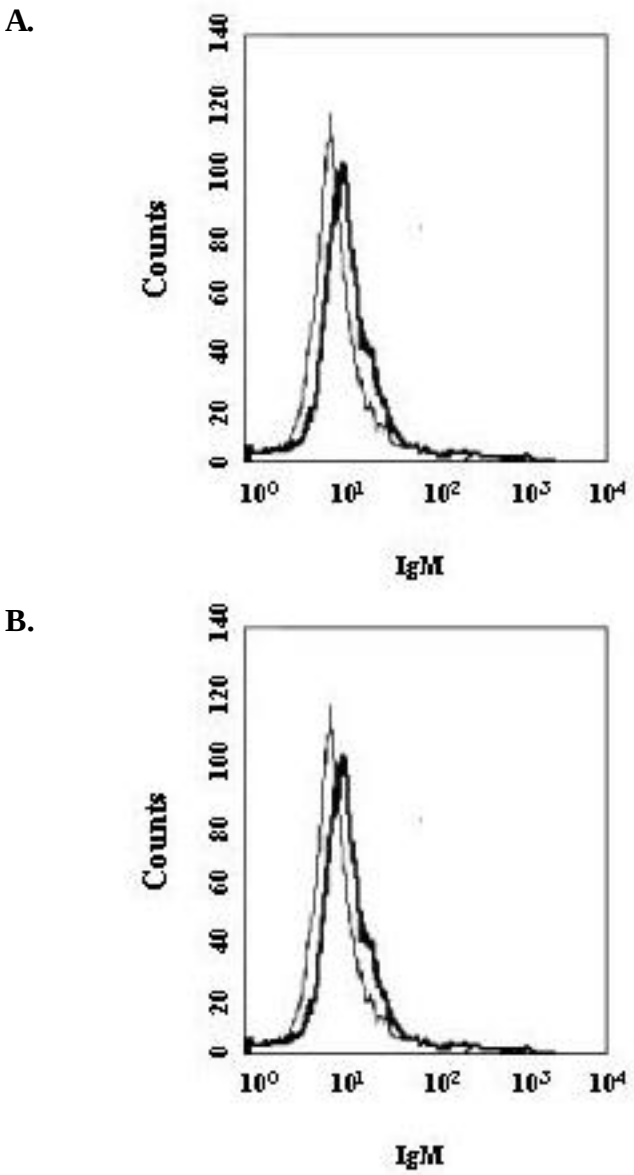

Figure 38. Flow cytometry analysis of IgM expression in K-562 cells. **A.** Control K-562 cells and **B.** K-562 cells transfected with the Ii expression vector. The thin histogram represents the γ1/γ2a control for non-specific binding and the thick histogram represents the IgD stained cells.

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T cell growth conditioned-media-induced changes in K-562 cells

K-562 cells are pluripotent hematopoietic stem cells, and their ability to differentiate along multiple pathways has been well documented (Alitalo, 1990; Andersson et al., 1979; Baker et al., 2000; Benz, 1980; Ichiki, 1987; Lozzio, 1983; Lozzio et al., 1981; Sutherland et al., 1986). In addition to differentiation along cell-specific lineages, various methods have been employed to change the morphology of the cells, including the induction of adherence and aggregation (Fitter et al., 1999; Hickstein et al., 1993; Jarvinen et al., 1993; Lundell et al., 1996). As well as providing a physical barrier to invading pathogens, cellular aggregation allows for communication between the cells of the immune system. This communication mediates cellular migration and proliferation. For these reasons, the adhesive properties and the pluripotent characteristics of K-562 cells provide researchers with valuable information concerning the possible methods of treatment of CML and, as such, are important aspects of leukemia research.

T cell growth-conditioned media induces homotypic aggregation and adherence in K-562 cells

Under normal conditions, K-562 cells grow in suspension culture, with little to no aggregation or adherence to the culture flask (Figure 39 A). However, culturing the cells with 50% T cell growth-conditioned media (CM) resulted in homotypic aggregation and

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Figure 39. Light microscopy of K-562 cells. **A.** Control K-562 cells, **B.** 50% GCM K-562 cells and **C.** trypsinized 50% GCM adherent K-562 cells.

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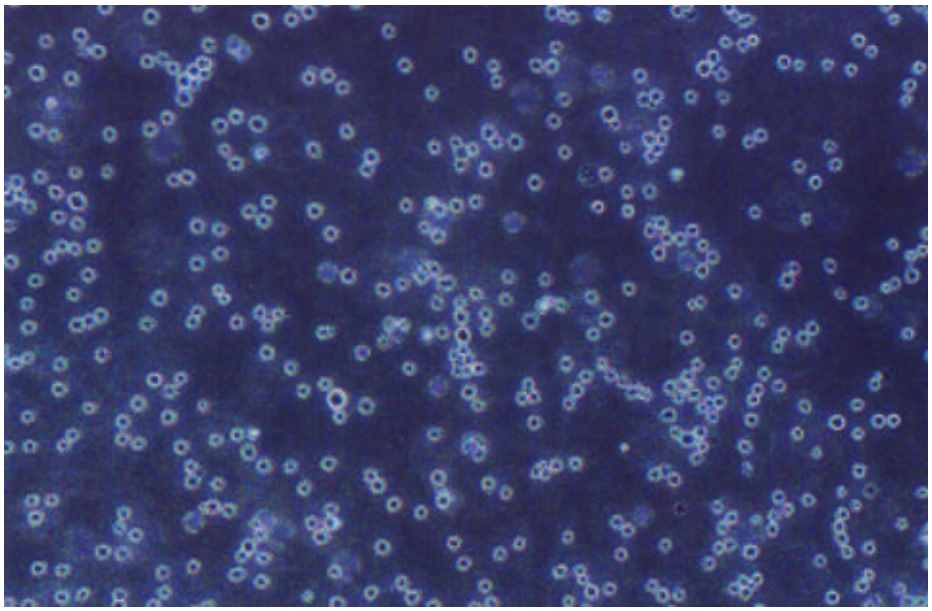
A.



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B.

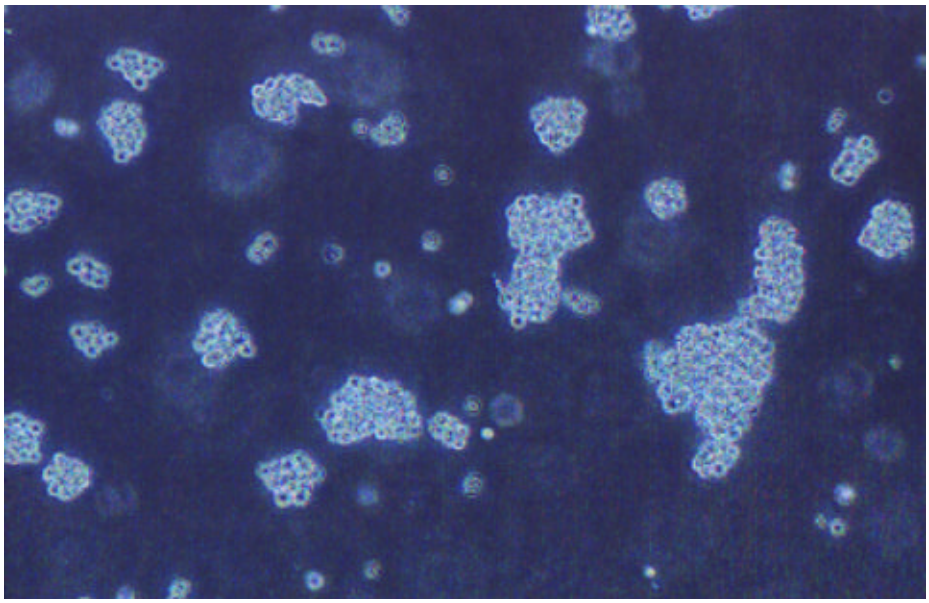

Figure 39, continued

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C.

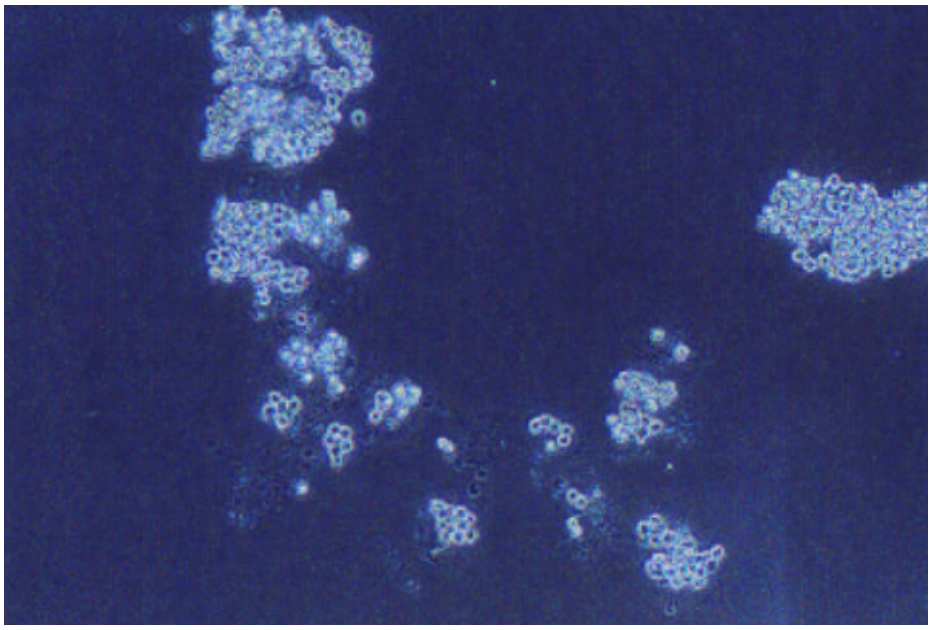

Figure 39, continued

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adherence to the culture flask within 24 hours (Figure 39 B). The media was produced by stimulating peripheral blood mononuclear cells (PBMC), isolated from healthy volunteers, with a crude extract of phytohemagglutinin (PHA) (5 µg/ml). The adherent cells appeared to be well anchored to the flask, as vigorous washing and trypsinization failed to remove them. Moreover, treatment of the cells with trypsin resulted in the breakdown of the cellular structure (Figure 39 C). Although it was previously reported that the induction of adherence in K-562 cells results in a decrease in proliferation (Lundell et al., 1996), a proliferation assay revealed that the CM cultured cells were proliferating at a slightly higher rate than the untreated cells, and that the rate of proliferation is maintained for at least 48 hours (Figure 40).

K-562 aggregates exhibit unusual morphology

Electron microscopy of the CM-cultured, aggregating K-562 cells revealed various morphological changes. As compared to control cells (Figure 41 A), the CM cultured cells (Figure 41 B) showed an increased number of mitochondria and golgi complexes, as well as cytoplasmic extrusions at the cell-cell junctions and the formation of intracellular vacuoles. Consistent with the results of the proliferation assay, these characteristics suggested that the aggregating cells were highly active.

CD15s is upregulated in CM-cultured K-562 cells

To identify the adhesion molecules involved in the aggregation and adhesion of the CM-cultured K-562 cells, flow cytometry was performed with a panel of monoclonal

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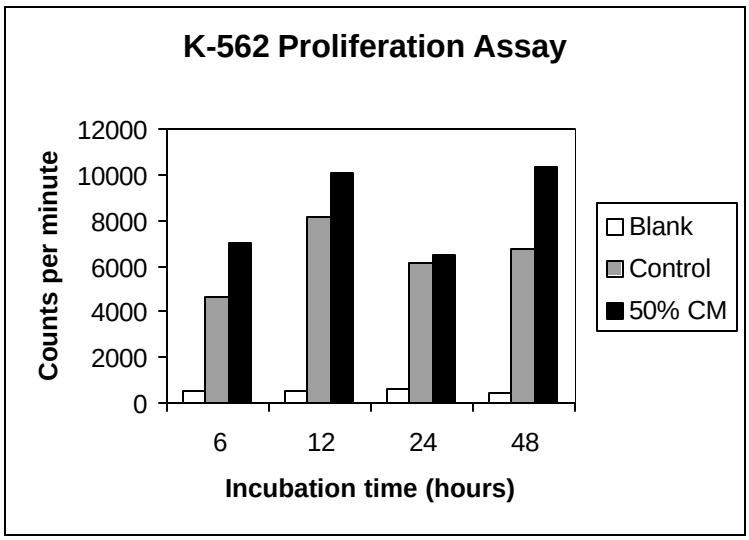

Figure 40. Proliferation assay of the 50% CM-treated K-562 cells. The blank sample is media alone, the control is untreated cells and the 50% CM sample is cells incubated in condtitioned media for 24 hours.

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Figure 41. Electon microscopy of K-562 cells. **A.** Control K-562 cells and **B.** 50% CM K-562 cells.

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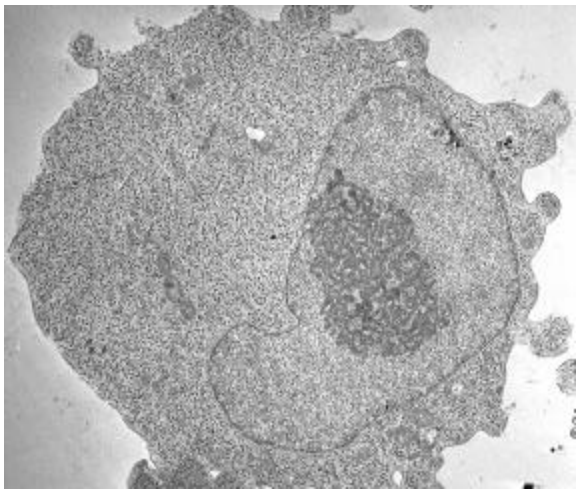
A.



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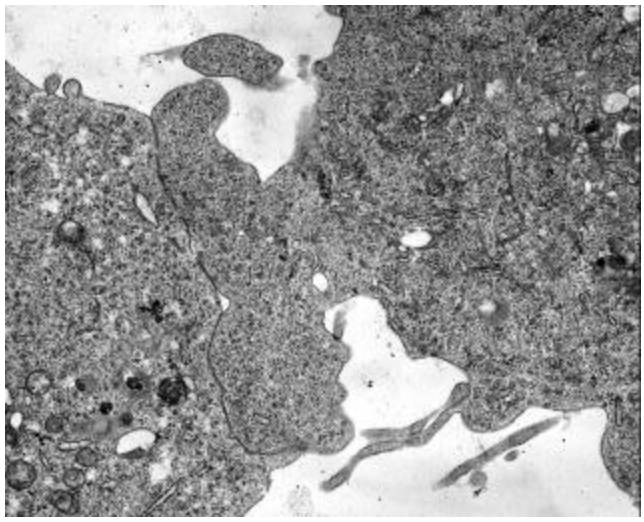

Figure 41, continued

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antibodies. The results showed that compared to control cells, ICAM-1 (CD54), GPIIIa (β3 integrin, CD61), PECAM-1 (CD31) and LFA-1 (CD11a) were not upregulated (data not shown); however, the expression of CD15s was upregulated in the treated cells (Figure 42). CD15s, also known as sialyl lewis X, is a sialomucin involved in the early stages of leukocyte adhesion (Tetteroo et al., 1984b). It serves as a ligand for E-selectin (CD62E) and P-selectin (CD62P), and could be involved in the homotypic aggregation of K-562 cells.

CM induces HLA-DR expression in a small population of K-562 cells

A 1990 study by Ress *et al.* found that culturing K-562 cells with 50% CM resulted in the expression of HLA-DR in approximately 45% of the cells. In an attempt to reproduce this result, K-562 cells were cultured in CM and analyzed by flow cytometry for the surface expression of HLA-DR. The results indicated that approximately 20% of the cells stained positive with the antibody (Figure 43). Furthermore, the forward and side scatter analysis (Cellquest software, Becton Dickinson) of the cells revealed a different pattern for the CM cells, as compared to control cells (Figure 44), indicating that a population of the CM cells had become larger and more complex. The Paint-a-gate option (Cellquest software, Becton Dickinson) was utilized to determine what relationship existed between the differential forward and side scatter patterns and the expression of HLA-DR. The results showed that the enlarged, more complex population of cells was the same as the population that stained positively

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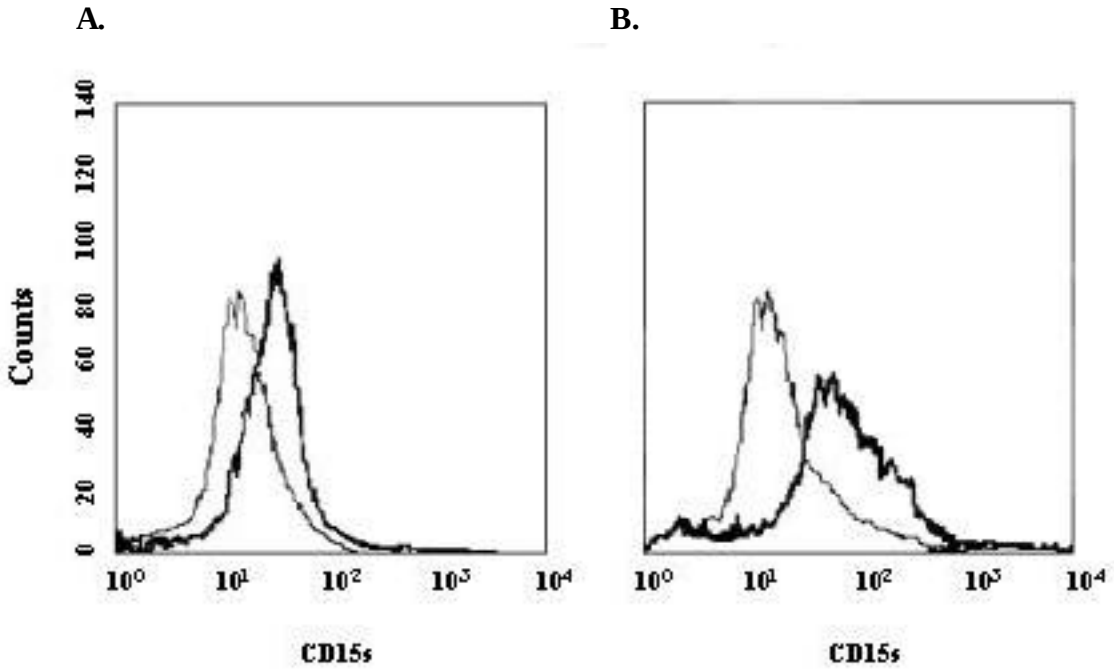

Figure 42. Flow cytometry analysis of the expression of CD15s in K-562 cells. **A.** Untreated and **B.** 50% CM-treated K-562 cells. The thin histogram represents the γ1/γ2a control for non-specific binding and the thick histogram represents the CD15s stained cells.

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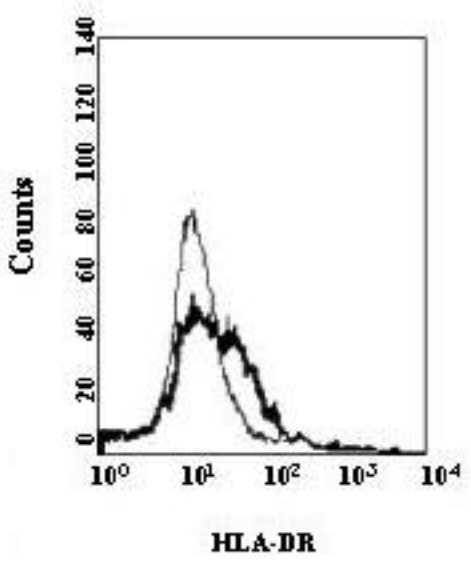

Figure 43. Flow cytometry analysis of the expression of HLA-DR in K-562 cells. Untreated cells are represented by the thin histogram and 50% CM-treated cells are represented by the thick histogram.

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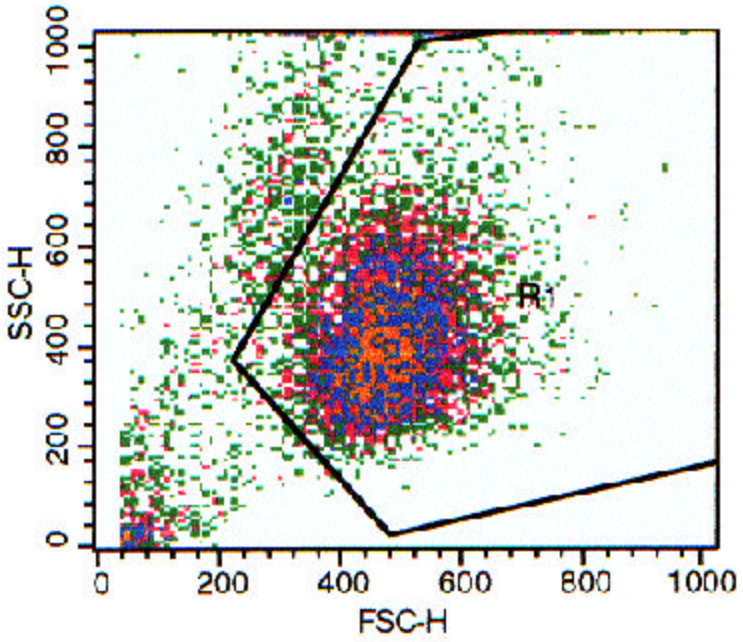
A.



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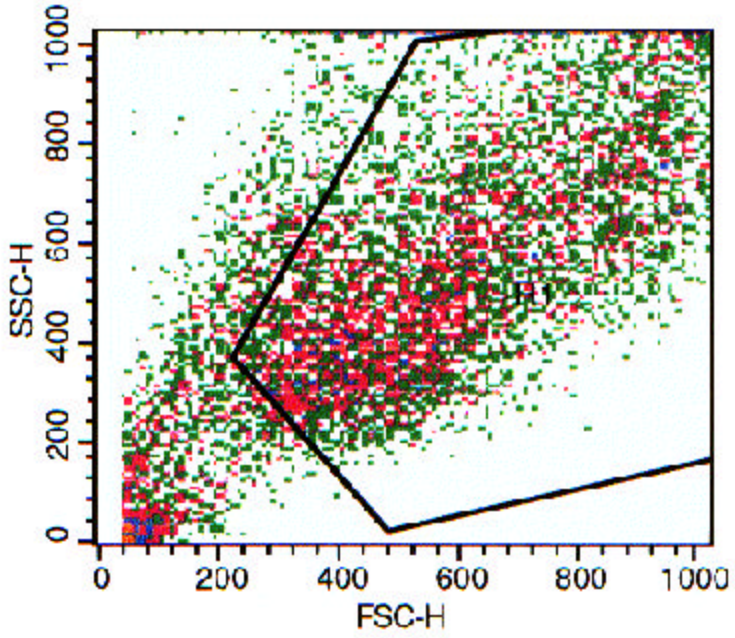

Figure 44. Forward and side scatter analysis of K-562 cells. **A.** Control and **B.** 50% CM-treated K-562 cells.

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for HLA-DR (Figure 45). However, the HLA-DR molecule was apparently not functional, as the CM-cultured K-562 cells failed to activate T cells in a T cell-activation assay (data not shown).

Caspase activity as a measure of apoptosis

When the observation was made that K-562 cell cultures had a consistently increasing number of cells, it was thought that the cells were proliferating excessively. However, subsequent studies revealed that the cells were resistant to apoptosis, or programmed cell death (McGahon et al., 1994; McGahon, 1997), causing an increase in the number of cells, and creating the illusion of excessive proliferation. Although K-562 cells can be induced to undergo terminal differentiation, they lack the DNA ladder pattern known as the hallmark of apoptosis, making the detection of apoptosis difficult. For various reasons, the ability to induce and detect apoptosis is essential to researchers. To that end, the presence of caspase-3 is proposed as an alternative to the DNA ladder pattern as a method of identifying the process of apoptosis in K-562 cells.

Detection of caspase-3 activity in K-562 cells

It has been established that PMA induces the terminal differentiation of K-562 cells (Sutherland et al., 1986; Tetteroo et al., 1984a); however there remains a need for a reliable method of detecting the resulting apoptosis. Caspase-3 plays a major role in one

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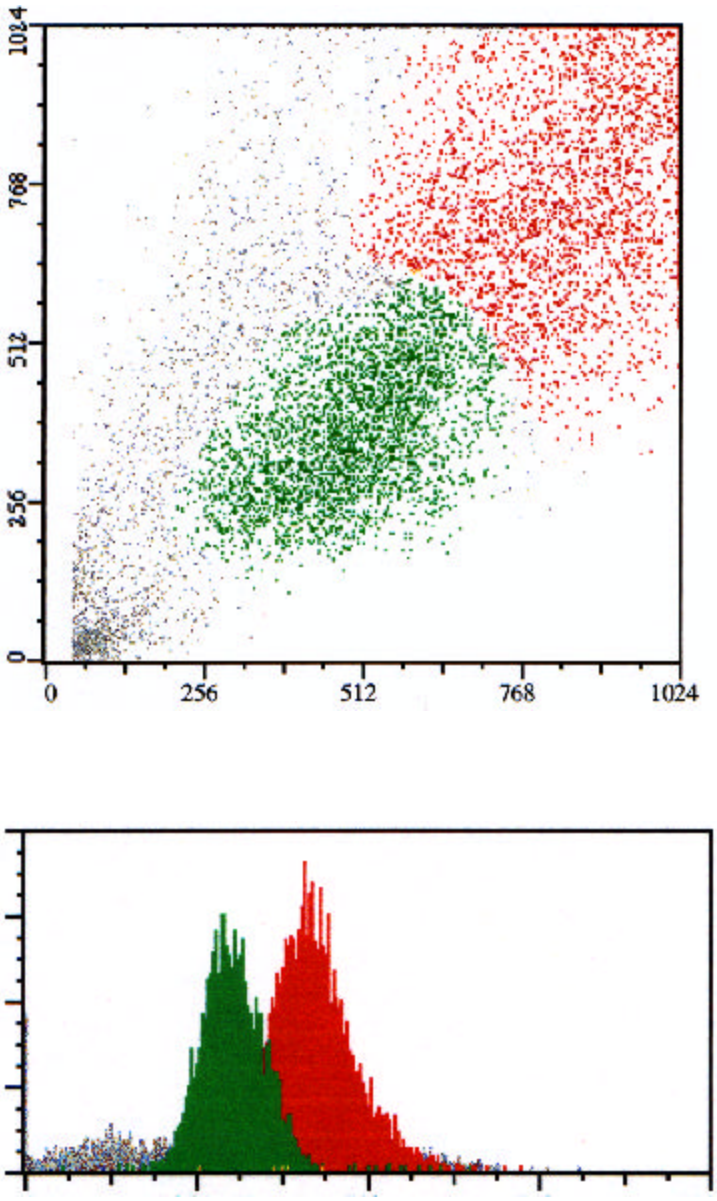

Figure 45. Paint-a-gate analysis of the 50% CM-treated cells. The larger, more complex 50% CM-treated cells correspond to the HLA-DR positive area of the histogram.

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of the pathways of apoptosis, and provides a valuable tool for the detection of the process (Lazebnik et al., 1994; Nicholson et al., 1995). The cleavage of procaspase-3 results in caspase-3, which then cleaves the inhibitor of caspase-activated DNase (ICAD) to CAD. CAD then translocates to the nucleus and begins fragmenting the DNA. A caspase-3 detection assay revealed that the peak level of caspase-3 in K-562 cells occurred approximately six hours post-treatment with PMA (Figure 46 A). The hypothesis was that this peak in caspase-3 could be correlated with an increase of apoptosis.

An increase in caspase-3 activity correlates with an increase in apoptosis

To establish a link between the increase in caspase-3 and apoptosis, PMA-treated K-562 cells were harvested at various time points and analyzed by flow cytometry. The Apo-direct assay (Pharmingen) detects apoptosis by labeling the 3' hydroxyl ends of the fragment DNA with dUTP-FITC. As expected, the peak in apoptosis followed the peak caspase-3 level by approximately two hours (Figure 46 B). This data verifies the usefulness of caspase-3 levels as a method of detecting apoptosis in K-562 cells.

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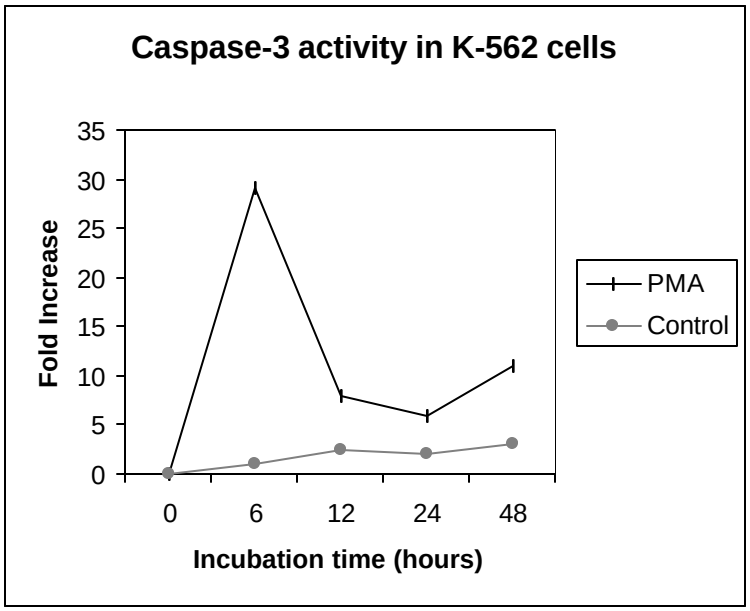
A.



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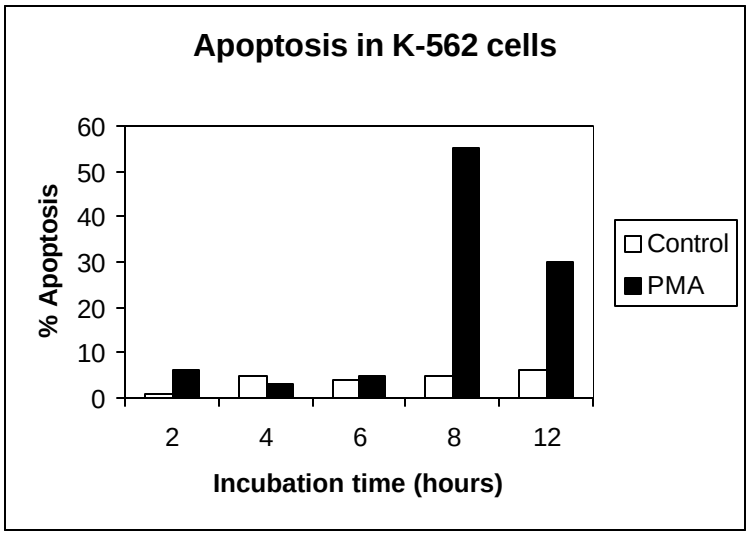

Figure 46. Caspase-3 activity correlates with apoptosis in K-562 cells. **A.** Caspase-3 activity in untreated and PMA-treated K-562 cells. **B.** Apoptosis in untreated and PMA-treated K-562 cells.

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Chapter 4

Discussion

Although the regulation of the MHC genes has been a topic of interest to immunologists for many years, the field of MHC research changed greatly with the discovery of CIITA (Steimle et al., 1993). The importance of CIITA to the antigen presenting capabilities of immune cells has been amply demonstrated by many different researchers (Peijnenburg et al., 2000; Quan et al., 1999; Steimle et al., 1993; Wiszniewski et al., 2001), and has provided insights into the factors underlying immunodeficiency.

The highly undifferentiated, pluripotent characteristics of the K-562 cell line lead to the conclusion that the cell line was derived from a pluripotential hematopoietic stem cell (Lozzio et al., 1981). Although the cells do not express MHC molecules under normal conditions, MHC class I expression can be induced in K-562 cells by treatment with IFN-γ; however, a reliable method for inducing MHC class II expression remains to be identified. Although culturing K-562 cells with T cell growth-conditioned media can induce HLA-DR expression (Ress et al., 1990), attempts to reproduce this observation in our laboratory have failed to produce consistent results. Moreover, we have found that the HLA-DR molecule induced on these cells fails to activate T cells in a T cell activation assay, suggesting that the cells are still not functioning as APC. This discrepancy suggests the possibility that, in spite of the reactivity of the anti-HLA-DR antibody, the MHC class II molecule is unable to function as a bridge to the T cell receptor. The

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absence of MHC class II expression in K-562 cells, combined with studies demonstrating the reported ability of these cells to function as accessory cells in T cell activation assays, raises the question of the role of the cell line in the immune response. Compromised MHC class II expression has been shown to result in severe immunodeficiency, caused by inefficient T cell activation and a diminished T cell repertoire. In the case of CML, the malignant cells can slowly replace the hematopoietic stem cells, leading to diminished immunity in the blast phase. For this reason, it is essential to identify the factors involved in the null expression of MHC class II genes in K-562 cells and, as such, this was the focus of the present study.

Liu *et al.* (1999), observed that CIITA mRNA was not expressed in K-562 cells, and that a complementation assay with the wild-type CIITA resulted in the recovery of MHC class II expression in K-562 cells, leading to the conclusion that the lack of MHC class II expression was caused by the absence of CIITA expression. In our laboratory, however, we have identified CIITA mRNA in a subline of K-562 cells. The discrepancy between our observations and those of Liu *et al*. is likely a result of the apparent secondary structure of the K-562 CIITA, which leads to the requirement of an unusually high temperature for reverse transcription. Still, the results of the complementation assay by Liu *et al*. suggested that there was some malfunction of the CIITA gene. This information, combined with observations demonstrating the importance of the individual protein domains of CIITA and the deleterious effects of mutations, provides evidence that CIITA is a likely culprit in the negative MHC class II expression of these cells.

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In this report we have shown that the CIITA transcript is alternatively spliced in K-562 cells, resulting in an insertion of a genomic sequence, which harbors a stop codon. We can confidently rule out the possibility of genomic DNA contamination of the RNA preparation, as the samples were treated with Rnase free Dnase prior to PCR. The insertion of the premature stop codon results in a truncated protein and the loss of several crucial protein domains, including a self-association domain and the carboxy-terminal LRRs. Although the alternatively spliced protein is able to associate with the MHC class II promoter and the RFX complex *in vivo*, the transactivation ability of the transcription factor is abolished. For this reason, the alternative CIITA transcript alone is insufficient for MHC class II expression in K-562 cells. Furthermore, there are low levels of constitutive activation of both CIITA PIII and PIV, although the relative levels are approximately 40% higher for PIII. However, upon treatment with IFN-γ, PIV is upregulated to surpass the activity of PIII and results in a relative activity that is as much as 95% higher than the vector alone. This data suggests that there should be both constitutive (PIII) and IFN-γ inducible (PIV) MHC class II expression in K-562 cells; however, consistent with previous reports, the surface expression of HLA-DR remains negative even after treatment with IFN-γ. This further supports the hypothesis that the cause of the negative expression of MHC class II genes in K-562 cells is a defect in CIITA.

The presence of multiple CIITA isoforms was first observed in MHC class II-positive Raji cells by Riley *et al*. (1995). It was suggested then that the alternatively spliced CIITA of Raji cells might serve a different function than that of the wild-type

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CIITA. This possibility cannot be ruled out at this time, and the presence of the alternative transcript in MHC class II-positive cells, combined with the lack of a mutation at the CIITA splice site, provide support for this hypothesis. If, in fact, the function of the alternative transcript is different than that of the wild-type, K-562 cells provide an excellent model for studying this alternative protein. This type of study could prove to be difficult in Raji cells, as they express both isoforms of the gene. However, K-562 cells do not express the wild-type, making a study of the protein function more practical. Although it was not included in this study, an experiment in which the alternative CIITA transcript is mutated in K-562 cells would be essential to identifying the function of this protein. The identification of the alternative transcript in Raji cells by real-time PCR, but not by RT-PCR, is likely due to the fact that the real-time PCR primers were designed for a region not present in the more abundant wild-type transcript, thereby eliminating the competition for binding that would exist in RT-PCR. Furthermore, real-time PCR is more sensitive because the results are detected by fluorescence emission, rather than by the human eye, as is the case with the visualization of an EtBr-stained agarose gel of RT-PCR products.

Many studies have demonstrated that the presence of CIITA is not sufficient for fully functional APCs (Bontron et al., 1997a; Bontron et al., 1997b; Hake et al., 2000; Linhoff et al., 2001; Quan et al., 1999). One reason for this is that the integrity of the carboxy-terminal domains of CIITA is essential for the transactivation of MHC class II genes (Hake et al., 2000; Linhoff et al., 2001; Quan et al., 1999). In specific cases, analysis of immunodeficient patients has revealed that a single amino acid substitution or

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deletion of a splice donor site can diminish the transactivating ability of CIITA. Furthermore, mutants in which the LRRs beyond amino acid 980 are altered or deleted have no transactivating activity (Bontron et al., 1997a; Bontron et al., 1997b). Because the carboxy-terminal domains are deleted, the premature stop codon in the K-562 CIITA cDNA results in the same CIITA expression pattern as each of these examples.

The alternative CIITA protein of K-562 cells is not the first case in which the transcription factor is able to associate with the MHC class II promoter, but does not result in the transcription of the class II gene (Hake et al., 2000). This suggests that the transactivating ability of CIITA is regulated by the carboxy-terminal domains of the protein, while the recruitment of CIITA to the class II promoter is regulated by a region not affected by the stop codon (amino acids 1-932). Likewise, since MHC class I expression is IFN-γ-inducible in K-562 cells, the CIITA domain involved in the regulation of class II expression is likely located within the first 932 amino acids. An investigation on these points would require further mutational analysis of CIITA, in which the functions of the domains at the amino end of the transcript are disrupted. The CIITA DNA fragment that was precipitated with the CIITA antibody indicates that the PCR primers encompassed a region involved in the self-association of CIITA. Although this self-association has been previously documented, the purpose of the association has not yet been identified (Linhoff et al., 2001; Sisk et al., 2001). It is possible that the transcription of the CIITA gene is, in part, regulated by the levels of the CIITA protein, but this remains to be verified. One way to verify this would be to over-express the CIITA protein and monitor the levels of endogenous CIITA production.

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We have shown that CIITA mRNA is transcribed in K-562 cells, which indicates that at least one of the four CIITA promoters is active in the cells. The pluripotential characteristics of the cell line, combined with the presence of the CIITA mRNA in K-562 cells, poses the question of which CIITA promoter will be active in these cells. Although the sequence of PIV is identical in Raji and K-562 cells, including the GAS element, E box and IRF, the activity in vivo is quite different, suggesting that there are additional factors regulating the CIITA promoter. The intriguing possibility of additional regulatory proteins binding the CIITA promoter of K-562 cells is not without precedent, as the BLIMP1 protein serves to extinguish PIII activity in plasma cells (Piskurich et al., 2000). The possibility also exists that K-562 cells might be deficient in some regulatory factor that inhibits the activation of PIV in normal cells. A DNA footprint of PIV from both cell lines would be necessary to determine if there are cell-specific proteins or regulatory factors associating with the promoter, resulting in the different patterns of activity.

Melanoma cells are the only other cell line in which PIV has been identified as constitutively active (Goodwin et al., 2001). In the case of melanoma cells, it is common to find constitutive expression of HLA-DR, which has been linked to a poorer prognosis. In melanoma cells, the constant activity of PIII was attributed to factors acting on the upstream enhancer, known as the IFN-γ response sequence. The unprecedented constitutive activation of PIV was thought to be activated by IRF-2, as IRF-1 was not constitutively expressed in the melanoma cells. As of yet, the expression of IRF-1, IRF-2, and the factors acting on the PIII IFN-γ-response sequence have not been investigated in K-562 cells. It is of notable interest that the only two cases in which PIV is not

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dependent on IFN-γ, and there are two active CIITA promoters, are both malignant cell lines. The present data provides insight into the role of CIITA in malignant cells and the effects of aberrant regulation of MHC class II expression.

There have been no previous reports in which the most active CIITA promoter changes upon treatment with IFN-γ, or any other inducing agent. This discovery in K-562 cells presents a paradox, as IFN-γ treatment leads to the upregulation of the CIITA promoter, but does not result in HLA-DR expression on the cell surface. Furthermore, the activity of PIII in K-562 cells would seem to suggest that there should also be constitutive expression of the MHC class II genes, but this is not the case. These observations support the hypothesis that the lack of MHC class II expression in K-562 cells is the result of the inactivity of CIITA.

We do not attempt to explain the lack of immunoglobulin expression on the K-562 cell surface, as the activity of PIII suggests that the cells are of the B lymphoid origin. The seemingly low level of expression of Ii, which is required for B cell maturation (Matza et al., 2001), was not surprising as previous reports of CIITA-deficient cells documented low levels of Ii (Chang et al., 1996; Lee et al., 1997), but transfection of the cells with the Ii expression vector did not restore immunoglobulin expression to the cells. This data indicates that there is another, possibly unrelated, reason for the lack of B cell markers on K-562 cells.

In addition to MHC expression, other regulatory processes are essential to the function of the immune system. Among these processes, apoptosis is important to control the balance in cell number between different cell types. Although the treatment

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of K-562 cells with PMA provides a means of inducing apoptosis, the process is difficult to detect, due to the lack of a DNA ladder pattern (McGahon et al., 1994; Sutherland et al., 1986; Tetteroo et al., 1984a). We have identified a correlation between the caspase-3 activity of PMA-treated cells and apoptosis, which could serve as a convenient method for detecting apoptosis in K-562 cells, making the study of apoptosis more practical for leukemia researchers.

During the course of an immune reaction, cellular adherence can be a vital factor to the success of the response. The adherent properties of certain cell types have been linked to the proliferation rate of the cells (Lundell et al., 1996). In fact, the induction of adherence of K-562 cells to fibronectin has been shown to result in a reduced rate of proliferation. Surprisingly, the treatment of K-562 cells with CM resulted in an increased rate of proliferation and the appearance of highly active cells, as seen by electron microscopy. The possibility that these observations were the effect of some external factor present in the PBMC sample can be ruled out, as the PBMC of several different volunteers were used on many separate occasions. Although increasing the number of leukemia cells is not a desirable outcome, it could serve as a tool for distinguishing between malignant cells and normal cells for treatments where this ability is essential, such as chemotherapy.

In addition to affecting the rate of proliferation, cellular aggregation provides a barrier to invading pathogens and allows for communication between cells. This communication is crucial to the recruitment of cells to the site of the immune response and its impairment could result in a diminished response. For these reasons, the ability to

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induce cellular aggregation in K-562 cells with CM is of significant importance to researchers, as well as clinicians. The upregulation of CD15s is common in neutrophils during the immune response and is essential to the trafficking of the cells. The role of CD15s in the aggregation of K-562 cells has not been identified, but it is possible that either E selectin or P selectin is also upregulated on the cells and is recognized by the CD15s, resulting in homotypic aggregation.

As a follow up to this report, future experiments could follow several different pathways. Initially, it would be important to assess the full range of effects that the mutation we have identified has on the function of CIITA. For example, the alternative CIITA could be over-expressed in cells that are MHC class II-positive under normal conditions, to determine its effect on MHC class II expression. The expectation is that the cells would be unable to transcribe the MHC class II genes and, subsequently, would not express MHC class II molecules on the cell surface. Furthermore, fluorescence microscopy of K-562 and Raji cells stained with an anti-CIITA antibody would provide information as to where the protein is localized in the MHC class II-negative K-562 cells, as compared to the MHC class-II positive Raji cells. Although Hake *et al*. (2000) found that mutating the LRRs of CIITA impaired nuclear localization, this is not expected to be the case, as we have demonstrated by chromatin immunoprecipitation that the alternative transcript does associate with the MHC class II promoter. It seems more likely that some other function of CIITA is abrogated in the alternative transcript, such as the histone acetyl transferase (HAT) activity. If this were the case, the transcription factor might associate with the promoter, but would not be able to remodel the chromatin, resulting in

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the inability to transcribe the MHC class II gene. A HAT assay of both the wild-type and alternative CIITA proteins would provide the necessary information to determine if this is the case with the K-562 CIITA protein. The possibility that this protein does serve some function unrelated to MHC expression is entirely feasible, and somewhat intriguing. To determine if the alternative CIITA has some function other than the coactivation of MHC class II genes, the first step would be to mutate the gene in K-562 cells and observe the cells for any changes in their established characteristics. Additionally, the alternative CIITA protein should be introduced into a cell line where it is not normally expressed, followed by observation of the cell-specific characteristics. Concerning the promoter activity of the MHC class II genes and CIITA, a luciferase assay in which the K-562 HLA-DR promoter is transfected into MHC class-II positive Raji cells would provide important information regarding the functional capacity of the K-562 MHC class II promoter. Likewise, a DNA footprint of the Raji and K-562 CIITA PIV would be essential to determining the presence of additional factors regulating the activity of the IFN-γ-inducible CIITA promoter.

As an extension of the CM study, it would be important to identify the factors involved in the induction of the adherence and aggregation of the CM-treated K-562 cells. Confocal microscopy using an antibody for CD15s would show the localization of the adhesion molecule in the aggregating cells. We can speculate that the antibody would stain most heavily at the cell-cell junctions, indicating that CD15s is involved in the homotypic aggregation of CM-treated K-562 cells. To determine if the upregulated CD15s molecule is acting by associating with either E selectin or P selectin, flow

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cytometry could be performed using a monoclonal antibody for either of these molecules (CD62E and CD62P). Although these adhesion molecules are not normally expressed on K-562 cells, it is conceivable that one, or both of them, might be upregulated upon treatment with CM. Lastly, cytokine capture beads could be used in combination with flow cytometry to determine what cytokines are present in the CM.

In support of the hypothesis that the negative MHC class II expression of K-562 cells is caused by a defect in CIITA, a debilitating premature stop codon in the K-562 CIITA transcript was identified. We have demonstrated that the activity of the alternative CIITA promoters is unique to malignant cells and should result in MHC class II expression. Furthermore, we have described a means of detecting apoptosis in K-562 cells and identified a method of inducing adherence and aggregation in these suspension culture cells. This data provides information that could be valuable not only to the field of leukemia researcher, but could also be applied to the study of MHC class II expression.

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Vita

Noel Elyssa Day was born on December 15, 1974, in Athens, Georgia. In 1993, after graduating with an honors diploma from Farragut High School, Knoxville, Tennessee, she enrolled at the University of Tennessee, Knoxville. In 1997 she received a Bachelor of Arts degree, cum laude, in Psychology. On January 3rd, 1998 Noel married her longtime friend and boyfriend, Brad. In August of the same year, she began her graduate studies in the Comparative and Experimental Medicine program in the University of Tennessee Graduate School of Medicine. She worked as a pre-doctoral student in the laboratory of Dr. Albert Ichiki at the University of Tennessee Medical Center as a graduate research assistant until December of 1999. At that time, Noel relocated with her husband to Tsukuba Science City, Japan. Supported by a National Science Foundation pre-doctoral grant, she carried out her research in the laboratory of Dr. Kazunari Yokoyama at the RIKEN Institute for Physical and Chemical Research. In December of 2001, she received her Ph.D. from The University of Tennessee, with a concentration in immunology. She will continue her interests in science as a Life Sciences Specialist at the law firm of Pillsbury Winthrop in San Francisco, California.

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