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MHC Gene Regulation

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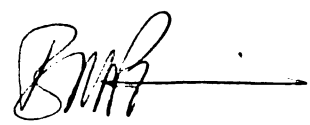
ABSTRACT

DQα and DQβ genes were systematically screened for the presence of regulatory elements able to stimulate transcription from a heterologous promoter in transient transfections. Two regions were identified in DQα which fulfilled the criteria for tissue specific enhancers. These enhancers function in both orientations and independently of position. They exhibit some tissue specificity but are not responsive to γ-interferon. RNase protection analysis demonstrated that they increase transcription arising from a downstream thymidine kinase promoter. One DQα enhancer is located in the promoter region and the other is found in the first intron. The first intron enhancer was found to be associated with a DNase I hypersensitive site in a constitutively expressing cell line. An enhancer was also found in the second and third introns of DQβ. These enhancers may play a role in the control of tissue specific expression of DQ.



MHC GENE REGULATION

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MHC GENE REGULATION

KATE SULLIVAN

THESIS INTRODUCTION

MHC GENE REGULATION

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GENE REGULATION

In eukaryotic cells, regulation of gene expression may occur in many guises. Transcriptional regulation appears to be the most common mechanism for controlling the abundance of individual protein products, however many examples of alternate regulatory strategies have been described. A well characterized example of gene regulation by poly(A) addition occurs in B cells where the switch from membrane bound IgM production to secreted IgM production is controlled at the level of poly(A) addition. This is reflected in an altered splice pattern but the developmental control of IgM RNA is believed to rely on the addition of poly(A) 3' to either exon 4 or exon 6. Addition of poly(A) after exon 4 results in secretory IgM production. Poly(A) addition after exon 6 results in 2 more exons being spliced into the message and membrane bound IgM production. (1)

An important example of regulation by splicing concerns the gene troponin T (TnT). This protein regulates the contractile function in striated muscle. Two tissue specific and developmentally regulated mRNAs are produced from a single gene. The TnT gene consists of 9 exons on 5kb of DNA. The αTnT message is found in adult striated muscle and the alternate form, ßTnT, is found predominantly in fetal and newborn muscle. The αTnT message consists of exons 1-7 and exon 9. The ßTnT message consists of exons 1-6 and exons 8 and 9. This mutually exclusive splicing pattern is determined by the incompatability of the splice junctions for the α7 and ß8 exons. Thus, splicing of all nine exons is impossible.(2) Other examples of

splicing heterogeneity occur in the family of contractile proteins but this is the clearest example of regulated splicing.(3,4)

Two examples of regulation by message turnover have been described recently. Interestingly both occur in the immune system where flexibility and responsiveness to stimuli are of singular importance. In the immunoglobulin heavy chain, the transcription rate appears to be determined primarily by the activity of its intronic enhancer. When tested in transient assays the heavy chain enhancer functions as well in immature PreB cells as well as fully mature plasma cells.(5) This is reflected in nuclear run-on assays which demonstrate similar transcription rates in preB cells and plasma cells.(6) However, when steady state total or poly(A) RNA is measured, plasma cells are found to have fifty times the level of preB cells. Consequently, preB cells have low levels of immunoglobulin heavy chain while plasma cells secrete large amounts of it. This suggests that immunoglobulin heavy chain message is turned over more rapidly in preB cells. The mechanism of this turnover is not known and may reflect a more proximal effect. For example, differences in polyadenylation or splicing might result in a shorter half life for immunoglobulin heavy chain message in preB cells. Similar examples of regulation by message turnover are found in the histone H4 (7), glyceraldehyde-3-phosphate-dehydrogenase (8), α amylase (9), and the thymidine kinase gene systems.(10)

The second example concerns a conserved sequence element found in the 3' untranslated portion of many lymphokine messages. The presence of this sequence element confers instability on those messages. The

conserved sequence is AU rich and can confer instability on heterologous messages. PMA and cycloheximide appear able to abrogate the destabilizing effects of this sequence suggesting that the instability is mediated by a specific factor with a short half life.(11) The evolutionary significance of this sequence is presumably to maintain transiently expressed messages within a narrow window of activity.

Regulation at the level of translation is unusual in eukaryotes. The translational machinery may not be flexible enough to regulate the synthesis of individual proteins, however viral gene products often repress translation of host mRNAs as a mechanism to focus the synthetic machinery on virus production. Polio virus infection results in cleavage of p220 of eukaryotic initiation factor eIF-4F and prevents the translation of capped mRNAs.(12) Since polio message is not capped it is translated at high levels in these cells. Other viruses appear to use different strategies to repress host mRNA translation, however in general, all host messages appear to be affected equally.

One of the activities of interferon involves the induction of the double stranded RNA activated inhibitor of protein synthesis (DAI). DAI is a kinase which phosphorylates the initiation factor eIF-2. When eIF-2 is phosphorylated it is unable to recycle and begin new initiation complexes.(13) The activity of interferon generally results not only in inhibition of viral synthesis but also a decrease in host cell protein synthesis and eventual cell death. Adenovirus has evolved a mechanism for evading this inhibition and in the process

releases the inhibition of host cell protein synthesis. Adenovirus VA RNA_1 is transcribed by RNA polymerase III and it accumulates in high levels in the late phase of infection. VA RNA_1 blocks the activation of DAI by double stranded RNA and prevents the inhibition of protein synthesis.(14) This is another example of translational regulation on a global scale.

There is some evidence that specific translational regulation accounts for the pattern of collagen synthesis in chondrocytes. Chondrocytes do not synthesize type I collagen which is found predominantly in epidermal or tendonous tissues. Yet, chondrocytes have type I collagen message levels comparable to fibroblastic cells. This message is able to be translated in an in vitro system but is not translated in chondrocytes.(15) The N-terminal propeptide of type I collagen acts to inhibit type I collagen translation in vitro and in vivo.(16) Similarly type II collagen inhibits its own translation in vitro.(17) The mechanism of action is unknown, however the propeptides may interact with the 5' region of the collagen message which has a very interesting structure. Two AUG codons are found in the 5' region. The second AUG is utilized for initiation and it is found in the stem of a very stable stem-loop structure. All collagen genes sequenced thus far share this unusual feature.(18) In chondrocytes, ribosomes may be directed to the first AUG from which protein synthesis does not occur. Other tissues may allow utilization of the second AUG by destabilizing the stem-loop structure.

A final example of nontranscriptional regulation concerns a very distal

effect of the adenovirus 2 protein, E3/19K, on the α chain of human MHC class I antigens. The adenovirus family seems to have evolved mechanisms to evade the immune system. Adenovirus 12 infection is known to specifically decrease class I α chain message levels and this effect has been mapped to the Ela and Elb genes.(19) Interestingly, a non-oncogenic adenovirus (Ad5) does not have the capacity for decreasing class I message. This finding stimulated speculation that oncogenic adenoviruses had evolved a mechanism to inhibit class I expression as a means to evade detection by the immune system. The finding of a second mechanism to inhibit class I expression within the adenovirus family would seem to support that idea. Adenovirus 2 produces a protein known as E3/19K which binds tightly to MHC class I α chains and prevents their glycosylation.(20) The complexed class I antigens are not expressed on the surface and as a result adenovirus 2 infected cells express only 10% the level of surface class I compared to uninfected cells. This may explain why adenovirus 2 is particularly prone to producing latent infections. A large decrease in class I expression may allow infected cells to propagate and escape lysis by cytotoxic T cells.

By far the most information about gene regulation has accumulated in the field of transcriptional regulation. Transcriptional control elements generally fall into three somewhat overlapping catergories. Genes may utilize any combination of these cis-acting elements to achieve the desired control of transcription. A promoter region directs RNA polymerase to the correct transcripion start site. There may be one or more sequence elements responsible for this

activity.(rev. 21) The second category consists of elements known as enhancers. Enhancers are cis-acting DNA sequences that increase the frequency of initiation from a linked homologous or heterologous promoter.(rev. 22) They are orientation and distance independent and they may be tissue specific, inducible or promiscuous. The third category of cis-acting elements consists of the repressors or blockers.(23) They repress transcription in certain tissues while allowing transcription in other circumstances. The activity of all of these cis-acting elements is dependent on the presence of trans-acting factors and a great deal of energy has been devoted to the isolation and characterization of those factors.

Genes transcribed by RNA polymerase I and III appear to be regulated by very simple mechanisms. Tremendous progress has been made in characterizing the requirements for polymerase III transcription. Three factors in addition to RNA polymerase III bind at an internal promoter and are sufficient for Xenopus 5S RNA transcription.(24) The switch from oocyte + somatic gene transcription to somatic gene transcription only is regulated at least in part by the concentration of transcription factor TFIIIA.(25) Transcription of oocyte 5S RNA genes requires a high concentration of TFIIIA because the internal promoter of oocyte genes binds TFIIIA with a lower avidity. Thus, developmentally controlled regulation may be determined by the concentration of a constitutive transcription factor.

Acanthamoeba ribosomal RNA gene transcription by RNA polymerase I has recently been investigated. It represents a similarly uncomplicated

regulatory strategy. Initiation of transcription requires binding of transcription initiation factor (TIF) to a promoter region and subsequent binding of RNA polymerase I.(26) Starvation of Acanthamoeba results in cyst formation and cessation of ribosomal gene transcription. Cyst TIF appears to be transcriptionally competant but the RNA polymerase I from cysts is not active. Examination of cyst RNA polymerase I suggested covalent modification which inhibited its ability to initiate transcription of ribosomal genes while preserving its ability to elongate in nonspecific assays.(27) Therefore, modification of a constitutive transcription factor represents another simple form of regulation.

Promoters transcribed by RNA polymerase II often contain a TATA box located approximately 30bp upstream of the transcription start site. This common promoter element binds a factor found in all cell lines which have been examined.(28, 29) Analysis of the TATA element has revealed that it is important in specifying the transcription start site. Mutation or deletion of the TATA box results in heterogeneous initiation while often grossly preserving the rate of RNA synthesis.(30) The TATA homology resembles the Pribnow box found in bacterial genes that is recognized by prokaryotic polymerase. While clearly evolved from its prokaryotic cousin, the TATA box in eukaryotes is probably not directly bound by RNA polymerase II. In fact, there are a variety of genes including an MHC class II gene, DQα, which are believed to be transcribed by RNA polymerase II but which do not contain a recognizable TATA box. Eukaryotic genes, in establishing the complex regulatory strategies required by multicellular organisms, seem

to have evolved numerous mechanisms for designating start sites and polymerase loading frequency. Genes which lack a TATA box may utilize a sequence element originally identified in viral genes. This element is known as the GC hexanucleotide or GC box and binds a factor known as SP1.(31) The GC box is often repeated in promoter regions and may be found in addition to the TATA box and other conserved sequences. When found in addition to the TATA box, the repeated GC motifs function as un upstream sequence.(32) When found in promoters that lack a TATA box, the repeated GC motifs appear able to designate a transcription start, performing the function of the missing TATA box.(33) The GC box was originally defined and characterized in the viral SV40 early promoter and in the herpes simplex virus thymidine kinase gene.(31, 32) Removal of individual GC boxes reduced transcriptional efficiency but no individual element appeared to be critical for transcription.(34) SP1-DNA contacts detected by dimethylsulphate protection have been identified only in the major groove of the DNA and it is curious that the GC box and its inverse function equally well in spite of sequence asymmetry.(31) A variety of eukaryotic genes contain multiple promoter GC boxes and their common characteristic appears to be that they are all housekeeping genes. HMG CoA reductase(33), adenosine deaminase(35), dihydrofolate reductase(36), superoxide dismutase(37) and hypoxanthine phosphoribosyltransferase(38) are all genes which utilize GC box containing promoters and are constitutively expressed. It seems efficient for constitutively expressed genes to have evolved a common mechanism for transcription. Viruses would naturally evolve to exploit such mechanisms, explaining the prevalence of GC boxes in viral promoters.(39, 40) Variations in the

transcription rates of genes which utilize a common transcription factor such as SP1 may be mediated by the number and/or position of binding sites. Alternatively, other regulatory strategies may be superimposed on an SP1 driven promoter.

A sequence found upstream of genes induced by heat shock is an example of an inducible upstream element. It interacts with a factor known as heat shock transcription factor (HSTF) in a manner analogous to SP1.(41) HSTF is found in many cells, however binding to the conserved sequence is restricted to stressed cells.(42) The conserved heat shock element (HSE) occurs multiply in a heat shock inducible promoter and can confer heat shock inducibility on a heterologous promoter.(43) In contrast to the GC box, HSTF binding sites are utilized in an ordered fashion and contribute to the transcriptional efficiency differentially.(43) Binding to the two proximal HSEs is cooperative and successive. Binding of HSTF to the HSEs does not require RNA polymerase II. Studies on heat shock typically utilize Xenopus or drosophila cells because they are more tolerant of temperature changes, however, the heat shock response is conserved throughout evolution. The drosophila genes induced by heat shock fall into three categories based on regulatory patterns. One subset of heat shock genes are expressed only in response to stress. Another subset is expressed at low levels constitutively with induction by stress. The final category includes genes which are expressed not only in response to stress but also in response to developmental cues. In an effort to address whether HSTF was involved in the developmental control of drosophila hsp26, a series of hsp26 deletion variants were transformed into

drosophila germ lines. It was demonstrated that the cis-acting DNA sequences responsible for ovarian expression of hsp26 were separate from the sequences responsible for heat induction.(44) Therefore, it seems likely that HSTF regulation of heat shock genes is limited to the stress response. The eukaryotic heat shock reponse is clearly evolved from the prokaryotic system. In E. coli, heat shock or other stress causes the appearance of damaged proteins. These aberrant proteins in some way result in expression or activation of the htpR gene product which is a sigma factor necessary for the transcription of heat shock genes under heat shock conditions. Many of the genes induced by heat shock are proteases which presumably degrade the aberrant proteins which stimulated the response.(45)

Heat shock genes are merely one group which contain inducible promoter sequences. Other genes which have well defined inducible upstream sequences include metallothionein (46), mouse mammary tumor virus (47), and class I genes of the major histocompatibilty complex (48). Metallothionein gene transcription is induced by steroids, heavy metals, interferon and lipopolysaccharide. The regulatory sequences mediating those effects have been determined for steroids, heavy metals and interferon and are distinguishable suggesting that each inducing agent acts via a separate regulatory factor.(46) The long terminal repeat of the mouse mammary tumor virus contains a sequence which stimulates transcription only in the presence of an active glucocorticoid hormone receptor.(47) Class I genes of the major histocompatibility complex respond to interferons by increasing their transcription. Interestingly, all three interferons (α,β, and γ)

act via the same upstream interferon response sequence, in spite of the fact that the three interferons utilize distinct surface receptors.(48) Many other inducible sequences have been localized to the promoter region. It seems efficient to locate regulatory elements near the loading site for RNA polymerase since it is that action which presumably is being regulated. This is of course not always the case, and the possible mechanisms of regulation from a distance will be discussed later.

If inducible response sequences are often localized to the promoter, tissue specific elements are less specifically located, with examples being found in the 3' flanking region (49), intronically (50), 5' flanking region (51) and even intragenically (52). Many tissue specific elements qualify as enhancers and will be discussed in that section. Other tissue specific elements have been termed upstream promoter elements but further investigation may reveal that they behave like enhancers. This confusion arises because of overlapping functional definitions for enhancers and upstream sequences.

One element which appears to confer tissue specificty is particularly interesting because of its ubiquitous nature. The CCAAT box is typically located about 80bp upstream of the transcription start site and deletion studies have demonstrated a deleterious effect of removal of this element.(53) Promoters from diverse genes utilize CCAAT boxes as functional upstream elements. Their precise function is unknown but long distance activation of a promoter by an enhancer requires an intact CCAAT box.(53) From this information it was believed that the

CCAAT box resembled the TATA box in that it was very common, found as a single unit, important for transcription and relatively passive in its effects. That is to say, the CCAAT box and TATA box are required for complete promoter competancy but that they are not sites important for the regulation of transcription from those promoters. Recently, a series of deletion studies on the Xenopus hsp70 gene have indicated that the CCAAT box may play a more active role in gene regulation. The hsp70 gene is transcribed constitutively in oocytes and after heat shock in both oocytes and somatic cells. The key finding was that oocyte specific expression was abolished by the removal of a CCAAT box while heat shock inducible transcription was retained. A second CCAAT box located upstream was retained. An attempt to reconstruct the promoter synthetically revealed that the combination of a duplicated heat shock element and a CCAAT box driving a thymidine kinase promoter recapitulated the transcriptional phenotype of the wild type hsp70 promoter.(54) This raises the question of how an ubiquitous conserved element may play a role in the tissue specific expression of a gene. One possibility is that the CCAAT box is bound by an ubiquitous factor which is in turn bound by a more tissue specific factor. Such higher order interactions have not yet been described in eukaryotes but they almost surely exist. A second possibility is that different tissues produce different factors binding common sequences. This has been demonstrated for the immunoglobulin octamer. In this case, an element originally detected in the promoters of all immunoglobulin genes was later found in such diverse promoters as the HSV thymidine kinase and histone H4A.(55) The octamer, important for B cell specific expression of the immunoglobulin gens, could not be performing an equivalent

function in the other genes. There appear to be two distinct factors which bind the octamer. One is found constitutively in non B cells and the other is found only in B cells.(56) It is this specific factor which interacts with the immunoglobulin octamer and confers tissue specific expression. Further investigation of the CCAAT binding factors may reveal a picture similar to that of the octamer.

Enhancers are cis-acting elements which potentiate transcription from promoters independently of orientation and distance. The initial description of an enhancer may have been by Grosschedl and Birnstiel who described an upstream element from the sea urchin histone H2A gene which was necessary for full transcriptional activity and could function in either orientation. (30) The definition and characterization of the enhancing effect fell to three labs working with the SV40 early promoter region. (57,58, 59) Sequences upstream of the promoter were found to stimulate transcription of a linked ß-globin gene. Experiments with this system have given us the now classic criteria for an enhancer: stimulation of transcription of a linked gene from the correct initiation site, stimulation independently of orientation, ability to function over long distances and the ability to function both upstream and downstream of the transcription start site. The SV40 enhancer has been extensively characterized and some small progress has been made in delineating the mechanism of the enhancing effect.

The enhancing region of SV40 contains a duplicated 72bp element which includes a core consensus sequence and a short stretch of alternating

purines and pyrimidines. The core consensus sequence is so named because it appears in a number of enhancers.(60) Not all enhancers contain this sequence but it is found commonly enough to warrant attention. Naturally occurring variants of SV40 with only one 72bp element demonstrated that the duplication was not essential for complete function.(61) Therefore, most experimentation on this region utilized SV40 variants with a single 72bp element. In an effort to delineate the structure of the SV40 enhancer, a complete dissection of that region was undertaken by two labs. The first approach utilized base specific mutagenesis by sodium bisulfite. This technique converts cytosines to uracils in single stranded DNA. By saturating a single 72bp repeat they were able to map two important regions. Mutation in either of these regions decreased transcriptional activity as measured in transient ransfection experiments utilizing a linked chloramphenicol acetyltransferase gene. Importantly, none of the mutations completely abolished activity. Mutation in the core consensus also reduced replication.(60) Their conclusions were that at least two regions were involved in establishing the enhancing effect.

The second lab to attempt characterization of the SV40 enhancer utilized oligonucleotide site directed mutagenesis. Three regions were found to be necessary for replication and transcriptional activation of a linked ß-globin gene. Two regions corresponded to those identified in the previous experiments and a third was found just upstream of the 72bp element. Although this region was not part of the 72bp element, it did contain a core consesus sequence. To identify the functional contributions of each of these regions, revertant SV40 virus was grown

out of CV-1 cells. Mutation of any one region was typically compensated by duplication of the remaining unmutated region. When all three regions were mutated, revertants consisted of double duplications in which a mutated region was duplicated with a correction of the mutation and then that corrected region was reduplicated.(62) The presence of three independent elements may seem unusually profligate but SV40 is an unusual virus in its relative lack of tissue specificity. The SV40 enhancer is similarly promiscuous. It was proposed that the presence of three elements increased the tissue range of the enhancer. Recent evidence suggests that may be the case. Competition experiments demonstrated that the activity of the SV40 enhancer required a limiting trans-acting factor.(63) Mixed competition experiments demonstrated that some enhancers share common binding factors. For example, the immunoglobulin C_{μ} enhancer can compete out factors necessary for SV40 and LPV enhancers but only in cells which utilize the C_{μ} enhancer.(64) An examination of the actual binding of factors to the SV40 72bp region by DNase I protection and DMS methylation protection revealed a slightly different pattern of binding with Hela Cell extracts compared to B cell extracts. An octameric motif found in the SV40 72bp element is bound in BJAB, a B cell line, but not Hela, an endothelial cell line. This is consistant with its utilization as a B cell specific enhancer in immunolgobulin genes. Another region near the octamer is bound consistantly in Hela but not in BJAB. The other differences relate more to the size of the protected region indicating different proteins may bind the same sequence in different tissues.(65)

Because the SV40 enhancer has been so well characterized, it has been utilized in a number of mechanistic investigations. Unfortunately, it is not yet understood how enhancers exert their effect on promoters which may lie some distance away. Several lines of investigation have given us tantalizing clues, however. After the description of the enhancer effect it seemed clear that the presence of an enhancer must somehow increase the loading efficiency of RNA polymerase II. It was however, a formal possibility that enhancers stabilized nascent ranscripts. A series of nuclear transcription assays clearly demonstrated that the SV40 enhancer increased the number of transcripts arising from a linked ß-globin gene.(66)

The next series of experiments devoted to understanding the mechanism of enhancer activity focussed on the chromatin structure of enahncers. The SV40 enhancer was found to be associated with left handed Z DNA (67), DNase I hypersensitive sites (68), and hypomethylation of cytosine bases (69). DNase I hypersensitivity has been found associated with many enhancers such as the immunoglobulin kappa enhancer (70), mouse mammary tumor virus glucocorticoid responsive enhancer (71), and the lysozyme enhancer (72). The other two characteristics have not been as thoroughly investigated. Additionally the SV40 enhancer is associated with a nucleosome free region.(73) All of these experiments suggest that there may be chromatin perturbations at the site of enhancers but give us no real insight into the mechanism of action.

Another approach to understanding enhancers involves constructs which

have been perturbed in some way to change the topological relationship of the enhancer to the promoter. Placing the SV40 enhancer on a hairpin loop did nothing to inhibit its effect suggesting that local changes in supercoiling are not the mechanism by which enhancers exert their effect.(74) Placing psoralen monoadducts between the SV40 enhancer and the ß-globin gene with plasmid sequences on the other side severely inhibited enhancer activity. Intervening vector sequences and psoralen adducts but not ß-globin sequences inhibited activity.(75) These results suggest that there is communication of some nature along the helix from enhancer to promoter. This is difficult to imagine since enhancers function over such long distances. Perhaps plasmid bound enhancers require close proximity or some element of chromatin structure to appose enhancer and promoter. This might explain why in vitro transcriptional enhancement requires the enhancer to reside close to the promoter.(76)

Finally, all mechanistic proposals must take into account the fact that enhancers may be dispensible once they have established a high transcriptional level. This was originally proposed for the immunoglobulin heavy chain enhancer where naturally occurring enhancer deletants were identified which retained full transcriptional activity.(77) The SV40 enhancer was examined using a temperature sensitive cos cell line which allowed temperature dependent replication of vectors containing an SV40 origin. They were able to demonstrate that competition for enhancer factors has no effect after 12 hours suggesting that once high level transcription is established, titration of enhancing factors off of the transcriptional unit has no effect.

Conversely, such factors are required during the establishment of such activity.(78) The 12 hour time limit is somewhat puzzling since initiation complexes are formed within minutes. It may represent a characteristic of the transfection protocol used.

The immunoglobulin heavy chain (IgH) enhancer was the first cellular enhancer described.(79) The procedures used to characterize the SV40 enhancer were applied to the immunoglobulin enhancer with similarly gratifying results. In contrast to the SV40 enhancer, the IgH enhancer is quite tissue specific. It requires factors present in B cells in order to exert its full activity.(50) Using a gel retardation assay, three binding sites were identified in the IgH enhancer region. Binding was confirmed by DMS protection studies. One of the sites contained an octamer sequence which is known to bind both B cell specific proteins and ubiquitous proteins. The other two sites bound proteins found in a variety of cells.(80) It would appear that the tissue specific nature of the IgH enhancer may be conferred by the octamer sequence. In spite of the narrow range of expression it is noteworthy that the IgH enhancer, like the SV40 enhancer, relies on multiple protein binding sites. It is this combinatorial binding which has complicated the boundary defintion of many cellular enhancers. In general, cellular enhancer regions are more diffuse than viral enhancers. That characteristic was difficult to understand until the notion of multiple binding sites was introduced.

Many other cellular enhancers have now been identified. The advent of transient transfections has allowed the easy analysis of such

enhancers. In general, such studies have merely identified the presence of an enhancing effect in a particular region of the gene. Much work is needed before an understanding of physiologic importance is gained. Enhancers in such diverse genes as insulin (81), chymotrypsin (82), lysozyme (83), elastase (84), α-fetoprotein (85), and α-crystallin (86), appear to function as tissue specific control elements but their precise contribution is not known.

The inverse of the enhancer is the suppressor or silencer. Its actions are even less well understood than those of enhancers and their actual contribution to the regulation of a gene is not well established. Their existance is implied by a number of experiments in which transcription from a normally silent gene is superinduced upon treatment with cycloheximide. This is true for c-myc expression (87), immunoglobulin kappa (88), interleukin 2 (89), and ß-interferon (90). It also seems to make sense that genes are not only controlled by positive regulatory factors. Two eukaryotic genes whose negative control elements have been characterized are ß-interferon and HMG CoA reductase. ß-interferon is highly inducible with double stranded RNA and this induction does not require protein synthesis. The upstream region of the ß-interferon gene contains a double stranded RNA inducible region. Within the inducible region is embedded a constitutive enhancer and a negative regulatory element. Deletion of the negative regulatory element leads to high level expression in the absence of an inducer. Consistant with this are results from DNase I footprinting studies which demonstrate a factor bound to the negative control region in uninduced cells which is not present in induced

cells.(91)

HMG CoA reductase is an example of a gene with an inducible negative control element. Sterols suppress HMG CoA reductase transcription by 70-90% in L-cells. The sequence responsible for this suppression is found embedded in the promoter region of this gene.(92)

Two other examples of negative control are the rat α-fetoprotein gene which combines a tissue specific negative control element with a tissue specific enhancer. The negative control element is able to confer down regulation on a heterologous promoter.(93) The final example of negative control concerns adenovirus 2 E1A products which appear to repress the activation of transcription by SV40, polyoma, IgH and adenovirus enahncers.(94) This activity may have evolved to control E1A transcription during a lytic infection but its effect on heterologous enhancers is more difficult to imagine unless a common binding site is involved.

This brief survey of gene regulation has demonstrated the many networks involved in the regulaton of eukaryotic genes. Even single functional units such as enhancers may represent the combined effects of several trans-acting factors. There remains the possibility that higher order protein-protein interactions may play a role in gene regulation. New assays will have to be devised to unravel that problem.

MHC CLASS I REGULATION

MHC class I antigens were originally identified as transplantation antigens but are now known to act principally as restriction elements for cytotoxic T cells which lyse virally infected targets. They are composed of a polymorphic 43Kd α chain and a constant 12Kd ß chain which is not encoded in the major histocompatibility complex. In man the major antigens are known as HLA-A, B and C and are encoded on chromosome six.(95) Other members of the class I antigen family constitute a nebulous Qa/Tla related grouping whose function is unknown. Virtually all cells are susceptible to viral infection and for this reason it is necessary that the expression of class I antigens be constitutive. In fact, after gastrulation all nucleated cells express class I antigens. However, not all cells express equivalent densities. Most cells express low levels with a significant capacity for induction by α, ß, or γ interferons.(96) B cells and T cells exhibit a much higher baseline expression and are correspondingly less inducible.(97) Aside from tissue specific differences and regulation by interferons, a third level of control appears to be mediated by mitochondria in certain inbred mouse strains. The structural gene for a class I protein called MTA (maternally transmitted antigen) is located in the Tla region of the major histocompatibility complex but its expression is determined by the inheritance of a mitochondrially encoded gene named mtf (maternally transmitted factor).(98) The mechanism and purpose of this regulatory strategy remain to be identified.

The major effector of regulation of MHC class I antigens appears to be the lymphokine family of interferons. Leukocyte (IFN-α), fibroblast (IFN-ß) and immune (IFN-γ) interferons all have antiviral activities. They also inhibit cell growth, alter the intrinsic charge of the cell membrane, induce 2'-5' oligoadenylate synthetase and increase class I antigen expression.(96, 99) The kinetics of class I antigen induction by any of the three interferons are coordinate and remarkably quick.(100) Steady state message levels are increased five minutes after interferon exposure and are about five-fold increased within one hour of exposure. The message level remains high for approximately 24 hours. The kinetics of 2'-5' oligoadenylate synthetase are very similar.(100) Interestingly, when interferon resistant lines are isolated on the basis of their escape from interferon mediated growth inhibition, they are also resistant to class I antigen induction and the antiviral effects of interferon.(101) This suggests that all three activities share a common induction pathway and that all three interferons must act on that same pathway.

IFN-α is known to induce the expression of 10-15 new proteins. Induction of transcription of all of these genes appears to be coordinate, however there are large differences in dosage sensitivity. It was hypothesized that some of the genes for these proteins might share regulatory regions important for their induction by IFN-α. Comparisons of HLA-DRα, metallothionein II (MT2), and two MHC class I genes, revealed a conserved sequence. The core of this consensus is AGTTTCTCC/TTCTC and it is found approximately 145bp upstream of the TATA box in the two class I genes and approximately 600bp upstream in

DRα and MT2.(100)

Dissection of this promoter region in the mouse H-2K^b gene revealed that the sequence identified by computer analysis was indeed required for IFN-α responsiveness. Not surprisingly, that sequence was also required for IFN-β and IFN-γ induction in transient assays.(102) The interferon responsive sequence (IRS) slightly overlaps an element which independently fulfills the criteria for a tissue specific enhancer.(103) Both the IRS and enhancer are required for function and together they can confer interferon responsiveness on a heterologous promoter. Neither the SV40 enhancer nor a different class I enhancer can substitute for the IRS associated enhancer. A class I gene, Q10, from the Qa region contains an IRS but has a severely altered IRS associated enhancer. The Q10 enhancer alone is five times less active than the corresponding sequence from H-2 K^b in transient assays and the Q10 upstream region is unable to confer interferon responsiveness on a heterologous promoter.(102) This suggests that a functional IRS associated enhancer is required for the full function of the interferon responsive sequence. MT2 and DRα genes which contain the IRS do not have any sequences corresponding to the IRS associated enhancer. Significantly, DRα is not IFN-α inducible and MT2 is inducible only with high concentrations of IFN-α.

This functional dissection appears to offer an explanation as to why all three interferons have such a similar effect on MHC class I induction. Their action on a single cis-acting element would result in the same profile of induction. The tissue specific differences in the

level of expression may relate to the function of the IRS associated enhancer. Many enhancers exhibit tissue specificity and if either of the two H-2 K^b enhancers function more efficiently in lymphoid cells, the baseline expression would be significantly increased. These results may also offer new insight into the bare lymphocyte syndrome. This syndrome is described in chapter 1 of this thesis.(104) Briefly, it consists of an inherited immunodeficiency coupled with a lack of expression of MHC class I antigens. This defect in expression was limited to T cells, B cells, and macrophages, with platelets and granulocytes expressing normal levels. On the basis of northern blots demonstrating parallel decreases in MHC class I and β_2 microglobulin messages in affected cells, we postulated a defect in a shared trans-acting factor required for high level expression in those tissues. Low level constitutive expression appeared to be preserved. This trans-acting factor may act on the IRS associated enhancer although the sequence is not perfectly conserved in β_2 microglobulin. Possibly different tissues produce different proteins which interact with the enhancer. An alternative hypothesis is that lymphoid cells may modify a trans-acting factor such that it exerts a more potent effect.

MHC CLASS II GENE REGULATION

The class II region of the major histocompatibilty complex (MHC) was first defined in the early 1970s as a region controlling immune responsiveness to certain polypeptide antigens.(105) Since then it has been shown to be coincident with a region which determines reactivity in mixed lymphocyte reactions.(106) This same region is also felt to play a role in the susceptability to certain diseases.(107) A fourth function for this region was delineated in 1975 when mixing studies demonstrated MHC class II restriction for antigen presenting cell- T cell and T and B cell interactions.(108, 109) It is not surprising that a set of genes with such protean manifestations has been subjected to intense scrutiny in the ensuing years. Mixed lymphocyte reactivity and disease susceptibilty have been found to be primarily determined by the inherited haplotype. Immune responsiveness is also determined primarily by the inherited haplotype but certain F_1 nonresponder mice express a responder haplotype at greatly reduced levels suggesting that quantitative variation is important in immune responsiveness.(110) Antigen presentation and T cell help assays clearly correlate class II antigen density with efficient function.(111) Loss of quantitative control of class II antigen expression is believed to play a role in a variety of autoimmune diseases (112) and certain maternal immune responses directed against fetal tissues (113). In contrast, patients with MHC class II deficient combined immunodeficiency (bare lymphocyte syndrome II) express low or undetectable amounts of class II antigens and are unable to mount effective immune responses.(114) Therefore the study of MHC class II gene regulation is central to the problem of

immune responsiveness. The second chapter of this thesis contains a more detailed review of recent contributions to the field of MHC class II gene regulation. The third chapter presents data suggesting that tissue specific expression of class II antigens relies on intronic enhancers which function in class II positive cell lines. This is reminiscient of the regulation of immunoglobulin genes discussed earlier. The final chapter studies the physiologic importance of these intronic enhancers by demonstrating loss of activity in a class II negative mutant cell line. The implications of these studies on our understanding of gene regulation is also discussed.

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MOLECULAR ANALYSIS OF THE BARE LYMPHOCYTE SYNDROME[1]

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Running Title: The Bare Lymphocyte Syndrome

ABSTRACT The Bare Lymphocyte Syndrome is a disorder in which Class I histocompatability antigens fail to be expressed normally on the surface of lymphocytes. Utilizing cDNA probes for both B_2 microglobulin and Class I genes, the molecular basis for this syndrome was investigated in a family with two siblings exhibiting the Bare Lymphocyte Syndrome. Southern blot analysis demonstrated no gross internal defect in either Class I or B_2 microglobulin genes. Northern blot analysis of Class I and B_2 microglobulin messenger RNAs also revealed no qualitative difference between affected and unaffected family members. In contrast, quantitation of both Class I and B_2 microglobulin transcripts demonstrated each to be decreased in patients when compared to controls. Moreover, the decrease in both transcripts was coordinate. These results suggest that the Bare Lymphocyte Syndrome may represent a pretranslational regulatory defect of both Class I and B_2 microglobulin gene expression.

INTRODUCTION

The Bare Lymphocyte Syndrome is defined as a syndrome in which a patient's lymphocytes cannot be tissue typed using standard serological cytotoxicity tests. Touraine desribed a child presenting with severe combined immunodeficiency in the initial case report of 1978.(1) Since then, several forms of the syndrome have been identified. A defect in Class I antigen expression has been described with and without concomitant severe combined immunodeficiency. (2-7) Several case reports of a Class II defect have appeared recently. This Class II defect may be isolated (8) or associated with a defect in Class I expression (9,10,11,) and has a variable phenotype. We have previously described two siblings without immunodeficiency who exhibit the Bare Lymphocyte Syndrome. (7) Analysis of these patients may help clarify the factors involved in the regulation of cell surface expression of Class I antigens and may contribute to our understanding of Class I antigen functions. Here, for the first time is a demonstration of a pretranslational defect in both Class I and B_2 microglobulin gene expression associated with the Bare Lymphocyte Syndrome.

METHODS

Cells Family "L", previously described (7), was used as the sole source of "bare lymphocytes" for the experiments described. An affected child (AC2, see Fig. 1) was identified during routine tissue typing in consideration for bone marrow transplant as treatment for aplastic anemia. At that time, another affected child (AC4) was also identified as having the Bare Lymphocyte Syndrome. Fresh peripheral blood mononuclear cells, consisting of lymphocytes and monocytes (PBMC) were isolated through a Ficoll Hypaque gradient. Fresh circulating leukocytes (lymphocytes, monocytes and granulocytes) were isolated in 1.5% dextran (200,000mw). EBV transformed lymphocytes from the family members were continuously maintained in RPMI 1640 supplemented with 10% fetal calf serum and the usual antibiotics.

Northern and RNA Dot Blot Analysis Three probes were utilized in these analyses. The B_2 microglobulin probe (gift of K. Itakura) is a 550bp cDNA clone.(12) The Class I probe (gift of S. Weissman) is homologous to HLA B7 but cross hybridizes to all Class I genes.(13) The Actin probe is a 1.5kb cDNA clone (gift of P. Gunning).(14) The gift of ribosomal cDNA was generated from L cells by Andree Dozy. RNA was isolated by the guanidinium method.(15) For the Northern blot in figure 2, 25 micrograms of total RNA was isolated from the EBV transformed lines, denatured in formaldehyde as previously described (16,17) and applied to a .8% agarose gel. For the Northern blot in figure 5, 5 micrograms of total RNA isolated from PBMC was used. The gel was photographed using a silica TLC plate to identify the positions of the 18s and 28s bands. Blots were baked and incubated with a 50% formamide solution at 42°, as previously described.(17) 50ng of

probe, nick translated to a specific activity of 10^8 cpm/microgram was denatured by boiling and added to 10ml of fresh formamide solution. After 24 hrs incubation the blots were washed in .1x SSC, .1% SDS at 55° as previously described.(17) Northern dot blots utilized total RNA isolated as above from fresh total white cells. For the dot blots, 1 microgram of RNA was denatured as above. Two fold dilutions of RNA were made into microtiter wells with 20x SSC. The RNA was then applied to nitrocellulose and probed as above.

Fluorescent Analysis Three antibodies from Bectin Dickinson (Mountain View) were used in the identification of subpopulations of PBMC. Leu1 (clone L17F12) depicts most T cells.(18) DR (clone L243) specific antibody binds nonpolymorphic regions of Class II molecules.(19) The M3 (clone Mϕ P9) antibody is specific for 70 - 90% of mature forms of circulating monocytes.(20) These three antibodies are linked to phycoerythrin, which emits at a wavelength distinct from that of fluorescein isothiocyanate (FITC) and allows simultaneous two color analysis.(21) Two other antibodies were used to analyze the display of Class I antigens on the surface of PBMC. Anti B_2 microglobulin (clone L368) linked to FITC was purchased from Bectin Dickinson.(19) W6/32 is a monoclonal antibody which recognizes a nonpolymorphic determinant on Class I molecules.(23) Fluorescent analysis using W6/32 was performed using an indirect immunofluorescent technique. Flow cytometry was performed on a FACS IV as previously described. (21, 23).

RESULTS

To examine the structure of the Class I and the B_2 microglobulin genes, Southern blots were prepared from DNA isolated from the family members PBMC. Southern blot analysis demonstrated no gross internal rearrangements in either the Class I genes or the B_2 microglobulin genes of the affected children when compared to the normal siblings or the parents. (Data not shown and ref. 7.) In an effort to analyze the transcripts from these genes, RNA was isolated from the EBV transformed cells of the family. Six weeks after EBV transformation, "bare lymphocytes" have been found to have normal Class I surface expression. (5, 7) Hybridization of Northern blots with the B_2 microglobulin probe revealed bands approximately one kilobase in length for all family members. (Fig. 2) There was no qualitative difference between the affected and the unaffected family members. When the Northern blots were hybridized with the B7 probe, specific for Class I antigens, a single band approximately 1.7 kb in length was found for all family members. (Fig. 2) Therefore, it appears that neither the genes nor the transcripts for Class I and B_2 microglobulin are qualitatively abnormal in the Bare Lymphocyte Syndrome.

Quantitative analysis by Northern dot blots was undertaken in order to evaluate the levels of Class I and B_2 microglobulin transcripts in the circulating leukocytes from the affected and unaffected family members. One microgram of total RNA isolated from the white cells of the family members was dotted onto nitrocellulose. This dot blot was then hybridized with either the Class I, B_2 microglobulin or an actin control probe. (Fig. 3) Class I and B_2 microglobulin transcripts were found to be 1.5 to 3 fold lower in the

affected children than in the unaffected family members. Actin transcripts were only slightly decreased. Thus, the Bare Lymphocyte Syndrome is associated with a quantitative defect in the number of Class I and B_2 microglobulin transcripts.

Decreased levels of RNAs specific for Class I and B_2 microglobulin could indicate a general diminution among all cells or represent the averaging of different levels among subpopulations of cells. The density of Class I antigens on subpopulations of mononuclear cells was analyzed by flow cytometry. PBMC from the affected family members stained with either Class I or B_2 microglobulin monoclonal antibodies exhibited a density of each molecule which was ten to twenty percent of that seen in unaffected family members or unrelated controls. Class I and B_2 microglobulin staining on platelets identified by forward light scatter demonstrated no difference between affected and unaffected family members (data not shown). Specific subpopulations of cells were identified by means of phycoerythrin labeled antibodies (i.e. Phycoerythrin Leu 1, Phycoerythrin DR, and Phycoerythrin M3) and the relative Class I and B_2 microglobulin density on the cells of each subpopulation was determined using FITC linked antibodies. (Fig. 4) In an affected child, Class I and B_2 microglobulin specific fluorescence associated with the T cell population was 10.5 fold less than the mother or an unrelated control. Staining in the DR and M3 positive population was 6 fold less in the affected child than in the control.(Table 1)

These findings suggest that not all cells are equally affected and that RNA levels might correspond more closely to surface expression if only affected cells are analyzed. A Northern blot analysis of PBMC

consisting of just lymphocytes and monocytes demonstrated a ten fold decrease of Class I levels in an affected child when densitometry levels were normalized to ribosomal RNA levels. Once again, Actin levels were slightly decreased in the affected child, but only two fold. (Fig. 5) Therefore, transcript levels associated with PBMC are ten fold decreased compared to controls, whereas leukocyte transcript levels were only threefold decreased.

DISCUSSION These experiments have demonstrated a pretranslational defect in Class I and B_2 microglobulin expression. This defect is differentially expressed in different subpopulations of cells and it is possible that only mononuclear cells are affected. When Class I and B_2 microglobulin transcript levels are analyzed from total leukocytes they are found to be only 1.5 to 3 fold decreased. Mononuclear cell transcripts, on the other hand, are ten fold decreased in the affected children. This suggests granulocyte expression may be normal in these children. Platelet expression was found to be normal and others have reported normal Class I surface expression on fibroblasts granulocytes and platelets. (1-6, 24)

Both Class I and B_2 microglobulin specific transcripts were found to be decreased in the mononuclear cells of these affected children. Since these genes reside on chromosomes six and fifteen, respectively and since EBV reverses the defect, it is unlikely that mutations in either gene could account for our findings. Instead, it is likely that the Bare Lymphocyte Syndrome represents a defect in the regulation of Class I and B_2 microglobulin gene expression. A related syndrome with a Class II defect is also thought to represent a defect in regulation. (9)

That these genes share common regulatory pathways is suggested by the finding that they are coordinately expressed in development. Class I and B_2 microglobulin antigens are absent from fertilized mouse eggs but appear later in development.(25, 26) This developmental control is reproduced in embryonal carcinoma (EC) cells.(27) EC cells do not express Class I antigens on their surface nor do they have detectable levels of transcript for either gene.(28, 29, 30) Differentiation of

these cells is accompanied by coordinate accumulation of messenger RNAs and surface expression of Class I molecules.(27) From these experiments it is clear that Class I and B_2 microglobulin genes are simultaneously induced during development.

Experiments investigating the regulation of expression of Class I and B_2 microglobulin genes in a mature, expressing cell, also suggest that Class I and B_2 microglobulin genes share common regulatory pathways. Lymphoblastoid and melanoma cell lines as well as PBMC have been induced to increase their Class I and B_2 microglobulin surface expression after administration of interferon.(31, 32, 33) The sequences responsible for the regulation of Class I transcription may reside in the 5' flanking regions of those genes. In transfection experiments, interferon was shown to increase Class I surface expression, when the 5' flanking region was included and this increase was correlated with an increase in the number of Class I transcripts. (34, 35)

These experiments suggest that Class I and B_2 microglobulin genes share common regulatory pathways. The Bare Lymphocyte Syndrome could represent a defect at some step leading to coordinate gene expression. First, it could represent an abnormality in interferon production or its tissue specific effects. In patients with combined Class I and Class II defects, alpha interferon was able to restore Class I expression. (4, 36) Preliminary experiments in which α interferon was administered to isolated "bare lymphocytes" failed to reverse the defect (data not presented). However, B cells and monocytes express normal levels of Class II molecules, making a defect in γ interferon unlikely. Secondly, a synthetic or structural

abnormality in the α or ß interferon receptor could make "bare lymphocytes" refractory to those lymphokines. Thirdly, the effector molecules that mediate the intracellular effects of interferons may be defective. These may include DNA binding proteins that recognize regulatory sites flanking the Class I and B_2 microglobulin genes. If these tissue specific enhancers of transcription are shared between these genes, one could postulate a lesion in a single protein that binds a specific DNA sequence. The low levels of Class I and B_2 microglobulin transcripts found in these patients may be products of a constitutive pathway unaffected by the binding protein or the effect of a defective binding protein with a lower affinity. Finally, although the "bare lymphocytes" display normal lymphocyte and monocyte differentiation markers, it is possible that the Class I and B_2 microglobulin genes are not fully differentiated in these cells.

The levels of Class I and B_2 microglobulin transcripts in the "bare lymphocytes" were approximately tenfold lower than in normal controls. The levels of surface Class I molecules were from six to ten fold diminished when affected and unaffected family members were compared. Actin levels were slightly decreased compared to controls and this may reflect altered metabolism in these cells. Therefore a pretranslational defect in Class I and B_2 microglobulin certainly constitutes a major factor in the Bare Lymphocyte syndrome but at this time is not possible to rule out other effects. Lastly, it should be stressed that the defect observed in this family with the Bare Lymphocyte Syndrome without immunodeficiency may not reflect that found in other cases of the Bare Lymphocyte Syndrome with immunodeficiency. Only through further study of this and similar disorders will the true

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FIGURE LEGENDS

Figure 1.

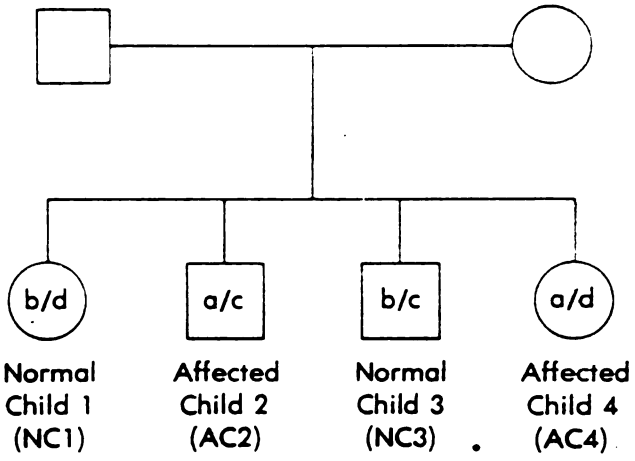
Pedigree. Family "L" consists of the unaffected mother and father (M, F), two unaffected children (NC1, NC3), and two affected children (AC2, AC4).

Figure 2.

Northern blot of transcripts isolated from EBV transformed cells of family "L". 25 microgram samples of total RNA were electrophoresed on a Northern formaldehyde gel to assess transcript length differences in the affected children. Shown in this figure are bands hybridizing with Class I and B_2 microglobulin (B_2 μ) specific probes for two unaffected family members (M, NC1) and two affected family members (AC2, AC4). There are no apparent differences in transcript length when affected are compared to unaffected.

Pedigree of the Family

Father					Mother				
a	A2	Cw4	Bw35	Bw6 DR3	c	A3	C-	B7	Bw6 DR4
b	A2	C-	Bw44	Bw4 DR4	d	Aw24	Cw6	Bw57	Bw4 DR7



NORTHERN BLOT ANALYSIS OF CLASS I AND β_2 MICROGLOBULIN TRANSCRIPTS

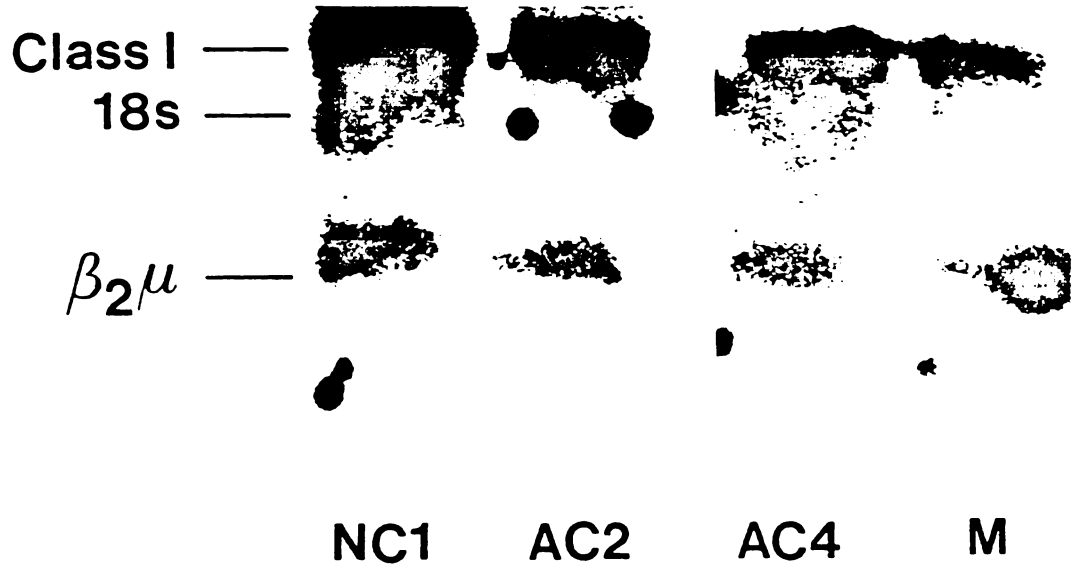


Figure 3.

Quantitative analysis of Class I and B_2 microglobulin transcripts. RNA was isolated as described from circulating leukocytes and dotted onto nitrocellulose in twofold dilutions of 1 microgram of RNA. This blot was probed with CLass I, B_2 microglobulin (B_2 μ) or actin specific probes as indicated.

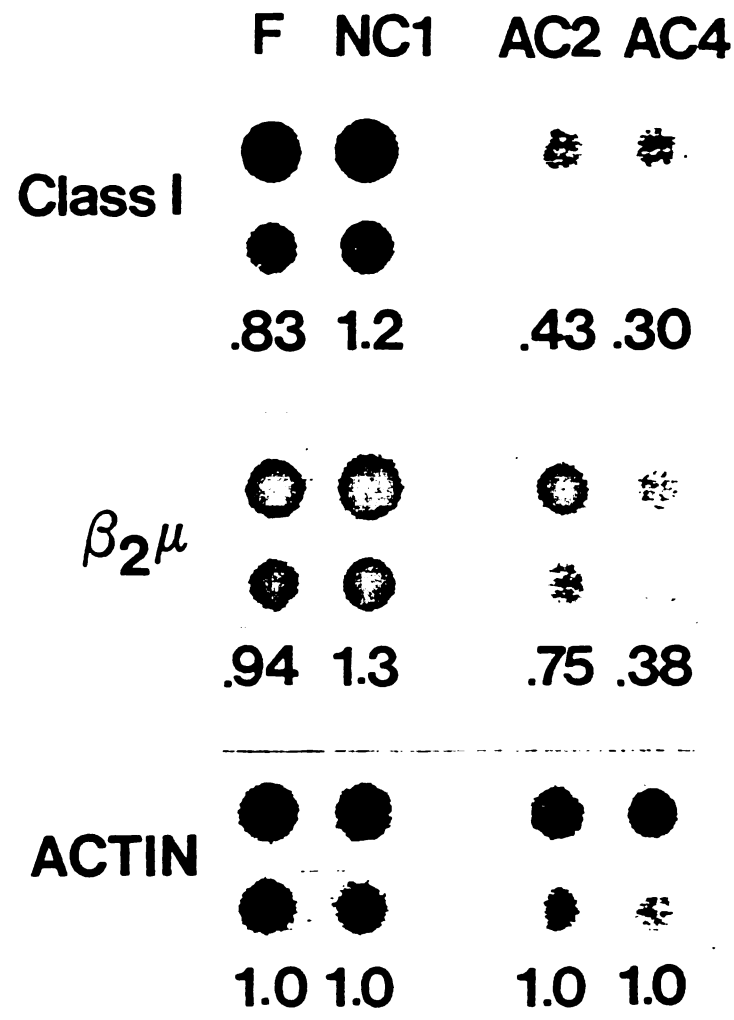
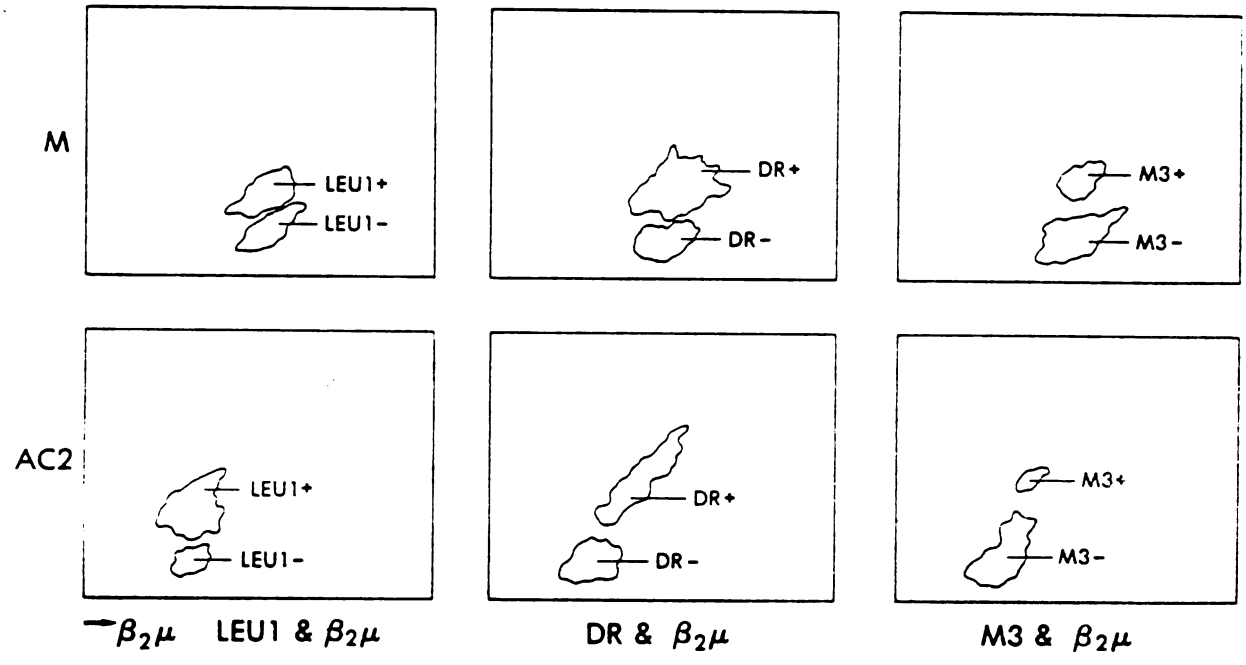

F
NC1
AC2
AC4
Class I
.83
1.2
.43
.30
$\beta_2\mu$
.94
1.3
.75
.38
ACTIN
1.0
1.0
1.0
1.0

Figure 4.

Two color Fluorescent cytometry analysis of B_2 microglobulin expression on peripheral blood mononuclear cell subsets. This figure compares B_2 microglobulin staining of an unaffected family member's PBMC subsets (M) and those of an affected family member (AC2). The vertical axis depicts staining with either Leu 1, anti- DR or M3 as indicated. Therefore, subsets are separated on the vertical axis, with the uppermost population staining positive with the indicated antibody. The horizontal axis depicts staining with anti- B_2 microglobulin. All subsets in the affected children exhibit decreased staining with anti- B_2 microglobulin with the greatest decrease apparent in T cells depicted by Leu 1. This is quantified in Table 1.

Figure 5.

Northern Blot and Densitometry of Transcripts Isolated from the PBMC (Lymphocytes and Monocytes) of Three Family Members. Shown in this figure are bands hybridizing to the Class I specific probe from two unaffected family members (M, and NC1) and one affected family member (AC2). Class I specific transcripts are 10 fold less abundant in the affected child when normalized to ribosomal RNA levels. The densitometry table also reveals a slight decrease in actin transcript levels in the affected child. Notice the apparent difference in M and NC1 Class I levels disappears when densitometry levels are normalized to correct for loading error.


M
AC2
LEU1+
LEU1-
DR+
DR-
M3+
M3-
LEU1+
LEU1-
DR+
DR-
M3+
M3-
$\beta_2\mu$
LEU1 & $\beta_2\mu$
DR & $\beta_2\mu$
M3 & $\beta_2\mu$

DENSITOMETRY ANALYSIS

	M	NCI	AC2
CLASS I	8.8	6.3	1.0
ACTIN	7.5	5.2	3.5
RIBOSOMAL	4.5	3.1	5.1
$\frac{\text{CLASS I}}{\text{RIBOSOMAL}}$	1.95	2.05	.19
$\frac{\text{ACTIN}}{\text{RIBOSOMAL}}$	1.67	1.67	.69

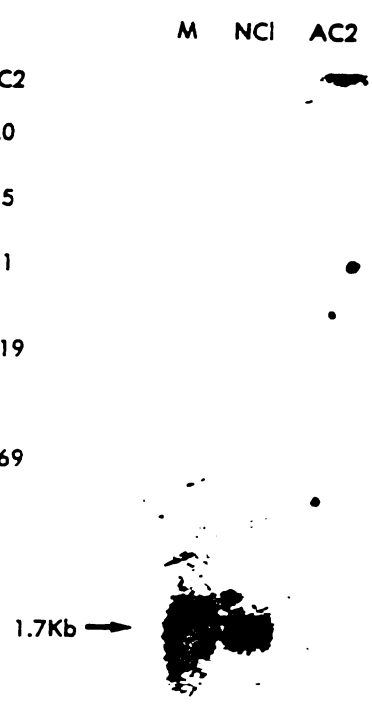


Table 1

PEAK CHANNEL VALUES FOR PBMC SUBPOPULATIONS

	CELL SURFACE MOLECULE DETECTED		
	Leu1	DR	M3
SOURCE:			
Unrelated control	140	139	138
Mother	134	133	134
AC2	76	100	102

In this table, values are given for the peak channel of fluorescence corresponding to the intensity of B_2 microglobulin staining. Different subpopulations of cells were detected with a counterstain. Leu 1 depicts most T cells, DR depicts B cells and monocytes, and M3 depicts mature monocytes. Here, the intensity of B_2 microglobulin staining in the Leu 1 population can be seen to be much less in the affected child (AC 2) than in the mother (M). On this scale 57 channels corresponds to roughly a ten fold difference in fluorescent intensity. Thus, in the Leu 1 population the affected childs cells express only one tenth the density of B_2 microglobulin as the Mother. In the DR + and M3 population, the affected childs cells express approximately six fold less B_2 microglobulin than the unaffected Mother.

A MODEL FOR THE TRANSCRIPTIONAL REGULATION OF MHC CLASS II GENES

K. E. Sullivan and B. M. Peterlin

INTRODUCTION

Class II antigens of the major histocompatibility complex (MHC class II, Ia) are a polymorphic group of molecules required for the presentation of antigen to T lymphocytes. In humans these molecules are encoded on the short arm of chromosome six and consist of at least three α ß heterodimers known as DP, DR and DQ. The α and ß chains associate noncovalently at the cell surface and contain membrane proximal immunoglobulin like domains. Assembly of the α and ß chains appears to involve an additional molecule called the invariant chain (Ii) which is coregulated with class II molecules but encoded on a separate chromosome (1). In mice the MHC class II genes are located on chromosome 17 and encode two heterodimers known as IE and IA (2). Regulation of class II molecules appears to be largely transcriptional although the T cell lymphokine γ-interferon (IFN-γ) may act through additional mechanisms (3,4). Different cell types exhibit different patterns of regulation. There are essentially three phenotypes with respect to MHC class II gene expression: non-inducible, inducible and constitutive. Many cell lines are class II negative and are not inducible by any known lymphokine. Monocytes and certain other human cells which are class II negative can be induced with IFN-γ to transiently express class II molecules (5). Human T cells express class II molecules after activation; however, murine T cells do not exhibit this phenomenon (6). Mature B cells constitutively express high levels of these antigens.

Regulation of MHC class II antigens on the surfaces of antigen

presenting cells plays an important role in an evolving immune response (rev. 7). Different class II antigens are expressed at different stages in B cell ontogeny and monocyte maturation (8). DP and DR antigens are expressed earlier than DQ and because certain antigens are presented more efficiently in the context of DQ, this may serve to restrict the type of cell involved in a particular immune response (9). Quantitatively, the level of MHC class II expression on the surface of antigen presenting cells correlates with the efficiency of antigen presentation (10). Therefore, an understanding of the regulation of MHC class II gene expression will help define the factors involved in normal and abnormal immune responses. In this paper, a model will be presented to account for the tissue specific expression and IFN-γ inducible expression of MHC class II antigens.

STRUCTURAL STUDIES

The human class II genes comprise a closely related family of at least three α chain genes and three ß chain genes. (Fig. 1A) Each gene consists of five exons with the first exon encoding the leader peptide, the second and third exons encoding the external domains, the fourth exon encoding the transmembrane and cytoplasmic domains and the fifth exon encoding the untranslated 3' sequences. (Fig. 1B) (1) The α and the ß chain genes are evolutionarily related and have similar promoters and membrane proximal domains (11). The class II promoters exhibit several features that are believed to be important in gene regulation. In Figure 1C a consensus structure for class II promoters developed by Kelly and Trowsdale, includes three conserved sequence elements (11). A CCAAT box which is found upstream of many transcription start sites is found in MHC class II promoters at about -50 in relation to the cap site. DRα, IEα and DQα do not contain a CCAAT box, but in the same location contain an element found in the immunoglobulin heavy and light chain promoters called the immunoglobulin octamer (12). Two other consensus sequences, which we will call MHC class II consensus elements A and B, are found upstream of all class II genes as well as H2A histone genes. They are located near positions -70 and -90 respectively. Their function is unknown, however their strict sequence conservation suggests an important role in the expression of class II genes.

The role of DNA methylation in the regulation of class II genes has

been investigated. In many systems a correlation between hypomethylation of a gene and its expression has been established. Initial studies found that certain cell lines expressing DRα have hypomethylated regions within the DRα gene compared to nonexpressing cell lines (13). However, extensive mapping of many potential methylation sites in various cell lines revealed no correlation between DNA methylation and DRα expression. Instead, it appears that DRα is demethylated in a region around the promoter in all cell lines regardless of DRα expression (Fig. 1D) (14). However, the 5' flanking, exons 2-5, and 3' flanking regions of the DRα gene are methylated. Demethylation of a region may specify a gap in the nucleosomal array that renders DNA accessible to binding by regulatory proteins (15). Therefore, the methylation analysis suggests that the DRα promoter is accessible in many cell lines. This hypothesis is further supported by work with pancreatic deoxyribonuclease I (DNaseI).

Chromatin structure analysis of the DRα gene revealed DNase I hypersensitive promoter sites in all lymphoid (B and T lineage) and nonlymphoid cell lines investigated, confirming that the promoter is accessible in many different cell lines regardless of DRα transcription (16). DNase I hypersensitive sites, like demethylated regions, are believed to represent local interruptions in the nucleosomal arrays (15). For several genes, an association between DNase I hypersensitive sites, and binding of regulatory proteins to enhancers has been demonstrated. In SV40, the enhancer binds several proteins in a nucleosome free region which can be shown to be DNase I hypersensitive (17, 18). A similar relationship has been demonstrated

for the glucocorticoid dependent enhancer of the mouse mammary tumor virus (19). Therefore, the finding of DNase I hypersensitive sites within a gene is suggestive of protein-DNA interactions at that site. Such interactions occur in promoters and enhancers as the regulatory proteins exert their effects. Examination of the DRα gene revealed the striking finding that constitutive expressors of DR exhibit a different chromatin configuration than cell lines which have been induced to express DR with IFN-γ. Cell lines which have been induced to express DR with IFN-γ exhibit only the promoter associated hypersensitive site. However, constitutive expressors of DRα have three hypersensitive sites, suggesting that three regulatory regions may exist in the DRα gene. One site maps near the promoter and the other two sites to the first intron (Fig. 1E) (16). This suggests that IFN-γ mediated induction may utilize a distinct set of regulatory regions to control MHC class II gene expression than those utilized by constitutively expressing cells.

These structural studies lead to the conclusion that the DRα promoter is accessible in many cells and that the induction of class II gene transcription by IFN-γ may not require binding of regulatory factors in the first intron. However, constitutive expression of class II does seem to rely on these intronic binding factors. Therefore, a minimum of three regulatory regions controlling the expression of DRα can be postulated.

CIS- AND TRANS- ACTING ELEMENTS

An examination of MHC class II deficient mutants sheds light on the trans- acting regulatory factors required for MHC class II gene expression. Three mutant lines have been characterized. They fall into two groups based on their expression of the class II associated invariant chain (Ii). A mouse Ia negative B cell line, R8205, expresses neither IA, IE nor Ii. Administration of IL-4 but not IFN-γ rescues the defect and leads to a high level of Ia expression (20). IL-4 is known to increase MHC class II expression in resting murine B cells and may lead to B cell differentiation (21). In contrast, both human Ia negative mutant B cell lines, 6.1.6 and RJ 2.2.5, are Ii positive. Whereas 6.1.6 can be rescued by fusion with either Ia positive B cell lines or Ia negative T cell lines (22), RJ 2.2.5 can be rescued only by fusion with Ia positive B cells or splenocytes (23). The high rate of revertants in 6.1.6 make the data difficult to interpret, however it appears that 6.1.6 and RJ 2.2.5 represent different complementation groups. The administration of IFN-γ has no effect on these mutant cell lines and the effects of IL-4 are unknown. In addition, the gene encoding the trans-acting factor defective in RJ 2.2.5 has been mapped to mouse chromosome 16. (24) Since these three mutant cell lines exhibit different phenotypes, it is possible that they represent mutations in three different factors required for constitutive expression of MHC class II genes. It is not clear whether these three trans-acting factors are related to the three potential regulatory sites identified by DNase I analysis. DNase I analysis of the mutant cell lines will help clarify the relationship.

The use of transgenic animals has enabled the investigation of MHC class II promoter structures in a cellular environment which mimics that of the native state. In mice lacking IE expression, the introduction of Eα into the germline led to tissue specific and IFN-γ regulated expression (25). Whereas constructions using 2 kb of 5' flanking information completely reconstituted IE expression, truncations of the 5' end to 1400bp abolished B cell expression while retaining expression in the thymus, spleen, and peritoneal exudate cells (26). This suggests that distant 5' upstream elements play an important role in IE regulation and that multiple elements may control the tissue specific expression of MHC class II genes.

In an effort to study cis-acting elements, transient transfection experiments using fragments of class II genes linked to a reporter gene have been utilized. The effect of the various fragments on the transcription of the linked gene can be measured in different cell types and in the presence of various inducing agents. We have systematically examined 30 kilobases of the DQ complex for the presence of cis-acting elements able to stimulate transcription of the chloramphenicol acetyltransferase gene from a thymidine kinase promoter. Using such techniques we have recently defined in the DQα gene a distinct promoter element which stimulates transcription in lymphoid cell lines as well as two intron associated tissue specific enhancers (27). Similarly, the DRα gene contains a complex of two intronic enhancers which correspond to the DNase I hypersensitive sites (27). Enhancers are genetic elements which are able to stimulate

transcription of a linked gene in a position, orientation and promoter independent fashion. The DQß gene also contains a tissue specific intronic enhancer (27). These enhancers function well in lymphoid cells, but not in nonlymphoid cells. They are not responsive to IFN-γ.

This type of analysis has also been used to dissect the functional elements in MHC class II promoters. Two separate elements within 1 kb of DRα 5'flanking sequence were found. An element responsive to IFN-γ can be functionally isolated from another element which seems to control constitutive transcription. The responsive element functions only in inducible cell lines (28). An element identified in the 5' flanking region of the IEß gene by stable transfection assays has yet to be characterized in terms of function (29). It may be related to the B cell specific element identified in IEα by analysis with transgenic mice (26). Therefore, transfection assays have identified upstream and intronic enhancers in several class II genes and a promoter associated IFN-γ responsive sequence which functions in inducible cells. The intronic and promoter enhancers appear to control tissue specific and constitutive expression of class II genes while the IFN-γ responsive element allows for inducible expression of class II genes.

MODEL

These data lead to the following model for MHC class II gene expression. Here, we assume that murine and human class II genes are regulated similarly. The structural and functional data obtained from various class II genes has been assimilated into a model for an MHC class II regulon.

The three constitutive control elements defined functionally in transient transfection assays map to the same location as the three DNase I hypersensitive sites in B cell lines. This suggests that in Ia positive B cells the three constitutive control elements are bound by regulatory factors. This situation is depicted in Figure 2A. DP and DR genes may bind the regulatory factors more avidly than DQ, explaining why they are expressed earlier in ontogeny and at higher levels than DQ. Each of the elements defined functionally may actually bind one or more regulatory proteins. For example, the immunoglobulin heavy chain enhancer and the SV40 enhancer each bind multiple proteins in order to exert their effects (30, 31). For simplicity however, we will assume that each of these elements binds a single factor. Therefore, in B cells which transcribe class II genes constitutively, the two intronic enhancers and the upstream enhancer are bound by regulatory proteins. The distant upstream elements identified in IEα and IEß genes may also be elements controlling constitutive tissue specific expression.

The binding pattern in Ia negative T cells is less well defined. From

the DNase I data, it would appear that only the upstream element is accessible since there is only the single promoter hypersensitive site. Yet, the transient transfection data suggest that the intronic and upstream binding factors are present in some T cell lines. Two pieces of evidence suggest that multiple regulatory binding factors exist within T cell lines and yet somehow fail to activate transcription. Each of the DQ and DR intronic enhancers function well in both T and B cell lines. Additionally, the class II negative mutant line 6.1.6 can be rescued by fusion with an Ia negative T cell line. There are three possible explanations for this paradox. First, it is possible that a necessary regulatory factor, as yet unrecognized, is missing from unactivated T cells. Second, T cell lines may contain a repressing factor in addition to the constitutive regulatory factors. Finally, the three baseline factors may exist within the cell but are unable to bind due to an inaccessible chromatin configuration. We favor the latter hypothesis since T cell lines exhibit only the single promoter DNaseI hypersensitive site. Activation of T cells and induction of MHC class II gene transcription would then require primarily a change in chromatin configuration (Fig. 2B). The altered chromatin structure would then be accessible to the regulatory proteins already present.

In the next example (Fig. 2C), activation of macrophages or induction of any inducible cell line with IFN-γ adds only the binding of the IFN-γ inducible factor. The transient transfection data using DQ intronic and upstream enhancers suggests that they are not functional in inducible cell lines either before or after IFN-γ administration.

From DNase I studies we know that IFN-γ induction does not require intronic binding factors. This binding pattern is similar to that of resting T cells, however T cells seem unable to utilize the IFN-γ responsive element in transient assays (27). This may represent specific repression or simply the lack of that inducible factor.

Non-inducible cell lines are not able to utilize the intronic or upstream enhancers in transient transfections assays. This suggests that the regulatory factors associated with those enhancers are absent in non-inducible cells. (Fig. 2D)

Does this model give us any insight into the defects represented by the various class II deficient mutant cell lines? The cell lines can be divided into two groups on the basis of class II associated invariant chain transcription. The murine cell line R8205 lacks Ii transcription while the human cell lines 6.1.6 and RJ 2.2.5 contain normal or near normal levels of invariant chain mRNA. We propose that a defect in invariant chain transcription implies that the mutation in the cell line R8205 eliminates the activity of a factor which is shared between Ii and class II genes. The promoter region of the invariant chain contains the class II consensus elements A and B. It is likely that the mutation in R8205 affects a protein that binds to either of the class II consensus elements or some other shared sequence. The cell lines which do contain detectable invariant chain message, 6.1.6 and RJ2.2.5, are proposed to have defects either in the intronic binding proteins or in a promoter binding protein which is not required for invariant chain transcription.

We have presented a molecular model to account for various recent experimental observations. Like all models, it is incomplete, however it does begin to address the multiple binding effects and interactions which must take place in order for MHC class II gene transcription to occur. It should not be surprising that this pattern of regulation resembles that of the immunoglobulin genes since they are evolutionarily related. Immunoglobulin genes are regulated by the combined effects of tissue specific intronic enhancers and promoter elements (32). It should also not be surprising that the control of expression requires many interactions since inappropriate expression of Class II molecules may have deleterious effects in the host. The requirement for multiple interacting regulatory elements makes it less likely that accidental expression of these genes could occur. It also allows for flexibility in the regulation of these genes since the induction of certain proteins may be able to compensate for the absence of others, as appears to be the case with IFN-γ induction. It is likely that as our understanding of enhancers and promoter structures increases, we will be forced to formulate an even more complex model to account for the regulation of MHC class II genes.

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FIGURE 1

In panel A of the figure, the human MHC class II region is shown. The DP, DQ and DR genes are arrayed on chromosome six and consist of multiple α and ß chain genes. The vertical lines separate the DP, DQ and DR subregions. DZα has been recently cloned, however, a protein product remains to be identified. Panel B depicts the exon arrangement for typical class II α and ß genes. The MHC class II promoter consensus is shown in panel C. Methylation analysis of the DRα gene has generated the map shown in panel D. A region surrounding the first exon is demethylated in all cell lines investigated. Panel E shows the DNaseI hypersensitive sites in the DRα gene. Site I is present in all cell lines investigated and sites II and III are found only in cells constitutively expressing DR.

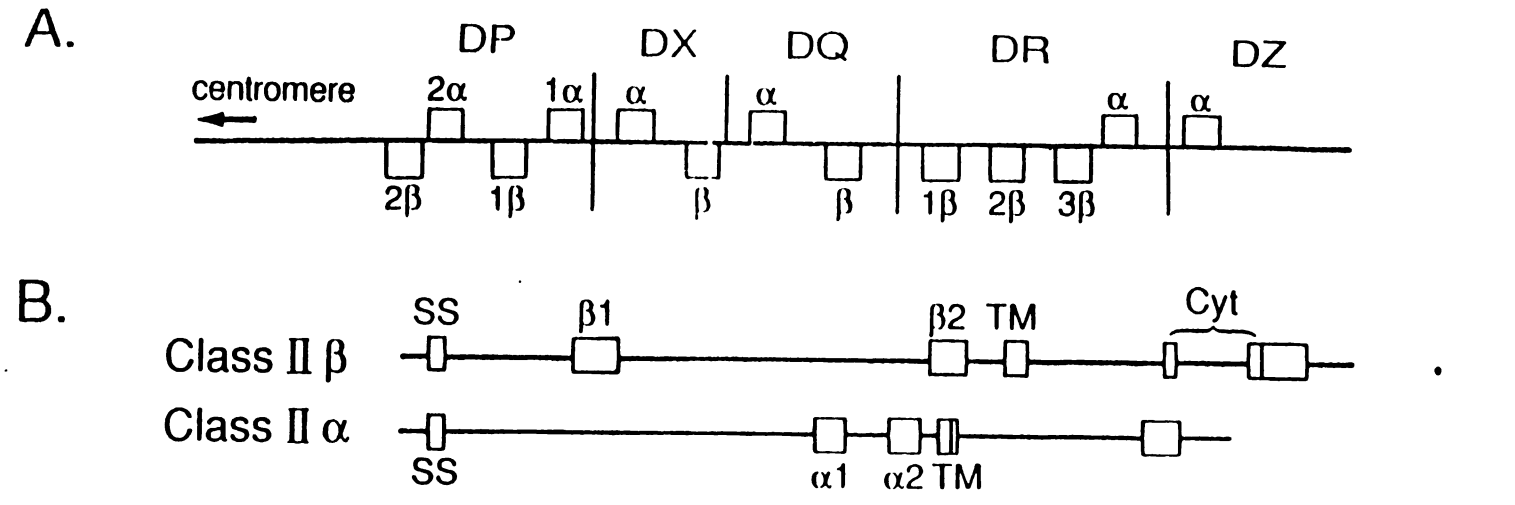
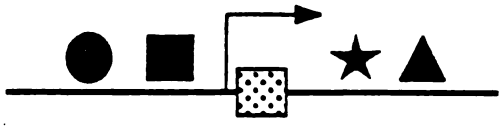

A.
DP
DX
DQ
DR
DZ
centromere
2α
1α
α
α
α
α
2β
1β
β
β
1β
2β
3β
B.
SS
β1
β2
TM
Cyt
Class II β
Class II α
SS
α1
α2
TM
C.
Consensus A
Consensus B
CAT Consensus
Transcription Start
Initiation Codon
A C/A -- T T/C T-CC T/C AG-A G/A ACAGAT
-15/16 bp-
T/C -- T T/G CTGATTGG T/C T
-13-21bp-
CCAATCC
-34-63 bp-
-47-78 bp-
ATG
D. DRα
M
M
DDD
M
M
M
M
M
1
2
3
4
5
M=methylated site
D=demethylated site
E. DRα
I
II
III
1
2
3
4
5
I, II, III : DNase I hypersensitive sites

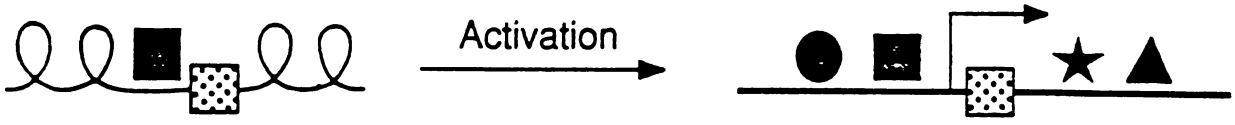
FIGURE 2

In this figure, a schematic representation of the regulatory regions and their binding factors is presented. Exon 1 of a generalized class II gene is shown as a hatched box. The key for each of the symbols appears at the bottom of the figure. The intronic enhancer binding factors (the star and the triangle) are shown as separate entities, however, they may comprise a single functional unit. In part A of the figure, the binding pattern in a constitutive MHC class II expressor is shown. All of the regulatory factors are utilized in generating this high level of transcription. In panel B, the binding pattern in T cells before and after activation is shown. T cell activation results in a change in chromatin structure which allows the regulatory factors to bind. In panel C, induction of MHC class II expression by IFN-γ is shown. The intronic binding factors are not necessary for the induction because the IFN-γ inducible factor can somehow compensate for them. In panel D, the binding pattern for non-inducible cell lines is shown. None of the regulatory factors are present.

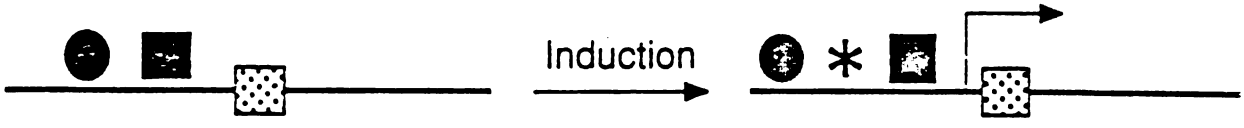
A. Constitutive expression (i.e. B cells)



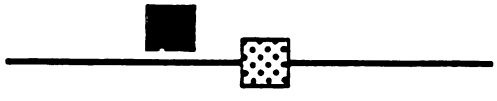
B. T cells



C. Inducible cell lines



D. Non-inducible cell lines



- Upstream binding factor
- Promoter factors (i.e. TATA, CAT binding proteins)
- Intronic enhancer binding factor
- Intronic enhancer binding factor
- IFN-γ inducible factor
- Transcription start

HLA-DQ REGULATION BY TISSUE SPECIFIC ENHANCERS

Running title: HLA-DQ REGULATION BY TISSUE SPECIFIC ENHANCERS

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ABSTRACT

DQα and DQß genes were systematically screened for the presence of regulatory elements able to stimulate transcription from a heterologous promoter in transient transfections. Two regions were identified in DQα which fulfilled the criteria for tissue specific enhancers. These enhancers function in both orientations and independently of position. They exhibit some tissue specificity but are not responsive to γ-interferon. RNase protection analysis demonstrated that they increase transcription arising from a downstream thymidine kinase promoter. One DQα enhancer is located in the promoter region and the other is found in the first intron. The first intron enhancer was found to be associated with a DNase I hypersensitive site in a constitutively expressing cell line. An enhancer was also found in the second and third introns of DQß. These enhancers may play a role in the control of tissue specific expression of DQ.

INTRODUCTION

The class II antigens of the major histocompatibility complex (MHC) are transmembrane glycoproteins composed of an α chain (34kd) and a ß chain (29kd). They are normally expressed on B lymphocytes, activated T cells and certain antigen presenting cells such as dendritic cells.(23,33) However, macrophages and various epithelial and endothelial cells can be induced to express class II antigens with γ-interferon (IFN-γ).(3) These antigens function in the regulation of the immune response by restricting the presentation of antigen to T lymphocytes and by mediating communication between lymphocytes.(14) Three families of class II antigens have been characterized so far and their cognate genes have been cloned.(2,4,20,42) These three families; DP, DQ and DR, have very similar protein structures and function similarly in antigen presentation assays. The DQ subregion of the MHC class II region consists of four genes.(25) DQα and DQß genes encode the antigen known as DQ. DXα and DXß genes appear to have no abnormal features to prevent transcription, however DX mRNA has never been demonstrated in vivo and they may represent psuedogenes.(2)

The availability of cDNA clones specific for the various class II antigens has greatly aided the study of the regulation of class II antigens. Class II gene expression is primarily regulated at the level of transcription although IFN-γ may act through additional mechanisms.(3,31,39) MHC class II antigens are expressed in a tissue restricted manner, are developmentally regulated (12) and can be coordinately induced in appropriate cells with IFN-γ.(5)

Constitutive expression of class II antigens is limited to mature B cells and perhaps certain antigen presenting cells. PreB cells and end stage antibody secreting myeloma cells are class II negative suggesting that expression is dependent on developmental cues.(37) During the ontogeny of B cells the class II antigens appear in an ordered fashion with DP and DR expressed first and then DQ.(12) The maturation of macrophages is also characterized by an ordered expression with only a subset expressing DQ at any time.(8) However, once expressed, all three antigens are coordinately induced by IFN-γ. The tissue specific expression of class II antigens is required to prevent the inappropriate presentation of antigen. Conversely, IFN-γ responsiveness allows for the augmentation of a given immune response by increasing class II expression and thus making antigen presentation more efficient. Of the class II antigens, DQ is the most restricted in its tissue distribution. It is found primarily on mature B cells. Activated T cells express class II antigens but rarely express DQ. Similarly, IFN-γ induced endothelial cells may express certain class II antigens but DQ is rarely detected and if present, is usually found in low levels. Certain antigens are presented more efficiently in the context of DQ and the expression of DQ may need to be limited to certain subsets of antigen presenting cells.(8)

We chose to analyze the regulation of DQ transcription by utilizing a transient transfection protocol. DQα and DQß genes were systematically screened for elements able to stimulate transcription from a heterologous promoter. Two enhancers identified in the DQα gene were implicated in the tissue specific expression of DQα. One

enhancer mapped to the promoter and was coincident with a sequence known as the class II consensus. The other enhancer mapped to the 3' end of the first intron. DQß also contained a tissue specific intronic enhancer, however it was localized to the second and third introns. None of the enhancers were responsive to IFN-γ induction. The enhancers appear to control the tissue specific expression of DQ and may be similar to the control elements regulating the expression of DR.

MATERIALS AND METHODS

CELLS AND CELL LINES. BJAB is a non-EBV transformed human B cell line which constitutively expresses DR and DQ antigens.(17) It was kindly provided by P. Gruss. Jurkat cells were derived from those originally obtained from K. Smith and have been repeatedly characterized as a DR and DQ negative human T cell line. The clone E6-1 was used for our studies.(22) HeLa cells are a cervical carcinoma line and were obtained from ATCC. They are DR and DQ negative in their resting state. Treatment for 48 hours with 100 U/ml of IFN-γ (provided by Genentech) results in DR expression and limited DQ expression. HUT-78 cells were provided by R. Gallo and represent a partially activated human T cell line.(19) They are DR+ and DQ-. Raji cells were obtained from D. Char and represent an EBV transformed human B lymphoblastoid line which constitutively expresses DR and DQ antigens. Three murine cell lines were used in the transfection assays. P3X63Ag8 is an IE-, IA- (murine MHC class II) myeloma line derived from PK3. It was a gift of T. Parslow. The cell lines 2PK3 and L10A were obtained from A. DeFranco and represent mature B cell lines which are IA and IE positive.(18)

PLASMID CONSTRUCTIONS. The control plasmids tk-CAT (PTE2 Δ Sal Nru) and RSV CAT (11) were provided by M. Walker. PTE2 Δ Sal Nru consists of 149 bp of the herpes simplex thymidine kinase (tk) promoter upstream of the Tn9 chloramphenicol acetyl transferase gene and SV40 small T antigen splices and polyadenylation site.(10) These have been cloned into a puc 19 vector (fig. 1c). Immediately upstream of the tk promoter lies a polylinker into which the DQ restriction fragments from

the cosmid clone Hlla were cloned.(25) Fragments which could not be directly cloned into the polylinker were blunt ended with T_4 polymerase and cloned into the blunt ended BglII site of the polylinker. DQ constructions identified as downstream or 3' position plasmids utilized restriction fragments which were blunt ended with T4 polymerase and cloned into the Sma I site immediately downstream of the polyadenylation site. The control plasmid RSV ßGAL consists of the RSV LTR cloned upstream of the ß galactosidase gene.(6) Plasmids designated as polyoma replicons have had 3.6kb of the polyoma early region blunt ended with T_4 polymerase and inserted into the SmaI site of each of the indicated constructs.(30) Polyoma constructs are indicated with the suffix "poly". The orientation of the fragment is such that the early region is transcribed in the opposite direction to the CAT transcripts. The plasmids described as the class II consensus constructs are named OTK and consist of a 48bp oligomer containing the sequences described as the class II consensus boxes by Okada et al.(25) The DQα promoter contains the class II consensus however there are several bases which do not conform to the canonical sequence and these are indicated with a dot in figure 3. The oligomer was synthesized with PstI and ApaI ends and was cloned into the puc based vector pIBI-76. The tk CAT fragment was excised and inserted on either side of the oligomer.

TRANSFECTIONS AND ASSAYS. Cells were transfected by suspending 2-4 x 10^7 cells with 2 µg of test plasmid and 2 µg of RSV ßGAL plasmid in 1 ml of serum free media with .1mM chloroquin and 300 µg/ml DEAE dextran. The cells were incubated for 2-3 hours at 37°, washed and resuspended in RPMI with 10% fetal calf serum. After 3 days the cells

were harvested, split into two halves, washed and resuspended in either 110 µl of .5M Tris-Cl pH 7.5 for CAT assays or .5 ml Z buffer (60mM Na_2PO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM Mg_2SO_4, 50mM ß-mercaptoethanol pH 7.0) for the ß galactosidase assay. Three cycles of freezing and thawing were used to disrupt the cells and a five minute incubation at 60° was used to inhibit proteases for the CAT assays. After spinning out debris, a protein determination was made using the BioRad coomassie assay. The amount of protein in each assay was standardized and the CAT assays consisted of lysate, 4mM acetyl coenzyme A (Sigma) and .2 µCi ^{14}C chloramphenicol (Amersham). The assays were incubated at 37° for four hours. The chromatography was performed as described elsewhere.(10) The transfections using polyoma replicons were performed identically, however the CAT assay incubation times were shortened to maintain the CAT signals within the linear range (i.e. less than 50% conversion to acetylated forms). The spots were cut out and dpms were counted in Ecolite. ß galactosidase assays were not performed when polyoma constructs were transfected. For the remaining transfections, the ß galactosidase assays were completed by adding o-nitrophenyl-ß-D-galactoside to 4 mg/ml and incubating at 28° overnight. Cellular debris were removed by centrifugation and optical density readings were taken at 420nm and 550nm as described.(1) A control of mock transfected cells was used.

<u>DNASE I HYPERSENSITIVE ANALYSIS</u>. The DNA used in this analysis was kindly prepared by A. Larsen. The conditions for nuclei isolation and DNase I digestion have been previously described. (Peterlin, et al. in press MCB) Briefly, nuclei were treated with 0-50 U/ml of DNase I for 3 minutes at room temperature. The digestions were stopped with

proteinase K and the DNA was extracted. The DNA was restricted with EcoRI and prepared for southern blotting.

RIBOPROBE PROTECTION. RNA was isolated in guanidine isothiocyanate according to Chirgwin. 50 µg of total RNA was incubated for 12 hours with 5 x 10^5 cpm of ^{32}P-UTP labelled, gel purified probe complementary to the tk CAT portion of the test plasmids. Hybridization occured in 80% formamide, .4M NaCl, 20mM Tris-Cl (7.5), .1mM EDTA and .1% SDS. 150 µl of RNase A (100 µg/ml) and RNase T1 (10 U/ml) in RNase buffer (300mM NaCl, 10mM Tris-Cl 7.5, and 5mM EDTA) were added. Digestion occured at room temperature for 90 minutes. The RNA was extracted, ethanol precipitated and run on a 6% urea-acrylamide sequencing gel.

RESULTS

SCREENING THE DQ SUBREGION

The DQα and DQß genes of the major histocompatibility complex were systematically screened for DNA elements able to stimulate transcription from the thymidine kinase promoter. Plasmids were constructed which contained a restriction fragment derived from the DQα or DQß gene upstream of the herpes simplex virus thymidine kinase (tk) promoter. The chloramphenicol acetyl transferase (CAT) gene was inserted directly downstream of the tk promoter. The rate of transcription of the CAT gene is accurately reflected in an enzymatic assay for the presence of CAT enzyme since translation and message turnover are constant.(10) Utilizing this transient transfection CAT assay system we were able to screen a large number of DQ restriction fragments for their ability to stimulate transcription of the CAT gene over and above that level arising from the tk promoter alone. The cosmid clone H11a (25) was subdivided initially into fourteen restriction fragments spanning the DQα and DQß genes. Figure 1a is a diagram indicating the origin of each of the restriction fragments. A map of the parent plasmid is shown in figure 1c and the results of the transfections of each of the screening plasmids are shown in figure 1b. The plasmids are named according to the restriction sites demarking their ends, their length, and their orientation is indicated as a subscript. The two cell lines chosen for screening were a lymphoblastoid B cell line, BJAB, which is a constitutive expressor of

DQ and a T cell line, Jurkat, which is DQ negative. Both cell lines were transfected with DEAE dextran and chloroquin and assayed on day three for the presence of CAT. An internal control plasmid, RSV ßGAL, was included in each transfection as an independent analysis of transfection efficiency. The results in figure 1b are therefore standardized for the amount of protein in each assay and the results of the ß galactosidase assay. The final values are averages of at least three experiments and are expressed in terms of the tk-CAT result, where the relative CAT value for tk-CAT is set equal to one. From the figure, it is clear that most DQ derived restriction fragments fail to stimulate transcription above that generated by the tk promoter alone. However, there are certain plasmids which do give relative CAT values above baseline. Unexpectedly, the plasmids seem to function equally well in both BJAB, the DQ+ cell line and Jurkat, the DQ- cell line. This issue is addressed in the tissue specificity analysis. In figure 2, CAT assays from the transfections of some of these plasmids are shown. CAT results from plasmids XS 2.5_{53}, XS 2.5_{35}, BB 1.8_{35}, BB 3.0_{53}, BB 3.0_{35}, BB $.8_{53}$, BB $.8_{35}$, XX 2.3_{53}, XX 2.2_{53}, and XX 2.2_{35} were felt to represent significant stimulation of transcription from the tk promoter and were chosen for further analysis.

CHARACTERIZATION

The DQ restriction fragments which stimulated transcription in transient transfections were further characterized in terms of localization within DQ and position and orientation independence. Figure 3 is a diagram of the various constructions which were tested in transient assays. The distant upstream fragment from DQα, XS 2.5, was tested in both orientations and downstream of the CAT gene (fig. 3a). It functioned well in both orientations but not at all in the downstream position. Therefore, this element fails to fulfill the criteria for an enhancer. Whether it may play a role in the regulation of DQ is difficult to judge. Its distant upstream position is unusual, however a similarly positioned enhancer for the murine MHC class II E_{β} gene has been described.(7)

A promoter associated enhancer was dissected out of the fragment BB 1.8 by a combination of deletion analysis and the use of a cloned synthetic 48bp oligomer (fig. 3b). This oligomer contains the canonical class II consensus elements as desribed by Okada et al. The complete sequence is given in the figure with the X and Y boxes enclosed and the bases where the DQα region differs from the consensus marked with dots. This region is highly conserved among MHC class II genes and has been termed the class II consensus. It consists of two highly conserved elements named the X and Y boxes with an intervening region which is less conserved. It fulfills the criteria for a tissue specific enhancer by demonstrating position, orientation and promoter-type independence. This element is quite strong when it is in the correct

orientation. However, it may not be the only promoter element important in the regulation of DQα expression. Two deletions of the original BB 1.8 fragment were tested and suggest that the sequences proximal to the X and Y boxes may also stimulate transcription. BB 1.8 ΔBal is a deletion of BB 1.8 where both the X and Y boxes as well as 500bp of 5' flanking sequences were removed. This deletion retains all of the sequences between the class II consensus and the transcription start site.. This deletion retains significant function in our CAT assays. In fact, it retains about 50% the activity of the parent fragment, BB 1.8. BB 1.8 ΔSty is a 126bp deletion of sequences from the transcription start site through the Y box. The X box and all the 5' flanking sequences are retained in this construct. This deletion removes all activity from the parent fragment and suggests that sequences 3' to the class II consensus may be important in the regulation of DQα expression. This region was not further investigated, however it is noteworthy that downstream of the Y box lies an element known as the immunoglobulin octamer.(29) It is important for the B cell specific expression of immunoglobulin genes. MHC class II genes are also expressed in B cells and it is possible that certain trans-acting regulatory factors are shared between the immunoglobulin and class II genes. The role of the octamer in MHC class II gene expression is not clear. Not all class II genes contain this element and even in DQα, the octamer is incomplete. Protein-DNA binding studies have shown that a single mismatch is enough to inhibit binding of the octamer binding factor, so it is difficult to judge the contribution of the octamer to class II gene expression.(35) Nevertheless, the deletion studies suggest that sequences downstream of

the class II consensus may play a role in DQ transcription.

In figure 3c, a restriction fragment spanning most of the first intron of DQα was analyzed. Curiously, it functions better in the downstream position. The finding of a preference for the downstream position may reflect a mechanism peculiar to dowstream enhancers. Alternatively, it may reflect the artificial nature of this assay system. BB 3.0 may only be a portion of a larger functional element which includes this restriction fragment and the adjacent fragment BB .8. The position and orientation analysis of BB .8 is shown in figure 3d. This element also appears to fulfill the criteria for being an enhancer. In the 5' position the function of BB .8 is extremely asymmetric. This asymmetry of function disappears in the 3' position, however. The activity of the enhancer may be artificially augmented when it is in a particular configuration with the promoter and the constructs utilizing BB .8 in the 3' position may give a more accurate representation of the strength of the enhancer contained in BB .8 since its natural location is 3' to the promoter.

The DQß fragments which tested positive in the initial screen were also characterized. No promoter element was identified, although the DQß promoter is known to include the same class II consensus element as DQα. It may be that the DQß end of the cosmid clone H11a is too truncated to reveal a promoter element. Unlike DQα, no first intron fragments tested positive in the initial screen. Instead, the fragments identified in DQß are found in the second, third and fourth introns. XX 2.3 from the DQß gene does not fulfill the criteria for an

enhancer but functions extremely well in one orientation and displays the expected tissue specificity (fig. 3e and table 1). In spite of its unidirectional function it does not appear to be acting as a promoter since the riboprobe analysis demonstrated that it stimulated transcription from the tk promoter. It is included in our analysis because it may represent an extension of the more 5' element found in XX 2.2. XX 2.2 (fig. 3f) appears to fulfill the requirements for an enhancer by stimulating transcription in both orientations upstream of the promoter and by functioning downstream of the promoter. It is unusual in that it functions in BJAB only in the downstream location. Again, this may represent a peculiarity of downstream enhancers or an artifact of the transient transfection protocol. It is worth noting that in these small puc based plasmids, an element located downstream of the CAT gene is approximately 2kb away from the promoter downstream and only 3kb away from the promoter on the upstream side. In circular plasmids the position independence criteria is most readily interpreted as distance independence.

Perhaps the regulatory region for DQß has been divided into a true enhancer with limited tissue specificity and an incomplete enhancer with appropriate tissue specificity. The original XX 2.3 element was whittled down to 700bp (BD 700_{53}) and not only retained its full activity in that orientation but actually became a stronger element with each paring down. In fact, that was a characteristic of each of the elements which were examined by resections. As the element became smaller it functioned better in the transient assays. It is known that plasmid sequences can have a damping effect on enhancer function. It

is possible that these intronic sequences may have a similar nonspecific damping effect. As the enhancer becomes more isolated from the surrounding intronic sequences its effect may become more pronounced.

The large number of potential regulatory regions in the DQ genes might reflect the presence of small internal genes unrelated to MHC class II but utilizing regulatory regions revealed by our screening. To investigate this possibility, northerns were probed with each of the fragments identified in the initial screen and in no case were any non-DQ bands observed (data not shown).

TISSUE SPECIFICITY

In an effort to examine whether these restriction fragments were able to stimulate transcription in a tissue specific fashion, a variety of cell lines were transfected. Tissue specific utilization of enhancers may imply physiologic importance. The plasmids which were tested utilized restriction fragments identified in the screening or characterization process. The cell lines into which the test plasmids were transfected were HeLa, a DQ negative human cervical carcinoma line which is MHC class II inducible with IFN-γ; HUT-78, a DQ negative human T cell line; Raji, a DQ positive B lymphoblastoid line; P3X63Ag8, an MHC class II negative murine myeloma line; 2PK3, an MHC class II positive murine B cell line; and L10A, another MHC class II positive murine B cell line. The human cell lines (HeLa, HUT-78, and Raji) were transfected using the same protocol as was used for BJAB and Jurkat. (Table 1) P3X63Ag8 was transfected using that procedure and also using a set of plasmids to which the polyoma early region was added downstream of the CAT gene. These constructs are indicated with the "poly" suffix. This rendered the plasmids replication competant in murine cell lines and significantly boosted the CAT signal. Addition of the polyoma early region does not significantly alter the relative CAT values in P3X63Ag8 cells. The cell lines L10A and 2PK3 were also transfected with the polyoma early region containing plasmids. (Table2) In the tables the results of those transfections are shown along with the results from BJAB and Jurkat for comparison. A DQ distant upstream fragment, XS 2.5_{35}, stimulates transcription in all class II positive B cell lines and a class II negative T cell line,

Jurkat. It does not function well in the other class II negative cell lines HUT-78, HeLa, or P3X63Ag8. Similarly, fragments from the first intron of DQα, BB 3.0_{53} and BB $.8_{35}$ function in the class II positive lines and Jurkat but not in the other class II negative lines. The fragment BB 1.8_{35} from the promoter region is able to stimulate transcription in the class II positive lines as well as Jurkat and HeLa but not in HUT-78 or P3X63Ag8. Notably, the class II consensus construct OTK_{53} functioned well in all six lines which were tested. This may imply a negative control element found in BB 1.8 which is not included in the 48bp element which blocked enhancer activity in HUT-78 and P3X63Ag8 cell lines. Alternatively, it may reflect the few bases which differ between the canonical class II consensus in the OTK constructs and the endogenous DQ region in the BB 1.8 construct.

Two fragments from DQß were also tested. A fragment from the fourth intron of DQß, XH 1.3_{53}, is able to stimulate transcription in the class II positive lines and Jurkat but not in the other class II negative lines. A fragment from the second and third introns of DQß functions in Jurkat, L10A, 2PK3, and Raji, but not in BJAB, HeLa, HUT-78 or P3X63Ag8. However, XX 2.2 does function in BJAB when located downstream of the promoter. These results may be more readily interpreted when one considers the cell lines as two groups. The cell lines in group 1 consist of the class II positive lines (BJAB, Raji, L10A, and 2PK3) and Jurkat. The restriction fragments in the table all stimulate transcription in these lines with the exception of XX 2.2 which does not function well in BJAB in the 5' position. The cell

lines in group 2 consist of the class II negative lines HeLa, HUT-78 and P3X63Ag8. The restriction fragments give baseline CAT assay values in these cell lines with the exception of BB 1.8 which functions in HeLa cells. BB 1.8 contains the class II consensus which may be important in mediating the IFN-γ response in HeLa cells. Therefore, with that exception, the restriction fragments identified in the initial screen function similarly in terms of tissue specificity. In general, they are able to function in this transient assay system in DQ positive cell lines and not in DQ negative cell lines. They are also able to function in Jurkat which is a DQ negative T cell line. The Jurkat clone used in these experiments, E6-1, was repeatedly characterized as DR and DQ negative. Activated human T cells do express MHC class II antigens so it is possible that Jurkat produces the regulatory factor(s) required for the transcription of DQ. The native class II genes in Jurkat may not be able to utilize those factor(s) because of unfavorable chromatin configuration. Therefore, with the exception of Jurkat, the ability of these fragments to stimulate transcription in various cell lines correlates with the expression of the endogenous DQ genes. The failure of any of the elements to function in the murine myeloma line, P3X63Ag8, suggests that immunoglobulin secreting cells which are class II negative do not produce the trans-acting factors required for class II expression.

MHC class gene expression can be induced in certain cells with IFN-γ. The effect of IFN-γ on the DQ constructs listed in Table 1 was examined by transfection of those constructs into HeLa cells followed by 48 hours of 100 U/ml of IFN-γ. This treatment is

sufficient to induce the endogenous class II expression in HeLa but no increase in CAT values were seen after treatment for any of the constructs (data not shown).

RIBOPROBE ANALYSIS

To confirm that the elements identified as potential enhancers were actually stimulating transcription from the tk promoter, a series of transfections into the cell line 2PK3 were performed. These transfections utilized the polyoma replicons to generate RNA levels detectable by riboprobe analysis. RNA was isolated from the transfections on the second day and 50 μg from each transfection was hybridized to complementary ^{32}P labelled tk-CAT RNA generated from an SP6 promoter. Unhybridized RNA was digested away with RNase A and RNase T_1. Urea-acrylamide gel analysis allowed precise mapping of the 5' ends of the CAT transcripts. In all cases the fragments stimulated transcription from the tk promoter. In no case was a cryptic promoter revealed. This was an important consideration for those fragments which functioned asymmetrically or only in one position. Not only did the fragments stimulate transcription from the appropriate promoter, but the RNA levels corresponded to the CAT signals obtained for the 2PK3 cell line.

DNASE I ANALYSIS

DNase I hypersensitive sites have been correlated with the presence of enhancers in many genes.(28,34,36,40) They may be associated with locally demethylated regions or nucleosome free regions.(15) They are believed to represent perturbations in the chromatin structure and may be associated with protein binding. In an effort to determine whether such a perturbation might be present in the DQα gene, nuclei were digested with varying concentrations of DNase I for 3 minutes and the extracted DNA was digested with EcoRI. Southern analysis revealed a 6.5kb parent fragment generated by EcoRI digestion in both BJAB and Jurkat cell lines. A clear sub-band is visible in the BJAB lanes which is approximately 3.5kb in length. Long exposures of the Jurkat blot did not reveal any corresponding bands. Jurkat DNase I digestion was judged to be equivalent to the BJAB digestion on the basis of ethidium bromide staining. Since the probe hybridized to only the 3' end of the fragments, the DNase I hypersensitive site in BJAB could easily be mapped to the 3' end of the first intron. It is located approximately at the boundary between BB 3.0 and BB .8 which may explain why both fragments can independently function as enhancers. This result would suggest that they belong to the same functional unit. It also suggests that these elements are not mere artifacts but actually perform some function in DQα transcriptional regulation. A Dnase I hypersensitive site in a similar location has been described for DRα.(Peterlin, et al. in press MCB) A similar analysis with the DQß gene might have been enlightening, however restriction site polymorphisms prevented the generation of an appropriate fragment from which to observe the

sub-bands. The DNase I hypersensitivity analysis of DQ is also complicated by cross hybridization with DX. DXα and DXß are highly homologous to DQα and DQß genes. The intron from which the probe was generated is 88% homologous to DX.(2) DXα bands were identified on our southerns from known polymorphisms (21) and disappearance with high stringency washing. Jurkat exhibits a DXα EcoRI-EcoRI restriction fragment about 8kb in length just above the DQα band. BJAB exhibits a DXα band of higher molecular weight which does not appear in this figure. It is not formally possible to exclude the possibility that the identified sub-band in BJAB was actually generated from DXα. We believe it arose from DQα because it corresponds with our functional data and because the sub-bands wash off at higher stringencies than the DXα band. Finally, there is a hint of a sub-band directly underneath the EcoRI-EcoRI parent fragment in BJAB which may correspond to a promoter hypersensitive site. Further investigation of this will require the identification of a smaller more 5' restriction fragment.

DISCUSSION

This study utilized a transient transfection protocol to screen the DQα and DQß genes for potential regulatory elements. Three regions were identified as being potentially important in the regulation of DQ expression. Two regions were identified in DQα. The promoter and first intron each contain a distinct tissue specific enhancer. The promoter associated enhancer appears to be coincident with a region known as the class II consensus boxes X and Y. This consensus element is conserved among class II genes and the invariant chain gene.(16,27) The invariant chain gene is coregulated with MHC class II genes and may have a functional role in the transport of class II proteins to the surface. In DQß an enhancer was found in the second and third introns. The tissue specificities of these elements were not absolute, however their activity conformed in a general way with endogenous DQ expression. In particular it was not expected that the elements would function in Jurkat, a DQ- T cell line. The fact that all of the elements functioned in Jurkat may be interpreted three ways. Firstly, it is possible that a negative control element suppresses transcription in this T cell line. Secondly, it is possible that an element, yet to be identified, is not active in this T cell line and it is that element which is critical for expression of DQ. Finally, it is possible that the positive regulatory factors exist within Jurkat but are excluded from binding by an unfavorable chromatin configuration. We favor the latter hypothesis based on the DNase I study which demonstrated no DQ hypersensitive sites in Jurkat. On the other hand, the data from the promoter region suggests that a negative control element may exist. A

tissue specific negative control element in combination with the class II consensus enhancer may regulate expression. Negative control elements have been described for HMG CoA reductase (26), ß interferon (9), and α-fetoprotein (24). The negative control element in α-fetoprotein is tissue specific so there is precedence for the a notion that a negative control element suppresses transcription of DQ in certain cell lines. The lack of endogenous DQ expression in Jurkat may be due to any one or a combination of features such as these.

Aside from Jurkat, the intronic enhancers all functioned in DQ+ cell lines and not in DQ- cell lines. The intronic enhancers most likely are important in B cell specific expression. In contrast, the class II consensus element functioned in all cell lines tested although a larger fragment failed to function in two DQ- cell lines, HUT-78 and P3X63Ag8. This may reflect the presence of a negative control element in the larger fragment, BB 1.8. Our deletion studies also suggest another positive control element downstream of the class II consensus in a region yet to be defined. The promoter region is very likely to consist of a pastiche of several elements. Additionally, we identified another potential regulatory element 3kb upstream of the DQα gene. It was not extensively characterized however it may play a role in DQ expression since it exhibited the same tissue specificity as the intronic enhancers. Distant upstream enhancers have been identified in α-fetoprotein (24) and the MHC E_{β} gene (7). Whether they play a role in the regulation of transcription remains to be determined. Further deletion studies may reveal the function of these distant upstream elements.

The intronic enhancers appear to be quite diffuse. Both the first intron enhancer from DQα and the second and third intron enhancer from DQß were split between two restriction fragments. The DQα intronic enhancer appeared in the BB 3.0 and BB .8 fragments and in each case functioned appropriately. That is to say each fragment exhibited the expected tissue specificity and each fragment independently fulfilled the criteria for an enhancer. We believe them to belong to a single functional unit on the basis of the DNase I study which showed a hypersensitive site near the BB 3.0 and BB .8 boundary. However, it is not formally possible to exclude that they are two distinct entities.

The DQß enhancer also appeared in two fragments, XX 2.3 and XX 2.2. In this case, only one of the fragments, XX 2.2, fulfilled the criteria for an enhancer. The other fragment, XX 2.3 or XH 1.3 functioned only upstream of the promoter and only on the 5' to 3' orientation. Those characteristics are very suggestive of a crytic promoter, however riboprobe protection revealed that the CAT transcripts did arise from the tk promoter in the XH 1.3_{53}poly construct. Its unidirectional activity in the CAT assays may reflect the fact that it is an incomplete element, lacking at least some of the binding sites for crucial trans-acting factors. There is precedence for the notion that large cellular enhancers when divided into smaller fragments lose some of the characteristics of the intact enhancer. The immunoglobulin heavy chain enhancer when divided into several parts was found to consist of a central element which functioned as an enhancer in non-B

cell lines while the fragments on either side functioned as enhancers only in B cell lines. The tissue non-specific fragment contained a core enhancer sequence while the two B cell specific fragments contained the immunoglobulin octamer sequence.(41) The whole enhancer has since been shown to bind three ubiqitous factors and one B cell specific factor.(32) If the DQ intronic enhancers also bind several factors then it is possible that our restriction fragments have inadvertantly separated some of the binding sites, thus giving more that one fragment enhancing characteristics. It also seems clear that enhancers function best with their own homologous promoter.(38) The enhancing effect might be stronger and the tissue specificity more absolute when the intronic enhancers are stimulating a homologous DQ promoter. This will be difficult to test in DQ because the promoter does not contain a TATA box or CCAAT box and is very poorly defined.

In conclusion, the data presented in this paper suggest that the expression of DQα and DQβ genes in B cells may be regulated by tissue specific enhancers located intronically. Inducible expression of DQ in the endothelial line Hela appeared not to utilize the intronic enhancers. Inducible expression may be controlled through the promoter region, where another enhancer is located. This enhancer appears to be coincident with the class II consensus and is not tissue specific. The class II consensus consists of two highly conserved elements, X and Y, with a slightly less conserved intervening sequence. The Y box contains a CCAAT element flanked by several conserved bases. CCAAT binding factors are ubiquitous and if they play a role in the activity of the class II consensus it may explain the lack of tissue

specificity. A large fragment from the promoter region of DQα, BB 1.8, functioned in B cell lines and the inducible line, HeLa, suggesting that there may be a tissue specific negative control element which acts in concert with the class II consensus element to control expression. This is reminiscient of the the regulatory strategy of the ß interferon gene. Its induction by double stranded RNA relies on the dissociation of a negative trans-acting factor and the unmasking of a constitutive enhancer.(9) IFN-γ inducible expression may also rely on a combination of promoter elements since only the promoter elements BB 1.8 and OTK functioned in the inducible line, HeLa. α,ß or γ interferon induction of MHC class I genes relies on an interferon responsive sequence which overlaps one of the two promoter associated enhancers in the class I genes. The enhancer is required for interferon induction and heterologous enhancers cannot substitute for it.(13) Perhaps the class II consensus of MHC class II genes is a functional correlate of the MHC class I promoter associated enhancer which is required for interferon induction. Because MHC class II gene expression needs to be finely regulated to prevent the inappropriate presentation of antigen, it should come as no surprise that the regulation of these genes requires interacting regulatory elements.

TABLE 1

CONSTRUCT	HELA		HUT-78		JURKAT		BJAB		P3X63AG8	
	CAT	s.d.	CAT	s.d.	CAT	s.d.	CAT	s.d.	CAT	s.d.
XS 2.5_{35}	1.14	.68	.92	.07	6.21	3.62	4.86	.49	1.10	.07
BB 1.8_{35}	2.34	1.13	1.30	.33	4.98	1.84	3.75	1.05	.99	.05
BB 3.0_{53}	1.54	.60	1.11	.22	2.79	.65	2.70	1.07	1.04	.04
BB $.8_{35}$	1.01	.37	1.25	.09	3.20	1.40	3.76	.99	1.25	.44
XH 1.3_{53}	.66	.24	.69	.14	5.96	2.39	4.76	2.43	1.44	.06
XX 2.2_{53}	1.41	.28	.55	.14	3.10	.67	1.06	.25	1.30	.47
OTK_{53}	2.72	.56	2.27	.46	10.72	1.59	48.42	12.85	2.43	.82
RSV-CAT	126.33	103.84	24.33	1.53	62.33	12.67	121.67	29.73	6.79	.00

TABLE 2

CONSTRUCT	P3X63AG8		2PK3		L10A	
	CAT	s.d.	CAT	s.d.	CAT	s.d
$XS2.5_{35}poly$	1.02	.07	6.68	2.63	8.19	.60
$BB1.8_{35}poly$	.52	.15	3.23	.86	4.10	1.06
$BB3.0_{53}poly$	1.13	.08	4.58	.67	3.92	1.18
$BB.8_{35}poly$	1.09	.02	3.22	1.19	4.97	1.21
$XH1.3_{53}poly$	.89	.13	1.82	.22	2.13	.00
$XX2.2_{53}poly$	1.11	.10	1.42	.29	3.83	.31

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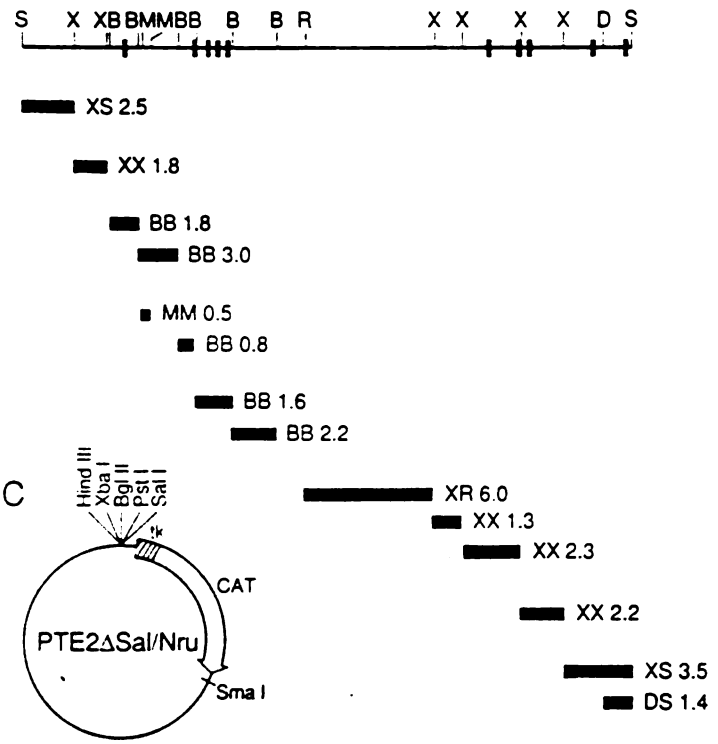
FIGURE LEGENDS

Figure 1:

Figure 1a is a map of the cosmid clone H11a. It was initially divided into 14 restriction fragments which were cloned into the CAT vector PTE2 Δ Sal Nru, called tk-CAT in this paper, shown in figure 1c. Their ability to stimulate transcription of the CAT gene from the thymidine kinase (tk) promoter was measured in transient transfection assays. The results for each of the fragments are shown in figure 1b. The DQ constructs were tested in two cell lines. BJAB is a DQ+ B cell line. Jurkat is a DQ- T cell line. The relative CAT values and their standard deviations are shown. The constructions are named according to the restriction sites demarking their ends, their length and their orientation with respect to the CAT gene. B=BglII, D=HindIII, M=BamHI, R=EcoRV, S=SalI, X=XbaI.

A



C

B

	BJAB		JURKAT	
	Rel CAT	*SD*	*Rel CAT*	*SD*
$XS2.5_{53}$	4.27	1.87	3.39	0.62
$XS2.5_{35}$	4.86	0.49	6.21	3.62
$XX1.8_{53}$	1.18	0.16	1.39	0.44
$XX1.8_{35}$	0.85	0.15	1.10	0.36
$BB1.8_{35}$	3.75	1.05	4.98	1.84
$BB3.0_{53}$	2.70	1.07	2.79	0.65
$BB3.0_{35}$	0.64	0.04	1.19	0.17
$MM0.5_{53}$	1.03	0.31	0.93	0.20
$BB0.8_{53}$	46.24	11.02	41.33	2.55
$BB0.8_{35}$	3.76	0.99	3.20	1.40
$BB1.6_{53}$	1.35	0.41	1.04	0.34
$BB2.2_{53}$	1.30	0.07	1.49	0.30
$BB2.2_{35}$	1.21	0.34	1.00	0.12
$XR6.0_{53}$	0.98	0.19	1.21	0.29
$XX1.3_{53}$	1.16	0.51	1.59	0.32
$XX2.3_{53}$	3.57	0.11	3.45	0.38
$XX2.3_{35}$	0.67	0.19	1.54	0.21
$XX2.2_{53}$	1.06	0.25	3.04	0.67
$XX2.2_{35}$	1.60	0.40	4.52	0.61
$XS3.5_{35}$	0.78	0.02	1.04	0.10
$DS1.4_{35}$	1.09	0.34	1.40	0.10

Figure 2:

Figure 2 demonstrates several CAT assays from the transfection of constructs which were felt to represent significant stimulation of transcription. Panel represents CAT assays from transfections into BJAB cells. Panel B presents CAT assays from transfections into Jurkat. 1, tk-CAT; 2, RSV-CAT; 3, XS 2.5_{35}; 4, BB 1.8_{35}; 5, BB 3.0_{53}; 6, BB $.8_{53}$; 7, XH 1.3_{53}; 8, XX 2.2_{53}. The series of spots above the origin are unacetylated chloramphenicol and the two series of spots above that are the monoacetylated forms of chloramphenicol.

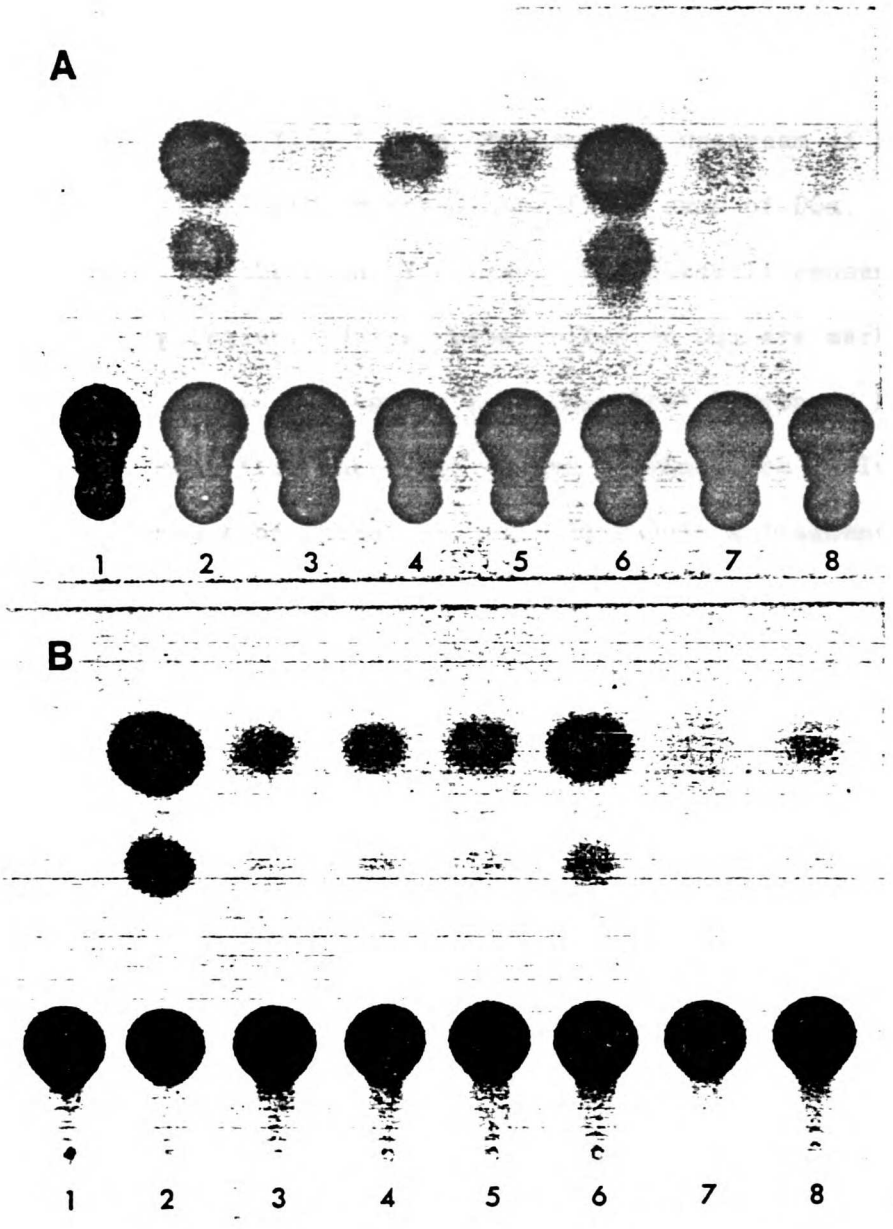
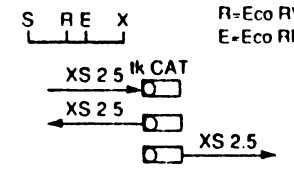

A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8

Figure 3:

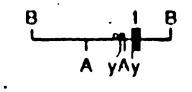
Figure 3 shows the constructs which were used to help characterize potential regulatory regions. A diagram of each construct appears on the left and the relative CAT values with their standard deviations appear on the right. 3a. XS 2.5 is a fragment 3kb upstream of DQα. 3b. BB 1.8 is a fragment which brackets the first exon of DQα. The 48bp oligomer consist of the X and Y boxes of the class II consensus and their intervening region. Bases which differ in DQα are marked with a dot. 3c. BB 3.0 is a fragment from the first intron of DQα. 3d. BB .8 is a fragment from the first intron of DQα which includes exon 2 and a small amount of intron 2. 3e. XX 2.3 is a fragment which brackets the last exon of DQß. Its activity appeared to localize to the fourth intron. 3f. XX 2.2 contains exons 3 and 4, intron 3 and part of intron 2 of DQß.

A. XS 2 5



	BJAB		JURKAT	
	Relative CAT	*SD*	*Relative CAT*	*SD*
XS 2 5 tk CAT →	4.27	1 87	3 39	0.62
XS 2 5 ←	4.86	0.49	6.21	3.62
→ XS 2.5	0.41	0.08	0.59	0 29

B. BB 1.8



BB 1.8 ←	3.75	1.05	4.98	1.84
BB 1.8 ΔBal ←	1.97	0.67	2.42	0 24
BB 1.8 ΔSty ←	0.91	0.19	1.05	0.32
Bal 500 →	4.45	0.50	6.46	0.72
Bal 500 ←	4.57	0.72	6.03	1.39
→ Bal 500	1.10	0.21	1.13	0.18

48 oligomer

48-mer →	48 42	12.85	10.72	1.59
48-mer ←	2.61	0.35	3 60	0 47
← 48-mer	13.28	6.02	6.96	1.82

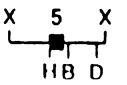
C. BB 3.0

BB 3.0 →	2.70	1.07	2 79	0 65
BB 3.0 ←	0 64	0.04	1.19	0.17
→ BB 3.0	10.89	3 26	7.49	3.18
→ BB 3.0	2.78	0 45	3 01	0.33
MM 0 5 →	1.03	0.31	0.93	0.20

D. BB 0.8

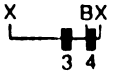
	BJAB		JURKAT	
	Relative CAT	*SD*	*Relative CAT*	*SD*
BB 0.8 →	46.24	11.02	41.33	2.55
BB 0 8 ←	3.76	0.99	3 20	1.40
→ BB 0.8	2.55	0.48	4 65	1.14
← BB 0.8	2.40	0.49	4.37	0.42

E. XX 2.3



XX 2.3 →	3.57	0.11	3.45	0.38
XX 2.3 ←	1.60	0.19	1.54	0.21
→ XX 2.3	0.45	0.09	0.62	0.30
XX 2.3 ←	0.58	0.16	0.81	0.10
XH 1.1 ←	1.38	0.30	1 08	0.42
XH 1.3 →	4.76	2.43	5.96	2.39
BD 700 →	18.77	3.89	3.43	1.66
← BD 700	1.18	0.11	0.28	0.16

F. XX 2.2



XX 2.2 →	1.06	0.25	3.04	0.67
XX 2.2 ←	1.60	0.40	4.52	0.61
→ XX 2.2	3.90	0.62	7.69	0.94

Figure 4:

In this figure riboprobe protection analyses of RNA from several transfections are shown. The length of the probe is 300bp and the length of the protected fragments generated from transcripts arising from the tk promoter are 220bp. The markers are ^{32}P labelled φX HaeIII fragments. The lanes are as indicated.

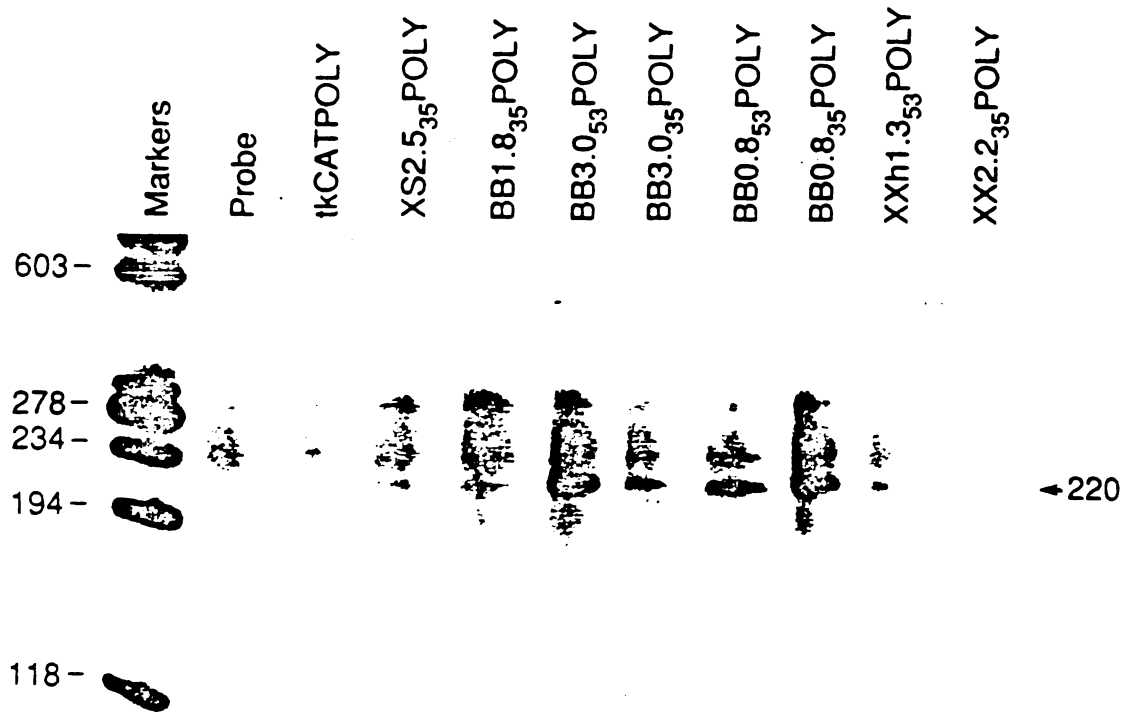
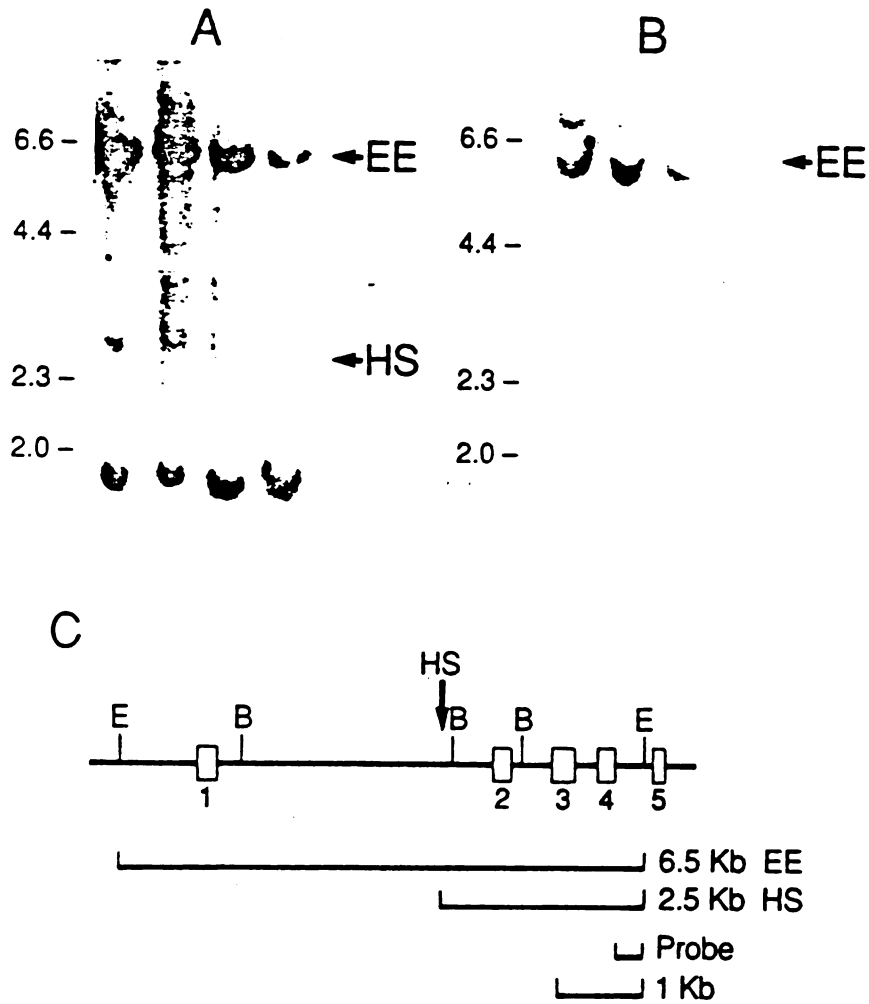

Markers
Probe
tkCATPOLY
XS2.5$_{35}$POLY
BB1.8$_{35}$POLY
BB3.0$_{53}$POLY
BB3.0$_{35}$POLY
BB0.8$_{53}$POLY
BB0.8$_{35}$POLY
XXh1.3$_{53}$POLY
XX2.2$_{35}$POLY
603–
278–
234–
194–
118–
220

Figure 5:

5a and 5b represent DNaseI hypersensitivity analyses of BJAB and Jurkat, respectively. BJAB is a DQ+ B cell line and Jurkat is a DQ- T cell line. The parent EcoRI-EcoRI 6.5kb fragment is marked EE. The band corresponding to the DNase generated fragment is marked HS. A map of the fragments is shown in 5c.


A
B
6.6 –
4.4 –
2.3 –
2.0 –
EE
HS
C
HS
E
B
B
B
E
1
2
3
4
5
6.5 Kb EE
2.5 Kb HS
Probe
1 Kb

MHC CLASS II ENHANCER FUNCTION IN TWO MHC CLASS II DEFICIENT MUTANT CELL LINES

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ABSTRACT

MHC class II enhancer function was examined in Raji, an MHC class II positive B lymphoblastoid line, and two MHC class II deficient mutant cell lines derived from Raji. Enhancer funtion was assayed in transient transfections of enhancer linked chloramphenicol acetyltransferase genes (CAT). One enhancer is located 3kb upstream of DQα, one enhancer, divided into two constructs, is located in the first intron of DQα and a third enhancer consists of the class II consensus boxes X and Y from the promoter region. Enhancers derived from DQα, rous sarcoma virus (RSV), and murine Igκ genes functioned well in Raji and Raji mutant 3 (RM3). In contrast, the intronic and upstream enhancers from DQα failed to function in Raji mutant 2 (RM2). The class II consensus, RSV and Igκ enhancers retained activity in this cell line suggesting a specific defect in a trans-acting factor required for DQα intronic and upstream enhancer function. This suggests that the intronic and upstream enhancers play a crucial role in the expression of DQα. Results with the class II consensus enhancer further suggest that DQα contains at least two separate types of enhancers which are separately regulated. Furthermore, the experiments with RM3 imply that there is at least one more regulatory region yet to be defined in DQα. These experiments are the first demonstration of a mutation in a eukaryotic trans-acting regulatory factor.

INTRODUCTION

Class II antigens of the major histocompatibilty complex (MHC) are transmembrane glycoproteins which function in the presentation of antigen to T lymphocytes (16). They are composed of a 48kd α chain and a 34kd ß chain which are expressed on the surfaces of antigen presenting cells as heterodimers (9). The regulation of expression of these antigens is primarily transcriptional and extremely complex (8). Three families of α and ß chain genes exist and two unpaired genes have recently been described (9,17,18,19). The three families of genes, DP, DQ and DR are expressed primarily on B cells, activated T cells and antigen presenting cells such as dendritic cells. Other tissues can be induced to express class II antigens transiently with γ-interferon (IFN-γ) (2). Expression of these antigens in the ontogeny of B cells occurs in an ordered fashion with DP and DR expressed initially, followed by DQ expression (7). Therefore, the expression of MHC class II antigens involves tissue specificity, coordinate induction by a lymphokine and dissociated expression in development. Additionally, the invariant chain (Ii), an unlinked gene encoding a protein which may be involved in the intracellular transport of class II antigens, is coregulated with class II genes (2). These features contribute to the complexity of the regulation of MHC class II antigens.

Class II gene expression appears to be regulated at least in part by tissue specific enhancers. Intronic enhancers have been described for DQα, DQß, and DRα (17,20). These enhancers are tissue specific and

appear to function primarily in the constitutive expression of of class II antigens in B cells. Another regulatory region, which has been termed the class II consensus X and Y boxes, functions as an enhancer but is not tissue specific (17). The class II consensus is a promoter element which is conserved among all of the class II genes and the Ii gene (10,12). It may play a role in the IFN-γ induction of class II expression. A distant upstream element has also been described for DQα. The function of this element is unclear, however it exhibits the same tissue specificity as the DQα intronic enhancer and may have a similar role (17).

This paper utilizes Raji, a B lymphoblastoid cell line, and two MHC class II deficient cell lines derived from it. The two mutant cell lines, RM2 and RM3, have been characterized as transcriptionally deficient for MHC class II gene expression. RM3 expresses no detectable message for DPß, DQα, DQß, DRα, or DRß. Ii message is only slighly decreased in RM3 compared to Raji. RM2 expresses very low levels of DPß, DQß, DRα and DRß and no detectable DQα. Ii is also slightly decreased. Run-off transcription assays and actinomycin D experiments demonstrated that class II message stability is normal in the two mutant cell lines but initiation of transcription is greatly reduced. Message levels for Igκ and MHC class I genes are normal in the two mutant lines suggesting that it is a specific defect in class II gene expression. All of the class II genes were affected by these mutations implying loss of a shared trans-acting factor required for the transcription of these genes (1). Several other cell lines exist in which the loss of a trans-acting factor specific for class II genes

is implied by the defect in their transcription (3,6,13,15). In one case, an MHC class II negative variant of Raji was shown to be complemented by a murine locus on chromosome 16 (6). In another case, expression of class II genes in a mutant murine cell line could be rescued by treatment with IL-4 (BSF-1) (13). Yet a third type of mutant cell line could be complemented by fusion with a class II negative T cell line (15). These class II deficient mutant cell lines will prove useful in the investigation of trans-acting factors required for the transcription of MHC class II genes. The findings presented here suggest that one of the class II deficient mutant cell lines, RM2, lacks a factor required for class II enhancer function. The other mutant line, RM3, appears to be defective for a different trans-acting factor.

MATERIALS AND METHODS

CELL LINES AND TRANSFECTIONS The characterization of these cell lines has been presented elsewhere (1). They were maintained in RPMI 1640 with 10% fetal calf serum, 50 U/ml penicillin G and 50 µg/ml streptomycin. Transfection of the cells took place in serum free media with .1mM chloroquin and 300 µg/ml DEAE dextran and 2µg of test plasmid. 2µg of RSV ßGAL was included in each transfection as a control for transfection efficiency (17). After 3 hours at 37°, the cells were washed and resuspended in media for three days. Assays for chloramphenicol acetyltransferase (CAT), protein determinations and ß galactosidase assays were performed as described (4,17). The relative CAT value for each transfection represents the percent acetylation standardized to the ß galactosidase value and then expressed in terms of the tk-CAT value which is set equal to one.

PLASMID CONSTRUCTIONS The enhancers were all cloned into a CAT vector called PTE2 Δ Sal Nru which will be known as tk-CAT in this paper (17). The DQα enhancers were derived as indicated in figure 1. The class II consensus was synthesized as a 48bp oligomer (17). The murine Igκ enhancer and the RSV enhancer have been described (5,14). Each plasmid was prepared by alkaline lysis and the supercoiled form was isolated on a cesium gradient (11).

RESULTS

In figure 2 representative CAT assays demonstrate that the Igκ, RSV, and DQ enhancers function well in Raji and RM3. In the mutant line RM2 the Igκ, RSV and class II consensus enhancers give values well above the tk-CAT baseline but the intronic and upstream enhancers from DQα, BB 3.0_{53}, BB $.8_{53}$ and XS 2.5_{35} do not. Averages of at least three transfections of each construct are shown in table 1. The dissociation of the upstream and intronic enhancer activities from the activity of the class II consensus suggests that at least two separate types of regulation exist for DQα. In previous studies, the class II consensus was shown to be tissue non-specific, while the upstream and intronic enhancers functioned primarily in mature B cell lines. The results with RM2 suggest that the class II consensus does not require a trans-acting factor which is required by the intronic and upstream enhancers. The activity of the endogenous class II consensus may explain why RM2 shows low levels of class II message. It may also explain why Ii message levels are not decreased in parallel with class II. The Ii gene contains a class II consensus in its promoter but may not rely on regulatory regions analogous to the intronic enhancers. Thus, in RM2, its transcription would be expected to be grossly intact. The defect in RM3 was not elucidated by these transfections suggesting that there are other regulatory elements yet to be identified.

DISCUSSION

In this report we have demonstrated that the defect in an MHC class II deficient mutant cell line affects the utilization of upstream and intronic enhancers but not a promoter associated enhancer known as the class II consensus. The mutants were generated by ethane methyl sulfonate mutagenesis and selection with CA 206, a monoclonal antibody against a monomorphic class II determinant (1). Both mutants have been characterized as defective for the transcription of class II genes, and southerns have demonstrated that this is not due to deletion of the genes (1). It is unlikely that more than one mutation accounts for the phenotype of the mutants and it has been hypothesized that each mutant cell line represents a defect in a trans-acting regulatory factor required for the transcription of class II genes. These experiments demonstrate that intronic and upstream enhancer function is crucial for the constitutive expression of DQα in a B lyphoblastoid cell line and that mutation in a trans-acting regulatory factor can completely abolish enhancer activity. Enhancer elements apparently have no intrinsic ability to stimulate transcription. Their activity relies on the binding of regulatory factors. The enhancing activity found in these intronic and upstream constructs was coordinately abolished in the RM2 cell line suggesting that they share the binding of at least one regulatory factor. Repeated motifs are a feature of enhancers but it is not clear why two separate regions of the gene would perform the same function. Perhaps the upstream region and the intronic enhancer perform distinct functions in certain circumstances. These two MHC deficient mutant cell lines should enable the characterization and

cloning of trans-acting regulatory factors required for the transcription of MHC class II genes.

TABLE 1

CONSTRUCT	Raji		RM3		RM2	
XS 2.5_{35}	2.06	.24	3.77	1.68	1.00	.15
BB 3.0_{53}	2.42	.61	1.95	.48	1.21	.14
BB $.8_{53}$	3.49	.80	2.85	.46	1.07	.27
OTK_{53}	5.39	.61	3.97	.96	2.27	.16
Igκ E	3.17	1.11	6.10	1.48	2.67	.51
RSV-CAT	20.33	8.39	28.18	6.23	22.76	8.82

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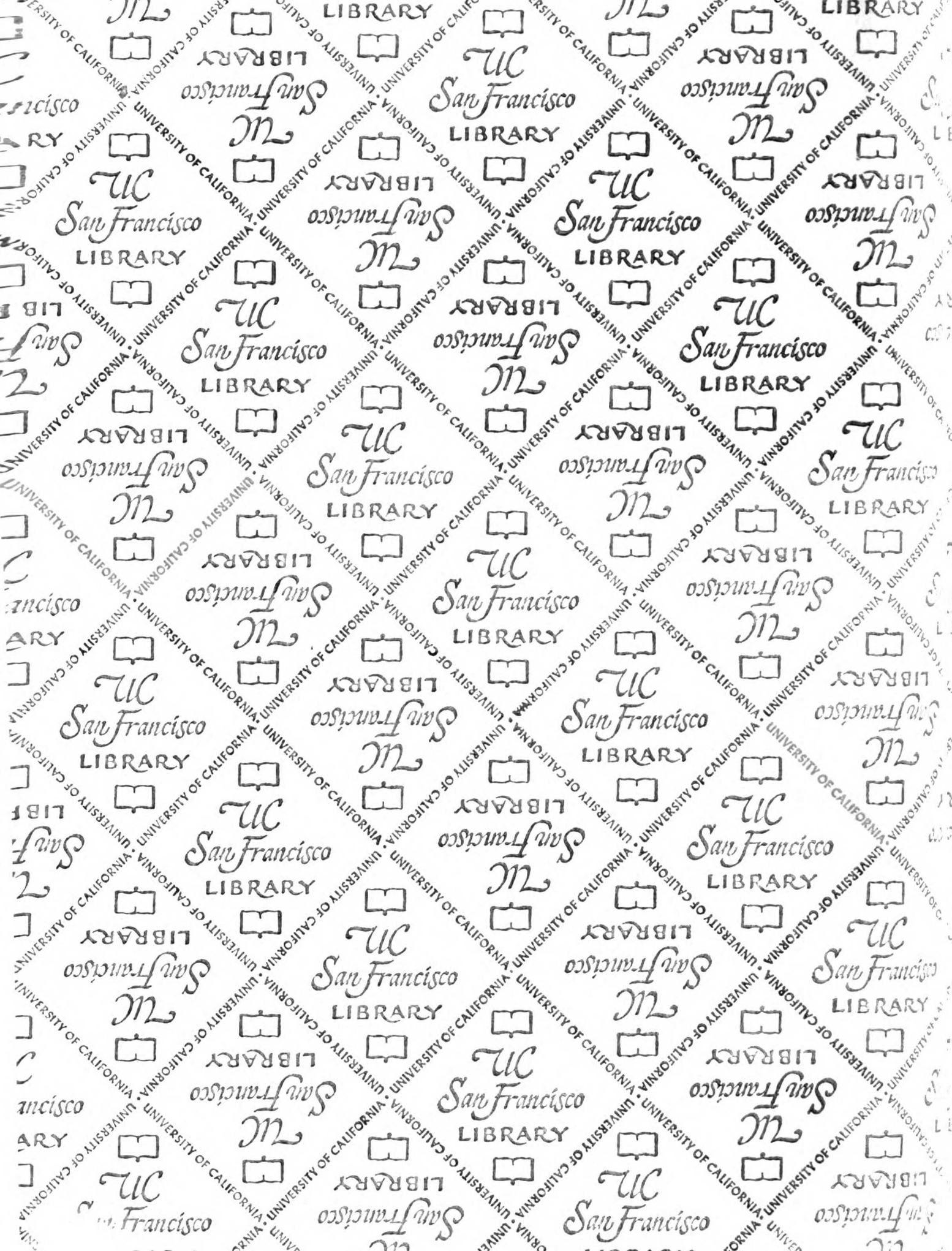
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