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Nuclear Protein Binding Patterns in the 5′-Upstream Regulatory Elements of HLA Class I Genes

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The expression of MHC class I genes has been thought to be regulated by two major cis-acting regulatory elements. The first region, enhancer A (Enh A) spanning from positions -210 to -165 contains perfect palindrome (PP), TGGGGATTCCCCA. The PP is well-conserved both in mouse and human MHC class I genes, even though the PP is disrupted by 2 bp substitutions (TGAGGATTCT CCA) in HLA-C genes. Three proteins binding to the Enh A of HLA-A and -B locus genes, but very weakly or nearly not to the Enh A of HLA-C locus gene have been identified. To determine functional importance of the PP for binding of trans-acting protein, mutant DNA probes were made by site-directed in vitro mutagenesis and then electrophoretic mobility shift assay was performed. HLA-A mutant DNA probe, in which the PP is disrupted, shows the same nuclear protein binding pattern as that of the HLA-C gene, and HLA-C mutant DNA probe, in which the PP is introduced, shows the same nuclear protein binding pattern as that of the wild type HLA-A gene. These data suggest that the perfect palindrome and its cognate DNA binding nuclear protein play an important role in the HLA class I gene regulation, and thus the lower expression of HLA-C antigen may be ascribed to no or very weak factor binding to the nonpalindromic sequences of HLA-C upstream DNA.

Key Words: Electrophoretic mobility shift assay, enhancer A, perfect palindrome, site-directed in vitro mutagenesis, HLA class I gene regulation

The expression of major histocompatibility complex (MHC) class I gene products participates in presentation of antigens on viral infected cells or tumor cells for mediation of the activation of cytotoxic T lymphocytes, and therefore, the regulation of MHC class I gene expression plays a key role in the control of the immune surveillance (Klein *et al.* 1983; Wake, 1986). In addition, the aberrant expression of class I antigens is thought to be involved in the oncogenicity and metastatic potentials of certain tumors by escaping the cellular immune surveillance (Schmidt and Festenstein, 1982; Wallich *et al.* 1985). MHC class I antigens are transmembrane glycoproteins consisting of α chain and β2-microglobulin (β2m) (Koeller and Ozato, 1986). In human, class I genes are clustered in the HLA-A, -B, and -C regions of the MHC on chromosome 6. In most instances, the entire familiy of class I α chain and β2m genes are coordinately regulated (Hood *et al.* 1983).

The expression of class I genes is tightly regulated in terms of tissue distribution and developmental control (Morello *et al.* 1985;

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Ozato *et al.* 1985). Most nucleated cells express class I antigens (Hood *et al.* 1983). Certain specialized cell types, such as trophoblasts (Faulk and McIntyre, 1983; Oudejans *et al.* 1989), embryonic cells (Ozato *et al.* 1985), and central nervous system tissues (Wong *et al.* 1984) have been known not to express class I genes. However, it has been reported that class I gene expression can be induced in a number of class I negative cells by stimulation with interferon-γ (IFN-γ) (Fellows *et al.* 1982; Wong *et al.* 1984; Wan *et al.* 1987).

Transcriptional regulation of most eukaryotic genes is known to be controlled by trans-acting factors which recognize the sequences in the 5′ flanking region of the structural genes and enhancers (Dynan and Tijan, 1985; Ptashne, 1986; Sassone-Coris and Borrelli, 1986; Maniatis *et al.* 1987). The on- and off-expression of class I genes is thought to be controlled by cis-acting elements and their interaction with trans-acting factors as in other eukaryotic genes (Kimura *et al.* 1986; Shirayoshi *et al.* 1987; Obata *et al.* 1988). In murine systems, two enhancer-like regions, A and B have been identified and whose importance for the expression and regulation of MHC class I genes through specific binding of nuclear proteins has been postulated. Actually some of these proteins have been identified and purified (Burke and Ozato, 1989; David-Watine *et al.* 1990). Recently two cis-acting regulatory elements (Enh A and B) showing transcriptional enhancing activity in chloramphenicol acetyltransferase (CAT) assays, were also identified in a human MHC class I gene. The Enh A consists of two palindromes, one of which is a perfect palindrome, and the other one is an imperfect palindrome which is juxtaposed to the perfect palindrome. Trans-acting protein complexes that recognize the Enh A have been identified by electrophoretic mobility shift assays (EMSA). When their DNA binding sequences are localized by DNase I footprinting assay and competition assays, they are similar to those of mouse MHC class I genes. Two discrete nuclear complexes were identified ; one recognizes the perfect palindrome while the other recognizes both palindromes (Park *et al.* 1993). Sequence comparison shows that the perfect palindrome is highly conserved between human and murine MHC class I genes with the exceptions of HLA-C genes which do not have the perfect palindrome due to the two base pair (bp) substitutions. Actually the expression level of class I gene in HLA-C is very weak (Mizuno *et al.* 1989) while those in HLA-A and -B are relatively strong. Therefore, different DNA-protein interaction within the Enh A region between in different loci may be involved in the differential regulation in HLA class I genes.

To understand the mechanisms controlling differential expression and/or suppression of the MHC class I locus genes in humans, EMSA and competition assays were performed using several wild type and mutant DNA probes derived from each of the HLA class I gene. Oligonucleotide probes of known factor binding motifs such as AP2, β2m, NF-κ B, and CA were also used to determine interaction of the Enh A-binding factors with promoters of other genes. β2m promoter exhibited a shifted band pattern similar to that of the Enh A, indicating that the Enh A-binding factors can also bind to the β2m promoter and may play a role in the coordinate regulation of MHC class I and β2m genes. Our results show that three nuclear proteins can also bind to the Enh A of HLA-A and -B locus gene, but very weakly or not to that of HLA-C locus gene, and a factor binding to the perfect palindrome controls the interaction of DNA-binding proteins. We suggest that the perfect palindrome is essential for the recognition of factor-binding and that there is a direct correlation between the activity of class I genes and the presence of a specific DNA sequence, the perfect palindrome, and its binding protein.

MATERIALS AND METHODS

Cell line and preparation of nuclear extract

A human B-lymphoblastoid cell line (B-LCL, BH) was grown in RPMI 1640 medium containing 10% fetal bovine serum supplemented with penicillin and streptomycin. For the nuclear extract preparation, all procedures were

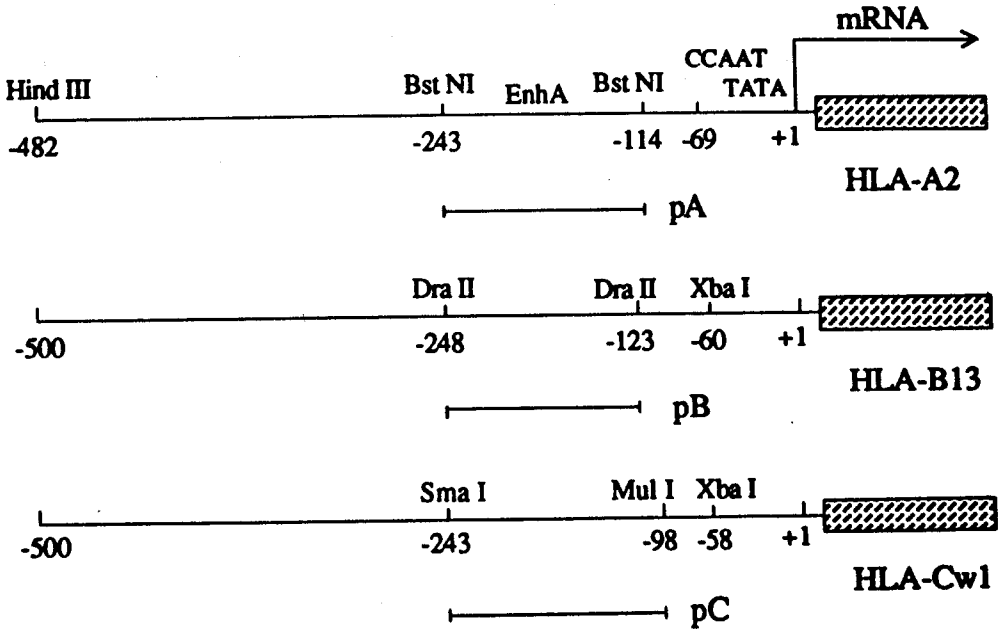

Fig. 1. *The probes derived from 5′ upstream sequence of each HLA class I locus gene are shown. The numbers represent relative nucleotide positions to the transcription initiation site (+1).*

performed at 4℃. Added to buffers before use were 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (containing antipain, aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor, 5 μg/ml each) (Ballard *et al.* 1990). Essential preparation of nuclei and nuclear extract is described elsewhere (Dignam *et al.* 1983) except, that buffer A contained no NP-40. Protein concentration was measured by the Bradford method (Bradford, 1976).

Plasmids and plasmid construction

Plasmid DNA pHLA-2a (Koller and Orr, 1985) was digested with HindIII and AhaII to generate approximately 500 bp DNA fragment containing 5′-upstream sequence of HLA-A2 gene. The gaps of this DNA fragment were filled with the complementary dNTPs by large fragment of DNA polymerase I (Klenow enzyme) and subcloned into SmaI site of pUC 18 plasmid vector (named pA2HA). pA2HA was digested with BstNI to generate approximately 120 bp fragment containing the Enh A of HLA-A2 (named pA) and the gaps of DNA fragment were filled by Klenow enzyme and subcloned into SmaI site of pUC 18 plasmid vector. Approximately 500 bp fragment of 5′-upstream DNA of HLA-B13 and HLA-Cw1 genes (Yang, unpublished data) was obtained from polymerase chain reactions (PCR) by using EcoRI tailed primers and was subcloned into the EcoRI site of pUC 18 (named pB13Up and pCw$_1$Up respectively). From this pB13Up, approximately 120 bp DNA fragment containing the Enh A of HLA-B13 (named pB) was generated by digestion with DraII. pCw1Up was digested with SmaI and MluI to generate approximately 120 bp DNA fragment containing the Enh A of HLA-Cw1 (named pC). Each DNA fragment was blunt-ended with Klenow enzyme and the blunt ended DNA fragments were ligated with the SmaI site of pUC 18 plasmid vector and subcloned (Fig. 1).

Site-directed in vitro mutagenesis

Mutant DNA probes were generated by site-directed in vitro mutagenesis using Muta-Gene

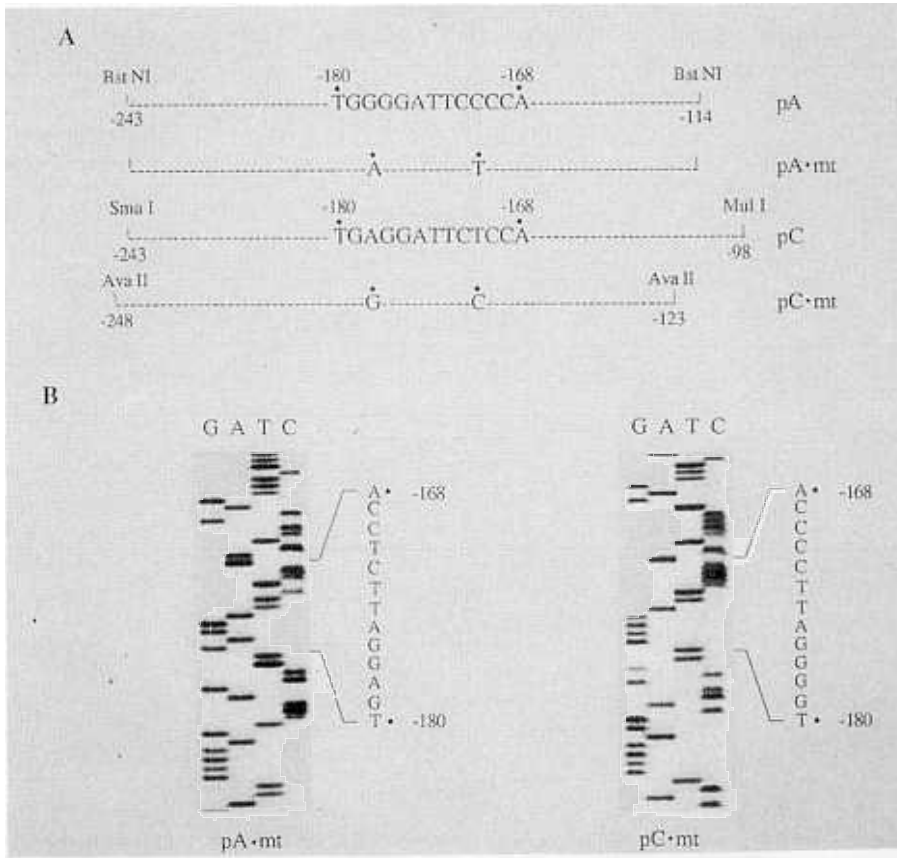

Fig. 2. *Mutant DNA probes generated by site-directed in vitro mutagenesis. (A) By two base pairs substitution, the perfect palindrome of pA was changed to the sequence of pC (pA·mt) and the perfect palindrome was generated in pC·mt. (B) The nucleotide sequences of changed (pA·mt) and generated (pC·mt) perfect palindrome were demonstrated by sequencing.*

M13 in vitro mutagenesis kit (Bio-Rad Laboratories, Inc., Hercules, CA., USA) according to the manufacturer's instructions. Briefly, EcoRI-PstI DNA fragment of pA2HA and EcoRI DNA fragment of pCw1Up were cloned into M13mp18 RF DNA respectively, and mutated with mutagenic oligonucleotide. Sequence and orientation of each mutant DNA were analyzed by DNA sequencing with chain-termination method (Sanger *et al.* 1977). RF DNA from each mutated clone was redigested with BstNI (named pA·mt) and, AvaII (named pC·mt). The gaps of pA·mt and pC·mt were filled and subcloned at SmaI site of plasmid vector pUC 18 (Fig. 2).

Electrophoretic mobility shift assay and competition assay

Binding reactions were performed using the procedure of Strauss and Varshavsky (1984) with minor modifications. Briefly, 5 μg of crude nuclear extract was incubated with 10,000 cpm of 5′-end labeled probe DNA (0.2~0.3 ng) for 20 minutes at room temperature in the presence of 3 μg of poly (dI-dC) (Pharmacia) which acted as a bulk carrier DNA (Sen and Baltimore, 1986a), 80 mM KCl, 0.1% (w/v) triton X-100, 4% glycerol, 1 mM EDTA, 10 mM β-mercaptoethanol, 10 mM Tris-Cl, pH7.5, 1 mM ATP, 300 μg/ml bovine serum albumin,

10 μM ZnSO4, and 0.5 mM spermidine. The reaction mixtures were electrophoresed through a 5% nondenaturating polyacrylamide gel containing Tris-glycine buffer. To prepare duplex oligonucleotides, complementary strands were synthesized on a DNA synthesizer (Applied Biosystems) using the phosphoramidite method as suggested by the supplier. The two strands were combined, boiled for 10 minutes in 10 mM Tris- Cl, pH 7.5, 1 mM EDTA, 50 mM NaCl, and allowed to anneal by cooling to room temperature over a period of 4 hours. For the competition assay, 100 molar excess amounts of cold dulplex oligonucleotides were added to binding reaction mixtures (Shirayoshi *et al.* 1987).

RESULTS

Sequence comparison of the Enh A region of MHC class I genes between mouse and human

Several conserved DNA sequence motifs are found in the 5′-upstream DNA of MHC class I genes (Fig. 3). A TATA box, thought to be important for accurate initiation of transcription (Hood *et al.* 1983; Kvist *et al.* 1983), is found at positions −25 to −20 upstream of the cap site. A CCAAT box is located at positions −54 to −50. Upstream of the CCAAT motif, there are two sequences called the enhancer A (Enh A, −202 to −168) or class I regulatory element (CRE) and the enhancer B (−80 to −70). These two enhancer sequences are strongly conserved among all murine and human class I genes. They appear to be cis-acting elements involved in the regulation of class I gene expression.

Nucleotide sequences of the Enh A region were further compared. Figure 4 presents the Enh A sequences of mouse and human MHC class I genes. The cis-acting regulatory elements, region I, II, III of the CRE proposed by Shirayoshi *et al.* (1987) are indicated. Despite the highly polymorphic coding region of these genes, the Enh A is well conserved among MHC class I genes. Both in mouse and human class I genes examined, the perfect palindrome (RI) is almost perfectly conserved except for the HLA-C alleles. Two base pair substitutions in this perfect palindrome were observed in the HLA-C alleles. In addition, sequence of half of the perfect palindrome (half palindrome) located in the RIII shows the high degree of homology between two species, although a sequence similar to region II is not conserved.

To identify the locus-specific differences in terms of the factor binding patterns, DNA probes containing the Enh A and its mutant DNA probes were prepared from 5′upstream DNA of each HLA class I locus gene.

Patterns of nuclear proteins binding to the Enh A region of each HLA class I locus gene

To detect and compare proteins that bind to the Enh A region of the each HLA class I locus gene, binding ability of nuclear factors to three probes (pA, pB, and pC) derived from each HLA class I locus gene was determined

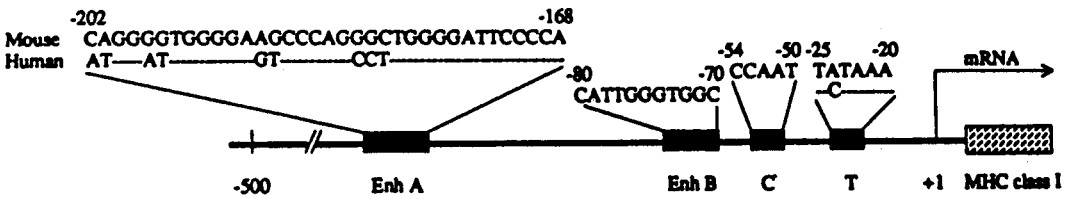

Fig. 3. Schematic diagram of the 5′ upstream region of the MHC class I gene. The solid boxes represent regulatory elements; two major transcription enhancing elements (Enh A and B), the CCAAT box (C), and the TATA box (T). Comparison of the consensus nucleotide sequences of these elements between mouse and human and their relative positions to the transcription initiation site (+1) are shown above the map.

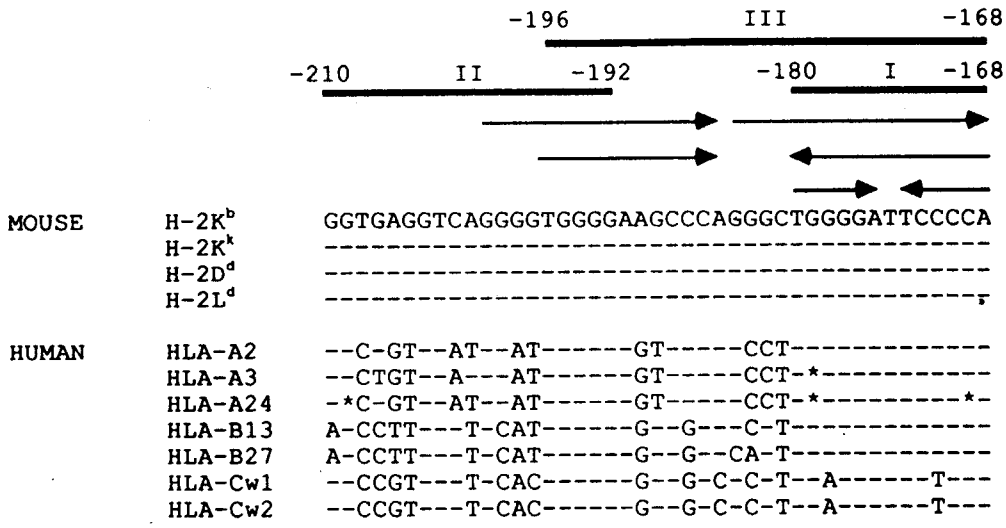

Fig. 4. *The comparison and alignment of the nucleotide sequences corresponding to the murine and human Enh A region. Dash (−) represents nucleotide identical to the H-2K^b gene. Asterisk (*) indicates missing nucleotide. Inverted repeats and tandem repeats indicated by arrows and three subregion of the Enh A proposed by Shirayoshi et al. (1987) were shown above the sequences. RI, the region I protein binding sequence, RII, the region II protein binding sequence, and RIII, the region III protein binding sequence. H-2K^b, K^k, L^d, and D^d (Kimura et al. 1986). HLA-A2 (Koller and Orr, 1985), -A3 (Strachan et al. 1984), -A24 (N'Guyen et al. 1985), -B13 (Yang, unpublished data), -B27 (Weiss et al 1985), -Cw1 (Yang, unpublished data), and -Cw2 (Gussow et al. 1987).*

by means of EMSA. As shown in figure 5 at least three distinct protein-DNA complexes, BI to BIII, are observed reproducibly with pA and pB. The pattern observed with the pA (Fig. 5, lane 2) is exactly the same as the one obtained with pB (lane 6), where BI is the most abundant retarded complex. Even though the half palindrome is perfectly conserved and the sequences between the half and perfect palindromes are highly conserved in HLA-C genes comparing to those of HLA-A and HLA-B locus genes (see Fig. 4 for sequence comparison), no or very weak protein-DNA complexes were shown when pC was used as a probe (Fig. 5, lane 8), that is, protein binding pattern is totally different from those with pA and pB (Fig. 5, lanes 2 and 6).

Cell surface expression of HLA-C molecules has been known to be consistently lower than any other class I antigen expression (Mizuno *et al.* 1989). To circumscribe a conceivability if the lower expression of HLA-C antigen could be due to the absence of perfect palindrome and its binding protein, two mutant probes were generated and tested as probes in EMSA; a mutant DNA probe of pA (named pA·mt) substituted to HLA-C nonpalindromic sequence and the other mutant DNA probe of pC (named pC·mt) substituted to perfect palindrome. As expected, all complexes are totally missing in pA·mt (Fig. 5, lane 4) as in the wild type pC, and all of them are reappeared using pC·mt (Fig 5, lane 10) as in the wild type pA. These suggest that pA·mt and pC are defective in the perfect palindrome and the perfect palindrome is responsible for the formation of protein-DNA complexes as well as protein-protein interaction.

BI requires the perfect palindrome while BII and BIII bind both palindromes

With a series of cold duplex oligonucleotide competitors, competition experiments were carried out using pA and pC·mt to determine which of the complexes identified by EMSA were due to the binding of nuclear factors to

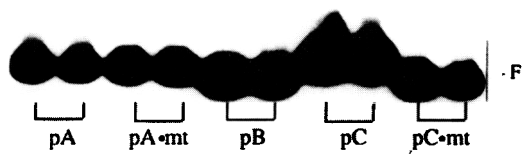

Fig. 5. Electrophoretic mobility shift assy using wild type DNA probes (pA, pB and pC) and mutant DNA probes (pA·mt and pC·mt) derived from each HLA class I locus gene. 5′ end-labelled probe DNA was incubated with (+) or without (−) 5 μg of nuclear extract prepared from a B lymphoblastoid cell line transformed by EBV and electrophoresed in a 5% polyacrylamide gel. Three shifted bands (BI to BIII) representing specific DNA-protein complexes were shown in pA, pB, and pC·mt which have the perfect palindrome. No or very faint band was shown in pA·mt and pC which do not have the perfect palindrome. The bottom bands (F) represent free unbound DNA.

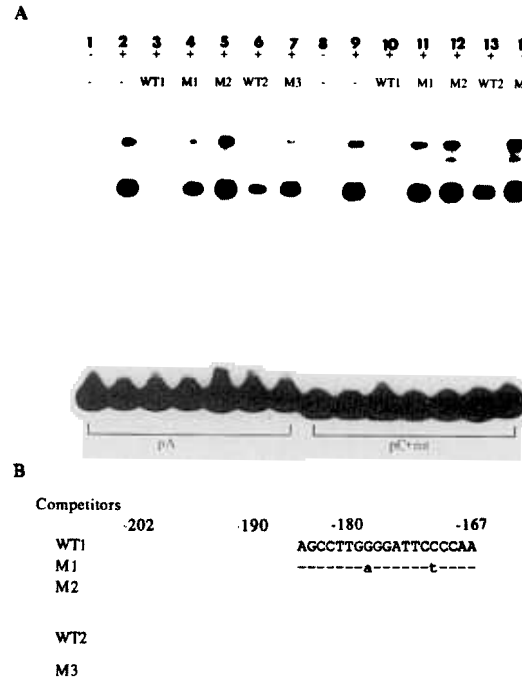

Fig. 6. Mobility shift competition assay using pA and pC·mt. (A) Binding reactions were performed with nuclear extract from a B lymphoblastoid cell line in either the absence or presence of several duplex oligonucleotide competitors. Oligonucleotide competitors were added at 100 fold molar excess. (B) The sequences of the oligonucleotide competitors.

each of the palindrome. When binding reaction is done with either pA or pC·mt in the presence of an excess of a duplex oligonucleotide containing the perfect palindrome, all compexes are competed out specifically (Fig. 6, lane 3 and 10). The same result is obtained when pB is used as probe DNA (data not shown). It can be concluded that all complexes are due to proteins that bind to the perfect palindrome. When binding reactions are performed in the presence of an oligonucleotide competitor covering the half palindrome, complex BII and BIII are specifically competed out (Fig. 6, lane 6 and 13). The loss of BII and BIII indicates that their formation involves the binding of nuclear factors to the half palindrome. Thus while BI is due to the binding of a factor to the perfect palindrome alone, the BII and BIII are bound to both palindromes. Protein complexes are competed out when the binding reaction is done in the presence of a competitor covering the perfect (Fig. 6, lane 3 and 10) or half palindrome (Fig. 6, lane 6 and 13), while the presence of an equivalent excess of a competitor derived from HLA-C (Fig. 6, lane 4 and 11) or mutant competitors (Fig. 6, lane 5, 7 and lane 12, 14) has no effect. These three bands are

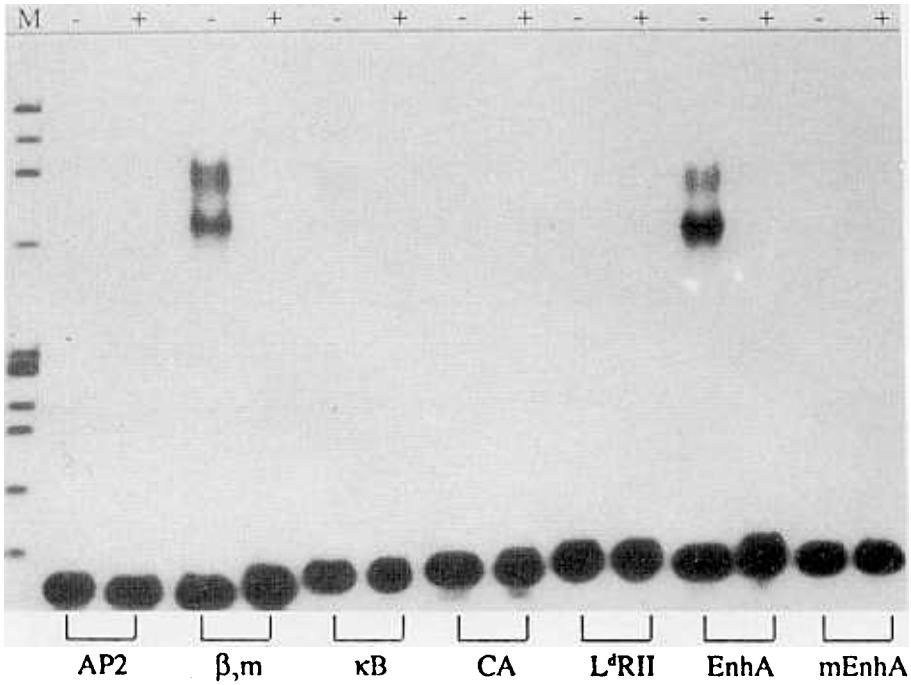

Fig. 7. *The interaction of the Enh A binding protein to the known regulatory elements. Mobility shift competition assay was performed as described in either the absence (−) or presence (+) of the oligonucleotide compertitior WT1 which was also used in Fig. 6. AP2, oligonucleotide from AP2 binding consensus sequence, GAACTGACCGCCCGCGGCCCGTG (Isreal et al. 1989); β2m, the promoter region of the β2m gene, GAAAGGGACTTTCCCAT (Isreal et al. 1987); κB, NF-κB binding motif, GACAGAGGGGACTTTCCGAGAGG (Hazan et al. 1990); CA, oligonucleotide from cAMP responsive element of the somatostatin gene, GTTGGCTGACGTCAGAGAGAG (Montminy et al. 1986); L^{d}R II, region II protein binding sequence of H-2L^d, TTGCCAGGCGGTGAGGTCAGGGGTGGGGAA (Shirayoshi et al. 1987); Enh A, the Enh A region of HLA-A2 gene, GATTGGGGAGTCCCAGCCTTGGGGATTCCCCAA; mEnh A, mutant oligonucleotide of the Enh A region of HLA-A2 gene, GATTGGGGAGTCCCAGCCTTGAGGATTCTCCAA.*

therefore sequence-specific protein-DNA complexes.

β2m gene enhancer shows the same factor binding patterns of the Enh A of HLA class I gene.

To identify other regulatory sequences that interact with the nuclear factors binding to the Enh A, EMSA was performed using oligonucleotide probes of AP2 (Israel *et al.* 1989), NF-κB (Hazan *et al.* 1990), CA (Montminy *et al.* 1987) binding motif, promoter of β2m gene (Israel *et al.* 1987), and L^{d}R II (Shirayoshi *et al.* 1987), in pararell with oligonucleotide probes of the Enh A of HLA-A and its mutant (Fig. 7). Only β2m promoter shows the similar shifted protein-DNA complex as those of the Enh A of HLA-A gene (Fig. 7, lane 4 and 12). Furthermore, the shifted complexes between β2m promoter and nuclear proteins are abolished by the Enh A wild type competitor oligonucleotide (Fig. 7 lane 5 and 13), suggesting that a factor binding to β2m promoter is also capable of binding to MHC class I gene promoter. And this is consistent with the observation that similar nuclear factor controls transcriptional regulation of the β2m and MHC class I genes (Israel *et al.* 1987). On the other hand, NF-κB shows only one band corresponding to BII in the

Enh A (Fig. 7, lane 6) while no band was appeared in AP2, CA, and L^{d}RII.

From these data obtained by EMSA and competition assay, several conclusions can be drawn. First, the protein-DNA complexes BII and BIII are due to the binding of factors to the conserved half palindrome. Second, complex BI is due to binding of a factor to the perfect palindrome. The same proteins are probably involved in binding to the Enh A of HLA-A and κB locus genes, since the EMSA profiles and the competition assay results are the same in pA and pB. Binding of nuclear proteins is not limited to the Enh A since they can bind to other promoters such as β2m and weakly to NF-κB. Finally, there is no proteins that recognize the pA·mt and pC DNA sequence in EMSA. This implies that the perfect palindrome, which contributes to the formation of all three bands, is essential for factor binding and for controllng the protein-protein interaction. The absence of perfect palindrome may therefore explain why HLA-C antigen expression is lower than any other class I antigen expression. The perfect palindrome and regulatory factors binding to it are essential for the constitutive and coordinate transcription of MHC class I genes.

DISCUSSION

5′-upstream DNA of MHC class I genes shows a remarkable degree of sequence homology. In particular, two short DNA segments called the Enh A and B are found in the promoters of human and murine class I genes examined (Fig. 3). Their sequence conservation and relative location to the genes suggest that they are cis-acting regulatory elements involved in both constitutive and inducible expression. However, no similiar sequence especially the perfect palindrome is found at equivalent position in HLA-C genes (Fig. 4). We have compared the interaction of regulatory factors with the Enh A using probe DNA derived from each HLA class I locus gene. This comparison has allowed us to elucidate that the perfect palindrome and regulatory factors binding to it are important for the regulation of HLA class I gene expression.

By detailed analysis of the mouse MHC class I promoter, it has been revealed that several cis-acting regulatory regions are involved in the expression and regulation of MHC class I genes (Burke and Ozato, 1989; David-Watine *et al.* 1990). Among these cis-acting regulatory regions, two enhancer-like sequences, A and B are postulated to be important for the regulation of MHC class I genes. Both enhancers are conserved within the promoter of several genes coding for classical class I antigens. The enhancer A (Kimura *et al.* 1986), also called as class I regulatory element (CRE) (Shirayoshi *et al.* 1987) has shown that there is specific binding of nuclear proteins to it (Baldwin and Sharp, 1987; Israel *et al.* 1987; Shirayoshi et al., 1987). Binding sites were mapped, and further divided into three distinct binding sequences called region I, II, and III (Shirayoshi *et al.* 1987).

Two major enhancing regions were detected by means of transient chloramphenicol acetyltransferase (CAT) assays with a series of deletion mutants placed in the 5′-upstream of a HLA class I gene fused to the CAT reporter gene. The first region, the Enh A, spanning from positions -210 to -165 shows to act as a typical enhancer capable of enhancing transcription. The functional significance of the Enh A deserves to be investigated since the Enh A is highly conserved among MHC class I genes. The conservation of the Enh A in human and mouse suggests that this cis-acting regulatory elements play a crucial role in controlling expression of the MHC class I genes and that the structural and functional properties of trans-acting factors that bind to it are also shared in both species. DNase I footprinting analysis showed that the binding sequence of nuclear proteins coincided with enhancing activity region and displayed homology with such known sequences as in mouse MHC class I genes, Enh A or CRE. The Enh A region can be dissected into a series of overlapping palindromes. The core region of the Enh A is a perfect palindrome, and an imperfect copy of it lies a few nucleotides upstream of the perfect palindrome (Park *et al.* 1993).

Another region, called the Enh B, is located

approximately 70 bp upstream from the transcription start site and its functional significance remains to be examined.

To study the functional role of the Enh A and regulatory complexes interacting with it, wild type DNA probes derived from each HLA class I locus gene were generated. And also the perfect palindrome was disrupted in HLA-A gene and the perfect palindrome was introduced in HLA-C gene by site-directed mutagenesis. Each mutated DNA probe was tested with wild type DNA probes. By using EMSA, three nuclear protein complexes, BI to BIII were found to bind to the Enh A (Fig. 5). BI was the most abundant one, and that is consistent with that the predominant complex was the factor binding to the perfect palindrome in murine liver and spleen tissues (Burke and Ozato, 1989). EMSA has shown that nuclear protein binding patterns with DNA probes defective in the perfect palindrome, such as the mutated Enh A of HLA-A gene (pA·mt) and probe DNA derived from HLA-C gene (pC), in which all three complexes are not formed or very weakly formed, are totally different from those with probe DNA with the perfect palindrome, such as the Enh A of HLA-A (pA) and -B (pB) locus genes and mutant DNA probe derived from HLA-C (pC · mt). The absence of perfect palindrome is correlated with no factor binding to it. It must be proposed that the perfect palindrome and regulatory factors binding to it are essential for constitutive transcription of class I genes. There are a few lines of evidence that indicate the functional importance of the perfect palindrome and its binding protein. A CAT construct containing 2 bp substitutions placed in the perfect palindrome of HLA-A2 but not in other regions of the Enh A, elicit markedly lower CAT activity in HeLa cells, and a CAT construct containing the perfect palindrome by 2 bp substitutions in HLA-Cw1 shows restored CAT activity to the level of HLA-A. (Park,unpublished data). It has been suggested that absence or presence of very low level of perfect palindrome binding factor in the prenatal liver may contribute to the undetectability of class I mRNA and antigens during very early embryonic stage (Miyazaki *et al.* 1986). It has also known that a factor recognizing the perfect palindrome is missing in the brain to show low class I gene expression (Burke and Ozato, 1989). Therefore, presence of the prefect palindrome and level of the perfect palindrome binding factor correlate with those of class I gene expression.

Results derived from competition experiment indicate that there are at least two different proteins that recognize the Enh A element; a perfect palindrome binding protein, and half palindrome binding proteins (Fig. 6). The BI complex was formed by recognizing the perfect palindrome whose sequence was identical to the region I of CRE. This perfect palindrome binding protein may control the binding of other complexes via cooperative interaction. More detailed kinetic analysis and study of the relative stability of BI, BII, and BIII at different salt concentrations should help to clarify this question. The BII and BIII are found to bind to the half palindrome that is a κB-like sequence and juxtaposed to the BI binding motif. This indicates that the half palindromes are responsible for stable binding of BII and BIII. Functions of BII and BIII binding are less clear at present.

It appears that a number of different proteins are capable of interacting with the Enh A. It is of interest that the Enh A falls within a region exhibiting striking homology among different promoters. The nuclear proteins AP1 and AP2, both of which bind the SV 40 viral enhancer, as well as some cellular genes, are also capable of binding the Enh A (Angel *et al.* 1987; Lee *et al.* 1987). However, reciprocal competition between the Enh A and AP1 or AP2 consensus sequences was not observed (Park *et al.* 1993), and no factor binding can be seen in the AP2 binding motif with nuclear extract containing the Enh A binding proteins, indicating that the AP1 and AP2 proteins appear to be distinct from the factors demonstrated in figure 5. NF-κB, originally identified as binding to the Igκ-chain gene enhancer was also demonstrated to show binding activity to the Enh A of the mouse MHC class I gene (Sen and Baltimore, 1986b; Baldwin and Sharp, 1988). Although the NF-κB promoter contains a sequence homolgous to the Enh A, BI and BIII are absent in NF-κB promoter and only very faint BII is observed.

NF-κB does not therefore seem to be essential for class I gene expression, and BI must be distinct from NF-κB and this perfect palindrome is unique in MHC class I gene. On the other hand, β2m enhancer displays the same retarded bands as those shown in the Enh A, suggesting that similar factors can bind to both promoters. These results are in accordance with data obtained from other investigators. A nuclear factor, KBF1, has been known to interact specifically with both palindromes with better affinity to the perfect palindrome. This factor also binds with a similar affinity to the promoter of the mouse β2m gemes and acts as a positive factor in the expression of H-2 class I and β2m genes, suggesting that MHC class I and β2m gene are regulated by common regulatory mechanism (Israel *et al.* 1987). It has been demonstrated that KBF1-binding activity is absent in undifferentiated EC cells where class I and β2m genes are silent and it is induced when the cells are triggered to differentiate (Yano *et al.* 1987). KBF1-like binding activity is undetectable in brain nuclear extracts, which correlates well with the lack of H-2 class I antigens in this organ (Burke *et al.* 1989). Another factor, H-2 transcription factor (H2TF1) binds to the Enh A recognizing exactly the same nucleotide residues as KBF1 (Baldwin and Sharp, 1987). Both H2TF1 and KBF1 bind poorly to the Igκ enhancer-derived site. Consequently, the perfect palindrome-binding protein identified here (BI, BII, and BIII) may represent the common or closely related factor to KBF1 or H2TF1. For the precise characterization and determination of the relationship between these proteins and the palindrome-binding protein identified here, isolation of the cDNA and/or purification of protein should be done.

We described elucidation of highly conserved cis-acting regulatory sequences in the 5′-upstream of the HLA classical class I gene. Our studies are consistent with the notion that trans-acting nuclear proteins which specifically interact with the Enh A elicit positive effects on the transcription of class I genes. Furthermore, a direct correlation between factor binding to the Enh A and regulated expression of the HLA class I gene is observed, which serves as evidence for functional importance of protein binding to the Enh A.

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