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Influence of the Sequence Variations of the HLA-DR Promoters Derived from Human Melanoma Cell Lines on Nuclear Protein Binding and Promoter Activity

Tae Jin Lee*, Se Jong Kim, and Jeon Han Park

Abstract

In previous studies we reported that the expression of HLA-DR on melanoma cell lines was differentially modulated by IFN-γ and that the transcription rate was responsible for this differential modulation. We have also reported the nucleotide sequence variations in the promoter region of HLA-DR genes, and proposed that differences in the promoter activity by the sequence variations of the HLA-DR promoters might contribute to such a differential transcriptional regulation at the promoter level. In this study, in order to assess whether the sequence variations of the HLA-DR promoters affect the factor binding and exert influence on the promoter activity, nuclear factor binding to our previous six HLA-DRA and fourteen HLA-DRB promoter clones was evaluated with the nuclear protein extracted from a B-lymphoblastoid cell line (BLCL), BH, together with the chloramphenicol acetyltransferase (CAT) reporter assay. In the HLA-DRA promoters, clone #35 containing one bp nucleotide sequence variation at the octamer binding site (OCT) (GATTTGC to GATCTGC) showed relatively weak factor binding. In the HLA-DRB promoters, clusters I, III, and IV of our previous HLA-DRB promoter homologues, containing one bp nucleotide sequence variation (GATTCG) in their Y boxes exhibited weak factor binding and CAT activity compared to other clusters (GATTGG) that showed strong factor binding and CAT activity. This data suggests that the binding patterns of transcription factors influenced by the nucleotide sequence variations of the HLA-DR promoter could affect the promoter activity and the DNA sequence elements in the HLA-DR promoter could mediate transcriptional regulation.

Key Words: HLA-DR promoter, sequence variations, factor binding, promoter activity, transcriptional regulation

INTRODUCTION

The recognition of antigen presented to CD 4+ T lymphocytes by major histocompatibility complex (MHC) class II molecules results in the specific regulation of immune responses and T cell function. Because of their critical role in T cell regulation, MHC class II molecules are tightly controlled.[1] MHC class II molecules are expressed on the surface of antigen-presenting cells such as macrophages, B cells, thymic epithelia, and activated T cells. MHC class II molecules consist of an α and a β chain encoded by separate genes. A wide variety of cytokines can stimulate or induce the class II expression, and the most potent one of which is IFN-γ. Inducing the class II expression by IFN-γ can affect many cell types and provide these cells with the ability to present antigenic peptides.[2-4]

Sequence and mutational analyses of the 5′ flanking region of the MHC class II genes have identified several compact, conserved multicomponent motifs comprised of four sub-elements termed the W/Z/S, X1, X2, and Y boxes that are responsible for MHC class II gene regulation. Thus, the presence of these conserved sequences allows the MHC class II gene to be regulated in a coordinate manner.[5,6]

Because many DNA-binding proteins have been found to be associated with HLA-DR regulatory sequences, and because these bindings have been found to influence the promoter activity, this study

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Department of Microbiology, Institute for Immunology and Immunological Diseases, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea.
*Current Address: Institute of Basic Medicine, Yeungnam University.

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Address reprint requests to Dr. J. H. Park, Department of Microbiology, Institute for Immunology and Immunological Diseases, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea. Tel: 82-2-361-5286, Fax: 82-2-392-7088, E-mail: jhpark5277@yumc.yonsei.ac.kr

focused on the influence of the sequence variations of the HLA-DR promoters derived from a panel of human melanoma cell lines on the nuclear protein binding and promoter activity. We reported previously that the expression of HLA-DR was differentially modulated by IFN-γ in a panel of human malignant melanoma cell lines, and that this differential modulation was mainly due to the mRNA transcription rate of the HLA-DR gene. We also proposed that the sequence variations of the HLA-DR promoter might contribute to such a differential transcriptional regulation at the promoter level.[3,4] To understand the differential modulation of HLA-DR expression caused by sequence variations in the promoter region, we examined HLA-DR promoter activity as well as binding patterns of nuclear protein extracted from a B lymphoblastoid cell line (BLCL) to the HLA-DR promoters cloned from the melanoma cell lines in our previous study.[4] In this study, we report that the DNA sequence variations in HLA-DR promoters might affect transcriptional regulation by influencing the nuclear protein binding and the promoter activity.

MATERIALS AND METHODS

Cell lines

A human malignant melanoma cell line, SK-Mel-24 (ATCC HTB71), was cultured with the Eagle's minimum essential medium supplemented with 15 mM N-2-hydroxyethylpiperazine-N-ethane sulfonic acid (HEPES) (Hazleton), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (FBS) (Gibco BRL). A BLCL, BH, was cultured with RPMI containing 10% FBS.

Probes and CAT constructs

Six HLA-DRA and fourteen HLA-DRB promoter clones reported in our previous study[4] were used as probes for the electrophoretic mobility shift assay (EMSA). For the EMSA, each probe was labeled with ^{32}P using Klenow enzyme as described previously.[7-9]

For the construction of CAT reporter plasmid, plasmid DNA of our previous HLA-DRB promoter clones, clone #17, 20, 24, 68, 58, and 22, which were the six representative clones of the HLA-DRB promoter, was digested with *Hind* III and *Xba* I, and then cloned into the *Hind* III and *Xba* I site of the pCAT^{TM}-Basic plasmid (Promega). The nucleotide sequences and orientation of each CAT construct were confirmed by the dideoxy-chain-termination sequencing.[10]

Extraction of nuclear protein

Nuclear protein was extracted from the BH cells by the Dignam method with minor modifications as described[7-9] and the protein concentration was measured by the Bradford method.[11]

EMSA

EMSA was performed according to previous studies.[7-9] Briefly, 5 μg nuclear protein was incubated with 10,000 cpm of the ^{32}P-labeled probe at room temperature for 20 min. The reaction mixtures were electrophoresed in a 5% polyacrylamide gel with Tris-glycine buffer. The gel was dried under a vacuum onto filter paper and autoradiographed.

Transfection and CAT assay

The transient transfection was carried out with SK-Mel-24 cells by the calcium phosphate method with minor modification.[7,9] Approximately 10^6 cells were seeded in a 10 cm dish 16 h before transfection. The medium was changed 4 h before transfection. Ten microgram CAT construct and 1 μg pCMV-β-gal plasmid DNA were cotransfected. After 48 h, the CAT activity and β-galactosidase activity were measured, and the CAT activity measurements were normalized to β-galactosidase activity.

RESULTS

Patterns of nuclear protein binding to the HLA-DR promoter

Binding patterns of nuclear protein extracted from the BH cells were determined by EMSA with six HLA-DRA and fourteen HLA-DRB promoter sequences cloned in our previous study.[4] As shown in Fig. 1, the six HLA-DRA promoter clones showed a high degree of sequence homology with one bp nucleotide

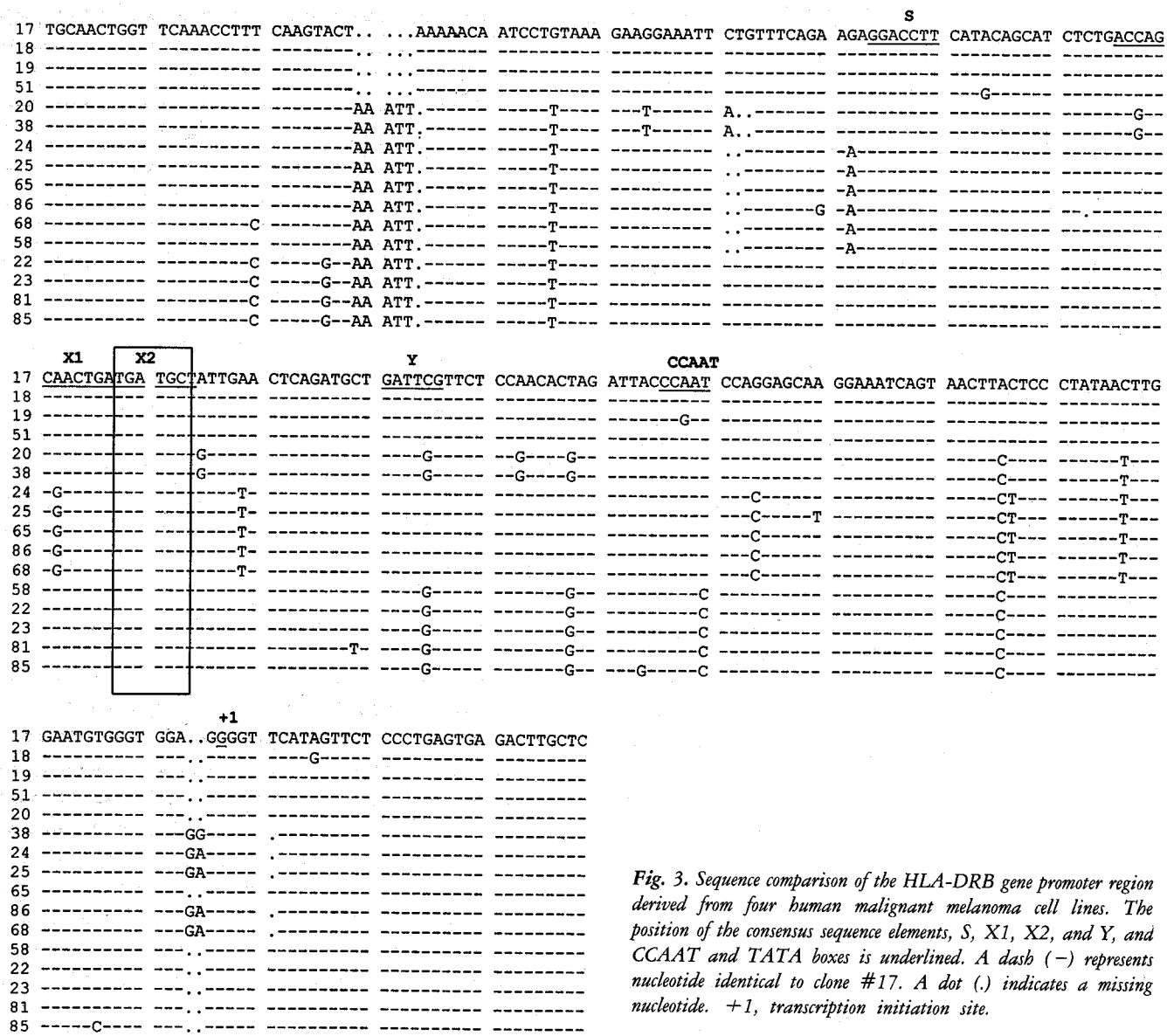

Fig. 3. Sequence comparison of the HLA-DRB gene promoter region derived from four human malignant melanoma cell lines. The position of the consensus sequence elements, S, X1, X2, and Y, and CCAAT and TATA boxes is underlined. A dash (−) represents nucleotide identical to clone #17. A dot (.) indicates a missing nucleotide. +1, transcription initiation site.

Promoter activity of HLA-DRB promoter

To determine the promoter activity of the HLA-DRB promoter, CAT reporter plasmids were constructed by cloning the promoter region of clone #17, 20, 24, 68, 58, and 22 into the pCAT™-Basic plasmid. These were the six representative clones of HLA-DRB promoter homologue clusters.[4] The CAT activity was measured 48 h after transfection and the activity was normalized against the β-galactosidase activity Fig. 5 shows the schematic map of the consensus *cis*-acting elements in the HLA-DRB promoters and the results of the CAT assays, which were performed in SK-Mel-24 cells expressing a high level of HLA-DR.[4] Approximately a five fold increase in CAT activity was observed in clone #20, 58, 22 which showed three protein-DNA complexes and strong factor binding, whereas a two fold increase was observed in clone #24, 68 which showed weak factor binding. These CAT assay results correlated to the nuclear protein binding patterns, suggesting that the nucleotide sequence variations of HLA-DR promoters influence the binding of transcription factors. These in turn could affect the promoter activity such that the DNA sequence elements in the HLA-DR promoter mediate transcriptional regulation.

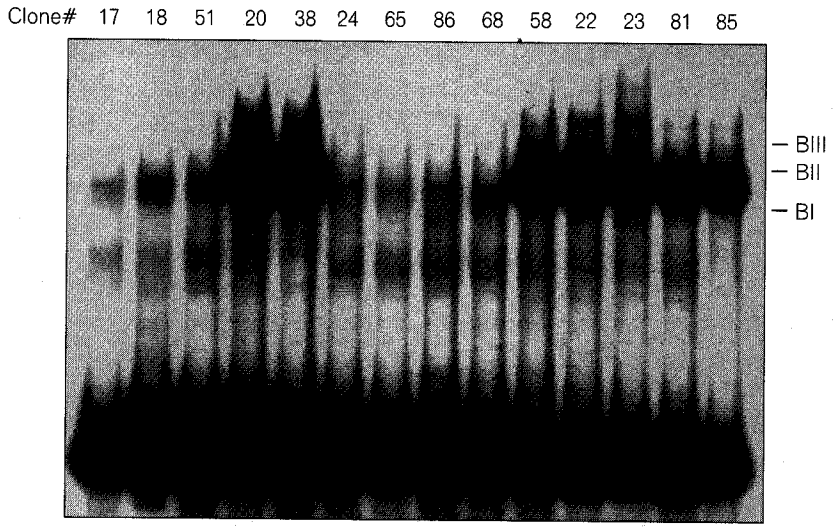

Fig. 4. EMSA performed with fourteen HLA-DRB gene promoter probes of Fig. 3. The assay was conducted as described in Materials and Methods using the nuclear extract from a BLCL, BH.

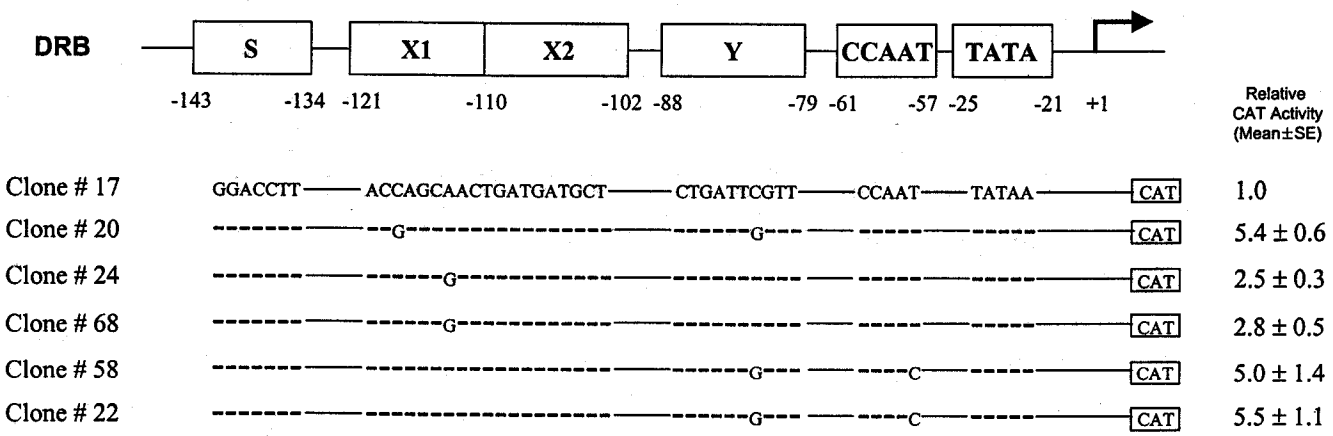

Fig. 5. CAT reporter gene expression in transiently transfected SK-Mel-24 cells. The CAT activity was measured for the six representative clones of the HLA-DRB gene promoter region, and was normalized against the β-galactosidase activity elicited by the cotransfected CMV-β-gal. The CAT activity levels shown are relative to the activity of clone #17. Each value represents a mean ± standard error of six independent experiments. A schematic map of the consensus sequence elements of the HLA-DRB gene promoter region is shown on the top.

DISCUSSION

We reported previously that the differential expression of HLA-DR on the melanoma cell lines is transcriptionally regulated, and also reported the sequence variations of *cis*-acting elements in HLA-DR promoters. We proposed that these sequence variations might contribute to the differential transcriptional regulation of HLA-DR genes.[3,4] In the present study, we report that the nucleotide sequence variations of the HLA-DR promoter affect the factor binding and promoter activity.

Regulated transcription depends on the interaction of *cis*-acting elements and *trans*-acting sequence-specific DNA binding proteins.[12] While the class II antigens of the HLA-DR of the MHC are regarded as extensively polymorphic cell surface molecules, the promoters of the various subloci are highly conserved. Several conserved *cis*-acting elements within the proximal promoter regions of the class II genes, termed X, Y, and the octamer region, were reported.

These elements are critical for the expression of the optimal class II gene in the cell-specific constitutive expression and inducible expression.[5,13] Our previous molecular cloning and sequence analyses revealed that the nucleotide sequences of the HLA-DRA proximal promoter region are quite similar to each other, while those of the HLA-DRB proximal promoter region are

relatively diverse.[4] For the analysis to clarify the mechanism of the differential modulation of the HLA-DR gene depending on the sequence variations, we performed the EMSA and CAT assay to determine the interaction of *cis*-acting elements and *trans*-acting factors.

We identified the distinct difference in the nuclear protein binding patterns according to the sequence variations in the HLA-DR promoters. For the DRA promoters, clone #1, 2, 3, 4, and 6 showed at least three protein-DNA complexes. However, clone #35 in which one bp nucleotide sequence variation was found in the OCT (GATTTGC to GATCTGC), showed only one single faint band. This finding is likely due to the incompetent binding of the octamer binding protein to the OCT, thus leading to the incompetent interaction with other proteins. This suggests that the sequence variation in the OCT found in clone #35 influenced the binding pattern and/or protein-protein interaction.

For the HLA-DRB promoters, clone #20, 38, clone #58, and clone #22, 23, 81, and 85 showed at least three protein-DNA complexes and strong factor binding in EMSA. However, two DNA-protein complex bands (BII, BIII) were not identified in clone #17, 18, 51, clone #24, 65, and clone #68. Moreover, the fast migrating band, BI, was weaker in band intensity than those of clones showing three bands. This finding is likely due to the less accessibility of the Y box binding protein to the Y box caused by sequence variation and the incompetent interaction with other proteins. The nucleotide sequences of the Y boxes of the clones showing three bands and the strong factor binding were conserved and their sequences were GATTGG. However, one bp nucleotide sequence variations (GATTGG to GATTCG) were found in the Y boxes of the clones showing weak factor binding. These results suggest that the nucelotide sequences of the Y box control the factor binding and/or protein-protein interaction.

The transient transfection assays showed that the Y box is essential for driving the expression of a reporter gene. Deletion or mutation of the Y box rendered the promoter inactive.[14] In this study, the transient transfection CAT assays showed that the clones showing three protein-DNA complexes and strong factor binding exhibited strong CAT activity. The clones showing weak factor binding exhibited relatively weak CAT activity. These findings revealed that sequence variations in the HLA-DRB promoter region might influence the factor binding and/or protein-protein interaction, thus possibly displaying an ability to promoter activity.

Genetic and biochemical analyses have identified multiple DNA-binding and non-DNA-binding proteins that functionally regulate the MHC class II genes, including RFX, X2BP, NF-Y, CIITA, Oct-2a and OBF-1. It was reported that one of the non-DNA-binding proteins, CIITA, appeared to function as a limiting switch that is responsible for controlling the class II expression and the regulation of the expression by IFN-γ.[6,15-18] Two models for class II regulation have been postulated. In the first model, an RFX-X2BP-DNA complex forms and is stabilized by NF-Y binding. The RFX-X2BP-NF-Y-DNA complex recruits and binds the CIITA. Once bound, the CIITA activates transcription. In the second model, the CIITA functions enzymatically, modifying the structure of the DNA-bound factors.[6]

Considering in concert the reports of other investigators, our findings show the likelihood that in the HLA-DRA promoter, the octamer binding protein is not capable of binding to the OCT due to the nucleotide variation in clone #35 thus leading to the formation of the only one single faint protein-DNA complex band in EMSA. The DRA gene is the only class II gene containing an OCT between the Y box and the TATA box, which decreases the level of DRA transcription when mutated.[19-23] Thus, it could be speculated that the transcription level of the HLA-DRA gene containing a similar sequence variation in the OCT region would decrease. In fact, clone #35 was cloned from IGR3 whose HLA-DR expression was neither detectable nor inducible by IFN-γ.[4] Hence, in the HLA-DRB promoter it could be speculated that the Y box binding protein did not bind to the Y box, so the incompetent formation of the multiprotein-DNA complexes led to weak factor binding and promoter activity. However, in our melanoma cell lines model, difference in the promoter activity by the sequence variations of the HLA-DR promoters is not explained solely by the differential modulation of the HLA-DR expression. In fact, it was also reported that a lack of induction of the MHC class II molecules within many human tumor cell lines was directly associated with the defective expression of the CIITA.[24] Thus, a defective expression of the CIITA could not be ruled out as a possible explanation for the lack of HLA-DR expression in IGR3.

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1  GCCGTGATTG ACTAACAGTC TTAAATACTT GATTTGTTGT TGCTGTTGTC CTGTTTGTTT AAGAACTTTA CTTCTTTATC CAATGAACGG AGTATCTTGT
2  ---------- ---------- ---------- ---------- --T------- ---------- ---------- ---------- ---------- ----------
3  ---------- ---------- ---------- ---------- --T------- ---------- ---------- ---------- ---------- ----------
4  ---------- ---------- ---------- ---------- --T------- ---------- ---------- ---------- ---------- ----------
6  ---------- ---------- ---------- ---------- --T------- ---------- ---------C- ---------- ---------- ----------
35 ---------- ---------- ---------- ---------- --T------- ---------- ---------- ---------- ---------- ----------
        S                          X1         X2                          Y                          OCT
1  GTCCTGGACC CTTTGCAAGA ACCCTTCCCC TAGCAACAGA TGCGCCATCT CAAAATATTT TTCTGATTGG CCAAAGAGTA ATTGATTTGC ATTTTAATGG
2  ---------- ---------- ---------- ---------- ----T----- ---------- ---------- ---------- ---------- ----------
3  ---------- ---------- ---------- ---------- ----T----- ---------- ---------- ---------- ---------- ----------
4  ---------- ---------- ---------- ---------- ----T----- ---------- ---------- ---------- ---------- ----------
6  ---------- ---------- ---------- ---------- ----T----- ---------- ---------- ---------- ---------- ----------
35 ---------- ---------- ---------- ---------- ----T----- ---------- ---------- ---------- ------C--- ----------
                                    +1
1  TCAGACTCTA TTACACCCCA CATTCTCTTT TCTTTTATTC TTGTCTGTTC TGCCTC.ACT CCCGAGCTCT A
2  ---------- ---------- ---------- --------C- ---------- ------.--- ---------- -
3  ---------- ---------- ---------- ---------- ---------- ------C--- ---------- -
4  ---------- ---------- ---------- ---------- ---------- ------.--- ---------- -
6  ---------- ---------- ---------- ---------- ---------- ------.--- ---------- -
35 ---------- ---------- ---------- ---------- ---------- ------.--- ---------- -
```

Fig. 1. Sequence comparison of the HLA-DRA gene promoter region derived from four human malignant melanoma cell lines. The position of the consensus sequence elements, S, X1, X2, and Y boxes, and the octamer binding site (OCT) is underlined. A dash (-) represents a nucleotide identical to clone #1. A dot (.) indicates a missing nucleotide. +1, transcription initiation site.

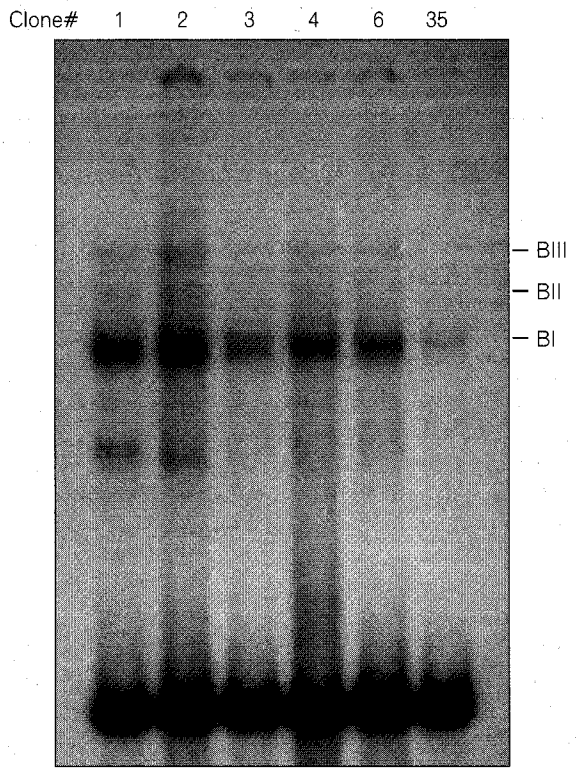

Fig. 2. EMSA performed with six HLA-DRA gene promoter probes of Fig. 1. The assay was conducted as described in Materials and Methods using the nuclear extract from a BLCL, BH.

sequence variations in the X2 box (clone #1: TGCGTCA to TGCGCCA) and the octamer binding site (OCT) (clone #35: GATTTGC to GATCTGC). At least three distinct protein-DNA complexes were observed with the HLA-DRA promoters. However, only one single faint band (BI) was found with clone #35 (Fig. 2), implying that a sequence change in the OCT mainly influences the binding of nuclear protein to the HLA-DRA promoter and/or protein-protein interaction.

More diverse nucleotide sequence variations were found in the *cis*-acting elements of the HLA-DRB promoters. One bp nucleotide sequence variations were shown in the X1 boxes of clone #20, 38 (ACCAGCAACTGATG to ACGAGCAACTGATG) and clone #24, 25, 65, 86, and 68 (ACCAGCAACTGATG to ACCAGCGACTGATG). Single bp changes were found in the Y boxes (GATTCG to GATTGG) of clone #20, 38, 58, 22, 23, 81, and 85 and the CCAAT boxes (CCGAT in clone #19; CCAAC in clone #58, 22, 23, 81, and 85) (Fig. 3).

Diverse nuclear binding patterns were observed. At least three distinct protein-DNA complexes were observed in clone # 20, 38, 58, 22, 23, 81, and 85. However, the two protein-DNA complex bands (BII, BIII) were not found in clone #17, 18, 51, 24, 65, 86, and 68. In these clones the BI protein-DNA complex showed weaker band intensities than those of the clones showing three protein-DNA complexes (Fig. 4). It is interesting to note that the nucleotide sequences of the Y boxes of the clones showing three protein-DNA complexes and strong factor binding were GATTGG, while those of the clones showing weak factor binding were GATTCG (Fig. 3). These results suggest that sequences of the Y box mainly affect the nuclear protein binding and/or protein-protein interaction.