A Binding Protein to the DNase I Hypersensitive Site II in HLA-DRα Gene Was Identified as NF90

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ABSTRACT: We previously observed that IFNγ-inducible expression of the human MHC class II, HLA-DRα, gene was enhanced by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) only in human monocytic leukemia THP-1 cells, but not in HeLa cells. In the HLA-DRα gene, three DNase I hypersensitive sites (DHS) are known to be present in the promoter region (DHS-I) and first intron (DHS-II and -III) and are assumed to be involved in HLA-DRα gene regulation. In this study, we found a binding factor which recognized a unique palindrome sequence (DHS-22) in the region of the DHS II site of the HLA-DRα gene in THP-1 cells and HeLa cells. The binding activity of this factor was decreased by TPA treatment in THP-1 cells, but not in HeLa cells. This binding activity was also detectable in nuclear extracts of bovine brains. Thus, we isolated the DHS-22 binding factor from bovine brain nuclear extracts and finally identified it as NF90 on the basis of molecular mass analysis of Lys-C-digested fragments and amino acid sequences of the two peptides of the trypsin-digested binding protein. The DHS-22 binding protein(s) in THP-1 cells is (are) further confirmed by reactivity to an antibody against NF90, and we have demonstrated that the GST fusion protein of NF90 interacts with DHS-22 by electrophoretic gel mobility shift assay (EMSA). The mRNA of NF90 was decreased by TPA treatment in THP-1 cells but not in HeLa cells. These results suggest that the binding of NF90 to the DNase I hypersensitive site II of HLA-DRα gene seems to negatively regulate HLA-DRα gene expression.

Major histocompatibility complex (MHC) class II gene products are members of an immunogloburin supergene family and function in the recognition of self versus nonself in the immune system. MHC class II genes are constitutively expressed in B-cells, and activated T cells and are inducible by IFNγ in macrophages and some other antigen-presenting cells (*1*, *2*). The appropriate constitutive and inducible expression of a class II gene is essential for normal immune function, whereas aberrant expression in various tissues has been implicated in the pathogenesis of autoimmune diseases such as rheumatoid arthritis, autoimmune nephritis, insulin-dependent diabetes mellitus, inflammatory bowel disease, and multiple sclerosis (*3*). Conversely, lack of MHC class II gene expression results in a severe combined immunodeficiency disease known as bare lymphocyte syndrome (BLS) (*2*, *4*).

Class II gene expression is controlled at the level of transcription. The proximal promoter region of the class II gene contains several cis-elements, termed W, X, and Y-box (*5*). These elements are well studied, and numerous binding factors have been identified (*2*, *6*). Human MHC class II HLA-DRα gene contains three DNase I hypersensitive sites (DHS-I, -II, -III)[1] (*7*). DNase I hypersensitive sites are believed to represent the "open windows" that allow enhanced access of crucial resident cis-acting DNA sequences to trans-acting factors in chromatin (*8*). HLA-DRα is inducible by IFNγ in Jurkat, HL-60 and THP-1 cells. Noteworthy, in Jurkat cells and HL-60 cells, there is only one DNase I hypersensitive site (DHS-I) in the promoter region, whereas two additional DNase I hypersensitive sites (DHS-II, -III) were found in the first intron in B-cells, which express HLA-DRα gene constitutively (*7*). Cis-acting elements containing DNase I hypersensitive sites were placed 5′ or 3′ to the chloramphenicol acetyltransferase (CAT) reporter gene, the transcription of which was initiated from the Herpes Simplex thymidine kinase (TK) promoter. In transient experiment assay, the cis-elements around DHS-II were able to increase CAT activity (*9*). Thus, the cis-element in the first intron was believed to be involved in cell type-specific HLA-DRα gene regulation (*5*, *9*). But cell type-specific DRα trans-acting factors to these cis-acting elements have not clarified yet. In this study, we focused on a unique parindromic sequence (DHS-22) around DHS-II in the first intron, which is a part of the transcriptional enhancer region in the first intron of DRα gene (*9*), and found a binding protein to this DNA. We isolated this binding protein and identified it as NF90.

EXPERIMENTAL PROCEDURES

Preparation of Nuclear Extracts. Nuclear extracts were prepared according to the procedure of Dignam et al. (*10*).

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[1] Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DHS, DNase I hypersensitive site(s); CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; EMSA, electrophoretic mobility shift assay; G418, 2-deoxystreptamine antibiotic; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; GST, glutathione *S*-transferase.

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THP-1 cells and HeLa cells (2×10^7 cells) were grown in RPMI 1640 supplemented with 10% fetal calf serum. In the case of TPA-treated cells, cells were treated for 1 day with 10 ng/mL TPA. Cells were harvested, washed twice with PBS, and spun down. Then cells were resuspended in 5 volumes of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF) and incubated for 10 min on ice. Afterward, the cells were spun down, resuspended in 4 volumes of buffer A, and homogenized in a Dounce homogenizer equipped with a B-type pestle. Homogenates were centrifuged at 2400 rpm for 5 min and washed once with buffer A supplemented with 10 mM KCl. The crude nuclear pellet was then resuspended in 4 volumes of buffer C (20 mM Hepes, pH 7.4, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF) and rotated on a tube rotator at 4 °C for 1 h. Chromatins were sedimented by centrifugation at 10 000 rpm for 20 min. The supernatant was dialyzed against 500 volumes of buffer D (20 mM Hepes, pH 7.4, 20% glycerol, 0.1 M NaCl, 0.2 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF) for 6 h, and insoluble proteins were removed by centrifugation at 10 000 rpm for 20 min. In the case of bovine brain, brain (100 g) was homogenized with 3 volumes of homogenization buffer (2.5 M sucrose, 11.1 mM $MgCl_2$, 11.1 mM Tris-HCl, pH 7.5) by a Polytron homogenizer for 15 min on ice. A homogenate was centrifuged at 22 500 rpm for 1 h, and the resultant nuclear pellets were treated as above. Protein concentrations were determined by the Bradford method (*11*).

Probe DNA. Double-stranded DHS-22 was synthesized by PCR using pAKR4705 as a template (*7*, *9*, *12*, *13*). The cosmid pAKR4705 contained human genomic DNA containing the whole HLA-DRα region, and it was provided by Dr. H. Inoko, Department of Transplantation, Tokai University School of Medicine (*14*). The PCR product was ligated with the T-cloning site of pT7Blue T-vector (Novagen). The resultant plasmid was termed pT7Blue-DHS-22. To synthesize a ^{32}P-labeled double-stranded DHS-22 DNA fragment, 0.2 mM each of sense and antisense primers of DHS-22 was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs). ^{32}P-end-labeled DHS-22 primers were precipitated by ethanol to remove free [γ-^{32}P]ATP. ^{32}P-labeled double-stranded DHS-22 was synthesized by PCR using these labeled primers and pT7Blue-DHS-22 as a template. The PCR reaction solution contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, these labeled primers, pT7Blue-DHS-22 (85 ng), and 1 unit of Taq DNA polymerase in a final volume of 50 μL. The amplification cycle was carried out at 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 90 s. This ^{32}P-labeled double-stranded DHS-22 was used or stored at −20 °C. DHS-22 primer sequences were as follows: DHS-22 sense primer, 5′-AATATTGTA-AGTCAAATTTGGTTT; antisense primer, 5′-AATATTGCT-TGTA-GCAGGACCGTG.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed as described previously (*15*) with some modifications. Nuclear extracts (1.5 μg) were incubated with ^{32}P-labeled double-stranded DHS-22 DNA fragments (10 000 cpm/1 ng), and poly(dI-dC) (0.5 μg) was incubated in binding buffer (10 mM Tris-HCl, pH 7.5, 5 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 5% glycerol, 1 mM EDTA) at 37 °C for 30 min. The mixture was then loaded on a 3.5% polyacrylamide gel in 0.5 × TBE at 100 V for 1 h. After electrophoresis, the gel was dried and exposed to a X-ray film. During the purification process, EMSA was performed as above except that 3 μL/fraction of protein and 0.05 μg of poly(dI-dC) were used.

Southwestern Blotting. Southwestern blot analysis was performed as previously described (*16*) with slight modifications. Proteins (15 μg) were subjected to 10% SDS−PAGE. After electro-transfer, the PVDF membrane was incubated with denaturation buffer (6 M guanidine-hydrochloride,, 20 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) for 15 min at 4 °C and renatured with Hepes−Salt buffer (20 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT). Then the membrane was incubated with 5% skim milk/Hepes−Salt buffer for 30 min at room temperature. The membrane was incubated at 4 °C for 24 h with ^{32}P-end-labeled DHS-22 oligonucleotide probe (1×10^6 cpm/mL) in 0.5% skim milk/Hepes−Salt buffer. The membrane was washed 3 times (15 min) using the same buffer at room temperature. ^{32}P-labeled bands were visualized by autoradiography.

Purification of DHS-22 Binding Protein. Nuclear proteins (5.5 mg) from bovine brain nuclei were separated on a hydroxylapatite column (Williamspora, PA) equilibrated with 5 mM potassium phosphate buffer, pH 7.5, containing 1 M KCl, washed with 5 mM potassium phosphate buffer, pH 7.5, containing 0.1M KCl, and eluted with 500 mM potassium phosphate buffer, pH 7.5, containing 0.1 M KCl. DHS-22 binding activity was examined by EMSA using DHS-22 DNA as the probe. Then positive fractions were diluted 10 times with 50 mM potassium phosphate buffer, pH 7.5, and loaded onto the Mono-Q column (SMART system, Pharmacia Biotech), equilibrated by 50 mM potassium phosphate buffer, pH 7.5, containing 0.1 M KCl, and washed with the same buffer. Proteins were eluted using a KCl gradient (0.05−1 M) in the same buffer. Active fractions were collected, and used for further experiments. All procedures were performed at 4 °C.

Mass Analysis. An active protein band was cut from a SDS gel and homogenized in elution buffer (1% SDS, 20 mM Tris-HCl, pH 8.0), and the protein was extracted by shaking vigorously overnight at room temperature. The protein was separated from the gel fragments by ultra free-C3-GV (Millipore). To precipitate the protein, the sample was incubated at −70 °C for 1 h in cold acetone. Precipitated protein was dissolved in digestion buffer (1 M Tris-HCl, pH 9.0) and digested by Lys-C (Boehringer Mannheim). The digested sample was analyzed by Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems).

Protein Sequencing. Partially purified proteins (50 μg) in MonoQ fractions were further separated by 10% SDS−PAGE and transferred to a PVDF membrane. After electroblotting, proteins were visualized by Coomassie brilliant blue, and the stained protein, corresponding to an active band by Southwestern blot analysis on the membrane, was cut and digested with trypsin (1 μg) in 0.1 M Tris-HCl, pH 8.5. Peptides released from the membrane were separated by μ reverse-phase high-performance liquid chromatography, using a C-18 column (SMART-system, Pharmacia Biotech). The peptides were eluted with a linear gradient of acetonitrile concentration from 10% to 70% in the presence of 0.1% trifluoroacetic acid. Eluates were monitored at 215 nm. An

eluate was further separated by a μ blotter (Applied Biosystems). Amino acid sequences of the peptides were determined with an amino acid sequencer Model 491cLC type (Applied Biosystems).

Western Blot Analysis. Western blot analysis was performed according to the method of A. Domingo and R. Marco (*17*). Nuclear proteins (10 μg) from THP-1 cells or the affinity-purified GST−NF90 (10 μg) and GST (10 μg) were subjected to 10% SDS−PAGE and electrotransferred onto a PVDF membrane; the membrane was incubated with an antiserum against NF90 or GST (Pharmacia Biotech) in GENT solution (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton-X100), and then horseradish peroxidase-labeled second antibody (Zymed Laboratories, Inc., San Francisco, CA). NF90 or GST and its derivatives on the membrane were visualized by staining using a chemiluminescence detection (ECL) system (Amersham). Rabbit polyclonal antisera were generated against NF90 by Peter Kao (Division of Pulmonary and Critical Care Medicine, Stanford University) (*18*) and kindly provided by him.

Construction of GST−NF90 Fusion Protein. The full-length cDNA of NF90 was synthesized by PCR using human B-cell cDNAs of λgt 11 phage library (Promega) as a template. The PCR product was ligated with *Bam*HI and *Kpn*I sites of pQE-32 vector after digestion of the PCR product with *Bam*HI and *Kpn*I. To obtain the NF90 fragment, the pQE-NF90 was digested by *Bam*HI and *Sma*I sites, and the fragment was ligated with *Bam*HI and *Sma*I sites of the pGEX-5X-1 expression vector. The resultant plasmid, pGEX-NF90, was transformed into competent cells of *E. coli* BL21. A single colony of pGEX-NF90-containing bacteria was grown in 200 mL of LB medium supplemented with 25 μg/mL ampicillin until an absorbance at 600 nm reached approximately 1.2. After the addition of IPTG to a final concentration of 1 mM, the bacteria were grown for 2 h and harvested by centrifugation. Lysates were prepared by resuspending the pellets in 20 mL of ice-cold PBS containing 0.1 mM PMSF and 30% glycerol, followed by sonication on ice. Triton X-100 was added to a concentration of 1% and incubated for 30 min on ice. After centrifugation at 12 000 rpm at 4 °C for 10 min, the supernatant was loaded onto a glutathione−Sepharose 4B affinity column (Pharmacia), equilibrated with the same buffer, and washed 3 times with the same buffer. Bound proteins were eluted from the column with 500 mL of elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0). The eluates were dialyzed against PBS, glycerol was then added to a concentration of 20%, and the proteins were used immediately or stored at −80 °C. NF90 primer sequences were as follows: sense, 5′-CGGGATCCTAAAAATGCGTCCAATGC; antisense, 5′-GGGGTACCAGCTTCCATACCCAGCAC.

RT-PCR. Total RNA was extracted from cells by acid guanidinum thiocyanate−phenol−chloroform extraction (*19*). RT-PCR was carried out as previously described (*20*, *21*) with some modifications. Total RNA was annealed with 2.5 μM random 9-mers (Takara Biomedicals, Tokyo) in a total volume of 20 μL and was reverse-transcribed with 5 units of AMV reverse transcriptase XL (Takara Biomedicals, Tokyo) at 42 °C for 30 min. Then 10 μL of the reaction product was added to a PCR reaction solution, which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 25 mM each of dATP, dCTP, dGTP, and dTTP, 0.2 mM each of sense and antisense primers of NF90, and 1 unit of Taq DNA polymerase in a final volume of 50 μL. The amplification cycle was carried out at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s. Reaction products after the 20th, 22nd, 25th, and 28th cycles were subjected to 2% agarose gel electrophoresis, and bands were visualized by ethidium bromide staining and quantified by an image analysis (Bio-rad). NF90 primers used were: NF90 sense primer, 5′-ATGCCCCCATCTTTACCA; antisense primer, 5′-TTCGGTCCCCCTCTGACG.

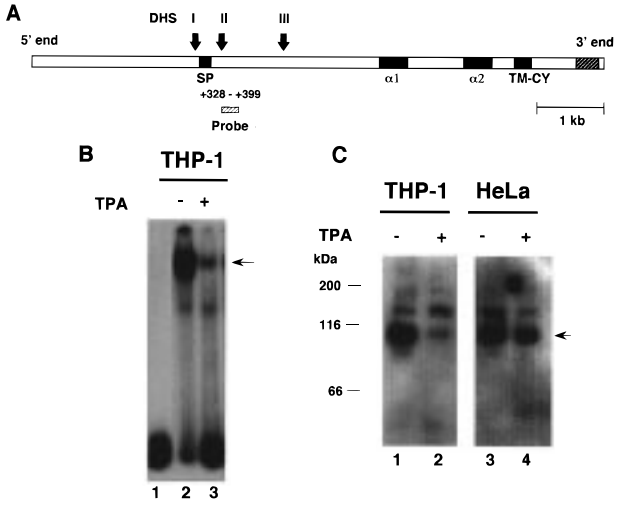

FIGURE 1: Effect of TPA on DHS-22 binding activity. (A) Schematic presentation of DNase I hypersensitive sites (DHS-I, -II, -III) of the HLA-DRα gene: SP, signal peptide; α1, α1 subunit; α2, α2 subunit; TM-CY, transmembrane region and cytoplasmic region. A DNA fragment (+328 to +399), DHS-22, located around the DNase I hypersensitive site II was used as a probe for EMSA and Southwestern blot analysis. (B) EMSA was performed using nuclear proteins (2 μg) of THP-1 cells treated without (−) or with (+) 10 ng/mL TPA for 1 day as described under Experimental Procedures. An arrow indicates the DHS-22 binding protein (C) Southwestern blot analysis of DHS-22 binding protein(s). THP-1 cells (lanes 1, 2) or HeLa cells (lanes 3, 4) were cultured for 1 day in the absence (lanes 1, 3) or presence (lanes 2, 4) of 10 ng/mL TPA. Nuclear proteins (15 μg) were subjected to SDS−PAGE and transferred to a PVDF membrane. The membrane was incubated with ^{32}P-labeled DHS-22 probe DNA. An arrow indicates the DHS-22 binding protein.

RESULTS

Effect of TPA Treatment on DHS-22 Binding Factor in THP-1 Cells and HeLa Cells. Since intronic hypersensitive sites have been found in the HLA-DRα gene, it has been suggested that regulatory elements are present in the body of the HLA-DRα gene. Therefore, we focused on a unique parindoromic sequence (DHS-22) around DHS II (Figure 1A) and attempted to find trans-acting factors which interacted with DHS-22. Using EMSA probed with DHS-22, we found a DHS-22 binding protein in nuclear extracts of THP-1 cells. Interestingly, the binding activity of this factor was decreased in activated THP-1 cells by TPA treatment (Figure 1B). Moreover, we found 110 kDa DHS-22 binding protein (P110) in both nuclear extracts of THP-1 cells and HeLa cells using Southwestern blot analysis probed with DHS-22 (Figure 1C, lanes 1, 3). The DHS-22 binding activity of P110 was abolished by TPA treatment in THP-1 cells (Figure 1C, lane 2), but not in HeLa cells (Figure 1C, lane 4). This result

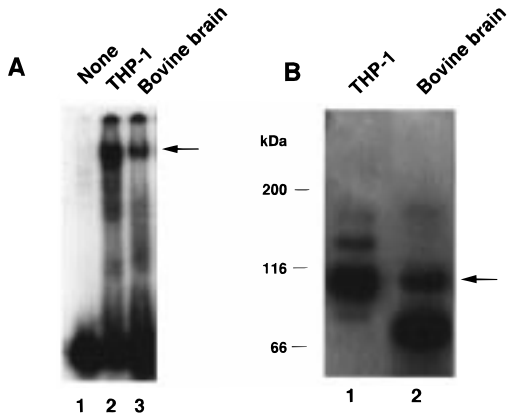

FIGURE 2: DHS-22 binding activity in bovine brain nuclear extract. (A) EMSA of nuclear proteins (2 μg) from THP-1 cells (lane 2) and bovine brain (lane 3) with DHS-22 as a probe. An arrow indicates the DHS-22 binding protein. (B) Southwestern blot analysis of nuclear extract (15 μg) from THP-1 cells (lane 1) or bovine brain (lane 2) as described under Experimental Procedures. Nuclear proteins were subjected to 10% SDS−PAGE and transferred onto a PVDF membrane. The membrane was incubated with ^{32}P-labeled DHS-22 probe. An arrow indicates the DHS-22 binding protein (P110).

suggests that the binding activity of P110 seems to be involved in the amplification of HLA-DRα in THP-1 cells by TPA treatment.

Purification and Characterization of DHS-22 Binding Factor (P110). Southwestern analysis and EMSA probed with DHS-22 indicate that a protein corresponding to the DHS-22 binding protein (P110) in THP-1 cells was also present in nuclear extracts of bovine brains (Figure 2A,B). Thus, we used bovine brain nuclear extracts to purify this factor. First, the nuclear extracts were loaded onto hydroxylapatite columns as described under Experimental Procedures. The pooled positive fractions eluted by hydroxylapatite chromatography were applied onto the Mono-Q column, and proteins were eluted by a linear KCl gradient (Figure 3A). The majority of the DHS-22 binding activity was found in fractions 4−7 (Figure 3B). Figure 4 shows the protein-stained pattern of SDS−PAGE and Southwestern blot analysis of fractions 4, 5, and 6 eluted from the Mono-Q column. Since fractions 5 and 6 mostly contained the DHS-22 binding protein (P110), we cleaved the gel corresponding to P110 and then extracted the P110 from the gel. The P110 protein was digested by Lys-C, and the molecular masses of the fragments were analyzed by MALDI-TOF mass spectroscopy (Figure 5A). The molecular masses of the fragments were 2613.3, 2748.74, 2984.59, 3925.78, 4443.11, 4754.42, and 4977.2 Da. Then, proteins in Mono-Q fractions 5 and 6 were subjected to SDS−PAGE, elecrtroblotted onto PVDF, and stained by Coomassie brilliant blue. The membrane corresponding to the P110 protein was cut, the P110 protein on the membrane was digested with trypsin, and the resulting peptides were separated by reverse-phase (C18) column chromatography and μ blotter (Figure 5B). The internal amino acid sequences of P110 were determined from two peptide fragments as shown by P1 and P2 (Table 1). Database search of Mass-Fit on the basis of these data indicates that NF90 was the only protein which matches the mass of Lys-C fragments and amino acid sequences.

Immunological Confirmation of the DHS-22 Binding Protein(s) in THP-1 Cells with Antisera to NF90. We purified

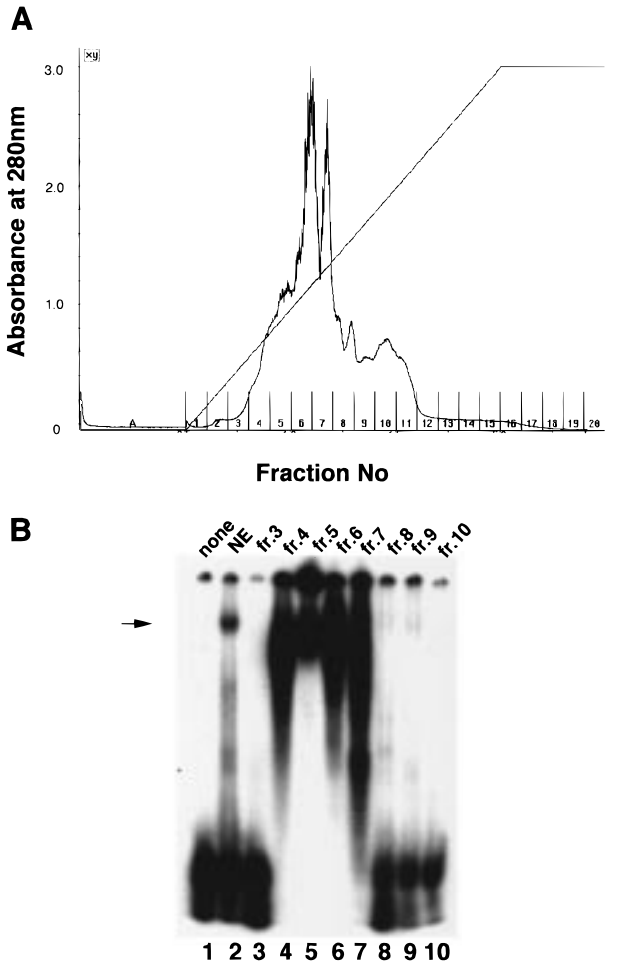

FIGURE 3: Separation and detection of DHS-22 binding proteins. (A) Typical elution pattern of bovine brain nuclear protein(s) from the Mono-Q column. The absorbance at 280 nm of the eluate was automatically recorded. The changes in KCl concentration were also monitored and are shown by the linear line. (B) DHS-22 binding activity of each fraction was examined by EMSA. Lanes 1, 2, and 3−10 represent probe DNA alone, bovine nuclear extracts, and fractions 3−10, respectively. An arrow indicates the DHS-22 binding protein.

the DHS-22 binding protein(s) from bovine brain nuclear extracts and identified it (them) as NF90. Thus, we need to confirm that the DHS-22 binding protein(s) in THP-1 cells is (are) also NF90. Western blot analysis using anti-NF90 sera shows that the bands corresponding to the DHS-22 binding protein of THP-1 cells reacted with anti-NF90 sera (*18*), which was provided by Peter Kao (Division of Pulmonary and Critical Care Medicine, Stanford University) (Figure 6A). Figure 6B shows the comparison of the DHS-22 DNA sequence to the antigen receptor response element (ARRE) of IL-2 promoter, which is known to interact with NF90. This suggests that the common sequence AAAACTG seems to be the binding site for NF90. Interestingly, the upper band reacted with anti-NF90 sera was enhanced by TPA treatment in THP-1 cells (Figure 6, lanes 3 and 4), whereas the bound ^{32}P-labeled DHS-22 was not changed with or without TPA treatment. (Figure 6, lanes 1 and 2). These results show that NF90 was somewhat modified by TPA treatment in THP-1 cells and the binding activity of NF90 to DHS-22 might decrease by some modification on NF90.

Interaction of GST Fusion Protein of NF90 with DHS-22. To confirm whether NF90 interacts with DHS-22, we

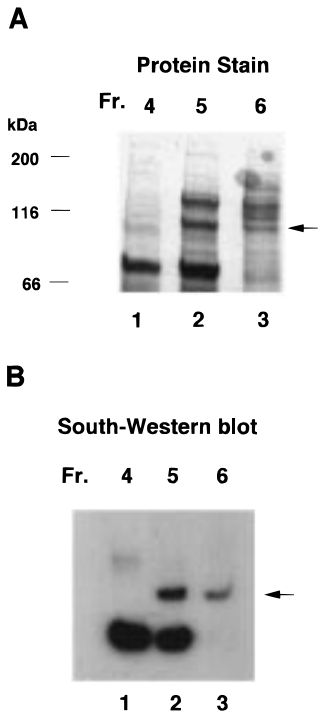

FIGURE 4: Analysis of partially purified DHS-22 binding proteins. Partially purified nuclear proteins (10 μg) of fractions 4, 5, and 6 (lanes 1, 2, and 3) were subjected to SDS−PAGE and stained with Coomassie brilliant blue (A) or examined by Southwestern blot analysis (B). Experimental conditions are described under Experimental Procedures. An arrow indicates the P110 protein.

prepared GST−NF90 fusion protein (GST−NF90) and assayed its binding activity by EMSA probed with DHS-22. The fusion protein migrated with an apparent molecular mass of 116 kDa, which was in agreement with the size deduced from the NF90 cDNA. Western blot analysis using an anti-GST antibody shows that all of the bands in addition to GST−NF90 reacted with anti-GST (Figure 7A, lane 3). These results indicate that the rest of the smaller proteins were the degraded GST−NF90 products or incomplete products (Figure 7A, lane 1). In the EMSA, GST−NF90 interacted with DHS-22, whereas GST itself did not interact with the probe (Figure 7B). These results indicate that NF90 recognized the unique palindoromic sequence (DHS-22) in the HLA-DRα gene.

Effect of TPA Treatment on the Expression of NF90 in THP-1 Cells and HeLa Cells. We examined why the binding activity of NF90 to DHS-22 was decreased by TPA treatment in THP-1 cells. First we examined the effect of phosphorylation on NF90 protein by TPA treatment. We did not detect significant amounts of phosphorylated NF90 in TPA-treated THP-1 cells by Western blot analysis of an anti-phosphoserine antibody. Phosphatase treatment of nuclear proteins in TPA-treated THP-1 cells did not recover the binding activity of NF90 with DHS-22 (data not shown). Then we measured the mRNA of NF90 by RT-PCR and found that NF90 mRNA was decreased by TPA treatment in THP-1 cells (Figure 8, lanes 1 and 2), whereas that in HeLa cells was the same regardless of treatment (Figure 8, lanes 3 and 4) (Table 2). These results indicate that the decrease in DHS-22 binding activity by TPA treatment in THP-1 cells was due to the decrease of NF90 mRNA and some modifications on NF90 except for the phosphorylation of serine residues.

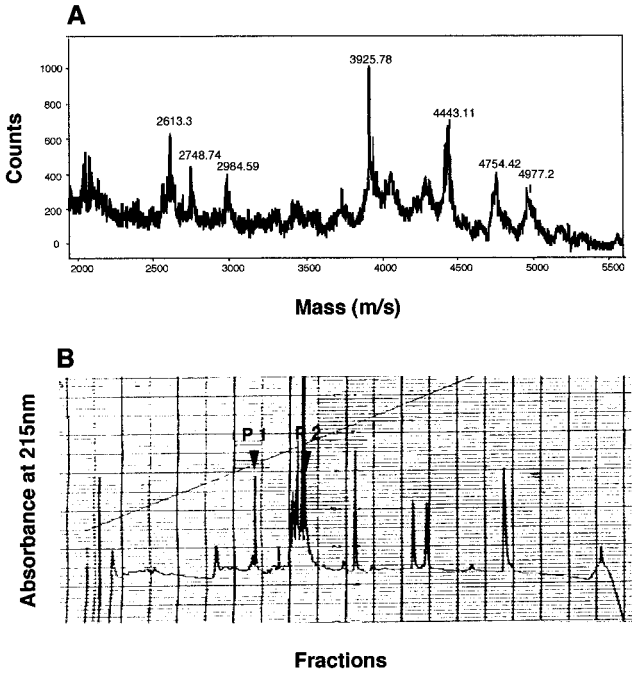

FIGURE 5: Identification of a purified DHS-22 binding protein. (A) Mass analysis of lysylpeptidase (Lys-C)-digested P110. A band corresponding to P110 on the SDS gel was cleaved, and the P110 protein was isolated as described under Experimental Procedures. The isolated P110 protein was digested with lysylpeptidase C in 1 M Tris-HCl (pH 9.0). The mass of the digested fragments was determined by a TOF-mass spectrometer (Perseptive Biosystems). (B) Elution pattern of trypsin-digested P110 by μ blotter. The isolated P110 protein was digested with trypsin, and the peptides were eluted from a reverse-phase C-18 column with a linear gradient of acetonitrile concentration from 5% to 45% in the presence of 0.1% trifluoroacetic acid. Peptides were monitored by the UV absorption at 215 nm, and the change in acetonitrile concentration is indicated by a straight line. An arrowhead indicates peaks 1 (P1) and 2 (P2), whose anino acid sequences are shown in Table 1.

Table 1: Internal Amino Acid Sequences of the DHS-22 Binding Protein (P110)[a]

peptide	sequence
P1	GQLHK
P2	KYELISETGGSHD

[a] The peptide fragments of the trypsin-digested DHS-22 binding protein (P110) were separated by reverse-phase chromatography (μ blotter, Applied Biosystems), and the amino acid sequences of two peptides, P1 and P2 in Figure 5B, were determined using an amino acid sequencer Model 491cLC type (Applied Biosystems) as described under Experimental Procedures.

DISCUSSION

Expression of MHC class II genes is strictly restricted in some specific cell types. In B cells and activated T cells, class II genes are constitutively expressed. In some other antigen-presenting cells, class II genes are inducible by IFNγ or other cytokines. To address the question of the restriction in class II expression, DNase I hypersensitive sites of the HLA-DRα gene were investigated. In all cells, a DNase I hypersensitive site (DHS) is found in the promoter, whereas B cells were observed to contain two other DHS in the first intron of the HLA-DRα gene (*7*). Although THP-1 cells are macrophage-like cells, they also contain two other DHS in the first intron of the HLA-DRα gene (*7*). The cis-acting elements around DHS of the HLA-DRα gene, which are

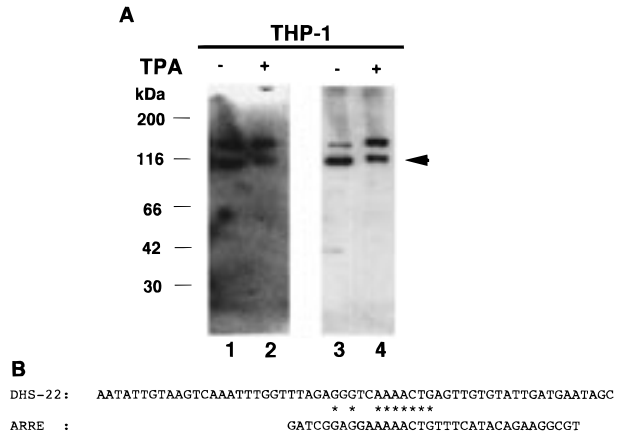

FIGURE 6: Southwestern blot analysis and Western blot analysis of the DHS-22 binding protein(s) in THP-1 cells. (A) Nuclear proteins (10 μg) from THP-1 cells with or without TPA treatment were subjected to 10% SDS−PAGE and transferred onto a PVDF membrane. The membrane was incubated with ^{32}P-labeled DHS-22 DNA probe (lanes 1 and 2). The same membrane, on which ^{32}P-labeled DHS-22 probe was removed, was stained immunologically by anti-NF90 sera (lanes 3 and 4). An arrow indicates P110. Precise procedures are described under Experimental Procedures. (B) Comparison of the DHS-22 DNA sequence to the ARRE-2 DNA sequence, which is located in the IL-2 promoter (−255 to ∼−285), and known as NF90 binding motif.

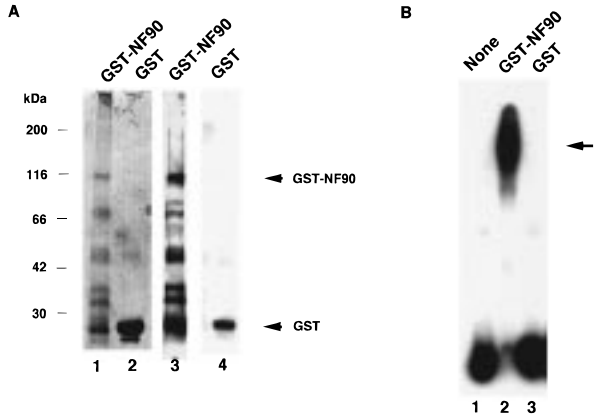

FIGURE 7: Interaction of the GST fusion protein of NF90 and DHS-22. (A) Affinity-purified GST−NF90 (10 μg) (lane 1) and GST (20 μg) (lane 2) were subjected to 10% SDS−PAGE and stained with Coomassie brilliant blue. The deduced positions of GST−NF90 and GST are marked by arrowheads. Standard molecular sizes of marker proteins are shown. GST−NF90 (10 μg) (lane 3) and GST (10 μg) (lane 4) were separated on SDS−PAGE and transferred to a PVDF membrane. GST and its derivatives on the membrane were immunologically stained with an antibody against GST as described under Experimental Procedures. (B) EMSA of GST−NF90 and GST with ^{32}P-labeled DHS-22 DNA as a probe. GST-NF90 (6 μg) (lane 2) and GST (20 μg) (lane 3) were incubated with ^{32}P-labeled DHS-22 probe DNA.

placed 5′ or 3′ to the TK-CAT reporter gene, were able to increase the reporter gene activity (*9*). But the tissue-specific trans-acting factors to the cis-acting elements have not clarified yet. In this study, we used a unique parindoromic sequence (DHS-22), which is a part of the transcriptional enhancer region around DHS-II in the first intron of HLA-DRα gene (*9*), and found a binding protein to DHS-22 in the nuclear extract of THP-1 cells, HeLa cells, and bovine brains. This binding protein was characterized as NF90 (*18*). SDS−PAGE and Southwestern blot analysis of DHS-22 binding protein indicated that the molecular mass of the

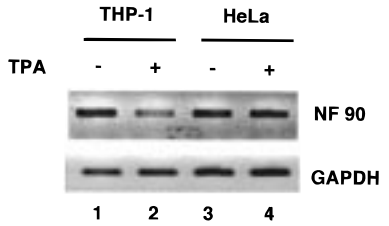

FIGURE 8: Effect of TPA on the expression of NF90 gene in THP-1 cells and HeLa cells. RT-PCR analysis was performed by using primers for NF90 genes as described under Experimental Procedures. THP-1 cells were incubated with medium alone (lane 1) or 10 ng/mL TPA for 1 day (lane 2), and HeLa cells were incubated with medium alone (lane 3) or 10 ng/mL TPA for 1 day (lane 4). A typical pattern of ethidium bromide-stained PCR products on a agarose gel is depicted.

Table 2: Effect of TPA Treatment on the Level of NF90 mRNA in THP-1 Cells and HeLa Cells[a]

cells	TPA	NF90	GAPDH	NF90/ GAPDH	% inhibition
THP-1	−	27.2	59.9	0.45	100
	+	13.2	70.6	0.19	42
HeLa	−	34.1	91.3	0.37	100
	+	30.6	100.0	0.31	84

[a] RT-PCR was performed as described under Experimental Procedures. PCR proceeded linearly until the 25th cycle; thus, the result of the 22nd cycle was presented in Figure 8, and ethidium bromide-stained bands were densitometrically quantified. The relative intensity of the band is presented in an arbitrary units. Experiments were repeated 3 times independently, and the values of % inhibition were within 5% error.

binding protein in THP-1 cells and bovine brain nuclei was 110 kDa, which does not correlate with the 90 kDa NF90 protein purified from Jurkat T cells (*18*, *22*). This difference may be accounted for as the protein in THP-1 cells seems to be covalently modified. The binding activity of NF90 to DHS-22 was decreased by TPA treatment in THP-1 cells, but not in HeLa cells (Figure 2). This phenomenon seems to be related to our previous observation that IFNγ-inducible expression of the DRα gene was enhanced when THP-1 cells were treated with TPA, but this enhancement was not observed in cell lines of other lineages, such as HeLa cells (*23*). Enhanced expression of HLA-DRα was also observed when B cell line Raji cells were treated with TPA (*24*). These results suggest that the binding of NF90 to the DHS II cis-element results in down-regulation of the HLA-DRα gene. However, NF90 was first identified as a nuclear factor that binds to a cis-acting response element of the IL-2 promoter, known as the antigen receptor response element (ARRE), in T cells to activate the expression of IL-2 (*18*, *22*). In Jurkat T cells, NF90 was induced by treatment with TPA and inonmycin and repressed by treatment with cyclosporin and FK506. But NF90 was also found in the nucleus of nonstimulated cells (*22*), suggesting that NF90 may be widely expressed. Thus, the precise mechanisms of action of NF90 in the expression of the IL2 gene and HLA-DRα gene are still to be elucidated.

Recently, Ting et al. also reported that NF90 was a substrate for DNA-PK in vitro and promoted the formation of a complex between DNA-PKcs and Ku. They also demonstrated that NF90 interacted with DNA-PKcs in vitro (*25*). Considering the fact that sequence-specific DNA binding of Ku/DNA-PK repressed glucocorticoid-induced MMTV transcription (*26*), binding of NF90 to the DHS-II

in the first intron of the DRα gene and association of Ku/DNA-PK with the bound NF90 may affect negatively the expression of the DRα gene. The TPA-induced decrease in binding activity of NF90 in THP-1 cells results in dissociation of NF90 from the DHS-II cis-element, and this may cause an amplification of IFNγ-inducible DRα gene expression by TPA treatment in THP-1 cells.

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