A Novel Cysteine-rich Sequence-specific DNA-binding Protein Interacts with the Conserved X-box Motif of the Human Major Histocompatibility Complex Class II Genes Via a Repeated Cys-His Domain and Functions as a Transcriptional Repressor

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Summary

The class II major histocompatibility complex (MHC) molecules function in the presentation of processed peptides to helper T cells. As most mammalian cells can endocytose and process foreign antigen, the critical determinant of an antigen-presenting cell is its ability to express class II MHC molecules. Expression of these molecules is usually restricted to cells of the immune system and dysregulated expression is hypothesized to contribute to the pathogenesis of a severe combined immunodeficiency syndrome and certain autoimmune diseases. Human complementary DNA clones encoding a newly identified, cysteine-rich transcription factor, NF-X1, which binds to the conserved X-box motif of class II MHC genes, were obtained, and the primary amino acid sequence deduced. The major open reading frame encodes a polypeptide of 1,104 amino acids with a symmetrical organization. A central cysteine-rich portion encodes the DNA-binding domain, and is subdivided into seven repeated motifs. This motif is similar to but distinct from the LIM domain and the RING finger family, and is reminiscent of known metal-binding regions. The unique arrangement of cysteines indicates that the consensus sequence $CX_3CXL\text{-}XCGX_{1-5}HXCX_3CHXGXC$ represents a novel cysteine-rich motif. Two lines of evidence indicate that the polypeptide encodes a potent and biologically relevant repressor of HLA-DRA transcription: (a) overexpression of NF-X1 from a retroviral construct strongly decreases transcription from the HLA-DRA promoter; and (b) the NF-X1 transcript is markedly induced late after induction with interferon γ (IFN-γ), coinciding with postinduction attenuation of HLA-DRA transcription. The NF-X1 protein may therefore play an important role in regulating the duration of an inflammatory response by limiting the period in which class II MHC molecules are induced by IFN-γ.

The class II region of the human MHC encodes three heterodimeric molecules: HLA-DR, -DQ, and -DP composed of α and β chain polypeptides with an approximate molecular weight of 60,000 (1). Peptides derived from extracellular antigens are recognized by helper T cells in the context of these molecules (2). These molecules are expressed constitutively on professional APCs such as macrophages, dendritic cells, and B cells and their biosynthesis is inducible on other cells upon binding of certain lymphokines such as IFN-γ, IL-4, and TNF-α to their respective receptors (3, 4). Class

II MHC genes are inactive in plasma cells, and cell fusion experiments indicate that a dominant repressor protein actively inhibits transcription of these genes (5). These highly polymorphic molecules determine the ability of an individual to respond to a given antigen, and the molecular basis of this lies in the differential capacity of allelic forms of these molecules to bind particular peptides (6). Because of the central role these molecules play in the initiation of the immune response, considerable effort is focused on elucidating the mechanisms governing the proper tissue-specific and developmental regulation of the class II MHC genes (7, 8).

Expression of the class II MHC genes is controlled primarily at the transcriptional level (9, 10). Systematic dele-

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tion and mutagenesis of the proximal promoters of the human and murine class II genes have identified two highly conserved cis-acting elements called the X and Y boxes that bind several transcription factors that participate in the regulation of these genes (11–14). These regions are occupied by DNA-binding proteins in class II–positive cells but not in class II–negative or in certain Bare Lymphocyte Syndrome cell lines (15, 16). The X-box is further subdivided into an upstream X1 box (5'CCTAGCAACAGATG3') and an X2 box (5'CGTCATC3') located immediately 3' of the X1 box (5). A family of genes encoding X1 box binding proteins have been cloned (RFX1-5) and at least one of these, RFX5, appears to be required for class II MHC gene transcription (17, 18). At least three factors (hXBP1, hXBP2, and c-jun) can interact directly with the X2 box, with the product of the c-fos proto-oncogene being a likely partner (19–23). The Y box is in fact an inverted CCAAT box which can bind a multiplicity of factors. Two factors, YB-1 and NF-Y, have been implicated in class II MHC gene regulation. YB-1 appears to encode a potent repressor of IFN-γ–induced class II gene expression, whereas the heterodimeric NF-Y encodes an activator (24–26). The Y-box may therefore act as a bifunctional cis-element, binding both an activator and repressor of class II MHC gene expression.

Recently, a novel factor (CIITA) required for both constitutive and IFN-γ–mediated expression of all of the class II MCH genes, has been isolated by complementation cloning using a mutant B-lymphoblastoid cell line (27, 28). This factor does not appear to interact directly with the class II MHC proximal promoter, but CIITA transactivation is mediated by the proximal promoter (presumably via protein–protein interactions between CIITA and other class II promoter binding proteins). In this report, we describe a unique X1 box binding protein, NF-X1, which represses class II MHC gene expression and provides an initial structure/function analysis of the protein.

Materials and Methods

Cell Lines. Raji and Jijoye cells are MHC class II–positive EBV-transformed human B lymphoblastoid cell lines. Jurkat is a class II–negative human T cell line. All three lymphoid cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated FCS, 20 mM Hepes, penicillin/streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate. HeLa cells were maintained in DMEM media. Transcription of the HLA-DRA gene and cell surface expression of the HLA-DR molecule is inducible by addition of rIFN-γ to these cells at a concentration of 100 U/ml for 24–48 h.

cDNA Cloning and DNA Sequence Analysis. Infection, plating, fusion protein induction, and nitrocellulose filter lifts were performed as described in (29). The oligonucleotides used to probe the immobilized recombinant fusion proteins were: HLA-DQB X (AAAATCTGCCCAGAGACAGATGAGGTCCTT) and HLA-DPB X (ACTTTCTGCCTAGTGAGCAATGACTCATAC). A HLA-DRA S box probe (TGTGTCCTGGACCCTTTGCAAGA) was also included in the screens. Double-stranded oligonucleotides were endlabeled with γ-[32]ATP using T4 polynucleotide kinase and subsequently concatenated with DNA ligase. Ligation efficiency was monitored by gel electrophoresis. Oligonucleotides were synthesized on a DNA synthesizer (model 391; Applied Biosystems,

Inc., Foster City, CA). Potential positive clones were subjected to secondary and tertiary screens. Insert cDNAs were then subcloned into pBluescript cloning/sequencing vectors, and restriction maps were generated. Bidirectional exonuclease deletions of the full-length clone-16 insert were generated and both strands were sequenced by the dideoxy method. A few regions that were between deletion points were sequenced using complementary oligonucleotides.

Southern and Northern Blot Analysis. High molecular weight DNA was isolated from the indicated cell lines as previously described (30). After restriction digestion with the indicated enzymes, DNA was transferred to Gene Screen Plus hybridization membranes and prehybridized, hybridized, and washed according to the manufacturer's guidelines (DuPont-NEN, Boston, MA). The hybridization probe was the EcoRI insert from the clone-16 bacteriophage. Northern blotting was also performed using the same membranes according to manufacturer's protocols using the same DNA probe. The HLA-DRA–specific probe is as previously described (8). RNA was isolated using the guanidium thiocyanate procedure (31). 20 μg total cellular RNA was separated after denaturation on an agarose/formaldehyde gel, transferred to hybridization membrane, and probed with the NF-X1 cDNA. The Northern blots were washed at high stringency and subjected to autoradiography.

RT-PCR and RNase Protection Analysis. RT-PCR was performed on samples of HeLa, Jurkat, and Jijoye total RNA isolated using the Guanidium isothiocyanate/cesium chloride (GIT/CsCl) procedure. cDNA synthesis was performed with 10 μg total RNA in 50 mM Tris-HCl, pH 8.3, 10 mM MgCl₂, 100 mM KCl, 10 mM dithiothreitol, and 500 μmol of each dNTP, 25 pmol 3' PCR primer, 7 U AMV-RT, and 1 μl RNAsin (Promega, Madison, WI). The total reaction volume was 11 μl. After incubation at 37°C for 1 h, 5 μl cDNA reaction mixture was used for amplification via PCR using internal NF-X1 antiparallel oligonucleotide primers. For RNase protection analysis, an antisense probe for the human γ-actin gene was synthesized by linearizing the plasmid SP6-γ-actin (32) with HinfI (New England Biolabs, Inc., Beverly, MA) followed by transcription in vitro using SP6 RNA polymerase (GIBCO-BRL, Gaithersburg, MD) and [32P]CTP (800 Ci/mmol; DuPont-NEN). 3.2 kb of the NF-X1 cDNA was subcloned into pBluescript to generate pBSclone-16. The antisense probe for NF-X1 was prepared by first linearizing the pBSclone-16 plasmid with AatII and transcribed using T7 RNA polymerase. 25 μg of each total RNA preparation was lypholized and hybridized at 45°C with 500,000 cpm of each labeled riboprobe. Hybrids were digested for 30 min at 30°C with RNase One (Promega) as recommended by the manufacturer.

Recombinant Protein Production and Electrophoretic Mobility Shift Assay. Recombinant proteins were produced essentially as previously described in (33). NF-X1 was produced either as a lysogen as described in the text, or from the T7 expression system (34). Protein was partially purified as previously described (35) and dialyzed against 50 mM Tris, pH 7.9, 0.5 M NaCl, 10% glycerol, and 1 mM PMSF. Gel mobility shift assays were performed by incubating bacterially produced NF-X1 with endlabeled probes for 30 min at room temperature in binding buffer consisting of 13 mM Tris, pH 7.9, 60 mM KCl, 12.5 mM NaCl, 12% glycerol, and 75 μg/ml poly:dIdC. The binding reactions were then resolved on low ionic strength 5% nondenaturing polyacrylamide gels and electrophoresed at 10 V/cm at room temperature (36).

Construction of NF-X1 Truncation Vectors, Filter Lift DNA-binding Analysis. Truncated NF-X1 cDNA fragments were first subcloned into appropriate pRSET vectors (Invitrogen, San Diego, CA) to place a methionine residue NH₂-terminal and in-frame with the fragment. NF-X1.A was subcloned into the pRSET.C vector with

an NH₂-terminal EcoRI site, NF-X1.(B-E) were subcloned into pRSET.B with an NH₂-terminal BamHI site, NF-X1.F was subcloned into pRSET.C with an NH₂-terminal SspI site, and NF-X1.G was subcloned into pRSET.A with an NH₂-terminal PstI site. PCR was used to amplify the resulting expression casettes and to insert terminal EcoRI recognition sites where appropriate. These products were purified by binding to glass beads and ligated to λgt11 bacteriophage arms (Stratagene, La Jolla, CA). The ligation reaction was then packaged (Gigapack Gold; Stratagene) and plated on Y1090 bacteria (37). Individual plaques were purified and assessed for insert orientation and binding ability in filter binding assays. After plaque lifting, filters were subjected to stepwise denaturation–renaturation and screened with multimerized radiolabeled probes as previously described (38). The binding buffer consisted of 12 mM Tris pH 7.9, 40 mM KCl, 0.12 mM EDTA, 30 μM ZnSO₄, and 400 μM β-MER. BSA (fraction V) was used in place of dried nonfat milk as a blocking agent. The binding and washing reactions were performed at 4°C. Autoradiographs were exposed overnight with intensifying screens.

Transient Transfections and CAT Assays. Transfections were either performed using the DEAE dextran method, as previously described (8), or using the lipofectamine reagent (GIBCO-BRL) according to the manufacturer's specifications. Typical transfections included varying amounts of effector plasmid (1–15 μg), 1–5 μg of reporter construct, and 5 μg of thymidine kinase/human growth hormone (tkHGH) transfection control plasmid. 48 h after transfection, cells were harvested by centrifugation, washed twice, and extracts prepared by multiple cycles of freeze/thaw. Chloramphenicol transferase (CAT) assays were performed as previously described (8, 22, 23). Each transfection experiment was performed five times to calculate standard errors.

Results

Isolation of cDNA Clones Encoding NF-X1. A number of overlapping complementary DNA clones encoding a newly identified human X1 box binding protein have been isolated by screening a Raji cell λgt11 expression library with a mixture of multimerized, radiolabeled, double-stranded oligonucleotides spanning the X boxes (and surrounding nucleotides) of the human class II MHC genes HLA-DQB and -DPB (38). Through restriction mapping and dideoxy sequence analysis, six overlapping cDNAs have been found to encode the same DNA-binding protein, designated NF-X1. The restriction map and the sizes of the overlapping clones are shown

in Fig. 1. One of these clones: clone-16, encodes a full-length (or nearly full-length) copy of the NF-X1 mRNA as estimated by Northern blot analysis (see Fig. 6, and data not shown). The complete nucleotide sequence of the clone-16 bacteriophage insert has been determined by (*a*) sequencing exonuclease generated truncations of the insert subcloned into the pBluescript vector (Stratagene), and (*b*) using oligonucleotide primers complementary to various locations within the cDNA to derive additional sequence information. Each portion of the cDNA has been sequenced multiple times and on both strands. Clone-16 extends 4,053 nucleotides beyond a short 5' untranslated region, including a 3,312-base largest open reading frame (ORF)[1], and 741 bases 3' of the termination codon. This clone contains the entire 3' untranslated region and contains a Poly(A) tail. We have identified two other types of clones that contain shorter 3' untranslated regions followed by long Poly(A) tails which presumably result from distinct Poly(A) addition sites. The complete nucleotide sequence of an mRNA containing the first polyadenylation site is shown in Fig. 2 *A*.

Primary Structure Analysis of NF-X1. The single large ORF encodes a polypeptide of 1,104 amino acids. The complete deduced amino acid sequence of this ORF is shown in Fig. 2 *A*. The estimated molecular weight of the polypeptide is 121,440. Primary structural analysis of NF-X1 indicates that the protein has a general symmetrical organization (Fig. 2 *B*). The polypeptide has four potential sites for N-linked glycosylation and 52 potential cAMP, CK2, and PKC phosphorylation and myristylation sites scattered throughout the polypeptide (positions are available upon request). This high density of potential posttranslational modification sites suggests that these modifications may play an important role in regulating the function of NF-X1. A large central region of 550 residues is rich in cysteine (17%). Hydropathy analysis indicates that the polypeptide is generally hydrophobic with the exception of the central domain (residues 430 to 680) which is less hydrophobic (Fig. 2 *C*). Seven repeated domains with the general consensus sequence CxxxCxLx-CGx₁₋₅HxCxxxCHxGxC are found in this region and

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[1] *Abbreviations used in this paper:* ORF, open reading frame.

CLONE-16
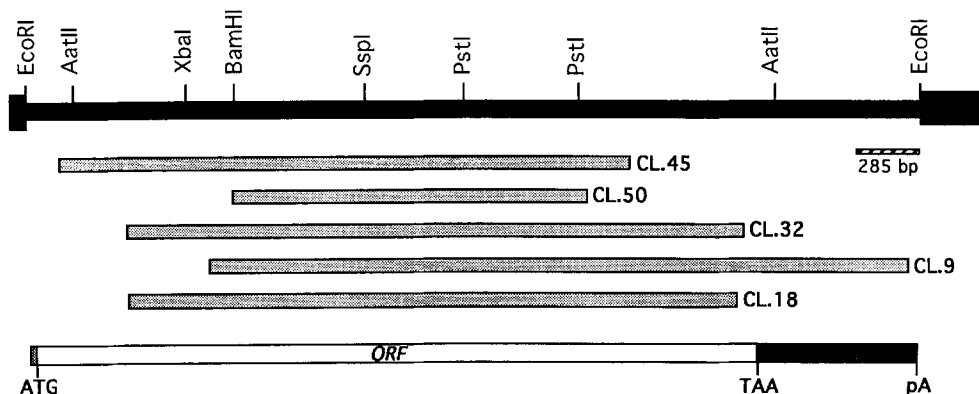
Figure 1. Overlapping λ-gt11 cDNA clones encoding NF-X1, the restriction map of clone-16, and the NF-X1 mRNA structure. Six overlapping cDNA inserts of bacteriophage clones encoding portions of a newly identified MHC class II X1-box binding protein, NF-X1, are shown. Bacteriophage clone-16, contains an insert of 4,053 nucleotides which encompasses all of the other cDNA inserts. A restriction map of the clone-16 EcoRI cDNA insert is shown. The mRNA contains a long ORF frame of 1,104 amino acids with a short 5' untranslated region and a 741-base 3'-UT. Clone-16 contains a Poly(A) tail.

A

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ATGGAATTCAGCAGCATCTGTATTGAATTT AAAAGTACCTTGAGACAGGAGGCGCCTCCG CCATCCCGTGCCGCAGAACCTAGATCGAGC TGTACAGTTCACCACCTCCCTGTCACCTTT CCAGGCAGGTCCCTTATGATGAAATCTCTG   150
MetGluPheSerSerIleCysIleGluPhe LysSerThrLeuArgGlnGluAlaProPro ProSerArgAlaAlaGluProArgSerSer CysThrValHisHisLeuProValThrPhe ProGlyArgSerLeuMetMetLysSerLeu

CTGTTCATCAGCATAGTTATCATCCGTCAG GAAGGCAAACCTAAGAGTCAGCAGACGTCT TTCCAGTCCTCTCCTTGTAATAAATCGCCC AAGAGCCATGGCCTTCAGAATCAACCTTGG CAGAAATTGAGGAATGAGAAGCACCATATC   300
LeuPheIleSerIleValIleIleArgGln GluGlyLysProLysSerGlnGlnThrSer PheGlnSerSerProCysAsnLysSerPro LysSerHisGlyLeuGlnAsnGlnProTrp GlnLysLeuArgAsnGluLysHisHisIle

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ArgValLysLysAlaGlnSerLeuAlaGlu GlnThrSerAspThrAlaGlyLeuGluSer SerThrArgSerGluSerGlyThrAspLeu ArgGluHisSerProSerGluSerGluLys GluValValGlyAlaAspProArgGlyAla

AAACCCAAAAAAGCAACACAGTTTGTATAC AGCTATGGTAGAGGACCAAAAGTCAAGGAG AAACTCAAATGTGAATGGAGTAACCGAACA ACTCCAAAACCGGAGATGCTGGACCCGAAA GTACCAAACCTGTGGGGGTTTTCCACCCTG   600
LysProLysLysAlaThrGlnPheValTyr SerTyrGlyArgGlyProLysValLysGlu LysLeuLysCysGluTrpSerAsnArgThr ThrProLysProGluMetLeuAspProLys ValProAsnLeuTrpGlyPheSerThrLeu

ACTCTTCAGAGGCATCCTCTAGAAAAGGAG TATTGGATGGGTATGGAGCCAGACGAAATG AGCAGAGAAGATACCCACAGAAAAGGCCTC CCTGGGAAGTGGAGGGGGCCAGGCCACGAC CAGGCAGAAATCCACCAAAACAGGAGGGCC   750
ThrLeuGlnArgHisProLeuGluLysGlu TyrTrpMetGlyMetGluProAspGluMet SerArgGluAspThrHisArgLysGlyLeu ProGlyLysTrpArgGlyProGlyHisAsp GlnAlaGluIleHisGlnAsnArgArgAla

ACCGACATACAAACGCAGGACACACAGAACA ACATGGGCCCCATTCCAAAGTGATGACCTC AATGAAAGACCAGCAAAATCTACCTGTGAC AGTGAGAACTTGGCAGTCATCAACAAGTCT TCCAGGAGGGTTGACCAAGAGAAATGCACT   900
ThrAspIleGlnThrGlnAspThrGluThr ThrTrpAlaProPheGlnSerAspAspLeu AsnGluArgProAlaLysSerThrCysAsp SerGluAsnLeuAlaValIleAsnLysSer SerArgArgValAspGlnGluLysCysThr

GTACGGAGGCAGGATCCTCAAGTAGTATCT CCTTTCTCCCGAGGCAAACAGAACCATGTG CTAAAGAATGTGGAAACGCACACAGGTTCT CTAATTGAACAACTAACAACAGAAAAATAC GAGTGCATGGTGTGCTGTGAATTGGTTCGT   1050
ValArgArgGlnAspProGlnValValSer ProPheSerArgGlyLysGlnAsnHisVal LeuLysAsnValGluThrHisThrGlySer LeuIleGluGlnLeuThrThrGluLysTyr GluCysMetValCysCysGluLeuValArg

GTCACGGCCCCAGTGTGGAGTTGTCAGAGC TGTTACCATGTGTTTCATTTGAACTGCATA AAGAAATGGGCAAGGTCTCCAGCATCTCAA GCAGATGGCCAGAGTGGTTGGAGGTGCCCT GCCTGTCAGAATGTTTCTGCACATGTTCCT   1200
ValThrAlaProValTrpSerCysGlnSer CysTyrHisValPheHisLeuAsnCysIle LysLysTrpAlaArgSerProAlaSerGln AlaAspGlyGlnSerGlyTrpArgCysPro AlaCysGlnAsnValSerAlaHisValPro

AATACCTTCTCTTGTTTCTGTGGCAAGGTA AAGAATCCTGAGTGGAGCAGAAATGAAATT CCACATAGCTGTGGTGAGGTTTGTAGAAAG AAACAGCCTGGCCAGGACTGCCCACATTCG TGTAACCTTCTGTGCCATCCAGGACCCTGG   1350
AsnThrPheSerCysPheCysGlyLysVal LysAsnProGluTrpSerArgAsnGluIle ProHisSerCysGlyGluValCysArgLys LysGlnProGlyGlnAspCysProHisSer CysAsnLeuLeuCysHisProGlyProCys

CCACCCTGCCCTGCCTTATGACAAAAACA TGTGAATGTGGACGAACCAGGCACACAGTT CGGTGTGGTCAGGCTGTCTCAGTCCACTGT TCTAACCCATGTGAGAATATTTTGAACTGT GGTCAGCACCAGTGTGCTGAGCTGTGCCAT   1500
ProProCysProAlaPheMetThrLysThr CysGluCysGlyArgThrArgHisThrVal ArgCysGlyGlnAlaValSerValHisCys SerAsnProCysGluAsnIleLeuAsnCys GlyGlnHisGlnCysAlaGluLeuCysHis

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GlyGlyGlnCysGlnProCysGlnIleIle LeuAsnGlnValCysTyrCysGlySerThr SerArgAspValLeuCysGlyThrAspVal GlyLysSerAspGlyPheGlyAspPheSer CysLeuLysThrCysGlyLysAspLeuLys

TGCGGTAACCATACATGTTCGCAAGTGTGC CACCCTCAGCCCTGCCAGCAATGCCCACCG CTCCCCCAGCTGGTGCGCTGTTGCCCCTGT GGCAAACTCCTCTCAGCCAATTGCTAGAA CTTGGAAGTAGTAGTCGGAAAACATGCATG   1800
CysGlyAsnHisThrCysSerGlnValCys HisProGlnProCysGlnGlnCysProArg LeuProGlnLeuValArgCysCysProCys GlyGlnThrProLeuSerGlnLeuLeuGlu LeuGlySerSerSerArgLysThrCysMet

GACCCTGTTGCCTTCATGTGGAAAAGTGTGC GGCAAGCCTCTGCCTTCTGGTTGCCTTAGAT TTCATTCATACCTGTGAAAACCTCTGCCAT GAAGGAGACTCTGACCAGTCTCTCGCACA TCAGTTATTTCCTGCAGATGGTCTTTCAGA   1950
AspProValProSerCysGlyLysValCys GlyLysProLeuProCysGlySerLeuAsp PheIleHisThrCysGluLysLeuCysHis GluGluAspCysGlyProValSerArgThr SerValIleSerCysArgCysSerPheArg

ACAAAGGAGCTTCCATGTACCAGTCTCAAA AGTGAAGATGCTACATTTATGTGTGACAAG CGGTGTAACAAGAAACGGTTGTGGCACGG CATAAATGTAATGAGATATGCTGTCTTGGAT AAGGAGCACAAGTGTCCTTTGATTTGTGCG   2100
ThrLysGluLeuProCysThrSerLeuLys SerGluAspAlaThrPheMetCysAspLys ArgCysAsnLysLysArgLeuCysValArg HisLysCysAsnGluIleCysCysValAsp LysGluHisLysCysProLeuIleCysGly

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ArgLysLeuArgCysGlyLeuHisArgCys GluGluProCysHisArgGlyAsnCysGln ThrCysTrpGlnAlaSerPheAspGluLeu ThrCysHisCysGlyAlaSerValIleTyr ProProValProCysGlyThrArgProPro

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GluCysThrGlnThrCysAlaArgValHis GluCysAspHisProValTyrHisSerCys HisSerGluGluLysCysProProCysThr PheLeuThrGlnLysTrpCysMetGlyLys HisGluPheArgSerAsnIleProCysHis

CTGGTTGATATCCTTTGCGGATTACCCTGC AGTGCCAGGCTACCATGTGGGATGCACAAA TGTCAGAGACTCTGTCACAAAGGGGAGTGT CTTGTGGATGAGCCCTGCAAGCAGCCCTGC ACCACCCCCAGAGCTGACGTGTCGTCACCCG   2550
LeuValAspIleSerCysGlyLeuProCys SerAlaThrLeuProCysGlyMetHisLys CysGlnArgLeuCysHisLysGlyGluCys LeuValAspGluProCysLysGlnProCys ThrThrProArgAlaAspCysGlyHisPro

CCTATGGCACCCTGCCATACCAGCTCACCC TGCCCTGTCACTGCTTGTAAAGCTAAGCTA GAGCTACACGTGAATCGGGACCAAGAAAA GAGATGGTGATTTGCTCTGAAGCATCTAGT ACTTATCAAAGAATAGCTGCAATCTCCATG   2700
CysMetAlaProCysHisThrSerSerPro CysProValThrAlaCysLysAlaLysVal GluLeuGlnCysGluCysGlyArgArgLys GluMetValIleCysSerGluAlaSerSer ThrTyrGlnArgIleAlaAlaIleSerMet

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AlaSerLysIleThrAspMetGlnLeuGly GlySerValGluIleSerLysLeuIleThr LysLysGluValHisGlnAlaArgLeuGlu CysAspGluGluCysSerAlaLeuGluArg LysLysArgLeuAlaGluAlaPheHisIle

AGTGAGGATTCTGATCCTTTCAATATACGT TCTTCAGGGTCAAAATTCAGTGATAGTTTG AAAGAAGATGCCAGGAAGGACTTAAAGTTT GTCAGTGACGTTGAGAAGGAAATGGAAACC CTCGTGGAGGCCGTGAATAAGGGAAAGAAT   3000
SerGluAspSerAspProPheAsnIleArg SerSerGlySerLysPneSerAspSerLeu LysGluAspAlaArgLysAspLeuLysPhe ValSerAspValGluLysGluMetGluThr LeuValGluAlaValAsnLysGlyLysAsn

AGTAAGAAAAGCCACAGCTTCCCTCCCATG AACAGAGACCACCGCCGGATCATCCATGAC TTGGCCCAAGTTTATGGCCTGGAGAGCGTG AGCTATGACAGTGAACCGAAGCGCAATGTG GTGGTCACTGCCATCAGGGGGAAGTCCGTT   3150
SerLysLysSerHisSerPheProProMet AsnArgAspHisArgArgIleIleHisAsp LeuAlaGlnValTyrGlyLeuGluSerVal SerTyrAspSerGluProLysArgAsnVal ValValThrAlaIleArgGlyLysSerVal

TGTCCTTACCACGCTGACAGGTGTGCTT GAAAGGGAAATGCAGGCACGGCCTCCACCA CCGATTCCTCATCACAGACATCAGTCAGAC AAGAATCCTGGGAGCAGTAATTTACAGAAA ATAACCAAGGAGCCAATAATTGACTATTTT   3300
CysProProThrThrLeuThrGlyValLeu GluArgGluMetGlnAlaArgProProPro ProIleProHisHisArgHisGlnSerAsp LysAsnProGlySerSerAsnLeuGlnLys IleThrLysGluProIleIleAspTyrPhe

CACGTCCAGGACTAAGAAGATCATGATGCA CTTAGATAAAAGAATGATTAGGTATAGTGG AGACTTATTTGCCAGCAGATAAATCATGCC CGTTCCCCTCTGCCTGGCAGAATCACAGTC TCACATACTGTCTTGTACTGACACATCCAA   3450
AspValGlnAsp.

AGCATGAGTGTGTCAGAAATCCCTTGTCTA TTCCTGTCTGTATAAAGTGTTTCAGGATGA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAA   3543
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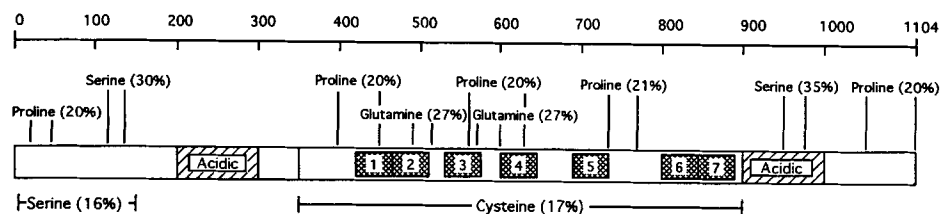
Figure 2. Deduced amino acid sequence and primary structural analysis of the NF-X1 polypeptide. (A) The complete deduced amino acid sequence of the largest ORF of the NF-X1 mRNA is shown from the first in-frame methionine to the most ORF-proximal polyadenylation site. No other significant ORFs are detected in either strand, and in vitro transcription/translation of NF-X1 cDNA fragments produces polypeptides of molecular weights in agreement with this ORF. Two additional polyadenylation sites are also observed in additional clones (data not shown). (B) Primary structure analysis of NF-X1. The entire amino acid sequence was subdivided into 22 fragments of 50 residues (with the final fragment containing 54 residues) and analyzed using the Gene Works software program (Intelligenetics, Mountain View, CA). Note the general symmetrical organization of the protein, with a 550-residue central cysteine-rich domain. Proline, serine, and glutamine-rich regions are also indicated, as are two acidic regions found at the NH2 and COOH termini. Seven repeats of approximately 40 residues in length are indicated within the cysteine-rich domain. (C) Hydropathy plot for the deduced amino acid sequence of NF-X1 using the algorithm of Kyte and Doolittle (57). (D) Amino acid homology alignment of the seven repeated domains within the cysteine-rich region. The repeat motif was detected by screening for internal homology using the FASTP program and the Gene Works software. The consensus sequence was derived by aligning the repeat motifs. The numbers to the left of the polypeptides indicate the amino acid positions included in each repeat, and the numbers below the consensus indicate the number of repeats that contain the consensus amino acid. These sequence data are available from EMBL/GenBank/DDBJ under accession number x79874.

these repeats are aligned in Fig. 2 D. The repeated domains are roughly 40 residues in length. The homology is greatest in the central portion of the repeat and decreases away from the center. However, several cysteine residues within the repeats appear to be conserved regardless of their distance from the central homologous region. It is possible that these conserved cysteines serve as a framework for the structure of the repeating domain via disulfide linkages, metal complexes, or an alternative mechanism.
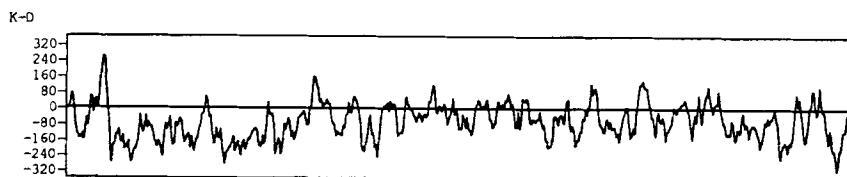
The existence of a cysteine-rich domain raises the possibility that this region might mediate sequence-specific binding via the formation of zinc finger(s). However, an exhaustive analysis for prototypical zinc-finger motifs does not reveal any typical zinc finger motifs of the C2C2 or C2H2 types. Although several cysteine and histidine residues do exist in the NF-X1 repeats, the distances between them and in potential linking regions are nonstandard (39). The repeated motif is itself most similar to but distinct from two previously de-

B


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      0    100   200   300   400   500   600   700   800   900  1000  1104
      |————|————|————|————|————|————|————|————|————|————|————|
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Serine (30%)
Proline (20%)   Proline (20%)   Proline (20%)   Proline (21%)   Serine (35%) Proline (20%)
Glutamine (27%) Glutamine (27%)

├Serine (16%)┤   ├——————————— Cysteine (17%) ———————————┤

1 cm = 50 residues
▨ = NFX Repeat

C

K-D


```
 320
 240
 160
  80
   0
 -80
-160
-240
-320
```

D

NFX REPEATS

Residue #:	Sequence
420/470:	PHSCGEVCRKKQPGQDCP-----HSCNLLCHPGPCPPCPAFMTKTCECGRT
473/520:	QAVSVHCSNPCENILNCGQ----HQCAELCHGGQCQPCQIILNQVCYCGST
533/581:	GFGDFSCLKTCGKDLKCGN----HTCSQVCHPQPCQQCPRLPQLVRCCPCG
599/647:	MDPVPSCGKVCGKPLPCGSLDFIHTCEKLCHEGDCGPVSRTSVISCRC
688/735:	VDKEHKCPLICGRKLRCGL----HRCEEPCHRGNCQTCWQASFDELTCHCG
799/839:	HLVDISCGLPCSATLPCGM----HKCQRLCHKGECLVDEPCKQP
840/876:	CTTPRADCG-----HPCMAPCHTSSPCPVTACKAKVELQCEC

| CONSENSUS: | C---C---L-CG-----H-C---CH-G-C-----------C |
| # MATCHES: | 5   5   5 76   7 7   77 5 6   5 |

scribed families of metal binding proteins, the LIM domain and the RING finger families, and is therefore likely to represent a novel metal binding domain (40, 41). The motif is highly significant since only seven proteins in the protein database contain stretches that are similar to the described motif, with the probability of detection being ~1.5 × 10⁻⁵. All of the proteins that contain related motifs (e.g. RAG-1, *Saccharomyces cerevisiae* RAD18, herpes simplex Ie110, the *ret* oncogene, the *Caenorhabditus elegans* developmental gene *lin-11*, and the insulin gene enhancer binding protein lsl-1) are thought to interact with DNA, although they are involved in the distinct enzymatic processes of recombination, repair, and transcriptional regulation. Further investigation is required to determine what sort of structures form in this region and how they might mediate sequence-specific binding.

The NF-X1 polypeptide contains several other features that are characteristic of transcription factors. Two acidic regions (between residues 200–300 and 900–1000) surround the cysteine-rich domain. Three regions rich in proline (>20%) and two regions rich in glutamine (27%) are located within the cysteine-rich domain. Two serine-rich regions (>30%) are located 100 residues from each terminus, and two proline-rich (>20%) segments are found at the termini of the polypeptide. Investigations are in progress to determine what regions of NF-X1 mediate its regulatory function.

Genomic Organization and Transcription of the NF-X1 Gene (42). Southern blot analysis of human genomic DNA digested with multiple restriction enzymes (using the clone-16 cDNA insert as a probe) reveals multiple bands ranging in size from ~4 to >23 kb in length (Fig. 3 *A*). As the sum of these fragments greatly exceeds the total length of the NF-X1 mRNA, we conclude that the NF-X1 gene is interrupted by introns of considerable size or that a related gene or genes (or pseudogenes) exists within the human genome. Southern blot analysis of genomic DNA isolated from mouse, Drosophila, and yeast cells detects homologous sequences in each organism and indicates that the NF-X1 gene (or a related gene) is evolutionarily conserved (data not shown). Northern blot analysis of total RNA isolated from B and T cell lines detects an mRNA of ~4 kb in size which is expressed at very low levels (data not shown). Since the NF-X1 mRNA is present in low abundance, both an RT-PCR and an RNase protection assay have been used for its detection and quantitation. cDNA synthesized from total RNA extracted from the class II–positive B lymphoblastoid cell line Jijoye, and the class II–negative cell lines Jurkat (T cell) and RJ2.2.5 (mutant B-LCL), has been used as substrates for PCR using two antiparallel oligonucleotides derived from the NF-X1 cDNA sequence. This nonquantitative RT-PCR detects NF-X1 mRNA in each cell line, regardless of class II phenotype

A

1 2 3 4

B

PHIX174 RF DNA/HaeIII

JIJOYE JURKAT 1 RJ 2.2.5 JURKAT 2

C

RNASE PROTECTION ANALYSIS

U937 W/ NFX
U937 W/ GAMMA-ACTIN
U937
CLONE-13
HELA
HELA + INF 1
HELA + INF 2
JIJOYE
JURKAT
RAJI
RJ 2.2.5

NFX →

GAMMA-
ACTIN →
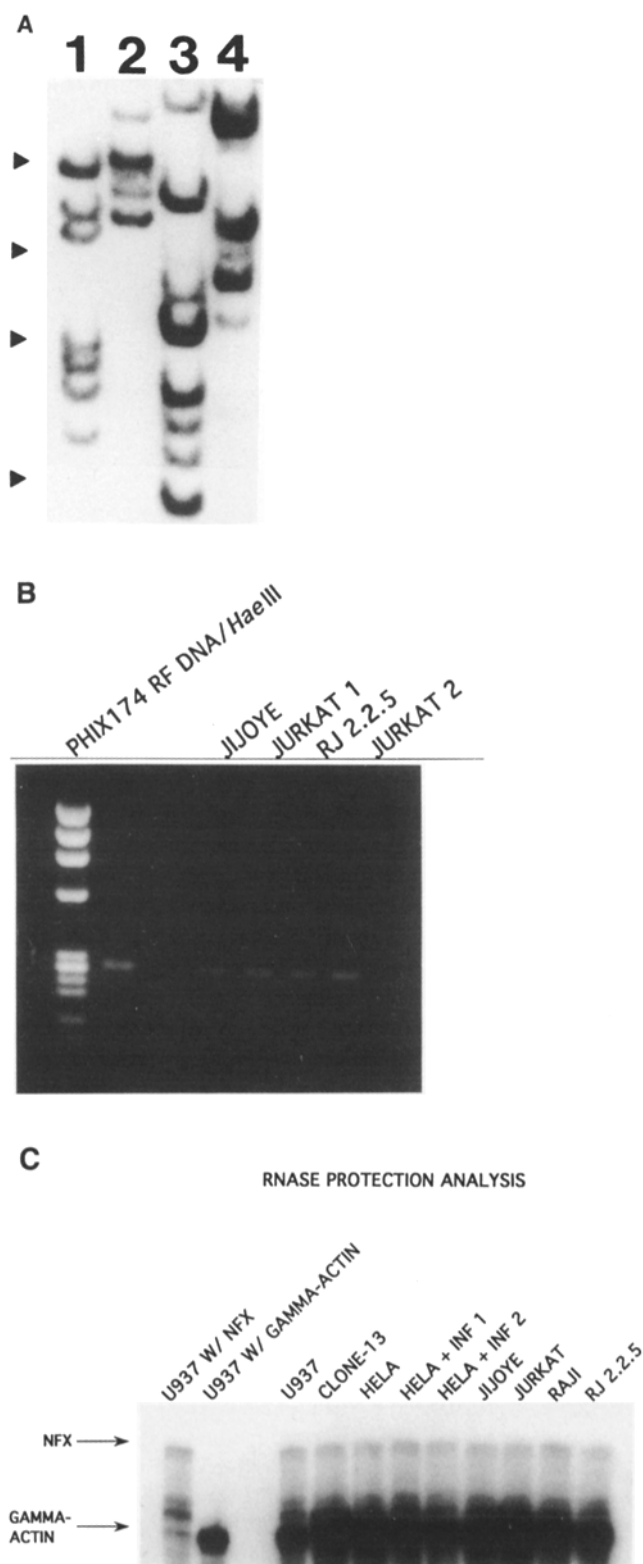
Figure 3. Genomic organization and transcription of the NF-X1 gene. (*A*) Genomic Southern blot probed with radiolabeled NF-X1 cDNA insert. High molecular weight DNA was isolated from murine splenocytes (lanes *1* and *2*) and from the human B-lymphoma cell line, clone-13 (lanes *3* and *4*). 20 µg DNA was digested to completion with EcoRI (lanes *1* and *3*) or BamHI (lanes *2* and *4*), resolved on a 1% agarose gel, transferred to a hybridization membrane, and probed with the radiolabeled clone-16

(Fig. 3 *B*). A larger panel of RNAs extracted from several class II–positive and –negative cell lines has also been analyzed for the presence of NF-X1 mRNA using a sensitive and quantitative RNase protection assay (Fig. 3 *C*). These analyses demonstrate that the NF-X1 mRNA is ubiquitously expressed in all cell lines tested and is present at an abundance ∼50-fold lower than an internal γ-actin control.

NF-X1 Encodes a Promiscuous X1 Box Binding Protein. Secondary and tertiary screens of bacteriophage clones isolated in this screening included an initial assessment of sequence specificity of encoded DNA-binding proteins by incubation of sections of nitrocellulose filter "lifts" with multiple radiolabeled recognition site probes. These analyses indicated that the NF-X1 protein interacts with both the DQB and DPB extended X box probes but not with the HLA-DRA S-box recognition site (data not shown). To allow further analysis of binding specificity, bacteriophage lysogens were constructed from the initial clone-16 bacteriophage using the method of Singh et al. (33). The binding specificity of isolated NF-X1 fusion protein induced after 1 h incubation in 10 mM isoprylthio-β-D-galactoside (IPTG) has been assessed by electrophoretic mobility shift analysis (Fig. 4 *A*). Recombinant NF-X1 binds to a radiolabeled, double-stranded oligonucleotide containing only the X1 box of the HLA-DRA gene promoter and seven nucleotides upstream of the element (5′CCCTTCCCCTAGCAAGAGATG3′). The shift is abolished by adding 100-fold excess unlabeled competitor oligonucleotides containing the analogous regions of the other human class II MHC gene promoters: HLA-DRB, -DPA, -DPB, -DQA, and -DQB. Addition of 100-fold excess unlabeled competitor oligonucleotide representing other elements in the HLA-DRA promoter (the Y-box and the S-box) or elements found in other promoters (the SP1 motif and the PRDII element of the human IFN-β gene promoter) does not influence the interaction of NF-X1 with its cognate recognition sequence. These data indicate that NF-X1 interacts sequence specifically with all human class II MHC X1 boxes.

Initial Deliniation of the DNA-binding Domain of NF-X1. Using the strategy of Keller and Maniatis (43), an initial definition of the NF-X1 DNA-binding domain has been accomplished (Fig. 4 *B*). Truncated forms of the NF-X1 cDNA were created by PCR and ligated into EcoRI-cleaved, phosphatased λgt11 DNA (44). The recombinant bacteriophage DNA was then packaged in high-efficiency phage packaging extract (Stratagene). The titer and frequency of recombination of the recombinant phage was determined by plating on Y1088 *Escherichia coli* with IPTG and X-Gal included in

cDNA insert. The Southern blot was subsequently washed at high stringency and subjected to autoradiography. The positions of DNA markers of HindIII-digested bacteriophage λ DNA are indicated by arrows. (*B*) RT-PCR analysis of total RNA isolated from Jijoye, Jurkat, and RJ2.2.5. Lane assignments are: (*1*) Lambda HindIII plus PhiX174/HaeIII, (*2*) positive control, (*3*) negative control (no template), (*4*) Jijoye template, (*5*) Jurkat template, and (*6*) RJ.2.2.5 template. (*C*) RNase protection analysis of total RNA isolated from class II MHC–positive and –negative cell lines. The RNAs utilized in the lanes labeled HELA + INF were extracted from HeLa cells incubated for 24 h with 250 U/ml IFN-γ.
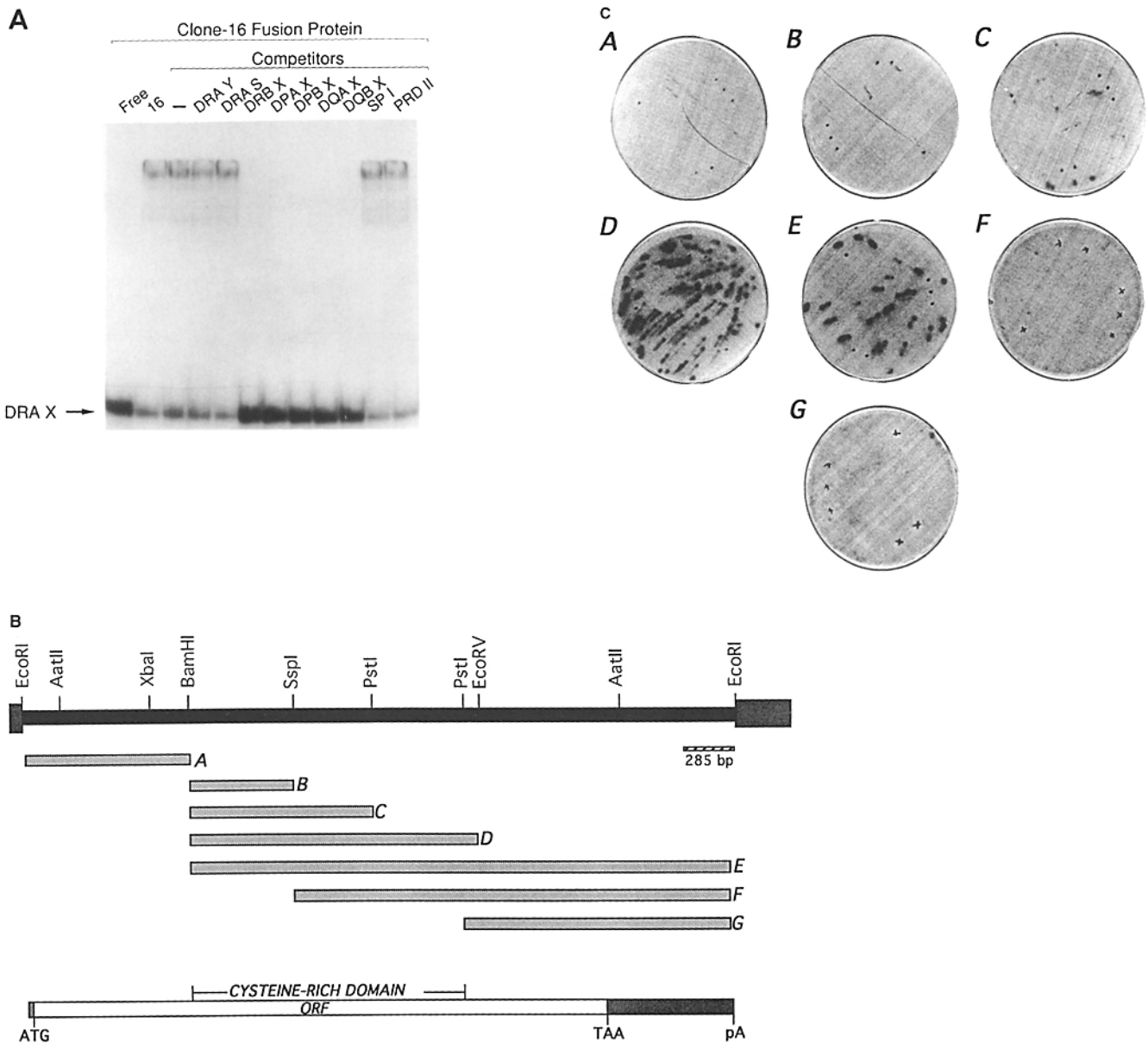
Figure 4. NF-X1 is a sequence-specific X1-box binding protein that requires its cysteine-rich domain for DNA binding. (*A*) Electrophoretic mobility shift analysis of recombinant NF-X1. Recombinant NF-X1 forms a specific complex with the double-stranded, DRA X1 box oligonucleotide (5'CCC-TTCCCCTAGCAAGAGATG3') which is competed for by 100-fold excess cold, double-stranded oligonucleotides containing the analogous regions from the HLA-DRB, -DPA, -DPB, -DQA, and -DQB promoters but not by HLA-DRA Y-box (5'AAATATTTTTCTGATTGGCCAAAGAGT3'), S-box (5'TGTGTCCTGGACCCTTTGCAAGA3'), SP1 (5'ATTCGATCGGGGCGGGGCGAGC3') or the IFN-β gene–positive regulatory domain II (PRDII) element (5'GTGGGAAATTCCGTGGGAAATTCCG3'). (*B*) Truncated forms of the NF-X1 cDNA were generated using the indicated restriction endonucleases, inserted into the pRSET series of bacterial expression vectors as described, and subsequently subcloned into the λgt11 bacteriophage and LNCX mammalian expression vectors. The ability of each subclone to generate the expected polypeptide was assessed by in vitro transcription/translation of linearized templates and analysis of translation products on SDS/polyacrylamide gels (data not shown). The relative positions of each fragment relative to the ORF are indicated. (*C*) In situ binding of induced fusion proteins encoded by λgt11 bacteriophage clones harboring the truncated NF-X1 polypeptides shown in *B*. After infection and induction, filters were processed as indicated (29, 44). Recombinant NF-X1 fusion proteins encoded by λgt11 phages D and E retain X1-box binding activity.

the plates. Several recombinant plaques were isolated for each construction and dideoxy sequence analysis of minipreparations of phage DNA was performed to identify bacteriophage particles harboring the NF-X1 truncations in frame with the NH₂-terminal portion of β-galactosidase. These phages were then plated on Y1090 bacteria, and the various truncated NF-X1 fusion proteins induced and assessed for their ability to bind radiolabeled DRA-X1 box oligonucleotides using the filter binding assay. The data presented in Fig. 4 *C* show that the entire cysteine-rich region spanning amino acids 420–900

A
LNCX

B

RAJI CELLS

HELA CELLS

C

RAJI CELLS
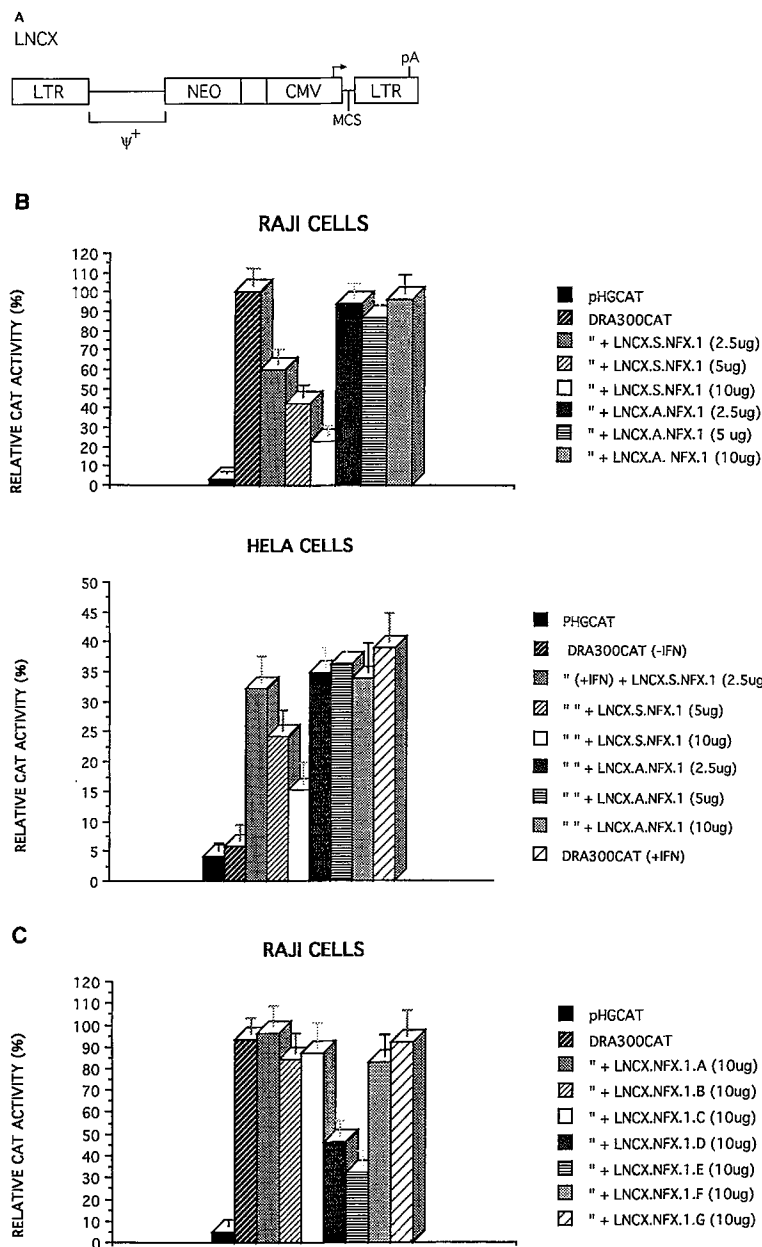
Figure 5. NF-X1 encodes a transcriptional repressor of HLA-DRA gene expression. (*A*) Map of the eukaryotic retroviral expression vector-LNCX indicating the neomycin resistance gene for selection of stable transformants, the potent cytomegalovirus promoter, the multiple cloning site for insertion of NF-X1 cDNA and indicated subfragments, and the retroviral LTRs. (*B*) Histograms showing the CAT activity produced in the class II MHC–positive cell line Raji and the class II–inducible cell line HeLa after cotransfection with the HLA-DRA promoter reporter construct DRA-300CAT and increasing amounts of the LNCX expression vectors containing the NF-X1 cDNA in either the sense or antisense orientations. CAT activities are normalized to a cotransfected HGH expression vector as described (8). (*C*) Transcriptional repression requires the DNA-binding domain of NF-X1. Histograms showing CAT activity in Raji cells after cotransfection with DRA300CAT and expression vectors containing the previously described subfragments of the NF-X1 cDNA. Only the LNCX.D and LNCX.E expression vectors mediate transcriptional repression from the DRA promoter.

is necessary and sufficient to mediate interaction with the HLA-DRA X1 box.

NF-X1 Encodes a Repressor of HLA-DRA Transcription and Requires the Cysteine-rich DNA-binding Domain for Regulatory Function. The regulatory function and effector domain(s) of NF-X1 have been investigated using mammalian expression vectors encoding the wild-type and six truncated NF-X1 polypeptides. The six truncated NF-X1 forms described in Fig. 4 *B* were generated by first subcloning the indicated NF-X1 restriction fragments in frame with the NH2-terminal peptide of the pRSET, A,B,C series of expression vectors (Invitrogen) to provide an NH2-terminal methionine residue to each NF-X1 subfragment. The resulting "expression cassettes" were then subcloned utilizing PCR methods into the retroviral vector pLNCX (Fig. 5 *A*) to generate a

series of mammalian expression vectors for cotransfection studies in mammalian cells (45). Each expression cassette was tested for its ability to direct the synthesis of the desired NF-X1 polypeptide by in vitro transcription and translation from linearized pRSET derivatives (data not shown). The regulatory function of NF-X1 has been assessed by cotransfection experiments where mammalian expression vectors encoding the wild-type and truncation derivatives of NF-X1 have been cotransfected with the HLA-DRA reporter construct DRA300CAT into a series of class II–positive, class II–negative, and IFN-γ–inducible cell lines (46). Wild-type NF-X1 has been found to encode a potent repressor of HLA-DRA transcription in the class II–positive cell Raji (Fig. 5 *B*). It also represses DRA transcription in IFN-γ–treated HeLa cells, but has no effect on DRA transcription in untreated HeLa
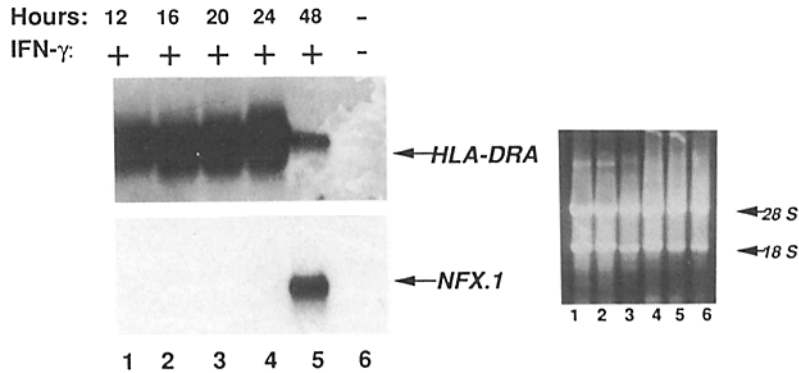
Figure 6. NFX.1 RNA is overexpressed late after incubation with IFN-γ and this coincides with reduction in HLA-DRA mRNA. HeLa cells were incubated with 250 U/ml rIFN-γ for the indicated number of hours before isolation of total RNA. 20 μg of total RNA from each sample was resolved by electrophoresis through a formaldehyde agarose gel and was transfered to a Gene Screen hybridization membrane by capillary transfer. A photograph of the ethidium bromide stained gel is presented to show that equivalent amounts of RNA were loaded for each sample. The same membrane was then probed with either a DRA- or NFX.1-specific radiolabeled probe, and the autoradiographs are presented. The DRA gene is strongly induced by IFN-γ and the level of DRA transcript increases to a maximal level 24 h postinduction. The level of DRA transcript is significantly reduced at 48 h after induction. The NFX.1 gene is expressed at very low levels as shown in Fig. 3 B, but is overexpressed at 48 h after induction.

cells and the class II–negative T cell line Jurkat (Fig. 5 B and data not shown). Overexpression of NF-X1 has no effect on transcription from reporter constructs that lack the X1 binding site such as a c-*fos* reporter construct, FC4, and RSVCAT (data not shown). Using the expression vectors encoding truncated NF-X1 forms, the cysteine-rich DNA-binding domain has been shown to be necessary and sufficient to mediate this transcriptional repression (Fig. 5 C). NF-X1 forms lacking significant regions of the DNA-binding domain cannot repress HLA-DRA transcription.

The NFX.1 mRNA Is Markedly Induced Late after Incubation with IFN-γ and this Coincides with Transcriptional Attenuation of the HLA-DRA Gene. Since artificial overexpression of the NFX.1 mRNA from retroviral constructs could repress transcription from the HLA-DRA promoter, a careful kinetic analysis of NFX.1 mRNA expression at several time points after incubation with IFN-γ was performed (Fig. 6). As is shown in Fig. 3 B, NFX.1 mRNA expression is difficult to detect in RNA derived from uninduced HeLa cells, and from HeLa cells incubated for short periods of time with 100–200 U/ml rIFN-γ. In contrast, the NFX.1 transcript is expressed at high levels in RNA derived from HeLa cells incubated for 48 h in the same concentration of IFN-γ. This overexpression of NFX.1 mRNA coincides with a marked reduction in the steady state level of HLA-DRA transcript. The level of DRA transcript increases during the first 24 h of incubation with IFN-γ and decreases significantly by 48 h after induction (Fig. 6 and our unpublished data). In view of the inhibitory effect of NFX.1 overexpression on HLA-DRA transcription (Fig. 5), these kinetic data strongly suggest that the NFX.1 protein functions in the postinduction turnoff of the HLA-DRA gene late after induction with IFN-γ.

Discussion

A newly identified, cysteine-rich polypeptide which interacts sequence specifically with the conserved X1 box regulatory element found in the proximal promoters of class II MHC genes was described, and its complementary DNA molecu-

larly cloned. The cysteine-rich domain contains a motif repeated seven times, and this entire region is necessary and sufficient for both sequence-specific binding and effector function. The motif is related to but distinct from the previously described metal-binding protein families LIM domain and RING finger. Overexpression of this protein strongly and specifically represses the transcription of the HLA-DRA gene in the class II–positive cell line Raji, and inhibits induction of the gene in the inducible cell line HeLa by IFN-γ, strongly suggesting that the NF-X1 protein encodes a transcriptional repressor. Additional evidence that NFX.1 is a biologically relevant repressor of HLA-DRA gene expression stems from the finding that the NFX.1 mRNA is markedly overexpressed late after induction of HeLa cells with IFN-γ, and that this overexpression coincides with a reduction in the level of HLA-DRA transcript in these cells. The identification of NF-X1 indicates that the X1 element, like the Y-box, can bind factors that can either activate or repress class II MHC gene expression.

A series of elegant classical genetic studies by Latron et al. (5) have previously demonstrated multiple genetic loci that encode either activators or repressors of class II MHC gene expression (for reviews see references 4, 5). These studies predicted the existence of two classes of genes termed *alr-1* and *slr-1* that encode either activator(s) or silencer(s) of class II MHC gene expression, respectively. The newly isolated cDNA, CIITA, located on human chromosome 16, appears to encode *alr-1* (27, 28). The *slr-1* gene or genes were identified in cell fusion experiments where factors expressed in the class II–negative plasmacytoma cell line P3-U1 were shown to rapidly and dominantly repress class II MHC transcription in the human B cell line Raji. Since the conserved X1 box of class II MHC genes plays a critical role in the transcriptional regulation of these genes, we are actively investigating whether NF-X1 is in fact the product of the slr-1 locus.

In addition to studies that will probe the interaction of NF-X1 with other known class II MHC and general promoter binding proteins, particular emphasis will be placed on studies to (*a*) elucidate how NF-X1 interacts sequence specifically with the X1 element and (*b*) probe how the effector function of NF-X1 is regulated in vivo. Three general models

of how transcriptional repressors act include: (a) direct competition for binding to a shared cis-element; (b) silencing (position-independent repression); and (c) neutralization (direct interaction with an essential activator), (47–53). The molecular cloning of NF-X1 should allow investigations into which mechanism is operating at the X1 box. With regard to regulation of effector function, two lines of investigation are required. First, a more extensive analysis of NF-X1 expression is required to determine whether the NF-X1 gene product might be overexpressed in other cell types or in response to physiological stimuli other than IFN-γ. The repressor of IFN-β gene expression, PRDI-BF1, is an example of a transcriptional repressor that binds to a positive regulatory element and is regulated by overexpression after the IFN-β gene has been induced (54, 55). This protein is therefore involved in the postinduction turn-off this gene (56). It is noteworthy that the class II MHC genes are also subject to

postinduction turn-off after activation with IFN-γ (9). The NFX.1 protein appears to have a similar role in HLA-DRA transcription that has been induced by IFN-γ. The second avenue of investigation involves the multiple sites of post-translational modification that may be involved in regulating effector function in other situations.

This information should contribute to our understanding of how class II MHC genes are regulated and may provide an avenue to manipulate the expression of these genes in disease states. Specifically, retroviral vectors (such as those described in this work) that can specifically repress the expression of class II MHC molecules might be useful as antiinflammatory reagents. These studies may also provide insight into the general problem of how the relative influence of two proteins that bind to the same cis-element, but which have opposing regulatory function, can be determined.

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