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Affinity Enrichment and Functional Characterization of TRAX1, a Novel Transcription Activator and X1-Sequence-Binding Protein of HLA-DRA

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The promoters of all class II major histocompatibility (MHC) genes contain a positive regulatory motif, the X element. The DNA-binding proteins specific for this element are presumed to play a critical role in gene expression, although there is a paucity of functional studies supporting this role. In this study, the X-box-binding proteins of HLA-DRA were affinity purified from HeLa nuclear extracts. Fractions 46 to 48 contained an X-box-binding activity and were determined by electrophoretic mobility shift assays to be specific for the X1 element. This X1 sequence-binding-protein, transcriptional activator X1 (TRAX1), was shown to be a specific transcriptional activator of the HLA-DRA promoter in an in vitro transcription assay. By UV cross-linking analysis, the approximate molecular mass of TRAX1 including the bound DNA was determined to be 40 kDa. When the TRAX1 complex was incubated with antibodies against a known recombinant X-box-binding protein, RFX1, and tested in electrophoretic mobility shift assays, TRAX1 was neither shifted nor blocked by the antibody. Further analysis with methylation interference showed that TRAX1 bound to the 5′ end of the X1 sequence at −109 and −108 and created hypersensitive sites at −114, −113, and −97. This methylation interference pattern is distinct from those of the known X1-binding proteins RFX1, RFX, NF-Xc, and NF-X. Taken together, our results indicate that TRAX1 is a novel X1-sequence-binding protein and transcription activator of HLA-DRA.

Major histocompatibility complex (MHC) class II molecules are transmembrane glycoproteins expressed on the cell surface as heterodimers of noncovalently associated A and B chains (9, 24, 58) and play important roles in immune function (reviewed in references 17 and 46). These molecules are involved in (i) the acquisition of the mature T-cell repertoire through positive and negative selection in the thymus (7, 34), (ii) the induction of lymphocyte and macrophage/monocyte activation and differentiation (6, 12), and (iii) the presentation of processed antigens to CD4-positive T lymphocytes (31). During an immune response, extracellular antigens are internalized by antigen-presenting cells and are processed and bound by class II MHC molecules as peptides (25). The peptide-bound class II molecules are then recognized by complementary T-cell receptors (10). The affinity between the T-cell receptor and a peptide-bound class II molecule is further enhanced by CD4 binding to a class II MHC molecule, which eventually results in the transduction of signals leading to T-cell activation and proliferation. Therefore, the regulation of MHC class II expression affects the extent of T-cell activation and the immune response.

In humans, class II MHC molecules are encoded by MHC class II genes (HLA-DR, -DP, and -DQ), and their expression is tissue restricted (reviewed in references 3, 5, and 23). They are constitutively expressed at high levels by B lymphocytes and dendritic cells but not by resting T lymphocytes, most peritoneal macrophages, and nonlymphoid cells (15). However, MHC class II molecules can be induced on T lymphocytes, macrophages, and nonlymphoid cells by a variety of different external stimuli; among these, gamma interferon (IFN-γ) is one of the most potent regulators of the class II MHC genes. These complex patterns of class II MHC gene expression have led to the intense investigation of *cis*-acting elements and *trans*-acting factors that are required for proper expression of the class II MHC genes (reviewed in references 14, 23, 68, and 70).

Sequence comparisons of the MHC class II promoters have revealed two highly conserved *cis* sequence motifs, the X and Y boxes, in all murine and human class II promoters (5, 38). Both the X and Y boxes are positive regulators of class II MHC gene expression and are required for accurate and efficient class II MHC gene expression (20, 60, 61, 70, 75). A critical role of the X-box-binding proteins for class II MHC expression has been demonstrated by in vivo footprinting (73), mutagenesis, and transient chloramphenicol acetyltransferase (CAT) expression analyses (4, 48, 62). In addition, a subset of patients with bare lymphocyte syndrome, a class II-negative hereditary immunodeficiency disease, exhibits a potential regulatory defect associated with the X1-sequence-binding protein, RFX (56).

By convention, the X box is subdivided into two target sequences, X1 and X2, which interact with several known proteins, including RFX (26, 28), RFX1 (29), NF-X (41, 43), NF-Xc (11), hXBP-1 (44, 45), X2BP (26), Jun/Fos (2), NF-S (40), YB-1 (18), and NF-X3/BCF-1 (72). RFX, RFX1, NF-X, and NF-Xc bind to the X1 sequence of the DRA promoter and have identical guanine and adenine methylation interference patterns. RFX is missing in class II-negative bare lymphocyte syndrome cell lines, but RFX1, NF-X, and NF-Xc are found in these extracts. It appears that RFX, RFX1, NF-X, and NF-Xc

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are closely related in terms of binding specificity, and most likely RFX1, NF-X, and NF-Xc are the same as determined by proteolytic digestion (27). hXBP-1, X2BP, Jun/Fos, and NF-S bind to the X2 sequence, which is similar to the tetradecanoyl phorbol acetate-responsive element and the cyclic AMP response element (16, 74). hXBP-1 is a bZIP protein which binds to the DRA promoter but not to the DRB, DQA, or DQB promoter and can form a heterodimer with c-Fos (but not with c-Jun) via the leucine zipper motif (49, 50). On the other hand, X2BP binds to the DRB and DRA promoters with a high affinity and does not heterodimerize with c-Fos. Jun and Fos are bZIP family proteins, and a Jun/Fos heterodimer binds to the X2 sequence of the DRA promoter. NF-S binds to the DQA and DPA promoters but weakly to the DRA promoter. YB-1 preferentially binds to the antisense strand of the X2 sequence of DRA (unpublished data) and is a negative regulator of DRA expression (14, 68, 69). NF-X3/BCF-1 is a B-cell-specific factor which binds to the X3 sequence (5′CAGATG3′, located from −100 to −95) of the DRA promoter and appears to be related to the helix-loop-helix proteins E12 and E47.

Although a large number of X-box-binding proteins have been characterized by DNA-binding analyses and λgt11 expression cloning, the functional significance of most X-box-binding proteins in class II MHC gene expression is not clear. To directly examine the functional roles of these X proteins in class II MHC gene expression, we sought to isolate X-box-specific transcription factors from crude nuclear extracts by using a combination of nonspecific and specific affinity columns. Here we describe the isolation of an X1-sequence-binding protein, transcriptional activator X1 (TRAX1), from HeLa nuclear extracts. We present evidence showing that TRAX1 (i) is a transcriptional activator as determined by in vitro transcription; (ii) has an approximate molecular mass of 40 kDa as determined by UV cross-linking; and (iii) specifically binds to the X1 sequence of DRA but is distinct from RFX1 as determined by antibody reactivity and from other known X1 proteins as determined by electrophoretic mobility shift assay (EMSA) and methylation interference. We also discuss the implications of how a ubiquitously expressed protein may regulate tissue-specific or inducible class II MHC gene expression.

MATERIALS AND METHODS

Cells and nuclear extracts. Namalwa B-lymphoblastoid cells (from R. Roeder, Rockefeller University) and Raji cells (human Epstein-Barr virus-positive Burkitt's lymphoma cell line) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% glutamine. HeLa (human cervical carcinoma cells) were cultured in suspension-minimum essential medium (S-MEM) supplemented with 5% horse serum, 5% fetal calf serum, and 1% glutamine.

Namalwa, Raji, and HeLa cells were used to prepare nuclear extracts, using a modification of Dignam's procedure as previously described (19, 66). Protein concentrations were determined by the Bio-Rad protein assay, and the extracts were frozen in aliquots at 70°C.

EMSAs and UV cross-linking. The X box and Y box of the DRA promoter were used as probes in mobility shift assays by the method described previously (28, 53). Four to 10 μg of nuclear extract was incubated with 2 μg of poly(dI-dC) · poly(dI-dC) and 50,000 to 100,000 cpm of ^{32}P-end-labeled DNA probes in a 20-μl reaction volume. After incubation for 20 min at room temperature, the complexes were resolved at room temperature on a 6% nondenaturing polyacrylamide gel containing 1× Tris-glycine. With affinity-purified protein, 5 μl of affinity-purified TRAX1 and 0.5 μg of poly(dI-dC) · poly(dI-dC) were added in the 20-μl reaction volume, the reaction mixture was incubated on ice for 30 min, and the TRAX1 complex was resolved at 4°C.

For EMSAs with anti-RFX1 antibody, the antibody was preincubated with TRAX1 or in vitro-transcribed and -translated RFX1 on ice for 1 h. The reaction mix was then processed according to the standard procedure with 20 min of incubation at room temperature before the complexes were resolved on the gel.

For UV cross-linking, the standard EMSA was scaled up 2.5-fold and bromodeoxyuridine-substituted 45-bp X1X2 oligonucleotides were used as probes. After a 20-min incubation, the reaction mixture was irradiated for 30 min in a cold room. To that mixture, 1 U of micrococcal nuclease (MNase), 4 μg of DNase I, and 1 μl of 0.5 M $CaCl_2$ were added, and the mixture was incubated at 37°C for 30 min. The mixture was then boiled at 95 to 100°C for 5 min and loaded onto a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel to identify the molecular weight of the cross-linked TRAX1.

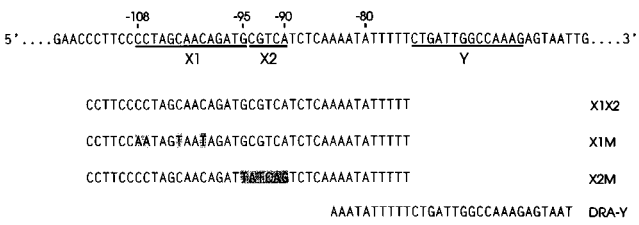

FIG. 1. Oligonucleotide map. Nucleotides are numbered relative to the cap site. The *cis*-acting X1, X2, and Y boxes are indicated. X1X2 represents the wild-type X sequence. The oligonucleotide with mutations in the X1 sequence is referred to as X1M, and that with mutations in the X2 sequence is designated X2M. The mutated nucleotides in the X1 and X2 sequences are in the shaded boxes.

Affinity purification of TRAX1. HeLa nuclear extracts were first chromatographed over a heparin-agarose (Sigma) column. The X-box-binding activity, monitored by EMSAs with X1X2 radiolabeled probes, was eluted with a linear gradient from 100 to 1,000 mM KCl in buffer containing 25 mM Tris-HCl (pH 8.0), 20% (vol/vol) glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM EDTA. Fractions were dialyzed against the same buffer containing 100 mM KCl. Fractions containing the X-box-binding activity were then chromatographed twice over an X1X2-specific affinity column prepared as described previously (30, 32, 75), using the 45-bp X1X2 oligonucleotides of the DRA promoter (Fig. 1). The X-box-binding activity was eluted with a linear gradient of 100 mM to 2 M KCl in the same buffer containing 0.1% Nonidet P-40. Bovine serum albumin (BSA) (0.3 mg/ml) was added to each fraction before the dialysis. Fractions were dialyzed against buffer containing 100 mM KCl and 0.1% Nonidet P-40, and fractions containing the X-box-binding activity determined by EMSAs were stored at −70°C.

Methylation interference assays. The methylation interference assay was performed as described previously (47, 59, 63), with a minor modification. Briefly, each strand of the 45-bp oligonucleotides spanning the X1 and X2 sequences of DRA was separately labeled with [γ-^{32}P]ATP at one end with T4 polynucleotide kinase (New England BioLabs). The labeled strand was then annealed to its complementary unlabeled strand and filled in with Sequenase (United States Biochemical). The labeled sense-strand probes were methylated with dimethyl sulfate for 1 min 10 s, and the labeled antisense-strand probes were methylated for 1.5 min at room temperature. The standard EMSA reactions were scaled up 10- to 12-fold for each strand, using 10^5 cpm of the methylated probes per reaction. The mobility shift reactions were resolved, and bound and free DNAs were excised from the gel. The bound and free DNAs were recovered by an Elutrap (Schleicher & Schuell) at 150 V for 2 h and purified by extraction with phenol-chloroform and then precipitation with 1/3 volume of 7.5 M ammonium acetate and 4 volumes of 100% ethanol. The bound and free DNAs were then cleaved with piperidine, and the products of the piperidine cleavage reactions were analyzed on a 12% polyacrylamide–8 M urea sequencing gel.

In vitro transcription. The standard transcription reaction was performed as described previously (66, 75), using 10 μl of Namalwa nuclear extract and 5′Δ-56SXY (4, 71) as a DNA template. 5′Δ-56SXY contains 141 bp of the DRA promoter linked to the CAT reporter gene, and *Nco*I-linearized 5′Δ-56SXY produces a 595-nucleotide (nt) CAT transcript in an in vitro transcription assay. A radiolabeled 494-nt Sp6 transcript (supplied by R. Kole, University of North Carolina) was added to each transcription reaction as an internal standard for sample recovery. To initiate the reconstitution reaction, Namalwa nuclear extract was first preincubated with cold 45-bp X1X2 oligonucleotides for 5 min at room temperature to absorb the X-binding proteins and thus to reduce the DRA X-dependent transcripts (75, 76). Transcription reactions were then reconstituted by adding affinity-enriched TRAX1. In another group of reconstitution experiments, competitive oligonucleotides were not added; instead, TRAX1 was directly added to an in vitro transcription reaction, and its capacity to enhance transcription was measured. In this protocol, the transcriptional cocktail contained 7 μl of nuclear extract which was determined by titration to produce a detectable but lowered level of ^{32}P-labeled transcripts. This lowered level of transcription was essential to detect the reconstituting activity of TRAX1. All reconstituted transcription reactions were performed in the same salt concentration containing 0.3 mg of BSA per ml to ensure that any difference observed was due to the reconstitution of the X-dependent activity alone. The final products were visualized by autoradiography.

RESULTS

The X-box-binding activities of HeLa and Raji nuclear extracts. The X-box-binding activities of crude nuclear extracts

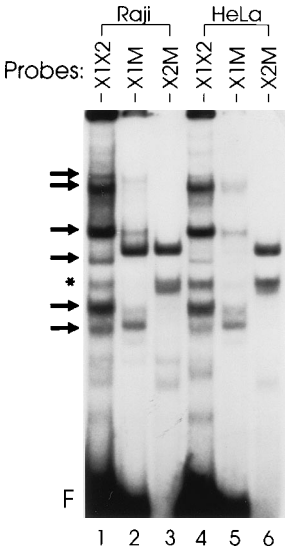

FIG. 2. Raji and HeLa extracts exhibit nearly identical EMSA patterns for the X-box-binding activities. Nuclear extracts were prepared from Raji and HeLa cells. The binding reaction mixture contained 12% glycerol, 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 60 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.3 mM dithiothreitol, 2 μg of poly(dI-dC) · poly(dI-dC), 4 to 8 μg of nuclear extract protein, and 10^5 cpm of labeled probe in a total volume of 20 μl. Eight micrograms of nuclear extract protein was used in lanes 1, 2, 4, and 5, and 4 μg of nuclear extract protein was used in lanes 3 and 6. Probes used were X1X2 (lanes 1 and 4), X1M (lanes 2 and 5), and X2M (lanes 3 and 6). The positions of the X1- and X2-sequence-binding activities are indicated by arrows, and that of the X1 sequence binding activity is indicated by an asterisk. F, free probe.

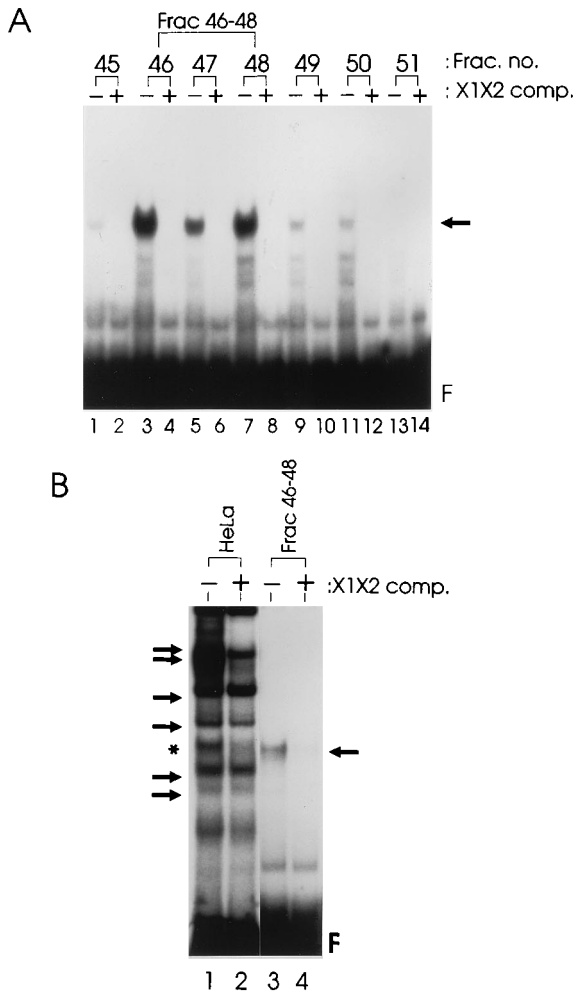

FIG. 3. Purification of DRA X-binding-protein, using an X-specific DNA affinity column. Crude HeLa nuclear extract was fractionated once on a heparin-agarose column and twice on an X1X2 DNA affinity column. At each step of purification, every third fraction was analyzed with ^{32}P-labeled X1X2 probe in EMSA. Fractions which contained the X-box-binding activity were then pooled and applied to the next column. (A) DRA X-box-binding activities in fractions (Frac) 46 to 48. Twice-X-affinity-purified proteins were analyzed in EMSA using ^{32}P-labeled X1X2 probe in the presence (+) or absence (−) of X1X2 cold competitor (comp.). Only the representative fractions from 45 to 51 out of 100 fractions collected are shown. The arrow indicates the X-specific complex. (B) Fractions 46 to 48 comigrate with the X1-sequence-specific protein in HeLa nuclear extract. HeLa nuclear extract (lanes 1 and 2) and fractions 46 to 48 (lanes 3 and 4) were run side by side to locate the corresponding complex in the HeLa nuclear extract. For an easy comparison with Fig. 2, the corresponding arrows and asterisk were placed on the left to mark the same complexes in both figures. F, free probes.

from HeLa and Raji cells were examined by EMSA with radiolabeled oligonucleotide probes consisting of wild-type X1X2, X1M (with mutations in the X1 sequence of the HLA-DR promoter), or X2M (with mutations in the X2 sequence of the HLA-DRA promoter) (Fig. 1). Figure 2 shows EMSA patterns that are nearly identical between Raji and HeLa extracts. There are several noticeable complexes which appear to be sensitive to both the X1 and X2 mutations (arrows) and one unique complex which is sensitive only to the X1 mutations and is present in both HeLa and Raji cells (asterisk). The sensitivity to both X1 and X2 mutations may be explained by the formation of complexes that require both X1 and X2. The requirement for both X1 and X2 in some complexes may be explained by cooperativity or mutual stabilization of some X1- and X2-binding proteins. Indeed, such a mutual dependency has been observed in an in vivo footprint and in vitro gel shift analyses wherein a mutation of X1 produced a diminution of binding at X2, and vice versa (55, 73). In lanes 2, 3, 5, and 6, there are a few complexes that are unique to the mutant probes and may be caused by the inadvertent generation of new binding sites.

Affinity purification of the X-box-binding proteins from HeLa nuclear extracts. HeLa nuclear extracts were chromatographed once through a heparin-agarose column and twice through an X1X2 affinity column to isolate the X-box-binding proteins. EMSAs were performed at each purification step to trace the X-specific activities in fractions. Figure 3A shows such an EMSA with twice-affinity-enriched fractions 45 to 51 out of 100 fractions collected. The addition of the 45-bp X1X2 oligonucleotide competitors resulted in the complete disappearance of the complex in fractions 46, 47, and 48 (compare lanes 3 and 4, 5 and 6, and 7 and 8). This result indicated that fractions 46 to 48 contained X-box-binding proteins. Consequently, fractions 46 to 48 were electrophoresed side by side with crude HeLa nuclear extracts (Fig. 3B). The affinity-enriched proteins in fractions 46 to 48 formed a complex that comigrated with the X1-sequence-specific proteins found in HeLa and Raji nuclear extracts (compare with Fig. 2). The fold enrichment of fractions 46 to 48 was further calculated. Fractions 46 to 48 were enriched 1.3×10^4-fold, with an increase in the specific activity from 9.75×10^1 (in crude nuclear extract) to 1.29×10^6 (in the enriched fractions 46 to 48) densitometric units of bound DNA per μg of protein.

Fractions 46 to 48 contain an X1-specific DNA-binding protein. Fractions 46 to 48 were further analyzed in EMSAs using various labeled oligonucleotides containing mutations in either

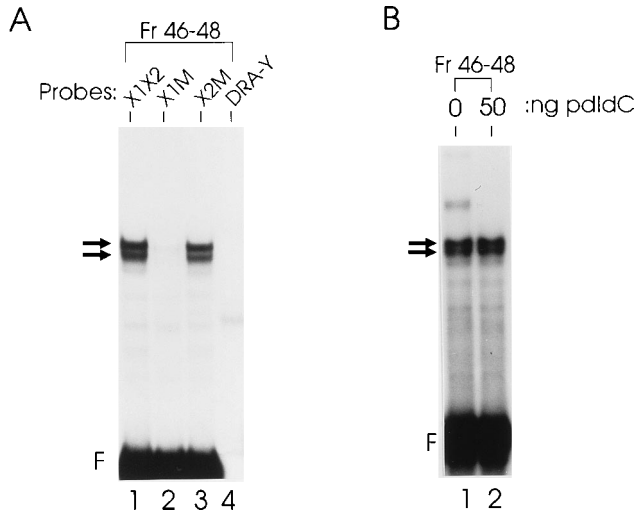

FIG. 4. Fractions 46 to 48 contain an X1-binding protein. (A) Fractions 46 to 48 were analyzed in EMSA using ^{32}P-labeled X1X2, X1M, X2M, and DRA-Y probes in parallel. Fractions (Fr) 46 to 48 are sensitive to mutations in the X1 sequence (lane 2) but not to the X2 sequence (lane 3) and do not bind to an unrelated Y sequence (lane 4). To increase the resolution of the X-specific complexes (arrows), DRA-Y free probe was electrophoresed off the gel. (B) Fractions 46 to 48 were analyzed in the absence or in the presence of 10-fold-less poly(dI-dC) · poly(dI-dC).

X1 or X2 to confirm the specificity (Fig. 4A). Fractions 46 to 48 bind to X1X2 and X2M ^{32}P-labeled probes (lanes 1 and 3) but not to X1M and unrelated DRA-Y ^{32}P-labeled probes (lanes 2 and 4). This finding demonstrates that the proteins in fractions 46 to 48 specifically recognize the wild-type X1 sequence but not the X2 or Y sequence of HLA-DRA. To evaluate the possibility that these fractions contain additional DNA-binding proteins that are blocked by the higher concentration (500 ng) of nonspecific competitor [poly(dI-dC · poly(dI-dC)] used for Fig. 4A, the experiment was repeated in the absence of or with 1/10 the concentration of poly(dI-dC) · poly(dI-dC). As seen in Fig. 4B, the pattern of the EMSA remains unchanged at this lower concentration of nonspecific competitor, demonstrating that the X1-box-specific proteins are the predominant binding activity in these fractions.

We have observed that fractions 46 to 48 formed either a single broad band (Fig. 3) or two sharp bands (Fig. 4) with the X1X2 probe in different experiments. Since the molecular weights of two forms were indistinguishable as determined by UV cross-linking analysis (unpublished observations), we believe these two forms may be different conformations or modifications of the same protein.

Fractions 46 to 48 contain an in vitro transcriptional activator of the DRA promoter. Several experiments were performed to determine if fractions 46 to 48 contain a transcriptional activator of the DRA promoter. In the first approach, fractions 46 to 48 were used to reconstitute a transcriptional extract that was functionally depleted of X-binding proteins. Namalwa nuclear extracts were used to supply necessary factors for transcription from the DRA promoter in vitro. The template plasmid, 5′Δ-56SXY (Fig. 5A), which contained 141 bp of the DRA promoter fused to the CAT gene, was predigested with *Nco*I to yield a 595-base known transcript in this in vitro transcription extract. To test the capacity of fractions 46 to 48 to reconstitute the transcriptional activity of DRA, in vitro transcription assays were performed in the presence of an excess of cold X1X2 oligonucleotides to functionally deplete the X-box-binding proteins in crude nuclear extracts. The amount of X1X2 oligonucleotide competitor was first titrated to achieve a significant but suboptimal level of competition (Fig. 5B); this suboptimal level of competition was necessary to ensure that both a negative and a positive effect of the proteins in fractions 46 to 48 can be detected in transcription assays. The X1X2 competitor is specific for the DRA promoter since it did not reduce the transcription of a dihydrofolate reductase (DHFR) promoter (data not shown).

Figure 5C shows the reconstitution of DRA transcription with fractions 46 to 48. In the presence of X1X2 oligonucleotides, the level of the 595-base DRA transcripts was reduced by approximately two-thirds (compare lanes 1 and 3). However, this reduction was not observed by an equivalent molar of an irrelevant competitor, the EL3noSP1 oligonucleotide (lane 2). When 5 μl of fractions 46 to 48 was added to the reaction, transcriptional activity of the DRA promoter was restored to its original level (lane 4), demonstrating that fractions 46 to 48 may contain a transcriptional activator.

The use of DNA competitors in the assay shown in Fig. 5C raises the criticism that fractions 46 to 48 may displace other DRA *trans*-activating factors from the competitor DNA such that the activation is indirect and due to a sequestration of competitor DNA. To address this possibility and to further evaluate the transcriptional activity and specificity of fractions 46 to 48, fractions 46 to 48 were directly added to a transcriptional cocktail containing 7 μl of Namalwa nuclear extract in the absence of the cold competitors (Fig. 5D). Results in Fig. 5D show that the addition of fractions 46 to 48 enhanced the DRA-specific 595-base transcripts (compare lanes 1 and 2), indicating that fractions 46 to 48 directly enhanced DRA transcription. As a specificity control, fractions 46 to 48 were also added to a reaction containing the DHFR promoter. The DHFR promoter has two start sites (66), and the products from these start sites, 976- and 1,016-base transcripts, were relatively unaffected by these fractions (compare lanes 3 and 4). It is important to note that Fig. 5D was exposed for a shorter time (one-third as long as Fig. 5C) to best demonstrate the clear differences in the enhancement between the DHFR- and DRA-specific transcripts. The 494-base (internal standard) and 595-base DRA-specific transcripts in Fig. 5D appear lighter than those in Fig. 5C because of this short exposure. To quantitate the results, a densitometric tracing of the DRA- or DHFR-specific transcript was normalized to the densitometric reading of the internal standard, the 494-base transcript in the same lane. Such an analysis shows that the DRA-specific transcript was enhanced approximately fivefold over the DHFR transcript. Therefore, fractions 46 to 48 contain the transcription activator with specificity for the DRA promoter. This transcription activator will be referred to as TRAX1.

TRAX1 is a unique X1-sequence-binding protein. To date, recombinant RFX (RFX1) is the only cloned X1-binding protein. An antiserum raised against recombinant RFX1 specifically immunoprecipitated a 116-kDa protein as well as the 140-kDa protein corresponding to RFX1 (29). A crucial issue is whether TRAX1 is RFX1. Affinity-enriched TRAX1 and the in vitro-translated RFX1 were preincubated with preimmune and anti-RFX1 immune sera in an EMSA (Fig. 6A). The complex formed with recombinant RFX1 was completely blocked by the anti-RFX1 antibody (compare lanes 4 and 5). In contrast, the anti-RFX1 antibody did not block or supershift TRAX1 (compare lanes 6 and 7), suggesting that TRAX1 is not related to RFX1.

The molecular weight of TRAX1 was estimated by UV cross-linking analysis (Fig. 6B). In the absence of nuclease treatment, a number of higher-molecular-weight complexes

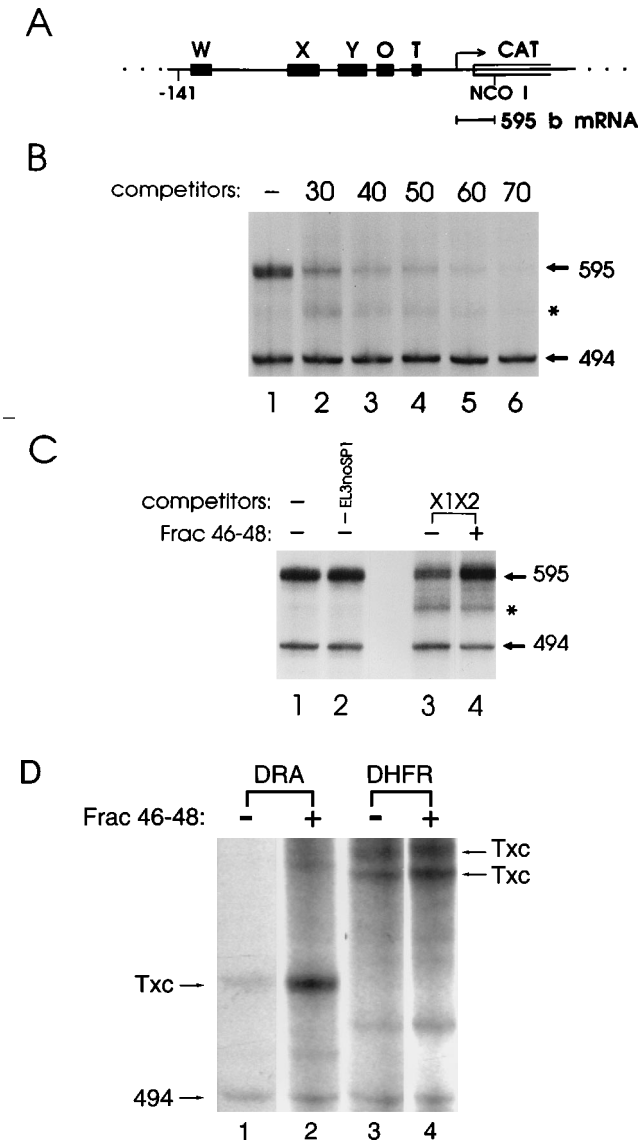

FIG. 5. Fractions 46 to 48 contain a DRA-specific transcriptional activator, TRAX1. (A) Map of DNA template 5′Δ-56SXY plasmid used in an in vitro transcription assay. 5′Δ-56SXY contains 141 bp of the DRA promoter including the TATA (T), octamer (O), Y, X, and W boxes cloned in front of the CAT gene of parent plasmid pD164-2 (4). b, base. (B) Titration of X1X2 cold competitors. Increasing amounts of double-stranded 45-bp X1X2 oligonucleotides (30- to 70-fold molar excess) were added to an in vitro transcription assay containing the template, 5′Δ-56SXY (see Materials and Methods). The upper arrow indicates a 595-base transcript initiated from the correct DRA transcriptional start site. This 595-base transcript was decreased by X1X2 cold competitors relative to an internal standard 494-base transcript (lower arrow). Lane 1, no competitor; lanes 2 to 6, reactions performed in the presence of 30, 40, 50, 60, and 70 molar excesses of cold 45-bp X1X2 oligonucleotides, respectively. (C) Fractions 46 to 48 reconstituted the transcriptional activity of DRA. Transcription reactions were supplemented with either 5 μl of the affinity column buffer containing 100 mM KCl plus 0.3 mg of BSA per ml (−) or fractions (Frac) 46 to 48 (+). The level of a DRA-dependent 595-base transcript (upper arrow) in each reaction was compared with the level of an internal standard 494-base transcript (lower arrow). Lane 1, no cold competitor was added; lane 2, a 50-fold molar excess of an irrelevant cold competitor oligonucleotide, ELSnoSp1, was added as a specificity control; lane 3, a 50-fold molar excess of X1X2 cold competitor was added to reduce the level of the 595-base transcripts; lane 4, a 50-fold molar excess of X1X2 cold competitor and 5 μl of fractions 46 to 48 were added to reconstitute the 595-base DRA-specific transcripts. The asterisks in panels B and C indicate an aberrant transcript that resulted from the depletion of factors with cold 45-bp X1X2 competitors. (D) Fractions 46 to 48 contained a DRA-specific transcriptional activator. In vitro transcription assays (see Materials and Methods) were performed in the absence of X1X2 cold competitor to detect the enhancement of DRA transcription by fractions 46 to 48. Under this condition, the DHFR- and DRA-driven templates were tested in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of fractions 46 to 48. The DHFR template was prepared by predigesting plasmid DHFR-239 with *Sca*I to produce 976- and 1,016-base transcripts (66). Densitometric tracings of the DRA- and DHFR-specific transcripts (arrows) were performed by NIH Image 1.47, and the results were normalized to the densitometric reading of the internal standard, the 494-base radiolabeled RNA. Txc, specific transcripts either from the DHFR (control)- or DRA (595-base)-specific template.

were observed in crude HeLa extract and TRAX1 (lanes 3 and 5). In the presence of both DNase and MNase, a major band of approximately 40 kDa appeared in crude HeLa extract as well as in TRAX1 (lanes 4 and 6). This size estimation includes protein and bound oligonucleotide that remained after nuclease treatment. The 40-kDa complex was efficiently abolished by a cold competitor that contained an intact X1 sequence (lane 7) but not one that contained a mutated X1 sequence (lane 8).

Another confirmation that TRAX1 is distinct from RFX1 or other known X1-sequence-binding proteins was derived from methylation interference analysis. This analysis was performed to identify the protected contact points involved in the formation of the TRAX1 complex (Fig. 7). Methylation at guanine residues and to a lesser extent adenine residues interferes with protein binding, and the band intensity is underrepresented in the population of the bound DNA and overrepresented in the population of free DNA. In agreement with the results of the gel mobility shift using X1M probes shown in Fig. 4, partial interference is located at −109 and −108 on the antisense strand and is absent on the sense strand. In addition, hypersensitive sites are observed at −114, −113, and −97 on the antisense strand. These hypersensitivity sites may be generated by an increased accessibility of dimethyl sulfate to the minor groove of DNA or local distortion in the three-dimensional structure of DNA around this region. This methylation interference profile of TRAX1 is different from previously published patterns for RFX, RFX1, NF-X, and NF-Xc. Together, these data indicate that TRAX1 is an X1-sequence-binding protein which has not been characterized previously.

DISCUSSION

Characterization of TRAX1. Using a combination of nonspecific and specific affinity columns, we have enriched TRAX1, a novel X1-sequence-binding protein, from HeLa nuclear extracts (Fig. 3 and 4). To determine its functional role in class II MHC gene regulation, TRAX1 was examined in an in vitro transcription assay and was determined to be a positive *trans*-acting factor of the DRA promoter (Fig. 5C and D). Analysis of TRAX1 by UV cross-linking showed that the molecular mass of TRAX1 is approximately 40 kDa (Fig. 6B). TRAX1 is distinct from RFX1, since an antiserum raised against recombinant RFX1 failed to block or supershift TRAX1 (Fig. 6A). In addition, the methylation interference pattern of TRAX1 is distinct from those of other known X1-sequence-binding proteins. TRAX1 interacts at the 5′ end of the X1 sequence (Fig. 7), and this methylation interference result is consistent with the specificity determined by EMSA (Fig. 4). Together, our findings indicate that TRAX1 is a novel X1-sequence-binding protein. This observation does not, however, exclude the possibility that TRAX1 is a subunit of RFX or other X-box-binding proteins.

The possible role of TRAX1 in HeLa cells. HeLa cells do not normally express class II molecules unless stimulated with cytokines such as IFN-γ (37), yet a DRA-specific transcriptional

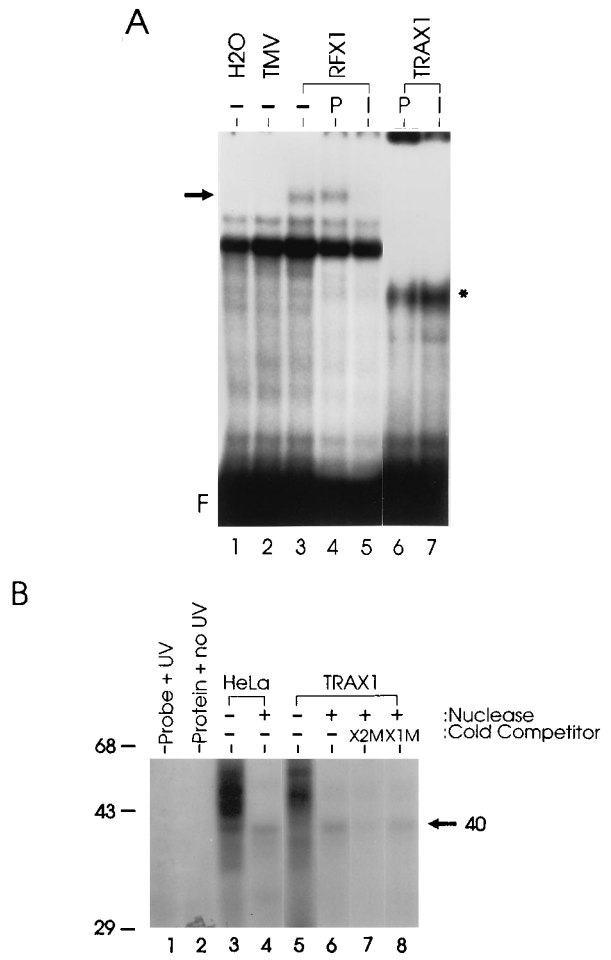

FIG. 6. (A) TRAX1 is not related to RFX1. Tobacco mosaic virus (TMV) RNA from Ambion Inc. (Austin, Tex.) and in vitro-transcribed RFX1 RNA were translated in vitro, using a Promega translation system. In vitro-translated proteins were then incubated with an X1X2 oligonucleotide and analyzed by EMSA. Lane 1, in vitro translation mix with no RNA added; lane 2, in vitro-translated TMV protein; lane 3, in vitro-translated RFX1; lane 4, in vitro-translated RFX1 incubated with preimmune serum (P); lane 5, in vitro-translated RFX1 incubated with anti-RFX1 antibody (I); lane 6, TRAX1 incubated with preimmune serum (P); lane 7, TRAX1 incubated with anti-RFX1 antibody (I). The arrow indicates the RFX1 complex, and the asterisk indicates the TRAX1 complex. F, free probes. The two bands below the RFX1 complex were detected in the negative controls that had H_2O or TMV mRNA and are inherent on the in vitro translation mixture. (B) TRAX1 is approximately 40 kDa. TRAX1 was UV cross-linked to the bromodeoxyuridine-substituted ^{32}P-labeled X1X2. The complex was then digested with nucleases, and the molecular weight of TRAX1 was visualized on an SDS–10% polyacrylamide gel. Lane 1, X1X2 probe subjected to UV cross-linking without added proteins; lane 2, proteins without UV cross-linking; lane 3, UV cross-linked crude HeLa nuclear extract; lane 4, UV cross-linked crude HeLa nuclear extract digested with DNase and MNase; lane 5, UV cross-linked TRAX1; lane 6, UV cross-linked TRAX1 digested with DNase and MNase; lane 7, UV cross-linked TRAX1 in the presence of X2M cold competitor followed by digestion with DNase and MNase; lane 8, UV cross-linked TRAX1 in the presence of X1M cold competitor followed by digestion with DNase and MNase. Positions of molecular mass markers (in kilodaltons) are at the left; the estimated molecular mass of TRAX1 is approximately 40 kDa.

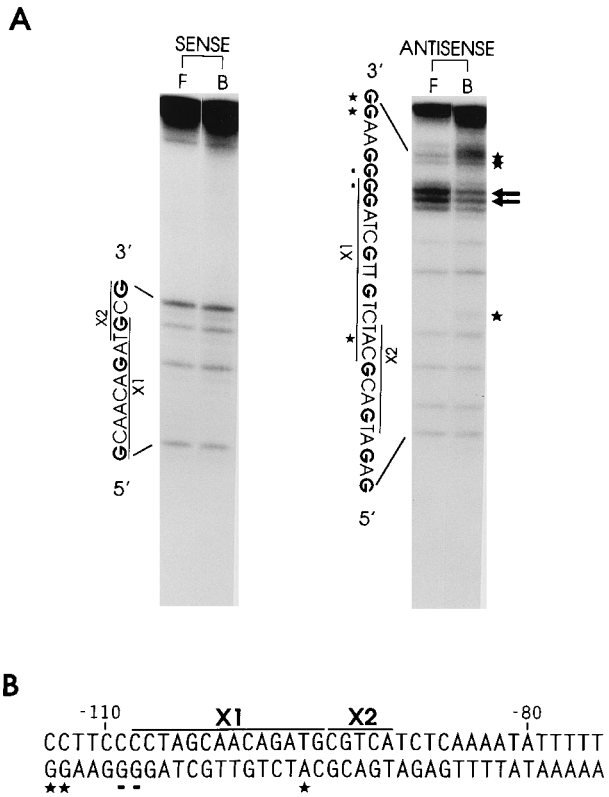

FIG. 7. Methylation interference analysis of TRAX1. (A) Methylation interference profile of TRAX1. Methylated 45-bp X1X2 oligonucleotide was used as a probe. Bound (B) and free (F) DNAs were separated on a nondenaturing polyacrylamide gel, isolated, purified, and cleaved with piperidine. The cleaved DNAs were then analyzed on a 12% sequencing gel. Arrows indicate residues at which methylation specifically interfered with protein binding; stars indicate residues which are hypersensitive. (B) Summary of the interference pattern of TRAX1. The locations of the X1 and X2 sequences, as well as the numbers of nucleotides relative to the cap site, are indicated. The dots indicate G residues at which methylation specifically interferes with protein binding, and the stars indicate residues which are enhanced.

activator, TRAX1, was affinity enriched from unstimulated HeLa cells. The in vivo footprinting analyses with IFN-γ-stimulated U373-MG (73) and HeLa cells (37) demonstrated that the X element of DRA was loaded with a protein in unstimulated HeLa cells but the protein loading to the X element became progressively stronger over time when the cells were stimulated with IFN-γ. These in vivo footprinting data suggest a model wherein the IFN-γ treatment results in a stronger and productive binding of TRAX1 to the X element, possibly through the interaction between preexisting TRAX1 and an IFN-γ-induced protein or the release of a repressor such as YB-1 or both (69). The formation of this productive interaction thus results in the production of DRA gene expression. Similarly, the requirement of an additional cellular factor for the formation of productive E47 and MyoD heterodimerization has been reported (67).

Importance of chromatin structure for cell-specific expression of class II. Although we have performed only limited analysis of TRAX1, TRAX1 appears to be expressed by cells that express DRA (Raji and Namalwa) as well as nonexpressors such as unstimulated HeLa cells. Most of the other X- and Y-element-binding proteins which have been described so far are ubiquitously expressed in cells as well. While the search for factors which may explain the tissue specificity of class II MHC gene expression has not revealed many such factors, there are two lines of evidence which may explain how ubiquitously expressed proteins may play a critical role in the proper coordinate regulation and in tissue-specific or inducible expression of the class II MHC genes. First, in vivo footprinting analysis shows that in some class II-negative mutant cell lines, the expression of class II MHC genes may depend on the accessi-

bility of the promoter by transcription factors. In the DR-negative Nacera, Ramia, BCH, and 6.6.1 cell lines, the DRA promoter lacks in vivo protein binding; however, integration of this promoter in another chromosomal site resulted in altered accessibility of the promoter and binding of proteins (35, 36). Second, a significant body of data suggests that chromatin structure plays an important role in gene activity and that the presence of core histone octamer and histone H1 can repress transcription factors from binding to a promoter (1, 21, 22, 39, 42). Combining these observations leads to a model wherein transcription factors compete with histone molecules for binding to the class II MHC promoters. The tissue-specific and coordinate regulation of MHC class II expression may rely on activators similar to glucocorticoid receptor (8, 51, 52), POH4 and POH2 in yeast cells (57), and GA14-VP16 (33), as a few examples, which can open the chromatin structure. Once the chromatin structure is opened by the activators, the class II transcription factors gain access to the promoters, which results in the activation of the class II MHC genes. According to this model, the defect in these DR-negative cell lines may depend upon activators which can open the chromatin structure. To test this hypothesis, TRAX1 and/or any other proteins specific for the class II MHC promoter can be examined to determine whether they can replace nucleosomes, using an in vitro transcription assay reconstituted with chromatin templates which contain physiological spacing of nucleosomes.

The newly described CIITA (MHC class II transactivator) demonstrates yet another level of control on MHC class II gene expression (64, 65). CIITA has been shown to fully restore HLA class II expression in RJ2.25 and BLS-2 cells without directly binding to DNA. RJ2.25 and BLS-2 are DR-negative cell lines with normal occupancy of transcription factors shown by in vivo footprinting. Presently, it is unclear how CIITA regulates MHC class II gene expression except that CIITA expression is correlated with and precedes class II MHC gene expression in IFN-γ-treated cells (13, 65). It will be interesting to test the effect of CIITA in TRAX1-induced class II MHC gene expression and the interaction between TRAX1 and CIITA.

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ADDENDUM

During revision of the manuscript, RFX purified from human cells was shown by Durand et al. to contain a 36-kDa DNA-binding subunit (20a).

ADDENDUM IN PROOF

Since the acceptance of the manuscript, another paper on CIITA and IFN-γ induction has been published (C.-H. Chang, J. D. Fontes, M. Peterlin, and R. A. Flavell, J. Exp. Med. **180:**1367–1374, 1994).

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