A Lipopolysaccharide-Induced DNA-Binding Protein for a Class II Gene in B Cells Is Distinct from NF- κ B

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Class II (Ia) major histocompatibility complex molecules are cell surface proteins normally expressed by a limited subset of cells of the immune system. These molecules regulate the activation of T cells and are required for the presentation of antigens and the initiation of immune responses. The expression of Ia in B cells is determined by both the developmental stage of the B cell and by certain external stimuli. It has been demonstrated previously that treatment of B cells with lipopolysaccharide (LPS) results in increased surface expression of Ia protein. However, we have confirmed that LPS treatment results in a significant decrease in mRNA encoding the Ia proteins which persists for at least 18 h. Within the upstream regulatory region of A_{α}^k , an NF- κ B-like binding site is present. We have identified an LPS-induced DNA-binding protein in extracts from athymic mice whose spleens consist predominantly of B cells. Binding activity is present in low levels in unstimulated spleen cells and is increased by LPS treatment. This protein binds to two sites in a regulatory region of the Ia A_{α}^k gene, one of which contains the NF- κ B-like binding site. DNA fragments containing these sites cross-compete for protein binding. Analysis by DNase I footprinting identified a target binding sequence, named the LPS-responsive element. Although this target sequence contains an NF- κ B-like binding site, competition with a mutant oligonucleotide demonstrated that bases critical for NF- κ B binding are not required for binding of the LPS-inducible protein. Therefore, we hypothesize that this inducible protein represents a new mediator of LPS action, distinct from NF- κ B, and may be one mechanism to account for the decrease in mRNA encoding the Ia proteins.

The class II (Ia) molecules are a family of cell surface glycoproteins encoded in the I subregion of the major histocompatibility complex (15) on murine chromosome 17. This subregion encodes four class II genes, A_{α} , A_{β} , E_{α} , and E_{β} , which are coexpressed. The cell surface proteins, I-A and I-E, are the products of noncovalent pairing of the α and β subunits. These proteins play a critical role in antigen presentation and immune-cell interactions (36). Increased levels of cell surface Ia on B cells increase the ability of these cells to present antigens to T lymphocytes (20). Expression of I-subregion proteins (Ia) and immunoglobulins in B cells is developmentally regulated. As B cells mature from pre-B to mature B cells, immunoglobulin synthesis is initiated and cells express specific immunoglobulin. These cells may differentiate further into antibody-producing plasma cells. Ia antigen is expressed as pre-B cells differentiate, persists in mature B cells, and is lost upon terminal differentiation to antibody-secreting plasma cells (18, 31). In addition, external stimuli, such as interleukin-4 (IL-4) (26) and antibodies to B-cell-differentiation antigens including surface immunoglobulin M (22) and immunoglobulin D (17), increase the level of expression of class II antigens on B cells. Although a number of stimuli have been shown to increase the expression of B-cell surface Ia, few signals have been shown to decrease its expression. Recently, however, corticosteroids were shown to reduce the expression of surface Ia on resting and activated murine B cells (21).

Lipopolysaccharide (LPS) is an integral component of the outer membrane of gram-negative bacteria and is a powerful B-cell mitogen. Approximately 30% of murine B cells respond to LPS by becoming blasts and secreting immunoglobulin (24). This program of immunoglobulin gene activation

may be mediated in part by the protein NF- κ B, which is involved in transcription of the κ -chain gene. The nuclear localization and binding of NF- κ B to a transcriptional motif within the κ light chain enhancer are induced by LPS in pre-B cells (4, 37, 38). Previous data from our laboratory obtained by using a panel of transformed pre-B cell lines suggested that class II major histocompatibility complex and immunoglobulin gene expression may be controlled by distinct pathways in early B cells (30). We therefore undertook the study of the effects of LPS on one of the class II major histocompatibility complex genes, A_{α} . Some information on the effect of LPS treatment on class II expression in B cells is available. Studies of the surface expression of Ia antigens in B cells after treatment with LPS has shown an apparent increase in surface expression which has been most convincingly documented at 36 to 64 h after treatment (9, 23, 41). However, it has also been demonstrated that LPS treatment leads to an early and significant decrease in levels of mRNA for A_{α} and A_{β} in murine spleen cells by 12 h (33).

The molecular mechanisms responsible for these effects are unknown. To study the early effects of LPS on class II gene expression and to compare these with the effects of LPS on immunoglobulin expression, the upstream region of the class II A_{α} gene was examined. This 1.25-kilobase (kb) region has previously been shown by us to be active in *cis* in murine B cells in a tissue-specific manner (5). Highly conserved transcriptional motifs called X and Y are present upstream of all class II genes sequenced to date. The conserved region containing these elements is found within bases -50 to -150 relative to the AUG initiator codon. The X motif is conserved and lies 19 to 21 base pairs (bp) upstream from the Y motif. Studies with transgenic mice (11) have shown that the X and Y motifs are essential *cis*-acting elements for the transcription of class II genes. They are

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required for the accurate initiation of transcription *in vivo* and bind sequence-specific proteins. However, these sequences alone cannot account for the *cis*-activating effect of the 1.25-kb A α upstream region (5, 6).

In this report, we demonstrate the presence of an LPS-inducible DNA-binding protein in nuclear extracts prepared from athymic (*nu/nu*) mouse splenocytes which binds to A α sequences upstream of the X and Y motifs. Northern (RNA) blot analysis of splenic RNA confirmed previous dot blot analyses that LPS exposure results in a rapid reduction in A α mRNA which persists throughout the 18 h culture period. Within the culture period, there was increased binding of the LPS-inducible protein to two sites in the upstream regulatory region of the A α gene. Binding to each site was specifically inhibited by each of the two DNA fragments containing these sequences. A target sequence for binding was determined by DNase I footprint analysis, and an oligonucleotide corresponding to this region prevented binding. Although an NF- κ B-like binding site is present within this target sequence, we have demonstrated that this site is not necessary for binding and that the true NF- κ B target sequence does not inhibit binding. Thus, the LPS-inducible protein which binds to the regulatory region of the class II A α gene is not NF- κ B.

MATERIALS AND METHODS

Cells and culture. Outbred *nu/nu* mice, 4 to 6 weeks old (obtained from R. Sedlacek, Massachusetts General Hospital, Boston), were killed by CO₂ asphyxiation. Spleens were harvested sterilely and crushed in complete media consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (catalog no. A-1111-L, lot no. 1111815; Hyclone Laboratories Inc.), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Whittaker M. A. Bio-products), 2 mM glutamine, 0.1 mM 2-mercaptoethanol, and 100 U of penicillin per ml and 100 μ g of streptomycin per ml (both from GIBCO Diagnostics). Hyclone serum and HEPES used in the complete media have negligible levels of LPS by assay. Media were tested by Chris Lu, Department of Medicine, Brigham and Women's Hospital, Boston, Mass., via the *Limulus* lysate assay (Cape Cod LEL Endotoxin Kit; Cape Cod Associates) and found to contain <0.32 ng of LPS per ml. Erythrocytes were lysed, and cells were washed with complete media, centrifuged, and suspended in complete media at a density of 2.5×10^6 /ml. For LPS stimulation, cell preparations were divided in half and cultured overnight in media alone or in media containing *Escherichia coli* LPS (Sigma Chemical Co.) to a final concentration of 50 μ g/ml. Cells were harvested for nuclear extract preparation after 18 h of overnight culture. Fluorescence-activated cell sorter analysis was performed on cells first incubated with a rat anti-Ia monoclonal antibody, M5/114 (14), followed by incubation with a fluorescein-conjugated goat anti-rat immunoglobulin G reagent to assess stimulation of surface Ia with a FACS analyzer (Becton Dickinson and Co.) as previously described (31). The M5/114 monoclonal antibody detects polymorphic determinants on spleen cells from mice of *H-2* haplotypes *b*, *d*, *q*, and *k*.

Nuclear extracts. Cells were harvested and washed in phosphate-buffered saline. Nuclear extracts (10) were prepared using 2×10^8 to 8×10^8 cells by a modification of the method of Dignam et al. as described elsewhere (6), except that the final salt concentration was adjusted to 0.5 M to allow for lysis for 45 min, centrifugation was at $150,000 \times g$ for 30 min, and 3 to 4 volumes of buffer C (20 mM HEPES

[pH 7.9], 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 25% glycerol [vol/vol]) per estimated cell volume was used.

DNA fragment and probe preparation. DNA fragments were prepared from a *Bg*/II-*Sac*I upstream fragment of the I-A α ^k gene extending 1.9 kb upstream of exon I and cloned in the *Bam*HI and *Sac*I sites of pUC18. The techniques used for fragment preparation, restriction digests, and gel sizing and separation were as described elsewhere (6). Probe DNA was 5'-end labeled after treatment with calf alkaline phosphatase by T4 polynucleotide kinase in the presence of [γ -³²P]ATP (3,000 to 6,000 Ci/mmol; Dupont, NEN Research Products). 3'-End labeling was performed with the Klenow fragment of DNA polymerase I in the presence of [α -³²P]ATP (3,000 Ci/mmol; NEN) under standard conditions. Labeled DNA was extracted sequentially with phenol-chloroform (1:1) and chloroform and separated on 5% polyacrylamide gels in 1 $\times$ Tris-borate-EDTA. Unlabeled DNA fragments were used in competition experiments.

Oligonucleotides were synthesized on a synthesizer (Bio-search) by standard phosphoramidite techniques. Overnight deprotection for 8 to 12 h was accomplished with fresh ammonium hydroxide at 55°C. After being vacuum dried and washed in sterile distilled water, 100 μ g of single-stranded DNA was loaded on an 8 M urea denaturing gel (12% polyacrylamide). DNA bands were visualized by UV shadowing, cut and eluted overnight in 0.5 M NaCl–10 mM MgCl₂–10 mM Tris hydrochloride (pH 7.6), and ethanol precipitated. Complementary strands were annealed in 0.6 M NaCl–20 mM Tris hydrochloride (pH 7.6)–1 mM EDTA by heating them to 95°C for 5 min, placing them in 65°C for 5 min, and cooling them to room temperature over 3 to 4 h. Annealed oligonucleotides were ethanol precipitated in the presence of 10 mM MgCl₂. For Northern blot hybridization, probes were an agarose gel-purified cDNA fragment of 1.0 kb for A α and a 1.1-kb fragment of A50 cDNA (25). These probes were labeled by nick translation (34) with [α -³²P]dCTP (3,000 Ci/mmol; NEN).

DNA-binding (mobility shift) assays. DNA-binding assays were performed as described elsewhere (6). Nuclear extracts were incubated with equal volumes of sterile water or competitor DNA at room temperature for 5 min. The remainder of the reaction components, including labeled fragments, were then added, with optimal poly(dI-dC) · (dI-dC) concentration being 0.33 mg/ml in a 12- μ l reaction, and incubated for 20 min. Samples were run on 4% polyacrylamide gel electrophoresis in 45 mM Tris-borate–1 mM EDTA at 200 V with cooling. Gels were then dried under vacuum, and autoradiography was performed. Nuclear extracts were normalized for protein concentration, and equal amounts of protein, usually 0.5 to 1.5 μ g, were added within experiments.

DNase I footprint analysis. DNA-binding reactions were performed as described above but were proportionally scaled up to a 25- to 30- μ l volume in each of five lanes. Optimal DNase I concentration was determined by concentration curves and was found to be 2 μ g/ml for the fragment II probes. Fragments were labeled at the 3' and 5' ends in separate experiments, and 500,000 Cerenkov cpm was used per total reaction. After standard incubation to allow binding, 3 μ l of 50 mM MgCl₂–50 mM CaCl₂ was added, with the subsequent addition of 37°C-activated DNase I. Samples were incubated for 90 s at room temperature, quenched with EDTA and EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] to 5 mM each and subjected to 4% polyacrylamide gel electrophoresis in 45 mM Tris-bo-

FIG. 1. FACS analysis of whole-spleen preparations from athymic mice with and without exposure to LPS at 50 μ g/ml. Control antibody is fluorescein-conjugated goat anti-rat immunoglobulin G. Anti-Ia antibody was M5/114, a monoclonal rat anti-Ia antibody (14). Cell surface staining was performed after 18 h of culture. Experiment 1 shows no induction of surface Ia with LPS exposure, as was seen in two of seven experiments. Experiment 2 shows a modest induction of surface Ia, as was seen in five of seven experiments.

rate-1 mM EDTA. Free and bound bands, identified by autoradiography of wet gels, were cut from the gels and eluted by diffusion. DNA was purified on Elutip columns (Schleicher & Schuell) and ethanol precipitated. Products of DNase I digestion were resolved on 6% polyacrylamide-8 M urea linear and wedge sequencing gels. An equal number of radioactive counts were loaded in each free and bound lane. Maxam-Gilbert ladders of unreacted probe were run simultaneously in order to identify sequences (13).

RNA analysis. In timed experiments, all cultures were maintained for a total of 18 h, regardless of treatment. Total cellular RNA was harvested from *nu/nu* mouse spleen cells by the guanidine isothiocyanate-cesium chloride density gradient method of Chirgwin et al. (8). Agarose gels (1.3%) containing 0.22 M formaldehyde were used for size fractionations. RNA was transferred in 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to Nytran filters as described elsewhere (8). Northern blots were prehybridized for 2 h at 42°C in 48% formamide-5 $\times$ SSC-10 mM Tris-0.1% sodium dodecyl sulfate-1 $\times$ Denhardt solution-10% dextran sulfate-100 μ g of denatured sheared herring sperm DNA per ml. Hybridization was for 36 h under identical conditions with the addition of 100 μ g of herring sperm DNA and 1 $\times$ 10⁶ to 3 $\times$ 10⁶ cpm of nick-translated cDNA probe per ml of final volume. Duplicate filters were hybridized to either A α probe or A50 probe. Filters were washed twice for 30 min each in 2 $\times$ SSC-0.1% sodium dodecyl sulfate at room temperature and then twice for 30 min each at 65°C in 0.2 $\times$ SSC-0.1% sodium dodecyl sulfate. Autoradiography was performed on wet filters. Filters were then washed under conditions to remove bound probe and rehybridized to the second probe.

RESULTS

LPS treatment minimally affects surface Ia expression and decreases level of Ia mRNA at early time points. Figure 1 shows representative results of FACS analysis experiments on expression of surface Ia antigens in whole-spleen preparations from athymic mice with and without LPS exposure at 18 h. These mice are maintained through life in a germfree

FIG. 2. Northern blot analysis of the time course of LPS effect on A α mRNA. Total cellular RNA was harvested from splenic lymphocytes of athymic mice after being cultured with 50 μ g/ml of LPS for 1 h (lanes 2 and 7), 4 h (lanes 3 and 8), 9 h (lanes 4 and 9), and 18 h (lanes 5 and 10). Total culture time for all samples was 18 h. Unstimulated spleen cells cultured for 18 h (lanes 1 and 6) served as control. Total RNA (10 μ g per lane, as measured by optical density at 260 nm) was used to prepare duplicate Northern blots which were hybridized to either A α probe or A50 probe. Rehybridization of the same filters with the second probe confirmed these results.

environment and thus are protected at baseline from exposure to endotoxin. In addition, athymic mice are T-cell deficient, and splenic lymphocytes are thus predominantly B cells. Flasks stimulated with LPS (50 μ g/ml) were examined microscopically after 18 h, and blast formation was present in all experiments. Although other investigators have shown an early two- to threefold increase in surface Ia antigen expression after treatment with LPS (9), we have seen a very modest increase in surface Ia expression at 18 h in five of seven experiments and no increase in expression in two experiments. One experiment from each group is shown. Since the FACS data are not normalized for the increase in cell surface area produced by LPS treatment, these data suggest that at early time points of stimulation, LPS is not a positive regulator of Ia expression.

Dot blot analysis of spleen cells treated with LPS (25 μ g/ml) for up to 12 h has suggested that such treatment leads to a decrease in mRNA encoding Ia proteins (33). We performed Northern blot analyses of total RNA from stimulated and unstimulated spleen cells (Fig. 2). RNA was prepared from cells incubated with LPS for 1, 4, 9, and 18 h. Filters were probed with A α cDNA and A50 cDNA probes. A50 contains sequences for a constitutively expressed mRNA (25) and serves as a control for sample concentration. Northern blot analysis demonstrated that treatment with LPS reduces the level of mature A α mRNA in comparison with unstimulated controls. Decrease in mRNA began by 1 h (Fig. 2, lane 2), continued at 4 h, and was maximal by 9 h. At 18 h, mRNA levels remained substantially reduced in comparison with unstimulated controls. Densitometry demonstrated a reduction in A α mRNA from baseline to 52% of baseline at 1 h, 23% of baseline at 4 h, 19% of baseline at 9 h, and 22% of baseline at 18 h. Levels of both mature A α mRNA and a larger presumed A α precursor mRNA were reduced. Hybridization of a duplicate filter and rehybridization of the same filter to the A50 cDNA probe indicate that A50 mRNA concentrations were comparable among samples and did not bias the results. In addition, levels of A50 mRNA

have remained constant with LPS stimulation. We conclude that the level of $A\alpha$ mRNA decreased with LPS treatment.

An LPS-inducible DNA-binding protein binds to an $A\alpha$ upstream sequence containing an NF- κ B-like binding site. To begin to examine the mechanism of LPS effects on class II gene expression, we sequenced 1.23 kb of DNA of the upstream regulatory region of $A\alpha^k$. Figure 3 shows this sequence, with the sequencing strategy shown below. The X and Y consensus motifs are noted just upstream of the ATG start codon. A reverse CCAAT box motif is also demarcated within the Y box region. Of note is the absence of a TATA box, as has been described in the upstream regions of other genes (7). A DNA-binding protein whose binding activity is influenced both by IL-4 and by differentiation in B cells has been described (5). The DNA sequences bound by this protein, BRE-1 and BRE-2, are noted. Of interest is that just upstream of BRE-2 is the sequence AAGGGAATTTTCC (Fig. 3A), which has a 10/12 sequence homology to the known binding site of NF- κ B, an LPS-inducible DNA-binding protein specific for the immunoglobulin κ gene enhancer and regulatory for κ light chain transcription in B cells (37, 38).

Since the control of gene transcription is usually mediated by *trans*-acting transcriptional factors which bind to upstream regulatory elements, we assayed nuclear extracts from unstimulated and LPS-stimulated splenic preparations from athymic mice for binding to seven DNA fragments prepared by *Hinf*I digestion of 1.9 kb from the upstream regulatory region of $A\alpha^k$ (Fig. 4A). Equal portions of unstimulated and stimulated extracts were added to each radiolabeled *Hinf*I fragment and assayed for binding activity by mobility shift assays (Fig. 4B). All fragments were assayed, including fragment IV, which contains the X-Y consensus sequence region. Binding to fragment IV occurs in both unstimulated and stimulated extracts (Fig. 4B, lanes 11 and 12) and represents constitutive binding to the X box and Y box motifs (6). This binding activity is equivalent in induced and uninduced spleen cells and serves as a control for protein concentration. Retarded bands representing DNA-protein complexes were present in LPS-induced extracts when restriction fragments I and II (Fig. 4B, lanes 3 and 6) were assayed with splenic nuclear extract. Binding activity was seen at low level in the uninduced extracts (Fig. 4B, lanes 2 and 5) but was present in much greater amounts in LPS-induced extracts. Treatment of extract with proteinase K abolished the DNA-protein complex (results not shown). These results demonstrate the presence of an LPS-inducible DNA-binding protein which binds to two sites in the upstream regulatory region of $A\alpha^k$.

To demonstrate specificity of protein binding to these $A\alpha^k$ regions, unlabeled competitor fragments were prepared from cloned, truncated subfragments of fragments I and II: I(t) and II(t) (Fig. 4A) (5). Mobility shift assays were performed after a 5-min preincubation with excess unlabeled competitor DNA (Fig. 5). Unlabeled fragment I(t) prevented the formation of a retarded complex when incubated with nuclear extract and radiolabeled fragment I (Fig. 5, lanes 3 and 11). Unlabeled unrelated competitor fragments did not prevent binding (Fig. 5, lanes 5 and 13), demonstrating that the protein binding is specific for fragment I. Unlabeled II(t) also prevented protein binding to radiolabeled fragment II (Fig. 5, lanes 8 and 16), but unrelated competitor DNA fragments did not (lanes 9 and 17). In addition, cross-competition occurred when unlabeled II(t) was used as a competitor for binding to fragment I (Fig. 5, lane 12) and when I(t) was used as competitor for binding to fragment II (lane 15). Because

FIG. 3. (A) Sequence of 1.23 kb of DNA of the upstream regulatory region of $A\alpha^k$. *Hinf*I restriction sites are noted by vertical bars. The target sequence of the LPS-induced DNA-binding protein is designated by the cross-hatched bar. The target sequences of the previously described (5) IL-4-responsive elements (BRE-1 and BRE-2) are designated by open bars. The NF- κ B-like sequence is starred. X and Y boxes are indicated. Note that there is a reverse CCAAT box motif located within the Y box, but there is no TATA box in the upstream sequence. Numbering is arbitrarily designated as 0 through 1230, since the actual site of initiation of transcription has not been determined. (B) Sequencing strategy. Both strands were sequenced by the Sanger dideoxy method with some overlap from Maxam-Gilbert ladders. This sequence has been submitted to GenBank and assigned accession number M24602.

FIG. 4. (A) Schematic representation of the *HinfI* restriction digest of the upstream region of *A α* . *HinfI* sites are marked with vertical hatches. Fragments are numbered according to size. Solid boxes identify the conserved X and Y box consensus sequences within fragment IV and the translation initiation of exon I leader peptide to the right. I(t) and II(t) denote the positions of cloned truncated subfragments of fragments I and II. (B) Mobility shift assays of unstimulated and LPS-stimulated extracts to fragments I through VII. Unstimulated (–) and LPS-stimulated (+) extracts are shown in pairs. Equal portions of extract were added to radiolabeled *HinfI* fragments from the upstream region of the *A α* ^k gene. Lanes 1, 4, 7, 10, 13, 16, and 19 contain no added nuclear extract. Retarded bands are present when LPS-induced extracts are incubated with fragments I and II (lanes 3 and 6) but not when they are incubated with other fragments. Binding to fragment IV (lanes 11 and 12) represents the constitutive X and Y box binding-protein activity which is present in all splenic nuclear extracts and serves as a control for protein concentration.

these cross-competition data implied that the same protein(s) binds to fragments I and II, and because the closest match to an NF- κ B target sequence was present in the fragment II DNA, we chose to focus on this fragment to identify the region of protein binding.

A target sequence of the LPS-inducible binding protein is identified. To determine the target sequence of the LPS-induced binding protein, we performed DNase I footprint analysis (13) using the fragment II(t) subfragment of fragment II. Fragment II(t) was labeled on both the coding and noncoding strands in separate experiments. DNA-protein complexes were formed and subjected to DNase I digestion. Radiolabeled bands representing free and complexed DNA were cut from the gel, eluted, and run in parallel on denaturing polyacrylamide gels. One distinct region of altered

FIG. 5. Competition and cross-competition experiments. LPS-induced nuclear extracts were incubated with radiolabeled fragment I (lanes 2 through 5 and 10 through 13) and fragment II (lanes 7 through 9 and 14 through 17). Lanes 1 and 6 contain unstimulated nuclear extract. Lanes 2, 7, 10, and 14 contain LPS-stimulated extract without competitor. Competitor fragments are I(t) (lanes 3, 11, and 15), 1.9-kb upstream fragment (lane 4), dimerized *PstI* fragment from fragment IV (lanes 5 and 9), fragment II(t) (lanes 8, 12, and 16), *A α* X box plus flanking sequence (44 bp) (lane 13), and randomly scrambled sequence of 27 bp from DR α X box (X' box) (lane 17). All competitor fragments were added in molar excess at 50 ng.

reactivity was consistently identified in fragment II(t) between free and protein-bound DNA in separate experiments (Fig. 6A). Binding of the LPS-induced protein to the non-coding strand produced three distinct hypersensitive sites. In the complementary region of the coding strand, binding of the LPS-induced protein led to a modest decrease in reactivity in the central portion of this complementary region. This decrease was seen reproducibly in three separate experiments. We have designated this region the LPS-responsive element (LRE). Additional attempts to obtain footprints by using the nuclease activity of 1,10-phenanthroline-copper ion (19) revealed no altered reactivity of bases in bound compared with free DNA (results not shown). Of interest is that in two of the three experiments with DNase I digestion, modest protection was also seen on the coding strand in the previously described B-cell-responsive element (BRE-2) (Fig. 6). This region, BRE-2 (5), represents a target sequence of a previously described IL-4-inducible DNA-binding protein, active at low levels in nuclear extracts from untreated spleen cells. The relative locations of the binding sites of the LPS-inducible and IL-4-inducible DNA-binding proteins are shown in Fig. 6B.

FIG. 8. Binding competition assay with NF-κB target sequence and oligonucleotides. (A) Oligonucleotide sequences used in competition assays. Note that the mutant LRE oligonucleotide has mutations in three key G residues. (B) Lane 1, Radiolabeled fragment II probe alone; lanes 2 through 11, LPS-induced nuclear extracts without (lane 2) and with (lanes 3 through 11) competitor. Lanes 3 through 5, True NF-κB target sequence at 25, 50, and 100 ng, respectively; lanes 6 through 8, mutant oligonucleotide spanning the LRE; lane 9, true LRE; lane 10, 27-bp oligonucleotide comprising the randomly scrambled sequence from the X box of DRα (X' box); lane 11, a second unrelated oligonucleotide spanning the DQB X box.

effects of LPS on expression of class II (Ia) antigen in cells of the B lineage.

The final effect of LPS-treatment of B cells on the surface expression of Ia antigens is undoubtedly complex. Several investigators have shown an increase in surface Ia expression after treatment with LPS. This effect appears to be cell cycle dependent and is probably most pronounced at 36 to 48 h (23), although some investigators have seen this effect as late as 64 to 84 h (41). In a purified B-cell population, Dennis et al. (9) have demonstrated an approximately twofold increase in surface Ia after treatment with LPS as early as 9 h. However, the increase in cell size and thus in cell surface area was not accounted for, raising the possibility that the number of Ia molecules per unit surface area was not increased at this early time point. The FACS data obtained in our laboratory demonstrate no substantial increase in surface Ia expression at 18 h. No increase in surface Ia expression was seen in two of seven experiments in splenic preparations from athymic mice, and only a modest increase was seen in five of seven experiments at 18 h. Furthermore, if the increase in surface area of LPS blasts is accounted for, there may be no significant change in the density of surface Ia antigen expression in whole spleens from athymic mice after 18 h of LPS stimulation. In contrast, IL-4 treatment of athymic mouse spleen preparations led to a significant increase in surface Ia expression in our hands (data not shown).

It is possible that some of this discrepancy is due to the difference in examining a purified B-cell population as op-

posed to a preparation of whole spleen as in our studies, although spleen cells from athymic mice are predominantly B cells. However, when steady-state mRNA levels for Aα were examined in these splenic preparations from athymic mice, a significant decrease in mRNA levels was seen by 4 h and was stable until at least 18 h. The level of mRNA for A50, a housekeeping gene, did not change with LPS treatment, arguing that the decrease in Aα mRNA is not an artifact of an increase in total mRNA. This had been previously demonstrated by dot blot analysis when mRNA levels for Aα and Aβ were examined in BALB/c spleen cells treated with LPS (33). In that study, a significant decrease in class II mRNA levels occurred by 5 h, and levels remained low at the last time point examined, 12 h. These data lead us to conclude that LPS may be a negative regulator of Ia expression in B cells at these early time points.

The regulation of class II gene steady-state mRNA in response to multiple stimuli has been shown to be controlled at the level of gene transcription (27, 32). Such transcriptional control is mediated by *cis*-acting DNA sequences and by transcription factors that bind to these sequences. In the instance of immunoglobulin κ-chain expression, some of these regulatory elements have been identified. In the pre-B cell line 70Z/3 the activation of transcription of the κ-chain gene by LPS is known to be mediated in part by the protein NF-κB, which binds to a transcriptional motif within the κ-chain enhancer (37). The DNA-binding activity of NF-κB is inducible by treatment with LPS (38). Sequence analysis of the 1.23 kb upstream of the Aα gene revealed the presence of an NF-κB-like binding site. Although an NF-κB-like binding sequence is present in the upstream region of the Eα gene, the Eα and immunoglobulin genes appear to rely on different factors to achieve B-cell-specific expression (12). We therefore chose to examine unstimulated and LPS-stimulated nuclear extracts from spleen preparations from athymic mice, which consist predominantly of B cells, in order to identify a nuclear factor analogous or identical to NF-κB which may bind to the upstream regulatory region of a class II (Ia) gene, Aα.

In the present report we demonstrate the presence of an LPS-inducible DNA-binding protein in nuclear extracts prepared from splenic preparations from athymic mice. This LPS-inducible protein binds to two fragments prepared from the upstream regulatory region of Aα^k, and these fragments cross-compete for binding. By using DNase I footprint analysis, a target binding sequence was identified in fragment II of the *Hinf*I digest of the upstream regulatory region of Aα^k. An oligonucleotide synthesized from this LRE successfully competes for binding to fragment II. Located within the target binding site of the LPS-inducible protein is a nucleotide sequence with a 10/12 homology to the binding site of the known LPS-inducible B-cell protein, NF-κB. Recent studies on transcription factors provide evidence that a factor which serves as an activator of gene expression can function as a repressor of gene expression when bound to a somewhat different target sequence (16). This raised the possibility that the LPS-inducible protein binding to be upstream region of Aα might be NF-κB even though NF-κB acts as a positive regulator in pre-B cells. However, an oligonucleotide containing the true NF-κB binding site was unable to compete for binding. In addition, three G residues which are essential for the binding of NF-κB have been identified (37). When the three G residues present in our NF-κB-like sequence were mutated from GGG to AAT, the mutated oligonucleotide was still able to prevent binding, suggesting that these G residues are not essential for the

binding of this protein. These data strongly suggest that this LPS-inducible protein is distinct from NF- κ B. Therefore, LPS leads to the induction of two distinct DNA-binding proteins in cells of the B lineage, one binding to a site in the κ -chain enhancer, NF- κ B, and acting as a positive regulator, and one binding upstream of an Ia gene, which we postulate may be a negative regulator. This provides further evidence for the hypothesis that B-cell expression of class II and immunoglobulin genes proceeds along distinct pathways.

Taken together, these data demonstrate that the LPS-inducible DNA-binding protein is distinct from NF- κ B and binds to A α upstream gene sequences. They further suggest that this LPS-inducible protein may be a factor in mediating the negative regulation of A α gene expression. Several examples of both *cis*-acting DNA elements and *trans*-acting factors which can repress gene transcription have already been described (35). An example of a *cis*-acting DNA element that exerts a negative regulatory effect on gene transcription is the DNA element in the upstream region of the HMG coenzyme A reductase gene, in which positive and negative regulatory elements are closely associated (28). The large T antigen of simian virus 40 is a *trans*-acting repressor which regulates the synthesis of virus-coded proteins by decreasing the rate of early-strand RNA synthesis (40). In addition, proteins which displace or prevent the binding of positive *trans*-acting factors have recently been elucidated. For example, a CCAAT displacement protein which is present only in nuclear extracts from embryos in which the sperm H2B gene is inactive has been described. This protein binds to sequences overlapping the proximal CCAAT element of the sperm H2B promoter and thus prevents the binding of the CCAAT-binding factor (3). Glucocorticoids have long been known to inhibit the transcription of several genes, including collagen and stromelysin (1, 42). It has now been demonstrated that the same glucocorticoid receptor known to activate gene expression can lead to negative regulation by interfering with the binding or activity of positively acting factors (1).

We have currently identified two target sequences for B-cell nuclear proteins within fragment II of a *Hinf*I digest of the 1.9-kb upstream regulatory region of the A α ^k gene. The LPS-inducible DNA-binding protein binds preferentially to one of these regions, designated LRE. The previously described IL-4-inducible DNA-binding protein binds to a second sequence called BRE-2 (5). These target sequences are separated by only 20 bp (Fig. 6B). We have preliminary data (E. Gravalles and L. Glimcher, unpublished results) demonstrating that the LRE sequence is able to prevent binding of the IL-4-induced binding protein to fragment II, and conversely, that BRE-2 is able to prevent binding of the LPS-induced protein to fragment II. In addition, in two experiments the region of DNase I protection delineated by the LPS-induced nuclear extract showed modest protection of the BRE-2 target site. This may represent constitutive binding of the IL-4-induced protein, which is present at baseline in unstimulated nuclear extracts (5). Alternatively, it may demonstrate the ability of the LPS-induced DNA-binding protein to bind to either the LRE or the BRE-2 site. One possible model for A α gene regulation would be that two inducible DNA-binding proteins result from treatment with either LPS or IL-4 and that each protein is able to bind to either the LRE or the BRE-2 site. The LPS-inducible DNA-binding protein binds preferentially to the LRE sequence, as indicated by its characteristic pattern of DNase I protection and hypersensitivity. It is possible that protein binding to this sequence prevents the binding of positive

regulatory elements such as the IL-4-inducible protein. The LPS-inducible DNA-binding protein could thus act as a negative regulator of A α gene expression. An alternative hypothesis is that a single protein is affected by both stimuli and binds preferentially to the LRE sequence only when affected by LPS. The target choice as well as the transcriptional effect would then depend upon the original external stimulus. Further studies are in progress to explore these possibilities.

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