The NF-κB and NF-IL6 elements have previously been shown to play an important role in regulation of both the mouse and human interleukin-6 gene. Between these two elements lies a G/C-rich sequence, which contains three repeats of the element CCACC, protein binding to which has not been previously characterized. In this study we demonstrate that the transcription factor Sp1 binds to these repeats and plays an important role in basal and in inducible expression of the murine interleukin-6 gene.

IL-6 is a cytokine with pleiomorphic biologic effects expressed in fibroblasts, monocytes/macrophages, endothelium, keratinocytes, and other mesenchymal and epithelial cells in response to a variety of noxious stimuli, including tumor necrosis factor-α, IL-1β, LPS, platelet-derived growth factor, and interferons (1). It is also produced constitutively in some lymphomas, sarcomas, and carcinomas and is elevated in patients with systemic bacterial infections (in patients with cardiac myxoma, rheumatoid arthritis, Castleman’s disease, mononuclear gammopathies, psoriasis, septic shock, and AIDS (2, 3)).

Isolation and analysis of human (4–6) and mouse (7) genomic IL-6 clones have revealed a highly similar structure of regulatory elements. These consist of a c-fos serum response-like element (8) shown to be the binding site for NF-IL6 (9), a cAMP response element binding protein site just upstream of the NF-IL6 site, and an NF-κB site (4–8, 10, 11). At a more upstream location are found putative multiresponse, glucocorticoid response, and ETS-responsive elements (12). Tanabe et al. (7) and Ray et al. (8) first noticed a GT-rich region lying between the NF-κB and NF-IL6 sites, which they felt may have homology to the fos retinoblastoma control element. However, no specific deletions or characterization of factor binding to this region were performed. Since then, there has been little further work on this region.

Studies with a wide variety of inducers of IL-6 (LPS, poly(I:C), phytohemagglutinin, and phorbol 12-myristate 13-acetate) have shown that induction correlates strongly with loading of NF-κB and that binding to this element in the IL-6 promoter is necessary for response to these agents (11). IL-1 and tumor necrosis factor (13) are probably the most potent cytokine up-modulators of IL-6 secretion in vivo and may be synergistic (14). Functionally significant up-modulation by IL-1 and tumor necrosis factor correlates with increased binding at the NF-IL6 site (15–17) and changes at the NF-κB site (18, 19).

We have previously demonstrated that mice expressing the HTLV-I tax gene develop fibroblastic tumors (20). These tumors show constitutive activation of the p50 and p65 subunits of NF-κB and constitutive activation of the IL-6 gene. Both IL-6 expression and tumorigenesis are highly dependent on NF-κB in this system since antisense inhibition of p65 profoundly affects both. By other approaches, we have demonstrated that IL-6 expression is an important autocrine growth factor for these transformed fibroblasts (21). In order to better understand transcriptional regulation of IL-6 we have performed promoter mapping and mutagenesis of the region immediately upstream of the NF-κB site. Here, we have identified Sp1 as prominently binding in this upstream region, which is functionally important in transcriptional regulation. Sp1 could serve as an important bridge in binding between NF-κB and C/EBP isoforms in the IL-6 promoter.

MATERIALS AND METHODS

Cell Lines and Nuclear Extracts—The fibroblastic B line cells were derived from LTR-tax transgenic mice (22). The B line tumor cells express high levels of Tax in the nucleus (23). In addition, all of these cells exhibit translocation of NF-κB p50 and p65 from the cytoplasm to the nucleus relative to nontransgenic fibroblasts.

B line cells were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. Antisense p50 and p65 treatments were as described previously (23). Treatments were at 40 μmol for 48 h.

Preparation and Treatment of Cell Lines for in Vivo Footprinting—Lysolecithin permeabilization of cells, DNase treatment, extraction of DNA, and IL-6 primer 1 extension were performed as described by Brown et al. (24). PCR reaction mixtures were prepared with the IL-6-2 primer using the hot start procedure. This was followed by 15 cycles of 94°C for 1 min, 68°C for 2 min, and 76°C for 3 min and then an additional step at 85°C for 5 min, during which time radiolabeled primer 3 was added. This was followed by 18 cycles in which the annealing temperature was raised to 72°C. The common linker and linker primer were the same as described previously (25). The following primers were complementary to the coding strand of the described sequences. The specific primers used for analysis of mouse IL-6 were: IL-6-1, 5′-GGAACTGCCTTCACTTAC-3′ (1347–1364 of GenBank locus MusIL6A, accession number M20572); (7); IL-6-2, 5′-GCAGAGAGGA-ACCTGATCGGGTTC-3′ (1319–1344); and IL-6-3, 5′-CTCTGCCT-TCTTGGTGGGCTCCAGAGCAGA-3′ (1281–1309). PCR products were separated on 6% denaturing polyacrylamide gels.

Electrophoretic Mobility Shift Assays (EMSA)—Gel shifts were performed as described previously (27). For supershift assays, 1 μl of anti-Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1 mg/ml affinity purified) or other high concentration antibodies (28) was pre-mixed with nuclear proteins for 20 min at room temperature, followed by the addition of the probe to the reaction mixture.

DNA Affinity Chromatography—CCACC binding protein was partially purified by phosphoelutose fractionation (P11, Whatman) before DNA affinity chromatography as described previously (28). We
have previously described the generation of affinity columns using double-stranded DNA coupled to bromoacetylagarose (29). The CCACC-containing oligonucleotide used for this experiment is shown in Fig. 1B. Efficiency of the affinity protein purification and its DNA binding activities were monitored by analysis of each eluted fraction using silver staining, EMSA, and Southwestern blotting.

Southwestern and Western Blot Analyses—The methods used were described previously (28). 6 \times 10^9 cpm of CCACC probe/ml plus 10 \mu g of poly(dI-dC)/ml were used in Southwestern blots. For Western blots, membranes were probed with an anti-Sp1 polyclonal antibody (PEP-2, Santa Cruz Biotechnology) followed by a horseradish peroxidase-conjugated secondary antibody. Visualization was by the ECL light system (Amersham Corp.).

PCR Generation of Luciferase Reporter Vector—Luciferase plasmids containing the IL-6 promoter with CCACC wild type or CCACC mutant were generated by cloning a proximal promoter-bracketing PCR product into the NheI and HindII sites of the pGL2-Basic Vector (Promega). The 5′-primer contained the NheI site (5′-TTGGATCTACGCGGTAGTGCA-3′) complementary to −179 → −158 of murine IL-6 promoter. The 3′-primer contained the HindII site (5′-TTGACTAGTTCGTG-3′) complementary to +34 → +12 of IL-6 promoter. The first round of PCR was performed using 100 pmol of each primer and 500 ng of mouse genomic DNA. After 30 cycles of amplification (94 °C × 30 s, 56 °C × 30 s, and 72 °C × 30 s), a 214-base pair fragment was recovered, which bracketed the downstream wild type promoter region of IL-6. For insertion of mutations, two directly adjacent but non-overlapping primers were synthesized. A 40-nucleotide internal primer 1 (5′-GGAGTTTGGGCTGAGCCAGGTGTTTGGGCTGATTGG-3′, −136 → −96) contained introduced mutations. Internal primer 2 (5′-AACCAGAGTTTATGACTCA-3′, −95 → −76) was just downstream of the CCACC region. These primers were paired with adjacent external primers, which allowed generation of two contiguous fragments that were ligated and cloned directionally. For a more detailed description of this cloning scheme see Ref. 56.

Transfections and Luciferase Assays—Wild type and mutant CCACC-containing plasmids were transfected into Balb cells using Lipofectamine (Life Technologies, Inc.). The ratio of DNA:lipofectamine was 1:4 (\mu g: \mu g). Amounts of DNA transfected are indicated in the figure legends. Forty-eight hours after transfection, cell extracts were prepared and luciferase activity was measured using the acetyl-CoA luciferase assay system (obtained from Promega). The level of luciferase expression was determined with a luminometer. Transfections were performed in duplicate or triplicate, and individual experiments were repeated. Derived ratios between mutant and wild type CCACC-CAT varied by less than 10% between experiments.

RESULTS

In Vivo Footprinting Suggests Cooperative Interactions with NF-κB and Prominent CCACC Element Binding—To understand transcription factor binding near the NF-κB region of the murine IL-6 promoter, we performed in vivo footprinting. This is shown in Fig. 1A. Previous studies have suggested that this band is the NF-κB site. As noted in the Introduction, this has not previously been appre-
associated as a strong site of transcription factor binding (see Ref. 11). It consisted of three repeats of the pentanucleotide sequence CCACC, similar to sequences previously shown to bind zinc finger proteins of the C2H2 type. The boundaries of these regions are shown schematically in Fig. 1B. The doubly underlined regions highlight the close proximity of the NF-IL6, CCACC, and NF-κB motifs. This region is highly conserved between mouse and human.

Gel Shift Analysis and Purification of CCACC Binding Factors—To obtain more detailed information about factors binding the CCACC sites, gel shift analyses were performed using short synthetic double-stranded probes bracketing the CCACC...
region. The synthesized native IL-6 CCACC probe is shown in Fig. 1B. Gel shift analysis using this probe and extracts from the murine B line (Fig. 2A, lane 1), shows strong specific binding of a complex at the top of the gel (indicated by arrow). Binding activity is predominantly nuclear (lanes 1 and 3 versus 2 and 4), does not vary significantly between tax-transfected B and Balb 3T3 cell lines (lane 1 versus 3), and is not inducible by phorbol or ionomycin (lanes 5–8). The complex is specifically competed by itself but not control sequences derived from the IL-6 or Ig regions (lanes 9–14). Enrichment by phosphocellulose fractionation shows elution of binding activity at 600 mM KCl (Fig. 2B, lanes 8 and 9), while the majority of total nuclear protein eluted at 400 mM KCl (Fig. 2B, lower panel). Gel binding activity was further enriched by passage across an affinity column composed of the CCACC probe. The column was prepared by rapid ligation to a bromoacetyl-agarose matrix (29). This approach has the advantage of defined terminal anchor-}

| Competitor | CACC | Sp1 | AP1 | AP2 |
|------------|------|-----|-----|-----|
| x 50       | x 100| x 100| x 50| x 100|
| x 50       | x 100| x 100| x 50| x 100|

Fig. 3. EMSA analysis using CCACC wild type probe. A, competition was done with cold CCACC probe itself; Sp1, AP1, and AP2 probes. Unlabeled CCACC could completely abolish the complexes, but the SV40 Sp1 could only compete the upper complex. AP2 had a partial effect, and AP1 did not compete any of the complexes. B, supershift assay with anti-Sp1 antibody. The upper complex was supershifted as indicated by the arrow.

Identification of the Binding Activity as Sp1—To confirm specificity of binding and facilitate identification of binding proteins, the CCACC probe was used in Southwestern blot analysis of unfraccionated and fractionated nuclear extracts (Fig. 2D). Four size classes of oligodeoxynucleotide binding proteins were identified in crude nuclear extracts (lane 1). The middle highly prevalent (approximately 40 kDa) protein failed to bind to phosphocellulose. The other two low molecular weight proteins were distributed throughout the flow-through and elution fractions of the phosphocellulose column (data not shown). When analyzed separately, proteins in the flow-through fraction failed to enrich when passed through the affinity column. This suggests that these proteins bind the CCACC probe in a nonspecific manner.

One protein, running at approximately 105 kDa on the Southwestern blot, was strongly enriched by both phosphocellulose and affinity fractionation (lanes 6 and 10). Based on the apparent size and target sequence, this protein was most consistent with an Sp1 family member. To further evaluate this, fractionated extracts were analyzed by Western blotting using a commercially available Sp1 antibody. The results are shown in Fig. 2E (lanes 4 and 5). Sp1 activity was shown to be highly enriched in these same fractions, further suggesting that the purified factor was indeed Sp1.

To confirm Sp1 binding activity, gel shift competitions were performed with probes conforming to known consensus sequences. When analyzed at high resolution, the CCACC binding complex could be resolved as two separate closely spaced bands (Fig. 3, A and B). Both bands were competed efficiently by native probe. Competition with the classic SV40 Sp1 probe (sequence shown in Fig. 1B) competed only the upper band (Fig. 3A), whereas another GC-rich target, AP2 (sequence shown in Fig. 1B), only partially competed the lower band and only at very high molar ratios (Fig. 3A). The AP1 probe was unable to compete. This also suggested that the upper band contained an Sp1 family member. To identify this member, an Sp1 specific antibody was used in supershift analysis (Fig. 3B). This was specifically able to shift the upper band, confirming the upper band as Sp1. Control NF-κB and cAMP response element binding protein antibodies had no effect on either band (data not shown).

CCACC Motifs Are Essential for Sp1 Binding and IL-6 Promoter Function—Initial definition of the Sp1 binding site was based on the GC-rich box of SV40 and other viral sequences (31). More recent analyses suggest tolerance for sequence variation in the central base (many references tabulated in Ref. 32). However the Cs flanking the variant base have been shown to be particularly critical in determining binding (33). We therefore placed C → A mutations in the flanking regions of the three CCACC repeats. The sequence of this mutant probe is shown in Fig. 1B. Gel shift analysis (Fig. 4) using the mutant probe shows complete inhibition of binding of upper bands. Cross-competition between mutant and wild type probes also demonstrates specificity, that is the mutant probe is unable to compete wild type binding. Therefore, these three repeats control specific binding to this region.

To analyze functional consequences of Sp1 binding to the CCACC region, a luciferase vector driven by the −179 to +34
region of the murine IL-6 promoter was constructed by PCR. This promoter therefore contained the NF-IL6, CCACC, and NF-κB elements as well as the native IL-6 TATA box. The mutant CCACC region was introduced by directional PCR cloning. Authentic sequences of both the wild-type and mutant promoter constructs were confirmed by automated sequencing.

The promoters were analyzed by transfection into both unstimulated and LPS-stimulated 3T3 fibroblasts. We have previously shown that Balb/c3T3 cells express low levels of IL-6 constitutively, but both NF-κB and IL-6 are highly inducible by LPS within 6 h. The results of these transfections are shown in Fig. 5. In unstimulated 3T3 cells, basal levels of luciferase for the wild type promoter were roughly 4 times that for the mutant at 24 h (Fig. 5B), increasing to 23 times when assayed at 48 h (Fig. 5A). This held true over a 3-fold range of plasmid dose. This difference was highly significant (p = 0.0062 (F(1,2) = 10610) by analysis of variance). Previous studies have demonstrated little induction in transient chloramphenicol acetyltransferase assays using LPS when assayed at 48 h (8). By performing these assays at 24 h and using the sensitive luciferase activity as a marker, we were able to demonstrate reproducible 2.6-fold induction of the wild type plasmid, which was significant (p < 0.05; t = 4.5), but only 1.5-fold induction in the mutant (Fig. 5B). Identical results were obtained with two different plasmid preps.

Taken together, these data show that Sp1 binding is important for both basal and inducible IL-6 expression.

**DISCUSSION**

Our in vivo footprinting studies suggested that the CCACC-containing region was constitutively occupied by transcription factors in expressing fibroblasts. This region consists of three repeats of the sequence CCACC. A promoter region with similar structure has also been identified in the human eosinophil peroxidase gene (34) and the c-fos promoter (35). Related sequences had previously been demonstrated to bind a variety of C/EBP type zinc finger-containing transcription factors, which include THP-1, GLI, htβ, Zif268 (ERG1), EKLF, Sp1-Sp4, and YY1 (36–39). Other proteins with similar binding targets also include Puf (40, 41), AP2 (42), and HATF-1 (43). Supershift, Southwestern, and affinity purification analyses suggest that the upper complex is Sp1. The identity of the lower complex remains to be determined. Luciferase experiments suggest that the binding of this complex is functionally very important in both basal and induced IL-6 regulation.

Both p50 and p65 antisense inhibition of NF-κB caused extensive and specific change to the footprint between the NF-IL6 and NF-κB sites. However, changes did not extend upstream of the NF-IL6 site. Ablation of the footprints in this chromatin domain could occur through numerous mechanisms. However, we feel it is most consistent with strongly cooperative binding of transcription factors bound by these elements. Cooperativity between NF-κB and NF-IL6 components has previously been demonstrated (44–48). Further, interactions between both the p50 and p65 subunits of NF-κB may have important interactions with NF-IL6 for at least two other genes (47, 48). Positive cooperativity between Sp1 and NF-κB has also been demonstrated to play an important role in regulation of the human immunodeficiency virus promoter (49). Interactions between Sp1 and NF-IL6 have not been previously described, and definition may have to await further mutagenic experiments. In the case of mouse IL-6, a variety of family members (C/EBP-α, -β, -γ, and -δ) may bind the NF-IL6 elements at various stages of the inflammatory response (19, 50, 51). In contrast, splicing variants of NF-IL6 may play a similar role in human cells (52). Similarly, a variety of NF-κB family members may bind the IL-6 NF-κB element during immune maturation.

In the case of the IL-6 promoter, Sp1 may provide the scaffolding to facilitate these interactions. Indeed, Stein and coworkers (53, 54) have shown that Sp1 may participate in matrix attachment sites. Such attachment sites could ensure poised IL-6 promoter for rapid response to inflammatory stimuli.
Binding and Functional Effects of Transcriptional Factor Sp1 on the Murine Interleukin-6 Promotor
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