NF-AB, a Liver-Specific and Cytokine-Inducible Nuclear Factor That Interacts with the Interleukin-1 Response Element of the Rat α_1 -Acid Glycoprotein Gene

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The 142-bp cytokine response element of the rat α_1 -acid glycoprotein (AGP) gene is a complex of several additively contributing regulatory sequences. By using deletions and point mutations, a minimal interleukin-1 (IL-1) response element was localized to the region from positions 1 to 36 within the 5'-most AB fragment of the cytokine response element. Two distinct sequence motifs were contained within this element, both of which were required to achieve full IL-1 response in rat and human hepatoma cells. This element showed a minor response to phorbol ester treatment only in human hepatoma cells. Southwestern (DNA-protein) blot analysis of nuclear proteins of rat liver and hepatoma cells revealed the presence of a heat-labile nuclear factor (NF-AB). NF-AB migrated as a basic protein with an apparent molecular mass of 37 kDa and bound specifically to the DNA sequence at positions 10 to 37 of the AB fragment. The NF-AB binding activity was detected neither in the cytoplasmic fraction of rat hepatoma cells nor in nuclear extracts from control or acute-phase rat kidney. The binding activity of NF-AB correlated with the transcriptional activity of the endogenous AGP gene in rat liver and hepatoma cells. Nuclear extract from human HepG2 cells showed a similar binding activity with an apparent molecular mass of 34.5 kDa. The human NF-AB binding activity was detectable only after 13 h of cytokine treatment and was not induced by phorbol ester. Tissue distribution, DNA sequence binding specificity, and kinetics of cytokine induction of NF-AB do not coincide with the characteristics of any other described factors that have been associated with cytokine regulation. Therefore, NF-AB is considered a new candidate involved in IL-1 regulation of the rat AGP gene.

Interleukin-1 (IL-1) interacts with many cell types and produces pleiotropic responses that include growth stimulation of fibroblasts and T lymphocytes and activation of acute-phase plasma protein genes in hepatocytes (25, 28). However, despite its central role in cellular communication, little is known about the intracellular signal pathway mediating IL-1 action.

IL-1 synergizes with tumor-promoting phorbol esters (12-O-tetradecanoyl-phorbol-13-acetate [TPA]) and the calcium ionophore A23187 to produce IL-2 in immature thymocytes (33). This IL-1 action was correlated with augmented levels of NF- κ B-like and AP-1-like factors that bind to IL-2 gene regulatory sequences (26). In the mouse pre-B-cell line 70Z/3, IL-1 can utilize cyclic AMP as a second messenger for the induction of κ immunoglobulin light-chain synthesis via the activation of NF- κ B or a related factor (37). Induction of human IL-6 gene expression by IL-1 was also ascribed to an NF- κ B-like factor (36) and to NF-IL6 in mouse fibroblasts (14). Cloning of NF-IL6 revealed high sequence homology with C/EBP (20) in its DNA-binding domain (1). Indeed, C/EBP protein binding sites have been identified in the IL-1 response elements (REs) of the rat angiotensinogen and human C3 genes (6, 41).

Previously, we have determined that the 5'-most 62-bp AB fragment of the cytokine response element (or distal regulatory element [DRE]) of the rat α_1 -acid glycoprotein (AGP) gene conveyed IL-1 regulation onto linked genes (42). However, no hormone-inducible binding activity was detected in

the nuclear extracts from rat liver and hepatoma cells by gel retardation analysis and DNase I protection assays, using either the entire AB sequence or various subfragments as probes (42, 42a).

Here we report that the region from positions 1 to 36 within the AB fragment represents the minimal IL-1 of the rat AGP gene. We identified by Southwestern (DNA-protein) blot analysis a hormone-inducible and heat-labile factor (NF-AB) present in nuclear extracts from rat liver and hepatoma cells that binds to a subfragment of the IL-1 RE. A point mutation that abolished the IL-1 response also led to the loss of NF-AB binding.

MATERIALS AND METHODS

Transfections and analysis of gene expression. H-35 (clone T-7-18 [32]) and HepG2 (16) cells were transiently transfected with chloramphenicol acetyltransferase (CAT) reporter plasmids as previously described (42). Thirty-six hours after transfection, cells were stimulated for 24 h with 500 U of recombinant human IL-1 α per ml, 250 U of recombinant IL-6 per ml, 1 μ M dexamethasone, or 0.25 μ M TPA and then harvested for CAT assays (11). For the analysis of endogenous AGP gene activation, confluent H-35 cells were treated for various lengths of time with hormones. Nuclei were prepared (2) and used for the run-on reaction (19). Equal amounts of ³²P-labeled transcripts (5×10^7 cpm) were hybridized to nitrocellulose strips carrying slot-blotted denatured genomic DNA encoding rat AGP (4). Cytoplasmic RNAs were isolated from postnuclear fractions of the cell lysates (8) and analyzed by Northern (RNA) blot hybridization for rat AGP mRNAs (30, 39). The change in the amount

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of secreted rat AGP was measured by rocket immunoelectrophoresis of culture medium (40).

Plasmid constructs. The simian virus 40 promoter-CAT constructs containing the DRE subfragments from positions 1 to 62 (AB), 1 to 28 (A), 29 to 62 (B), 10 to 37, 10 to 37 mut (change of T at position 28 to G), or 63 to 117 (C) have been described previously (42). The fragment from positions 1 to 36 was obtained by digestion with BAL 31 exonuclease. Three copies of this fragment (1-36) were inserted as *Clal* fragments into the *Clal* site of pSV-CAT, yielding p3x(1-36)-SV-CAT. p3x(1-36 mut)-SV-CAT contained the same sequence prepared as a synthetic oligonucleotide with a guanine at position 28 instead of thymidine.

Preparation of cell extracts. Extracts from whole cells (43) and nuclei (34) were prepared as described previously. Cytoplasmic extracts were obtained from the postnuclear fraction of cell homogenates. Acute phase was induced in Buffalo rats (3 months old, weighing 300 g) by subcutaneous injection of 250 μ l of turpentine. Nuclear extracts from rat liver and kidney were prepared according to Gorski et al. (12).

DNA binding assays. Southwestern blot analysis was performed with minor modifications of the methods of Miskimins et al. (24) and Silva et al. (38). Briefly, proteins (70 to 200 μ g) were separated on sodium dodecyl sulfate (SDS) (0.1%)–polyacrylamide (10%) gels, renatured, and electroblotted onto a nitrocellulose membrane. The membranes were blocked for 3 h at 4°C with 5% nonfat dry milk in the binding buffer (50 mM KCl, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.9], 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$). The binding of end-labeled DNA fragments (10⁶ cpm/ml) was carried out for at least 12 h at 4°C in binding buffer containing 0.25% nonfat dry milk, poly(dI-dC) (10 μ g/ml), and when necessary competitor DNA. The specific DRE subfragment probes were recovered from the respective CAT vectors. A synthetic double-stranded oligonucleotide of the rat haptoglobin A fragment (positions –165 to –147) served as an unrelated IL-1 response sequence (5). The filters were washed three times for 20 min at room temperature with binding buffer, rinsed briefly with 100 mM NaCl–10 mM Tris-HCl (pH 7.6)–1 mM EDTA, air dried, and exposed for autoradiography at –70°C with an intensifying screen.

[³⁵S]methionine labeling and two-dimensional gel analysis of nuclear proteins. Cells were labeled for 2 h in serum-free and methionine-free medium containing [³⁵S]methionine (45 μ Ci/ml; 800 Ci/mmol). Nuclear extracts were prepared, and 150- μ g samples of proteins were separated by two-dimensional polyacrylamide gel electrophoresis using non-equilibrium isoelectric focusing (from acidic to basic direction for 4,800 V · h) in the first dimension (27). The gel was either stained with silver, dried, and subjected to autoradiography or processed for Southwestern blot analysis.

RESULTS

IL-1-specific regulatory sequences within the AB fragment.

Previous functional analysis of the DRE allowed us to identify the 62-bp AB fragment as an IL-1 RE (42). Since this fragment included a NF- κ B-like sequence at positions 45 to 55, its significance for overall IL-1 response was determined by deletion. The sequence from 1 to 36, representing the major nuclear protein protected region in AB, was prepared, inserted into pSV-CAT (42), and tested for function in H-35 cells. A fivefold IL-1 stimulation was observed (Fig. 1), comparable to that achieved with AB (42). This suggested

FIG. 1. IL-1 response through fragment (1-36). H-35 cells were transfected with p3x(1-36)-SV-CAT, p3x(1-36 mut)-SV-CAT, or pSV-CAT. The cultures were subdivided and treated for 24 h with IL-1 and/or TPA, and CAT assays were performed with equal amounts of cell extract.

that the remaining AB sequences did not contribute significantly to IL-1 response. As previously shown, fragment (29-62) failed to respond to IL-1 (42). Since neither fragment (1-28) nor fragment (10-37) alone showed a detectable IL-1 response (42), it appears that either two separate elements (one at position around 10; the other at position around 28) or a single contiguous element (1-36) mediates the IL-1 response. This response was completely lost by a T-to-G substitution at position 28 [p3x(1-36 mut)-SV-CAT; Fig. 1]. Although the sequence from 1 to 36 was devoid of a NF- κ B binding site, a threefold stimulation by TPA was detected. However, the TPA effect could be fully accounted for by the response of the vector itself (Fig. 1). We concluded that the sequence from 1 to 36 within the DRE contains the minimal IL-1 RE and that it is not a detectable target for the protein kinase C signaling pathway in H-35 cells.

Identification of NF-AB in the nuclear extract from H-35 cells. To identify proteins that interact specifically with IL-1 response sequences, H-35 cells were treated with a combination of IL-1, IL-6, and dexamethasone to achieve maximal stimulation of AGP gene expression (3, 5). At various time points, nuclear proteins were extracted, denatured, separated on one-dimensional SDS-polyacrylamide gels, renatured and subjected to Southwestern blot analysis using a labeled AB fragment as a probe (Fig. 2C). A major binding activity migrating with an apparent molecular mass of 37 kDa was observed which in this particular experiment increased ninefold by 24 h of treatment with IL-1, IL-6, and dexamethasone. This 37-kDa binding activity was termed NF-AB (nuclear factor bound to AB). Besides NF-AB, several additional binding activities of larger size were detectable, but these binding activities were relatively minor and did not show reproducible changes following hormone treatment.

The hormone stimulation of NF-AB binding activity was temporally correlated with the transcriptional activation of the endogenous rat AGP gene. Characteristic for H-35 cells is that the onset of hormone-stimulated expression of AGP is delayed by 4.5 h (Fig. 2A). The NF-AB binding activity showed a similar delay, and its increase could be reproducibly measured only after 4.5-h treatment period.

FIG. 2. Time course for the induction of NF-AB binding activity and AGP gene expression. Duplicate confluent monolayers of H-35 cells were treated with IL-1, IL-6, and dexamethasone. At the times indicated, nuclear extracts were prepared from one set of cells, and the rate of AGP gene transcription and level of mRNA were determined from the other set. In addition to the AGP gene, run-on analysis (A) also included a probe for the triosephosphate isomerase gene as an internal reference. Each treatment yielded the same hybridization signal for the reference gene (see also references 3 and 5). RNA was analyzed by Northern blot hybridization (B). The major band represents the full-length cytoplasmic AGP mRNA of 850 nucleotides (35). Nuclear extracts (100 μ g) were subjected to Southwestern blot analysis using the 32 P-end-labeled AB fragment as a probe (C). The positions of molecular mass markers (in kilodaltons) are indicated at the right.

Characteristics of NF-AB binding activity. The sequence specificity of NF-AB binding was determined by competition assays using nuclear extracts from either cytokine-treated cells or acute-phase rat liver which also contains the NF-AB binding activity (see Fig. 7). Since identical results were obtained regardless of the source of nuclear extract, only representative examples are shown in Fig. 3. Excess amounts of unlabeled AB fragment competed effectively for binding, whereas the C fragment, which contains the IL-6-specific regulatory sequence (42), did not (Fig. 3A). Moreover, no specific binding activity on Southwestern blot was discernible when the blot was probed with a labeled C fragment.

The NF-AB binding site within AB was defined by using subfragments of AB as probes (Fig. 3B). The minimal IL-1 response sequence (1 to 36) bound as well to NF-AB as did intact AB. Neither subfragment A (1 to 28) nor B (29 to 62) served as a binding substrate for NF-AB. However, fragment (10-37), which covers the AB junction, was equally well recognized by NF-AB. NF-AB binding was almost completely lost by a T-to-G substitution at position 28 within fragment (10-37). This mutation when present in fragment (1-36) also abolished the IL-1 response (Fig. 1). We conclude that the NF-AB binding sequence is localized to the 3' half of the IL-1 RE and that its recognition by NF-AB is required but not sufficient for IL-1 regulatory function.

To determine whether NF-AB binding is more general to IL-1 signaling, the IL-1 response sequence of another acute-phase reactant gene was similarly tested. The IL-1 RE of the

FIG. 3. NF-AB binding activity specific to the AGP IL-1 RE. (A) Binding of nuclear extracts (100 μ g) from 13-h acute-phase rat liver to labeled fragment AB or C was carried out in the absence or presence of a 100-fold molar excess of unlabeled competitors. (B) Nuclear extracts (100 μ g) from H-35 cells treated with hormone for 4.5 h were incubated with the indicated end-labeled fragments. The specific activities of probes were 1.8×10^8 (AB; 1-62), 2.0×10^8 (C), 2.6×10^8 (1-36), 1.5×10^8 (10-37), 2.8×10^8 (10-37 mut), 1.7×10^8 (1-28), 2.5×10^8 (29-62), and 2.6×10^8 (Hp "A") cpm/ μ g. The position of NF-AB binding activity is indicated by arrow. Sizes of standards (Std) are indicated in kilodaltons.

rat haptoglobin gene (A element [5]) showed only a very weak interaction with NF-AB (Fig. 3B).

NF-AB binding activity to all possible substrates was completely abolished by heat treatment of the extract (85°C for 10 min) prior to gel electrophoresis (data not shown). NF-AB appears to differ in this respect from the highly heat stable C/EBP (20). Moreover, we also noticed that inclusion of the nonionic detergent Nonidet P-40 in cell lysis solutions drastically lowered the recovery of NF-AB binding activity (data not shown).

NF-AB is a minor component of the nuclear extract. Thus far, NF-AB was identified only as a binding activity on one-dimensional polyacrylamide gels. To assess the relative quantity of NF-AB protein, nuclear extracts from [35 S]methionine-labeled H-35 cells were analyzed by two-dimensional gel electrophoresis (Fig. 4). Duplicate gels were prepared; one gel was processed for silver staining followed by autoradiography, and the other gel was used for Southwestern blot analysis. The protein NF-AB appeared as single AB probe-binding spot migrating with basic charge and with an apparent molecular mass of 37 kDa (Fig. 4A). NF-AB was detectably labeled with [35 S]methionine and was barely visible by silver staining (Fig. 4B). Taken together, this two-dimensional gel analysis suggested that NF-AB is a minor component in H-35 nuclear extracts but appears to have an avid binding activity for the AB sequence.

Regulation of NF-AB binding activity by hormones. Since NF-AB binding activity increased in H-35 cells following treatment with the combination of IL-1, IL-6, and dexamethasone (Fig. 2), the contribution of each hormone to this regulation was determined and compared with the response

FIG. 4. Two-dimensional gel separation of NF-AB. (A) H-35 cells were treated for 4.5 h with IL-1, IL-6, and dexamethasone and labeled for 2 h with $[^{35}\text{S}]$ methionine. Nuclear proteins (150 μg) were electrophoresed in a nonequilibrium isoelectric focusing (NE-IEF) gel in the first dimension and then in a 10% polyacrylamide gel (SDS PAGE) in the second dimension. The gels were processed for silver staining or for Southwestern blot analysis. (B) Comparison of the boxed area in panel A in silver-stained and $[^{35}\text{S}]$ methionine- and ^{32}P -labeled patterns. One gel was silver stained and then autoradiographed. The other gel was used for protein transfer to nitrocellulose. The proteins were probed with ^{32}P -labeled AB fragment and exposed to two X-ray films separated by a piece of paper, with an enhancing screen against the second film. The film in direct contact with the gel shows both ^{35}S and ^{32}P pattern, while the second film shows only the ^{32}P pattern. All autoradiograms were exposed for 24 h. The position of NF-AB is marked by arrow.

of AGP gene elements. To do so, we transfected the p4xAB-SV-CAT plasmids into H-35 cells and then treated the cells for 24 h with dexamethasone, IL-1, and IL-6 separately or in combination (Fig. 5). The amount of rat AGP in the medium reflected the hormone-specific regulation of the endogenous AGP gene that was characterized by the strong synergistic action of dexamethasone with IL-1 and IL-6 (Fig. 5A). The CAT activity in the cell extract reflected the regulatory

FIG. 5. Hormone-dependent changes in AGP gene expression and NF-AB binding activity. One set of H-35 cells was transfected with p4xAB-SV-CAT, subdivided, and treated with the indicated factors for 24 h, and CAT activity was determined (B). Another set of H-35 cells was identically treated, and nuclear extracts were prepared and used for Southwestern blot analysis (C). The amount of rat AGP secreted in the medium was determined by rocket immunoelectrophoresis (A). Dex, dexamethasone. Sizes of standards (Std) are indicated in kilodaltons.

potential of just the AB region (Fig. 5B). In this experiment, CAT activity was increased fourfold by IL-1, fivefold by IL-6, and eightfold by IL-1 plus IL-6. Dexamethasone enhanced the effect of the cytokines approximately twofold. A maximal 13-fold increase was achieved with a combination of IL-1, IL-6, and dexamethasone. The NF-AB binding activity in the same cells was increased 1.6-fold by IL-1, 4-fold by IL-6, 7-fold by IL-1 plus IL-6, and 13-fold by the combination of dexamethasone, IL-1, and IL-6 (Fig. 5C). Some variations in the recovery of NF-AB activity from the cells treated with dexamethasone alone were observed. Although in the experiment shown in Fig. 5C the dexamethasone treatment resulted in a decreased binding activity, three additional hormone treatment experiments did not show a reproducible reduction of NF-AB by dexamethasone. The combined results of all of the hormone treatment experiments indicated that the changes in the NF-AB binding activity were highly correlated with transcriptional stimulation of the AGP gene elements.

In addition to NF-AB, there was a second, less abundant binding activity that migrated with an apparent mass of 20 kDa. Although the amount of the smaller form relative to the 37-kDa form was variable, a similar hormone-specific change was noted (Fig. 5 to 7). We assume that the 20-kDa form represents a degradation product of a 37-kDa species which might arise in the cells or during the nuclear isolation and sample preparation.

NF-AB is undetectable in cytoplasmic extract. To determine whether the increased NF-AB binding activity in hormone-

FIG. 6. Subcellular distribution of NF-AB binding activity. H-35 cell cultures were incubated for 14.5 h in the absence (–) and presence (+) of IL-1, IL-6, and dexamethasone. Whole cell extract (WCE), cytoplasmic (C), and nuclear (N) fractions were prepared, and 200 μ g of each was subjected to Southwestern blot analysis using fragment AB as a probe. Sizes of standards (Std) are indicated in kilodaltons.

treated cells was a consequence of translocation of the factor from the cytoplasm to the nucleus, we investigated the subcellular distribution of NF-AB in control and hormone-treated H-35 cells (Fig. 6). NF-AB binding activity detected in the whole cell extract could be fully accounted for by the change in nuclear concentration. No NF-AB binding activity was detectable in the cytoplasmic extracts from either un-

FIG. 7. NF-AB binding activity in rat liver. (A) Nuclear extracts were prepared from control and acute-phase liver and kidney and from H-35 cells treated for 14.5 h with IL-1, IL-6, and dexamethasone. Protein (70 μ g) of each nuclear extract was analyzed for NF-AB binding activity (arrow). Sizes are indicated in kilodaltons. (B) Nuclear extracts prepared from rat liver at various times following treatment with turpentine were analyzed for NF-AB binding activity by Southwestern blot analysis. Hybridization intensity was measured by densitometry of the autoradiogram and expressed in standardized densitometric units.

FIG. 8. Regulatory activity of region (1-36) in HepG2 cells. HepG2 cells were transfected with indicated plasmids, subdivided, and treated for 24 h with IL-1 and/or TPA as indicated. Equal amounts of cell extracts were assayed for CAT activity.

treated cells or cells treated with IL-1, IL-6, and dexamethasone.

NF-AB is not a ubiquitous factor. Since AGP is a major acute-phase plasma protein in the rat, we determined whether the NF-AB binding activity correlated with AGP gene expression or whether it was also present in nonhepatic rat tissues. Nuclear extracts were prepared from liver or kidney of control and acute-phase rats. Equal amounts of proteins were then analyzed by Southwestern blotting and compared with the pattern obtained with H-35 extract (Fig. 7A). A binding activity identical in molecular mass to NF-AB of H-35 cells was observed in liver nuclear extracts. The liver NF-AB binding activity increased gradually following initiation of the acute phase and reached the maximum by 12 h (Fig. 7B). The change in NF-AB activity was kinetically similar to the transcriptional activation of the endogenous AGP gene (18). Depending on the preparations, the peak level of NF-AB activity in liver was between 2 and 10 times lower than in H-35 cells (10 times lower in the example shown in Fig. 7A). In contrast to liver, no traces of binding activity with the electrophoretic mobility of NF-AB were found in kidney nuclear extracts from two rats prepared and tested separately (Fig. 7A). This result suggested that NF-AB is not a ubiquitous binding activity.

NF-AB binding activity in human hepatoma cells. Previous functional characterization of DRE sequences had been carried out in HepG2 cells (42). We therefore assumed that the human cells must have an NF-AB equivalent component. To ensure that region (1-36) also functioned as an IL-1 RE in HepG2 cells, p3X(1-36)-SV-CAT was transfected in these cells (Fig. 8). A 12-fold stimulation by IL-1 was achieved, which slightly exceeded that measured in H-35

FIG. 9. Stimulation of NF-AB binding activity in human hepatoma cells. Nuclear extracts from untreated HepG2 cells and from cells treated with TPA for 1 or 17 h or with IL-1, IL-6, and dexamethasone for 13 h were analyzed for NF-AB binding activity. End-labeled fragment (1-36) was used as a probe. The NF-AB binding activities from rat and human cells are indicated by closed and open arrows, respectively. Sizes are indicated in kilodaltons.

cells (Fig. 1). As expected, the reporter plasmid with the mutant element sequence did not respond to IL-1. The most prominent difference from H-35 regulation was that in HepG2 cells TPA stimulated CAT expression from the (1-36)-containing plasmid threefold above the level for either the mutant plasmid or the vector alone (Fig. 8). This TPA response of region (1-36) suggests that the signaling pathway involving protein kinase C might converge with that of IL-1 in HepG2 cells and that the NF-AB binding site involving base position 28 might be the ultimate target of those pathways. To identify the binding factor for cytokine and TPA response, Southwestern blot analysis was performed with fragment (1-36) as a probe as done for H-35 cell extracts (Fig. 9). No binding activity was detected until after 13-h hormone treatment with IL-1, IL-6, and dexamethasone. A specific binding activity appeared with a size approximately 2.5 kDa smaller than that of NF-AB found in rat liver and H-35 cells. Treatment of HepG2 cells with TPA for either 1 or 17 h did not result in the appearance of NF-AB binding activity, suggesting either that the magnitude of stimulation was still below the detection level of Southwestern blot analysis or that TPA response through the (1-36) sequence was independent of the human NF-AB.

DISCUSSION

In an effort to identify IL-1-inducible binding proteins, we performed Southwestern blot analysis and identified a nuclear protein (NF-AB) that binds to the IL-1 RE of the rat AGP gene. The functionality of NF-AB in the signal-dependent transcriptional regulation of the AGP gene is supported by the following observations: (i) the temporal change in NF-AB binding activity correlated with the transcriptional activation of the AGP gene following hormone treatment (Fig. 2) or acute phase (Fig. 7B), and (ii) a one-base-pair substitution abolished the NF-AB binding (Fig. 3B) and IL-1 response (Fig. 1 and 8).

Although the exact nature of increased binding activity of NF-AB is not known, a posttranslational activation as ex-

emplified by NF- κ B (10) is unlikely. No NF-AB binding activity was present in the cytoplasmic fraction when the proteins were separated under denaturing conditions (Fig. 6), suggesting that a cytoplasmic-to-nuclear translocation control does not apply to NF-AB. Another mechanism to posttranslationally activate transcription factor binding involves covalent modifications, such as phosphorylation and dephosphorylation (21, 23, 31, 44). Because TPA did not stimulate NF-AB binding (Fig. 1 and 9), control of its activity by a protein kinase C-dependent pathway seems unlikely. However, the possibility remains that NF-AB activity is regulated by another hormone-dependent kinase or phosphatase.

The minimal NF-AB binding site is located within fragment (10-37) (Fig. 3B), and the sequence from positions 25 to 37 is highly homologous to the NF-IL6 binding site in the IL-6 gene (1). We do not consider NF-AB to be identical to NF-IL6 for the following reasons: (i) unlike NF-AB, the binding activity of NF-IL6 has been reported to be present in kidney tissues from acute-phase animals and in untreated HepG2 cells, and (ii) NF-IL6 binding activity is heat stable whereas NF-AB binding activity is heat labile. However, functional data suggest that the action of NF-AB is similar to that of the products of the C/EBP gene family members. NF-IL6 (1), like rat IL-6 DBP (29) or LAP (9) and the mouse homolog to IL-6 DBP, called AGP/EBP (7), shares sequence homology with C/EBP (20) in its DNA binding domain. All of these factors have been described as binding to specific regulatory elements of several acute-phase genes and activating expression of linked reporter genes. In addition, an involvement of C/EBP or C/EBP-related proteins in the IL-1 response of the human C3 gene and the rat angiotensinogen gene has been inferred from the observation that C/EBP protein can bind in vitro to the sequence within the identified regulatory elements (6, 41). Even in the case of the IL-1 RE of the rat AGP gene, mouse C/EBP was highly effective in transactivating a cotransfected CAT reporter gene plasmid containing fragment (1-36) or (10-37) (5a). This action of mouse C/EBP via the NF-AB binding site suggests that NF-AB overlaps in binding specificity with C/EBP and might represent another member of that transcription factor family. There are precedents that distinct but structurally related protein factors can interact with identical DNA sequences, for instance, members of the Jun/Fos family (17) and the ATF/CREB family (13). Moreover, specificity and functionality of these nuclear factors are envisioned to be modulated by the formation of homo- or heterodimeric complexes (15). Conceivably, NF-AB, C/EBP, and C/EBP-like factors (e.g., NF-IL6 and IL-6 DBP) might form diverse dimers, and these might compete for binding sites within the AB fragment, leading to different functional activities. Further definition of NF-AB in AGP gene control and in relationship to other C/EBP-like factors must await the molecular cloning of NF-AB.

Our functional analysis showed conclusively that the NF-AB binding site (10 to 37) is not sufficient for the IL-1 response: an unidentified component has to interact with the sequence upstream of the NF-AB binding site to gain full function (Fig. 1 and 8; 42). Interestingly, the sequence at positions 13 to 4 (AATGTTGGAA) (the region suspected to be necessary for gaining full IL-1 response function) is also found at positions -80 to -71 within the IL-6 RE of the human C-reactive protein promoter (22) and is equivalent to the type B sequence found in other acute-phase protein genes (42). Since this sequence alone was also not sufficient for achieving an IL-1 response (42), a cooperative action of

separate binding proteins on fragment (1-36) is probably essential for conveying transcriptional activation by IL-1.

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