Multiple Elements within the Glucocorticoid Regulatory Unit of the Rat α1-Acid Glycoprotein Gene Are Recognition Sites for C/EBP*

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Sequences between −106 and −42, located immediately downstream of the glucocorticoid response element, are essential for efficient glucocorticoid-stimulated expression of the α1-acid glycoprotein (AGP) gene. We have used mobility shift assays with oligonucleotides bearing wild type and mutated sequences from segmented portions of this region to characterize the specific interaction of similar binding factors from rat liver and HTC rat hepatoma cell nuclear extracts. One of these factors, AGP nuclear factor 2 (ANF-2), appears capable of dual interaction with the homologous recognition sites, HA (−133 to −104) and HB (−81 to −72), which overlap and are located downstream of the glucocorticoid response element, respectively. Using an affinity matrix containing the HB sequence we have isolated ANF-2 from rat liver nuclear extracts. On the basis of immunological evidence rat liver ANF-2 was confirmed to be highly related and probably identical to CCAAT/enhancer-binding protein (C/EBP). Methylation protection analyses with partially purified, rat liver ANF-2 confirmed HA and HB as recognition sites for C/EBP-related factors and are consistent with the location of a third interaction site for these transactivating proteins at HX (−102 to −93). We propose that the sequences HA, HX, and HB, spanning residues −113 to −72 of the AGP promoter, might serve as recognition sites for a family of C/EBP-like nuclear factors that coordinate the glucocorticoid-mediated induction of the AGP gene.

As a reaction to inflammatory stimuli, the acute-phase response is characterized by increased hepatic production of a subset of plasma proteins known as the acute-phase reactants (for review, see Ref. 1). One of the major acute-phase proteins, α1-acid glycoprotein (AGP), appears to have a role in modulating the immune system during the acute-phase response (2). Among mammalian species, AGP is transcriptionally regulated by interleukins −1 and −6 and by glucocorticoids (3−13). A similar hormonal specificity has been indicated for the regulation of other acute-phase protein genes including those encoding haptoglobin, complement C3, serum amyloid A, and hemopexin, comprising the group of type 1 acute-phase proteins. Induction of AGP mRNA by dexamethasone is markedly reduced in cycloheximide-treated cells suggesting that efficient transcription of the AGP gene requires a pre-existing, labile protein in addition to the glucocorticoid receptor (3, 13, 14). The dexamethasone responsive region has been delineated between positions −121 and −42 of the AGP promoter (15) and contains a binding site for the glucocorticoid receptor centered approximately 110 base pairs upstream of the AGP transcription initiation site (15). Sequences between −106 and −42, located downstream of the glucocorticoid regulatory element (GRE), are essential for efficient glucocorticoid-stimulated expression from the AGP promoter (15). Additional results suggest that elements responsible for cycloheximide inhibition of this glucocorticoid-mediated induction reside within the same region (15).

In DNase I protection assays with nuclear extracts from HTC (JZ.1) rat hepatoma cells, workers from this laboratory have previously demonstrated a footprinted region spanning −110 to −68 of the AGP gene (15). The result suggested the presence of multiple elements within a control region known to be important for transcriptional activity of the AGP promoter in response to glucocorticoids (15). It was intriguing that these binding elements might represent interaction sites for positive factors that cooperate synergistically with the glucocorticoid receptor leading to induction of transcription by the AGP promoter. As an approach to further characterizing the structural and functional properties of the glucocorticoid regulatory unit of the rat AGP gene, we have used mobility shift assays with oligonucleotides bearing sequences from segmented portions of the DNase I footprinted region to demonstrate the specific interaction of similar factors from rat liver and rat hepatoma cell nuclear extracts. In this study we describe the isolation of one of these factors, designated AGP nuclear factor 2 (ANF-2), from rat liver nuclear extracts using sequence-specific DNA-affinity chromatography. We show that rat liver ANF-2 is immunologically indistinguishable from the CCAAT/enhancer-binding protein (C/EBP), a liver-specific transcription factor (16), and propose that these newly found DNA sequences, the most upstream one of which overlaps the GRE, might serve as recognition sites for a family of C/EBP-related nuclear factors that coordinate the regulation of AGP by glucocorticoids.

EXPERIMENTAL PROCEDURES

Material--[γ−32P]ATP (3000 Ci/mmol) was purchased from Du Pont, [α−32P]CTP (3000 Ci/mmol) from Amersham Corp., and [3H]-
Protein A (53.5 μCi/μg) from ICN; poly(dI-dC) and guanidine hydrochloride was from Boehringer Mannheim; Immobilon P was from Millipore; CNBr-activated Sepharose 4B, heparin-Sepharose 6B, T4 polynucleotide kinase, and protein molecular weight markers were from Pharmacia; and calf thymus DNA was from Bethesda Research Laboratories Inc.; carboxymethylidiamido-activated Sepharose was from Reactigel (6× 150/500 mm) (Pharmacia). DNA was eluted from CNBr activated Sepharose 4B with 50 mM of heparin-Sepharose previously equilibrated with the same buffer. The mixture was stirred at 4°C for 1 h after which the gel was filtered, washed three times with 100 ml of buffer B50, and packed in a 25 × 125-mm column. The column was washed successively with 100 ml of 0.2, 0.3, 0.45, and 0.6 M KCl in buffer B (buffer B100 without KCl). The peak protein fractions from each salt wash were combined (30 ml) and precipitated by mixing with 3.5 ml of 1 M ammonium acetate (pH 7.4) and 3.5 ml of 0.5 M NaCl. The mixture was shaken gently at 4°C for 1 h after which the gel was washed three times with 10 ml of buffer B100 by repeated centrifugation (3500 rpm/2 min/4°C), careful aspiration of the supernatant and mixing with buffer. The gel was packed in a 8.5 × 90-mm column and washed three times with 2 column volumes of 0.35 M KCl buffer B. ANF-2 was recovered by elution with 2 ml of buffer B containing 0.6 M KCl. In preparation for protein analysis by denaturing SDS-PAGE (20) recovered fractions were lyophilized after dialysis against 50 mM NH4HCO3, pH 8.0, 0.05% SDS. Proteins were detected by silver staining (21).

We observed during this study that the ability of ANF-2 to interact with sequence-specific DNA was maintained after exposure of rat nuclear extracts to heat at 85°C for 10 min. Denatured protein was cleared from the extract by centrifugation giving an ANF-2 purification of ~10-fold increased purity and suitable for direct DNA affinity chromatography. However, the protein was precipitated by quickly washing 5 ml of the resin with 25 ml of ice-cold water and suspending the gel in 9.5 ml of ice-cold coupling buffer. The annealed amino-linked oligonucleotide product was was added and reaction allowed to continue at 4°C for 2 h with gentle agitation. Unreacted oligonucleotide was removed by filtration of the suspension. Purification was achieved by electrophoresis through a 1X 4% polyacrylamide gel. A similar procedure was used to isolate coding strand sequences.

The first was synthesized by coupling multiple wild-type oligonucleotide (see Fig. 1) onto the base pair -90 to -64 sequence of the rat AGP promoter sequences (nucleotides -360 to -260). For the noncoding strand, HindIII digestion of the plasmid pAGPCATA20.1 (14) gave a 764-base pair fragment which was end-labeled with T4 polynucleotide kinase and protein molecular weight markers were from Pharmacia; and calf thymus DNA was from Bethesda Research Laboratories Inc.; carboxymethylidiamido-activated Sepharose was from Reactigel (6× 150/500 mm) (Pharmacia). DNA was eluted from CNBr activated Sepharose 4B with 50 mM of heparin-Sepharose previously equilibrated with the same buffer. The mixture was stirred at 4°C for 1 h after which the gel was filtered, washed three times with 100 ml of buffer B50, and packed in a 25 × 125-mm column. The column was washed successively with 100 ml of 0.2, 0.3, 0.45, and 0.6 M KCl in buffer B (buffer B100 without KCl). The peak protein fractions from each salt wash were combined (30 ml) and precipitated by mixing with 3.5 ml of 1 M ammonium acetate (pH 7.4) and 3.5 ml of 0.5 M NaCl. The mixture was shaken gently at 4°C for 1 h after which the gel was washed three times with 10 ml of buffer B100 by repeated centrifugation (3500 rpm/2 min/4°C), careful aspiration of the supernatant and mixing with buffer. The gel was packed in a 8.5 × 90-mm column and washed three times with 2 column volumes of 0.35 M KCl buffer B. ANF-2 was recovered by elution with 2 ml of buffer B containing 0.6 M KCl. In preparation for protein analysis by denaturing SDS-PAGE (20) recovered fractions were lyophilized after dialysis against 50 mM NH4HCO3, pH 8.0, 0.05% SDS. Proteins were detected by silver staining (21).

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template DNA after labeling with the Klenow fragment of DNA polymerase and [\(\alpha^32\)P]dCTP.

Footprint reactions were performed as described by Ohlsson and Edlund (19). Varying amounts (3.5-15 pg) of heat-treated rat liver polymerase and [\(\alpha^32\)P]CTP was continued for 20 min on ice. Reactions were placed at room temperature, the end-labeled DNA fragment (approximately 1 ng in 5 \(\mu\)l) was added and incubation was continued for 20 min on ice. Reactions were placed at room temperature for 2 min, and 5 \(\mu\)l of a 50 mM CaCl\(_2\), 100 mM MgCl\(_2\) solution was added followed immediately by 5 \(\mu\)l of DNAse I (10 ng/\(\mu\)l). Reactions were terminated after 60 s by addition of 100 \(\mu\)l of stop solution (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 100 \(\mu\)g/ml proteinase K, 50 \(\mu\)g/ml trRNA, and 1% SDS). After incubation at 37 \(^\circ\)C for 15 min, nucleic acids were recovered by phenol/chloroform extraction and ethanol precipitation. Pelleted DNA was precipitated with ethanol. Samples were treated as outlined for guanine cleavage in chemical DNA sequencing (25).

Western Analysis—Western blot analysis was performed as described by Towbin et al. (26) using \(^{32}\)P-protein A to visualize immunoreactive material. Samples of a purified, bacterially expressed C/EBP fusion protein and affinity purified ANF-2 from heated and nonheated nuclear extracts were submitted to SDS-PAGE on a 15% gel. After electrophoretic transfer to Immobilon P, C/EBP-related proteins were detected with a C/EBP-specific antibody directed against an internal 14-amino acid peptide (16).

RESULTS

Separate DNA binding studies with partially purified rat glucocorticoid receptor and nuclear extracts from the HTC (JZ.1) rat hepatoma cell line have previously revealed a combined region of protection from DNase I digestion extending between residues \(-121\) and \(-68\) (15). The footprinted region afforded by rat hepatoma HTC (JZ.1) cell nuclear extracts is shown divided into X, Y, and Z domains (Fig. 1A). Both Y and Z regions are included in the wild type oligonucleotide \((-90\) to \(-64\)) (Fig. 1A) used in mobility shift analyses with rat hepatoma cell nuclear extracts. The major retracted complex observed in these experiments was attributed to the specific binding protein, GAP nuclear factor 2 (ANF-2) (Fig. 1B). A 3-base substitution in the Z domain (ACA to GTG, Z mutant, see Fig. 1A) was sufficient to disrupt the specific interaction of ANF-2 (Fig. 1B). Close examination of the protected region within the rat AGP promoter revealed two regions of shared homology: HA, sequence \(-113\) to \(-104\), and HB, sequence \(-81\) to \(-72\) (Fig. 1A). It was of particular interest that the upstream homologous region overlaps the recognition sequence for the glucocorticoid receptor (Fig. 1A). The oligonucleotide, HA \((-120\) to \(-90\)), which includes both the glucocorticoid response element and the HA sequence (Fig. 1A), was shown to compete efficiently with the wild type oligonucleotide probe for binding to ANF-2 (Fig. 1B). In separate experiments, a similar sized oligonucleotide containing only the HA sequence flanked by a random selection of nucleotides displayed identical competition properties for ANF-2 (not shown). An extension of the same study showed that mutation of the bases AGA, within the HA sequence, effectively negated this competition confirming the importance of at least one of these bases for ANF-2 interaction (27). Within the HA sequence, this critical region was directly comparable in positional location to the triplet of nucleotides, ACA, found in the homologous HB sequence to be required for binding to ANF-2 (Fig. 1).
2-like factor with the appropriate DNA binding specificity (data not shown). Rat liver was then chosen as a convenient tissue source for the isolation of ANF-2 by sequence-specific DNA affinity chromatography. Two different affinity resins were employed. Based on the Kadonaga/Tjian methodology (17), we prepared a DNA affinity resin using the wild type oligonucleotide encompassing the Y and Z domains from −90 to −64 of the AGP promoter (Fig. 1). Our initial experience with this gel indicated considerable interference from non-specifically adsorbed proteins, probably due to the inherent charge effects resulting from the CNBr-activated mode of coupling (18). To overcome this problem and to eliminate the possible interaction of ANF-1, a factor with binding specificity for the Y domain, we prepared a novel gel from an amino-linked oligonucleotide containing a 3-base substitution in the Y region (see Y mutant, Fig. 1). Coupling of the amino-linked DNA was achieved via carbonyldiimidazole-activated Sepharose. The advantage of this coupling method is that, with extended incubation under the coupling conditions, remaining activated groups are hydrolyzed to the native Sepharose matrix leaving only the coupled oligonucleotide (18). Although the amount of coupled DNA in the conventional gel is 2–3 times higher (25 versus 9 μg/ml) than in the amino-linked resin, both gels appear to perform equally well after adequate washing with salt buffers.

Fig. 2 shows the results of a two-step purification for ANF-2 from rat liver nuclear extracts involving sequential heparin-Sepharose and DNA affinity chromatography. Mobility shift assays with the labeled, wild type oligonucleotide (Fig. 1) were used to monitor DNA binding activity in recovered fractions. A 0.45 M KCl eluate from batchwise heparin-Sepharose chromatography contained most of the ANF-2 activity which was shown to display the correct binding specificity by competition analysis with both wild type and mutated oligonucleotides (Fig. 2, A and B). This step afforded a 12–15-fold increase in ANF-2 purity, and after dialysis against B100 buffer the partially purified extract was equilibrated with nonspecific competitor poly(dI-dC) and submitted to sequence-specific DNA affinity chromatography. ANF-2 was recovered by elution with 0.6 M KCl buffer, and competition analysis confirmed the DNA binding specificity of the isolated fraction (Fig. 2, C and D). Denaturing SDS-PAGE of the 0.6 M KCl fraction, followed by silver staining, indicated the selective elution of three proteins corresponding to a major band migrating at 29 kDa and two minor bands at 43 and 20 kDa (data not shown).

The affinity-purified ANF-2 preparation was characterized further after denaturation-renaturation of proteins eluted from SDS-PAGE gel slices (22). The recovered proteins were precipitated with acetone, dissolved in 6 M guanidine hydrochloride and allowed to renature by dialysis against guanidine-free buffer. Mobility shift analysis of the renatured fractions confirmed the presence of DNA binding activity in slices 6, 7, 8, and 10 corresponding to molecular mass windows of 50–41, 41–34, 34–29, and less than 20 kDa, respectively (Fig. 3A). Although only the result of competition analysis for slice 10 activity is shown in Fig. 3B, specific DNA binding was demonstrated for both major activities observed in slices 6 and 10.

A survey of the literature indicated that the molecular sizes of the observed DNA binding fragments matched closely those reported for the CCAAT/enhancer-binding protein (C/EBP) (16) previously isolated from rat liver by Johnson et al. (24). Native C/EBP exists as a 42-kDa protein, but can be proteolyzed to smaller 30-, 20-, and 14-kDa fragments (16) (Fig. 4). A comparison of the C/EBP binding site and the homologous regions (HA, HB) on the AGP promoter indicated considerable similarity. The optimal C/EBP binding site consists of a dyad-symmetric recognition sequence containing the 5′-GCAAT-3′ half-site (28). On alignment the homologous regions, HA and HB, respectively, show a 7 and 6 out of 9 direct correspondence to the bases in the C/EBP site (Fig. 4).
Fig. 3. Renaturation of affinity-purified ANF-2 after denaturing SDS-PAGE. A, ANF-2, purified by sequential heparin-Sepharose and affinity chromatography, was electrophoresed through the length of the separating gel. Proteins eluted from slices taken throughout the length of the separating gel were renatured and analyzed by mobility shift assay. B, competition mobility shift analysis for renatured slice 10 activity. Aliquots containing the renatured protein were incubated in binding reactions with the labeled wild type oligonucleotide in the presence and absence of a 100-fold molar excess of competing oligonucleotide.

Fig. 4. Comparisons between ANF-2 and C/EBP. The consensus site for C/EBP has been compiled from several viral enhancers by Ryden and Beemon (29), while the optimal recognition sequence has been described by Vinson et al. (28).

However, by including the single bases adjacent 5' to both sequences in the AGP promoter, both regions display a 7 out of 10 identity to the C/EBP recognition sequence (Fig. 4). The upstream sequence (HA) conforms better with the consensus C/EBP site compiled from recognition sequences present in several genes (Fig. 4) (29). McKnight and collaborators (24) have reported that C/EBP DNA binding activity is substantially unaffected when heated to 80°C for 5–10 min. Heat treatment of rat liver nuclear extracts indicated a similar stability for ANF-2 (not shown). The chromatographic properties of ANF-2 activity from heated extracts were also shown to parallel those observed for unheated preparations (Fig. 5). Heated ANF-2 could be recovered from heparin-Sepharose with buffers containing 0.4–0.5 M KCl, similar to salt concentrations (0.45 M KCl) used for elution of ANF-2 during chromatography of nonheated nuclear extracts (see Figs. 2A and 5A). On heating, >80% of the protein in the nuclear extract is denatured and precipitated resulting in a preparation enriched 8–10-fold for ANF-2. This facilitated further purification by direct sequence-specific DNA affinity chromatography. As seen for unheated preparations, ANF-2 from heated extracts was eluted from the affinity column with 0.6 M KCl (see Figs. 2B and 5B). The appearance of ANF-2 activity in the flow-through fraction (Fig. 5B) was attributed to an overloading of the affinity column and could be prevented by increased gel volume and lower poly(dI-dC) concentration. Binding sequence specificity for the recovered ANF-2 was again confirmed by competition analysis (Fig. 5C).

Rat Liver ANF-2 Is Closely Identical to C/EBP—ANF-2 from rat liver was confirmed to be highly related and probably identical to C/EBP after Western blot analysis with a C/EBP-specific antibody raised against a 14-amino acid peptide situated N-terminal of the C/EBP DNA binding domain (16) (Fig. 6A). Major antibody-reacting proteins of approximately 40 and 30 kDa were observed for both heated and nonheated affinity-purified extracts, consistent with molecular sizes for native and proteolytically degraded C/EBP, respectively (Fig. 6A). In a separate experiment an affinity-purified extract from a heated nuclear preparation was submitted to denaturing SDS-PAGE followed by silver staining. Fig. 6B shows that most protein contaminants were removed by successive washing of the affinity resin with 0.35 M KCl buffer. On elution with 0.6 M KCl, five major bands were observed corresponding to proteins of approximately 40 (doublet), 30 (doublet), and 20 kDa in size (Fig. 6B, lane 4). We assumed initially that all of these proteins were C/EBP-related. However, after recovery of the individual proteins in these bands by elution, SDS-PAGE followed by Western analysis with the C/EBP peptide-specific antibody confirmed only the two 30-kDa proteins and the 20-kDa species as C/EBP-related fragments (not shown). The identity of the remaining 40-kDa proteins was not pursued. One possibility is that these proteins were also C/EBP-related, but that a low elution recovery from the SDS-PAGE gel may have prejudiced the signal intensity in Western analysis. Alternatively, the original affinity-purified extract may have contained only degraded C/EBP-related, fragments accounting for the observed antibody reactive proteins.

The Glucocorticoid Regulatory Unit of the AGP Promoter Contains Multiple Interaction Sites for C/EBP-like Factors—Using mobility shift analyses with the labeled wild type and HA (~120 to ~60) oligonucleotides (see Fig. 1) we were able to confirm the dual binding site specificity of an authentic, bacterially expressed C/EBP fusion protein for the homologous HA and HB regions (data not shown). To further assess the interaction of C/EBP-like factors with sequences within the glucocorticoid regulatory unit of the AGP promoter we performed DNase I footprint assays, on DNA templates spanning this region, using an ANF-2 preparation derived from heated rat liver nuclear extract. For both coding and noncoding strands the observed area of protection ranged from...
Fig. 5. Chromatographic properties of ANF-2 from heated rat liver nuclear extracts. After heat treatment (85 °C/10 min), precipitated protein was pelleted by centrifugation and the soluble extract was submitted separately to heparin-Sepharose and DNA affinity chromatography. The salt elution properties of ANF-2 from both adsorbants were monitored by mobility shift analysis. A, heparin-Sepharose chromatography. Load, heat-treated nuclear extract; F/T, flow-through; remaining lanes represent column elutes with 0.2, 0.3, 0.4, 0.5, and 0.6 M KCl, respectively. B, DNA affinity chromatography. Load, heat-treated nuclear extract; F/T, flow-through; fractions eluted successively with 0.2, 0.3, 0.4, and 0.6 M KCl are represented in the remaining lanes. C, competition mobility shift analysis for heated, affinity-purified ANF-2. Competing oligonucleotides were used in present column eluates with 0.2, 0.3, 0.4, and 0.5 M KCl, respectively. ANF-2 was recovered with a final 2-ml wash of 0.6 M KCl washes; remaining lanes represent column elutes with 0.2, 0.3, 0.4, 0.5, and 0.6 M KCl, respectively.

Fig. 6. Identification of ANF-2 as a C/EBP-related protein. A, Western blot analysis of affinity-purified ANF-2 from heated and nonheated rat liver nuclear extracts. Purified proteins were separated by gel electrophoresis and analyzed by Western blotting using C/EBP peptide specific antibody (16). Antibody reaction with an 11-kDa, bacterially expressed C/EBP fusion protein (16) is shown in the left-hand lane. B, analysis of affinity-purified ANF-2 from heated rat liver nuclear extracts by SDS-PAGE. Heated nuclear extract was cleared by centrifugation and added directly to amino-linked affinity resin (1 ml) for batchwise incubation. After extensive washing with buffer B100 the gel was packed in a column and eluted three times with 2 ml of 0.35 M KCl. ANF-2 was recovered with a final 2-ml wash of 0.6 M KCl. All 0.35 and 0.6 M KCl fractions were dialyzed against 50 mM NH4HCO3 buffer containing 0.05% SDS and lyophilized. After electrophoresis on a 15% (w/v) SDS-polyacrylamide gel, the separated proteins were visualized by silver staining. Lanes 1–3, proteins eluted with successive 0.35 M KCl washes; lane 4, protein in the 0.6 M KCl eluate.

residues -115 to -65 (Fig. 7A) and was identical to that previously reported by our group for crude extracts from rat hepatoma HTC (JZ.1) cells (15).

Rat liver ANF-2 interaction with recognition sequences within the footprinted region was further examined by methylation protection analysis. Occupancy of the HB site altered the pattern of methylation by DMS at four locations on the noncoding strand. ANF-2 binding partially inhibited the methylation of guanines at -73 and -75, while a modest enhancement of methylation was observed for the guanine residue at -76 and the adenine at -81 (Fig. 7B). There was no evidence of altered methylation on the noncoding strand for the RA recognition site (Fig. 7B). However, marked changes in methylation pattern were apparent for residues located immediately downstream of the homologous HA sequence (Fig. 7B). Bound protein strongly enhanced the methylation of the guanine at -97 (Fig. 7B). From an autoradiography obtained by shorter film exposure (not shown) it was also clearly evident that methylation of the adjacent guanine at -96 had been inhibited (Fig. 7B).

Methylation protection analysis of the coding strand revealed a weak protection, in the presence of rat liver ANF-2, of the guanine at -79 within the HB sequence (Fig. 7B). Immediately upstream of this binding element, methylation of the guanine residue at -83 was seen to be augmented (Fig. 7B). ANF-2 interaction with the HA recognition site resulted in less effective methylation of the guanine at -109 and was accompanied by increased alkylation of the guanine and adenine residues at -105 and -106, respectively (Fig. 7B). In between the homologous regions HA and HB, the pattern of methylation on the coding strand was unaltered by ANF-2 interaction except for enhanced methylation of the guanine at -93 and the adenine residue at -94 (Fig. 7B). A summary of the observed pattern of protection and enhancement on both strands is presented in Fig. 7C. Our results confirm HA and HB as recognition sites for C/EBP-like factors and are consistent with the presence of a third interaction site, designated HX, for the protein located between residues -102 and -93 (Fig. 7C). This putative binding sequence, ATTTCCCAAG, is closely comparable with the consensus recognition sequence for C/EBP (see Figs. 4 and 7C).

DISCUSSION

We have used affinity chromatography with a matrix containing the HB sequence, derived from the glucocorticoid regulatory unit of the AGP promoter, to isolate the nuclear
factor ANF-2 from rat liver. Several points of evidence indicate that the isolated protein is highly related to C/EBP, a heat-stable DNA-binding protein initially identified in rat liver nuclear extracts (16, 24). ANF-2 is similarly stable to thermal denaturation, and both proteins display a dual binding site specificity by interacting with both homologous regions, HA and HB, on the AGP promoter. ANF-2 was confirmed by immunological analysis to be very similar and probably identical to C/EBP. Our evidence from methylation protection analysis suggests that C/EBP also recognizes a third binding sequence, HX, located between the homologous regions, HA and HB. Chung et al. (30) have described three analogous motifs for C/EBP-like factors in the corresponding region of the mouse AGP gene. These authors have recently identified the murine C/EBP-related factor, AGP/EBP, which was shown to interact with an oligonucleotide incorporating sequences equivalent to HA and HX on the mouse AGP promoter (30). Whether this interaction involves one or other of these recognition sites, or both, was not clearly demonstrated.

While C/EBP appears capable of interacting with DNA sequences that significantly diverge from its consensus binding site (TGNNNGTAAG) (29) the homologous sequences, HA and HB within the AGP promoter, match very closely the optimal recognition site (ATTGCGCAAT) (28) for C/EBP. C/EBP is a major regulatory protein with a basic DNA binding region related in amino acid sequence to the transforming proteins Myc, Fos, and Jun (16). A common structural motif, termed the “leucine zipper,” facilitates the sequence-specific interaction of these proteins with DNA (28, 31). In early studies, Southern blot analysis, using the conserved DNA binding/leucine zipper domain of C/EBP, suggested the existence of additional C/EBP-related proteins.2

Indeed cDNA clones encoding a number of these factors have recently been isolated and characterized (32–35). DBP, a recently described liver-specific transcription factor (34), shares the conserved basic domain, but lacks a leucine zipper structure, and in this respect differs from other members of the C/EBP family (34). However, NF-IL6, an interleukin-1-inducible factor (32); Ig/EBP-1, a ubiquitously expressed immunoglobulin enhancer-binding protein (35); and LAP (liver activating protein) (the AGP/EBP rat homolog), a factor found to be highly enriched in rat liver nuclei (33), all contain potential leucine zippers and are highly homologous to C/EBP in the C-terminal DNA binding region. Both Ig/EBP-1 and LAP have a demonstrated ability to form heterodimeric complexes with C/EBP (33, 35). Additionally, all of these proteins, including DBP (D binding protein), share common recognition sequences (32–35). Of considerable interest is the observation that NF-IL6 binds to interleukin-6 responsive elements in the regulatory regions of several acute-phase proteins implying a role for NF-IL6 in gene regulation during the acute-phase reaction (32). The recognition sequence (GTGTGGCAAT) for NF-IL6, pertaining to the interleukin-6 responsive element on the AGP promoter, lies approximately 5 kilobases upstream of the transcriptional initiation site (11) and is closely comparable with the homologous binding sites, HA and HB, located within the glucocorticoid regulatory unit (see Fig. 4).

This report presents another example of the proximal location of recognition sequences for C/EBP-related nuclear factors and a glucocorticoid responsive element. Glucocorticoid-induced transcription of the rat angiotensinogen gene is

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1. S. L. McKnight, personal communication.
mediated by a multimodular enhancer consisting of an acute-phase response element flanked on both sides by two functionally distinct GREs (36). The acute-phase response element has been shown to bind a cytokine-inducible NF-κB-like factor and constitutive C/EBP-like proteins in a mutually exclusive manner (37). Complete glucocorticoid responsiveness of the angiotensinogen gene appears to be dependent on the association of either one of these DNA-binding proteins with the acute-phase response element leading to the formation of a synergistic enhancer complex together with activated glucocorticoid receptor (36). It is of note that the angiotensinogen acute-phase response element contains the sequence ATTTCCTCAAC which is homologous to region HX on the AGP promoter. Our results suggest that HX may be an ATTTCCCAAC which is homologous to region HX on the glucocorticoid receptor with the acute-phase response element leading to the formation of a cytokine-inducible NF-κB-like factor and constitutive C/EBP-like proteins in a mutually exclusive manner (37). Functional analysis of the C/EBP binding sites on the AGP promoter, are presented in a separate report (45).

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