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Identification of a C/EBP-Rel Complex in Avian Lymphoid Cells

J. ALAN DIEHL AND MARK HANNINK*

Biochemistry Department, University of Missouri—Columbia, Columbia, Missouri 65212

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Protein-protein interactions between the CCAAT box enhancer-binding proteins (C/EBP) and the Rel family of transcription factors have been implicated in the regulation of cytokine gene expression. We have used sequence-specific DNA affinity chromatography to purify a complex from avian T cells that binds to a consensus C/EBP motif. Our results provide evidence that Rel-related proteins are components of the C/EBP-DNA complex as a result of protein-protein interactions with the C/EBP proteins. A polyclonal antiserum raised against the Rel homology domain of v-Rel and antisera raised against two human RelA-derived peptides specifically induced a supershift of the C/EBP-DNA complex in mobility shift assays using the affinity-purified C/EBP. In addition, several κB-binding proteins copurified with the avian C/EBP complex through two rounds of sequence-specific DNA affinity chromatography. The κB-binding proteins are distinct from the C/EBP proteins that directly contact DNA containing the C/EBP binding site. The identification of a protein complex that binds specifically to a consensus C/EBP site and contains both C/EBP and Rel family members suggests a novel mechanism for regulation of gene expression by Rel family proteins.

Inducible expression of interleukin 6 (IL-6) and IL-8 is mediated by several transcription factors that are activated in response to tumor necrosis factor alpha and IL-1 (1, 18, 33, 42). One transcription factor that is important for inducible expression of IL-6 and IL-8 is C/EBP-β, a member of the CCAAT box enhancer-binding protein (C/EBP) family (1, 26, 42). The C/EBP family currently consists of four highly related proteins (isoforms) termed C/EBP-α, C/EBP-β, C/EBP-δ, and C/EBP-γ (1, 6, 7, 20, 23, 39). The C/EBP family members are defined by the presence of a conserved C-terminal bZIP domain that is responsible for DNA binding and dimerization, while their divergent N-terminal regions typically contain a transactivation domain. Additional C/EBP-related proteins with distinct regulatory functions are also generated through the use of alternative translational start sites (11).

A second family of transcription factors that are involved in regulation of inducible IL-6 and IL-8 gene expression is the Rel family of transcription factors (23, 26, 42). The Rel family is defined by a highly conserved 300-amino-acid domain, the Rel homology domain, that is located near the amino termini of all family members. Within the Rel homology domain lie the amino acid residues necessary for sequence-specific DNA binding, dimerization with other Rel family members, and association with members of the IκB family of proteins (inhibitor of κB; reviewed in reference 31). Members of the Rel family include p50 (the proteolytic product of NF-κB1) and RelA (formerly referred to as p65); p52 (the proteolytic product of NF-κB2); an internally truncated form of RelA (Δp65); a leucine zipper-containing protein (RelB, I-*rel*); the v-Rel oncoprotein and c-Rel, its cellular counterpart; and the *dorsal* morphogen (3–5, 8, 15, 22, 27, 29, 30, 32, 35–38, 40, 41, 44, 46).

Heteromeric association between C/EBP and Rel proteins in vitro suggests that protein-protein interactions between C/EBP and Rel may be important for transcriptional regulation in vivo (24, 42). The presence of consensus Rel- and C/EBP-binding sites in the promoters of several genes, including the IL-6 and IL-8 genes, is consistent with this suggestion (26, 28, 42). At least two possible mechanisms can be envisioned by which protein-protein interactions between C/EBP and Rel proteins might regulate gene expression. In one mechanism, C/EBP and Rel proteins bind to their respective DNA-binding sites in a single promoter and synergistically regulate gene expression. In support of this hypothesis, Rel and C/EBP family members bind to adjacent binding sites in the IL-8 promoter (26, 42), and binding of Rel family members to the κB site enhances binding of C/EBP-β to the adjacent C/EBP-binding site (42). Alternatively, C/EBP and Rel proteins might associate to form a DNA-binding complex in which only one of the components makes direct contact with its cognate DNA motif. The ability of Rel family members to activate C/EBP-dependent gene expression through the consensus C/EBP-binding site without binding directly to this site is consistent with this model (42).

We have used oligonucleotides containing the C/EBP-β-binding site from the human IL-6 promoter in electrophoretic gel mobility shift assays (EMSA) to identify and purify a complex from an avian T-cell line that contains both Rel and C/EBP family members. Several avian C/EBP-related (aC/EBP) proteins were purified by affinity chromatography. The purified C/EBP proteins were characterized by a combination of immunoblot analysis, Southwestern (DNA-protein) blot analysis, and UV cross-linking experiments. The stable association of an avian RelA-related protein with the aC/EBP was demonstrated by the ability of antisera raised against human RelA-derived peptides to induce a supershift of the protein-DNA complex containing purified aC/EBP. Two proteins of approximately 80 and 66 kDa that copurified with the C/EBP DNA-binding activity were identified by their ability to bind specifically to DNA containing a consensus κB-binding site. Peptide mapping of the 66-kDa copurifying κB-binding protein suggests that it may be the avian RelA homolog or a closely related protein. Our results demonstrate that C/EBP and a RelA-related protein associate to form a stable heteromeric

* Corresponding author. Phone: (314) 882-7971. Fax: (314) 884-4597.

complex that binds specifically to a consensus C/EBP motif in avian T cells.

MATERIALS AND METHODS

Recombinant DNA methodology. Conventional methodologies were used in the construction of all plasmids. The Rel expression vectors used in these experiment are derived from pJD214, a spleen necrosis virus-derived retroviral vector (14). The pTATA-Inr luciferase reporter plasmid was obtained from the laboratory of Bill Fahl. pTATA-Inr was constructed by insertion of a *Bgl*II-to-*Hin*dIII fragment that contained a minimal TATA-Inr sequence into the pGL2 vector (Promega). The wild-type human C/EBP-β (hC/EBP-β) luciferase plasmid (pwt-hC/EBP-β-TATA-Inr) was constructed by insertion of a double-stranded oligonucleotide containing three copies of the human C/EBP-β-binding site from the human IL-6 promoter into the *Bgl*II site of pTATA-Inr, 5′ to the TATA-Inr sequences. The sequence of the coding strand of this oligonucleotide is 5′-ACATTGCACAATCTACATTGCACAATCTAC ATTGCACAATCT-3′. The mutant hC/EBP-β luciferase plasmid (pmt-hC/EBP-β-TATA-Inr) was constructed by insertion of a double-stranded oligonucleotide containing three copies of a mutated, nonfunctional C/EBP-β consensus sequence into the *Bgl*II site of pTATA-Inr. The sequence of the coding strand of the mutant oligonucleotide is 5′-ACACTACAAA CTCTACACTACAAACTCTACACTACAAACTCT-3′. The plasmids were confirmed by sequence analysis.

Transfection of eukaryotic cells and reporter assays. MSB-1 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum. MSB-1 cells were electroporated with a Bio-Rad Gene Pulser. MSB-1 cells were resuspended at 6.5×10^7 cells per ml, and 300 μl of the cells was added to 500 μl of medium containing 15 μg of the appropriate DNA. After a 5-min incubation on ice, the cells were electroporated at 960 μF and 350 V and placed on ice for 5 min. The cells were then placed in 2 ml of prewarmed RPMI 1640 containing 10% fetal calf serum and harvested 2 days later by two washes in phosphate-buffered saline before lysis in luciferase lysis buffer (25 mM glycylglycine, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40). Samples were transfected in duplicate for each experiment.

Luciferase assays were performed essentially as previously described (16). A luminometer from Turner Designs (Sunnyvale, Calif.) was used. Typically, 100 μl of 1 mM luciferin was injected into a tube containing 20 μl of cell lysate, 10 μl of 2.5 mM ATP, 10 μl of 1 M K_2PO_4 (pH 7.8), 10 μl of 100 mM $MgCl_2$, and 50 μl of H_2O. The light output, measured over a 15-s interval, was given in arbitrary units and is therefore expressed relative to that of the basic reporter construct, pTATA-Inr. pCMV-CAT, containing the immediate-early promoter from human cytomegalovirus (45), was included in all transfections as an internal control. Transfections were normalized to levels of chloramphenicol acetyltransferase activity present in each cell lysate.

Chicken embryo fibroblasts (CEF) were obtained from SPAFAS and cultured in medium 199 supplemented with 5% tryptose phosphate broth, 5% fetal calf serum, and 1% chicken serum. CEF were transfected by the calcium phosphate coprecipitation protocol (25). CEF were plated at 2×10^5 per 60-mm-diameter dish the day before transfection. Cells were typically transfected with 10.0 μg of retroviral plasmids and 1.0 μg of pSW253, a plasmid DNA containing a replication-competent clone of reticuloendotheliosis virus strain A. The transfected cells were grown for 4 days in complete medium, at which time they were lysed in ELB buffer and used for DNA binding experiments as previously described (12).

DNA binding analysis of aC/EBP. Typically, 10 μg of nuclear extract was incubated with 10^5 cpm of a ^{32}P-labeled oligonucleotide containing the hC/EBP-β-binding motif and 1.0 μg of poly(dI-dC) · poly(dI-dC) in CDB buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 50 mM NaCl, 5 mM Tris-Cl [pH 7.0], 1 mM dithiothreitol, 15 mM EDTA, 10% glycerol [1]). The DNA-binding probe was synthesized by primer extension with a 1:1 molar ratio of bromodeoxyuridine-thymidine and radiolabeled with [^{32}P]dCTP and [^{32}P]dGTP. The coding strand of the hC/EBP-β probe is 5′-TGAGACATTGCACAATCTGCAT-3′, and the primer sequence is 5′-ATGCAGAT-3′. The underlined 14 nucleotides comprise the human C/EBP-β-binding site as determined in reference 1, and the flanking sequences were selected at random. For competition experiments, complementary oligonucleotides containing either the wild-type or mutant C/EBP-binding sites were annealed by heating to 80°C and slowly cooled over a 1-h period. The sense-strand sequence of the wild-type competitor is 5′-TGAGACATTGCACAATCTG CAT-3′, and the sense-strand sequence of the mutant competitor is 5′-GGACGTCACAcTaCAaAcTCTTAATAA-3′. The 14 nucleotides that comprise the C/EBP-β site are underlined; lowercase letters represent those nucleotides that have previously been demonstrated to abolish binding to the human C/EBP-β site (1). The protein-DNA complexes were allowed to form for 20 min at room temperature before electrophoresis through a 5% polyacrylamide gel.

aC/EBP was tested for dual DNA-binding specificity by using a double-stranded oligonucleotide that contained adjacent hC/EBP-β and palindromic κB sites. The sequence of the coding strand is 5′-CCGAACATTGCACAATCTGGGAAT TCCC-3′; the sequence of the noncoding strand is 5′-TCGG GGGAATTCCCAGATTCTGCAATGT-3′. The underlined nucleotides comprise the hC/EBP-β site and the palindromic κB site. The oligonucleotide was labeled by filling in the 5′ overhang with [^{32}P]dGTP and [^{32}P]dCTP. Double-stranded oligonucleotides in which either the κB site (IL-X-mt-κB) or the C/EBP site (IL-X-mt-C/EBP) was mutated were also synthesized for use in competition experiments. The sequence of the IL-X-mt-κB coding strand is 5′-CCGAACATTGCA CAATCTatctATctCC-3′; the sequence of the IL-X-mt-C/EBP coding strand is 5′-CCGAACACTaCAaAcTCTGGGAATT CCC-3′. The underlined sequences denote the DNA-binding site that has been mutated, and lowercase letters denote the specific nucleotides that have been mutated. The protein-DNA complexes were allowed to form for 20 min at room temperature before electrophoresis through a 5% polyacrylamide gel.

For in situ UV cross-linking, DNA binding reaction mixtures were irradiated with UV light for 10 min before separation through a 5% native polyacrylamide gel. The protein-DNA complexes were visualized by autoradiography at 4°C. The protein-DNA complexes of interest were subsequently excised and irradiated with UV light for an additional 15 min. The gel slices were then placed in the wells of a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel with 50 μl of 2× SDS sample buffer. The complexes were electrophoresed at a constant voltage for 3 h and subsequently visualized by autoradiography.

In some experiments, nuclear extracts were incubated for 45 min on ice with various antisera prior to the DNA binding reaction. The R2 antiserum was raised against amino acids 92 to 456 of v-Rel. The N-terminal anti-RelA peptide serum was obtained from Santa Cruz Biotechnology. The C-terminal anti-RelA peptide serum was raised against a peptide corresponding to C-terminal sequences of human RelA and was obtained from the laboratory of Warner Greene. Antisera

raised against rat C/EBP-α, -β, and -δ were obtained from the laboratory of Steve McKnight. The pan-CRP antiserum (an antiserum raised against a basic-region peptide that is evolutionarily conserved in all C/EBP-related proteins) was obtained from Peter Johnson.

Affinity purification of aC/EBP. MSB-1 nuclear extracts were prepared as described by Dignam et al. (13), and protein concentrations were determined by the Bio-Rad protein assay kit. For affinity purification, the hC/EBP-β oligonucleotide was oligomerized as previously described (19), and the 5′ overhanging ends were filled in with Klenow fragment in the presence of dATP, dCTP, [^{32}P]dGTP as a radiolabel trace, and biotin-11-UTP (Boehringer Mannheim). The hC/EBP-β affinity matrix was prepared by coupling the oligomerized hC/EBP-β motif to streptavidin paramagnetic beads (Promega).

Nuclear extracts were passed over a single-stranded DNA-agarose affinity column (U.S. Biochemical) to remove nonspecific DNA-binding proteins. Fractions containing aC/EBP DNA-binding activity were pooled and added to the hC/EBP-β affinity matrix in the presence of 20 ng of poly(dI-dC) · poly(dI-dC) per 1.0 μg of partially purified MSB-1 nuclear extract. The C/EBP complexes were eluted from the hC/EBP-β affinity matrix by washes with CDB buffer supplemented with increasing increments of KCl.

Rel, Ap2, and Sp1 DNA binding analysis and V8 proteolysis. κB-binding proteins were detected by solution UV cross-linking to a palindromic κB site or a mutant κB site in HDKE buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 10 mM EDTA, 5% glycerol)–0.1 μg of poly(dI-dC) · poly(dI-dC) in a final reaction volume of 20 μl. The coding strand of the palindromic κB probe is 5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′; the sequence of the coding strand of the mutant κB probe is 5′-GAGGTCGacAAagatCTCCCCCCCCCC-3′. The palindromic κB site has previously been demonstrated to be a high-affinity binding site for Rel proteins (2). The mutant κB site is derived from the κB site in the avian c-Rel promoter (16); the sequences which have been changed specifically disrupt binding by Rel proteins, while the sequences flanking the site do not contribute to DNA binding by Rel proteins (data not shown). The probes were synthesized by primer extension with a 1:1 molar ratio of bromodeoxyuridine-thymidine and were radiolabeled with [^{32}P]dCTP and [^{32}P]dGTP. The sequence of the primer for the palindromic κB oligonucleotide is 5′-AAGGAGAGGG-3′; the sequence of the primer specific for the mutant κB oligonucleotide is 5′-GGGGG GGGGG-3′. The reaction mix was irradiated with UV light for 10 min, and an equal volume of 2× sample buffer was added. The samples were then boiled for 3 min and electrophoresed through an SDS–7.5% polyacrylamide gel, and the protein-DNA adducts were visualized by autoradiography.

In situ V8 protease digestions were performed as described by Cleveland et al. (10). Proteins were labeled by solution UV cross-linking to a ^{32}P-labeled oligonucleotide containing a palindromic κB-binding site, and the protein-DNA adducts were electrophoresed through a preparative SDS-polyacrylamide gel. The protein-DNA adducts were excised and soaked in overlay/rehydration buffer (125 mM Tris-HCl [pH 6.8], 1 mM EDTA, 0.1% SDS, 1 mM 2-β-mercaptoethanol, 30% glycerol, bromphenol blue) for 1 min. The gel slices were subsequently placed in the wells of an SDS–12.5% polyacrylamide gel and overlaid with 10 μl of overlay/rehydration buffer and 10 μl of enzyme buffer (125 mM Tris-HCl [pH 6.8], 1 mM EDTA, 0.1% SDS, 1 mM 2-β-mercaptoethanol, 10% glycerol, phenol red) containing V8 protease. The protein-DNA adducts and V8 protease were electrophoresed into the gel, and the reaction was stopped for 30 min to allow digestion to occur. Following digestion, labeled peptides were separated by electrophoresis and visualized by autoradiography.

Ap2 and Sp1 DNA-binding activities were detected by EMSA using a ^{32}P-labeled doubled-stranded oligonucleotide containing a consensus Ap2-binding site or a consensus Sp1-binding site. The double-stranded oligonucleotides were radiolabeled by filling in 5′ overhangs in the presence of [^{32}P]dGTP or [^{32}P]dCTP, respectively. The sequence of the coding strand of the Ap2 oligonucleotide is 5′-TCGACTCCTGGGGAC-3′, and the sequence of the noncoding strand of the Ap2 oligonucleotide is 5′-TCGACTCCCCAGGCG-3′. The sequence of the coding strand of the Sp1 oligonucleotide is 5′-TCGAGC CCCCGCCCCG-3′, and the sequence of the noncoding strand of the Sp1 oligonucleotide is 5′-TCGACGGGGCGGGGGC-3′. DNA binding was carried out in CDB buffer in the presence of 0.1 μg of poly(dI-dC) · poly(dI-dC). The protein-DNA complexes were allowed to form for 20 min at room temperature before electrophoresis through a 5% polyacrylamide gel.

Immunoblot and Southwestern blot analysis. Immunoblot analysis was carried with the ECL (enhanced chemiluminescence) Western blotting (immunoblotting) kit (Amersham) as recommended by the manufacturer. For Southwestern blot analysis, protein samples were electrophoresed through a 10% polyacrylamide gel and transferred to nitrocellulose. The proteins immobilized on the nitrocellulose were subjected to a denaturation-renaturation step with guanidine-HCl. The filter was incubated in binding buffer (25 mM HEPES, 10% glycerol, 100 mM KCl, 5 mM MgCl, 0.1 mM EDTA, 0.1% Nonidet P-40) containing 5% nonfat dried milk for 1 h at room temperature. The filter was washed for 10 min in binding buffer and incubated in binding buffer containing 0.5% nonfat dried milk, 25 μg of poly(dI-dC) · poly(dI-dC) per ml, and the oligomerized hC/EBP-β oligonucleotide labeled with [^{32}P]dGTP for 1 h at room temperature. The nitrocellulose filter was then subjected to two 15-min washes with binding buffer, and the protein-DNA complexes were visualized by autoradiography.

RESULTS

The C/EBP-binding site from the human IL-6 promoter is a functional enhancer element in avian T cells. To determine the level of C/EBP DNA-binding activity in avian cells, nuclear extracts were prepared from several avian cell lines and examined by EMSA with a ^{32}P-labeled oligonucleotide containing the hC/EBP-β DNA-binding site. MSB-1 cells (an avian T-cell line) were found to contain the highest level of C/EBP DNA-binding activity and were therefore chosen for further characterization (the proteins in avian cells that specifically interact with the hC/EBP-β-binding site are referred to as aC/EBP). One major protein-DNA complex (I) and two minor complexes (II and III) were detected in MSB-1 nuclear extracts (Fig. 1A, lane 1). Some variability in the levels of complexes II and III was detected in different extract preparations, and binding by complexes II and III, but not complex I, could be inhibited by increasing the concentration of poly(dI-dC) · poly(dI-dC) (data not shown). The DNA-binding specificity of the aC/EBP was determined in competition experiments using excess unlabeled wild-type hC/EBP-β and mutant hC/EBP-β oligonucleotides. A 50-fold excess of the unlabeled wild-type hC/EBP-β oligonucleotide abolished DNA binding by complexes I, II, and III (Fig. 1A, lane 2). A 50-fold molar excess of an unlabeled oligonucleotide containing a mutant hC/EBP-β oligonucleotide did not efficiently compete for binding by the aC/EBP (Fig. 1A, lane 3).

To determine if the aC/EBP sequence-specific DNA-binding activity in MSB-1 cells correlated with transcriptional activa-

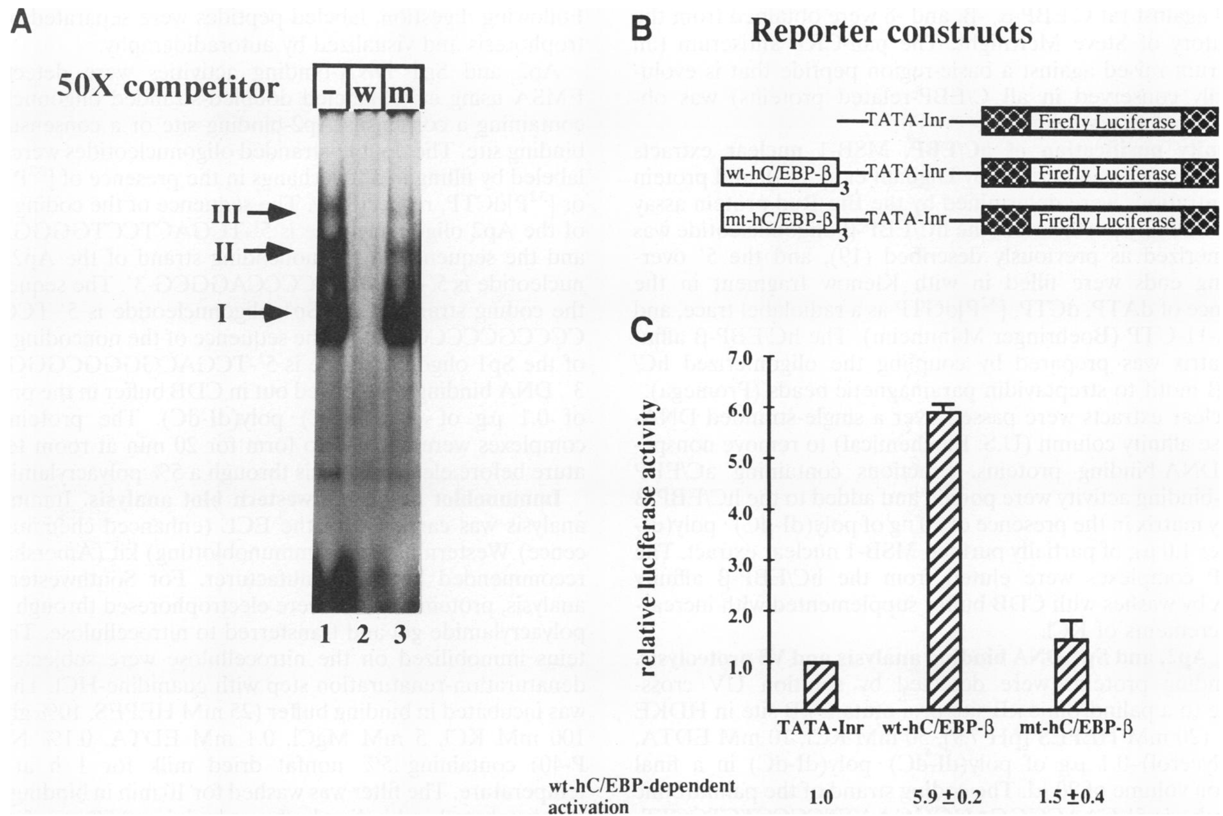

FIG. 1. (A) hC/EBP-β DNA-binding activity in nuclear extracts of MSB-1 cells. MSB-1 nuclear extracts were incubated with the ^{32}P-labeled hC/EBP-β oligonucleotide in the absence of competitor (lanes 1) or in the presence of a 50-fold molar excess of unlabeled wild-type hC/EBP-β oligonucleotide (w; lane 2) or a 50-fold molar excess of a mutant hC/EBP-β oligonucleotide (m; lane 3). Three specific protein-DNA complexes were observed in MSB-1 nuclear extracts. Complexes I to III are indicated at the left. (B) Structures of the luciferase reporter plasmids. An oligonucleotide containing three copies of the wild-type 14-bp hC/EBP-β-binding site was cloned into a plasmid containing a minimal TATA box and initiator element (pTATA-Inr) in front of the firefly luciferase reporter gene (pwt-hC/EBP-β-TATA-Inr). An oligonucleotide containing three copies of a mutant hC/EBP-β-binding site was also cloned into pTATA-Inr (pmt-hC/EBP-β-TATA-Inr). (C) Transcriptional activation through the wild-type hC/EBP-β in MSB-1 cells. Fifteen micrograms of each reporter construct was introduced into 2×10^7 MSB-1 cells by electroporation along with 3.0 μg of plasmid pCMV-CAT as an internal control. The luciferase activities, shown below the graph, are averages of two (pmt-hC/EBP-β-TATA-Inr) or three (pwt-hC/EBP-β-TATA-Inr) independent experiments and are expressed relative to activity of the basic vector (pTATA-Inr). Luciferase expression was normalized to chloramphenicol acetyltransferase activity.

tion, an oligonucleotide containing three copies of the wild-type hC/EBP-β-binding site was cloned into pTATA-Inr, a plasmid containing a minimal promoter driving expression of the firefly luciferase gene (pwt-hC/EBP-β-TATA-Inr; Fig. 1B). An oligonucleotide containing three copies of the mutant hC/EBP-β-binding site was also cloned into pTATA-Inr (pmt-hC/EBP-β-TATA-Inr; Fig. 1B). The ability of each of these constructs to drive expression of the reporter gene was then tested in transient transfection experiments.

Plasmids pwt-hC/EBP-β-TATA-Inr and pmt-hC/EBP-β-TATA-Inr were electroporated into MSB-1 cells along with pCMV-CAT as an internal control. The lysates were collected 36 to 48 h after electroporation, and the level of luciferase activity present in each lysate was determined. Insertion of the wild-type hC/EBP-β trimer resulted in a sixfold activation of luciferase expression relative to the minimal pTATA-Inr vector (Fig. 1C). Insertion of the mutant hC/EBP-β trimer resulted in less than a twofold activation of luciferase expression relative to the minimal pTATA-Inr vector (Fig. 1C). These data demonstrate that the consensus hC/EBP-β-binding site is a functional transcriptional enhancer element in avian T cells.

Presence of a Rel-related protein in aC/EBP-DNA complex I. The ability of Rel proteins to associate with mammalian C/EBP isoforms in vitro and to modulate C/EBP-dependent transcriptional activation in vivo (24, 43) suggested that aC/EBP and Rel family members might associate to form DNA-binding complexes in vivo. Therefore, we examined MSB-1 nuclear extracts for aC/EBP-Rel complexes. We used a polyclonal antiserum raised against v-Rel (R2) in EMSA experiments. The R2 antiserum was raised against a portion of v-Rel that includes the highly conserved Rel homology domain and is able to recognize several Rel family members, including human p50 and human RelA (2). MSB-1 nuclear extracts were incubated with preimmune serum or the R2 antiserum prior to the addition of the ^{32}P-labeled hC/EBP-β oligonucleotide (Fig. 2A). Incubation with preimmune sera did not affect the mobility of the hC/EBP-β DNA-binding complexes, although it did result in a slight increase in binding to this oligonucleotide as a result of increased protein concentration (Fig. 2A, lane 2). Incubation with the R2 antiserum resulted in the formation of a new, more slowly migrating complex with a concomitant decrease in the binding intensity of complex I (complex IV; Fig. 2A, lane 3). Incubation of the ^{32}P-labeled hC/EBP-β

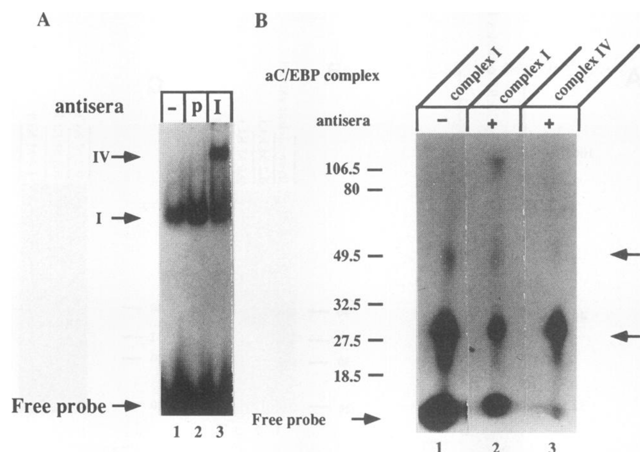

FIG. 2. (A) v-Rel antiserum induces a supershift of aC/EBP-DNA complex I. MSB-1 nuclear extracts were incubated on ice for 45 min in the absence of antiserum (−; lane 1), in the presence of preimmune serum (p; lane 2), or in the presence of immune serum (I; lane 3) prior to the addition of the ^{32}P-labeled hC/EBP-β oligonucleotide. The protein-DNA complexes were subsequently analyzed through a 5% polyacrylamide gel and visualized by autoradiography. Arrows indicate the positions of protein-DNA complexes I and IV. Free probe is indicated at the bottom of the gel. (B) In situ UV cross-linking of aC/EBP-DNA complexes I and IV from MSB-1 nuclear extracts. Nuclear extracts from MSB-1 cells were incubated on ice for 1 h in the absence (−) or presence (+) of the R2 serum prior to the addition of the ^{32}P-labeled hC/EBP-β oligonucleotide. The DNA binding reaction mixture was irradiated with UV light for 10 min prior to separation on a 5% native polyacrylamide gel. The protein-DNA complexes were visualized by autoradiography at 4°C, after which the protein-DNA complexes were cut out and irradiated with UV light for an additional 15 min. The gel slices containing aC/EBP-DNA complexes I and IV were then placed in the wells of an SDS–10% polyacrylamide gel with 50 μl of 2× SDS sample buffer and electrophoresed at constant voltage for 3 h, after which they were visualized by autoradiography. Arrows indicate the proteins of 20 and 43 kDa present in complex I and complex IV (lanes 1 to 3). Positions of molecular weight markers are indicated in kilodaltons at the left.

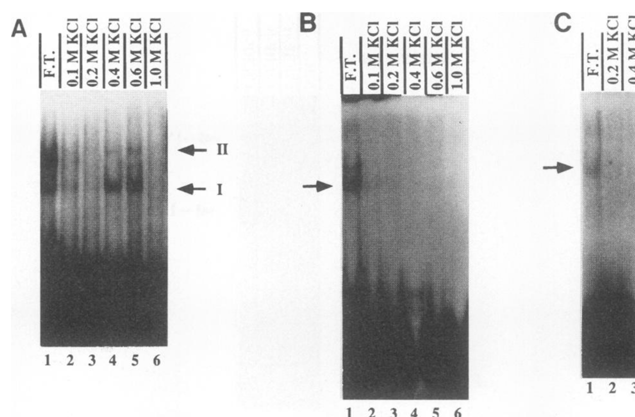

FIG. 3. (A) Profile of C/EBP DNA-binding activity after sequence-specific DNA affinity purification. aC/EBP was eluted from the hC/EBP-β affinity matrix with increasing concentrations of KCl (indicated at the top). Fractions containing aC/EBP DNA-binding activity were determined by subjecting a portion of each column fraction to EMSA with a ^{32}P-labeled hC/EBP-β probe. Complexes I and II are indicated by the arrows at the left. F.T., flowthrough (B) Detection of Ap2 DNA-binding activity. Column fractions from the hC/EBP-β affinity matrix were assayed for proteins that bound to an Ap2 consensus motif. The fractions are indicated at the top, and the arrow at the left identifies the Ap2-DNA complex. (C) Detection of Sp1 DNA-binding activity. Column fractions from the hC/EBP-β affinity matrix were assayed for proteins that bound to an Sp1 consensus motif. The fractions are indicated at the top, and the arrow at the left identifies the Ap2-DNA complex.

oligonucleotide with the R2 antiserum in the absence of nuclear extract confirmed that this antiserum does not bind directly to the hC/EBP-β oligonucleotide (data not shown).

The induction of complex IV by the R2 antiserum with the concomitant decrease in the binding intensity of complex I suggested that the antiserum might be binding to a Rel-related protein present in this aC/EBP-DNA complex, resulting in a protein-DNA complex of reduced mobility (supershift). An alternative explanation is that incubation of the nuclear extracts with the R2 antiserum might activate a new aC/EBP DNA-binding complex by inducing the dissociation of an inhibitory protein. To determine if the appearance of complex IV was the result of the ability of the R2 antiserum to supershift a portion of complex I or if it induced a new aC/EBP-binding activity, we performed in situ UV cross-linking on complex I from MSB-1 nuclear extracts before and after incubation with the R2 antiserum (Fig. 2B). In situ UV cross-linking of complex I prior to incubation with the R2 antiserum resulted in the labeling of proteins of approximately 20 and 40 kDa (after the contribution of the single-stranded oligonucleotide to the mobility of the protein-DNA complex is subtracted; Fig. 2B, lane 1). In situ UV cross-linking of complex I following incubation with the R2 antiserum resulted in the same pattern of labeled proteins as detected prior to incubation with the R2 antiserum (Fig. 2B; compare lanes 1 and 2). In situ UV cross-linking of complex IV resulted in labeled proteins identical in mobility to those cross-linked in complex I (Fig. 2B, lane 3), indicating that addition of the R2 antiserum induces a supershift of complex I rather than the induction of a new aC/EBP DNA-binding activity.

Purification of aC/EBP. The ability of the R2 antiserum to supershift a portion of aC/EBP complex I indicated that one or more Rel-related proteins are components of this DNA-binding complex. To further characterize the aC/EBP complex, we used sequence-specific DNA affinity chromatography to purify the aC/EBP complex from MSB-1 nuclear extracts. MSB-1 nuclear extracts were initially passed over a single-stranded DNA-agarose affinity column to remove nonspecific DNA-binding proteins; subsequently, fractions containing aC/EBP DNA-binding activity were pooled and subjected to affinity purification with an oligomerized hC/EBP-β-binding motif coupled to magnetic beads. The DNA affinity matrix was washed extensively with buffer containing increasing concentrations of salt; less than 1% of the cellular protein added to the C/EBP affinity matrix remained bound to the matrix after one wash with CDB buffer (data not shown). aC/EBP DNA-binding activity routinely eluted between the 0.4 and 0.6 M KCl washes (Fig. 3A, lanes 4 and 5). DNA binding by two other sequence-specific DNA-binding proteins, Ap2 and Sp1, was detected only in the flowthrough fraction (Fig. 3B and C, respectively).

We next used antisera raised against mammalian C/EBP-α, -β, and -δ in EMSA experiments to determine which C/EBP isoforms are present in the aC/EBP complex. The respective antisera were added to the purified protein prior to EMSA. Addition of the anti-C/EBP-α and anti-C/EBP-β sera did not affect the mobility of the aC/EBP-DNA complex (Fig. 4, lanes 2 and 3). However, addition of the anti-C/EBP-δ serum resulted in the induction of a weak supershift (Fig. 4, lane 4).

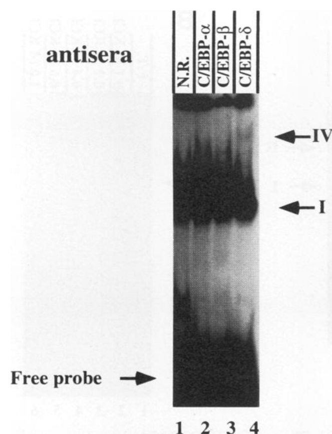

FIG. 4. Antiserum raised against rat C/EBP-δ induces a supershift of the aC/EBP-DNA complex. Purified aC/EBP was incubated with normal rabbit serum or antiserum raised against mammalian C/EBP for 45 min prior to incubation with the ^{32}P-labeled hC/EBP-β oligonucleotide. The antisera and amounts used were as follows: 2.0 μl of normal rabbit serum (N.R.; lane 1), 2.0 μl of anti-C/EBP-α serum (lane 2), 2.0 μl of anti-C/EBP-β serum (lane 3), and 2.0 μl of anti-C/EBP-δ serum (lane 4). The protein-DNA complexes were electrophoresed through a 5% polyacrylamide gel and visualized by autoradiography. The arrows indicate the positions of complexes I and IV.

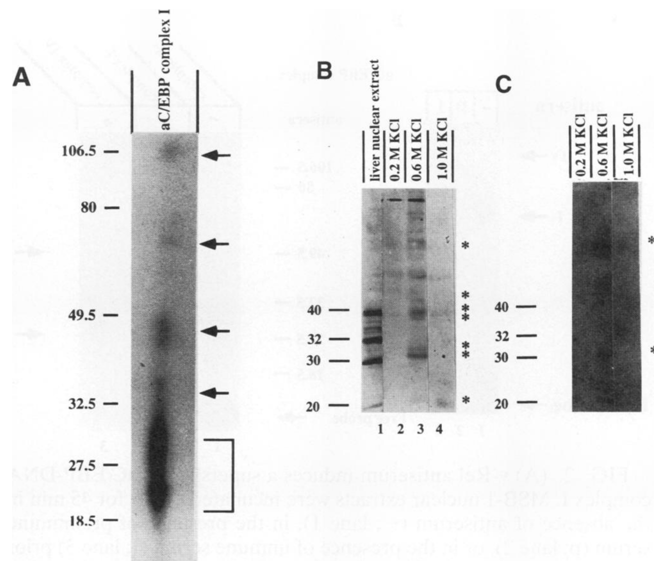

FIG. 5. (A) In situ cross-linking of purified aC/EBP-DNA complex I. Purified aC/EBP was subjected to a standard DNA binding reaction with the ^{32}P-labeled hC/EBP-β probe. Subsequently, the DNA binding reaction mixture was irradiated with UV light for 10 min prior to separation on a 5% native polyacrylamide gel. The aC/EBP-DNA complex I was visualized by autoradiography at 4°C, after which the protein-DNA complex was cut out and irradiated with UV light for an additional 15 min. The gel slice containing aC/EBP-DNA complex I was then placed in the well of an SDS–10% polyacrylamide gel with 50 μl of 2× SDS sample buffer and electrophoresed at constant voltage for 3 h, after which the protein-DNA adducts were visualized by autoradiography. Arrows indicate the proteins of approximately 30, 40, 55, and 100 kDa present in complex I, and the bracket indicates the cross-linked 20-kDa polypeptides. Positions of molecular weight markers (in kilodaltons) are indicated at the left. (B) Pan-CRP immunoblot of the purified aC/EBP. Equivalent volumes of the 0.2 (lane 2), 0.6 (lane 3), and 1.0 (lane 4) M KCl fractions from the hC/EBP-β affinity matrix or 2.0 μl of rabbit liver nuclear extract (lane 1) was electrophoresed through a 10% polyacrylamide gel, transferred to nitrocellulose, and blotted with the pan-CRP antiserum. The immune complexes were visualized by ECL (Amersham). Positions of four C/EBP family members recognized by the pan-CRP antiserum in the rabbit nuclear extract are indicated in kilodaltons at the left. Positions of the purified avian C/EBP-related proteins are indicated by the asterisks at the right. (C) Southwestern blot analysis of the purified aC/EBP. Subsequent to immunoblot analysis of the purified aC/EBP, the proteins immobilized on the filter were subjected to a denaturation-renaturation step with guanidine-HCl and probed with an oligomerized, ^{32}P-labeled hC/EBP-β probe. Positions of C/EBP family members from the rabbit liver nuclear extracts are indicated in kilodaltons at the left as molecular weight markers. Positions of the purified aC/EBPs that bind to the oligomerized hC/EBP-β site are indicated by the asterisks at the right.

The ability of the anti-C/EBP-δ serum to supershift the aC/EBP complex suggests that an avian C/EBP-δ-related protein is a component of this complex. The inability of the anti-C/EBP-δ serum to efficiently supershift the aC/EBP complex is likely a reflection of a weak antibody-antigen interaction due to cross-species variation. Alternatively, the lack of a quantitative supershift could reflect the presence of multiple protein-DNA complexes of similar electrophoretic mobilities.

To further characterize the affinity-purified aC/EBP, in situ UV cross-linking was performed. In situ UV cross-linking of the aC/EBP with a ^{32}P-labeled oligonucleotide containing the hC/EBP-β site resulted in the cross-linking of proteins of 20, 30, 40, 55, and 100 kDa (Fig. 5A). Thus, in addition to the 20- and 40-kDa proteins detected by in situ UV cross-linking of the crude nuclear extracts, additional proteins of 30, 55, 100 kDa are present in the purified aC/EBP and directly contact the C/EBP site. The failure to cross-link the 30-, 55-, and 100-kDa proteins in the crude nuclear extracts is likely due to the low level of sensitivity obtained with unpurified protein.

To confirm that the purified proteins were C/EBP family members, the 0.2, 0.6, and 1.0 M KCl washes of the C/EBP affinity matrix were examined by immunoblot analysis with the pan-CRP antiserum. This antiserum recognizes all mammalian C/EBP isoforms by immunoblot analysis (18a). Equivalent volumes of each of the fractions and a portion of a rabbit liver nuclear extract were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to nitrocellulose. Rabbit liver nuclear extract was used as a positive control for the immunoblot. The pan-CRP antiserum recognizes proteins of approximately 20, 30, 32, and 42 kDa in this extract (Fig. 5B, lane 1). Immunoblot analysis with the pan-CRP antiserum demonstrated that C/EBP-related proteins in the size range of 20, 30, and 40 kDa elute in the 0.6 M KCl fraction and to a lesser extent in the 1.0 M KCl fraction (Fig. 5B, lanes 3 to 5). Several proteins with relative mobilities greater than 100 kDa are also recognized by the pan-CRP anti-C/EBP-δ serum (Fig. 5B, lanes 3 to 5). Normal rabbit serum failed to recognize the 20-, 30-, or 40 kDa protein (data not shown). These results indicate the 20-, 30-, and 40-kDa proteins are C/EBP-related proteins.

Following immunoblot analysis, the nitrocellulose filter was subjected to Southwestern analysis with the ^{32}P-labeled hC/EBP-β oligonucleotide. Proteins of approximately 30 and 100 kDa bound to the hC/EBP-β oligonucleotide (Fig. 5C, lane 2). This result demonstrates that the purified 30-kDa C/EBP-related protein identified by immunoblot binds directly to the hC/EBP-β site. The inability of the other C/EBP-related proteins to bind to the C/EBP oligonucleotide by a Southwestern blot may reflect their inability to renature under the conditions of the experiment. The combined results from in

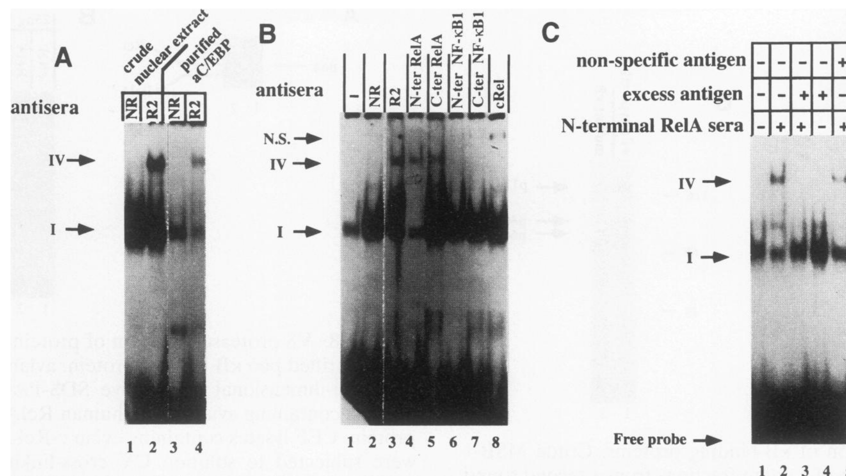

FIG. 6. (A) The aC/EBP-Rel complex remains intact throughout affinity purification. Eight micrograms of crude MSB-1 nuclear extract or 2.0 μl of purified aC/EBP was incubated with 1.0 μl of normal rabbit serum (NR; lanes 1 and 3) or 1.0 μl of the R2 antiserum (lanes 2 and 4) prior to incubation with the ^{32}P-labeled hC/EBP-β oligonucleotide. The protein-DNA complexes were electrophoresed through a 5% polyacrylamide gel and visualized by autoradiography. Arrows indicate the positions of complexes I and IV. (B) Antisera raised against two distinct human RelA-derived peptides also induce a supershift of the purified aC/EBP DNA-binding activity. Purified aC/EBP was incubated in the absence or presence of antisera for 45 min prior to incubation with the ^{32}P-labeled hC/EBP-β oligonucleotide. The antisera and amounts used were as follows: no antiserum (−; lane 1), 2.0 μl of normal rabbit serum (NR; lane 2), 1.0 μl of R2 antiserum (R2; lane 3), 2.0 μl of N-terminal human RelA antiserum (N-ter RelA; lane 4), 2.0 μl of C-terminal human RelA antiserum (C-ter RelA; lane 5), 2.0 μl of N-terminal human NF-κB1 antiserum (N-ter NF-κB1; lane 6), 2.0 μl of C-terminal human NF-κB1 antiserum (C-ter NF-κB2; lane 7), and 2.0 μl of C-terminal avian c-Rel antiserum (cRel); lane 8). The protein-DNA complexes were electrophoresed through a 5% polyacrylamide gel and visualized by autoradiography. Arrows indicate the positions of complexes I and IV and a nonspecific complex (N.S.) that is also induced by certain lots of normal rabbit sera. (C) Excess N-terminal RelA peptide antigen prevents the induction of the aC/EBP supershift by the N-terminal RelA peptide serum. The N-terminal RelA peptide serum was incubated with an excess of the peptide antigen to which it was raised or an excess of a nonspecific peptide antigen for 15 min prior to its addition to the purified aC/EBP. Subsequently, 2.0 μl of the RelA peptide serum (lane 2), 2.0 μl of the RelA peptide serum plus antigen (lane 3), antigen alone (lane 4), or 2.0 μl of the RelA peptide serum plus nonspecific antigen (lane 5) was added to the purified protein and incubated on ice for another 45 min prior to addition of the ^{32}P-labeled C/EBP oligonucleotide. The protein-DNA complexes were electrophoresed through a 5% polyacrylamide gel and visualized by autoradiography. Arrows indicate the positions of complexes I and IV. Free probe is indicated at the bottom of the gel.

situ UV cross-linking, immunoblot analysis, and Southwestern blotting demonstrate that avian C/EBP-related proteins of approximately 20, 30, and 40 kDa were purified by DNA affinity chromatography and are components of aC/EBP-DNA complex I.

A RelA-related protein is a stable component of the C/EBP-DNA complex. To determine if a Rel protein(s) was still associated with aC/EBP after sequence-specific DNA affinity purification, the purified aC/EBP DNA-binding activity was incubated with either normal rabbit serum or the R2 antiserum prior to EMSA. Incubation of the purified aC/EBP with the R2 antiserum but not normal rabbit serum induced a supershift of complex I (complex IV) that was indistinguishable in mobility from the supershift induced by the R2 antiserum in the crude MSB-1 nuclear extract (Fig. 6A; compare lanes 2 and 4). The inability of the R2 antiserum to quantitatively supershift complex I is perhaps due to the presence of a comigrating aC/EBP complex that does not contain a Rel-related protein that is recognized by the R2 antiserum. Alternatively, the inability of the R2 antiserum to quantitatively supershift complex I might be due to a low binding affinity of the R2 antiserum toward the Rel-related protein present in the aC/EBP-DNA complex. To determine which Rel family members were present in complex I, antisera raised against various Rel proteins were used in EMSA experiments (Fig. 6B). Antisera raised against human NF-κB1-derived peptides failed to affect the mobility of the aC/EBP-DNA complex (Fig. 6B, lanes 6 and 7). Antiserum raised against the C terminus of avian cRel also failed to affect the mobility of the aC/EBP-DNA complex (Fig. 6B, lane 8). Affinity-purified immunoglobulin G raised against an N-terminal human RelA-derived peptide and antiserum raised against a C-terminal human RelA-derived peptide induced a supershift of complex I identical in mobility to that induced by the R2 antiserum (Fig. 6B, lanes 3 to 5). Incubation of the N-terminal RelA peptide serum with excess cognate peptide prior to its addition to the purified protein efficiently inhibited the supershift induced by the N-terminal RelA peptide serum (Fig. 6C, lanes 1 to 3). Peptide alone had no effect on the mobility of the aC/EBP-DNA complex (Fig. 6C, lane 4). Addition of excess nonspecific peptide did not affect the supershift induced by the N-terminal RelA peptide serum (Fig. 6C, lane 5), thereby demonstrating the specificity of the antigen-antibody interaction. These results demonstrate that an avian protein antigenically related to RelA is a component of the aC/EBP-DNA complex.

The purified aC/EBP proteins were subjected to a second round of sequence-specific DNA affinity purification. aC/EBP DNA-binding activity was eluted at 0.6 M KCl, as determined by EMSA (data not shown). To determine which Rel protein(s) copurified with aC/EBP, fractions from the second round of purification were subjected to solution UV cross-linking to an oligonucleotide containing a palindromic κB site (Fig. 7A). κB-binding proteins were absent from the low-salt washes (Fig. 7A, lanes 2 to 4). κB-binding proteins of approximately 118 kDa (p118) and a group of four proteins whose molecular sizes ranged from 65 to 80 kDa were detected in the 0.6 M KCl fraction (after the contribution of the single-

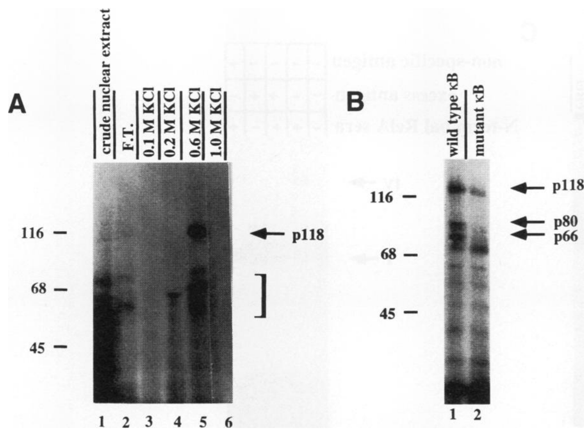

FIG. 7. (A) Copurification of κB-binding proteins. Crude MSB-1 nuclear extract and a portion of column fractions from a second round of DNA affinity purification were tested for proteins that bound to a palindromic κB motif. The KCl concentrations used to elute each fraction from the DNA affinity matrix are indicated at the top. κB-binding proteins eluted from the hC/EBP-β affinity matrix in the presence of 0.6 M KCl (lane 5). The arrow indicates a κB-binding protein of approximately 118 kDa that specifically copurified over the C/EBP DNA affinity matrix. The bracket indicates the approximate positions of four additional copurifying κB-binding proteins in the 65- to 80-kDa size range. Approximate positions of molecular weight markers are indicated in kilodaltons at the left. (B) DNA-binding specificity of the copurified κB-binding proteins. The fraction containing the purified aC/EBP DNA-binding activity following a single round of DNA affinity purification was subjected to solution UV cross-linking to an oligonucleotide containing either a palindromic κB site or a mutant κB site. The protein-DNA adducts were electrophoresed through a 7.5% polyacrylamide gel and visualized by autoradiography. Arrows indicate positions of the proteins that specifically bind to the palindromic κB site. Approximate positions of molecular weight markers are indicated in kilodaltons at the left.

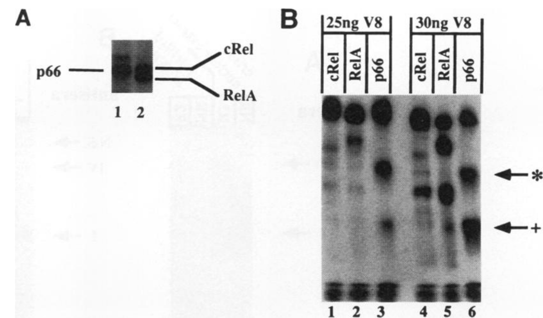

FIG. 8. V8 protease digestion of protein-DNA adducts containing the copurified p66 κB-binding protein, avian c-Rel, and human RelA. (A) One-dimensional preparative SDS-PAGE of the protein-DNA adducts containing avian c-Rel, human RelA, and the p66 κB-binding protein. CEF lysates containing avian c-Rel and human RelA (lane 2) were subjected to solution UV cross-linking to a palindromic κB-binding site in parallel with purified κB-binding proteins (lane 1). Protein-DNA adducts were electrophoresed through an SDS–7% polyacrylamide gel and visualized by autoradiography. The portion of the gel containing the indicated proteins is shown. The protein-DNA adducts containing c-Rel, RelA, and p66 are indicated. (B) Digestion of the protein-DNA adducts containing avian c-Rel, human RelA, and p66 with V8 protease. Gel slices containing the protein-DNA adducts of c-Rel (lanes 1 and 4), RelA (lanes 2 and 5), and p66 (lanes 3 and 6) were excised and placed in the wells of an SDS–12.5% polyacrylamide gel with 25 ng (lanes 1 to 3) or 30 ng (lanes 4 to 6) of V8 protease. The protein-DNA adducts and V8 protease were electrophoresed into the gel, and electrophoresis was stopped. After digestion, electrophoresis was resumed and the labeled peptides were visualized by autoradiography. The p66-derived peptide and c-Rel-derived peptide of similar mobilities are indicated by *; the p66-derived peptide and RelA-derived peptide of similar mobility are indicated by +.

stranded oligonucleotide to the mobility of the protein-DNA complex is subtracted [Fig. 7A, lane 5]).

To determine if the copurified κB-binding proteins bound specifically to the κB site, proteins in the 0.6 M KCl wash were subjected to solution UV cross-linking to an oligonucleotide containing either the palindromic κB site or a mutant κB site (Fig. 7B). κB-binding proteins of approximately 80 kDa (p80) and 66 kDa (p66) that bound to the palindromic κB site but not the mutant κB site were identified (Fig. 7B, lane 1). The relative mobility of p66 suggested that it may be the avian RelA homolog. Thus, we compared the mobility of the p66-DNA adduct with that of the avian c-Rel and human RelA protein-DNA adducts. A retroviral vector encoding human RelA was transfected into CEF, and whole cell lysates were collected and used for solution UV cross-linking experiments in parallel with the copurified κB-binding proteins. The p66-DNA adduct had an electrophoretic mobility intermediate to those of the avian c-Rel and the human RelA protein-DNA adducts (Fig. 8A). The protein-DNA adducts containing avian c-Rel, human RelA, and p66 were excised from the gel and subjected to in situ digestion with V8 protease. In situ digestion of these protein-DNA adducts with 10 or 30 ng of V8 protease resulted in distinct peptide patterns for each of these three proteins (Fig. 8B), thereby distinguishing p66 from human RelA and avian c-Rel. p66 does have one peptide fragment that comigrates with a RelA-derived peptide (Fig. 8B, lanes 5 and 6, +) and one peptide fragment that comigrates with a c-Rel-derived peptide (Fig. 8B, lanes 1 and 3, *).

In addition to p66 and p80, proteins of approximately 118 kDa bound to the palindromic κB site and to the mutant κB site (Fig. 7B, lanes 1 and 2). Electrophoresis of p118 cross-linked to the palindromic κB site revealed that it could be resolved into a doublet of two distinct proteins (p118A and p118B; Fig. 9A, lane 1). As the apparent molecular size of the p118 proteins was close to that predicted for NF-κB1 or NF-κB2, the mobility of the p118-DNA adducts was compared with those of the protein-DNA adducts of NF-κB1 and NF-κB2 (Fig. 9A). To determine if p118A or p118B was identical to either NF-κB1 or NF-κB2, the protein-DNA adducts were excised from the gel and subjected to digestion with V8 protease (Fig. 9B). Partial in situ digestion of the p118A-DNA adduct and the p118B-DNA adduct with either 25 or 100 ng of V8 protease resulted in the appearance of distinct patterns of peptides (Fig. 9B, lanes 1, 2, 5, and 6), confirming that p118A and p118B are distinct proteins. Partial digestion of the NF-κB1–DNA adduct and the NF-κB2–DNA adduct with V8 protease also resulted in the appearance of distinct patterns of peptides (Fig. 9B, lanes 3, 4, 7, and 8). Comparison of the proteolytic fragments of the p118A-DNA and p118B-DNA adducts with those of the NF-κB1–DNA and NF-κB2–DNA adducts indicates that p118A and p118B are distinct from NF-κB1 and NF-κB2 (Fig. 9B, lanes 1 to 8).

The aC/EBP-RelA complex exhibits singular DNA-binding specificity. The results obtained from UV cross-linking experiments indicate that the Rel family members present in the purified aC/EBP-Rel complex do not directly contact the C/EBP site. However, DNA binding by several copurifying κB-binding proteins can be detected by solution UV cross-

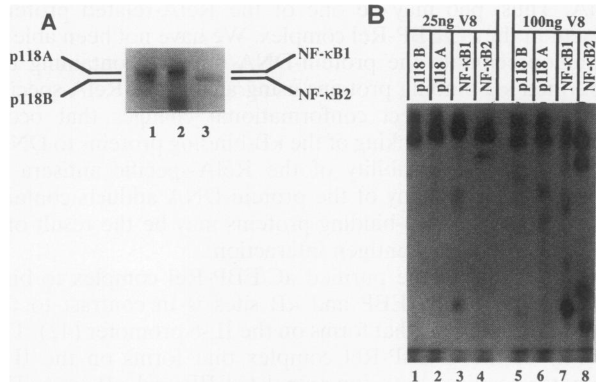

FIG. 9. V8 protease digestion of protein-DNA adducts containing the copurified p118 κB-binding proteins, avian NF-κB1, and NF-κB2. (A) One-dimensional preparative SDS-PAGE of the protein-DNA adducts containing avian NF-κB1, NF-κB2, and the p118 κB-binding proteins. CEF lysates containing avian NF-κB1 and NF-κB2 (lanes 2 and 3) were subjected to solution UV cross-linking to a palindromic κB-binding site in parallel with copurified κB-binding proteins (lane 1). Protein-DNA adducts were electrophoresed through an SDS–7% polyacrylamide gel and visualized by autoradiography. The portion of the gel containing the indicated proteins is shown. The bands corresponding to the protein-DNA adducts containing p118A, p118B, NF-κB1, and NF-κB2 are indicated. (B) Digestion of the protein-DNA adducts containing p118A, p118B, NF-κB1, and NF-κB2 in situ with V8 protease. The protein-DNA adducts from the one-dimensional SDS-polyacrylamide gel containing p118B (lanes 1 and 5), p118A (lanes 2 and 6), NF-κB1 (lanes 3 and 7), and NF-κB2 (lanes 4 and 8) were excised and placed in the wells of an SDS–12.5% polyacrylamide gel with 25 ng (lanes 1 to 4) or 100 ng (lanes 4 to 8) of V8 protease. The protein-DNA adducts and V8 protease were electrophoresed into the stacking gel, and electrophoresis was stopped for 30 min. After digestion, electrophoresis was resumed and the labeled peptides were visualized by autoradiography.

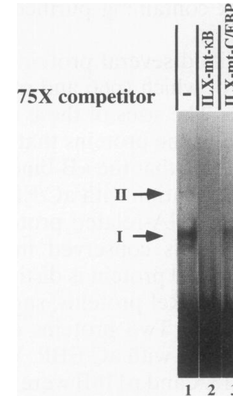

FIG. 10. The aC/EBP-Rel complex exhibits DNA-binding specificity for a consensus C/EBP site rather than a κB site. The purified aC/EBP-Rel complex was incubated with a ^{32}P-labeled oligonucleotide containing adjacent hC/EBP-β and κB sites (IL-X) in the absence of competitor (−; lanes 1) or in the presence of a 75-fold molar excess of the unlabeled oligonucleotide IL-X containing a mutated κB site (IL-X-mt-κB; lane 2) or a 75-fold molar excess of the IL-X oligonucleotide containing a mutated hC/EBP-β site (IL-X-mt-C/EBP; lane 3). The protein-DNA complexes were electrophoresed through a 5% polyacrylamide gel and visualized by autoradiography. Arrows at the left indicate the positions of the protein-DNA complexes.

linking using an oligonucleotide containing a κB site. These results suggested that the C/EBP-Rel complex might have a dual DNA-binding specificity for both C/EBP and κB sites. To examine this possibility, we synthesized an oligonucleotide in which a κB site was placed immediately adjacent to a C/EBP-binding site (IL-X), analogous to the spatial arrangement found in the IL-8 promoter. Two mutant versions of the IL-X oligonucleotide were also synthesized for use as competitors: one in which the κB site was mutated, leaving only a functional C/EBP site (IL-X-mt-κB), and one in which the C/EBP site was mutated (IL-X-mt-C/EBP), leaving only a functional κB site. Use of the ^{32}P-labeled IL-X oligonucleotide as an EMSA probe with the purified aC/EBP-Rel complex resulted in the formation of two complexes with electrophoretic mobilities similar to that of complexes I and II that form on the hC/EBP-β oligonucleotide (Fig. 10, lane 1, and data not shown). Addition of a 75-fold molar excess of unlabeled IL-X-mt-κB oligonucleotide resulted in the complete inhibition of both complexes (Fig. 10, lane 2), while addition of a 75-fold molar excess of the IL-X-mt-C/EBP did not compete for binding to the IL-X oligonucleotide (Fig. 10, lane 3). Experiments using a ^{32}P-labeled IL-X-mt-κB oligonucleotide or a ^{32}P-labeled IL-X-mt-C/EBP oligonucleotide as the EMSA probe with the purified aC/EBP-Rel complex indicated that the κB site does not contribute to the binding affinity of the aC/EBP-Rel complex I to the IL-X probe (data not shown). These results demonstrate that the aC/EBP-Rel complex I does not have a dual DNA-binding specificity for both C/EBP and κB sites.

DISCUSSION

Identification of an in vivo-derived C/EBP-Rel complex. We have purified a protein complex from an avian T-cell line that contains both C/EBP and Rel family members. We have purified this complex by virtue of its sequence-specific DNA-binding activity toward a consensus C/EBP site. That the DNA-binding components of this protein complex are avian C/EBP family members was established by several criteria. First, the complex binds in a sequence-specific manner to a consensus C/EBP site. Second, three of the proteins purified by sequence-specific DNA affinity chromatography were recognized by an antiserum that recognizes all known mammalian C/EBP-related proteins. A combination of in situ UV cross-linking and Southwestern blotting analysis confirmed that the proteins recognized by the pan-CRP antiserum bound directly to DNA containing a C/EBP site. Third, an antiserum raised against rat C/EBP-δ induced a supershift of the aC/EBP-DNA complex in EMSA experiments, suggesting that a C/EBP-δ-related protein is a component of the aC/EBP-DNA complex.

The results presented in this report indicate that an avian RelA-related protein stably associates with aC/EBP. An anti-peptide serum raised against an N-terminal human RelA-derived peptide induced a supershift of the purified aC/EBP-DNA complex that was specifically blocked by excess peptide antigen. As Rel proteins do not bind to DNA containing C/EBP sites (reference 42 and data not shown), it is likely that this RelA-related protein is a component of the aC/EBP-DNA complex by virtue of direct protein-protein interactions with aC/EBP.

Multiple C/EBP-Rel complexes. Several avian C/EBP-related proteins, ranging in size from 20 to 100 kDa, were purified by DNA affinity chromatography. These proteins are recognized by anti-C/EBP sera and bind directly to DNA containing a consensus C/EBP site. As mammalian C/EBP can form both hetero- and homodimeric complexes, it is likely that aC/EBP-DNA complex I consists of several dimeric combinations of the avian C/EBP. The ability of an antiserum raised against rat C/EBP-δ to induce only a partial supershift of the

protein-DNA complex containing purified aC/EBP is consistent with this hypothesis.

We have also identified several proteins that copurify with aC/EBP, at least two of which (p66 and p80) bind specifically to a consensus κB site. The sizes of these κB-binding proteins are distinct from those of the proteins that directly contact the C/EBP site, demonstrating that the κB-binding proteins copurify by virtue of their association with aC/EBP. The p66 protein is likely to be an avian RelA-related protein, as it contains a V8-derived peptide that is conserved in the human RelA protein. The size of the p80 protein is distinct from that of any currently identified avian Rel proteins, suggesting it may be a novel Rel-related protein. Two proteins of 118 kDa (p118A and p118B) also copurified with aC/EBP. V8 peptide mapping confirmed that the p118A and p118B were distinct proteins. In addition, the V8 peptide maps of both p118 proteins are distinct from those of other known Rel family members of comparable size. The p118 proteins may represent novel Rel-related family members, or alternatively, they may be non-Rel-related proteins (9). A copurified protein of approximately 118 kDa also bound to the mutant κB site. The mobility of the nonspecific p118 DNA-binding protein was slightly faster than that of the p118A/B doublet (Fig. 7B), suggesting that the p118 protein that bound to the mutant κB site is distinct from the p118 proteins that bound to the palindromic κB site. Alternatively, p118A or p118B may possess nonspecific DNA-binding activity.

C/EBP and Rel proteins form a variety of inter- and intrafamily heteromeric complexes in vitro (7, 23, 31, 47). The copurification of multiple C/EBP and κB-binding proteins in concert with the ability of C/EBP and Rel to form heteromeric complexes in vitro suggests that multiple C/EBP-Rel complexes likely exist in vivo. Consistent with this hypothesis, the R2 and RelA-specific antisera induced only a partial supershift of the aC/EBP-DNA complex I. In addition, aC/EBP-DNA complex I can be resolved into two closely migrating complexes in some EMSA experiments (Fig. 10). Both of these complexes appear to contain a Rel-related protein(s) (data not shown). Thus, aC/EBP complex I is likely to be a heterogeneous mixture of aC/EBP-Rel complexes that exhibit sequence-specific DNA-binding activity for a consensus C/EBP site.

Functional significance of C/EBP-Rel association. Although the purified aC/EBP-Rel complex contains both C/EBP and Rel family members, it does not have a dual DNA-binding specificity for both C/EBP and κB sites. The ability of the purified aC/EBP-Rel complex to bind DNA in EMSA experiments is strictly dependent upon a functional C/EBP site. The inability of the aC/EBP-Rel complex to bind DNA through a κB site demonstrates that the associated Rel protein(s) does not bind directly to a κB site when present as a component of the C/EBP-Rel complex. The apparent ability of the C/EBP component of this complex to inhibit DNA binding by the associated Rel-related proteins is consistent with the observed repression of κB-dependent gene expression by C/EBP (42, 43) and suggests a possible biochemical mechanism for the observed repression of κB-dependent gene expression.

We were able to detect sequence-specific DNA binding by two copurified proteins (p66 and p80) to a κB site by solution UV cross-linking. The ability to detect κB DNA binding by solution UV cross-linking experiments may reflect the ability of the solution UV cross-linking experiment to covalently capture weak protein-DNA interactions. In contrast, a weak protein-DNA interaction may not withstand electrophoresis during EMSA. One of the copurified κB-binding proteins, p66, is of the expected molecular size for avian RelA (17) and contains a V8-derived peptide that is conserved with human RelA. Thus, p66 may be one of the RelA-related proteins present in the aC/EBP-Rel complex. We have not been able to immunoprecipitate the protein-DNA adducts containing the copurified κB-binding proteins using any of the RelA-specific sera. This may reflect conformational changes that occur following UV cross-linking of the κB-binding proteins to DNA. Alternatively, the inability of the RelA-specific antisera to immunoprecipitate any of the protein-DNA adducts containing the copurified κB-binding proteins may be the result of a low-affinity antibody-antigen interaction.

The inability of the purified aC/EBP-Rel complex to bind simultaneously to C/EBP and κB sites is in contrast to the C/EBP-Rel complex that forms on the IL-8 promoter (42). The stability of the C/EBP-Rel complex that forms on the IL-8 promoter requires both functional C/EBP and κB sites. The DNA-binding specificity and the stability of the aC/EBP-Rel complex that we have purified versus that of the C/EBP-Rel complex that forms on the IL-8 promoter may reflect differences in composition of the complexes. While C/EBP-β is the C/EBP component of the IL-8 complex, we have been unable to detect C/EBP-β in the aC/EBP-Rel complex. Instead, our results indicate that a C/EBP-δ-related protein is a component of the aC/EBP-Rel complex. Alternatively, the differences in the stability and DNA-binding specificity of these two distinct C/EBP-Rel complexes may reflect cell-type-specific posttranslational modifications to one or more of the components.

An increasing body of evidence indicates that constitutive expression of inflammatory cytokines contributes to various malignancies and autoimmune diseases (1). Regulation of both IL-6 and IL-8 gene expression is mediated, in part, by the synergistic actions of two distinct transcription factor families, C/EBP and Rel (26, 28, 42). Several mechanisms can now be developed in which interactions between C/EBP and Rel family members might modulate expression of genes containing C/EBP and/or Rel-binding sites. First, C/EBP and Rel family members can cooperatively bind to adjacent motifs and thereby direct transcription of the corresponding gene, as has been demonstrated for IL-8 (26, 43). Second, RelA can enhance binding by C/EBP-β to DNA in vitro without stably associating with the C/EBP-DNA complex. Third, the presence in a C/EBP-DNA complex of Rel-related proteins that do not directly contact the C/EBP site is consistent with an adaptor function for Rel proteins. The ability of certain Rel family members to associate with the TATA-binding protein and TFIIB further supports the potential role of Rel family members as adaptor proteins (21, 48). Other roles for the Rel-related components of the C/EBP-DNA complex, including stabilization of DNA binding by the C/EBP proteins or alteration of sequence specificity, can also be envisioned.

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