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Identification of Heterogeneous Nuclear Ribonucleoprotein K (hnRNP K) as a Repressor of C/EBPβ-mediated Gene Activation*

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Transcription factor C/EBPβ has been known to regulate a wide array of genes including those involved in the acute-phase response. One of the molecular mechanisms underlying transcription activation by C/EBPβ is through protein-protein interaction with other transcription factors. Here we report the identification and characterization of physical and functional interactions between C/EBPβ and heterogeneous nuclear ribonucleoprotein (hnRNP) K. This interaction results in the repression of C/EBPβ-dependent trans-activation of the *agp* gene. Footprinting assays indicate that hnRNP K cannot bind to the promoter region of *agp* gene or interfere with the binding of C/EBPβ to its cognate DNA site. Furthermore, *agp* gene activation by the synergistic interaction of Nopp140 and C/EBPβ is abolished by hnRNP K. The kinetics of appearance of C/EBPβ-hnRNP K complex in the nuclear extract after initiation of acute-phase reaction indicates that hnRNP K functions as a negative regulator of C/EBPβ-mediated activation of *agp* gene.

C/EBPβ (also called AGP/EBP, NF-IL6, IL6-DBP, CRP2) (1–5) belongs to the C/EBP transcription factor family which includes C/EBPα (6), C/EBPγ (7), C/EBPδ (8), and CHOP (9). C/EBPβ is a key transcription factor involved in induction of genes during acute-phase or immune response (10). Responding to extracellular stimuli, C/EBPβ may form heterodimers with other C/EBP family members or interact with other transcription factors such as members of the NF-κB family (11–13), glucocorticoid receptor (14), AP-1 (15), Sp1 (16), and p53 (17). These interactions may result in cross-communication between transcription factors of different family members and thus increase the flexibility of gene regulation through combinatorial mechanisms.

In eukaryotic cells, nascent RNA transcripts are associated with large, multiprotein complexes called heterogeneous nuclear ribonucleoprotein complexes (hnRNP)[1] (18). These hnRNP proteins bind pre-mRNAs and appear to facilitate various stages of mRNA biogenesis such as pre-mRNA processing and mRNA transport from the nucleus to cytoplasm (18). Among these hnRNP proteins, hnRNP K is known to be the major poly(rC)-binding protein in HeLa cells (19), and possesses an unusual structure comparing with other hnRNP proteins. Nucleic acid binding activity of hnRNP K is not mediated by an RNA-binding consensus sequence, but by three repeats of motifs termed the KH (K homology) domain (20). These repeated motifs also be found in other proteins, including Ri autoantigen (21), fragile-X protein (22), and MER (23), which are all nucleic acid-binding proteins, suggest that KH motif may be involved in nucleic acid binding. The competition experiments revealed DNA rather than RNA to be the preferred ligand for hnRNP K binding *in vitro* (24). Thus, it is not surprising that hnRNP K has been repeatedly identified as a sequence-specific DNA-binding protein (25–28). Recently, several reports have shown that hnRNP K can bind to a cis-element within the human c-*myc* promoter and activates c-*myc* expression (24, 29). Thus, hnRNP K appears to be involved in transcriptional regulation. Direct protein-protein interaction between hnRNP K and some proto-oncogene provides evidence that hnRNP K acts as a docking platform to facilitate molecular interactions (30–32). Thus, in addition to an architectural component of hnRNP complexes, hnRNP K is also involved in other process such as transcriptional regulation and signal transduction.

To systematically search for proteins that interact with C/EBPβ, rat liver nuclear extracts were fractionated with anti-C/EBPβ immunoaffinity column chromatography and SDS-PAGE followed by LC/MS/MS analysis. A number of proteins were identified to be putative C/EBPβ-interacting partners. Among them, a phosphoprotein of 140 kDa, Nopp140, was identified to be interacting with C/EBPβ synergistically in activating *agp* gene expression (33). In this report, we describe the identification and characterization of hnRNP K to be another C/EBPβ-interacting protein. This protein-protein interaction results in the repression of C/EBPβ-dependent transactivation of *agp* gene. During the acute-phase reaction, the kinetics of the decrease of hnRNP K-C/EBPβ complex appears to correlate with the increase of *agp* gene expression. These results suggest that hnRNP K is a negative regulator of C/EBPβ-mediated *agp* gene activation.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length hnRNP K cDNA was isolated by reverse transcriptase-polymerase chain reaction from rat liver RNA and cloned into pCRII T vector (Invitrogen). The cDNA was analyzed by restriction enzymes mapping and partial sequencing to confirm that it is cDNA of hnRNP K. The *Eco*RI fragment from pCRII/hnRNP K was subcloned into CMV expression vector (pcDNA3, Invitrogen), and also subcloned into pGEX vector (Pharmacia) for GST fusion protein production. For the deletion analysis, *Eco*RI-*Nde*I fragment (amino acids 1–380) or *Eco*RI-*Hin*dIII fragment (amino acids 1–180) were blunt-ended and ligated to pcDNA3 and pGEX vectors. Other plasmids, AGP-CAT, C/EBPβ-CAT, and CMV-Nopp140, and CMV-C/EBPβ constructs were

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[1] The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein K; PAGE, polyacrylamide gel electrophoresis; LC/MS, liquid chromatography/mass spectroscopy; CMV, cytomegalovirus; GST, glutathione *S*-transferase; BHK, baby hamster kidney; WT, wild-type; CAT, chloramphenicol acetyltransferase; LPS, lipopolysaccharide.

as described (33), AGP/D-CAT was constructed by oligomerized D site of *agp* gene ligated to minimal promoter region of *agp* gene (34). Deletion mutants of C/EBPβ from pRSET vector (Invitrogen) were created by *NcoI/HindIII* digestion for C/EBPβ-N (from amino acids 21 to 146) and by *PvuII/HindIII* digestion for C/EBPβ-P (from amino acids 21 to 265). CMV-Nopp140/BS was created by ligation of the *BamHI/SacI* fragment of Nopp140 cDNA (containing amino acids 1–169) to a CMV expression vector.

Recombinant Proteins and Antibodies—Recombinant hnRNP K, Nopp140, and C/EBPβ (both full-length and truncated forms) from pRSET vector were expressed in *Escherichia coli* BL21 (DE3, pLysS) and purified by a nickel column. GST-Nopp140 and GST-hnRNP K from the pGEX vector (Phamacia) were induced in *E. coli* DH5α. Rabbit anti-hnRNP K, anti-Nopp140, and anti-C/EBPβ antibodies were produced by immunizing the rabbit with purified recombinant proteins. The monoclonal antibodies to C/EBPβ were as described (2). The specificities of these antibodies were characterized by Western blot analysis using liver nuclear extracts. These antibodies were monospecific and no cross-reactivities could be detected.

Nuclear Extract Preparation, Immunoprecipitation, and Western Blot—Nuclear extracts from rat liver were prepared as detailed elsewhere (3). For immunoprecipitation analysis, 100 μg of liver nuclear extracts were incubated with 5 μg of anti-Nopp140, anti-hnRNP K, or anti-C/EBPβ antibody in 1 ml of IP buffer (10 mM Tris, pH 7.5, 200 mM NaCl, 0.1% Nonidet P-40) and mixed constantly at 4 °C overnight. The immune complexes were reacted with protein A-Sepharose at 4 °C for 2 h, washed four times with IP buffer, and resuspended in SDS loading buffer and subjected to SDS-PAGE. For Western blot analysis, the separated polypeptides were blotted into Hybond-C membrane (Amersham) using a semi-dry transfer unit (CBS, Del Mar, CA) at 1 mA/cm^2 constant current for 1 h. The membrane was probed with antibody and detected using the enhanced chemiluminescence kit (Amersham).

Transient Transfection and Chloramphenicol Acetyltransferase (CAT) Assay—Baby hamster kidney (BHK) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For transfection experiments, the cells were plated on 6-cm diameter Petri dish at about 30% confluence and transfected the next day using the calcium phosphate precipitation method. The amounts of CAT reporter and expression vectors used are detailed in the figure legends. pCMV/SEAP (1 μg, which encodes secreted form of alkaline phosphatase, from Tropix) was included in each transfection as an internal control for transfection efficiency. pCMV plasmid DNA, which contains the CMV promoter only, was used to bring the total DNA to 5 μg. 48 h later, the culture supernatants were collected for alkaline phosphatase detection assays (35). The cells were harvested and CAT assays were performed as described (33). The conversion activities were quantitated with image analyzer (Fuji, BAS 1000) and normalized with alkaline phosphatase activity. All transfection experiments were done with duplicates and repeated two to four times. The relative CAT activities were shown as an average of these independent duplicate experiments. The *error bars* refer to standard deviation.

Gel Mobility Shift and Footprinting Assays—The AGP-CAT plasmid that contained the *agp* gene promoter region (−180 to +60) was digested with *HindIII*, blunt-ended with Klenow fragment in the presence of [α-^{32}P]dCTP and dNTPs. The 5′-end-labeled DNA fragment was then cut out by *XbaI* and purified from agarose gel with Nucleotrap (Macherey-Nagel). The probe (20,000 cpm/ng) was added to a 20-μl reaction mixture (25 mM HEPES, pH 7.8, 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, and 1 μg of poly(dI-dC)). Liver nuclear extracts or recombinant proteins were added and the binding reaction was allowed to proceed for 1 h on ice. Then DNase I (1 mg/ml, freshly diluted in 10 mM $MgCl_2$ and 5 mM $CaCl_2$) was added. DNA fragment was digested at room temperature for 1 min followed by addition of 80 μl of stop buffer (75 μg/ml sonicated *E. coli* DNA, 20 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K). The samples were then incubated for 30 min at 65 °C, extracted twice with phenol-chloroform, and precipitated with ethanol at −70 °C. The DNA pellets were analyzed by denaturing gel. The gel retardation assays were performed as described (2). The oligonucleotide of C/EBPβ binding motifs was used as probes and labeled with Klenow fragment in the presence of [α-^{32}P]dCTP.

Protein-Protein Interaction Assay—Glutathione-Sepharose 8A beads (Pharmacia) were mixed with 3 μg of either recombinant GST-hnRNP K or GST-Nopp140 fusion proteins, or GST only, in 500 μl of phosphate-buffered saline, 1% Triton X-100 on a rotary shaker for 20 min at room temperature. The beads were washed three times with phosphate-buffered saline and combined with 100 ng of recombinant full-length C/EBPβ, C/EBPβ-N, or C/EBPβ-P in a final volume of 200 μl of binding buffer (phosphate-buffered saline, 0.1% Triton X-100) and incubated by shaking on a rotary shaker for 2 h at 4 °C. The beads were washed four times with binding buffer. The bound proteins were eluted by boiling in SDS-PAGE loading buffer and subjected to SDS-PAGE for Western blot.

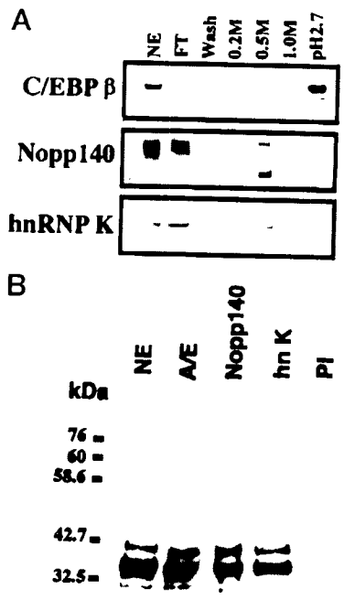

FIG. 1. **hnRNP K exists in C/EBPβ-containing complex in liver nuclear extract.** *A*, Western blot analysis of a series of eluted fractions from the anti-C/EBPβ immunoaffinity column. Rat liver nuclear extracts and different eluted fractions from anti-C/EBPβ antibody affinity column were separated by SDS-PAGE. Western blot was performed with anti-C/EBPβ, anti-Nopp140, or anti-hnRNP K antibody. *B*, immunoprecipitation of C/EBPβ by anti-hnRNP K antibody. Rat liver nuclear extracts were immunoprecipitated with anti-C/EBPβ (A/E), anti-Nopp140 (Nopp140), anti-hnRNP K (*hn K*), or control (*PI*) antibody, and subjected to SDS-PAGE followed by Western blot assay with anti-C/EBPβ monoclonal antibody. *NE* denotes direct loading of 20 μg of liver nuclear extract.

RNA Extraction and Northern Blot Analysis—Total rat liver RNA was extracted from rat liver as described previously (2). For Northern blot analysis, 10 μg of total RNA per lane was resolved by electrophoresis using 1.5% agarose gel containing 2.2 M formaldehyde. RNAs were transferred to Hybond N membrane (Amersham), and hybridized with ^{32}P-labeled C/EBPβ cDNA probe (2 × 10^6 cpm/ml).

Affinity Column Chromatography—A monoclonal anti-C/EBPβ antibody was purified with protein G affinity column. The purified immunoglobulin G (IgG) was then immobilized to protein A-Sepharose CL-6B (Pharmacia Biotech Inc.) and cross-linked by the cross-linking agent dithiobis(succinimidyl propionate) (Pierce). IgG of other monoclonal antibody was used as a nonspecific immunoaffinity column control. The column was equilibrated with buffer A (50 mM HEPES, pH 7.9, 20% glycerol, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) containing 0.1 M NaCl. Rat liver nuclear extract (50 mg) was loaded onto a 1-ml column with constant recirculation of the flow-through fraction for 30 min at 4 °C. The column was then washed with buffer A containing 0.1 M NaCl. Stepwise elution was performed with 3 ml of buffer A containing 0.2, 0.5, and 1 M NaCl followed by 0.1 M glycine buffer, pH 2.7.

LC/MS/MS Analysis of the Purified Protein—Rat liver nuclear extracts were purified by anti-C/EBPβ antibody column chromatography and SDS-PAGE. The major polypeptide detected by silver staining of SDS gel was recovered by extracting the gel slice with buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 0.1% SDS) at 37 °C overnight. The extracted protein was precipitated with acetone, washed with 70% ethanol, dissolved in 20 μl of 10 mM Tris-HCl, 0.5 μl of 1 M $CaCl_2$, 0.2 μg of trypsin, and digested for 18 h at 37 °C. Liquid chromatography-tandem mass spectrometry (LC/MS/MS, quadrupole spetrometer) is used for sequencing of short peptides with high sensitivity. This analysis was performed by Dr. John Yates's Lab at the Department of Molecular Biotechnology, University of Washington, Seattle, WA.

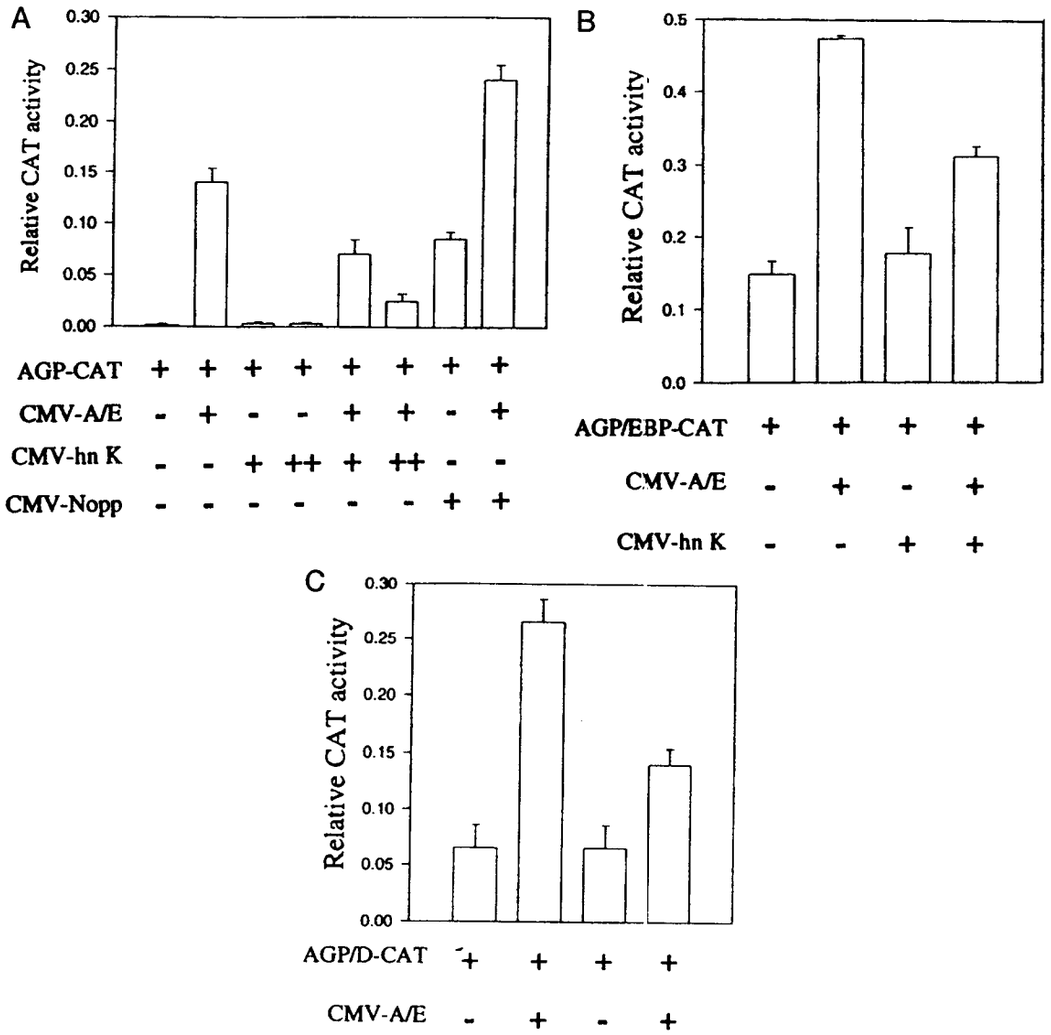

FIG. 2. **hnRNP K can repress C/EBPβ-mediated gene activation.** *A*, BHK cells were transfected with 1 μg of AGP-CAT in the absence or presence of CMV-C/EBPβ (CMV-A/E, 0.1 μg), CMV-Nopp140 (CMV-Nopp, 0.5 μg), or CMV-hnRNP K (CMV-hnK, 1 or 2 μg), CMV-C/EBPβ and CMV Nopp140, or CMV-C/EBPβ and CMV-hnRNP K. *B*, C/EBPβ-CAT (AGP/EBP-CAT, 1 μg); *C*, AGP/D-CAT (1 μg) was transfected into BHK cells in the presence of CMV-C/EBPβ (0.1 μg), CMV-hnRNP K (2 μg), or both together. The relative CAT activity was presented as an average of two independent duplicate experiments and normalized by internal control of alkaline phosphatase activity.

RESULTS

Identification of hnRNP K in a Complex Containing C/EBPβ—In our previous report (33), a number of C/EBPβ-interacting proteins have been identified from anti-C/EBPβ antibody affinity column. Briefly, one of the specific polypeptides retained by the anti-C/EBPβ immunoaffinity column and eluted at 0.5 M NaCl has a molecular mass of approximately 55 kDa in SDS-PAGE. This polypeptide was eluted from SDS gel and subjected to trypsin digestion followed by LC/MS/MS analysis (detailed under "Experimental Procedures"). The sequence of one of the tryptic peptides matched to the rat hnRNP K (amino acids 377–396, RGSYGDLGGPIITTQVTIPK), a component of heterogeneous nuclear ribonucleoprotein complex. The full-length cDNA of hnRNP K was isolated by reverse transcriptase-polymerase chain reaction and the recombinant hnRNP K was expressed in *E. coli*. The predicted open reading frame of hnRNP K cDNA is a polypeptide of 464 amino acids which correlates well with the purified 55-kDa protein. Western blot analysis with anti-hnRNP K antibody was performed on eluted fractions of anti-C/EBPβ affinity column. The results demonstrated that both hnRNP K and Nopp140 can be retained by the C/EBPβ antibody affinity column and eluted in the 0.5 M NaCl fractions (Fig. 1*A*). However, C/EBPβ was eluted at pH 2.7 glycine buffer. To further analyze the biochemical nature for the retention of hnRNP K by the C/EBPβ immunoaffinity column, we performed immunoprecipitation using rat liver nuclear extracts. Polyclonal antibody to C/EBPβ, Nopp140, or hnRNP K, but not preimmune serum, can bring down C/EBPβ from rat liver nuclear extract (Fig. 1*B*). These results indicate the co-existence of C/EBPβ-hnRNP K in a complex in the nuclear extract.

hnRNP K Functions as a Repressor of C/EBPβ-mediated Transactivation of agp Gene—To further address the possible functional interaction between hnRNP K and C/EBPβ, we performed transfection experiments using expression plasmids of hnRNP K and C/EBPβ and reporter AGP-CAT. hnRNP K alone does not have any apparent effect on AGP-CAT activity; in contrast, Nopp140 or C/EBPβ could activate AGP-CAT expression (Fig. 2*A*). However, when BHK cells were co-transfected with hnRNP K and C/EBPβ, C/EBPβ-mediated activation of AGP-CAT is repressed by hnRNP K in a dose-dependent manner (Fig. 2*A*). To delineate the promoter specificity of this

repression, we used promoter from the *C/EBPβ* gene for the transfection assay. Similarly, hnRNP K can repress C/EBPβ-mediated activation of C/EBPβ-CAT reporter (Fig. 2*B*). To further test the involvement of the C/EBPβ-binding motif in the repression of the C/EBPβ-mediated transactivation of target gene by hnRNP K, we conducted transfection experiments using CAT reporter containing the oligomerized C/EBPβ-binding motif (*i.e.* AGP/D-CAT, Ref. 34). Again, hnRNP K could repress C/EBPβ-mediated activation of the artificial reporter (Fig. 2*C*). Taken together, these results suggest that hnRNP K functions as a negative regulator for C/EBPβ-dependent genes activation.

Direct Protein-Protein Interaction between hnRNP K and C/EBPβ—Results from the previous section suggest that hnRNP K and C/EBPβ may exist in a complex in the nuclear extract. To further characterize their interaction biochemically, we employed GST-hnRNP K fusion protein as a bait for probing the recombinant C/EBPβ (Fig. 3*A*). Wild-type (WT) or truncated forms (C/EBPβ-P and C/EBPβ-N) of recombinant C/EBPβ were incubated with GST-Nopp140, GST-hnRNP K, or GST alone. Both wild-type and C/EBPβ-P could bind GST-hnRNP K specifically (Fig. 3*A*, *lanes 10* and *12*), however, C/EBPβ-N failed to interact with hnRNP K (Fig. 3*A*, *lane 11*). The C/EBPβ-N is a bZIP domain-truncated mutant which retains amino acids 21–146, while C/EBPβ-P is a leucine zipper-deleted basic amino acid region-intact mutant which includes amino acids 21–265. Thus, these results suggest that like Nopp140, the basic amino acid domain of C/EBPβ is important for its interaction with hnRNP K. To further investigate the physical and functional interactions of C/EBPβ and hnRNP K, we established two deletion mutants (hnk(380) and hnk(180)) of hnRNP K for protein-protein interaction and transient transfection assay. Both deletion mutants could interact with C/EBPβ, albeit with apparent lower affinity than the wild-type protein (hnk(wt)) (Fig. 3*B*). However, the results from the co-transfection assays showed that the sequential deletions from the C terminus results in decreasing repressive activity (Fig. 3*C*). Taken together, these results suggest that the intact molecule of hnRNP K is essential for full activity of C/EBPβ-binding and repression of C/EBPβ-mediated activation.

hnRNP K has been identified as a DNA-binding protein. To address the possibility that the repression of C/EBPβ-mediated activation of its target gene by hnRNP K is dependent on sequence-specific binding of hnRNP K, we performed footprinting analysis. As demonstrated in Fig. 4*A*, specific protection could be detected by recombinant C/EBPβ but not by hnRNP K using a probe derived from the DNA fragment of *agp* promoter. When the recombinant hnRNP K was incubated with recombinant C/EBPβ, the footprinting pattern was not affected. In a parallel experiment, footprinting was performed using liver nuclear extract in the presence or absence of recombinant hnRNP K (Fig. 4*B*). Both control and recombinant hnRNP K (100, 200, and 400 ng, respectively) proteins failed to interfere with the known protection pattern mediated by members of the C/EBP family. These results indicate that the hnRNP K may complex with C/EBPβ independent of the C/EBPβ-binding motif. DNA binding activity of C/EBPβ is not affected by hnRNP K. To determine the sequence specific binding activity of hnRNP K·C/EBPβ complex, we performed gel retardation experiments. As shown in Fig. 4*C*, antibodies to C/EBPβ (*lane 3*), Nopp140 (*lane 4*), and hnRNP K (*lane 5*) can all supershift a retarded band formed by a probe of C/EBPβ-binding motif and nuclear extract. Preimmune serum (*lane 6*) fails to produce such supershift. This result suggests that hnRNP K exists in the C/EBPβ-containing complex. Taken together, these results indicate that hnRNP K and C/EBPβ exists in a complex by direct interaction between them.

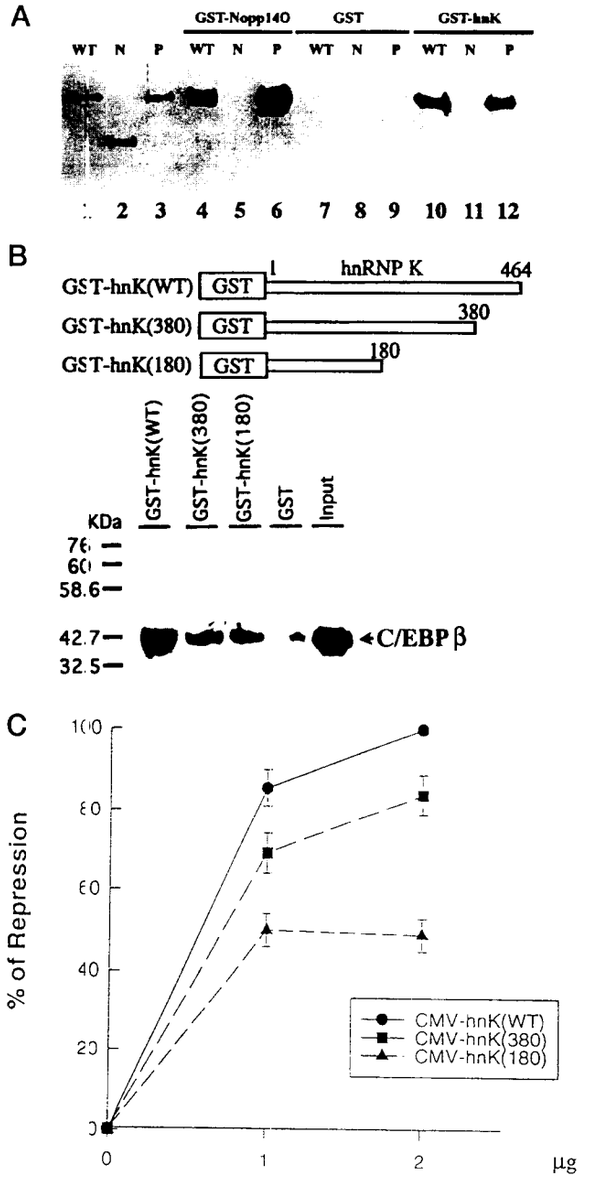

FIG. 3. **Biochemical and functional characterization of interaction of C/EBPβ and hnRNP K.** *A*, several deletion constructs of recombinant C/EBPβ were incubated with glutathione bead immobilized GST-Nopp140 (*lanes 4–6*), GST (*lanes 7–9*), or GST-hnRNP K (*lanes 10–12*) fusion proteins at 4 °C for 2 h. After an extensive wash, the protein complexes were subjected to SDS-PAGE and immunoblotted with anti-C/EBPβ antibody. *Lanes 1–3* represent direct loading of different recombinant C/EBPβ. *WT*, *N*, and *P* represent wild-type C/EBPβ, C/EBPβ-N and C/EBPβ-P which were described under "Experimental Procedures." *B*, *upper panel*, schematic representation of wild-type GST-hnRNP K and deletion mutants of GST-hnK(380) and GST-hnK(180). *Lower panel*, 3 μg of recombinant GST-hnRNP K (wild-type or deletion mutants) or GST were incubated with wild-type recombinant C/EBPβ (100 ng). After an extensive wash, the protein complexes were analyzed by Western blot with anti-C/EBPβ antibody. *C*, transient transfection assays demonstrate the functional domain of hnRNP K that interact with C/EBPβ. BHK cells were transfected with 1 μg of AGP-CAT and wild-type or deletion mutants of hnRNP K (CMV-hnK(WT), CMV-hnK(380), or CMV-hnK(180), 1 or 2 μg) in the presence of CMV-C/EBPβ (0.25 μg). The results were presented by the percent of repression of hnRNP K.

The Kinetic Change of Levels of hnRNP K-C/EBPβ Complex during Acute-phase Response—C/EBPβ is one of the key tran-

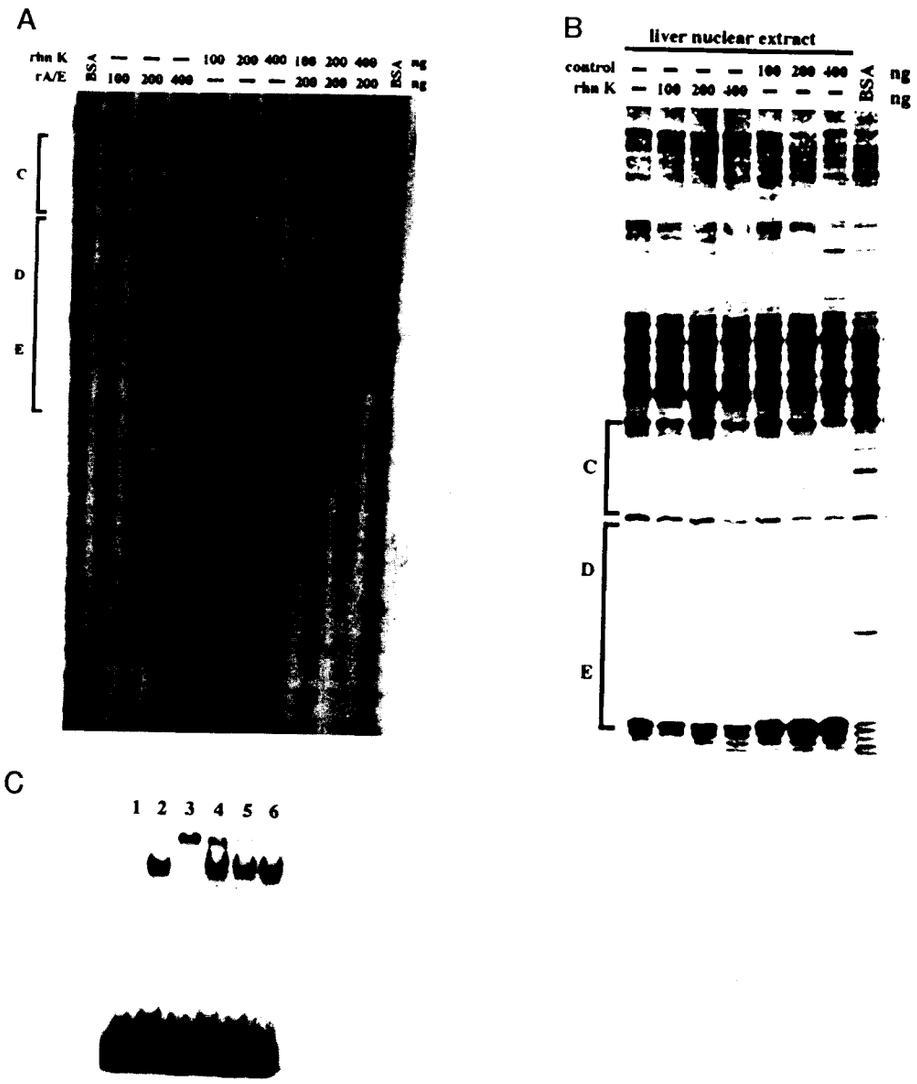

FIG. 4. **hnRNP K does not bind to the promoter region of the *agp* gene or interfere with the DNA binding activity of C/EBPβ.** *A*, probe derived from DNA fragment spanning −180 to +60 of the *agp* gene promoter was used for footprinting analysis. DNA probe was incubated with bovine serum albumin (*BSA*), recombinant C/EBPβ, hnRNP K, or both C/EBPβ and hnRNP K as indicated. After DNase I digestion, the DNA fragments were analyzed by denaturing gel. *B*, DNA probe described in *A* was incubated with liver nuclear extracts in the absence or presence of recombinant hnRNP K or control recombinant protein (baculovirus p35 protein) and followed the procedures which were the same as *A*. *C*, gel mobility shift assay of hnRNP K-C/EBPβ binding complexes. An oligonucleotide probe corresponding to the C/EBPβ-binding motif was incubated with liver nuclear extract at room temperature for 20 min, followed by adding the anti-C/EBPβ (*lane 3*), anti-Nopp140 (*lane 4*), anti-hnRNP K (*lane 5*), or preimmune serum (*lane 6*) antibody and incubation continued for 10 min. DNA-protein complexes were separated by native gel and analyzed by autoradiography. *Lane 1* is probe alone, and *lane 2* is without antibody control.

scription factors responsible for the induction of genes during acute-phase response. When the animals were treated with LPS, AGP RNA expression was induced dramatically after 30 min (Fig. 5*A*). The results of Western blot analysis showed that the expression of C/EBPβ increased only slightly while hnRNP K remained unchanged (Fig. 5*B*). Stat 3 (APRF), a known transcription factor induced by LPS treatment of animals, was used as a control (Fig. 5*B*). To assess the levels of hnRNP K during the acute-phase reaction, we analyzed the kinetics of appearance of the hnRNP K-C/EBPβ complex in the nuclear extracts from normal and LPS-treated rat liver. Both normal and LPS-treated nuclear extracts were immunoprecipitated with anti-C/EBPβ (BR) or anti-hnRNP K (hn K) antibody (Fig. 5*C*). The level of C/EBPβ precipitated by anti-C/EBPβ antibody was about the same in the nuclear extract of normal and LPS-treated rat liver. However, C/EBPβ precipitated by anti-hnRNP K antibody decreased at 30 min and increased thereafter. Taken together, these results suggest that the decrease in the level of hnRNP K-C/EBPβ complex correlated with the induction of the acute-phase response gene (*e.g. agp*). hnRNP K-C/EBPβ complex may have a negative effect on C/EBPβ-mediated gene activation.

Activation of agp Gene by Synergistic Interaction between Nopp140 and C/EBPβ May be Inhibited by hnRNP K—Our previous results showed that Nopp140 could interact with C/EBPβ and activate *agp* gene synergistically. To test the effect of hnRNP K on the synergistic activation of AGP-CAT by Nopp140 and C/EBPβ, we performed transfection experiments using expression vectors of Nopp140 and C/EBPβ in the presence of increasing amounts of hnRNP K. The results showed that hnRNP K acts similarly as the dominant-negative mutant of Nopp140 Nopp140/BS (*i.e.* hnRNP K could abolish the syn-

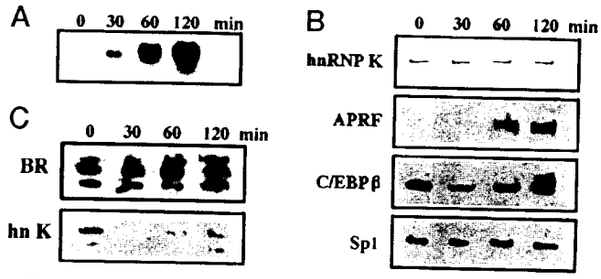

FIG. 5. **Kinetic appearance of expression of the *agp* gene and C/EBPβ·hnRNP K complex during the acute-phase reaction.** *A*, Northern blot analysis of *agp* gene expression. The rats were injected with LPS (5 mg) intraperitoneally. After the indicated time, total RNA was isolated and Northern blot was performed using AGP cDNA probe. *B*, Western blot analysis. Normal and LPS-treated liver nuclear extracts (20 μg each lane) were separated by SDS-PAGE, blotted onto membrane, and detected by Western blot analysis using anti-C/EBPβ, anti-hnRNP K, anti-APRF, or anti-Sp1 antibody. *C*, kinetic change of C/EBPβ·hnRNP K complex during acute-phase reaction. Normal and LPS-treated liver nuclear extracts were immunoprecipitated with anti-C/EBPβ (*BR*) or anti-hnRNP K (*hn K*) antibody. Western blot was probed with anti- C/EBPβ monoclonal antibody.

ergistic activation of AGP-CAT by Nopp140 and C/EBPβ in a dose-dependent manner) (Fig. 6*A*). The overexpression of increasing amounts of C/EBPβ or Nopp140 could also overcome the repression of hnRNP K (Fig. 6*B*). To further characterize the biochemical relationship of hnRNP K, Nopp140, and C/EBPβ, we performed the glutathione bead pull-down assay. Recombinant C/EBPβ was incubated with GST-Nopp140 in the presence of increasing amounts of recombinant hnRNP K (Fig. 6*C*). hnRNP K can abolish the interaction of Nopp140 and C/EBPβ in a dose-dependent manner. There is good correlation between the disruption of Nopp140 and C/EBPβ complex formation by hnRNP K and the repressive effect of hnRNP K on the synergistic activation of AGP-CAT by Nopp140 and C/EBPβ.

DISCUSSION

C/EBPβ is a key transcription factor responsible for regulating genes involved in inflammatory and acute-phase responses. Responding to extracellular stimuli, C/EBPβ may cooperate with other transcription factors in activating its target genes (11–14). Established results indicate that C/EBPβ interacts with a number of transcription factors physically and functionally (11–17). Several lines of evidence showed that there are physical and functional interactions between hnRNP K and C/EBPβ. 1) hnRNP K was retarded by anti-C/EBPβ antibody affinity column (Fig. 1). 2) C/EBPβ can be immunoprecipitated by anti-hnRNP K antibody from the nuclear extract (Fig. 1). 3) hnRNP K could repress C/EBPβ-mediated gene activation in a C/EBPβ-binding motif dependent manner (Fig. 2). 4) Direct interaction between hnRNP K and C/EBPβ (Fig. 3). 5) The co-existence of hnRNP K and C/EBPβ in the complex formed with C/EBPβ-binding motif (Fig. 4).

hnRNP K was first discovered as a component of the hnRNP particle (18). Recently, hnRNP K has been identified as a DNA-binding factor involved in transcription regulation. hnRNP K has been identified as human c-*myc* CT-element binding protein. Transfection and *in vitro* transcription assays indicated that hnRNP K could activate gene expression in a CT-element dependent manner (24, 28, 29). Thus hnRNP K functions as a transcription factor when it binds to the CT-element of c-*myc* promoter. Using G-rich oligonucleotides derived from catalase gene silencer element as probe for binding screening of the expression cDNA library, hnRNP K was isolated from a rat hepatoma cell line (26, 36). cDNA encoding a 65-kDa κB-motif binding phosphoprotein had been cloned and identified to be the murine homolog of human hnRNP K (27). It was shown that hnRNP K could bind to the κB-motif in a sequence-specific manner. Furthermore, nuclear protein H16, a simian homolog of human hnRNP K, binds specifically *in vitro* to the late coding SV-40 virus DNA strand in the region of transcription control without binding to the complementary strand (25, 37, 38). Collectively, these observations provide evidence that the hnRNP K protein is involved in regulation of gene expression by binding to the specific DNA motif of its target gene. In this report, we provide evidence that hnRNP K can participate in gene regulation through protein-protein interaction with C/EBPβ without binding to the specific DNA sequence (39).

We have demonstrated two distinct C/EBPβ-containing complexes in rat liver nuclear extract. One of these complexes, C/EBPβ-Nopp140, functions as an activator, while the other, C/EBPβ-hnRNP K, functions as a repressor for C/EBPβ-dependent gene transcription. hnRNP K functions as a dominant-negative regulator by disrupting the synergistic interaction between Nopp140 and C/EBPβ through complex formation between Nopp140 and C/EBPβ (Fig. 6). During the acute-phase reaction, the decrease of hnRNP K·C/EBPβ complex coincides with the increase of AGP mRNA (Fig. 5). These results together with those of transfection assays, suggest that the hnRNP K·C/EBPβ complex may serve as a negative homeostatic regulator of *agp* gene expression.

One of the possible mechanisms of repression by hnRNP K may be the block of functional transcription preinitiation complex formation. In our previous report (33), we suggest that Nopp140 may function as a coactivator by interacting with both C/EBPβ and TFIIB. We have also identified a dominant-negative mutant, Nopp140/BS, which failed to interact with TFIIB but still could interact with C/EBPβ (33). Co-transfection of Nopp140/BS with Nopp140 and C/EBPβ abolished the synergistic activation of the *agp* gene by Nopp140 and C/EBPβ. The dominant-negative function of Nopp140/BS may be substituted by hnRNP K for blocking the synergistic activation of the *agp* gene by C/EBPβ and Nopp140 (Fig. 6). These results imdicated that interruption of the proper link between C/EBPβ and general transcription factors or other components of the transcription machinery might be a mechanism of repressed C/EBPβ function. Although there is no direct evidence to suggest that the mechanism for inhibition of these two factors is the same. Mutational analysis of hnRNP K indicates that amino acids from 380 to 464 are essential for full C/EBPβ binding. The decreasing of C/EBPβ binding activity of sequential deletions from the C terminus of hnRNP K correlates with the diminishing of their repressive activity. Thus, intact hnRNP K is essential for full C/EBPβ binding and repressive activity. The involvement of other repressors in the hnRNP K-mediated inhibitory effect may also exist. For example, a novel hnRNP K-interacting protein, Zik1, has been identified as a transcriptional repressor and the ZiK1-binding region of hnRNP K has been identified between amino acid 209 to 307 (40).

The molecular mechanisms of kinetic change in the levels of hnRNP K·C/EBPβ complex during the acute-phase reaction are unclear. Recently, an interleukin-1-responsive serine/threonine kinase has been described to associate and phosphorylate hnRNP K (41). Post-translational modification of hnRNP K by a kinase (*i.e.* interleukin-1-responsive kinase) may affect the stability and activity of the hnRNP K·C/EBPβ complex. The level of hnRNP K·C/EBPβ complex was decreased by 30 min after the initiation of the acute-phase response and was restored gradually by 60 min. As a control, no apparent change of the levels of Nopp140·C/EBPβ complex was observed (data not shown). The transcriptional activity of Nopp140·C/EBPβ com-

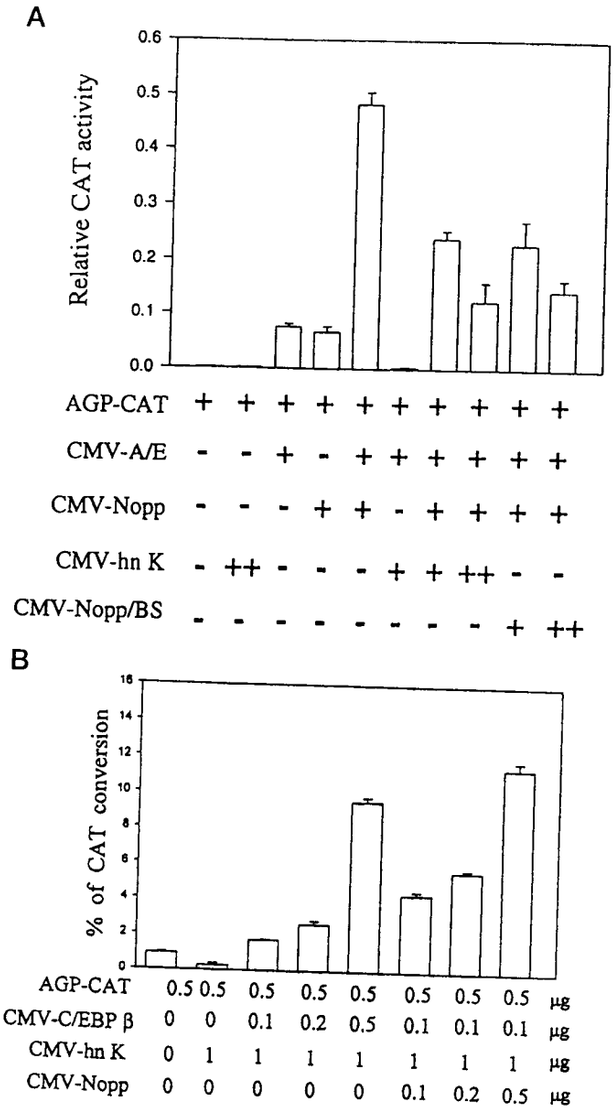

FIG. 6. **hnRNP K could repress the synergistic interaction between C/EBPβ and Nopp140.** *A*, BHK cells were co-transfected with AGP-CAT (1 μg) in the absence or presence of CMV-C/EBPβ (CMV-A/E, 0.05 μg), CMV-Nopp140 (CMV-Nopp, 0.5 μg), or CMV-hnRNP K (CMV-hnK, 2 μg) or CMV-C/EBPβ and CMV-Nopp140, or CMV-C/EBPβ and CMV-hnRNP K, or CMV-C/EBPβ, CMV-Nopp140 and CMV-hnRNP K (1 or 2 μg), or CMV-C/EBPβ and CMV-Nopp140 and CMV-Nopp/BS (1 or 2 μg) as indicated. The relative CAT activities were presented by an average of two independent duplicate experiments. *B*, BHK cells were co-transfected with AGP-CAT (0.5 μg) and increasing amounts of CMV-C/EBPβ (0.1, 0.2, and 0.5 μg), or 0.1 μg of CMV-C/EBPβ and increasing amounts of CMV-Nopp140 (0.1, 0.2, and 0.5 μg) in the presence of 1 μg of CMV-hnRNP K. The CAT conversion was shown as an average of two independent duplicate experiments. *C*, GST-Nopp140 (3 μg each lane) was immobilized on glutathione beads and incubated with 100 ng of recombinant C/EBPβ in the presence of 2 or 4 μg of recombinant hnRNP K or control proteins. The binding proteins were washed and subjected to SDS-PAGE and then immunoblotted with anti-C/EBPβ monoclonal antibody (*upper panel*). *Lower panel* represents Amido Black staining of blotted GST-Nopp140.

plex may be modulated by phosphorylation of Nopp140 during the acute-phase reaction.[2] In addition to the post-translational modifications, alternative splicing forms of hnRNP K have been reported. These derivatives may also be involved in transcriptional regulation of the *agp* gene (42).

Two repressors, nucleolin and hnRNP K, have been identified to be involved in the regulation of the *agp* gene. Nucleolin can bind to a specific DNA motif and inhibit *agp* gene expression (43). While the repressor function of hnRNP K is mediated by its interaction with C/EBPβ. hnRNP K may have important homeostatic function by maintaining the basal activity of C/EBPβ-inducible genes under normal conditions. We have also demonstrated that hnRNP K can interact with C/EBPα and repress C/EBPα-mediated activation of target genes (data not shown). During the acute-phase reaction, as contrary to the increase of C/EBPβ, C/EBPα is decreased (44). Thus, it is likely that the complex of C/EBPα and hnRNP K plays somes role for regulating genes before the initiation of the acute-phase response. Taken together, these results suggest that hnRNP K may be involved in the regulation of genes induced by transcription factors of C/EBP family.

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