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DNA Binding Activity of C/EBPβ and C/EBPδ for the Rat α_2-Macroglobulin Gene Promoter Is Regulated in an Acute-Phase Dependent Manner

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Abstract—Turpentine-induced acute-phase (AP) response in rats is followed by transcriptional activation of the α_2-macroglobulin (MG) gene mediated by cytokine interleukin-6 (IL-6) and glucocorticoids. Based on nucleotide sequence analysis of the α_2-MG gene promoter regions responsive to IL-6, we postulated that binding of members of the liver-enriched CCAAT-enhancer-binding proteins (C/EBP) family of transcription factors to the type I IL-6 responsive element (IL-6RE) may participate in the transcriptional activation of this gene during AP response. Results of Western immunoblot and Northern-blot assays revealed coordinate changes in the pool levels of C/EBPα, -β, and -δ protein isoforms and their genes expression in liver in response to turpentine. By means of an *in vitro* phosphorylation assay, South-Western blot, and selective proteolysis we have also found that only abilities of 35-kD C/EBPβ and 27-kD C/EBPδ to bind to the α_2-MG gene promoter were affected by phosphorylation. Based on these data we concluded that transcriptional induction of the rat α_2-MG gene during AP response correlates with both increased synthesis and phosphorylation-induced binding of 35-kD C/EBPβ and 27-kD C/EBPδ.

Key words: acute-phase response, turpentine, α_2-macroglobulin gene, C/EBPα, C/EBPβ, C/EBPδ, DNA-binding activity, phosphorylation

The acute-phase (AP) response is a set of complex and systemic reactions following tissue injury, trauma, or infection. It is mediated by cytokines and signaling molecules generated at the site of injury and distributed via the blood stream to hepatocytes where they activate the expression of AP protein (APP) genes [1]. α_2-Macroglobulin (α_2-MG), a plasma glycoprotein that functions as protease inhibitor, is the major and typical positive APP in rats [2]. Nearly one hundred-fold increase in α_2-MG plasma concentration during AP response is preceded by transcriptional activation of its gene mediated by cytokine interleukin-6 (IL-6) and glucocorticoids [3]. IL-6 promotes the full expression of α_2-MG gene via two *cis*-acting promoter regions responsive to IL-6, located at the −852/−777 and −404/−165 bp positions relative to the transcription initiation site [4]. Sequence analysis of these two regions revealed the existence of distinct functional elements implicating the involvement of different IL-6 activated transcription factors in the regulation of the rat α_2-MG gene expression.

Two families of transcription factors that play important roles in mediating the changes in APP genes expression in response to IL-6 include members of the CCAAT/Enhancer Binding Protein (C/EBP) [5] and Signal Transducers and Activators of Transcription (STAT) [6] protein families. STAT proteins have been shown to regulate the expression of α_2-MG [7], γ-fibrinogen [8], serine protease inhibitor-3 [9], C-reactive protein [10], and haptoglobin [11] genes by means of binding to the type II IL-6RE in the promoter. On the other hand, members of the C/EBP protein family recognize the type I IL-6RE which conforms to the consensus sequence T/(T/G)NNG(A/C/T)AA(T/G) [12].

C/EBP family members are important basic region-leucine zipper (bZIP) motif containing liver-enriched transcription factors. Through interaction of their leucine-zipper domain, they form active homo- and hetero-dimers, bind to specific DNA recognition sequences through interactions with basic regions and function as transcription regulators via their activation domains [13]. This family of transcription factors encompasses both constitutive and inducible isoforms of α, β, δ, γ, ε, and ξ

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proteins [14], which are expressed *in vivo* and *in vitro* [11, 15-18]. Survey shows that activity of different C/EBPs is primarily regulated at the transcriptional level [19]. In addition, synthesis of specific isoforms of C/EBPα and -β proteins is regulated through the mechanism of an alternative translational initiation of multiple AUG initiation sites within each mRNA [20]. Several lines of evidence suggest that C/EBPα, -β, and -δ are primary mediators of the IL-6 dependent transcriptional induction of various APP genes [21] and initiation of AP response in liver [22].

Based on these data and identification of type I IL-6RE in the rat α_2-MG gene promoter, we proposed that C/EBPα, -β, and/or -δ proteins could contribute to the rat α_2-MG gene induction during AP response. Therefore, we studied the turpentine-mediated regulation of C/EBPα, -β, and -δ protein pool levels and DNA-binding activities toward the α_2-MG promoter in rat liver. We have also investigated an effect of phosphorylation on C/EBPα, -β, and -δ DNA-binding activities during turpentine-induced AP response.

MATERIALS AND METHODS

Animals. Knowing that stimulatory effect of IL-6 on rat hepatocytes function *in vitro* can be successfully mimicked by injecting rats with turpentine oil [23], AP response in two-months old male Wistar rats was elicited by a subcutaneous injection of turpentine in the lumbar region (1 μg/g body weight). The animals were sacrificed 12 h later when maximal transcription rate of the rat α_2-MG gene was established [24].

Probes. Nucleotide sequence of the rat α_2-MG gene promoter (spanning from −852 to +12 bp relative to the transcription initiation site), subcloned into *Hinc*II site of pUC_{13}, was kindly donated by Dr. Peter Heinrich (Institute für Biochemie an der RWTH Aachen, Aachen, Germany). For the determination of C/EBPα, -β, and -δ mRNA levels in Northern blot analysis, cDNA inserts were isolated from each plasmid (a generous gift from Dr. S. L. McKnight) by digesting $pEMBL_{19}$-C/EBPα with *Hind*III, Bluescript KS^+-C/EBPβ and Bluescript KS^+-C/EBPδ with *Eco*RI–*Hind*III. All DNA probes were labeled by a random priming technique using [α-^{32}P]dCTP (Amersham, England).

Preparation of rat liver nuclear extracts. Nuclear extracts (NEs) were prepared from the pool of five livers from control and 12 h turpentine-treated rats [25] and their protein content was measured subsequently [26].

Western immunoblot assay of rat liver NEs. Rat liver NEs were separated by 12% SDS-PAGE [27], transferred to Hybond P membranes (Amersham) and probed with rabbit polyclonal antibodies (Santa Cruz Biochemicals Inc., USA) specific to C/EBPα (14AA, dilution 1 : 1000), C/EBPβ (C-19, dilution 1 : 1000), and C/EBPδ (C-22, dilution 1 : 750). Immunoreactive proteins were detected according to the procedure recommended by the supplier of ECL Western immunoblot kit RPN 2108 (Amersham). Each filter was consecutively reprobed with all three C/EBP antibodies according to the reprobing protocol.

South-Western blot analysis of NEs. Following SDS-PAGE, NEs were transferred onto Hybond^{TM}-C nitrocellulose membrane (45 μm; Amersham) and probed with 10^6 cpm/lane random-prime [α-^{32}P]dCTP labeled α_2-MG gene promoter sequence [28]. DNA-binding proteins were identified after exposing dried membrane to X-ray film (Kodak, USA) for 1-4 days.

RNA isolation and Northern blot analysis. Total RNA was isolated from the pool of five livers from control and 12 h turpentine-treated rats using RNAeasy Mini Kit 74104 (Qiagen, USA). Total RNAs (20 μg) were loaded on 1.2% agarose–2.2 M formaldehyde gel and transferred onto a nylon membrane (Hybond N, Amersham). To confirm equivalent RNA loading and transfer, membranes were rinsed with 0.04% methylene blue and photographed. Membranes with equivalent amounts of RNA were hybridized to a random-prime labeled C/EBPα, -β, and -δ cDNA probes (10^6 cpm/ml) according to the membrane manufacturer's instructions. The levels of C/EBPα, -β, and -δ mRNAs in the livers of 12 h turpentine-treated rats were normalized to their levels in the control livers (100%) by densitometry.

Phosphorylation and dephosphorylation of proteins in rat liver NEs. Rat liver NEs were phosphorylated *in vitro* in the presence of endogenous protein kinases and [γ-^{32}P]ATP according to the *in vitro* phosphorylation assay [29] and separated in 12% SDS-PAGE system. After exposing dried gel to a phosphor screen (Phosphorimager, Molecular Dynamics, USA), quantitative measurement of the separated samples was performed by volume integration of the selected region by using the Image Quant software package. The pattern of *in vitro* phosphorylated proteins was visualized after exposing dried gels to X-ray film (Kodak, USA). In the dephosphorylation assay, liver NPs from control and turpentine treated rats were dephosphorylated with bovine intestinal alkaline phosphatase (Boehringer Mannheim, Germany; 2.5 U/10 μg protein). For South-Western analysis, NEs were phosphorylated following the same protocol with 50 μM unlabeled ATP instead of [γ-^{32}P]ATP.

Peptide mapping protocol. NEs from the livers of control and 12 h turpentine-treated rats as well as *in vitro* phosphorylated control NEs were separated by 12% SDS-PAGE and after Coomassie brilliant blue staining, band corresponding to the 27-kD C/EBPδ protein was cut out and eluted from the gel [30]. 27-kD C/EBPδ protein eluted from the livers of control and 12 h turpentine-treated rats as well as *in vitro* phosphorylated control NEs were submitted to selective proteolysis by V8 protease originating from *Staphylococcus aureus* (2 μg, 1 h, 37°C) [31]. Peptide fragments were then separated in the 15% SDS-PAGE system and visualized after silver staining [32].

RESULTS AND DISCUSSION

Identification of proteins that bind to the α_2-MG gene promoter. The proposed AP-related participation of C/EBPα, -β, and -δ proteins in the formation of DNA–protein complexes with the rat α_2-MG gene promoter was estimated by South-Western analysis of rat liver *trans*-acting nuclear proteins (NPs) molecular masses and their pattern of DNA-binding.

Figure 1 shows that α_2-MG gene promoter sequence interacts with several liver *trans*-acting NPs with molecular masses ranging from 29 to 97 kD (Fig. 1, lane *1*). AP response was followed by an increase in the binding of 29-, 32-, 35-, 45-, and 68-70-kD NPs for the α_2-MG gene promoter and an induction of that of 27-kD NP (Fig. 1, lane *2*). At the same time, the intensity of the 42-kD band, which is the molecular mass reported for full-length C/EBPα [33] and C/EBPβ [34], remained unchanged. The molecular mass and higher intensity of 32-kD and 35-kD protein bands correspond to the behavior of equivalent C/EBPβ isoforms in response to lipopolysaccharide (LPS) [20] and aging [35]. The band at 27 kD, which is in the molecular mass range reported for C/EBPδ [13], exhibited well-known strong inflammatory agent-dependent change of intensity suggesting that it could play a significant role in mediating α_2-MG gene transcriptional activation (Fig. 1, lane *2*).

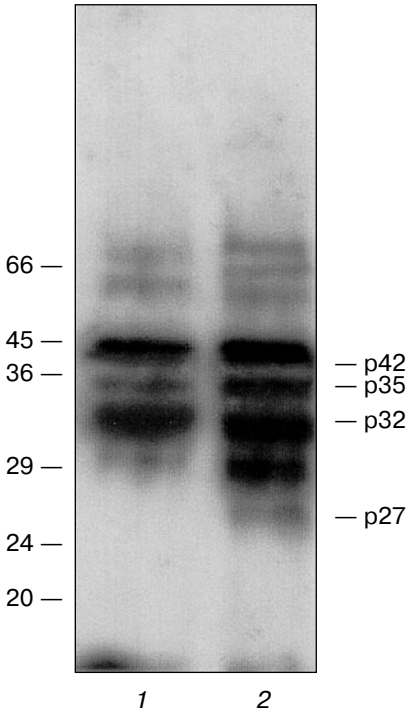

Fig. 1. South-Western blot analysis of liver nuclear proteins that bind to the α_2-MG gene promoter sequence. Male Wistar rats were injected with turpentine in the lumbar region (1 μg/g body weight) and sacrificed 12 h later. Nuclear extracts (150 μg) prepared from control (lane *1*) and 12 h turpentine-treated (lane *2*) livers were subjected to 12% SDS-PAGE, blotted onto Hybond^{TM}-C nitrocellulose membrane and probed with 10^6 cpm/lane of [α-^{32}P]dCTP labeled α_2-MG gene promoter sequence as described in "Material and Methods". The locations of the molecular size standards in kD are shown on the left. The arrows on the right indicate the positions of bands formed between α_2-MG gene promoter sequence and *trans*-acting nuclear proteins with molecular masses 27, 32, 35, and 42 kD.

Since qualitative and quantitative changes in the binding of 27-, 32-, 35-, and 42-kD NPs to the α_2-MG gene promoter follow the pattern already established for corresponding C/EBPα, -β, and -δ isoforms [13, 20, 35], we assumed that some of them could indeed belong to the C/EBP family of transcription factors.

Nucleotide sequence analysis of the rat α_2-MG gene promoter. To determine whether the rat α_2-MG gene promoter does contain the type I IL-6RE we explored the nucleotide sequence of the two of its regions involved in the α_2-MG gene induction by IL-6, located between –852 and –777 bp as well as –404 and –165 bp [36]. Results shown in Fig. 2 have disclosed the existence of the two sequences which are homologous to the C/EBPβ binding site in the human IL-6 gene promoter and also match the consensus sequence T/(T/G)NNG (A/C/T)AA(T/G) of the type I IL-6RE [12] within both IL-6 dependent regions. On the basis of α_2-MG promoter sequence analysis (Fig. 2) and South-Western assay (Fig. 1), we are of an opinion that binding of C/EBPα, -β, and -δ to the type I IL-RE in the α_2-MG promoter could participate in the turpentine-mediated transcriptional activation of this gene. In addition to that, identification of type I of IL-6RE interacting with C/EBPs in promoters of numerous APPs genes, including that of α_2-MG, implied a coordinate regulation of their transcription by a common set of IL-6 activated *trans*-acting factors.

Analysis of the rat liver pool levels of C/EBPα, -β, and -δ isoforms in response to turpentine. Three principal and closely related C/EBP family members, α, β, and δ have molecular weight ranging from 20 to 45 kD [37] that corresponds to those of some *trans*-acting NPs found to bind the rat α_2-MG promoter (Fig. 1). To firstly identify whether and which of them are present in rat liver NE, we performed Western immunoblot assay with rabbit polyclonal anti-C/EBPα, -β, and -δ antibodies.

Western immunoblotting with anti-C/EBPα antibody revealed six isoforms of this protein (C/EBPαs) with molecular weight of 42, 38, 35, 30, 20, and 14 kD (Fig. 3a, lane *1*). Following an induction of AP response, pool levels of all C/EBPαs were significantly reduced, with the pool level of 30-kD C/EBPα isoform slightly higher than that of the other C/EBPαs (Fig. 3a, lane *2*).

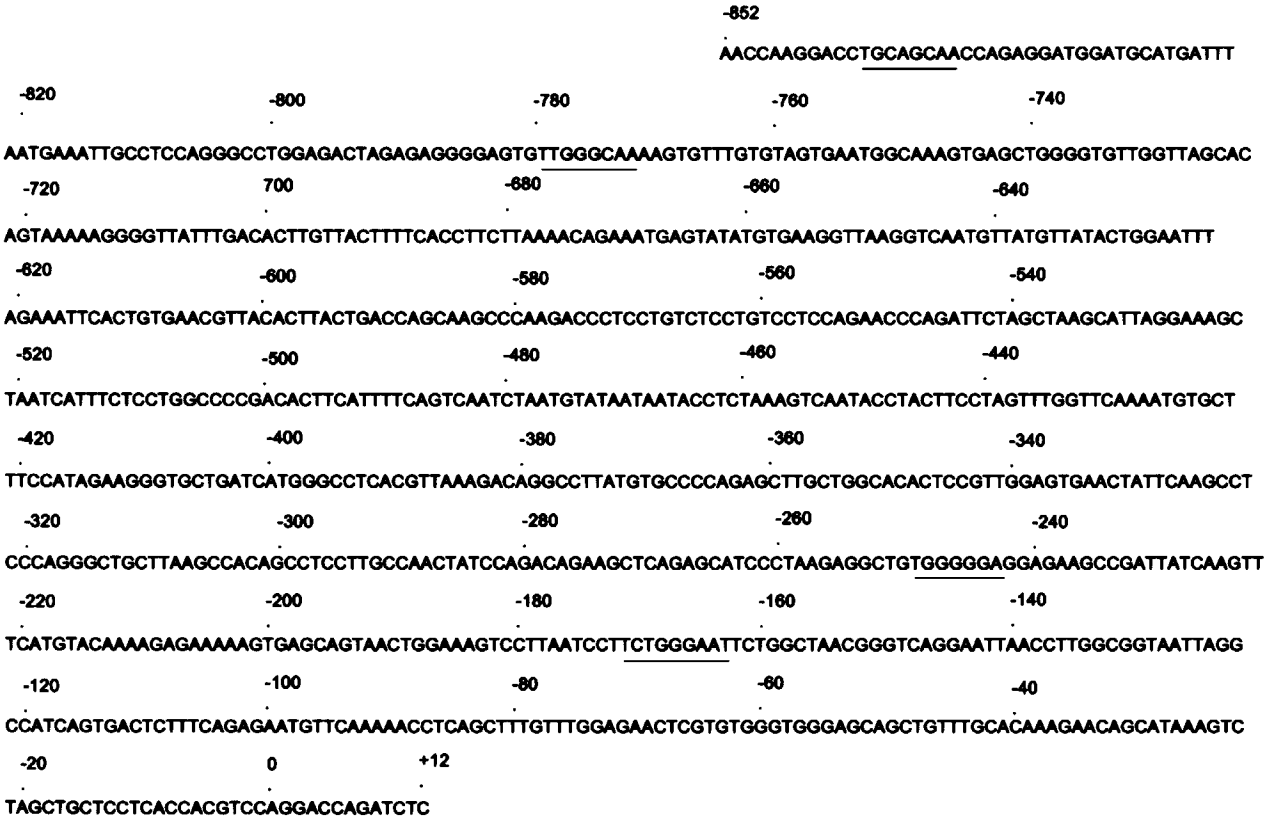

Fig. 2. DNA sequence of the rat α_2-MG gene promoter [36]. Consensus sequence T/(T/G)NNG(A/C/T)AA(T/G) of the type I IL-6RE found within both IL-6 dependent regions (−852/−777 and −404/−165 bp) is underlined.

Anti-C/EBPβ antibody detected four isoforms of 42, 35, 32, and 20 kD (Fig. 3b, lane *1*). In contrast to the pool level of 42-kD C/EBPβ isoform, which remained unaffected by turpentine treatment (Fig. 3b, lane *2*), the pool levels of other C/EBPβs increased in relation to the control (Fig. 3b, lane *1*).

C/EBPδ antisera recognized only one protein with molecular weight of 27 kD in both NEs of control (Fig. 3c, lane *1*) and turpentine-treated (Fig. 3c, lane *2*) rats amount of which increased in response to turpentine.

Our study demonstrated that C/EBPα is a predominant C/EBP protein under normal conditions, which is in accordance with the prevailing view that C/EBPα is involved in the homeostatic maintenance of APPs genes expression [19]. The fact that C/EBPβ and -δ become major isoforms after turpentine treatment suggests that changes in C/EBPα to C/EBPβ and/or -δ ratio may be a part of regulatory processes during AP response to turpentine. Our finding that among the multiple isoforms detected with both C/EBPα and C/EBPβ antibodies, 42-kD, 35-kD, and 20-kD ones are shared between them is consistent with the alternative mRNA translational initiation mechanism [20]. That this feature is not a result of proteolytic cleavage as could be expected for adult rat liver was proven after consecutive reprobing of each membrane with anti-C/EBPα, -β, and -δ antibodies and confirmed with non-immune probe detection (data not shown). Namely, unlike the mechanism of alternative translation, the cleavage mechanism was established to be specific only for prenatal and newborn rat livers where it simultaneously decreased the level of 38-kD C/EBPβ and 35-kD C/EBPβ while increasing the level of 20-kD C/EBPβ [16].

Expression of C/EBPα, -β, and -δ genes in rat liver. Here described AP-related changes in the binding of C/EBPβ and -δ, but not C/EBPα to the α_2-MG gene promoter (Fig. 1) paralleled alterations of their pool levels in NE (Fig. 3) and thus may be determined by the level of the C/EBPα, -β, and -δ genes expression in rat liver. To determine if the changes in C/EBPα, -β, and -δ pool levels in NE correlated with their mRNA levels, we performed Northern blot hybridization of total RNA from the livers of control (Fig. 4, a-c, lanes *1*) and turpentine-treated (Fig. 4, a-c, lanes *2*) rats with C/EBPα, -β, and -δ cDNA probes. Following an induction of AP response, C/EBPα mRNA level decreased 14% while the levels of

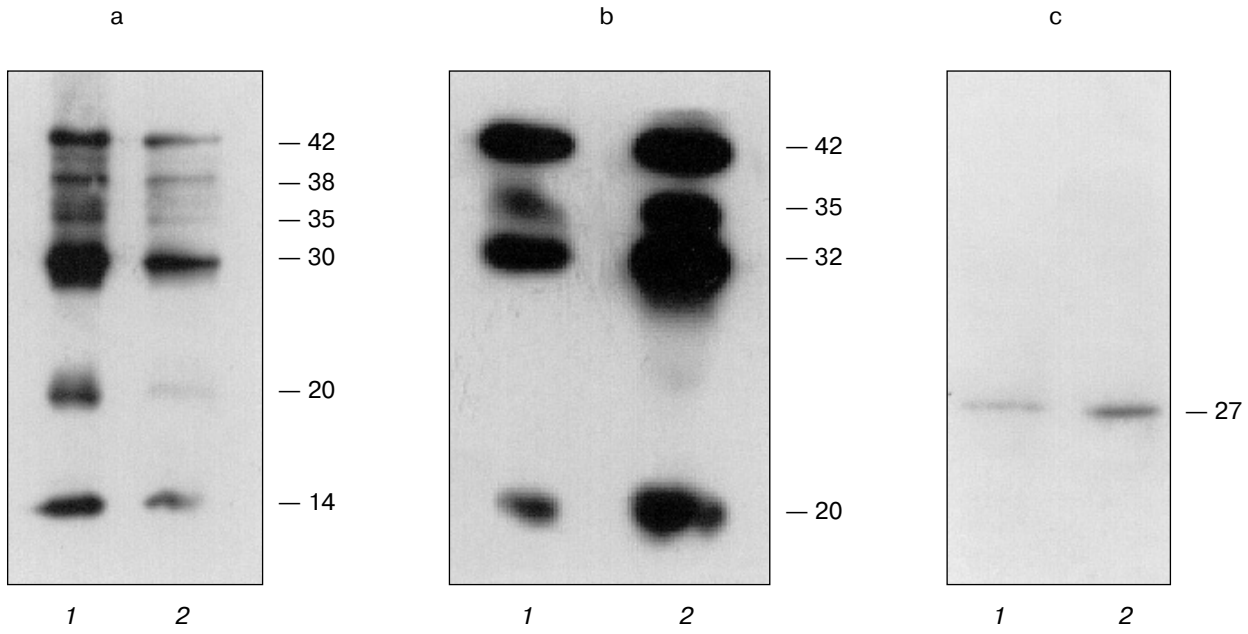

Fig. 3. Western blot assay of the levels of C/EBPα, -β, and -δ protein isoforms in rat liver in response to turpentine treatment. Livers nuclear extracts (20 μg) from control and 12-h turpentine-treated rats were loaded on lanes *1* and *2*, respectively, subjected to 12% SDS-PAGE, and transferred to nitrocellulose membranes. Immunoblots were incubated with rabbit polyclonal anti-C/EBPα (a), C/EBPβ (b), and C/EBPδ (c) antibodies or with non-immune serum as a control. Molecular masses of antigen–antibody complexes detected in each immunoblot with ECL Western immunoblot kit are shown on the right in kD. No bands were detected with non-immune serum (data not shown).

C/EBPβ and -δ mRNAs were up regulated 53 and 144% in relation to the control, respectively.

Thus, turpentine-induced changes in C/EBPα, -β, and -δ mRNA pool levels in rat liver reflect an adjustment of their gene expression levels during AP response and are accompanied by adequate modifications of their protein pool levels in NE (Fig. 3) and DNA-binding activity (Fig. 1).

C/EBPβ and -δ require phosphorylation in order to bind to the α_2-MG promoter. Given that cytokine-induced transcription of many genes is mediated by a signal-transducing protein kinase cascade [38] and the fact that phosphorylation of C/EBPβ and C/EBPδ is regarded as a major mechanism to control their activity in regulation of gene expression [39], we wanted to examine whether DNA-binding activities of the rat liver C/EBPβs and C/EBPδ towards the α_2-MG gene promoter are affected by a phosphorylation event during AP response.

To establish the optimal time necessary for the majority of rat liver NPs to reach their maximum extent of phosphorylation, they were incubated with [γ-^{32}P]ATP for different times. Figure 5 illustrates the time course of *in vitro* phosphorylation of control NPs by the action of endogenous rat liver protein kinases. Results obtained by quantification of radioactive band intensities of the examined proteins on Phosphorimager revealed that they become phosphorylated after 30 sec (Fig. 5, 0.5 min) and that their extent of phosphorylation increased reaching the highest level in the fifth minute of the reaction (Fig. 5, 5 min). Prolonged duration of the reaction resulted in a decrease of the radioactive band intensity of all proteins towards the 30 min period (Fig. 5, 30 min) when their capacity to incorporate radioactive phosphorus was lower than in the initial time interval. In accordance with this finding, the 5-min interval was appraised as the optimal time necessary for all rat liver NPs to reach their highest level of [γ-^{32}P]ATP incorporation under *in vitro* conditions.

By comparing the capacity of NPs from the livers of turpentine treated rats (Fig. 6a, lane *2*) to incorporate [γ-^{32}P]ATP to that of control proteins (Fig. 6a, lane *1*), it was discovered that they exhibited some differences. This was particularly noticeable for 27-kD NP whose capacity to incorporate radioactive phosphorus was higher in relation to the control. Since nuclear amount of 27-kD C/EBPδ in the liver NEs from turpentine-treated rats increases in response to turpentine treatment, we suggest that this change could explain increased capacity to incorporate radioactive phosphorus of this protein. Incorporated [γ-^{32}P]ATP was removed from both samples by their dephosphorylation with bovine intestinal alkaline phosphatase (Fig. 6a, lanes *3* and *4*).

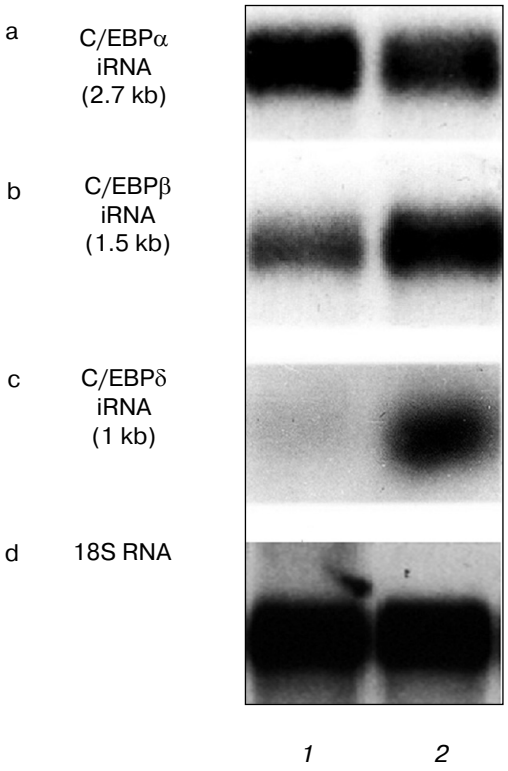

Fig. 4. C/EBPα, -β, and -δ mRNA levels in rat liver in response to turpentine treatment. Total RNA (20 μg) isolated from livers of control (panels a-c, lanes *1*) and 12 h turpentine-treated (panels a-c, lanes *2*) rats were subjected to 1.2% agarose–formaldehyde gels, transferred to nylon membranes and hybridized with [α-^{32}P]dCTP labeled C/EBPα (a), C/EBPβ (b), and C/EBPδ (c) cDNA probes. The sizes of the individual mRNAs in kb are shown on the left. d) 18-S RNA on a representative methylene blue-stained blot confirms that equivalent amounts of RNA are in each lane.

To investigate the effect of phosphorylation on DNA binding capacity of C/EBPβ and C/EBPδ, we performed the South-Western analysis with phosphorylated or dephosphorylated NPs either from the control or from turpentine treated rat livers (Fig. 6b). South-Western analysis of DNA binding ability of C/EBPβ and C/EBPδ after *in vitro* phosphorylation of NEs with unlabeled ATP revealed that phosphorylation caused increased binding of control 35-kD C/EBPβ and at the same time induced binding of 27-kD C/EBPδ isoform (Fig. 6b, lane *6*). Dephosphorylation of *in vitro* phosphorylated control NE resulted in a decline of the DNA binding activity of the former proteins and a loss of ability of the latter protein to bind to the α_2-MG promoter (Fig. 6b, lane *7*). Phosphorylation of NEs from turpentine-treated rats (Fig. 6b, lane *3*) raises DNA-binding ability of the same NPs observed with NEs from treated rats (Fig, 6b, lane *2*). This was particularly noticeable for 27-kD and 35-kD C/EBPs. Dephosphorylation of NEs from turpentine-treated rat livers (Fig. 6b, lane *4*) as well as *in vitro* phosphorylated NEs from treated rat livers (Fig. 6b, lane *5*), led to the loss of 27-kD C/EBPδ and decline of 35-kD C/EBPβ DNA-binding activity.

This result clearly showed that DNA-binding activities of 35-kD C/EBPβ and 27-kD C/EBPδ isoforms are not only regulated at the level of their genes expression in rat liver but also by their post-translational modification by phosphorylation. Such a result also served as an indirect proof of *trans*-acting 35- and 27-kD NPs being 35-kD C/EBPβ and 27-kD C/EBPδ proteins. Thus, both increased synthesis of 35-kD C/EBPβ and 27-kD C/EBPδ, as well as their activation via phosphorylation during AP response, can be considered as a prerequisite for α_2-MG gene transcriptional activation.

Peptide mapping of 27-kD C/EBPδ protein. We therefore postulated that native forms of 27-kD C/EBPδ and 35-kD C/EBPβ undergo conformational changes due to phosphorylation resulting in revealing region(s) that can better adopt to the type I IL-6RE in the rat α_2-MG promoter. Since this has already been demonstrated for 35-kD C/EBPβ isoform [11], only 27-kD C/EBPδ

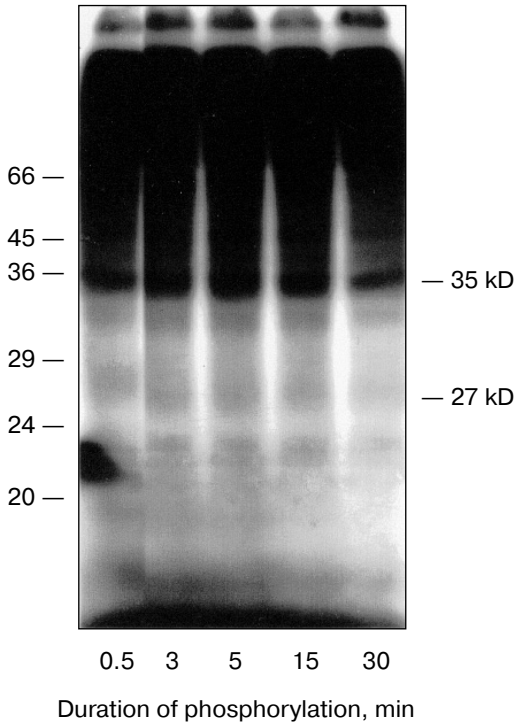

Fig. 5. Time course of *in vitro* phosphorylation of control rat liver nuclear proteins. Nuclear extracts (150 μg) were incubated with [γ-^{32}P]ATP for different times (0.5, 3, 5, 15, and 30 min) as described in "Materials and Methods". Autoradiogram of the gel with phosphorylated proteins, resolved by 12% SDS-PAGE, is shown. Time of incubation of the reaction mixture is indicated in each lane. The locations of the molecular size standards in kD are shown on the left.

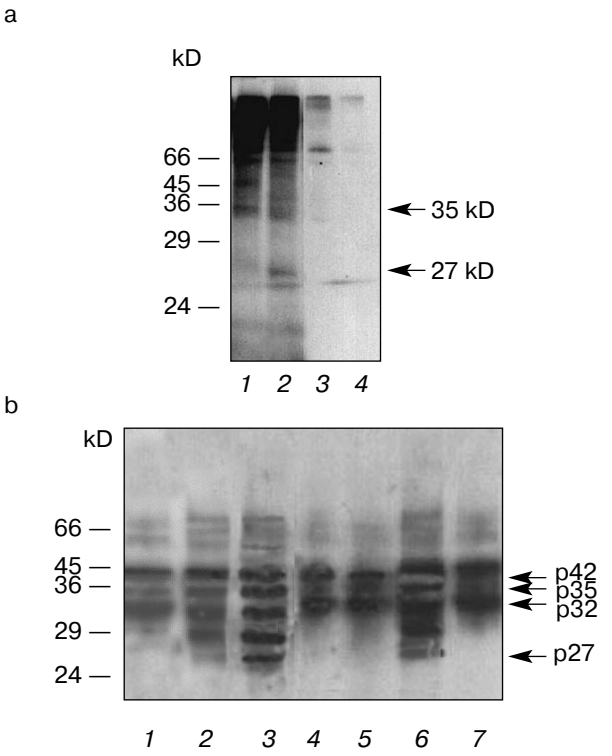

Fig. 6. a) Autoradiogram of the gel with separated *in vitro* phosphorylated control (lane *1*) and rat liver nuclear proteins from 12-h turpentine-treated rats (lane *2*) with [γ-^{32}P]ATP. Lanes *3* and *4* represent dephosphorylated liver nuclear proteins from control and turpentine-treated rats after their phosphorylation with radioactive phosphorus. b) The effect of phosphorylation/dephosphorylation of rat liver nuclear proteins on their South-Western pattern of DNA binding to the α_2-MG gene promoter sequence. Lanes *1* and *2* represent DNA-binding activities of control and nuclear proteins from 12-h turpentine-treated rat livers. Lanes *3* and *6* represent DNA binding of liver nuclear proteins from turpentine-treated and control rats that were *in vitro* phosphorylated with unlabeled ATP (50 μM) whereas lanes *4* and *7* contain liver nuclear proteins from turpentine-treated and control rats that were dephosphorylated with bovine intestine alkaline phosphatase (2.5 U per 10 μg protein) after their *in vitro* phosphorylation with unlabeled ATP. Lane *5* represents DNA binding of liver nuclear proteins from turpentine-treated rats after their dephosphorylation. All samples of nuclear proteins were size separated in 12% SDS-PAGE system, transferred onto Hybond^{TM}-C nitrocellulose membrane and probed with 10^6 cpm/lane of [α-^{32}P]dCTP labeled α_2-MG gene promoter sequence. The locations of the molecular size standards in kD are shown on the left. Arrows on the right indicate the position of the complexes formed with those nuclear proteins that belong to the C/EBP family.

was eluted from gels and subjected to V8 protease originating from *Staphylococcus aureus*. This particular protease was chosen for its ability to cleave N/Q-derived peptide bonds that are often positioned in the neighborhood of S/T residues, as protein kinases targets [40].

Comparative analysis of 27-kD C/EBPδ digestion patterns from turpentine-treated (Fig. 7, lane *2*) and control (Fig. 7, lane *1*) rat liver NEs revealed two new peptide fragments at 12- and 21-kD positions. Also, the number and the amount of observed peptide fragments of 27-kD C/EBPδ from *in vitro* phosphorylated control NEs (Fig. 7, lane *3*) were identical to those of protein from turpentine-treated rat liver NEs (Fig. 7, lane *2*).

On the basis of these experiments we believe that phosphorylation alters conformation of 27-kD C/EBPδ and 35-kD C/EBPβ in such a way that their DNA-binding activities are changed. According to the hypothetical model for positive regulation of transcription factors DNA-binding activity by phosphorylation proposed by

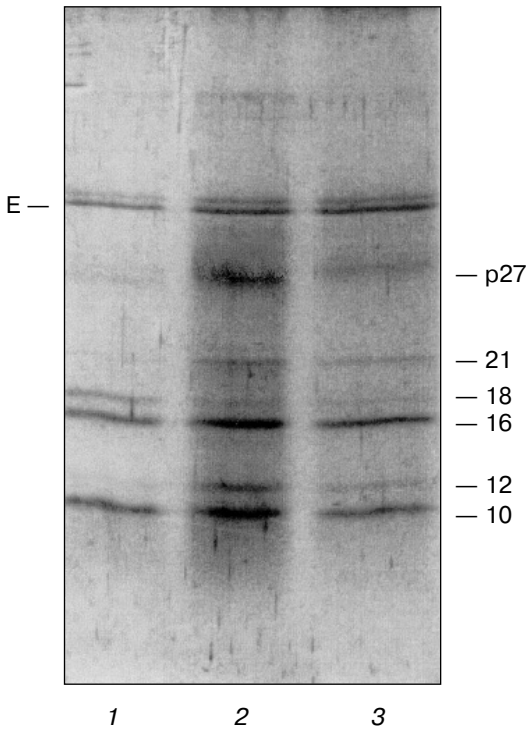

Fig. 7. One dimensional peptide map of 27-kD rat liver nuclear protein obtained after its partial proteolysis by V8 protease from *Staphylococcus aureus*. 27-kD rat liver nuclear protein was eluted from gels with size separated control (lane *1*), nuclear proteins from 12-h turpentine-treated rats (lane *2*) and control nuclear proteins *in vitro* phosphorylated with unlabeled ATP (50 μM) (lane *3*). Equal amounts (20 μg) of each of these three samples were digested with 2 μg of *Staphylococcus aureus* V8 protease (2.5 h; 37°C). Resulting peptide fragments were separated in 15% SDS-PAGE system and visualized after silver staining. Molecular masses of peptide fragments in kD are shown on the right. Arrow indicates the position of staphylococcal V8 protease.

Hunter and Karin, this occurs on phosphorylation sites quite far from their DNA-binding domain [41]. Besides, existence of five mutual peptide fragments with molecular masses 10, 12, 16, 18, and 21 kD additionally supports our postulation that 27- and 35-kD NPs are indeed members of the C/EBP transcription factors family.

Similar changes in the pool levels and DNA binding activities of C/EBPα, -β, and -δ have also been reported to be caused by stimuli other than turpentine, such as LPS [20], aging [35], and thermal injury [42]. It is interesting to notice that C/EBPδ is more strongly induced by turpentine compared to C/EBPβ isoforms, which also occurs after LPS treatment [43]. Besides the fact that turpentine induces much higher levels of C/EBPδ than LPS [5], such difference in behavior of C/EBPδ compared to C/EBPβ is thought to result from a higher binding affinity of C/EBPδ for C/EBP binding sites or stabilization of C/EBPδ binding by additional factors [13]. It can also be related to specific regulatory functions of certain C/EBP isoforms. Namely, 20-kD C/EBPβ termed liver-enriched inhibitory protein (LIP) is an amino-truncated version of the 35-kD C/EBPβ—liver-enriched activatory protein (LAP) [44]. LIP acts as a dominant negative inhibitor of C/EBP-mediated transcription through heterodimerization with other isoforms of C/EBPβ or with C/EBPδ [45]. Therefore, our result that LIP does not bind α_2-MG gene promoter may account for a significant increase of 35-kD C/EBPβ and induced binding of 27-kD C/EBPδ for α_2-MG gene promoter and consequent AP-related transcriptional activation of this gene. Besides rat, 20- and 35-kD C/EBPβ as well 27-kD C/EBPδ were all found in livers of rabbits [5] and mice [20, 35], regardless of the type of stress stimuli, strongly suggesting that a set of at least three such stress-response mediators contributes to the initial step of α_2-MG gene and other APPs genes expression.

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