Volume 291, number 1, 58–62 FEBS 10232 October 1991

ADONIS 001457939100942J

Augmentation of haptoglobin production in Hep3B cell line by a nuclear factor NF-IL6

Shunji Natsuka, Hiroshi Isshiki, Shizuo Akira and Tadamitsu Kishimoto

Institute for Molecular and Cellular Biology, Osaka University, 1-3, Yamada-oka, Suita, Osaka 565, Japan

Received 27 June 1991; revised version received 8 August 1991

The nuclear factor NF-IL6 had been suggested to be responsible for the IL-6-mediated induction of several acute-phase proteins. To obtain evidence for the involvement of NF-IL6 in the induction of acute-phase proteins, we introduced the NF-IL6 gene and its truncated mutant (delNFIL6) gene into a hepatoma cell line Hep3B. Then, we examined the effect of the overproduced NF-IL6 and delNFIL6 on the expression of haptoglobin, fibrinogen and albumin. As a result, basal production as well as induction of haptoglobin by IL-6 were augmented by the expression of NF-IL6, whereas delNFIL6 blocked the production of haptoglobin, fibrinogen and albumin.

Acute phase protein; NF-IL6; Interleukin-6; Gene expression; Haptoglobin; Hepatocyte

1. INTRODUCTION

Interleukin 6 (IL-6) is a cytokine with pleiotropic functions [1] and is a key mediator of the reaction regulating the expression of the genes encoding acute-phase proteins in the liver [2–4]. In a human hepatoma cell line, Hep3B, it has also been demonstrated that some acute-phase genes were activated at the transcriptional level by IL-6 [5]. However, the precise mechanisms through which the liver-specific expression of the acute-phase protein genes are coordinated during the acute-phase response are currently unknown.

Our previous report [6] showed that IL-1-dependent transcriptional activation of the IL-6 gene was regulated via a 14-bp palindrome sequence at position −150 bp relative to the cap site, and a novel nuclear factor (NF-IL6) was specifically able to recognize the sequence [6]. The molecular cloning of the NF-IL6 gene demonstrated the high homology of its leucine zipper sequence and basic domain with a liver-specific CCAAT/enhancer binding protein (C/EBP) [7].

Cortese and his colleagues [8,9] identified the IL-6-responsive elements (IL-6REs) in the haptoglobin, hemopexin and CRP genes in Hep3B cells. We showed that NF-IL6 was able to bind the IL-6REs in these genes [10]. Recently, Descombes et al. reported the isolation of a gene encoding a liver-enriched transcriptional factor (LAP) [11] and Poli et al. carried out the cloning of an IL-6-dependent DNA binding protein (IL-6DBP) [12]. These two molecules were the same and turned out to be a rat homolog of NF-IL6. In addition, Cheng et al. cloned a cDNA for AGP/EBP (α_1-acid glycoprotein gene/enhancer binding protein) [13] and it was also a mouse homolog of NF-IL6. These observations strongly suggested tht NF-IL6 was a *trans*-acting factor for some acute-phase proteins. However, it has not been shown whether the molecule works as a positive *trans*-acting factor on the endogenous genes for acute phase proteins in hepatocytes. In this report, we strongly suggested that NF-IL6 activated the endogenous gene for haptoglobin.

Correspondence address: T. Kishimoto, Institute for Molecular and Cellular Biology, Osaka University, 1-3, Yamada-oka, Suita, Osaka 565, Japan. Fax: (81) (6) 877 1955.

2. MATERIALS AND METHODS

2.1. *Cell culture and DNA transfection*

Hep3B cells [14] were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum.

DNA transfections were performed by the electroporation technique [5]. Stable transfections were selected with 1.2 mg/ml of G418. After the selection, the transfectants were maintained in medium with 0.6 mg/ml of G418.

2.2. *Measurement of acute-phase proteins*

The cells (1.0×10^6 cells) were cultured in 6-cm dishes for 48 h with or without 100 ng/ml of IL-6. The amount of acute-phase proteins in the culture supernatant was measured by the ELISA method.

2.3. *Construction of plasmids*

The mammalian expression vector BCMGNeo [16] was kindly donated by Dr H. Karasuyama (Tokyo University, Tokyo, Japan). A 1.5 kb fragment containing the full protein-coding region of NF-IL6 was inserted into a *Xho*I site of the BCMGNeo vector. The plasmid in which the NF-IL6 cDNA was inserted in a sense orientation was named BC/NFIL6. The 1.5-kb NF-IL6 cDNA fragment was digested with *Spl*I and self-ligated for deletion of 0.5-kbp *Spl*I fragment. The deletion mutant of the NF-IL6 gene (delNFIL6) was inserted into the BCMGNeo vector and the product was named BC/delNF. This deletion mutant lost the region from tyrosine-40 to proline-204 of NF-IL6.

Published by Elsevier Science Publishers B.V.

2.4 *Preparation of nuclear extract from Hep3B cells*

1×10^7 cells of Hep3B or transfectants were suspended in 1.5 ml of hypotonic buffer on ice. After standing on ice for 15 min, 100 μl of 10% Nonidet P-40 was added and mixed vigorously. Then the suspension was separated by centrifugation, and 200 μl of extraction buffer containing 0.4 M NaCl was added to the pellet. After shaking for 20 min, a soluble fraction was collected as nuclear extract.

2.5 *Western blot analysis*

Nuclear extract was subjected to SDS-polyacrylamide gel electrophoresis in a 10–20% gradient gel under reduced condition and electrotransferred to PVDF (polyvinylidene difluoride) membrane, Trans-Blot (Bio-Rad). Anti-NF-IL6-peptide antiserum [7] which was made against the peptide, SKAKKTVDKHSDEYKIRR in the DNA-binding domain of NF-IL6 was used as the first antibody. The alkali phosphatase method was used for the detection.

2.6. *Oligo-DNA binding assay*

Oligo-HpC (50 pmol) [8] was end-labeled with biothin, mixed nuclear extract from 1×10^7 cells and 20 μg of poly(dI-dC)(dI-dC), and incubated for 10 min at room temperature. Then, 20 μl of streptavidin-agarose was added and incubated for 20 min at room temperature. It was washed with Tris-buffered saline/0.05% Tween-20 three times. The proteins binding to it were subjected to SDS-PAGE, followed by Western blot analysis with anti-NF-IL6-peptide antiserum.

3. RESULTS AND DISCUSSIONS

3.1. *Transfection of NF-IL6 and delNFIL6 in Hep3B cells*

In Hep3B cells, NF-IL6 mRNA was constitutively expressed but its level was further augmented with IL-6 treatment (data not shown). At the same time, the production of the acute-phase proteins, haptoglobin and fibrinogen were augmented, but their fashions were different. Before IL-6 treatment, haptoglobin was expressed in a small amount and its production was drastically increased with IL-6 treatment, while fibrinogen was expressed fairly in untreated condition and an only twofold increase was observed after IL-6 treatment. The production of albumin was decreased in Hep3B cells (Fig. 1). We previously suggested that maximal transcriptional activation of NF-IL6 involved sufficient synthesis of NF-IL6 and its modification to a fully active form [10]. Therefore, we reasoned that overproduction of NF-IL6 in Hep3B cells, which expressed relatively low levels of NF-IL6, may lead to the transcriptional activation due to the increased levels of activated NF-IL6. In contrast, overproduction of a truncated mutant NF-IL6 (delNFIL6) may lead to the transcriptional suppression by competitive binding between the mutant and NF-IL6.

As a mammalian high expression vector, we used the BCMGNeo [16] which carried the neomycine resistant gene together with the bovine papilloma viral origin of replication. The NF-IL6 and delNFIL6 genes were inserted downstream of the cytomegalovirus promoter/enhancer of the BCMGNeo vector, respectively. The delNFIL6 gene deleted almost all the functional domain of NF-IL6. Its product had binding ability to DNA but not the transcriptional activity (our unpublished data). The BCMGNeo vectors containing the NF-IL6 and delNFIL6 genes were named BC/NFIL6 and BC/delNF, respectively. The vectors, BCMGNeo, BC/NFIL6 and BC/delNF were introduced into Hep3B cells and stable transfectants were obtained under G418 selection. These stable transfectants were named Hep/BC, Hep/NF and Hep/del, respectively.

The expression of the products from introduced genes was examined by Western blot analyses. The results of the representative clones from Hep/NF and Hep/del groups were shown in Fig. 2. In the case of Hep/NF, 44 kDa and 42 kDa bands were detected, whereas the 25 kDa and 22 kDa band were from Hep/del. The native NF-IL6 in Hep3B and HepG2 cells also showed two faint bands at the same locations as seen in Hep/NF (data not shown). The reason for the appearance of two bands is not clarified at present. The most likely explanation for the two forms of NF-IL6 may be due to two different translation starting points, because the second methionine exists at the 24th position of NF-IL6

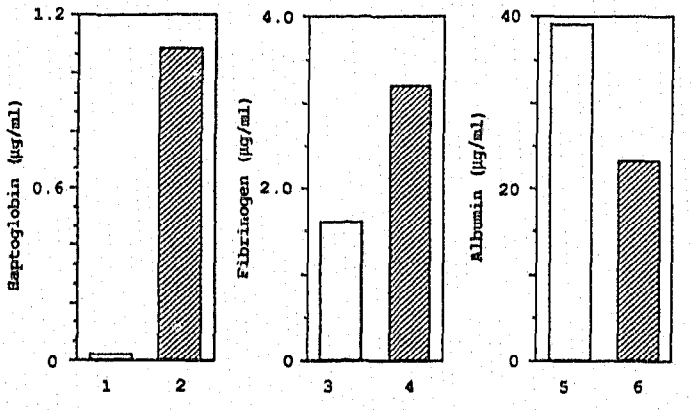


Fig. 1. Production of some acute-phase proteins from Hep3B cells. The amount of acute-phase proteins in their culture supernatants were measured with ELISA method. Columns 1, 3 and 5 are untreated and 2, 4 and 6 are IL-6-treated.

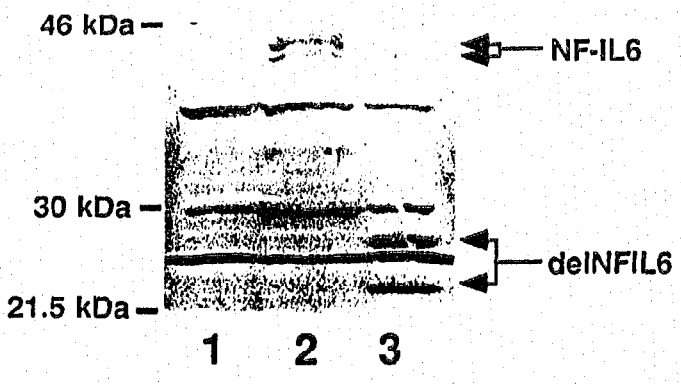
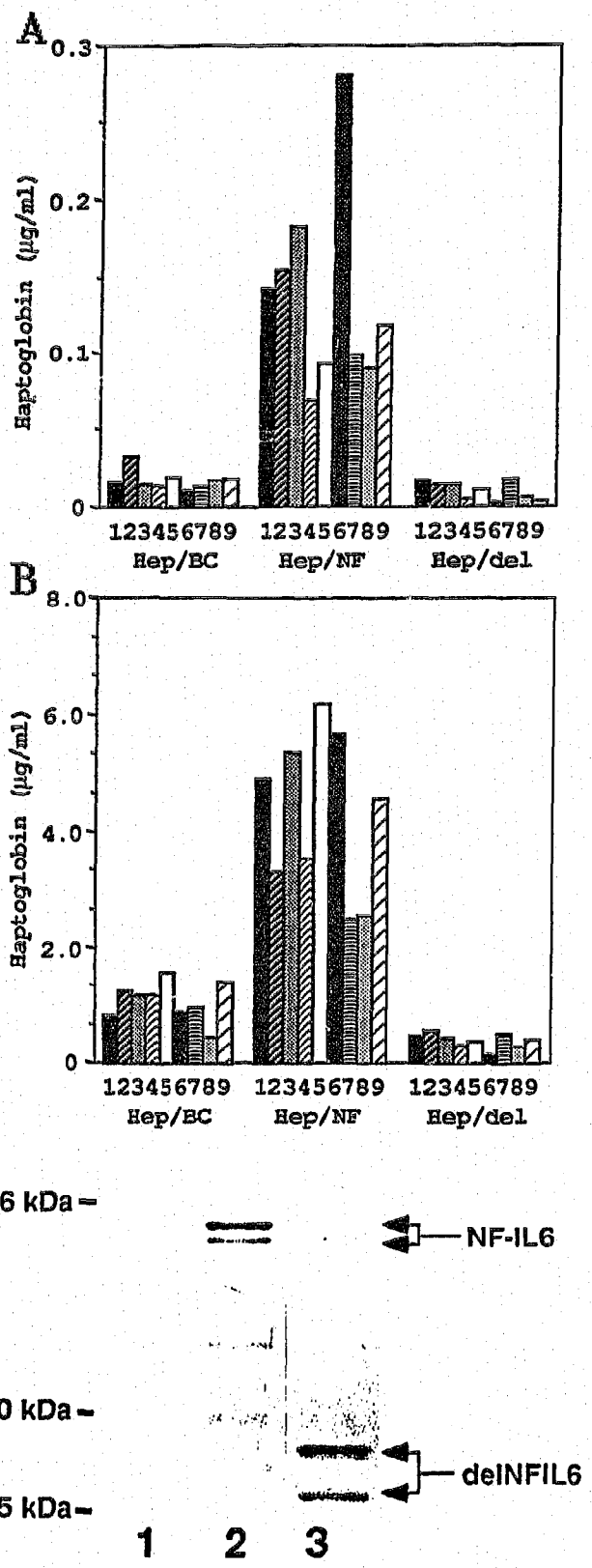


Fig. 2. Western blot analysis of the transfectants. Anti-NF-IL6-peptide antiserum was used for detection. Lane 1, 2 and 3 are Hep/BC-1 clone, Hep/NF-1 clone, and Hep/del-1 clone, respectively. For each lane, 100 μg of nuclear-extracted protein was loaded. Arrows indicate NF-IL6 and delNFIL6 products, respectively.



←

Fig. 3. Production of haptoglobin from the transfectants, Hep/BC, Hep/NF and Hep/del. The amount of haptoglobin in their culture supernatants was measured by ELISA method. Numbers 1 to 9 indicate individual clones from each group of the transfectants. Panels (A) and (B) show the result in untreated and IL-6-treated cases, respectively. In panel (A), the difference between Hep/BC and Hep/del was significant with $P<0.001$. (C) Western blot analysis of oligo-HpC binding proteins with anti-NF-IL6-peptide antiserum. The method was described in section 2. Lanes 1, 2 and 3 are Hep/BC-1, Hep/NF-1, and Hep/del-1 clones, respectively. Arrows indicate NF-IL6 and delNFIL6 products, respectively.

and the calculated molecular weight of 23 amino acids corresponds with the difference of the two products.

3.2 *Effects of the expression of NF-IL6 and delNFIL6 on acute-phase proteins*

We measured the production of haptoglobin in Hep/NF, Hep/BC and Hep/del. Each nine clones from them were used for the measurement of haptoglobin production. The results of ELISA for haptoglobin in their culture supernatants are shown in Fig. 3A. In both IL-6-treated and untreated cases, Hep/NF produced remarkably more haptoglobin than control Hep/BC (average, about 4-fold and 8-fold higher, respectively). On the other hand, in both cases, Hep/del produced it less than control Hep/BC (average, about 60% and 40% less, respectively).

To confirm the binding activity of expressed NF-IL6 and delNF-IL6 to regulatory *cis*-element of haptoglobin, we performed an oligo-DNA binding assay for nuclear extracts from the transfectants. As the oligo-DNA, we used oligo-HpC (26-mer) which was an essential *cis*-element for haptoglobin induction by IL-6 treatment [8]. The HpC-binding proteins in the transfectants were assayed by Western blotting using anti-NF-IL6 antiserum (Fig. 3C). NF-IL6 and delNFIL6-specific bands were observed in the lanes of Hep/NF-1 and Hep/del-1 clones, respectively. This result indicates that NF-IL6 and delNFIL6, expressed in the transfectants, are able to bind the HpC element in vitro, and suggests that they directly act on the haptoglobin gene.

The average amount of haptoglobin from untreated Hep/NF was about ten times less than that of control Hep/BC treated with IL-6 (Fig. 3A and B). These observations suggest two possibilities: (1) some other *cis*-elements and *trans*-acting factor may be involved in induction of haptoglobin, or (2) modification of NF-IL6 or association of other molecule(s) to NF-IL6 are necessary for maximal induction of haptoglobin.

We also measured the amounts of fibrinogen and albumin production in nine clones from each group, using the same supernatants used for the measurement of haptoglobin production. The results of ELISA for fibrinogen and albumin in their culture supernatants are

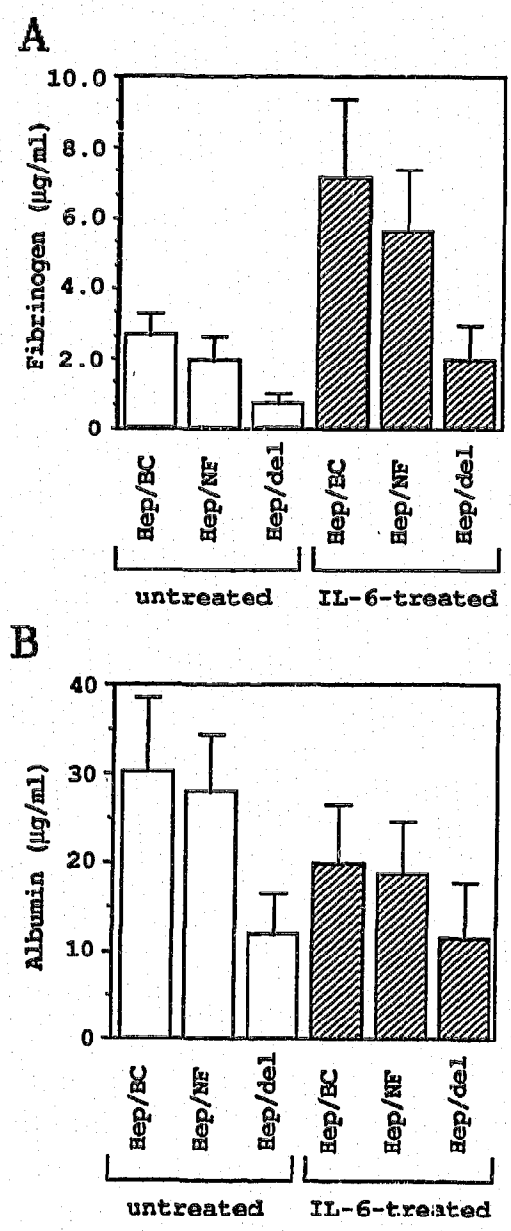


Fig. 4. Production of acute-phase proteins from the transfectants. The method of measurement was same as in Fig. 3A and B. Each bar indicates the average of nine clones from each group of the transfectants. Panels (A) and (B) indicate the production of fibrinogen and albumin, respectively. In panels (A) and (B), the differences between Hep/del and Hep/BC were significant with $P<0.001$ and $P<0.02$, respectively.

shown in Fig. 4. The production of fibrinogen from Hep/NF did not increase as compared to that from control Hep/BC. This result suggests that NF-IL6 does not have a strong transcriptional activity for the fibrinogen genes. Hep/del produced a significantly lower amount of it as compared to Hep/BC and Hep/NF, suggesting the possibility that delNFIL6 suppressed the expression of it through *cis*-elements recognized by NF-IL6. Although evidence that the 5'-regulatory regions of the fibrinogen genes contain an NF- IL6 binding motif has not been provided, our results suggest that *cis*-elements homologous to an NF-IL6 binding motif are important for the expression of fibronogen.

In the albumin gene, several *cis*-regulatory elements have been reported [17], and two of these elements, B and D, are particularly important for the liver-specific transcription of albumin. As transcription factors that recognized the D site, C/EBP and DBP were isolated [18,19]. NF-IL6 also binds to the D site [7]. We showed in this study that Hep/del produced less albumin than Hep/BC, confirming that the D site is an important positive *cis*-regulatory element in the albumin expression. If NF-IL6 is a negative regulator for the albumin gene, the suppression of albumin production would be augmented in Hep/NF as compared to Hep/BC. However, the amount of albumin produced by Hep/NF was comparable to that by Hep/BC. These results indicate that other IL-6-inducible molecule(s) which are able to bind tightly to the D site may be responsible for the transcriptional suppression of the albumin gene after IL-6-treatment.

We also measured the production of α_1-antitrypsin, the expression of which did not significantly change during IL-6-treatment in Hep3B cells [5]. The production of α_1-antitrypsin did not show any significant difference between Hep/ BC, Hep/NF and Hep/del, excluding the possibility that non-specific inhibition for protein synthesis by Hep/del might be responsible for the suppression of haptoglobin, fibrinogen and albumin production. Furthermore, to exclude the possibility that IL-6 induced by NF-IL6 might be responsible for the above results, we examined IL-6 production in Hep/BC, Hep/NF and Hep/del. IL-6 was undetectable in these culture supernatants with the ELISA method (<0.025 U/ml).

In this study, we strongly suggest that NF-IL6 actually worked on an endogenous gene of haptoglobin as a positive *trans*-acting factor, and suggest that some other factors may be necessary for maximal induction of haptoglobin.

Acknowledgements: We would like to thank Dr H. Karasuyama for the kind gift of plasmid, BCMGNeo, and also Ms. K. Kubota and Ms. K. Ono for their secretarial assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

REFERENCES

[1] Kishimoto, T. and Hirano, T. (1988) Annu. Rev. Immunol. 6, 485–512.
[2] Andus, T., Geiger, T., Hirano, T., Northoff, H., Ganter, U., Bauer, J., Kishimoto, T. and Heinrich, P.C. (1987) FEBS Lett. 221, 18–22.
[3] Castel, J.V. Gomez-Lechon, M.J., David, M., Hirano, T., Kishimoto, T. and Heinrich, P.C. (1988) FEBS Lett. 232, 347–350.
[4] Gauldie, J., Richards, C., Haarnish, D., Landsdorp, P. and Baumann, H. (1987) Proc. Natl. Acad. Sci. USA 84, 7251–7255.

[5] Morrone, G., Ciliberto, G., Oliviero, S., Arcone, R., Dente, L., Content, J. and Cortese, R. (1988) J. Biol. Chem. 263, 12554–12558.
[6] Isshiki, H., Akira, S., Tanabe, O., Nakajima, T., Shimamoto, T., Hirano, T. and Kishimoto, T. (1990) Mol. Cell Biol. 10, 2757–2764.
[7] Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T. and Kishimoto, T. (1990) EMBO J. 9, 1897–1906.
[8] Oliviero, S. and Cortese, R. (1989) EMBO J. 8, 1145–1151.
[9] Poli, V. and Cortese, R. (1989) Proc. Natl. Acad. Sci. USA 86, 7547–7551.
[10] Isshiki, H., Akira, S., Sugita, T., Nishio, Y., Hashimoto, S., Palowski, T., Suematsu, S. and Kishimoto, T. (1991) New Biol. 3, 1–8.
[11] Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E. and Schibler, U. (1990) Genes Dev. 4, 1541–1551.
[12] Poli, V., Mancini, F.P. and Cortese, R. (1990) Cell 63, 643–653.
[13] Chang, C.J., Chen, T.T., Lei, D.S. and Lee, S.C. (1990) Mol. Cell Biol. 10, 6642–6653.
[14] Knowles, B.B., Howe, C.C. and Aden, D.P. (1980) Science 209, 497–499.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[16] Karasuyama, H., Tohyama, N. and Tada, T. (1989) J. Exp. Med. 169, 13–25.
[17] Lichtsteiner, S., Wuarin, J. and Schibler, U. (1987) Cell 51, 963–973.
[18] Landschulz, W.H., Johnson, P.F., Adashi, E.Y., Graves, B.J. and McKnight, S.L. (1988) Genes Dev. 2, 786–800.
[19] Mueller, C.R., Maire, P. and Schibler, U. (1990) Cell 61, 279–291.