A heat-inducible nuclear factor that binds to the heat-shock element of the human haem oxygenase gene

Kinuko MITANI, Hiroyoshi FUJITA, Shigeru SASSA* and Attallah KAPPAS
The Rockefeller University Hospital, New York, NY 10021, U.S.A.

Haem oxygenase is a heat-shock protein in several rat tissues, as well as in certain human cells such as Hep3B hepatoma cells. In common with other heat-shock-protein genes, both the human and the rat haem oxygenase genes contain a heat-shock element (HSE) in their promoter regions. In the present study we have identified a factor in nuclear extracts of human Hep3B cells which binds specifically to the HSE of the human haem oxygenase gene. The factor in Hep3B cells was significantly induced within 1 h after heat-shock treatment, and the induction was blocked by treatment of cells with actinomycin D or cycloheximide. The factor was not detected in human HepG2 hepatoma cells, which exhibit the heat-mediated induction of heat-shock protein 70 mRNA, but not that of haem oxygenase mRNA. These findings suggest that the heat-inducible nuclear factor is increased at the level of transcription and that it may activate the human haem oxygenase gene via the HSE after heat treatment.

INTRODUCTION

Microsomal haem oxygenase (EC 1.14.99.3) is the rate-limiting enzyme in the oxidative catabolism of haem which yields biliverdin IX α [1]. The enzyme activity and the transcript of haem oxygenase can be increased in many cell types by treatment with haemin, the substrate for the enzyme, as well as various other non-haem substances [2–6]. Haem oxygenase has been reported to be a major 32 kDa stress protein, which can be induced in various cell lines of rats and humans by treatment with stress inducers such as Cd²⁺, As³⁺, thiol reagents and H₂O₂ [7–10]. Haem oxygenase has also been shown to be a heat-shock protein (hsp) in rat and mouse cells [7,11].

The human haem oxygenase gene contains, similarly to the rat haem oxygenase gene [12], a potential heat-shock element (HSE) in its 5'-flanking region (from –381 bp to –368 bp); however, heat-shock treatment failed to induce haem oxygenase in human macrophages [13], glioma [13], HeLa [9] and HL60 cells [9]. We have recently reported [10,15] that heat-shock treatment of Hep3B and HepG2 cells, i.e. the human hepatoma cell lines with well differentiated hepatocyte properties [14], induces hsp70 mRNA in both cell lines, whereas haem oxygenase mRNA was induced only in Hep3B, but not in HepG2, cells. Although a human heat-shock transcription factor (HSTF), which binds specifically to the HSE of hsp genes, has been purified [16], it is not known whether this, or a similar factor, is involved in the activation of the human haem oxygenase gene after heat-shock treatment in heat-responsive cells such as Hep3B cells. In order to examine this question, we incubated nuclear extracts from human hepatoma cells with radiolabelled oligonucleotides which contained a sequence corresponding to the HSEs in the promoter region of the human haem oxygenase [12] and the human hsp70 genes [17], and examined the formation of a sequence-specific DNA–protein complex. Our results indicate that a nuclear factor which binds to the HSE of the haem oxygenase gene is markedly induced in heat-shocked Hep3B cells, but not in heat-shocked HepG2 cells.

MATERIALS AND METHODS

Cell culture and heat treatment

Hep3B cells were obtained from American Type Culture Collection, Rockville, MD, U.S.A. HepG2 cells were kindly

provided by Dr. Barbara B. Knowles, The Wistar Institute, Philadelphia, PA, U.S.A. Hep3B and HepG2 cells were grown in 150 mm $\times$ 20 mm tissue-culture dishes (Corning, New York, NY, U.S.A.) in minimum essential medium with Earle's salts supplemented with 10% (v/v) defined calf serum (HyClone Laboratories, Logan, UT, U.S.A.), 100 units of penicillin/ml, 100 μ g of streptomycin/ml and 2 mM-glutamine. Cells were seeded into culture dishes at 12.5% confluence, followed by medium replenishment after 4 days of incubation, and the heat treatment was done 24 h after the medium replenishment. To treat cells with heat, the growth medium was removed and saved, the cells were incubated with Earle's buffer solution at 43.5 °C for 60 min. After heat treatment, Earle's buffer solution was removed and the growth medium was added back to the cells and incubation was continued at 37 °C for 3 h before the preparation of nuclear extracts.

Gel-retardation assay

Three synthetic oligonucleotides (A, B, and D) and a PCR-amplified DNA fragment (C) (Fig. 1a) were used in gel-retardation assays with nuclear extracts. Oligonucleotide A contained a 27 bp sequence harbouring the HSE and the IL-6-responsive element of the human haem oxygenase promoter region [12], and oligonucleotide B contained a 25 bp sequence containing only the IL-6-responsive element in a reverse orientation. The DNA fragment C contained bases –386 to –143 of the human haem oxygenase gene, with sequences corresponding to oligonucleotides A and B on each end of the fragment. Oligonucleotide D (–114 to –89) contained the HSE of the human hsp70 promoter region [17]. Sense-strand oligonucleotides were end-labelled with T4 polynucleotide kinase (Pharmacia, Piscataway, NJ, U.S.A.), and annealed to a 4-fold excess of the corresponding unlabelled antisense oligonucleotide. Nuclear extracts were prepared as described by Dignam *et al.* [18]. The binding buffer consisted of 10 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, 4% (w/v) Ficoll, 1 mM-dithiothreitol and 75 mM-KCl. An equimolar mixture of poly[d(I-C)] and poly[d(A-T)] (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) was used as a non-specific competitor at a concentration of 500 ng/ μ l. A 500 pg portion of double-strand probes was used in each binding reaction. A 200-fold excess of sequence-specific competitor was used for competition assays. Reaction mixtures, in a total volume

Abbreviations used: HSE, heat-shock element; hsp, heat-shock protein; HSTF, heat-shock transcription factor; IL-6, interleukin 6.

* To whom correspondence should be sent.

Fig. 1. Identification of a heat-inducible nuclear protein–DNA complex in Hep3B cells

(a) Fragments used in gel retardation analysis. (b) Gel-retardation analysis using nuclear extracts from Hep3B cells. (c) Gel-retardation analysis using nuclear extracts from HepG2 cells. Each lane contained 60 µg of protein. Heat treatment was at 43.5 °C, for 60 min, followed by incubation at 37 °C for 3 h. A specific protein–DNA complex is shown as Band H in (b). Abbreviation: IL-6-RE, IL-6-responsive element.

of 20 µl, were incubated for 15 min at 4 °C and electrophoresed on a 5% non-denaturing polyacrylamide gel in 0.25 × Tris/borate buffer (22 mM-Tris base/22 mM-boric acid/0.5 mM-EDTA, pH 8) at room temperature at 150 V for 1 h.

RESULTS
Induction of a nuclear protein which binds to the HSE of the human haem oxygenase gene after heat-shock treatment in Hep3B cells

Gel-retardation assays using nuclear extracts from untreated Hep3B cells showed the formation of a HSE-sequence specific

Fig. 2. Competition gel-retardation analysis with various oligonucleotides and nuclear extracts of Hep3B cells

Heat treatment was at 43.5 °C, for 60 min, followed by incubation at 37 °C for 3 h. End-labelled oligonucleotides were incubated with nuclear extracts, either in the absence of a competitor (–) or in the presence (+) of the indicated competitor at a 200-fold molar excess. Probe designation is shown in Fig. 1(a).

Fig. 3. Effect of actinomycin D and cycloheximide on the formation of the heat-inducible nuclear protein–DNA complex

End-labelled oligonucleotides used in the assay are shown at the top of the panel. –, no heat treatment; H, heat treatment at 43.5 °C, 60 min, followed by incubation at 37 °C for 3 h; AD, heat treatment in the presence of actinomycin D (0.5 µg/ml); CX, heat treatment in the presence of cycloheximide (2 µg/ml).

DNA-protein complex with oligonucleotide A which contained the HSE and the IL-6-responsive element of the human haem oxygenase promoter (Fig. 1b). Among several bands observed in nuclear extracts from untreated Hep3B cells, a band, termed 'H', was specifically increased by heat-shock treatment for 60 min at 43.5 °C (Fig. 1b). The intensity of band H increased linearly with increasing amounts of nuclear extracts from both untreated and heat-treated cells, indicating the quantitative validity of this assay. The level of band H at the lowest concentration of the nuclear extract from heat-treated cells (6 µg of protein/assay) was significantly greater than that observed at the highest concentration of the nuclear extract from untreated cells (60 µg of protein/assay) (Fig. 1b). In contrast with Hep3B cells, band H was not observed either in untreated or in heat-treated HepG2 cells (Fig. 1c), which are known to show no heat-mediated induction of haem oxygenase mRNA [15].

Specific binding of the factor to the HSE

Band H was clearly demonstrated in nuclear extracts of Hep3B cells after heat treatment when the probes containing the HSE were used, i.e. oligonucleotides A and D, but not when probe B, lacking the HSE, was used (Fig. 2). Competition of the DNA-protein complex-formation with the radiolabelled oligonucleotide A was also examined by adding unlabelled oligonucleotides A, B, D or fragment C to the reaction mixture. All oligonucleotides containing the HSE, i.e. A, D and the fragment C, significantly blocked complex-formation with the radiolabelled oligonucleotide A. However, oligonucleotide B, which lacks the HSE, did not compete for complex-formation (Fig. 2). These findings suggest that the formation of the DNA-nuclear protein complex is dependent on the presence of the HSE-specific sequence.

Blockade of the heat-mediated induction of the nuclear factor by actinomycin D and cycloheximide

When actinomycin D, a specific inhibitor of DNA-directed RNA synthesis, or cycloheximide, an inhibitor of protein synthesis, was added to Hep3B cells at the beginning of heat treatment, the heat-mediated induction of the nuclear factor was essentially abolished (Fig. 3), suggesting that the induction of the heat-specific nuclear factor is dependent on RNA and protein synthesis *de novo*.

DISCUSSION

The results of our experiments provide evidence for the induction of a nuclear factor(s) in human Hep3B cells in response to heat-shock treatment (Fig. 1). The factor was not detectable in HepG2 cells, which lack the heat-mediated induction of haem oxygenase [15]. A nuclear protein-DNA complex, termed 'H', was barely detectable in untreated Hep3B cells, but its level was markedly increased after heat treatment. The heat-mediated increase in the nuclear factor in Hep3B cells was rapid, occurring within 60 min. The complex-formation was highly specific to the HSE sequence, which was derived either from the human haem oxygenase gene (CTGGAACCTTCTGG) [12], or from the human hsp70 gene (CTGGAATATTCCCG) [17]. Simultaneous treatment of Hep3B cells with actinomycin D or cycloheximide significantly prevented the increase in the nuclear factor, whereas the same treatment did not influence the DNA-protein complex-formation in untreated control cells,

suggesting that the heat-mediated increase of the nuclear factor is dependent on the synthesis of RNA and protein *de novo*.

These findings are consistent with our previous studies, which demonstrated the transcriptional induction of haem oxygenase mRNA in Hep3B cells after heat treatment [15]. Similarly, hsp70 mRNA is induced in Hep3B cells by heat-shock treatment, and the induction is abrogated by treatment with actinomycin D or cycloheximide (results not shown). Thus it appears that gene activation of both haem oxygenase and hsp70 in Hep3B cells may be transcriptional.

The activation of the hsp70 gene in both HepG2 and Hep3B cells after heat-shock treatment is probably mediated by HSTF [16]. It is known that the induction of HSTF in human cells is not blocked by cycloheximide, suggesting that an initial event in the hsp70 gene activation involves the activation of a pre-existing regulatory factor [19]. The induction of band H, however, appears to be mediated by a different mechanism from that which involves HSTF, since its induction appears to occur at a transcriptional level (Fig. 1c). Thus the nuclear factor involved in the induction of band H, as well as the activation of the haem oxygenase gene, is likely to be distinct from HSTF. In addition, this nuclear factor which appears to be essential for haem oxygenase gene activation is not evidently required for the activation of the hsp70 gene. Further investigation of the nature of band H, e.g. purification of the nuclear factor, should yield useful information concerning the particular role of this factor in the activation of the human haem oxygenase gene.

This work was supported in part by grants from the U.S. Public Health Service (DK-32890, DK-39264 and ES-01055) and The Suntory Fund for Biomedical Research. The excellent technical assistance of Ms. Luba Garbaczewski is gratefully acknowledged.

REFERENCES

1. Tenhunen, R., Marver, H. S. & Schmid, R. (1969) *J. Biol. Chem.* **244**, 6388-6394
2. Kikuchi, G. & Yoshida, T. (1983) *Mol. Cell Biochem.* **53**, 163-183
3. Kappas, A. & Drummond, G. S. (1984) *Environ. Health Perspect.* **57**, 301-306
4. Maines, M. D. (1984) *CRC Crit. Rev. Toxicol.* **12**, 241-314
5. Kappas, A. & Drummond, G. S. (1986) *J. Clin. Invest.* **77**, 335-339
6. Shibahara, S. (1988) *Semin. Hematol.* **25**, 370-376
7. Taketani, S., Kohno, H., Yoshinaga, T. & Tokunaga, R. (1988) *Biochem. Int.* **17**, 665-672
8. Keyse, S. M. & Tyrrell, R. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 99-103
9. Taketani, S., Kohno, H., Yoshinaga, T. & Tokunaga, R. (1989) *FEBS Lett.* **245**, 173-176
10. Mitani, K., Fujita, H., Sassa, S. & Kappas, A. (1990) *Biochem. Biophys. Res. Commun.* **166**, 1429-1434
11. Shibahara, S., Muller, R. M. & Taguchi, H. (1987) *J. Biol. Chem.* **262**, 12889-12892
12. Shibahara, S., Sato, M., Muller, R. M. & Yoshida, T. (1989) *Eur. J. Biochem.* **179**, 557-563
13. Yoshida, T., Biro, P., Cohen, T., Muller, R. M. & Shibahara, S. (1988) *Eur. J. Biochem.* **171**, 457-461
14. Knowles, B. B., Howe, C. C. & Aden, D. P. (1980) *Science* **209**, 497-499
15. Mitani, K., Fujita, H., Sassa, S. & Kappas, A. (1989) *Biochem. Biophys. Res. Commun.* **165**, 437-441
16. Goldenberg, C. J., Luo, Y., Fenna, M., Baler, R., Weinmann, R. & Voellmy, R. (1988) *J. Biol. Chem.* **263**, 19734-19739
17. Wu, B. J., Kingston, R. E. & Morimoto, R. I. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 629-633
18. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475-1489
19. Kingston, R. E., Schuetz, T. J. & Larin, Z. (1987) *Mol. Cell Biol.* **7**, 1530-1534