FEBS Letters 457 (1999) 214–218 FEBS 22483

Coordinated regulation of ceruloplasmin and metallothionein mRNA by interleukin-1 and copper in HepG2 cells

Angela A.I. Daffada[1], Stephen P. Young*

Department of Rheumatology, Division of Immunity and Infection, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Received 21 July 1999

Abstract During the acute phase response, cytokines induce hepatic metallothionein and ceruloplasmin synthesis and the uptake of metals. We have investigated how copper and cytokines may interact in controlling ceruloplasmin (CP) and metallothionein mRNA in liver cells. We found that IL-1α, IL-1β and IL-6 increased both metallothionein-1 (MT-1) and metallothionein-2 (MT-2) mRNA in HepG2 cells. The time and pattern of induction was different, both IL-1α and IL-1β inducing two peaks of MT-1 and MT-2, with that of MT-2 being much larger. IL-6 induced only low levels of both MT-1 and MT-2 mRNA. CP mRNA was also increased after 16 h by IL-1β, whereas IL-1α induced two CP peaks at 8 and 20 h, while IL-6 had little effect. Copper administration gave rise to substantially increased MT-1 mRNA, a slightly lower increase in MT-2 and also a significant increase in CP mRNA with similar kinetics. These parallel increases in MT and CP mRNA suggest that the coordinated expression of these proteins may be important for their synthesis during the acute phase response.

Key words: Interleukin-1; Copper; Metallothionein; Ceruloplasmin; Interleukin-6; Liver; HepG2; Acute phase response

1. Introduction

During the acute phase of an inflammatory response, a substantial change occurs in the synthesis by the liver of the plasma cuproprotein ceruloplasmin (CP). This is a well-described acute phase protein which has anti-oxidant properties and plays an important role in controlling iron release from cells [1]. The oxidase function of CP is dependent on its multiple copper atoms [2] and yet, the presence of copper is reported not to influence the production of the protein by the liver, since protein synthesis occurs irrespective of the supply of copper [3,4]. In inflammation, however, metals accumulate in the liver, as a result of the increased synthesis of the low molecular weight metal-binding protein, metallothionein (MT). Cytokines, predominantly interleukin-1 (IL-1) and interleukin-6 (IL-6), have been shown to be responsible for the observed changes in liver protein synthesis [5]. The increased MT synthesis during inflammation results in an increase in the intracellular pool of copper available and may so facilitate the increased synthesis of fully functional holo-CP. While the functions of MT remain somewhat enigmatic, a role as a chaperone or reservoir of metals is generally accepted [6]. Cytoplasmic MT-derived copper may be released by reduction by glutathione, as occurs with zinc [7,8], and then transported into the Golgi or endoplasmic reticulum by the P-type ATP-ase copper transporter [9,10] for incorporation into CP. However, for this to be relevant to the acute phase response, a coordinated increase in the transcription of MT and CP would be expected, but there have been few studies investigating the relationship between the expression of these proteins. The aim of this study was to examine the kinetics of changes in MT and CP mRNA in response to inflammatory cytokines and copper in vitro, using the human hepatoma cell line HepG2. We found that both IL-1α, IL-1β and copper up-regulated mRNA for MT-1, MT-2 and CP while IL-6 had only a small effect. Their up-regulation followed similar kinetics, suggesting that their coordinated control may be important for optimal control of copper uptake and functional protein synthesis during the acute phase response.

2. Materials and methods

2.1. DNA probes

A cDNA probe for CP was provided by Dr D. Critchley, Leicester University, in the form of a lambda gt11 clone derived from a rat liver phage library. This was re-cloned into the *Eco*RI restriction sites of pUC18. Other probes used were specific for MT-1 and MT-2 mRNA (supplied by Dr P. Searle, Birmingham University) [11], actin mRNA [12] and 15 S rRNA (from Dr E. Murray, Roche Products, Welwyn Garden City). MT-l and MT-2 probes were previously shown to bind unique sequences [11] and this was confirmed here, since each bound to a single band, the size of which differed slightly on a Northern blot, and they did not compete for binding (not shown).

2.2. Cytokines

Human recombinant IL-1α and IL-1β were provided by Dr D. Westemacott, Roche Products, Welwyn Garden City. Human recombinant IL-6 was supplied by Prof. L. Aarden, The Netherlands.

2.3. Cell culture

HepG2 cells [13] were cultured in 60 mm^2 tissue culture plates (NUNC) containing RPMI medium (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 1.6 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (37°C in 5% CO_2/air). 1×10^6 cells were seeded per plate, allowed to recover for 24 h and stimulated with IL-1α (1000 U/ml), IL-1β (1000 U/ml), IL-6 (100 U/ml) or Cu (50 μM) for various times before the monolayer of cells was rinsed twice with 5 ml phosphate-buffered saline (PBS) and were recovered in 1 ml PBS by scraping. The cells were centrifuged at $2800\times g$ at 20°C and the cell pellet was frozen at −70°C.

2.4. mRNA extraction

mRNA was extracted essentially as described by Chomczynski and Sacchi [14]. The resulting RNA pellet was dried briefly after washing

*Corresponding author. Fax: (44) (0121) 414-6794.
E-mail: s.p.young@bham.ac.uk

[1] Present address: Academic Department of Biochemistry, Institute of Cancer Research, Royal Marsden Hospital, Fulham Road, London SW3 6JJ, UK.

PII: S0014-5793(99)01036-4

in 70% ethanol, re-suspended in 20 μl 0.5% SDS by heating to 65°C for 20 min and frozen at −70°C until use. Quantitation was performed by spectrophotometry.

2.5. Slot-blot analysis

20 μg (approximately 5 μl) total RNA was added to 95 μl DEPC-treated water using a 96 well plate (NUNC). An equal volume of ice cold 20 mM NaOH, 2 mM EDTA was added to denature the RNA. The RNA was then allowed to drain onto a nylon membrane (Bio-Rad) by gravity using a Bio-Rad slot-blot apparatus before being washed with 400 μl 10 mM NaOH, 1 mM EDTA by vacuum. The filter was then baked at 80°C for 1 h.

Probes were labelled with ^{32}P using an oligo-labelling kit (Amersham/Pharmacia) (CP, MT-1, MT-2 and actin cDNA) or end-labelled (rRNA oligonucleotide) using T4 kinase (Life Technologies) as recommended by the manufacturers. Pre-hybridization, hybridization and stringency washes were performed using a hybridization oven (Hybaid), as recommended by the manufacturers. Pre-hybridization (3 h) and hybridization (18 h) were carried out in 7% SDS, 1 mM EDTA and 10 mM sodium phosphate buffer (pH 7.3) at a temperature which was dependent on the probe being used (CP, actin, MT-1 and MT-2 at 62°C; rRNA at 60°C). Filters hybridized with the cDNAs were washed in 5% SDS, 1 mM EDTA and 10 mM sodium phosphate buffer (pH 7.3) for 60 min at 62°C, then, a further 30 min at 62°C with fresh buffer, followed by two more washes each with 1% SDS, 1 mM EDTA and 10 mM sodium phosphate buffer (pH 7.3) at 62°C for 30 min. When probed with rRNA, the filter was rinsed briefly in the 5% SDS wash buffer described above at 60°C, followed by fresh 5% SDS buffer for 3 min at 60°C. The filters were covered in cling-wrap and autoradiographed (Fuji X-ray film) at −70°C with intensifying screens (GRI). Quantification was performed using a LKB Ultroscan Laser Densitometer (absorbance units per mm^2) and the intensity of the bands was normalized to the control rRNA level after re-probing. All experiments were repeated on at least three occasions.

3. Results

3.1. IL-1α and IL-1β up-regulate MT-1 and MT-2 mRNA expression

When HepG2 cells were stimulated with IL-1α, an increase in MT-1 and MT-2 mRNA expression was observed. A 3-fold increase in MT-1 mRNA levels at 8–12 h post-stimulation with IL-1α was seen which was in contrast to the slightly decreased expression seen at those times in the unstimulated cells (Fig. 1A). A second peak (2.8-fold increase) was observed 20–24 h post-stimulation, which coincided with a 1.5-fold induction in MT-1 mRNA in the control cells. Similar results were seen with MT-2 mRNA expression, with a 4-fold peak induction at 8–12 h and a similar rise at 20–24 h post-stimulation with IL-1α (Fig. 1B).

IL-1β also induced the expression of MT-1 and MT-2 mRNA in the HepG2 cells with a 5-fold increase in MT-1 mRNA levels at 12–16 h post-stimulation with IL-1β (Fig. 1C). A second smaller peak of induction (2-fold) was seen at 48 h post-stimulation, while unstimulated cells produced a 2.4-fold peak induction of MT-1 at 24 h. MT-2 mRNA showed the greatest response with expression reaching a peak 19.3-fold increased at 8–16 h post-stimulation with IL-1β (Fig. 1D), compared to levels of 2.9-fold in the unstimulated control cells. Another smaller rise was observed 48 h post-stimulation which did not coincide with the small increase in MT-2 mRNA expression in the unstimulated cells. No significant changes in actin mRNA in response to cytokines were observed (data not shown).

3.2. IL-6 has a small effect on MT mRNA expression

IL-6 had a lesser stimulatory effect on MT-1 or MT-2 mRNA expression than either form of IL-1. A small transient increase in MT-1 mRNA (2.7-fold) was observed 4–8 h post-stimulation with IL-6 compared to a small decrease in MT-1 mRNA levels seen at these times in the unstimulated cells (Fig. 2A). The cytokine also produced a small increase in MT-2 mRNA expression (2.8-fold) at 4–8 h (Fig. 2B).

3.3. IL-1α and IL-1β up-regulate CP mRNA expression

When HepG2 cells were stimulated with IL-1α, a 6.5-fold induction of CP mRNA levels was observed (Fig. 3A). A second peak (6.4-fold) was seen at 20–24 h, which coincided with a 1.5-fold increase in CP mRNA levels in the control cells. IL-1β also produced an increase in CP mRNA expression (Fig. 3B) with a 3.7-fold induction at 12–16 h post-activation, compared to a 1.2-fold increase in unstimulated controls at this time. In contrast, only a very small increase in CP mRNA levels (1.7-fold) was observed, which occurred 4–8 h after stimulation with IL-6 (Fig. 3C).

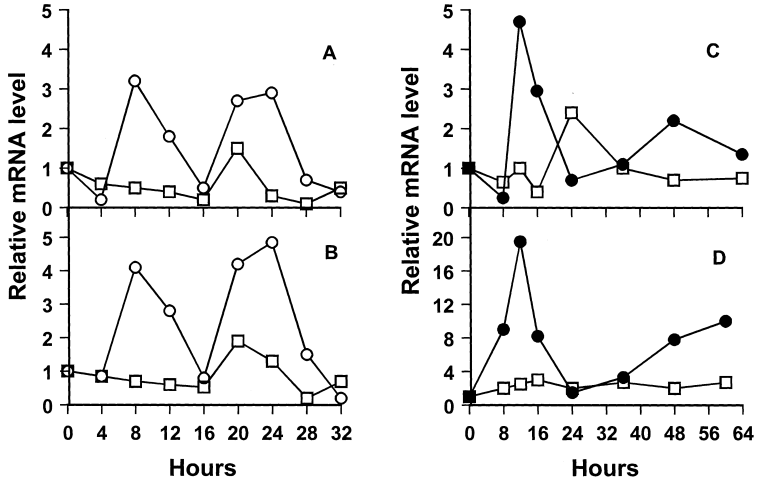

Fig. 1. Effects of IL-1α and IL-1β on MT-1 and MT-2 mRNA in HepG2 cells. HepG2 cells (1×10^6 cells) were stimulated with either IL-1α (1000 U/ml) or IL-1β (1000 U/ml), harvested and mRNA slot-blotted and probed with ^{32}P-labelled cDNA probes for MT-1 and MT-2. Actin was used as a control probe which showed no significant changes over the time of the experiment. After autoradiography, bands were quantitated by densitometry and normalized to the intensity of the rRNA probe with the same blots. A and C: MT-1. B and D: MT-2. No IL-1 (□), IL-1α (○), IL-1β (●).

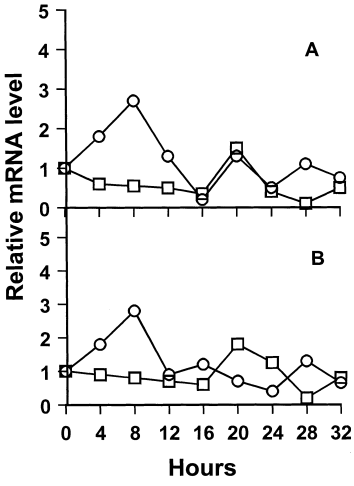

Fig. 2. Effects of IL-6 on MT-1 and MT-2 mRNA in HepG2 cells. As for Fig. 1, but cells were incubated with IL-6 (100 U/ml) A: MT-1. B: MT-2. No cytokine (□), +IL-6 (○).

3.4. Copper increases MT-1, MT-2 and CP mRNA expression

Upon stimulation of HepG2 cells with Cu ions, a substantial increase in MT-1 (16-fold) and MT-2 (10-fold) mRNA levels was observed (Fig. 4A and B, respectively). This induction was observed 4–12 h post-induction. Copper also produced an increase in CP mRNA levels (5.8-fold) which coincided with the peak in MT mRNA (Fig. 4C). A smaller second peak was seen from about 24 h with all three mRNAs.

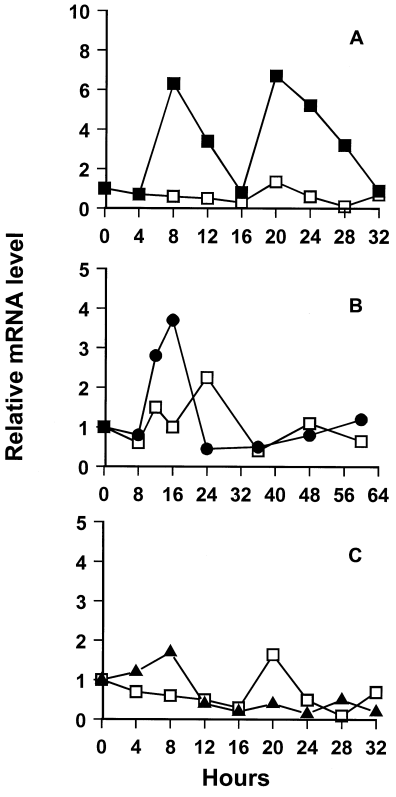

Fig. 3. Effects of IL-1 and IL-6 on CP mRNA in HepG2 cells. HepG2 cells were incubated with IL-1α (1000 U/ml), IL-1β (1000 U/ml), IL-6 (100 U/ml) and then probed using a CP cDNA. A: IL-1α. B: IL-1β. C: IL-6. Open symbols without cytokine, closed symbols with cytokine.

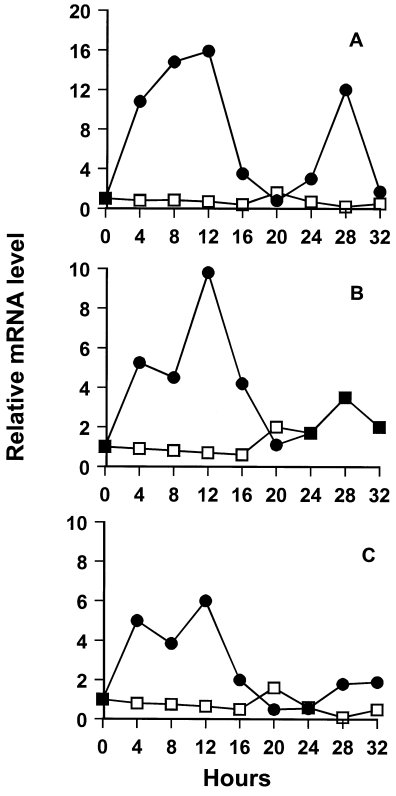

Fig. 4. Effects of Cu on MT-1, MT-2 and CP mRNA in HepG2 cells. HepG2 cells were incubated with Cu (50 μM), harvested and mRNA slot-blotted and probed with ^{32}P-labelled cDNA probes for MT-1, MT-2 and CP. A: MT-1. B: MT-2. C: CP. Open symbols without Cu, closed symbols with Cu.

4. Discussion

The control of APP synthesis is achieved largely through cytokine-induced alterations in mRNA expression of the APP genes in the liver. Although Karin [15] demonstrated that only MT-2 mRNA expression (not MT-1) was induced by IL-1 in HepG2 cells. The results presented here are in agreement with others who have shown that IL-l is capable of inducing MT-1, MT-2 and CP expression [16].

Here, we have shown that MT genes showed differential stimulation by IL-1α and IL-1β with the induction by IL-1α and IL-1β being greater for MT-2 (4- and 19-fold, respectively) (Fig. 1C and D) than MT-1 (3- and 5-fold, respectively (Fig. 1A and B). In addition, IL-1β was a better inducer of MT mRNA levels than IL-1α. In general, therefore, MT-2 was the more responsive gene and IL-1β the better stimulator of the two species.

Although the sequences of IL-1α and IL-1β are dissimilar [17] and they have both been found to bind the same receptor with different affinities (the binding affinity of IL-1α is less than that for IL-1β) [18], little information is available on differential actions of the two forms of IL-1 in signal transduction or APP synthesis.

The temporal and quantitative differences in induction of MT-1, MT-2 and CP mRNA expression could be due to a difference in the processing of the IL-1α/IL-1R and IL-1β/IL-1R complexes after internalization by the HepG2 cells and/or by different signal transduction mechanisms [19]. Differences

in processing or receptor recycling could also explain the second peak of mRNA expression. Upon binding of IL-1 to its cell surface receptors, internalization has been found to occur in a number of cell lines [20]. However, the fate of the IL-1R after internalization is dependent on the cell type. In fibroblasts, a continuous level of IL-1R on the cell surface and recycling of internalized receptors [21] has been noted, while in T- and B-cells, there is a reduction of receptors on the plasma membrane (75–80% remaining) [22], with no recycling of the protein [20]. The double peak in mRNA could therefore be explained by retro-endocytosis of the complex as can occur with, for instance, the transferrin receptor [23].

However, two forms of IL-1R have been shown to exist and to be expressed in a cell specific manner [22,24]. If both forms are expressed by HepG2 cells, then, complex responses to IL-1 would be expected. IL-1α has been found to migrate to the nucleus in B- and T-cells in the form of IL-1α/IL-1R complexes [20], implying a nuclear role for IL-lα in signal transduction. A mechanism similar to glucocorticoid signal transduction may be involved: the glucocorticoid receptor plus ligand migrates to the nucleus, binds to DNA and so modulates gene transcription on its own or in conjunction with other transcription factors [25]. No evidence for the migration of IL-1β/IL-1R complexes into the nucleus has been reported and this may explain the differences seen between the actions of IL-1α and IL-1β.

Since the second peak produced by IL-1α coincided with the peak mRNA expression in the unstimulated cells, it may represent an amplification of the normal up-regulation of MT that is seen during cell division. The HepG2 cells that were used here are adherent cells which were seeded from confluent flasks and are so likely to have been at least partially synchronized in the cell cycle and could so produce a simultaneous rise in the MT just prior to cell division as seen for other cells [26]. MT is an important housekeeping gene and induction of growth in rat hepatocytes has been shown to induce high levels of mRNA which were maximal at 48 h. It was also shown that the effects of growth and metals were additive which would support the observations that we have made here [27].

In contrast to IL-1, IL-6 was found to stimulate MT-1 and MT-2 mRNA levels (Fig. 2), but to have little effect on CP mRNA (Fig. 3). The increase in CP, MT-1 and MT-2 mRNA levels induced by IL-6 was observed at 4–8 h post-stimulation, which is earlier than the stimulatory effects of IL-1. Peak increases of MT-1 and MT-2 mRNA expression were 2.7- and 2.8-fold, respectively, while the induction of CP mRNA was only 1.7-fold. These are compared to the slight reduction of mRNA levels in the unstimulated cells at 8 h. These results agree with reports that IL-6 is capable of increasing the mRNA expression of MT-1 and MT-2 genes, although this effect was only reported to occur in the presence of dexamethasone [28,29]. The same study indicated that IL-1 had little effect on MT mRNA levels, which is in contrast to the present finding that IL-1 stimulates MT expression to a greater extent than IL-6 and to other reports of IL-1 stimulation of MT mRNA expression [15]. Up to 1000 U/ml IL-1 were used by Schroeder and Cousins [28] with no effect, while the increase in MT mRNA levels was demonstrated here with 100 U/ml (in both cases, U = lymphocyte activating factor units). The explanation for this difference is not obvious, but it may be a species difference, since both we and Karin [15] investigated human cells while Schroeder and Cousins used rat liver cells.

IL-1α was also the strongest inducer of CP mRNA, which occurred with similar kinetics to the MT. The second peak with IL-1α was seen as with MT. Since CP is unlikely to be a housekeeping gene in HepG2 cells, this suggests that there may be two cycles of IL-1α response. Again, IL-1β gave a monophasic response and since MT-1 was similarly up-regulated in these cells, this suggests that the biphasic response induced by IL-1α is a specific effect of this cytokine. IL-6 has been shown to increase the level of synthesis of CP protein [30] and it has been reported that IL-6 is an equal good inducer of CP expression as IL-1 [31]. The results obtained here indicate that any effect of IL-6 on CP is likely to occur at a post-transcriptional level, rather than transcriptionally, since we saw little effect of IL-6 on CP mRNA levels.

We also saw a significant effect of copper on CP mRNA. Previous reports on the role of metals in CP synthesis have observed little effect either in vivo [3,4] or in vitro in LEC-rat liver-derived cell lines [32] with apo-CP being produced even in the absence of copper. However, neither of these studies directly investigated the effects on CP mRNA. The CP gene contains a few superficially characterized control elements including a C/EBP consensus sequence [33] and one partially homologous to the rat albumin D site [34], although none through which a metal response might be controlled. However, our previous work might explain the mechanism involved. We showed that CAT constructs under the control of a promoter containing an AP-1 consensus sequence could be up-regulated by metals, whereas a construct containing an NFκB element was not [35]. Examination of the rat CP gene 5′ region [34] contains no obvious AP-1-binding site (unpublished observation). This suggests that the metal responses in the CP mRNA level we have seen probably arise due to induction of some other protein dependent either on a unique metal response element or on AP-1 which in turn enhances the transcription of the CP mRNA or its stabilization. The characterization of such a factor(s) awaits further work.

In summary, there is a complex interplay between copper and cytokines in the control of mRNA for MT and CP in liver cells. Other factors are also involved but the concomitant up-regulation of these copper-binding proteins may be important in their regulation and function during the inflammatory response.

Acknowledgements: This work was supported by the Arthritis Research Campaign and the Biotechnology and Biological Science Research Council.

References

[1] Young, S.P., Fahmy, M. and Golding, S. (1997) FEBS Lett. 411, 93–96.
[2] Gutteridge, J.M.C. and Stocks, J. (1981) CRC Crit. Rev. Clin. Med. Sci. 14, 257–329.
[3] Sato, M. and Gitlin, J.D. (1991) J. Biol. Chem. 266, 5128–5134.
[4] Gitlin, J.D., Schroeder, J.J., Leeambrose, L.M. and Cousins, R.J. (1992) Biochem. J. 282, 835–839.
[5] Baumann, H. and Gauldie, J. (1994) Immunol. Today 15, 74–80.
[6] Palmiter, R.D. (1998) Proc. Natl. Acad. Sci. USA 95, 8428–8430.
[7] Jacob, C., Maret, W. and Vallee, B.L. (1998) Proc. Natl. Acad. Sci. USA 95, 3489–3494.
[8] Jiang, L.J., Maret, W. and Vallee, B.L. (1998) Proc. Natl. Acad. Sci. USA 95, 3483–3488.
[9] Yang, X.L., Miura, N., Kawarada, Y., Terada, K., Petrukhin,

K., Gilliam, T. and Sugiyama, T. (1997) Biochem. J. 326, 897–902.
[10] Bingham, M.J., Burchell, A. and McArdle, H.J. (1995) J. Physiol. (Lond.) 482, 583–587.
[11] Searle, P.F., Davison, B.L., Stuart, G.W., Wilkie, T.M., Norstedt, G. and Palmiter, R.D. (1984) Mol. Cell. Biol. 4, 1221–1230.
[12] Alonso, S., Minty, A., Bourlet, Y. and Buckingham, M. (1986) J. Mol. Evol. 23, 11–22.
[13] Knowles, B.B., Howe, C.C. and Aden, D.P. (1980) Science 209, 497–499.
[14] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[15] Karin, M., Imbra, R.J., Heguy, A. and Wong, G. (1985) Mol. Cell. Biol. 5, 2866–2869.
[16] Cousins, R.J. and Leinart, A.S. (1988) FASEB. J. 2, 2884–2890.
[17] Aarden, L.A. (1979) J. Immunol. 123, 2928–2929.
[18] Matsushima, K., Akahoshi, T., Yamada, M., Furutani, Y. and Oppenheim, J.J. (1986) J. Immunol. 136, 4496–4502.
[19] Horuk, R. (1991) Biochem. J. 273, 79–83.
[20] Mizel, S.B., Kilian, P.L., Lewis, J.C., Paganelli, K.A. and Chizzonite, R.A. (1987) J. Immunol. 138, 2906–2912.
[21] Bird, T.A. and Saklatvala, J. (1987) J. Immunol. 139, 92–97.
[22] Horuk, R. and McCubrey, J.A. (1989) Biochem. J. 260, 657–663.
[23] Young, S.P. and Bomford, A. (1994) Biochem. J. 298, 165–170.
[24] Horuk, R., Huang, J.J., Covington, M. and Newton, R.C. (1987) J. Biol. Chem. 262, 16275–16278.
[25] Beato, M. (1989) Cell 56, 335–344.
[26] Studer, R., Vogt, C.P., Cavigelli, M., Hunziker, P.E. and Kagi, J.H. (1997) Biochem. J. 328, 63–67.
[27] Moffatt, P., Plaa, G.L. and Denizeau, F. (1995) Hepatology 21, 1038–1044.
[28] Schroeder, J.J. and Cousins, R.J. (1990) Proc. Natl. Acad. Sci. USA 87, 3137–3141.
[29] Itoh, N., Kasutani, K., Muto, N., Otaki, N., Kimura, M. and Tanaka, K. (1996) Toxicology 112, 29–36.
[30] Mackiewicz, A. and Kushner, I. (1989) Scand. J. Immunol. 29, 265–271.
[31] Ramadori, G., Van Damme, J., Rieder, H. and Meyer zum Buschenfelde, K.H. (1988) Eur. J. Immunol. 18, 1259–1264.
[32] Nakamura, K., Endo, F., Ueno, T., Awata, H., Tanoue, A. and Matsuda, I. (1995) J. Biol. Chem. 270, 7656–7660.
[33] Bingle, C.D., Fleming, R.E. and Gitlin, J.D. (1993) Biochem. J. 294, 473–479.
[34] Fleming, R.E. and Gitlin, J.D. (1992) J. Biol. Chem. 267, 479–486.
[35] Daffada, A.A.I., Murray, E.J. and Young, S.P. (1994) Biochim. Biophys. Acta 1222, 234–240.