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Transcriptional Control of the Heme Oxygenase Gene in Mouse M1 Cells During Their TPA-Induced Differentiation Into Macrophages

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Abstract It has long been known that heme oxygenase (HO) is a key enzyme in heme catabolism and recently it was also found to acts as an oxidative stress protein to produce carbon monoxide (CO), which has similar actions to those of nitrogen monoxide (NO). Therefore, we examined transcriptional control of the HO gene in mouse M1 (myeloleukemia) cells during their differentiation into macrophages. Since the promoter region of this gene is known to have a TPA-responsive element (TRE), its expression might be regulated by a C-kinase signal transduction pathway. Then we investigated the activation of the HO gene after treatment of M1 cells with TPA and inhibitors of C-kinase. When M1 cells were treated with TPA, they differentiated into macrophage-like cells. Upon treatment with TPA, H_2O_2 was produced first, the nuclear proto-oncogenes *fos* and *jun* were activated, and then the HO gene was activated. The extent of transcriptional activation of the *fos, jun,* and HO genes in M1 cells treated with TPA was reduced by a specific inhibitor of C-kinase and a scavenger of oxygen radicals. When M1 cells were treated with H_2O_2, essentially the same level of transcription of the HO gene was observed, but the extent of transcriptional activation of the *fos* and *jun* genes was about half of the treatment with TPA. Super-shift assays using the TRE of the HO gene revealed that the Fos and Jun proteins from nuclei of M1 cells treated with TPA bound to the TRE, and same assays using DNA with the NF-κB motif also revealed that the active NF-κB protein from M1 cells treated with H_2O_2 or TPA also bound to the corresponding motif. These results strongly suggest that the HO gene in M1 cells is activated by TPA through a production of H_2O_2, an oxidative activation pathway of NF-κB, and a signal-transduction pathway that involves C-kinase during the differentiation of macrophages that occurs upon treatment with TPA.

Key words: M1 cell, heme oxygenase, transcription, H_2O_2, TPA

INTRODUCTION

Mouse M1 cells are known to differentiate into macrophage-like cells upon treatment with TPA [Kurata and Nakajima, 1990]. An important function of macrophages is to scavenge aged erythrocytes and presumably to produce heme oxygenase (HO), a key enzyme in the degradation of heme [Tenhunen et al., 1968]. Therefore, it is of interest to investigate the mechanism of activation of the HO gene during differentiation of macrophages. Recently HO has been shown to act also as a scavenger of oxygen radicals [Stocker, 1990; Applegate et al., 1991]. During the degradation of heme, HO produces carbon monoxide (CO) which has a similar action to that of nitric oxide (NO) [Schmidt, 1992; Stevans and Wang, 1993; Zhuo et al., 1993]. It has long been thought that this is the only reaction that produces CO in higher organisms. We reported previously that transcription of the HO gene in mouse M1 cells is activated by treatment with TPA [Kurata and Nakajima, 1990]. Since the mechanism of this activation by TPA is unknown, in this study we examined the mechanism of this activation. The promoter region of the HO gene has a TPA-responsive element (TRE; [Alam and Zhining, 1992]), and TPA is a strong activator of C-kinases [Nishizuka, 1984]. Therefore we suspected that the C-kinase and Fos-Jun heterodimer complex (AP-1) might be involved in activation of the HO gene during the differentiation of M1 cells to macrophages that is induced by TPA. Then we

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also examined the activation of the *fos* and *jun* proto-oncogenes during TPA-induced differentiation of M1 cells to macrophages. We found that on treatment of cells with TPA, H_2O_2 was produced first then the *fos* and *jun* proto-oncogenes in the cells were transcribed after about 30 min, and the HO gene was transcribed 1.5 h later. On treatment of the cells with H_2O_2, essentially the same results for transcription of the HO gene were obtained, but the rates of transcription of the *fos* and *jun* oncogenes were about 50% of those observed with TPA treatment. Treatment with a C-kinase inhibitor (GF-109203) greatly reduced the transcriptional activation of these genes in M1 cells treated with TPA. In the case of treatment with H_2O_2, the rate of transcription of the HO gene was only reduced by about 30% of that in cells without the C-kinase inhibitor. When M1 cells were pretreated with N-acetyl-L-cysteine (NAC; a scavenger of oxygen radicals) [Roederer et al., 1990] the H_2O_2-induced transcriptional activation of the HO gene was greatly inhibited, but the TPA-induced transcription of this gene was only reduced by about 50% of that in cells without NAC. On treatment with both GF-109203X and NAC, the TPA-induced transcriptional activation of the HO gene was greatly reduced. Gel mobility shift assays revealed that the DNA of the TRE motif in the promoter region of the HO gene mainly bound to the protein in the nuclear extract of TPA-treated cells, and the NF-κB DNA motif mainly bound to the protein in the nuclear extract of H_2O_2-treated M1 cells. The binding proteins were found to be AP-1 and NF-κB-like protein by super-shift assays with specific antibodies. These findings strongly suggest that the mouse HO gene is activated by an AP-1 through the C-kinase signal-transduction pathway and by oxidative NF-κB-activation pathways upon treatment of M1 cells with TPA.

MATERIALS AND METHODS

Reagents

The DNA probe for the mouse HO gene was a gift from Dr. S. Sakiyama [Kageyama et al., 1988] and M1 cells were a gift from the Japanese Cancer Research Resources Bank. RPMI 1640 medium, fetal bovine serum (FBS), AP-1-binding DNA and NF-κB-binding DNA motifs were purchased from Gibco, and *fos* and *jun* DNA probes Fos- and Jun-specific antibodies were purchased from Oncogene Science. Antibodies against NF-κB p65 were purchased from Santa Cruz Biotechnology. TPA, H_2O_2, and the C-kinase inhibitor (CF-109203X) were from Sigma and serum free medium (Hymedium 606) was from Nipro. All other standard chemicals used were of reagent grade.

Cell Culture

M1 cells derived from a mouse myeloleukemia were grown in RPMI 1640 supplemented with 10% FBS under 5% CO_2 in air at 37°C. Routine subculture was performed at the late log phase. For experiments, cells were collected, washed three times with serum- and phenol red-free synthetic medium (Hymedium 606 buffered with 10 mM Hepes, pH 7.2), and incubated in the same medium containing test compounds [first treated with 1 μM C-kinase inhibitor (GF-109203X) or 20 mM NAC and 1 h after with 0.05 μg/ml TPA or 0.2 mM H_2O_2] under 5% CO_2 in air at 37°C. For treatment with H_2O_2, cells were pretreated with 0.2 mM H_2O_2 for 30 min. Then H_2O_2 was washed out and cells were used for experiments.

Slot Blot Analysis

Samples of 5×10^6 M1 cells were collected at intervals during treatment with test compounds, and RNA was isolated by the guanidium-isothiocyanate-cesium chloride method [Kurata and Nakajima, 1990]. Extracted RNA was denatured, spotted onto a nylon membrane, and hybridized with a ^{32}P-labeled mouse HO, c-*fos* or c-*jun* DNA probes for 24 h at 45°C. Then the filters were washed successively with 2 × SSC (0.3 M NaCl, 30 mM sodium citrate) that contained 0.1% SDS at 30°C, and with 0.1 × SSC that contained 0.1% SDS at 65°C for 0.5 h. The filters were exposed to X-ray film (Kodak-XAR) at −70°C for about 2 days. After autoradiography, the hybridized slots were removed and their radioactivities were quantitated in a liquid scintillation counter.

Assay of Nuclear Transcription In Vitro

Nuclear run-off assays were performed by the method of Groudine et al. [1981] and Kurata and Nakajima [1990] with minor modifications. In brief, M1 cells treated with appropriate agents were collected (1×10^7 cells) at intervals and treated with 100 μl of a solution of 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 (pH 7.4). The resultant suspension of nuclei was mixed with an equal volume of reac-

tion buffer [2.5 mM $MgCl_2$, 2 mM dithiothreitol, 20 units of RNase inhibitor and 0.4 mM NTP (ATP, CTP, and GTP)] that contained 200 μCi of [α-^{32}P]UTP. The mixture was incubated at 30°C for 15 min, and the reaction was terminated by the addition of DNase I (final conc., 10 μg/ml). After incubation for 10 min at 30°C, SDS, EDTA, and proteinase K were added and nuclear RNAs were extracted with phenol-chloroform in the presence of 10 μg of yeast tRNA. The labeled RNA was precipitated five times with 70% ethanol, and the final pellet of RNA was dissolved in hybridization buffer. Samples of 10 μg of *fos, jun,* and the HO gene probes were denatured, blotted onto a nylon membrane, and allowed to hybridize with the isolated RNA in 1 ml of hybridization buffer for 50 h at 45°C. The washing conditions were similar to those employed for slot hybridization. The filters were exposed to XAR film for 5 days at −70°C. After autoradiography, hybridized slots were removed and radioactivity was quantitated in a liquid scintillation counter.

Immunoblot Analysis

M1 cells treated with appropriate agents were collected at intervals and cell extracts were prepared by five cycles of freezing (−80°C) and thawing. Samples of 20 μg of protein from each extract of M1 cells were blotted onto a Millipore Immobilon polyvinylidene difluoride membranes. Each membrane was then blocked with 10% FBS in TTBS (0.05% Tween 20, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and treated with a primary antibody that had been diluted appropriately with TTBS for 18 h at room temperature. Membranes were then treated with ABC kit reagents (Vectastain) according to the supplier's recommended protocol. Immunoreactive HO, Fos, and Jun proteins were located with the substrate of the peroxidase reaction. Intentions of signals that appeared on the membranes were photographed and measured by densitometry.

Treatment With NAC and C-Kinase Inhibitor

M1 cells were collected, transferred to serum-free medium, and preincubated with 20 mM NAC or 1 μM GF-109203X [Toullec et al., 1991]. They were then cultured with TPA and H_2O_2 for 0.5 or 2 h, collected, and used for run-off assays, as described above. For H_2O_2 treatment, cells were pretreated with 0.2 mM H_2O_2 for 30 min. H_2O_2 was washed out and cells were used for experiments as described above.

Measurement of the Concentration of H_2O_2 in the Culture Medium

M1 cells were collected, washed with serum-free medium (Hymedium 606), and cultured in Hymedium 606 (serum- and phenol red-free) with TPA. At intervals, samples of medium were collected and their H_2O_2 content was determined by the method of Shibanuma et al. [1990] with slight modifications. In brief, an aliquot of the medium was incubated in a reaction mixture that consisted of 5 mM 3-(p-hydroxyphenol)-propionic acid (HPPA) in 0.1 M Tris-HCl (pH 8.5) for 5 min and then the fluorescence intensity was measured at 404 nm with excitation at 320 nm in a spectrofluorometer. The amount of H_2O_2 was determined from a calibration curve that demonstrated a linear relationship between the intensity of fluorescence and the concentration of H_2O_2 in the range of 0.2–10 μM.

Binding of Nuclear Proteins to the AP-1- and NF-κB-Binding Motifs

Nuclear proteins that bound to the AP-1 and NF-κB-binding DNA motifs were detected with a Gibco-BRL band shift assay kit. A 40-mer fragment of DNA containing two AP-1-binding motifs (TGACTCA) and a 42-mer DNA containing two NF-κB-binding DNA motifs (GGGGACTTTCC) were end-labeled with [γ-^{32}P]ATP for binding of proteins with nuclear extracts. Nuclear extracts were prepared at intervals by the method of Dignam et al. [1983] during treatment of cells with TPA or H_2O_2. Samples of 5 ng of end-labeled DNA fragments were incubated with 3 μg of nuclear proteins in a solution that contained 20 mM Hepes buffer (pH 7.9), 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 125 mM spermidine, and 3 μg of poly (dI-dC) for 20 min. The mixtures were then fractionated by electrophoresis in a 4% polyacrylamide gel in Tris-borate-EDTA buffer and subjected to autoradiography. For competition assays, excess amounts of unlabeled 40-mer fragments and synthetic mutant variants of the AP-1-binding motif, (AGCTGGACTCAGCCG) and of the NF-κB-binding motif (TCGACAGAATTCACTTTCCGAGAGGCTCGA; [Lee et al., 1987] were used for binding assays. For super-shift assays, 10-fold diluted rabbit antiserum against c-Fos, c-Jun, or the NF-κB (p65) protein was also added to the binding reaction (super-shift assay). The complexes of DNA, nuclear protein, and antibody were identified by electropho-

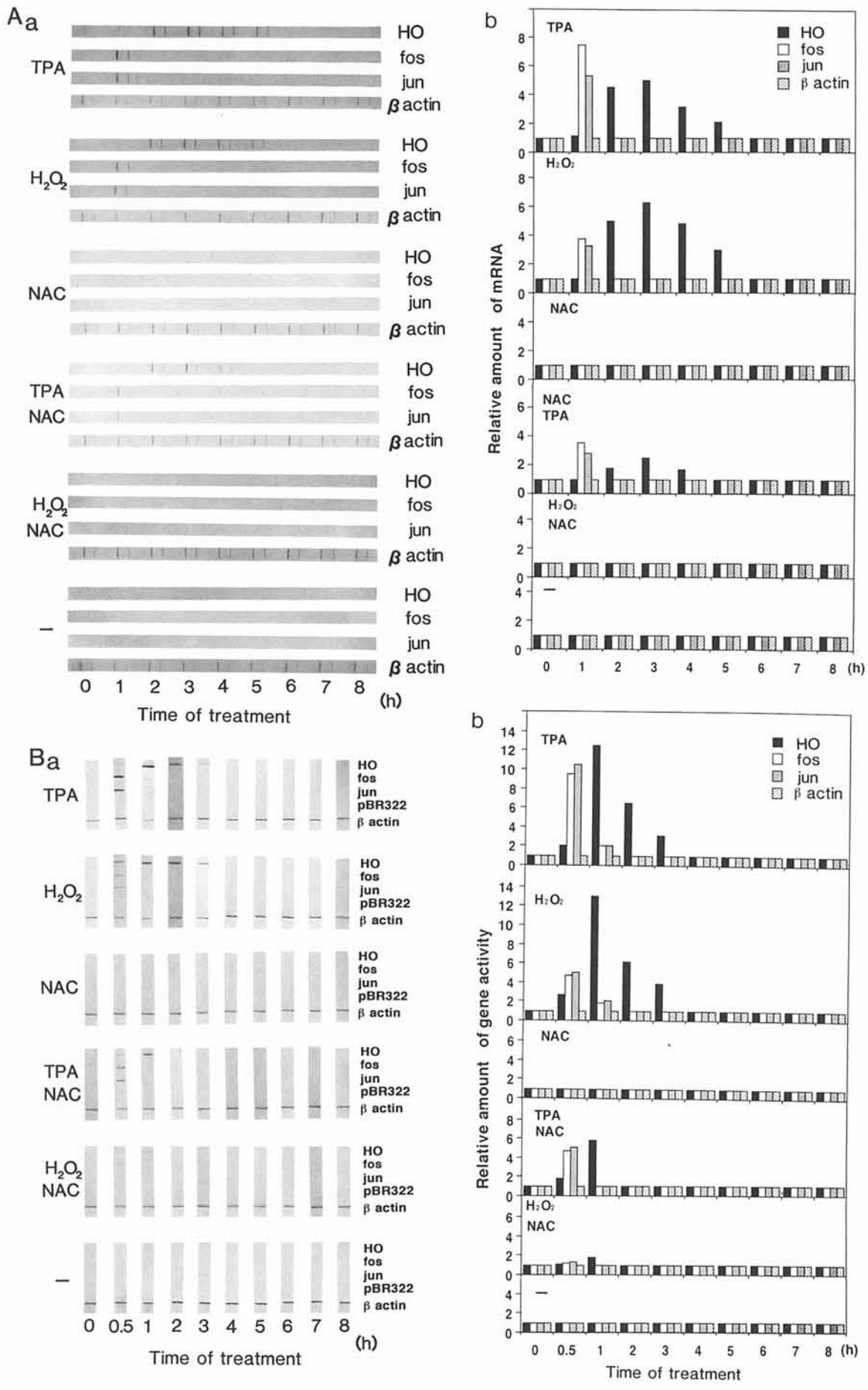
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Figure 1.

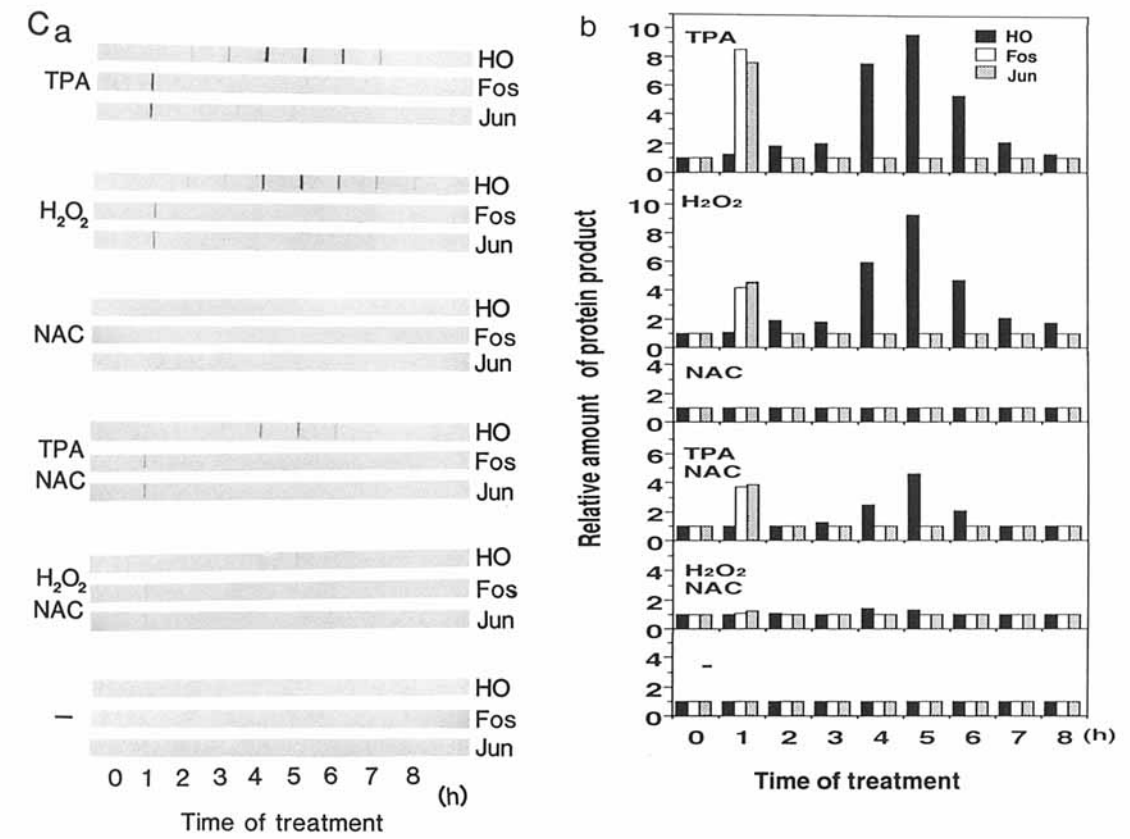

Fig. 1. Transcriptional activation of the *fos, jun,* and HO genes in M1 cells after treatment with TPA or H_2O_2 with or without pretreatment with NAC. **A:** Accumulation of *fos, jun,* and HO mRNAs in M1 cells after treatment with TPA or H_2O_2 with or without pretreatment with NAC. *a:* Slot hybridization: Cytoplasmic RNAs were isolated at intervals after the indicated treatments and samples of 4 μg or 1 μg of RNA were spotted onto nylon membrane filters. The filters were hybridized with probes for mouse *c-fos, c-jun,* HO, and β-actin genes. *b:* Pattern of accumulation: The radioactivity of the hybridized slots (4 μg of RNA) shown in "a" was measured with a liquid-scintillation counter and relative amounts of mRNAs were calculated by subtracting background values and normalizing to "0 h" values for the corresponding genes. **B:** Run-off assays for *fos, jun,* and HO genes in M1 cells after the various treatments. *a:* Run-off assays: Nuclei of M1 cells after various treatments were isolated and transcription was allowed to proceed for 15 min at 30°C in the presence of [α^{32}P]UTP. The RNA products were allowed to hybridize with *fos, jun,* HO, and β-actin DNA probes. *b:* Pattern of activation: Radioactivity of hybridized slots shown in "a" was measured with a liquid-scintillation counter and relative transcriptional activation was calculated as described in A. **C:** Accumulation of Fos, Jun, and HO proteins in M1 cells after the various treatments. *a:* Immunoblot analysis: Cytoplasmic extracts obtained from M1 cells treated with the indicated reagents were blotted onto the filters. The filters were examined with an ABC kit using antibodies against Fos, Jun, and HO proteins. *b:* Patterns of accumulation: After immunoblot analysis, filters were photographed and the amounts of Fos, Jun, and HO proteins were determined by densitometry. Relative amounts of these proteins were calculated almost the same as described in A.

resis and autoradiographed as described previously [Metzger et al., 1993].

Binding of Nuclear Proteins to the Promoter Region of the HO Gene

Two closely spaced DNA elements (TGAGTCA and TGTGTCA), located approximately 4 kb upstream of the mouse HO gene, resemble the consensus binding site of the AP-1 transcription factor. A 286-bp DNA fragment upstream of the mouse HO gene was end-labeled with [γ-^{32}P]ATP for binding of proteins with nuclear extracts. Band-shift assays and super-shift assays for binding of nuclear proteins from M1 cells treated with various reagents were performed as described above.

RESULTS

Activation of the *fos, jun,* and HO Genes by Treatment With TPA or H_2O_2

Mouse M1 cells were cultured in RPMI 1640 containing 10% FBS to the late log phase and then transferred to serum-free synthetic medium (Hymedium 606, without phenol red) and

treated with various reagents. The M1 cells were collected at intervals, RNA was extracted and amounts of HO, *fos,* and *jun* mRNAs were measured by slot hybridization (Fig. 1Aa). On treatment of TPA or H_2O_2, *fos,* and *jun* mRNAs appeared 1 h and then disappeared rapidly. However, after on treatment with H_2O_2, the levels of *fos* and *jun* mRNAs were about half those observed on treatment with TPA. Upon treatment with TPA or H_2O_2, HO mRNA was detectable after 1 h and its level reached a maximum after 3 h and then decreased. The amounts of HO mRNA during H_2O_2 and TPA treatment were almost the same in spite of the reduced amounts of *fos* and *jun* mRNAs observed during H_2O_2 treatment. NAC raises the intracellular level of glutathione and thereby protects cells from the effects of oxidative reagents. When M1 cells were pretreated with NAC, the amounts of *fos, jun,* and HO mRNAs induced by treatment of H_2O_2 were greatly reduced, being the same as in control M1 cultures. When NAC-pretreated M1 cells were treated with TPA, the amounts of *fos, jun,* and HO mRNAs were about half of those obtained from NAC-untreated cells. The time courses of changes in the relative amounts of *fos, jun,* and HO mRNAs are shown in Figure 1Ab.

For investigation of the way in which the activation of the *fos, jun,* and HO genes corresponds to the accumulation of these mRNAs in the steady state, samples of 1×10^7 M1 cells were collected at intervals during treatment with several reagents. The nuclei of the cells were then extracted and subjected to transcription assays in vitro (Fig. 1Ba). Upon treatment with TPA or H_2O_2, the rates of transcription of the *fos* and *jun* genes reached a maximum after 0.5 h and then rapidly decreased. On treatment with H_2O_2, the rates of transcription of the *fos* and *jun* genes were about half of those observed on treatment with TPA. There was no difference between the rate of transcription of the HO gene upon treatment with TPA and H_2O_2. During both treatments, the transcriptional activity of the HO gene appeared after 1 h, reached a maximum after 2 h and decreased after 3 h. When M1 cells were pretreated with NAC, no transcriptional activation of the *fos, jun,* or HO gene was observed on treatment with H_2O_2, and the rates of the transcription were the same as in control M1 cultures. However, when NAC-pretreated M1 cells were treated with TPA, the rates of transcription of the *fos, jun,* and HO genes were about half of those in NAC-untreated M1 cells. The time courses of changes in relative rates of transcription of these genes are shown in Figure 1Bb.

The accumulation of the protein products of the *fos, jun,* and HO genes was also measured by immunoblotting. 2×10^6 M1 cells were collected at intervals during treatments with several reagents, and equivalent numbers of cells extracts were spotted onto filters and immunostained with the antibodies against the HO [Kurata and Matsumoto, 1992], Fos and Jun proteins (Fig. 1Ca). Upon treatment with TPA or H_2O_2, the amounts of Fos and Jun proteins reached maxima after 1 h and then rapidly decreased. However, the amounts of Fos and Jun proteins on treatment with H_2O_2 were about half of those on treatment with TPA. During both treatments, the amounts of HO reached a maximum after 5 h and decreased after 7 h, and there was no difference between the amounts of HO in both treatments with TPA and H_2O_2. When M1 cells were pretreated with NAC, the amounts of Fos, Jun, and HO proteins did not increase on treatment with H_2O_2, being the same as in control M1 cultures. However, when NAC-pretreated M1 cells were treated with TPA, the amounts of Fos, Jun, and HO proteins were about half those in cells that had not been pretreated with NAC. The time courses of changes in the relative amounts of these proteins are shown in Figure 1Cb. The time courses of the accumulation patterns of the mRNAs, proteins and the transcriptional activations of the corresponding genes were closely correlated with each other. Thus, the amounts of Fos, Jun, and HO seemed to be controlled at the transcriptional level.

Production of H_2O_2

Activation of the HO gene by H_2O_2 was almost completely inhibited by NAC pretreatment. Interestingly, activation of the HO gene by TPA was also inhibited about 50% by NAC treatment. Thus, TPA might act as a kind of oxidative reagent. To examine this possibility, we measured the production of H_2O_2 in M1 cells during treatment with TPA. The serum- and phenol red-free medium (2 ml) of M1 cells was collected at intervals during TPA treatment and the H_2O_2 content of the medium was measured by the method of Zaitsu and Ohkura [1980]. The time course of changes in the H_2O_2 content of the medium is shown in Figure 2. The produc-

tion of H_2O_2 reached a maximum after treatment with TPA for 10 min, then decreased and reaching a low level after 40 min. Thus, treatment of M1 cells with TPA resulted in the production of H_2O_2 at a very early stage.

Inactivation of the HO Gene by Treatment With C-kinase Inhibitor

M1 cells were pretreated with an inhibitor (GF-109203X) of C-kinase and then treated with TPA or H_2O_2 with or without pretreatment with NAC, and the rate of transcription of the *fos, jun,* and HO genes was determined after 0.5 and 2 h. Pretreatment with GF-109203X reduced the rate of transcription of the *fos* and *jun* genes on TPA treatment by about 80% and that of the HO gene by about 60% (Fig. 3). However, it reduced the rate of transcription of the HO gene in H_2O_2-treated cells by only about 30%. Pretreatment of M1 cells with two reagents (GF-109203X and NAC) greatly reduced the rates of transcriptions of the *fos, jun,* and HO genes in M1 cells upon treatment with TPA and H_2O_2. These results indicate that the activation of transcription of the HO gene by TPA is mainly regulated by the C-kinase signal-transduction pathway and an oxidative activation process.

Binding of Nuclear Factors From M1 Cells Treated With TPA or H_2O_2 to the AP-1-Binding Motif and to the TRE-Like Sequence in the Upstream Region of the HO Gene

The mouse HO gene has a distal AP-1-binding motif in its promoter region [Alam and Zhining, 1992]. Two closely spaced DNA elements (TGAGTCA and TGTGTCA), located approximately 4 kb upstream of the mouse HO-1 gene, resemble the consensus binding site TGA (G/C) TCA of the AP-1 transcription factor. Binding of proteins to AP-1-binding DNA motif was detected with a Gibco-BRL kit. A 40-mer DNA containing TGACTCA motifs was end-labeled with [γ-^{32}P]ATP, and incubated with nuclear proteins extracted from M1 cells treated with TPA or H_2O_2, with or without NAC and GF-109203X. The 40-mer DNA fragments were then separated by electrophoresis and mobility shifts were examined (Fig. 4Aa). The percentage shifts of the bands were related to the extent of transcriptional activity of the HO gene (Fig. 4Ab; cf. Fig. 1). The shifted band disappeared on addition of a 50-fold excess of unlabelled 40-mer fragments but did not disappear on addition of a 25-fold excess of unlabelled synthetic variant of the AP-1 motif [Lee et al., 1987]. The shifted bands were super-shifted by antisera against Fos and Jun proteins, and several super-shifted bands appeared (Fig. 4Aa). These results suggest that M1 cells treated with TPA or H_2O_2 produce an AP-1-like trans-activating factor. However, on treatment with H_2O_2, only a small amount of the AP-1-like factor was detected (Fig. 4Aa).

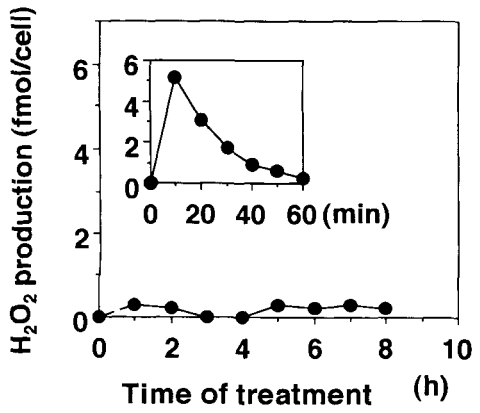

Fig. 2. Production of H_2O_2 in M1 cells after treatment with TPA. M1 cells were treated with TPA and aliquots of the medium were collected at intervals. H_2O_2 was quantitated as described in the text.

To examine the binding activity of the transcription-activating element in the upstream region of the HO gene, we prepared a 286-bp fragment (SacI-XbaI fragment from a site approximately 4 kb upstream of the mouse HO gene) that contained two AP-1-binding motifs, end-labeled it and incubated it with nuclear proteins from M1 cells treated with the various reagents as described above. The 286-bp fragments were then separated by electrophoresis and mobility shifts were examined (Fig. 4Ba,b). Essentially the same results were obtained as for the AP-1-binding motif (cf. Fig. 4A), and percentage shifts were related to the extent of transcriptional activation of the HO gene (Fig. 4Bb, cf. Fig. 1). These results indicate that M1 cells produce active AP-1 upon treatment with TPA or H_2O_2 and thereby activate the promoter of the HO gene. However, on H_2O_2 treatment, M1 cells produce smaller amounts of AP-1.

Binding of a Nuclear Factor From M1 Cells Treated With TPA or H_2O_2 to the NF-κB-Binding Motif

To examine the binding activity of a nuclear factor in M1 cells to the NF-κB-binding motif, we end-labeled a 42-mer NF-κB-binding frag-

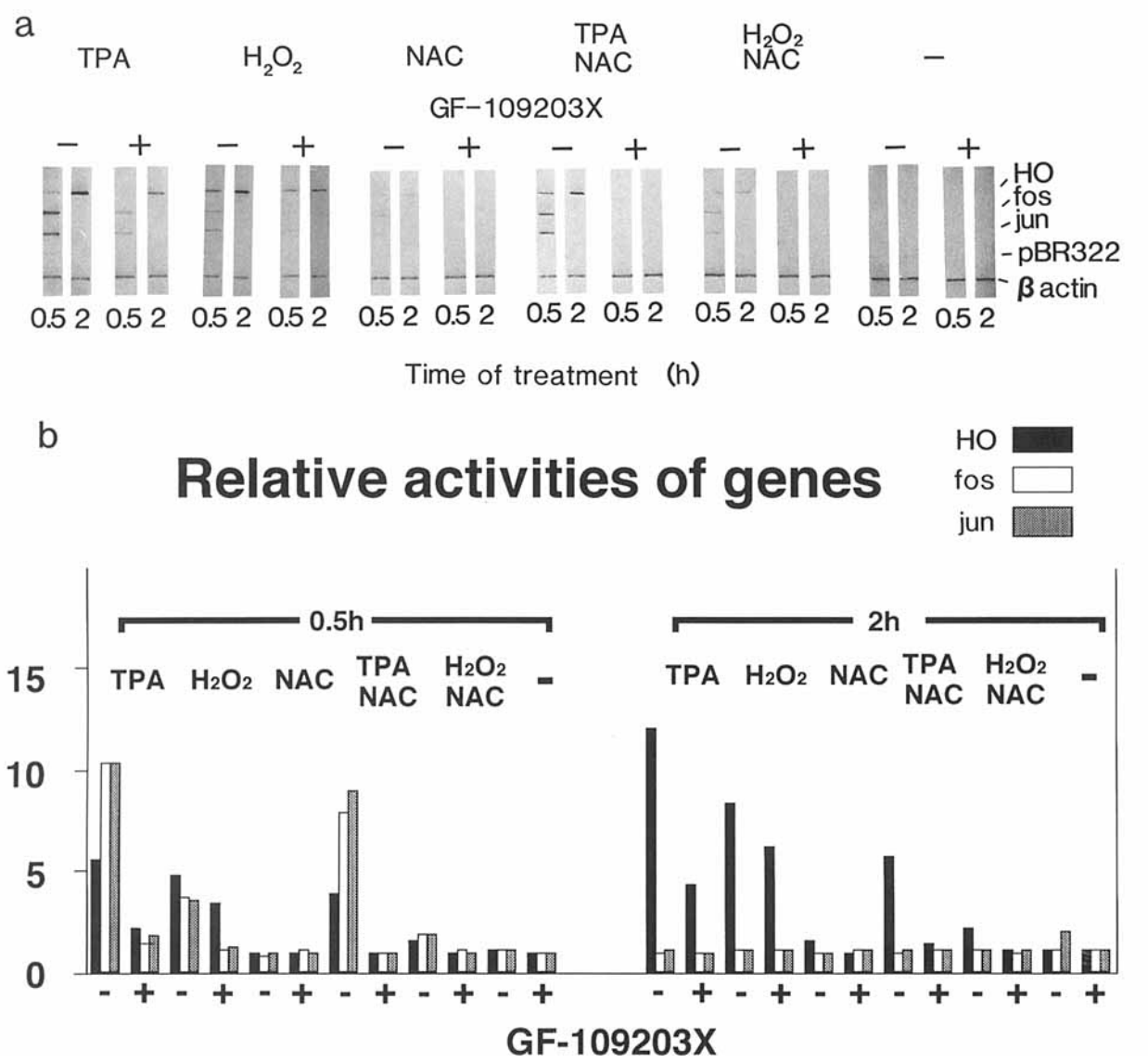

Fig. 3. Effects of GF-109203X on transcription of the *fos, jun,* and HO genes in M1 cells. **a:** Run-off assays: M1 cells were treated first with GF-109203X and then with TPA or H_2O_2 with or without NAC. After treatment for 0.5 or 2 h, nuclei of M1 cells were isolated and the transcriptional activity of the *fos, jun,* HO, and β-actin genes was determined by run-off assays (cf. Fig. 1B). **b:** Relative rates of transcription: Radioactivity of hybridized slots in "a" was measured and relative transcriptional activities were calculated by subtracting background values and normalizing to untreated control values after 0.5 and 2 h, respectively.

ment of DNA and incubated it with nuclear proteins from M1 cells treated with various reagents. The 42-mer DNA was then separated by electrophoresis and mobility shifts were examined (Fig. 5a). The percentage shift of the shifted band was related to the transcriptional activation of the HO gene (Fig. 5b cf; Fig. 1). The shifted band disappeared on addition of a 50-fold excess of an unlabelled 42-mer fragments but did not disappear on addition of a 25-fold excess of an unlabelled synthetic variant of the NF-κB motif [Lee et al., 1987]. The shifted bands were also super-shifted by antiserum against NF-κB p65 and several super-shifted bands appeared (Fig. 5a). These results suggest that M1 cells treated with TPA or H_2O_2 activate an NF-κB-like factors. However, on TPA treatment, M1 cells produce smaller amounts of this factor than they do on treatment with H_2O_2.

DISCUSSION

HO functions not only in the catabolism of heme but also in production of CO gas, which has similar actions to those of NO. This is the

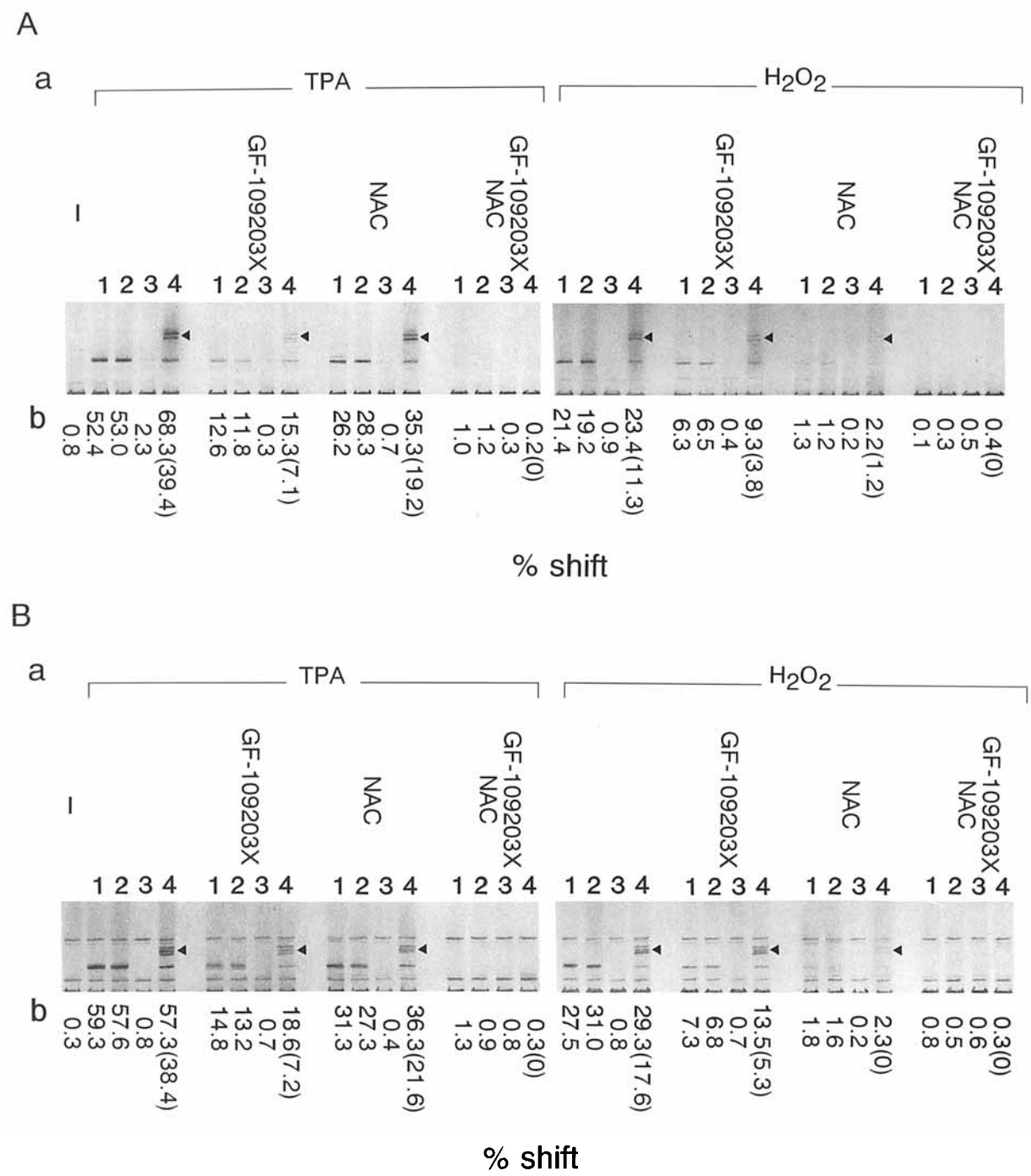

Fig. 4. Band-shift assays of the AP-1-binding motif and the HO promoter region. **A:** Band-shift assays of the AP-1 binding motif. *a:* Band-shift assays: Binding of the 40-mer AP-1-binding motif to nuclear proteins from M1 cells first treated with GF-109203X for 1 h and then with TPA or H_2O_2 for 1 h with or without NAC. A 40-mer DNA was end-labelled and used for binding assays. *Lane 1:* band-shift assay without competitor DNA and antibodies. *Lane 2:* a 25-fold excess of an unlabelled mutant of the AP-1-binding motif was added during the band-shift assay. *Lane 3:* a 50-fold excess of unlabelled 40-mer DNA was added during the band-shift assay. *Lane 4:* super-shift assay with Fos- and Jun-specific antibody. The arrowheads indicate the super-shifted bands. *b:* Percentage shift: Percentage shift of the complex of 40-mer DNA and nuclear proteins was calculated as follows:

$$\text{shift}(\%) = \frac{\text{cpm of shifted band}}{\text{total cpm}} \times 100$$

total cpm = cpm of shifted band + cpm of unshifted band. For lane 4, the total percentage shift and the percentage super-shift are shown. **B:** Band-shift assay of the HO promoter region. *a:* Band-shift assays: Binding of DNA fragments of the HO promoter region with nuclear proteins from M1 cells as in A. The 286-bp DNA fragment was labeled and used for binding assays. *Lane 1:* band-shift assay without competitor DNA and antibodies. *Lane 2:* a 25-fold excess of the unlabelled mutant of the AP-1-binding motif was added during the band-shift assay. *Lane 3:* a 50-fold excess of the unlabelled 286-bp DNA fragment was added during the band-shift assay. *Lane 4:* super-shift assay with Fos- and Jun-specific antibodies. The arrowheads indicate the super-shifted bands. **b:** Percentage shift: Percentage shift of the complex of the 286-bp DNA and nuclear proteins were examined as described above. For lane 4, the total percentage shift and the percentage super-shift are indicated.

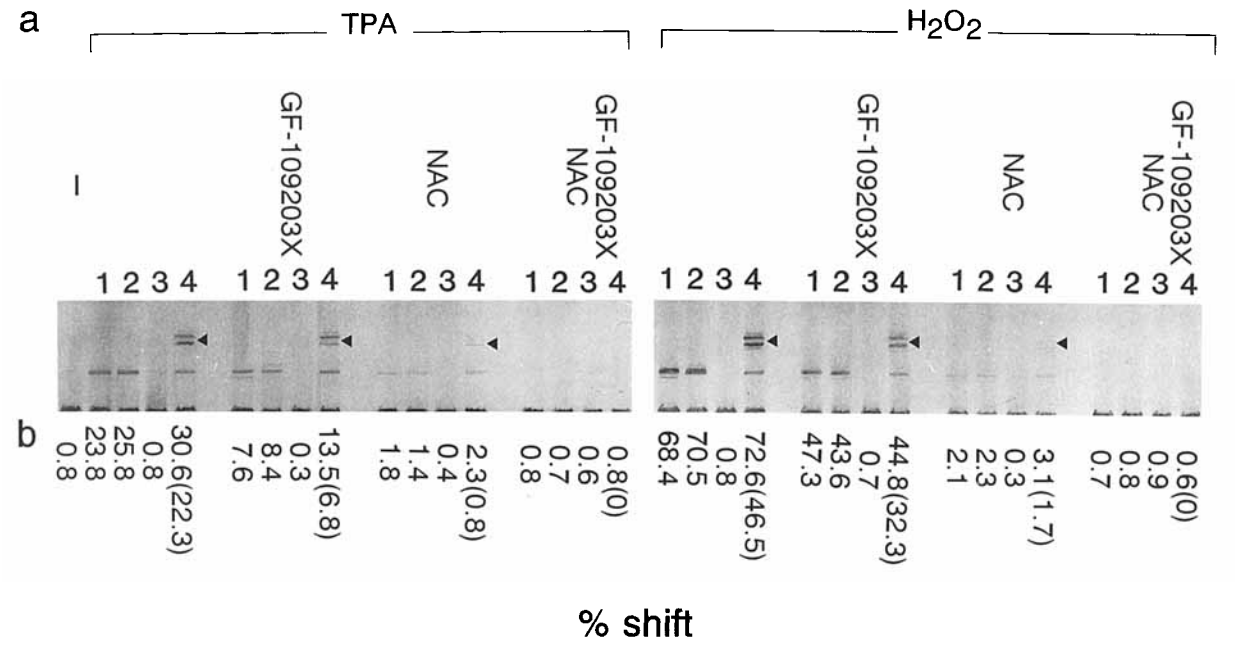

Fig. 5. Band-shift assays of the NF-κB-binding motif. **a:** Band-shift assays: Binding of the 42-mer DNA containing two NF-κB-binding motifs with nuclear proteins of M1 cells first treated with GF-109203X for 1 h and then with TPA or H_2O_2 for 1 h with or without NAC. A 42-mer DNA from a Gibco-BRL kit was labeled and used for binding assays. *Lane 1:* band-shift assay without competitor DNA and antibodies. *Lane 2:* a 25-fold excess of an unlabelled mutant of the NF-κB-binding motif was added during band-shift assay. *Lane 3:* a 50-fold excess of unlabelled 42-mer DNA was added during the band-shift assay. *Lane 4:* super-shift assay with NF-κB p65-specific antibodies. The *arrowheads* indicate the super-shifted bands. **b:** Percentage shift: Percentage shifts of the complex of 42-mer DNA and nuclear proteins were determined as in Fig. 4A. For lane 4, the total percentage shift and the percentage super-shift are indicated.

only reaction to produce CO in higher organisms. Thus it was of interest to investigate activation of the HO gene during differentiation of M1 cells into macrophages upon treatment with TPA. Transcription of the HO gene is known to be activated by TPA [Kurata and Nakajima, 1990], and this gene has a TRE element in its promoter region [Alam and Zhining, 1992]. Thus, this gene seemed likely to be regulated by a C-kinase signal-transduction pathway. In this study we investigated the mechanism of activation of the HO gene upon treatment of M1 cells with TPA. On treatment of M1 cells with TPA, we found that H_2O_2 was produced rapidly (within 10 min), transcription of the nuclear proto-oncogenes *fos* and *jun* was activated after 0.5 h, and transcription of the HO gene was activated after 2 h of treatment. Treatment of M1 cells with H_2O_2 resulted in almost the same patterns of activation of the *fos, jun,* and HO genes as treatment with TPA, but the amounts of Fos and Jun proteins were about half those observed on treatment with TPA. The mouse HO gene has a TRE in its promoter region, and its expression may be regulated by a *fos/jun* heterodimer complex (AP-1). When M1 cells were treated with TPA, transcription of the HO gene appeared to be activated by AP-1 (cf. Fig. 4), which is known to be activated by the C-kinase signal-transduction pathway.

As mentioned above, H_2O_2 was rapidly produced on treatment of M1 cells with TPA. Therefore, we examined the transcriptional activation of the above mentioned genes by H_2O_2. We found that H_2O_2 stimulated the production of active NF-κB, Fos, and Jun. Thus, rapidly produced H_2O_2 in response to TPA should also activate the production of these transcription-activating proteins. Since the human HO gene has an NF-κB-binding motif in its promoter region, the mouse HO gene may also have an NF-κB motif in its promoter region. H_2O_2 has been shown to be a strong activator of NF-κB [Schreck et al., 1991], and we found that the amount of active NF-κB in M1 cells was greatly elevated by H_2O_2 treatment. Thus, transcription of the mouse HO gene also may be activated by H_2O_2 via NF-κB, a transcription-activating protein. Pretreatment of M1 cells with an oxidant scavenger (NAC) strongly inhibited the transcriptional activation

of the *fos, jun,* and HO genes upon treatment with H_2O_2. However, pretreatment of M1 cells with NAC reduced the transcriptional activation of the *fos* and *jun* genes by TPA by only about 20% and the transcription of the HO gene was reduced by about 50%. These results indicate that activation of HO by TPA is due to two pathways; 1) transcriptional activation of the HO gene through active NF-κB that is induced by H_2O_2 or TPA; and 2) transcriptional activation of the HO gene by AP-1 through a C-kinase signal-transduction pathway. Therefore, we also examined the effect of a specific inhibitor of C-kinase. When M1 cells were pretreated with GF-109203X, transcriptional activation of the *fos* and *jun* genes was greatly reduced in TPA-treated M1 cells, but the rate of transcription of the HO gene was reduced by only about 50%. For further investigation, DNA from the upstream region of the HO gene containing a TRE-like sequence was incubated with nuclear extracts of M1 cells that had been treated with various reagents, and the resultant DNA-protein complexes were examined by electrophoresis. Super-shift assay revealed that the shifted bands contained Fos- and Jun-like proteins. We also examined the effects of incubation of NF-κB-binding motif with nuclear extracts of M1 cells that had been treated with various reagents, and examined the band shifts after electrophoresis. Super-shift assay revealed that the shifted bands contained NF-κB-like proteins. The sum of the percent shifts due to AP-1 and NF-κB (cf. Fig. 4, 5) correlated well with the pattern of transcriptional activation of the HO gene (cf. Fig. 1). These results support the hypothesis that transcription of the HO gene may be regulated by a C-kinase signal-transduction pathway via AP-1 and an oxidative activation pathway via NF-κB.

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