# Liver Cell Proliferation Induced by Nafenopin and Cyproterone Acetate Is Not Associated With Increases in Activation of Transcription Factors NF-κB and AP-1 or With Expression of Tumor Necrosis Factor α

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**Our previous studies have shown a different pattern of immediate early gene and growth factor gene expression between compensatory liver regeneration occurring after cell loss/death and direct hyperplasia induced by primary mitogens. In the present study, modifications in the activation of two transcription factors, NF-κB and AP-1; steady-state levels of tumor necrosis factor α (TNF-α) messenger RNA (mRNA); and induction of the inducible nitric oxide synthase (iNOS) were examined in rat liver during different types of cell proliferation. Compensatory regeneration was induced in male Wistar rats by partial hepatectomy of two thirds (PH) or a necrogenic dose of $CCl_4$ (2 mL/kg), whereas direct hyperplasia was induced by a single administration of the primary mitogens lead nitrate (LN, 100 μmol/kg), cyproterone acetate (CPA, 60 mg/kg), or nafenopin (NAF, 200 mg/kg). Liver regeneration after treatment with $CCl_4$ was associated with an increase in steady-state levels of TNF-α mRNA, activation of NF-κB and AP-1, and induction of iNOS. A strong and prolonged activation of NF-κB but not of AP-1 was observed in LN-induced hyperplasia. LN also induced an increase in hepatic levels of TNF-α and iNOS mRNA. On the other hand, direct hyperplasia induced by two other primary mitogens, NAF and CPA, occurred in the complete absence of modifications in the hepatic levels of TNF-α mRNA, activation of NF-κB and AP-1, or induction of iNOS, although the number of hepatocytes entering S phase 18 to 24 hours after NAF was similar to that seen after PH. These results add further support to the hypothesis that cell proliferation occurring in the absence of cell loss/death may be triggered by unknown signaling pathways different from those responsible for the transition of hepatocytes from G0 to G1 after PH or cell necrosis. (Hepatology 1997;25:585-592.)**

The unique ability of the liver to regenerate after partial removal or injury has been of interest to investigators for many years. Although considerable advances have been made in understanding the molecular mechanisms that control liver cell growth, the exact nature of the stimuli involved in the initiation and progression of the cell cycle has remained elusive. Activation of preexisting latent transcription factors recently has been claimed to play a major role in making the hepatocytes competent to proliferate (priming effect),[1] presumably via induction of the hepatic expression of several immediate early genes considered to be related to cell cycle.[2] One such transcription factor is NF-κB. NF-κB refers to members of a Rel family of transcription factors that were identified for their ability to bind and transactivate the enhancer of immunoglobulin κ gene in B cells. More recently, a role for NF-κB in liver regeneration was suggested based on the finding of a strong induction of DNA binding by NF-κB (p65/p50 heterodimer), p50/p50 homodimers, and posthepatectomy factor within 30 minutes after partial hepatectomy of two thirds (PH).[3,4] One of the most powerful inducers of NF-κB is tumor necrosis factor α (TNF-α).[5] This cytokine, which is involved in several pathophysiological processes, has been suggested to play a major role in liver cell proliferation based on the following: 1) pretreatment with anti–TNF-α antibodies inhibits liver regeneration after PH[6]; 2) a rapid increase in TNF-α messenger RNA (mRNA) is observed during liver cell proliferation induced by some primary mitogens[7,8]; and 3) single or repeated treatment with recombinant TNF-α induces proliferation of liver cells *in vivo*.[9-11] Among its effects, TNF-α induces the expression of inducible nitric oxide synthase (iNOS),[12] an enzyme catalyzing the synthesis of NO from L-arginine.[13] Induction of iNOS mRNA and enhanced production of NO have also been observed during liver regeneration after PH.[14,15] Irrespective of whether these changes are causally involved in liver cell proliferation occurring after surgical removal of part of the liver, it is becoming increasingly evident that the nature of the proliferative stimulus can alter the relationship of changes in cytokine levels, activation of transcription factors, and expression of immediate early genes and growth factor genes to hepatocyte replication. Indeed, whereas compensatory regeneration (cell proliferation occurring after loss/death of liver cells such as that observed after PH or $CCl_4$) is associated with increases in the steady-state hepatic levels of mRNA of immediate early genes such as c-*fos*, c-*jun*, *egr*-1, and c-*myc* and of growth factor genes (hepatocyte growth factor and transforming growth factor α),[16-20] no significant changes in the hepatic expression of these genes have been observed during direct hyperplasia (cell proliferation occurring in the absence of cell loss and resulting in an excess of tissue) induced by the primary mitogens nafenopin (NAF) and BR931,[21-23] suggesting the exis-

Abbreviations: PH, partial hepatectomy of two thirds; TNF-α, tumor necrosis factor α; mRNA, messenger RNA; iNOS, inducible nitric oxide synthase; NAF, nafenopin; cDNA, complementary DNA; EMSA, electrophoretic mobility shift assay; ABC, avidin-biotin-peroxidase complex; Ig, immunoglobulin; IL, interleukin; PPAR, peroxisome proliferator–activated receptor.

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tence of a different signaling pathway in the triggering of liver cell proliferation.

In a further attempt to characterize differences and similarities between compensatory regeneration and direct hyperplasia and to establish whether changes in the above-mentioned cytokines and transcription factors are an essential prerequisite for liver cell proliferation induced by stimuli of different nature, we determined the effect of direct hyperplasia induced by three liver mitogens, lead nitrate (LN), cyproterone acetate (CPA), and NAF, and compensatory regeneration induced by PH and $CCl_4$ on changes in the following: 1) hepatic expression of TNF-$\alpha$; 2) activation of NF-$\kappa$B; and 3) induction of the iNOS gene. In addition, activation of AP-1, the product of the Fos-Jun interaction, was also examined in virtue of its relationship to c-*myc* activation and cell proliferation.[24]

We show that although increased hepatic levels of TNF-$\alpha$ and iNOS mRNA and activation of NF-$\kappa$B and AP-1 are associated with compensatory regeneration after $CCl_4$, none of these changes occurred during direct liver hyperplasia induced by the peroxisome proliferator NAF and the antiandrogen CPA. However, another primary mitogen, LN, induced a pattern of response that is intermediate between the former two, suggesting the existence of at least two classes of primary mitogens.

## MATERIALS AND METHODS

Eight-week-old male Wistar rats (200 g) were purchased from Charles River (Milano, Italy). The animals were fed a laboratory chow diet provided by Ditta Piccioni (Brescia, Italy) and had free access to food and water. All animals were acclimated 1 week before treatment, and the National Research Council criteria for the care and use of laboratory animals in research were followed. $CCl_4$ (Sigma Chemical Co., St. Louis, MO; 2 mL/kg), NAF (a gift from Dr. V. Preat; 200 mg/kg), and CPA (Pentagone Pharmaceuticals Inc., Montreal, Canada; 60 mg/kg) were dissolved in corn oil and administered intragastrically. LN (Carlo Erba, Milano, Italy; 100 $\mu$mol/kg) dissolved in distilled water was injected intravenously through the femoral vein. PH was performed according to the method of Higgins and Anderson.[25] Most treatments were performed between 9 AM and 12 AM. Rats were killed at various times (range, 0.5-24 hours) thereafter. Immediately after death, liver sections were fixed in 10% buffered formalin and processed for H&E staining or for immunohistochemistry. The remaining liver was frozen in liquid nitrogen and stored at −80°C for future studies.

***Northern Blot Analysis.*** Total RNA was isolated from frozen liver by the procedure described by Chirgwin et al.[26] Forty micrograms of total RNA was separated on 1% agarose-formaldehyde gels and blotted on Hybond N membrane (Amersham, Buckinghamshire, UK). RNA concentration was determined spectrophotometrically at 260 nm. To control the amount of total RNA in each lane, the gels before blotting and the filters after transfer were stained with ethidium bromide. Poly(A)+ RNA, when needed, was isolated by two cycles of oligo(dT) cellulose affinity chromatography (Boehringer Mannheim, Germany).

UV-irradiated filters were then hybridized with a random-primed ($^{32}$P) rat iNOS complementary DNA (cDNA), a 700-bp, *Eco*RI fragment of rat liver iNOS cDNA[27]; TNF-$\alpha$, a 254-bp, *Kpn*I-*Hinc*II fragment of rat TNF-$\alpha$ gene cloned in a bluescript vector[28]; and glyceraldehyde-3-phosphate dehydrogenase, a 780-bp *Pstl*-*Xbal* fragment excised from the pHcGAP clone.[29] Membranes were exposed to radiographic film (Kodak Eastman, Rochester, NY), and the intensity of the bands was quantified by Phosphor Imager (Molecular Dynamics, Milano, Italy).

***EMSA.*** Nuclear extracts were prepared from 200 mg of liver tissue according to the method of Schreiber et al.[30] in the presence of 10 $\mu$g/mL leupeptin, 5 $\mu$g/mL antipain and pepstatin, and 1 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co.). Protein concentration in the nuclear extracts was determined using the method of Bradford.[31] Eight micrograms of nuclear extracts were incubated at room temperature for 30 minutes with (2-5 $\times$ $10^4$) of the $^{32}$P-labeled double-stranded oligonucleotide containing the consensus NF-$\kappa$B DNA-binding site from the immunoglobulin $\kappa$-light chain promoter (Ig-$\kappa$B) (5′ GATCCAGAGGGGACTTTCCGAGTAC 3′) or from the class I major histocompatibility complex (H2-$\kappa$B) (5′ GATCGGCTGGGGATTCCCCATCT 3′) or the AP-1 DNA-binding site from the collagenase promoter (5′ CTAGTGATGAGTCAGCCGGATC 3′), in a 15-$\mu$L reaction mixture containing 20 mmol/L HEPES, pH 7.9, 50 mmol/L KCl, 10% glycerol, 0.5 mmol/L dithiothreitol, 0.1 mmol/L ethylenediaminetetraacetic acid, 2 $\mu$g of poly(dI-dC), and 1 $\mu$g of salmon sperm DNA. Electrophoretic mobility shift assay (EMSA) was also performed with an oligonucleotide binding the transcription factor YY1 (5′ CTGCAGTAACGCCATTTTGCAAGGCATGAA 3′) from the murine leukemia virus upstream conserved region.[32] Products were fractionated on a nondenaturing 5% polyacrylamide gel. In competition assays, $\times$100 oligonucleotide competitor was added 15 minutes before addition of the labeled probe. The intensity of the retarded bands was measured by Phosphor Image.

***Supershift Assay.*** Supershift assay was performed by incubating 1 $\mu$L of antibody with nuclear extracts in binding buffer for 1 hour at 4°C before addition of labeled oligonucleotide. Polyclonal antibodies anti-p65 N terminal, p65 C terminal, p50, p52, c-rel, and Rel-B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

***Determination of Labeling Index of Hepatocytes After NAF or PH.*** Rats were subjected to PH or given a single dose of NAF. After 10, 16, and 22 hours, all animals received a single dose of BrdU (50 mg/kg intraperitoneally) and were killed 2 hours later. BrdU incorporation into nuclei was determined immunohistochemically by the avidin-biotin-peroxidase complex (ABC) method using a mouse anti-BrdU monoclonal antibody (Becton Dickinson Immunocytometry Systems, San José, CA) and Vectastain Elite ABC kit (PK-6102; Vector Laboratories Inc., Burlingame, CA). Briefly, tissue sections fixed in 10% formalin were embedded in paraffin, deparaffinized, exposed to 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase, treated with 2 N HCl, and incubated with trypsin 0.1% for 20 minutes and then with normal horse serum for 20 minutes at room temperature. The sections were then incubated for 2 hours with an anti-BrdU monoclonal antibody, followed by biotinylated horse anti-mouse immunoglobulin (Ig) G and ABC. The sites of peroxidase binding were detected with diaminobenzidine,[33] and the sections were counterstained with hematoxylin. A segment of duodenum, an organ with a high rate of cell proliferation, was included for each rat to confirm delivery of the DNA precursor. At least 3,000 hepatocyte nuclei per rat were scored.

## RESULTS

In agreement with previous reports,[34] single treatment with CPA, NAF, and LN induced an increase in liver weight, DNA content, and mitotic activity with no signs of cell necrosis (direct hyperplasia). On the contrary, $CCl_4$ treatment resulted in a severe centrilobular hepatic necrosis followed by regeneration beginning at about 36 to 40 hours (data not shown).

***Compensatory Regeneration After PH and $CCl_4$.*** Northern blot analysis of hepatic RNA from animals treated with $CCl_4$ showed a striking increase in the levels of TNF-$\alpha$ mRNA as early as 2 hours after treatment (Fig. 1). The increase in TNF-$\alpha$ mRNA level was accompanied by a transient induction at 12 and 24 hours of the iNOS gene (Fig. 2). In agreement with previous studies,[15] induction of hepatic levels of iNOS mRNA beginning at 4 hours was also seen in another model of compensatory regeneration, namely the one occurring after PH (data not shown). No signal was seen in the liver of control rats.

To search for an underlying pattern of transcriptional activation during compensatory regeneration, mobility shift assays were performed with oligonucleotide probes representing the binding sites for NF-$\kappa$B and AP-1 transcriptional factors. We analyzed the factors present in nuclear extracts that bind to the $\kappa$B site. A complex pattern of retarded bands was observed in EMSA using an oligonucleotide probe carrying the Ig-$\kappa$B site (Fig. 3). When competition experiments were performed, 100-fold excess of unlabeled oligonucleotide of Ig-$\kappa$B itself or of H2-$\kappa$B probe completely competed with complexes derived from labeled Ig-$\kappa$B oligonucleotide-protein interaction; on the contrary, they were not competed out by an unrelated unlabeled oligonucleotide, AP-1. At least three

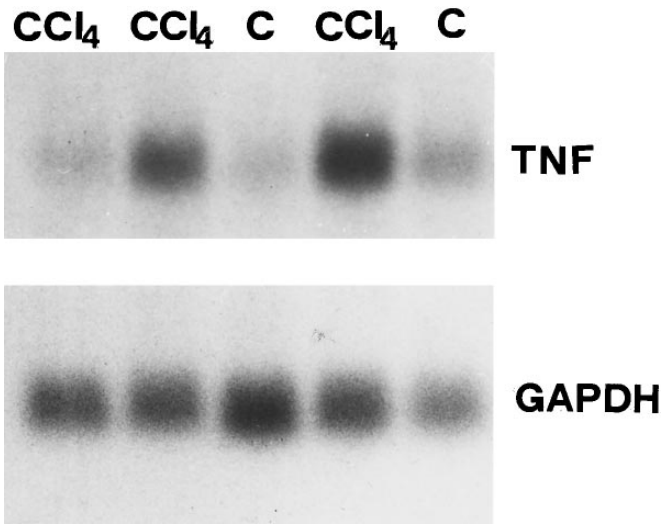


FIG. 1. Northern blot analysis of changes in TNF-$\alpha$ mRNA expression in rat hepatic tissue after $CCl_4$ administration (2 mL/kg, intragastrically in oil). Poly(A)$^+$ RNA was isolated by two cycles of oligo(dT) cellulose affinity chromatography from liver of $CCl_4$-treated rats or corn oil controls. Hybridization was to the murine TNF-$\alpha$ cDNA probe and a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lanes 1, 2 and 4, TNF-$\alpha$ mRNA 2, 3, and 5 hours after $CCl_4$ treatment; lanes 3 and 5, controls.

different complexes of proteins were found to bind to the $\kappa$B element. These complexes were also detected after long exposure to RX film, when nuclear extracts from control rat liver were used in EMSA (data not shown). As shown in Fig. 3, after $CCl_4$ administration there was an activation of NF-$\kappa$B approximately 4- and 10-fold greater than that of controls at 6 and 12 hours, respectively. A return to the basal level was observed at 24 hours.

In an effort to identify the subunit composition of different complexes that bind to -$\kappa$B element, we used polyclonal antibodies directed against members of the Rel family in a supershift assay. The anti-p50 antibody partially inhibited the formation of all types of DNA-protein complexes. Two different preparations of antibodies against the p65 subunit have been used—the antibody p65N, which is directed against an epitope corresponding to amino acids mapping within the amino terminal domain of NF-$\kappa$B, and a second antibody directed to an epitope corresponding to amino acids mapping at the carboxy terminus of NF-$\kappa$B p65 (p65C). As shown in Fig. 4, the anti-p65C antibody produced a supershifted band in EMSA, corresponding to the slower mobility complex; on the other hand, the anti-p65N partially inhibited the formation of DNA-protein complex of both the slower and the faster mobility bands. The anti–Rel-B inhibited the formation of the DNA-protein complex of the intermediate mobility in EMSA, whereas neither anti–c-Rel nor anti-p52 antibodies recognized any of the complexes that bind the -$\kappa$B sequence. Thus, from the data shown in Fig. 4, it is reasonable to conclude the following: 1) that the complex of slower mobility is a heterodimer p65/p50; and 2) that the intermediate mobility complex is a heterodimer Rel-B/p50. At present, we could not determine whether the faster mobility complex is a heterodimer of p50 with an amino terminal derivative of p65. Moreover, the presence of p50/p50 homodimers cannot be excluded.

In our previous studies, we have shown that a transient induction of c-*fos,* c-*jun,* and c-*myc* mRNA levels occurs at 1 to 4 hours after $CCl_4$.[21,22] It is known that the products of c-*fos* cooperate with c-*jun* proteins to encode the transcription complex AP-1. Figure 3 shows that the binding activity of AP-1 was markedly stimulated (20-fold) 6 hours after $CCl_4$, remained elevated at 12 hours, and declined to control values 24 hours after treatment.

***Direct Hyperplasia Induced by NAF and CPA.*** As shown in Fig. 5, no increase in TNF-$\alpha$ mRNA levels could be seen during liver cell proliferation induced by the mitogen NAF. The inability of NAF to induce TNF-$\alpha$ expression was accompanied by a lack of induction of iNOS gene (Fig. 6) and by the failure of this mitogen to activate NF-$\kappa$B (Fig. 7). Moreover, no activation of AP-1 was seen after treatment with this mitogenic agent (Fig. 7). Another primary mitogen, the synthetic steroid CPA, also failed to induce iNOS expression and NF-$\kappa$B and AP-1 activation (Figs. 6 and 7). The possibility that the lack of activation of these transcription factors by mitogens could be caused by degradation of the nuclear extracts from NAF, CPA was ruled out by the following: 1) longer exposure of nuclear extracts from NAF- and CPA-treated rat liver to RX films showed the same pattern of retarded bands and the same intensity as those from control rats (data not shown); and 2) a similar pattern of DNA-protein interaction in $CCl_4$-, NAF-, and CPA-treated rat livers was shown in EMSA performed using an oligonucleotide probe carrying the YY1 site (Fig. 8). Thus, these findings, together with previous data showing that NAF and CPA induce hepatocyte proliferation in the absence of increased levels of c-*fos* and c-

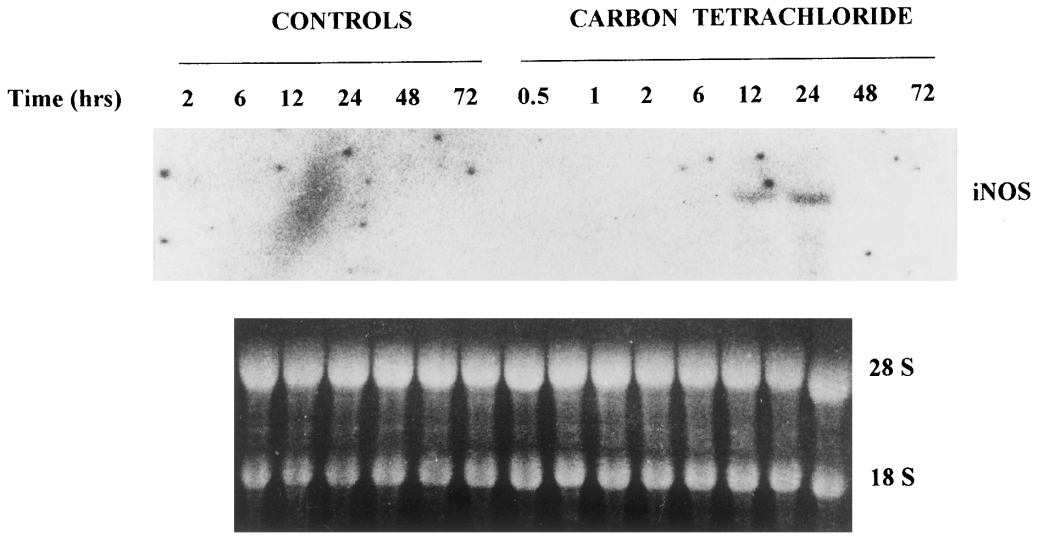


FIG. 2. Northern blot analysis of iNOS mRNA from livers of rats given corn oil or $CCl_4$ and killed at various times. The bottom panel shows ethidium bromide staining of the same gel.

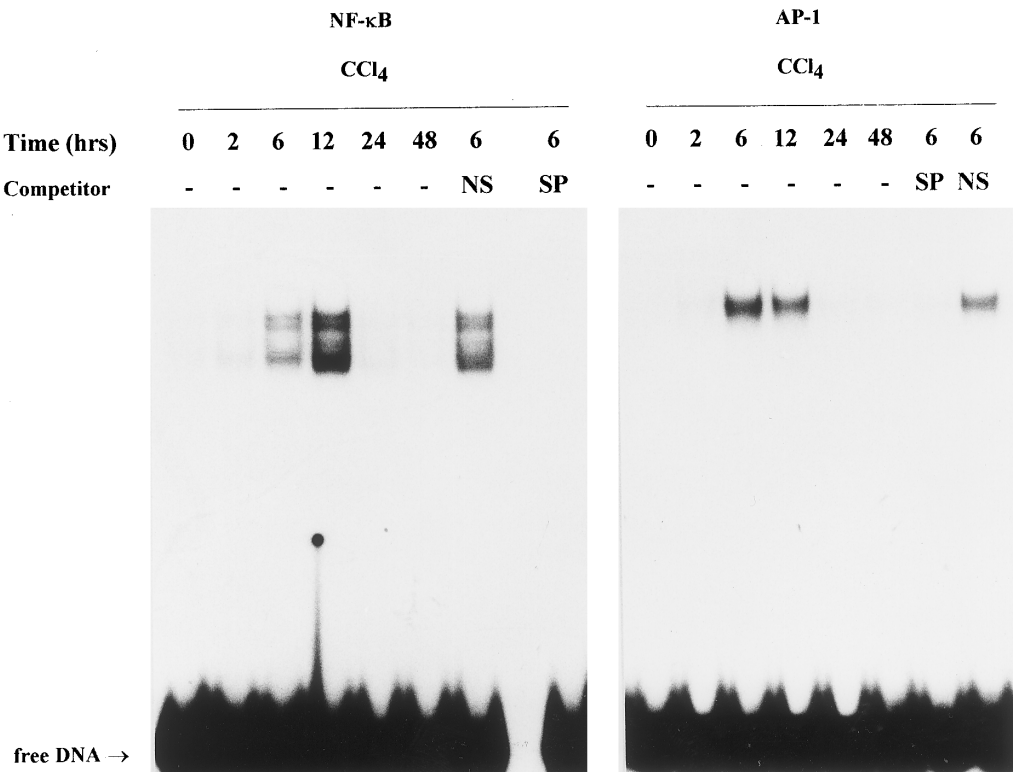


FIG. 3. DNA-binding activity of NF-κB and AP-1 transcription factors in the liver of $CCl_4$-treated rats. Nuclear extract from liver of rats killed 0, 2, 6, 12, 24, and 48 hours after $CCl_4$ administration were incubated with $^{32}P$-labeled double-stranded oligonucleotide containing the consensus sequence for the NF-κB or AP-1 binding site. The specificity of the bands was demonstrated by competing with 100-fold excess of either specific [SP] or nonspecific [NS] unlabeled oligonucleotides.

*jun* mRNA,[21,22] strongly suggest that hyperplasia induced by these mitogens occurs in the absence of some of the changes considered essential for the priming of the hepatocytes. Next, we examined the possibility that the lack of NF-κB and AP-1 activation by these mitogens could be caused by a lack of synchrony in the entry into S phase or an insufficient number of proliferating cells recruited into cell cycle by the mitogens. Therefore, the proliferative response of the liver to NAF treatment measured as the number of BrdU-positive hepatocyte nuclei was determined and compared with the response after PH. The results shown in Fig. 9 indicate that the number of hepatocytes entering S phase was basically similar in NAF-treated rats and those subjected to PH (12% and 14% of BrdU-positive hepatocyte at 18 and 24 hours after NAF and 6% and 16% of labeled hepatocytes 18 and 24 hours after PH). Interestingly, a lower but still significant number of BrdU-positive hepatocyte nuclei was seen as early as 12 hours after NAF treatment.

***Direct Hyperplasia Induced by LN.*** Unlike NAF and CPA, direct hyperplasia induced by LN was associated with an increase in TNF-α and iNOS mRNA levels (Figs. 5 and 10) and a rapid and prolonged activation of NF-κB (Fig. 11). Because LN does induce a marked increase in c-*jun* expression in the absence of significant increases in c-*fos* mRNA levels,[22,35] activation of AP-1 was monitored after treatment with this mitogen. EMSA of nuclear extracts showed only a very faint retarded band compared with that elicited by $CCl_4$ treatment (Fig. 11). In addition, this weak activation of AP-1 occurred much later than that of NF-κB.

## DISCUSSION

Induction of immediate early genes is one of the earliest events in liver regeneration occurring after PH or necrogenic

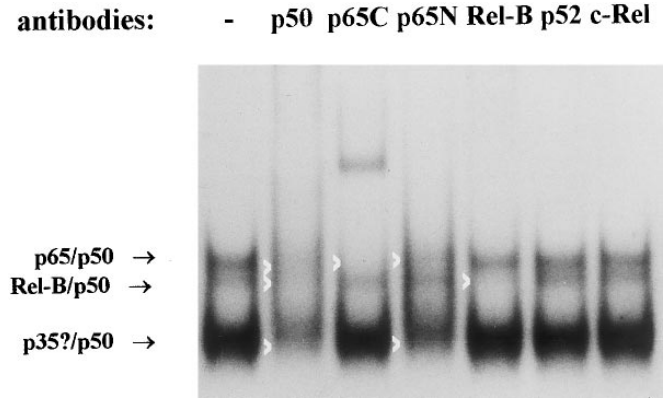


FIG. 4. Antibodies against the different subunits of the NF-κB/Rel family were used in a supershift assay of nuclear extracts from rat liver 12 hours after treatment with $CCl_4$. Antibodies used are indicated at the top of the figure. The nature of the complexes corresponding to different bands is reported on the left. *White arrows* indicate the position of the bands that showed complete or partial loss of intensity after supershift assay.

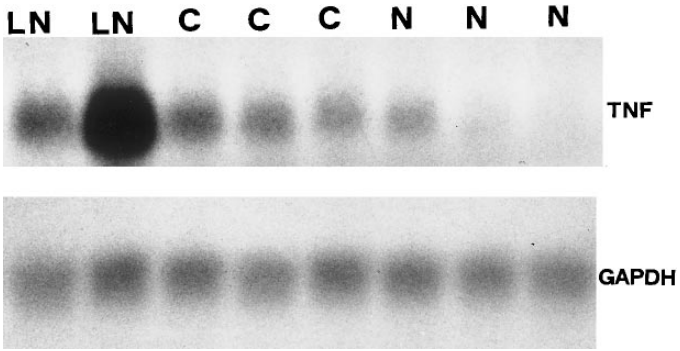


FIG. 5. Northern blot analysis of changes in TNF-α mRNA levels in rat liver after treatment with NAF (200 mg/kg) and LN (100 μmol/kg). Poly(A)$^+$ mRNA was isolated as described in the legend to Fig. 1. Lanes 1 and 2, TNF-α mRNA levels 1 and 4 hours after LN; lanes 3 to 5 [C], controls 0.5, 1, and 4 hours after corn oil; and lanes 6 to 8 [N], 0.5, 1, and 4 hours after NAF treatment.

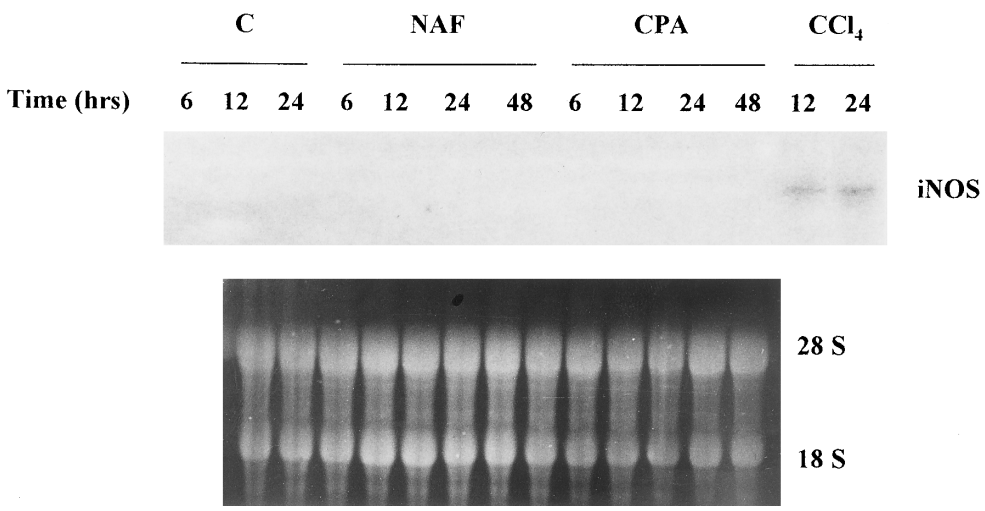


FIG. 6. Northern blot analysis of iNOS mRNA expression in rat liver after treatment with NAF, CPA, and $CCl_4$. C, corn oil controls.

compounds.[1,2] Because induction of these genes occurs in the absence of *de novo* protein synthesis, preexisting latent transcription factors must be modified via posttranslational modifications of the intracellular signals that initiate liver regeneration.[17] These intracellular signals are generated by the binding of specific growth factors or cytokines to the relevant extracellular receptors. One such latent transcription factor is NF-κB. Two NF-κB–putative binding sites are present in the promoter region of the gene coding for iNOS.[36] Xie et al.[37] have recently shown that the activation of NF-κB is necessary for iNOS promoter inducibility. Another interesting feature of this transcription factor is that, similar to Stat3, NF-κB is activated by cytokines such as TNF-α, interleukin (IL) 1, and IL-6.[5] These cytokines, especially TNF-α, recently have been implicated in liver regeneration after PH.[6,16] In this study we show that an increase in steady-state levels of TNF-α occurs during compensatory liver regeneration induced by a necrogenic dose of $CCl_4$. The increase in TNF-α hepatic expression was associated with induction of iNOS and activation of NF-κB, similar to those seen after PH. Our studies with antibodies directed to p50, p65 C terminal, and Rel-B

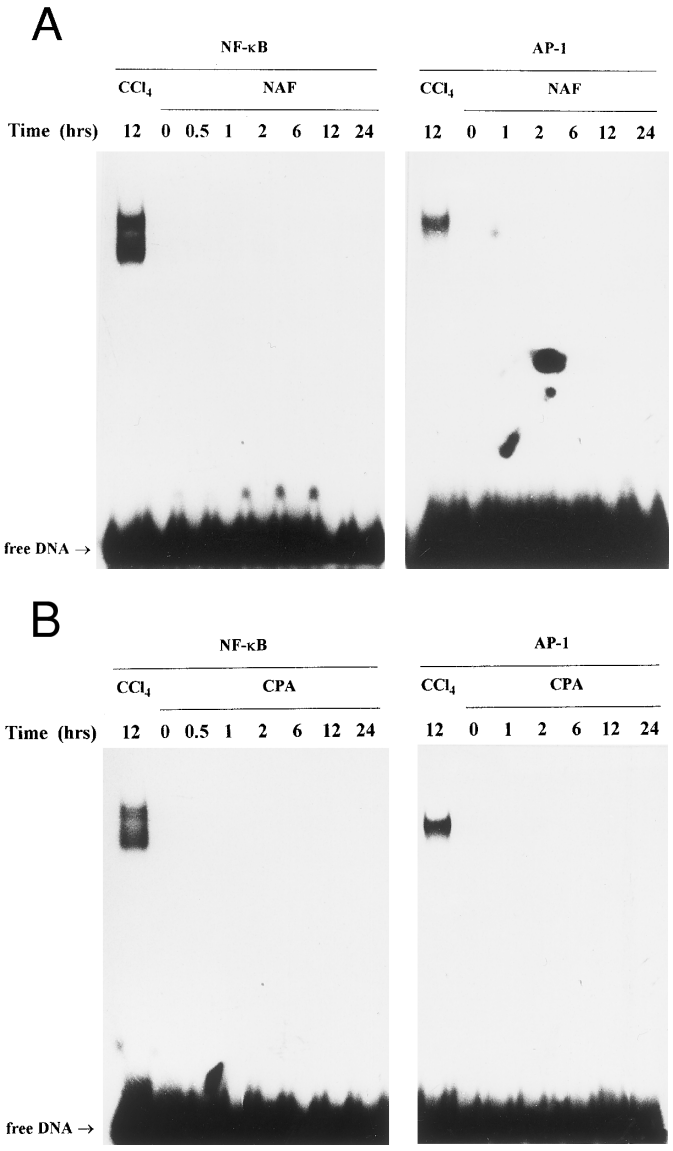


FIG. 7. DNA-binding activity of NF-κB and AP-1 transcription factors in the liver of (A) NAF- or (B) CPA-treated rats. Nuclear extracts from liver of rats killed 0, 0.5, 1, 2, 6, 12, and 24 hours after NAF and CPA administration were processed as described in Fig. 3. $CCl_4$, gel shift analysis from rat liver 12 hours after treatment.

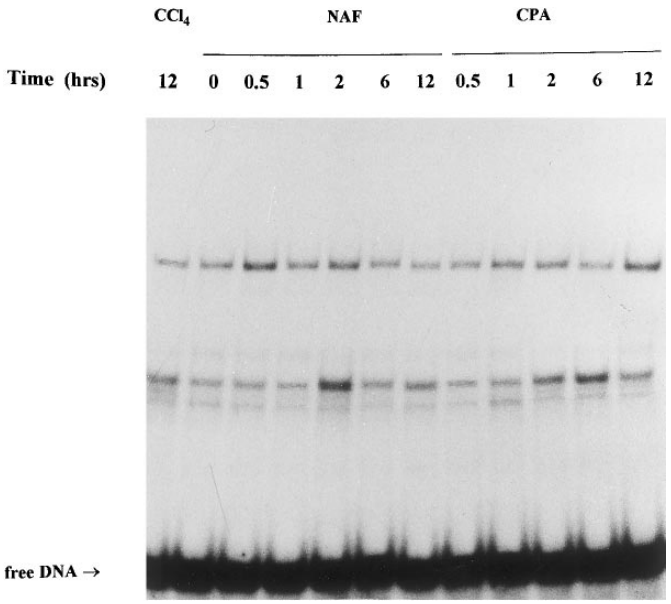


FIG. 8. DNA-binding activity of YY1 transcription factor. Nuclear extracts from liver of rats killed 0, 0.5, 1, 2, 6, and 12 hours after NAF and CPA administration were processed as described in Fig. 3. $CCl_4$, gel shift analysis from rat liver 12 hours after treatment.

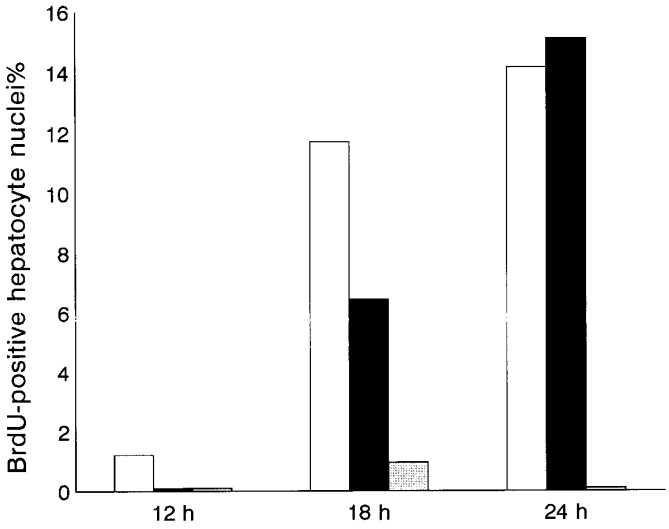


FIG. 9. Labeling index of hepatocytes from rats treated with a single dose of NAF (200 mg/kg) or subjected to PH and killed 12, 18, and 24 hours later. A single dose of BrdU was administered 2 hours before the animals were killed. Labeling index is expressed as the number of BrdU-positive hepatocyte nuclei per 100 hepatocytes. At least 3,000 hepatocyte nuclei per rat were scored. *Significantly different from control for at least $P < .050$. □, NAF; ■, PH; and ▨, oil.

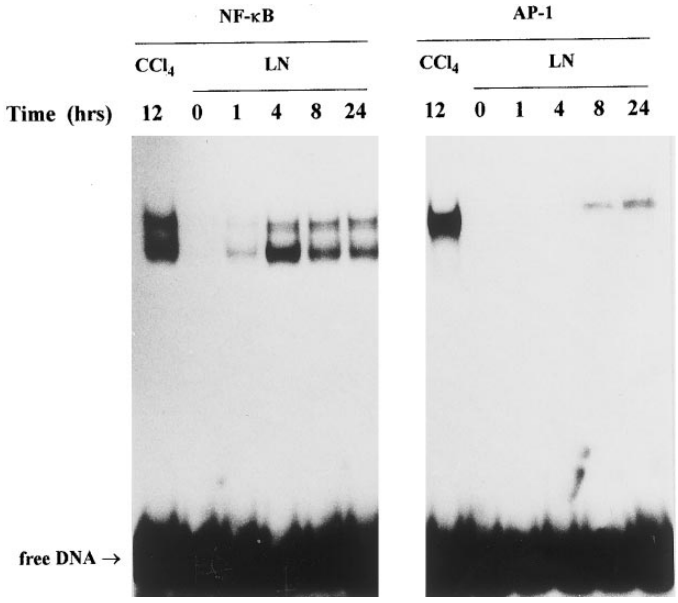


FIG. 11. DNA-binding activity of NF-κB and AP-1 transcription factors in the liver of LN-treated rats. Nuclear extracts from liver of rats killed 0, 1, 4, 8, and 24 hours after LN administration were processed as described in Fig. 3. $CCl_4$, gel shift analysis from rat liver 12 hours after treatment.

indicated that two of the three complexes detected by EMSA were p65/p50 and Rel-B/p50. These results agree with previous findings indicating a strong activation of DNA binding by activities consistent with the p65/p50 heterodimers.[3,4] As to the activation of Rel-B, it was previously reported that Rel-B is induced as an immediate early gene after PH,[3] and its critical function in the liver tissue was established on the basis of the severe histopathological abnormalities occurring in the liver of Rel-$B^{-/-}$ mice.[38] As far as the faster mobility complex is concerned, it is possible that it could represent a heterodimer p35/p50 resulting from the turnover of nuclear p65, via a degradative mechanism that converts it to p35 with κB binding activity, as suggested by Cressman and Taub.[39] In agreement with the same authors, the presence of these complexes in normal liver suggests that this pattern is a constitutive event in liver cells and that proliferation could increase this binding activity.

Because modifications in TNF-α, iNOS, and NF-κB seen in $CCl_4$-treated rat liver might also be associated with the induction of liver cell necrosis and inflammatory response elicited by this hepatotoxin, we examined changes in these parameters using a model whereby liver cell proliferation is not preceded by cell loss (direct hyperplasia). Interestingly, the primary mitogen LN, which induces a doubling of liver mass and DNA content within 3 days,[40] also induces TNF-α mRNA levels and activation of NF-κB and iNOS gene expression, suggesting that these changes are not necessarily caused by cell death. A possible role of TNF-α in triggering liver cell proliferation has been supported recently by the following: 1) germ-free rats show delayed regeneration of the liver after PH[41]; 2) anti–TNF-α antibodies inhibit liver regeneration after hepatectomy[6]; 3) treatment with recombinant TNF-α either in a single dose or by continuous infusion caused liver cell proliferation[9-11]; and 4) mitogens such as LN and ethylene dibromide induce TNF-α mRNA hepatic levels at a time that precedes DNA synthesis, and inhibition of TNF-α mRNA transcription by several treatments inhibits liver cell proliferation induced by these mitogens.[8,42] In addi-

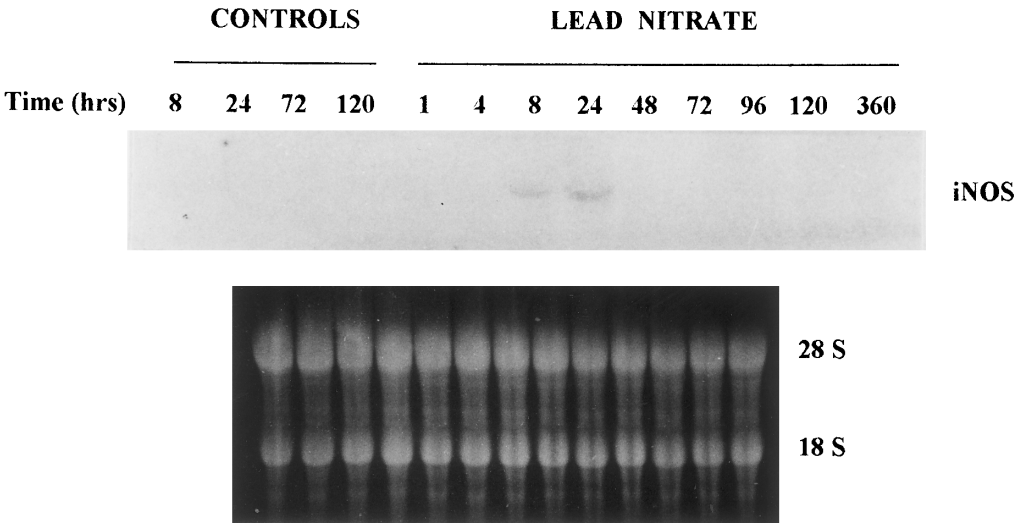


FIG. 10. Northern blot analysis of iNOS mRNA expression in rat liver after treatment with LN.

tion, we obtained preliminary evidence that mice lacking the receptor 1 of TNF-$\alpha$, unlike their wild-type counterpart, do not respond to hepatocyte proliferation induced by EDB.[43]

These results add support to the notion that TNF-$\alpha$ may be a critical factor in liver cell proliferation. It was recently shown that Kupffer cells increase in number during the first 24 hours after $CCl_4$,[44] and proliferation of nonparenchymal cells, including Kupffer cells, can also be seen after treatment with LN.[11,45] Kupffer cells are the main producers of TNF-$\alpha$, a strong inducer of NF-$\kappa$B, and Kupffer cell/hepatocyte cocultures release NO upon immunological activation by LPS.[46] Moreover, Kupffer cell products are required for NO production in hepatocytes.[47] These findings strongly suggest a role of Kupffer cells in triggering liver cell proliferation after administration of $CCl_4$ (a necrogenic agent) and LN (a primary mitogen). As already mentioned, after $CCl_4$ and LN administration an increase in Kupffer cells number precedes hepatocyte proliferation. It is possible that activation of Kupffer cells by these agents may explain the rather prolonged activation of NF-$\kappa$B as opposed to the transient activation observed after PH. Although these findings support the existence of a TNF-$\alpha$–mediated pathway leading to hepatocyte proliferation, it is possible that the increases in hepatic levels of TNF-$\alpha$ and iNOS mRNA and activation of NF-$\kappa$B reflect the results of metabolic changes not directly related to mitogenic events. For example, it may be hypothesized that increased production of reactive oxygen species caused by functional overload of the remnant liver after PH or by oxidative damage induced by $CCl_4$ and perhaps LN might be responsible for the increase in TNF-$\alpha$ production and consequently NF-$\kappa$B activation and NO production.

The possibility that increased expression of TNF-$\alpha$ and iNOS and NF-$\kappa$B activation might not be a necessary prerequisite for entry of hepatocytes into the cell cycle, or that alternative pathways leading to liver cell proliferation may exist, is supported by the finding that hepatocyte proliferation induced by two other primary mitogens, NAF and CPA, occurs in the complete absence of increased expression of TNF-$\alpha$ or iNOS or activation of NF-$\kappa$B and AP-1. The number of hepatocytes entering S phase 18 and 24 hours after NAF was similar to that seen after PH. These mitogens also fail to induce activation of immediate early genes such as c-*fos,* c-*jun,* and c-*myc,*[21,22] and a single administration of another peroxisome proliferator, BR931, does not result in increased expression of other immediate early genes such as egr-1, LRF-1, or growth factor genes such as hepatocyte growth factor or TGF-$\alpha$.[23] NAF and BR931 are part of a class of chemicals that bind to a nuclear receptor, peroxisome proliferator–activated receptor (PPAR), of the superfamily of hormone-nuclear receptors.[48] PPAR binds to the peroxisome-proliferator responsive elements as a heterodimer with the retinoic X receptor,[49] which in turn may act as a transcription factor. Thus, it is possible that an alternative pathway to that requiring cytokines and NF-$\kappa$B might be involved in liver cell proliferation induced by certain mitogens (hormone receptor-mediated cell proliferation) through a direct action on downstream molecules such as G1 cyclins. Mitogens such as NAF exert their proliferative effect mainly on hepatocytes, whereas the initial cell population target of LN appears to be nonparenchymal cells.[11] Further support for an alternative pathway is suggested by the findings that two physiological chemicals, T3 and 9-*cis* RA, both possessing a nuclear receptor, also induce liver cell proliferation.[50-52]

In conclusion, compensatory regeneration induced by $CCl_4$ is associated with changes in TNF-$\alpha$ and iNOS mRNA levels and to activation of transcription factors such as NF-$\kappa$B and AP-1. Similar changes, although with different timing, have been seen during liver regeneration after PH. On the contrary, another model of liver cell proliferation, namely direct hyperplasia induced by certain mitogens, occurs without any detectable increase in activation of the two preexisting transcription factors or in changes in steady-state levels of TNF-$\alpha$ and iNOS mRNA. These results indicate the existence of different signaling pathways in the triggering of cell proliferation, depending on the nature of the proliferative stimulus. Whether these differences may explain the different effect of the two proliferative stimuli on early stages of chemical hepatocarcinogenesis[34,53] is worthy of investigation.

Furthermore, our present data suggest that direct hyperplasia may be induced two different ways: via TNF-$\alpha$, such as in the case of LN, or through binding of the mitogens to nuclear receptors of the superfamily of the steroid hormones (TNF-$\alpha$–independent pathway). Whether this difference is caused by a difference in the initial target cell population in the liver (nonparenchymal cells vs. hepatocytes) is a hypothesis that needs to be tested.

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