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Lesion-induced increase in nerve growth factor mRNA is mediated by c-*fos*

(transcription factor AP-1/gene regulation)

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ABSTRACT Lesion of the sciatic nerve caused a rapid increase in c-*fos* and c-*jun* mRNA that was followed about 2 hr later by an increase in nerve growth factor (NGF) mRNA. To evaluate whether the initial increase in c-*fos* mRNA is causally related to the subsequent increase in NGF mRNA, we performed experiments with fibroblasts of transgenic mice carrying an exogenous c-*fos* gene under the control of a metallothionein promoter. In primary cultures of these fibroblasts, $CdCl_2$ evoked a rapid increase in exogenous c-*fos* mRNA, followed immediately by an increase in endogenous c-*jun* mRNA and with a slight delay by an increase in NGF mRNA. In fibroblasts of C3H control mice, $CdCl_2$ had no effect on the mRNA levels of the protooncogenes c-*fos* and c-*jun* or of NGF. Additional evidence for a causal relationship between c-*fos* induction and the subsequent increase in NGF mRNA was obtained in cotransfection experiments. Fibroblasts of C3H control mice were cotransfected with a metallothionein-promoter-driven c-*fos* expression vector and a NGF promoter–chloramphenicol acetyltransferase reporter gene construct. Induction of the exogenous c-*fos* by $CdCl_2$ resulted in increased activity of the NGF promoter. DNase I footprint experiments demonstrated that a binding site for transcription factor AP-1 (Fos/Jun heterodimer) in the first intron of the NGF gene was protected following c-*fos* induction. That this protected AP-1 site indeed was functional in the regulation of NGF expression was verified by deletion experiments and by a point mutation in the corresponding AP-1 binding region in the NGF promoter–chloramphenicol acetyltransferase reporter construct.

The protooncogene c-*fos* belongs to a family of genes that are rapidly and transiently activated by a great variety of stimuli such as growth factors, ionophores, and phorbol esters (1–5). The c-*fos* mRNA encodes the nuclear protein Fos, which is an intermediate in the signal transduction of external stimuli to the nucleus (6). It has been suggested that c-*fos* plays an essential role in cellular growth (3, 7) and differentiation (6, 8, 9). It has been shown that c-*fos* is involved in the transactivation of several genes (6, 10–15). Fos is known to associate with DNA in a complex with other proteins, initially termed p39 (16), one of which is Jun/AP-1 (17–19). The association of Fos and Jun in the heterodimeric complex (transcription factor AP-1) is mediated by regularly spaced leucine residues (20) and is required for functional activity (6, 19), apparently by binding to a specific recognition site in the promoter region of the corresponding genes (19, 21, 22).

We previously showed (23, 24) that transection of the sciatic nerve led to an enhanced synthesis of nerve growth factor (NGF) by the non-neuronal cells surrounding the axons. The rapid increase in NGF mRNA reached a maximum by 12 hr after lesion. Here we report that transection of the sciatic nerve also leads to an induction of c-*fos* and c-*jun* mRNA that precedes the increase in NGF mRNA in the injured nerve. To determine whether the lesion-mediated increases in c-*fos* and c-*jun* mRNAs were responsible for the subsequent increase in NGF mRNA, we used fibroblasts isolated from transgenic mice that carried an exogenous c-*fos* gene under the control of a human metallothionein promoter and upstream of the retroviral long terminal repeat (MT–c-*fos*–LTR; ref. 25). Induction of the exogenous c-*fos* mRNA by $CdCl_2$ was followed by an increase in NGF mRNA. An AP-1 consensus sequence in the first intron of the NGF gene§ was identified as the binding site of the Fos protein complex by DNase I footprint experiments. That this DNase I-protected sequence was indeed functional was verified by use of NGF promoter–chloramphenicol acetyltransferase (CAT) reporter gene constructs in which this region was deleted or the AP-1 consensus sequences was mutated from TGAGTCA to ATAGTCA.

MATERIALS AND METHODS

Mice. Transgenic mice carrying an exogenous c-*fos* gene under the control of a human MT promoter (construct p76/21; ref. 25) were used for primary cultures of skin and sciatic nerve fibroblasts.

Organ Cultures of Sciatic Nerve. Pieces of rat sciatic nerve (3 cm) were prepared and placed into culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After incubation for various times the tissue was rapidly frozen and total RNA was extracted (24).

Cell Culture and Transfection. Fibroblasts prepared from the skin of normal and transgenic mice by sequential collagenase (0.1%, 15 min) and trypsin (0.1%, 15 min) digestions (26) were cultured in DMEM with 10% FBS. Confluent cultures were stimulated with $CdCl_2$ (10 μM), after which total cellular RNA was prepared. For transfection experiments the fibroblasts were replated (2×10^5 cells per 60-mm dish) in DMEM with 5% FBS. After 2 days the cells were transfected for 14 hr with 6 μg of the NGF promoter–CAT reporter plasmid or 6 μg of the MT–c-*fos*–LTR expression plasmid plus 2 μg of the NGF promoter–CAT reporter plasmid by a modified calcium phosphate precipitation method (27). After washing, the cells were incubated in DMEM containing 0.5% FBS in the presence or absence of 5 μM $CdCl_2$ for 36 hr. The cells were harvested and equal

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Abbreviations: NGF, nerve growth factor; CAT, chloramphenicol acetyltransferase; MT, metallothionein; LTR, long terminal repeat.
§The sequence of the NGF promoter region reported in this paper has been deposited in the GenBank data base (accession no. M33683).

amounts of cell extract protein were assayed for CAT activity as described (28).

RNA Analyses. Total RNA was prepared (24, 26) and separated according to size in a 1.2% agarose gel. For measuring the efficiencies of RNA preparations, 20 pg of NGF recovery standard (a 510-nucleotide *in vitro* transcript in sense orientation) was added to each sample prior to extraction. Following vacuum blotting the nylon filters (Hybond N, Amersham) were hybridized with mouse c-*fos* and mouse NGF complementary RNA probes as described (26). The filters were then washed, treated with RNase A (0.1 μg/ml), and exposed to Fuji RX film. The c-*fos*- and NGF-specific transcripts were quantified by densitometric scanning of the autoradiograms.

DNA Construction and Site-Directed Mutagenesis. A 2.1-kilobase *Hin*cII-*Pvu* II fragment of the NGF promoter region was inserted between the *Xba* I and *Xho* I sites of the CAT expression vector pBLCAT3 (29) to produce pPNCAT. To obtain the deletion fragments, the promoter region was cut with restriction enzyme (*Eco*NI or *Hae* II), and the resulting fragments were again linked to the CAT reporter plasmid. To introduce the point mutation of the intronic AP-1 site in the construct pPN−58CAT, an oligodeoxynucleotide (AGCGCATCGGATAGTCAGGCTT) was extended by the polymerase chain reaction (30) to give plasmid pPN−58mACAT.

Preparation of Nuclear Extracts and DNase I Footprint Analysis. Skin fibroblasts of transgenic mice were prepared as described above, grown in DMEM with 5% FBS until they reached 90% confluency, and stimulated with 10 μM $CdCl_2$ for 8 hr. For control extracts, confluent NIH 3T3 fibroblasts were kept in DMEM with 0.5% FBS for 2 days before they were harvested. Nuclear extracts were prepared as described (31).

A 970-base-pair *Bgl* II fragment spanning the 5′ promoter region, exon 1b, and the AP-1 binding site was cloned into the multiple cloning site of a Bluescript SK(−) vector (Stratagene). *Eco*RI was used to label the fragment at either side by filling in with Klenow DNA polymerase, and two other restriction sites were used to remove the fragment from the vector. One to two nanograms of end-labeled fragment was treated with DNase I (Pharmacia) as described (32).

RESULTS

Lesion-Mediated Changes in mRNA Levels of c-*fos*, c-*jun*, and NGF. For reasons of convenience we performed the experiments with sciatic nerve pieces in culture, as it had been shown that the initial time course of NGF mRNA changes was virtually the same *in vivo* after nerve lesion and *in vitro* (24). As shown in Fig. 1 *A* and *B*, NGF mRNA began to increase after 2 hr in culture, attaining a maximal level by 12 hr of incubation. In contrast, c-*fos* mRNA was already significantly increased after 30 min and reached a peak by 2 hr. The time course of c-*jun* mRNA changes was very similar to that of c-*fos*, peaking also by 2 hr (Fig. 1 *B* and *C*). Cycloheximide (5 μg/ml), an inhibitor of protein synthesis, partially blocked the increase in NGF mRNA (data not shown). Under the same conditions c-*fos* mRNA was superinduced, in agreement with previous studies (3, 33).

$CdCl_2$ Induces an Increase in NGF mRNA in Transgenic Fibroblasts. To evaluate whether the increase in c-*fos* mRNA was causally related to NGF expression, we used fibroblasts of transgenic mice carrying an exogenous c-*fos* gene controlled by a human MT promoter (MT–c-*fos*–LTR). The 3′ nontranslated region of the mouse c-*fos* mRNA, which is responsible for its instability, is replaced in this construct by a retroviral LTR to assure a sustained high level of c-*fos* expression (construct p76/21; ref. 25). As shown by Rüther *et al.* (25) administration of $CdCl_2$ to these mice leads to >10-fold increase in c-*fos* mRNA in most tissues tested. In view of the high toxicity of $CdCl_2$ *in vivo*, we refrained from *in vivo* experiments. Since previous studies had shown that primary cultures of fibroblasts are a good system to study the regulation of NGF expression (26), we prepared fibroblast cultures from the MT–c-*fos*–LTR transgenic mice and corresponding control mice. Addition of 10 μM $CdCl_2$ to the transgenic fibroblast cultures resulted in a time-dependent increase in exogenous c-*fos* mRNA (Fig. 2*A*), accompanied by a virtually simultaneous increase in endogenous c-*jun* mRNA (Fig. 2*B*) and followed with some delay by an increase in NGF mRNA (Fig. 2*A*). In fibroblasts from control C3H mice the mRNA levels of c-*fos* and NGF were not significantly changed after the addition of $CdCl_2$, excluding the possibility that $CdCl_2$ had additional, nonspecific effects (Fig. 2*A Inset*).

c-*fos* Expression Stimulates NGF Promoter Activity. A genomic clone containing the promoter region of the mouse NGF gene, including part of the first intron, was isolated, sequenced (see Fig. 5*A*), and found to partially overlap the NGF promoter fragments published by Selby *et al.* (34) and Zheng and Heinrich (35) (positions −639 to +72). A 2.1-kilobase fragment was then linked to a plasmid carrying the CAT reporter gene for transfection studies (pPNCAT). The MT–c-*fos*–LTR transgenic fibroblasts were transfected with

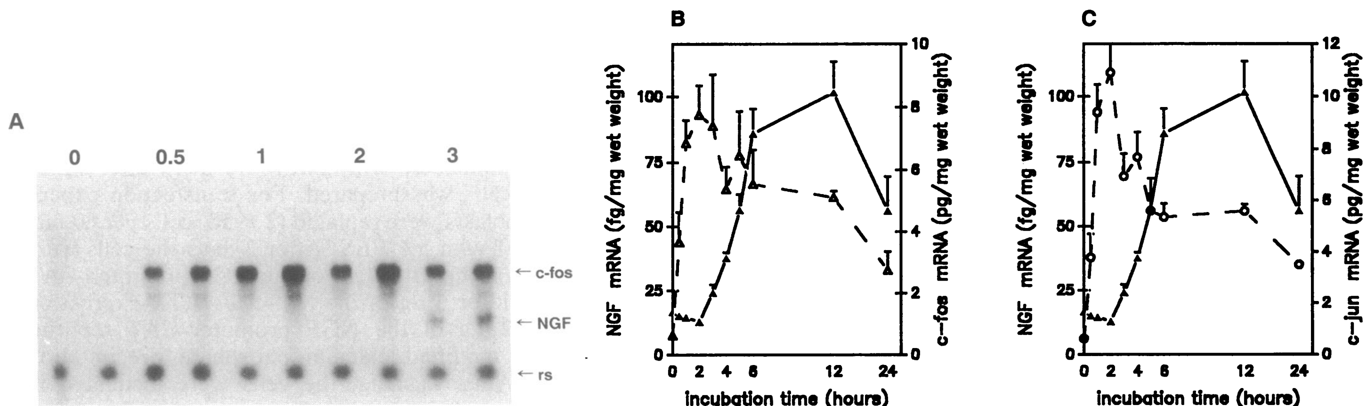

FIG. 1. Levels of c-*fos*, c-*jun*, and NGF mRNAs in rat sciatic nerve pieces after various times in culture. (*A*) Sciatic nerves were prepared and kept in culture for 0–3 hr. The levels of mRNAs encoding Fos and NGF were then determined by quantitative Northern blot analyses. For quantification a NGF recovery standard (rs) was added prior to RNA extraction (see *Materials and Methods*). Duplicates are shown for each time point. (*B* and *C*) mRNAs encoding Fos (△), Jun (○), and NGF (▲) were quantified by densitometry of the autoradiograms. For clarity, NGF/c-*fos* (*B*) and NGF/c-*jun* (*C*) comparisons are shown separately. Data represent the mean of three or more determinations at each time point.

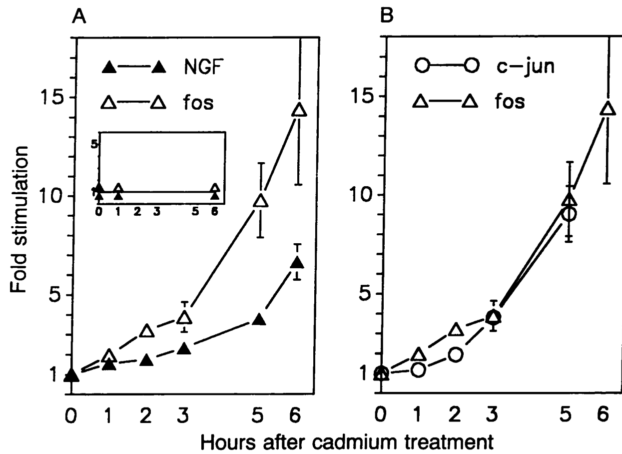

FIG. 2. Expression and inducibility of c-*fos*, c-*jun*, and NGF mRNAs in skin fibroblasts from MT–c-*fos*–LTR transgenic mice. Cells were incubated in the presence of 10 μM $CdCl_2$ to induce the c-*fos* transgene. Northern blot analyses were used for the determination of the changes in c-*fos* (△), c-*jun* (○), and NGF (▲) mRNA levels. Data represent the mean of two or more experiments at each time point. For clarity, c-*fos*/NGF (*A*) and c-*fos*/c-*jun* (*B*) comparisons are shown separately. *Inset* (in *A*) shows results obtained with C3H control fibroblasts; note that endogenous c-*fos* expression was not induced by $CdCl_2$.

the NGF promoter–CAT reporter plasmid pPNCAT and tested for CAT activity before and after $CdCl_2$ treatment. $CdCl_2$ increased the activity of pPNCAT about 6-fold in the transgenic fibroblasts (Fig. 3).

Fos Binding to the NGF Promoter. Several genes that are regulated by Fos share a motif similar to the AP-1 consensus sequence 5′-TGAGTCA-3′. Analysis of the sequence of the NGF promoter fragment used in the transfection studies revealed nine sequences resembling the AP-1 consensus sequence (see Fig. 5*A*). To evaluate whether a transcription factor(s) binds to all or part of these AP-1-like sequences in the NGF promoter region, we performed *in vitro* DNase I footprint experiments. Comparing the footprint patterns obtained with nuclear extracts from quiescent 3T3 fibroblasts and from $CdCl_2$-stimulated skin fibroblasts prepared from MT–c-*fos*–LTR transgenic mice, we found that c-*fos* induction resulted in only one additional protected site in the NGF promoter region between positions −1000 and +289. The region protected from DNase I, positions +33 to +50, contained the AP-1 consensus sequence TGAGTCA (Fig. 4*A*). None of the other sites resembling the AP-1 consensus sequence was protected from DNase I digestion in these experiments (Fig. 4*B*).

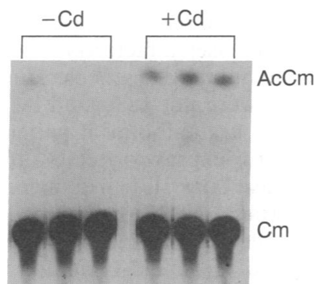

FIG. 3. Effect of $CdCl_2$ on the NGF promoter activity in transgenic fibroblasts. The transgenic skin fibroblasts were transfected with a construct containing the NGF promoter region linked to a CAT reporter gene (pPNCAT). Following incubation for 36 hr in the absence (−Cd) or presence (+Cd) of 10 μM $CdCl_2$, CAT activity in the lysed cells was determined by incubation with [^{14}C]chloramphenicol and TLC analysis of the reaction products. Autoradiograms show that acetylated chloramphenicol (AcCm) was produced from chloramphenicol (Cm) by the lysates from $CdCl_2$-treated cells. Results of three experiments are represented.

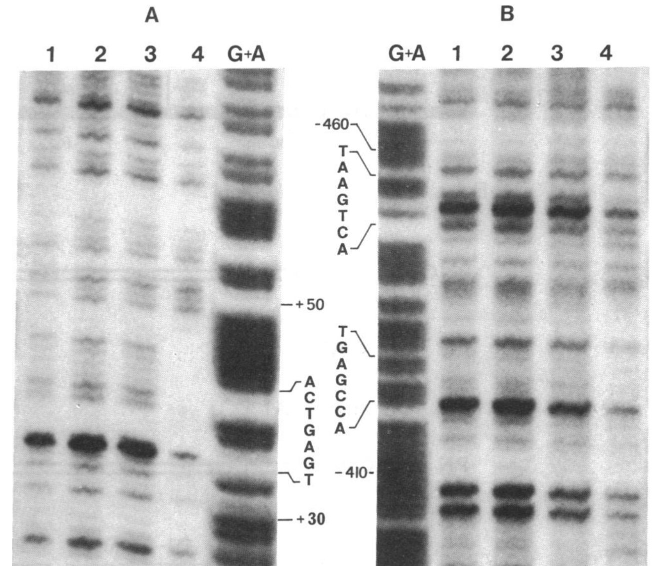

FIG. 4. Protein binding to the AP-1 consensus sequence located in the first intron of the NGF gene. The noncoding strand of a DNA fragment containing a sequence from position −251 to +143 and the coding strand of the fragment from −251 to −784 were labeled at position −251 with Klenow polymerase. This probe (2×10^5 cpm) was incubated without protein (lanes 1), with 100 μg of bovine serum albumin (lanes 2), with 100 μg of extract of quiescent 3T3 cells (lanes 3), or with 100 μg of extract of $CdCl_2$-stimulated transgenic fibroblasts expressing high levels of Fos (lanes 4). After digestion with DNase I the resulting fragments were electrophoresed in a 6% acrylamide/7 M urea gel. G+A chemical sequencing ladders were used as size markers (lanes G+A). Numbers indicate the positions relative to the transcription start site. (*A*) Protein binding to the intronic AP-1 site. (*B*) Example of no detectable protein binding to AP-1-like sequences in the NGF promoter.

Functional Activity of the Intronic AP-1 Site. The functional importance of this intronic AP-1 binding site for the regulation of the NGF promoter by c-*fos* was evaluated by cotransfection of fibroblasts with the MT–c-*fos*–LTR expression vector and various promoter-deletion mutants of the NGF promoter–CAT reporter plasmid (Fig. 5*B*). The construct pPNCAT contained a 2.1-kilobase fragment including the transcription initiation site and comprising both CAAT-box and TATA-box consensus sequences as well as 250 base pairs of the first intron, including the AP-1 binding site at position +35. Upon stimulation of the exogenous c-*fos* gene by the addition of $CdCl_2$ to the cotransfected cells, the transcriptional activity of the NGF promoter increased about 6-fold (Fig. 5*C*). Deleting the sequences 3′ to position +4 (pPN+4CAT) reduced the basal activity of the NGF promoter about 10-fold (data not shown) and eliminated its inducibility by c-*fos*; deleting the sequences 5′ to position −58 (pPN−58CAT) also reduced the basal activity of the remaining fragment, which included the two TATA-box consensus sequences and the AP-1 binding site, by a factor of about 30 (data not shown). In contrast to pPN+4CAT this construct still retained its inducibility by c-*fos*. To prove the functional role of the intronic AP-1 site, a point mutation changing the AP-1 binding sequence from TGAGTCA to ATAGTCA was introduced into pPN−58CAT. This mutant construct (pPN−58mACAT) lost its c-*fos*-inducibility and showed a decreased basal activity. Thus, the transcriptional regulation of NGF by c-*fos* is mediated by this AP-1 site located in the first intron of the NGF gene. Moreover, the intronic AP-1 site seems also to contribute to the basal NGF

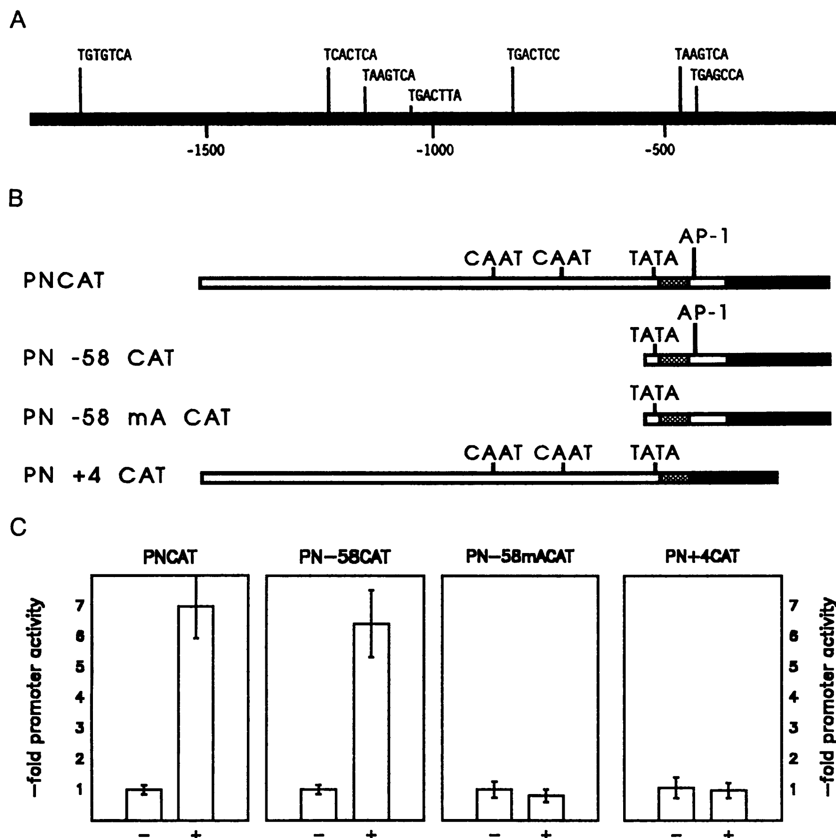

FIG. 5. Influence of various NGF promoter constructs on c-*fos* inducibility. (*A*) Location of AP-1-like sequences in the NGF promoter region between positions −1881 and +289. The complete sequence of this fragment has been submitted to GenBank. (*B*) Structure of the NGF promoter-deletion constructs. pPN+4CAT is missing the sequence from +4 to +214 of pPNCAT; pPN−58CAT is missing the sequence 5′ to position −58; pPN−58mACAT is similar to pPN−58CAT except that the sequence of the intronic AP-1 site has been mutated to prevent the binding of transcription factor AP-1. Open bar, NGF promoter region; cross-hatched bar, exon 1b; filled bar, CAT reporter. Positions of CAAT and TATA sequence elements are indicated. (*C*) Promoter activity of the deletion constructs. Fibroblasts were cotransfected with the $CdCl_2$-inducible expression vector MT–c-*fos*–LTR and the various deletion constructs. Bars represent the mean of three independent experiments, showing the activities of the different NGF promoter–CAT reporter constructs in the absence (−) or presence (+) of $CdCl_2$.

expression in this system. Other sequences in the NGF promoter region that resemble the AP-1 consensus sequence are not essential for the regulation of NGF mRNA by c-*fos*.

DISCUSSION

Our results provide evidence that the NGF gene is a target for c-*fos* activity. This conclusion is based upon the finding that selectively induced c-*fos* regulates NGF promoter activity. In primary cultures of skin fibroblasts of MT–c-*fos*–LTR transgenic mice, $CdCl_2$ treatment led to an increase in exogenous c-*fos* mRNA followed immediately by an increase in c-*jun* mRNA and with a slight delay by an increase in NGF mRNA. In control experiments with normal mouse fibroblasts, $CdCl_2$ did not affect the level of c-*fos* mRNA or NGF mRNA. This indicates that the observed induction of NGF mRNA was due to an activation of the MT promoter of the exogenous c-*fos* rather than to a nonspecific effect of $CdCl_2$. Moreover, $CdCl_2$ treatment induced CAT activity in normal C3H mouse fibroblasts that had been cotransfected with the NGF promoter–CAT reporter plasmid pPNCAT and the MT–c-*fos*–LTR expression plasmid, indicating that Fos can interact with the promoter region of the NGF gene (Fig. 5*C*). Our results support the assumption that the level of Jun protein does not represent a limiting factor under our experimental conditions. This might be due to either a sufficiently high steady-state level of Jun (36) or a c-*fos*-induced positive regulation of transcription of the c-*jun* protooncogene by its own product (37). Indeed, the addition of $CdCl_2$ to cultured fibroblasts of transgenic mice led not only to an increased level of exogenous c-*fos* mRNA but also to an increase in endogenous c-*jun* mRNA (Fig. 2*B*).

To identify AP-1-responsive elements in the NGF gene, DNase I footprint assays were performed. We looked for differences in the protection pattern of the NGF promoter region, comparing nuclear extract of quiescent 3T3 fibroblasts with that of MT–c-*fos*–LTR transgenic fibroblasts expressing high levels of Fos. No specifically induced footprints were observed 1000 bases upstream of the transcription start site: in both cases a factor(s) bound to the TATA box (data not shown) but the sequences resembling the AP-1 consensus sequence were not protected (see Fig. 4*B*). The only major difference in the footprint patterns was that the extract from the c-*fos*-expressing cells afforded a strong protection from DNase I digestion in a region (positions +33 to +50) centered around an AP-1 consensus sequence and directly adjacent to the exon 1b/intron border. These results indicate the presence of an AP-1 binding site in the first intron. The AP-1 binding sites of the collagen and transin genes are located at almost identical positions around −70. However, this position relative to the transcription start site does not seem to be mandatory to make an AP-1 binding site functional (32). For example, an enhancer element in the first intron of the 4F2 heavy-chain gene contains an AP-1 site that is necessary for full enhancer activity (38). The absence of AP-1 binding to the AP-1-like sequences in the NGF promoter region is in accordance with the findings of Risse *et al.* (39), who determined the capacity of systematically point-

mutated AP-1 sites to bind a Fos/Jun heterodimer. They also detected no binding to sites corresponding to AP-1-like sequences present 5′ to the transcriptional start site of the NGF promoter.

The mere presence of an AP-1 consensus sequence does not necessarily mean that it is active in regulation. A mutation of the AP-1 binding site in the simian virus 40 promoter prevents the binding of AP-1 but fails to affect the basal activity of the promoter and its inducibility by phorbol 12-myristate 13-acetate in transiently transfected Hep G2 hepatoma cells (40). The transactivating function of a DNA-bound factor can additionally be regulated by posttranslational modifications as in the case of the serum response factor (41).

To test the regulatory function of the intronic AP-1 binding site on NGF promoter activity, primary cultures of skin fibroblasts were cotransfected with a c-*fos* expression vector and CAT reporter constructs containing various fragments of the NGF promoter. The c-*fos* inducibility of the construct pPNCAT was lost in a similar construct that lacked only the sequence 3′ to position +4. In contrast, deleting the promoter sequences 5′ to position −58 did not eliminate the inducibility by c-*fos*. However, the mutation of the intronic AP-1 site in this construct resulted in a loss of c-*fos* responsiveness. These results demonstrate that the AP-1 complex not only binds to the sequence around position +35 but also has a positive regulatory function on the NGF promoter activity.

Deleting or mutating the intronic AP-1 site not only eliminated the inducibility of the NGF promoter by c-*fos* but also reduced the basal NGF promoter activity. This implies that AP-1 may also be involved in the regulation of the basal activity of the NGF promoter, but it has to be considered that the NGF promoter activity we define here as "basal" activity might reflect an already elevated activity of the NGF promoter. In pieces of the sciatic nerve kept in culture for 3 days the NGF mRNA level was about 8-fold higher than that in intact nerve (23). Thus, the fibroblasts used in the transfection experiments may have been in a similar activated state with an enhanced NGF mRNA expression. Consistent with this interpretation is the observation that in the transfection experiments with the pPNCAT vector, the NGF promoter activity was higher than that of other promoters (e.g., the thymidine kinase promoter; data not shown). This could also explain why the inducibility of the NGF promoter–CAT reporter construct was less pronounced (about 6-fold) than the observed increase in NGF mRNA when pieces of sciatic nerve were brought into culture (about 45-fold) (24).

We have provided evidence for a role of c-*fos* in the regulation of NGF expression under various experimental *in vitro* conditions. These results suggest that similar mechanisms might activate NGF expression *in vivo*. Transection of a peripheral nerve, like induction by serum (1), is followed by a rapid and transient increase in c-*fos* mRNA. The subsequent induction of NGF mRNA outlasts the typical short c-*fos* peak (Fig. 1*B*) and results in a local synthesis of NGF protein in the non-neuronal cells surrounding the axon (23). The nerve lesion coinduces c-*fos* and c-*jun*, which is a prerequisite for the formation of the biologically active AP-1 complex (19). These observations indicate that c-*fos* could also regulate NGF induction after sciatic nerve lesion *in vivo*. The nature of the signal that leads to the initial induction of c-*fos* remains to be identified; however, in long-term sciatic nerve cultures interleukin 1 seems to be the responsible agent regulating NGF mRNA levels (42).

In support of our data are the results presented by Moccetti *et al.* (43). They demonstrated an increase in c-*fos* mRNA followed by increased NGF mRNA levels after β-adrenergic stimulation of C6-2B astrocytoma cells.

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