Unusual c-*fos* induction upon chromaffin PC12 differentiation by sodium butyrate: loss of fos autoregulatory function

Jose R.Naranjo[1,2], Britt Mellström[1,2], Johan Auwerx[2], Faustino Mollinedo[3] and Paolo Sassone-Corsi[2]*

[1]Instituto Cajal de Neurociencia, Consejo Superior Investigaciones Cientificas, Av. Doctor Arce 37, 28002 Madrid, Spain, [2]Laboratoire de Genetique Moleculaire des Eucaryotes, CNRS, 11 rue Humann, 67085 Strasbourg Cedex, France and [3]Centro Investigaciones Biologicas, CSIC, C/ Velazquez 144, 28006 Madrid, Spain

Received February 8, 1990; Revised and Accepted May 11, 1990

ABSTRACT

Induction of PC12 pheochromocytoma cells neuronal differentiation upon treatment with nerve growth factor (NGF) is accompanied by a coupled stimulation of c-*fos* and c-*jun* oncogene transcription. We found that induction of c-*fos* and c-*jun* proto-oncogene mRNAs levels following the endocrine differentiation of PC12 cells by sodium butyrate is uncoupled. While c-*fos* mRNA level increased within minutes, the content of c-*jun* mRNA was significantly elevated only 24 hours after treatment. Continuous presence of sodium butyrate for 72 hours resulted in stable high levels of c-*fos* and c-*jun* mRNAs. Gene transcription of the other members of the *jun* family, *jun* B and *jun* D, was not significantly modified at any induction time. The early accumulation of c-*fos* mRNA was accompanied by increased levels of c-Fos protein. While the NGF-induced c-Fos protein migrates with an apparent homogeneous molecular weight of 62 kDa, the sodium butyrate-stimulated Fos protein is of heterogeneous lower molecular weight. The different gel mobility of the Fos immunoreactive bands induced by sodium butyrate and the sustained Fos mRNA levels after induction suggested that the sodium butyrate-induced c-Fos protein could be non-functional in the autoregulation of the c-*fos* gene. Gel shift analysis showed unimpaired capacity of the butyrate-induced c-Fos protein to participate in the formation of transcriptional complexes with the Jun/AP-1 protein. However, transfection experiments indicate that the sodium butyrate-induced c-Fos protein is not able to negatively trans-regulate the c-*fos* promoter.

INTRODUCTION

Phenotypic conversion between cell derivatives from the caudal part of the neural crest is a multidirectional phenomenon conducted by a discrete number of factors. Among these, glucocorticoids, nerve growth factor (NGF) and a 45-kDa glycoprotein (cholinergic neuronal differentiation factor), direct precursor cell differentiation to chromaffin cells, adrenergic and cholinergic neurons, respectively (1, 2).

PC12 pheochromocytoma cells, a cell line phylogenetically related to the sympathoadrenal precursor of the neural crest, offer a similar plasticity. Indeed, PC12 cells exhibit a well characterized neuronal phenotype upon treatment with NGF (3) and, more recently, it has been shown that exposure of PC12 cells to sodium butyrate stops cell division and leads to a form which resembles the chromaffin phenotype (4, 5). Morphologically, sodium butyrate-differentiated PC12 cells show a tendency to form clusters of pseudo-syncytial appearance without neurite formation (4). Several tissue-specific changes are observed in the pattern of gene expression following sodium butyrate treatment including an increased expression of the pre-proenkephalin gene (5), new expression of enolase immunoreactivity (4), a reduced expression of tyrosine hydroxylase and NGF receptor genes (J.R.N. and B.M., in preparation). Long exposure ($\geq$4 days) to glucocorticoids also results in a chromaffin phenotype which shows a decrease in the low affinity NGF receptor binding sites (6).

Expression of nuclear proto-oncogenes has been correlated with the induction of proliferation or differentiation in different cell systems (for review see ref. 7). Among these, c-*fos* is the most extensively studied. It has been shown that the Fos protein acts through the formation of specific complexes with other nuclear proteins bearing a 'leucine zipper' domain close to a DNA-binding basic motif (8–13). AP-1, a transcription factor identified as the product of the proto-oncogene c-*jun* (14, 15), associates with the c-Fos protein to bind and activate transcription from promoter elements responsive to phorbol-esters (8–13). Furthermore, the products of two more recently described genes with sequence similarity to c-*jun*, *jun* B and *jun* D, may also form nuclear complexes with c-Fos or Fos-related proteins and

* To whom correspondence should be addressed

in that way might combinatorially modulate gene expression and cell proliferation (16–18).

Previous studies have shown a transient coordinate induction of c-*fos*, c-*jun* and *jun* B when PC12 cells are induced to divide by epidermal growth factor or to differentiate along the neuronal pathway by NGF (3, 19–24). Moreover, c-*fos* (19, 24–29) and *jun* B (28) are induced in PC12 cells following membrane depolarization, while c-*jun* does not respond to this stimulus (28).

To better understand the possible physiological meaning of the changes in nuclear proto-oncogene expression in PC12 pheochromocytoma cells, we sought evidence for an involvement of *fos* and *jun* in the process of PC12 cells differentiation towards the endocrine (chromaffin) phenotype after sodium butyrate exposure. In this report, we show that the induction of the proto-oncogenes *fos* and *jun* upon sodium butyrate treatment of PC12 cells is uncoupled. In particular, c-*fos* mRNA level is elevated up to 72 hrs after induction. We also show that autoregulation by the Fos protein does not occur in sodium butyrate treated PC12 cells, and suggest that this is due to the fact that, upon sodium butyrate treatment, the Fos protein undergoes some important modifications.

MATERIALS AND METHODS

Cell culture

PC12 cells were grown directly on untreated plastic (Falcon) as described (6). Drugs (Sigma) were added to exponentially growing non-confluent cultures at concentrations and durations specified in the figure legends.

Northern blot analysis

Total cellular RNA was isolated by CsCl centrifugation of guanidium thiocyanate lysates. Size-fractionation of RNA was achieved by electrophoresis on agarose-formaldehyde gels as described (5). ^{32}P labeled cDNA probes were prepared using the random oligonucleotide priming method (Oligo-labeling kit, Pharmacia) to specific activity equal to or higher than 7×10^8 cpm/μg. Conditions for blot hybridization and washing have been described elsewhere (5).

Isolation of nuclei and Western blot analysis

Cell nuclei from induced and uninduced cells were isolated and lysates prepared as described (30). 10 μg of protein (Bradford micromethod, BioRad) from each sample were applied per well to a 10% $NaDodSO_4$-polyacrylamide gel. After electroblotting and blocking in 20% fetal calf serum and 5% defatted milk in TBST buffer (50 mM Tris HCl pH8.0, 150 mM NaCl, 0.005% Tween 20), the nitrocellulose membranes (High-bond, Amersham) were incubated with an affinity purified c-Fos antibody (a generous gift from Dr. Michael Iadarola) for one hour. Signal was developed using a goat anti-rabbit immunoglobulin coupled to alkaline phosphatase (pico Blue™ system, Stratagene) according to the manufacturer indications.

Gel retardation analysis

Five μg aliquots from each nuclear extract were used in each binding assay. The assays were performed as described (8, 31), using a 18 bp TRE oligodeoxynucleotide bearing the consensus TGACTCA sequence (8). Inhibition of complex formation with anti-Fos antibodies (against the M peptide, 32) and anti-Jun antibodies (against the PEP-1 peptide, 14) was performed as described (8, 9).

Transfection of cultured cells

Rat pheochromocytoma PC12 cells were plated in DMEM 10% fetal calf serum, 5% horse serum, at 1×10^6 cells per 10 cm tissue culture dish, 24 hr before DNA transfection. Cells were transfected by the calcium phosphate coprecipitation technique and CAT assays were performed as described (8). All recombinants used in this study have already been described (8). The BK28/Δ plasmid is a human c-*fos* expression vector with an internal deletion in the (3′ end carboxy terminus of the coding region) of the cDNA generated by excising a BstEII fragment. When less than 20 μg of specific DNA was used, pGEM4 plasmid DNA was added to give 20 μg of total transfected DNA. Inductions by NGF and sodium butyrate were performed 24–36 hrs after DNA transfection.

RESULTS

Uncoupling of fos and jun induction

To better understand the possible role of *fos* and *jun* protooncogenes in the process of PC12 cell differentiation, we studied their transcriptional induction upon treatment with sodium butyrate. Addition of sodium butyrate (6 mM) to PC12 cell cultures resulted in a rapid increase in c-*fos* mRNA accumulation. The increase in c-*fos* mRNA was significant within thirty minutes after sodium butyrate addition, and peaked three hours later. Six hours after sodium butyrate administration, the level of c-*fos* mRNA was still 6-fold higher than the uninduced control levels, and at 24 hours the induction was stabilized between 5 and 6 fold. In contrast, the levels of c-*jun*, *jun* B and *jun* D mRNA were unchanged or slightly reduced during the first three hours of treatment (Fig. 1). There is no detectable transcript of c-*jun* between 0 and 6 hours after induction (data not shown, see fig. 1B). Between six and twenty four hours following sodium butyrate treatment the level of c-*jun* mRNA increased 6 to 8-fold over control values. No induction of *jun* B or *jun* D was evident even at later times. Three days after sodium butyrate the content of c-*jun* mRNA had stabilized at a level 5-fold higher than that in uninduced cells (fig. 1B).

These results are quite distinct from the NGF induction characteristics of these genes in PC12 cells (see fig. 1B, refs 20–29). Effects of NGF administration were also measured at late times for comparative purposes. A 1.5-fold increase was observed for c-*fos* and c-*jun* mRNAs six hours after NGF administration and by 24 hours no increase of c-*fos* mRNA remained and a slight reduction in c-*jun* mRNA was observed (fig. 1B).

The effects of sodium butyrate on *fos* and *jun* expression were linear over the concentration range 0.1 to 6 mM (not shown), the same range which was previously described to efficiently stop cell division (4). Since sodium butyrate is unstable in the culture medium (4, 5), the highest concentration (6 mM) was used in all other experiments. For the same reason, in treatments longer than 24 hours, medium with sodium butyrate was replaced every day.

The effects of NGF and sodium butyrate on PC12 cell division and differentiation are dependent on the continuous presence of the drug in the culture medium and their removal reestablish mitosis (3–5). When PC12 cells were exposed to sodium butyrate for different time periods, after which the medium was removed and the cultures maintained in control medium, the levels of c-*fos* mRNA returned to basal values (results not shown).

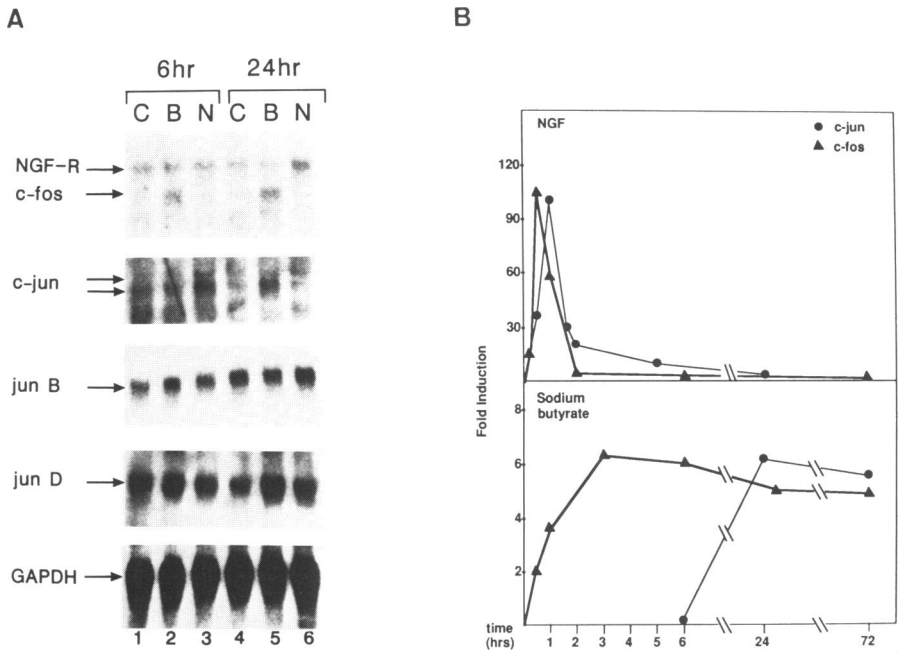

Figure 1. Induction of c-*fos* and members of the *jun* gene family upon sodium butyrate treatment of PC12 cells. *a*) Northern blot analysis of mRNA levels after 6 h and 24 h of sodium butyrate (B) or NGF (N) treatments (20 μg of total RNA were applied in each lane). Basal control levels are also indicated (C). *Fos* induction is sustained between 6 and 24 hrs; mRNA levels of NGF-receptor gene transcription (NGF-R) are also shown for comparison. NGF-receptor gene transcription decreases at 24 h treatment with sodium butyrate, but not with NGF (lanes 5−6, and B.M., in preparation). *b*) Time-course of c-*fos* and c-*jun* inductions upon NGF (top panel) and sodium butyrate (bottom) treatments. The values blotted are obtained after scanning of Northern blot autoradiograms and, in the case of NGF early induction times, also confirmed by published studies (23, 41).

Unusual modifications of Fos protein

Transcriptional autoregulation of both c-*fos* and c-*jun* expression has been demonstrated (31, 33). Through binding to an AP-1 site in the c-*jun* promoter, Fos/Jun complexes trans-activate the c-*jun* gene (33), while, in contrast, the dyad symmetry element (DSE) of the c-*fos* promoter mediates the trans-repression by Fos of the c-*fos* gene (31). This negative feed-back mechanism explains in part the transient nature of the c-*fos* mRNA or protein accumulation in different experimental models (19−28, 34, 35), and the phenomenon of superinduction of the c-*fos* gene after co-administration of protein synthesis inhibitors (22, 23, 36, 37). Furthermore, the reduction of c-Fos protein level may contribute to the termination of the auto-activation of c-*jun* (AP-1) (33). The possibility of a deficient process of translation of the c-*fos* mRNA induced by sodium butyrate, or a non-completed post-translational maturation of the c-Fos protein was investigated to explain the longer permanence of elevated levels of c-*fos* and c-*jun* mRNAs in PC12 cells after sodium butyrate administration.

Using Western blot analysis we observed an increase in c-Fos immunoreactivity following the accumulation of c-*fos* mRNA (fig. 2). In keeping with previous reports (25, 27, 38), nuclear extracts from normally growing cultures showed the presence of a group of c-Fos immunoreactive bands in the 30−40 kDa area but very low or undetectable levels of mature p62 c-Fos protein ($p55^{fos}$ is modified by phosphorylation to a protein apparently of 62 kDa, ref. 39 and data not shown). The exact nature of the immunoreactivity in the 40 kDa area is unclear though some have been identified as Fos-related proteins (Fos B and Fra-1, refs 38, 40) and as Jun/AP-1-associated polypeptides (8−14, 41). Thus, we wanted to compare the appearance of c-Fos protein after inductions with both NGF or sodium butyrate. In fig. 2A an analysis using high resolution acrylamide protein gel is shown. After administration of sodium butyrate, a broad immunoreactive band appears at an apparent molecular weight lower than mature p62 c-Fos protein (fig. 2A, lane 1), between 55 and 62 kDa. This is in contrast to the Fos protein obtained after NGF induction (lane 2). The immunoreactive bands in the 40 kDa area are only slightly modified by sodium butyrate treatment. These data suggest that a post-translational modification pattern of Fos has been altered in sodium butyrate-treated cells. The increase in c-Fos immunoreactivity was significant at 90 minutes and peaked at 3 hours after the initiation of the treatment (fig. 2B). The amount of c-Fos immunoreactivity and the pattern of bands in the higher molecular weight range remained elevated in sodium butyrate-treated cells up to 90 hours after the exposure (not shown, see also fig. 2A, lane 1).

The increase in c-Fos immunoreactivity was concentration-dependent between 0.1 and 6 mM (data not shown). NGF administration (100 ng/ml) resulted in the induction after 90 minutes of mature p62 c-Fos protein, without effect on the immunoreactivity in the 40 kDa protein range (fig. 2A).

Induction of c-fos and c-jun by sodium butyrate is independent of the calmodulin signal transduction pathway

It has previously been shown that the induction of c-*fos* gene expression by NGF is independent of the Ca^{2+}-calmodulin pathway, while the induction by depolarizing agents could be blocked by chlorpromazine or by the more specific calmodulin inhibitor W7 (25, 27). Administration of 10 μM of W7 for 24 hours or a few minutes before the addition of sodium butyrate

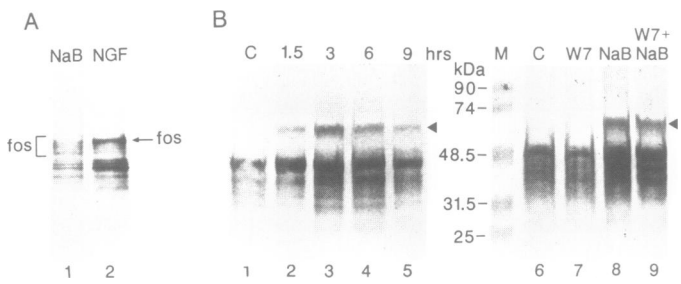

Figure 2. Analysis of Fos protein upon sodium butyrate induction of PC12 cells. *a*) Comparison of Fos protein appearance after sodium butyrate (NaB, lane 1) and NGF (lane 2) inductions after 1 h treatment. On this high- resolution protein gel the Fos protein appears as a broad, heterogenous band after sodium butyrate induction in contrast to the homogeneous Fos protein observed after NGF addition (lanes 1–2). *b*) Time-course of Fos protein appearance upon sodium butyrate addition. Levels of Fos protein are sustained also at late times, in contrast to NGF induction (lanes 1–5, ref. 22). The arrowhead indicate the Fos protein; the heterogenicity of Fos protein is less noticeable if high-resolution PAGE are not used. Lanes 6–9: Induction of c-Fos protein by sodium butyrate is independent of the calmodulin signal transduction pathway. Sodium butyrate induced protein after 3 h induction (NaB, lane 8). Addition of W7 calmodulin inhibitor had no effect on Fos protein production (W7+NaB, lane 9).

did not alter the pattern of accumulation of the c-*fos* and c-*jun* mRNAs by sodium butyrate alone (results not shown). Pretreatment with W7 also had no effect on the pattern and intensity of the c-Fos immunoreactive bands when compared to those obtained with sodium butyrate alone (fig. 2B, lanes 7–9).

Binding to an AP-1 recognition site

It has already been shown that binding to a TRE (TPA-responsive element), the AP-1 recognition site, is enhanced by TPA-treatment of fibroblasts and by NGF-treatment of PC12 cells (24). The increase is due to the rapid modification of both Fos and Jun oncoproteins induced by the activation of the relative signal transduction pathways. Because the Fos protein undergoes unusual modifications upon sodium butyrate induction of PC12 cells (fig. 2), we examined its ability to participate in a TRE-specific nucleoprotein complex formation. Cellular extracts were prepared from PC12 cells treated at different times (1 hr to 24 hrs) with sodium butyrate. The results, shown in figure 3, indicate that the Fos protein forms a specific nucleoprotein complex with the TRE oligodeoxynucleotide probe (lanes 1 to 5). No complex is formed when a mutated TRE probe (8) was used in the same gel retardation assay (not shown). There are two noteworthy features in this experiment. First, a significant increase in binding is detected with the extract from the 6 hrs sodium butyrate-induction time-point. This is in contrast with TPA or NGF induction where increase in binding is detectable after 1 h treatment. This result partly correlates with the delayed induction of c-*jun* reported in fig. 1. It has to be noted that the decrease in Fos protein after 9 hrs induction (see fig. 2) is likely to explain the absence of further increase in DNA-binding in extracts from cells induced after 6 hrs. In fact, the quantity of Fos protein peaks at 3–6 hrs (fig. 2) which correlates with increased binding. Fos protein might dimerize [with other available Jun proteins and bind to the TRE (such as JunB and JunD, see fig. 1)]. Second, there is a detectable increase in the mobility of the nucleoprotein complex, which might correlate with the decreased size of the Fos protein (see fig. 2A) or to preferential association of modified Fos with proteins of the Jun family. We confirmed with specific

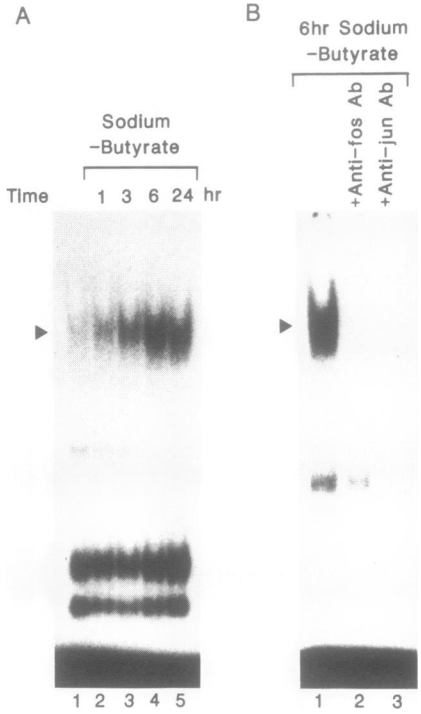

Figure 3. Binding to an AP-1 site. *a*) Nuclear extracts from sodium butyrate-induced PC12 cells were prepared at several time points (1–24 h); gel-retardation binding analysis was performed using an oligoprobe containing the consensus TGACTCA Jun/AP-1 binding site of the human metallothionein II gene (8). Induction of binding is detectable only at 6 h (lane 4) in contrast to NGF induced extracts in which induction is observed after 1 h induction (24). *b*) Binding inhibition using specific anti-Jun and anti-Fos antibodies was performed as described (8). Both Fos and Jun oncoproteins participate in the complex binding to the TRE using the PC12 nuclear extract from 6h-induced cells (compare lane 1 with lanes 2–3).

anti-Fos and anti-Jun antibodies that the DNA-binding complex required both Fos and Jun oncoproteins (fig. 3B). Non-specific antibodies were used in control experiments (not shown). These results imply that both Fos and Jun protein domains required for dimerization and DNA-binding were functional after sodium butyrate administration.

Lack of fos autoregulation upon sodium butyrate induction

The Fos oncoprotein trans-regulate the activity of the c-*fos* promoter by repressing the serum-inducibility (31). This mechanism, first studied in fibroblasts, is present also in PC12 cells upon NGF induction. In fig. 4, we show transfection experiments in which a c-*fos*-CAT reporter gene has been introduced in PC12 cells and the NGF inducibility of the promoter studied (29). The induction by NGF is about 8-fold and has previously been shown to require the SRE (serum responsive element) of the c-*fos* promoter (29; compare lanes 1 and 2). Co-transfection of a c-*fos* expression vector strongly inhibits the NGF induction (lane 3). This result is reminiscent of the autoregulation exerted by the Fos protein upon serum stimulation (31). A control expression vector which encodes a Fos protein mutated in the carboxy terminus has no inhibitory effect (lane 4). Because the different appearance of the Fos protein upon sodium butyrate induction and the sustained RNA levels observed after treatment of cultured PC12 cells, we wanted to test whether the modified

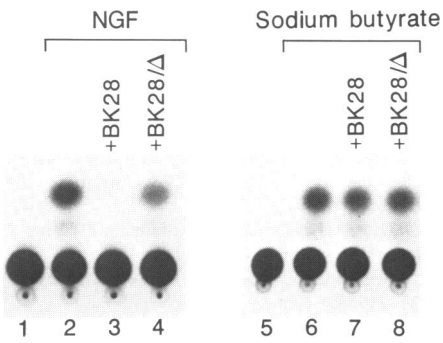

Figure 4. Lack of c-*fos* promoter down-regulation in PC12 cells treated with sodium butyrate. Lane 1−4 : The c-*fos*-CAT plasmid (containing c-*fos* promoter sequences from −404 to +42) transfected in PC12 cells is inducible by NGF (4h, ref. 29). Co-transfection with the c-*fos* expression vector BK28 (8) shows strong inhibition of NGF induction (compare lanes 2−3). Co-transfection with the BK28/? mutant (lacking the carboxy-terminus of the c-*fos* coding sequence, see ref. 31) shows lack of down-regulation (lane 4). Lanes 5−8: Induction with sodium butyrate (8h) is obtained after transfection of the c-*fos*-CAT plasmid (lanes 5−6); no down-regulation is observed after co-transfection of the c-*fos* expression vectors (lanes 7−8).

Fos protein would repress c-*fos* induced transcription. In cells transfected with the c-*fos*-CAT plasmid we obtained induction of the promoter after treatment with sodium butyrate (lanes 5−6). Co-transfection with a c-*fos* expression vector failed in showing autoregulation by Fos protein since no repression of the induced level was detectable (lane 7). This result suggest that one reason of the sustained c-*fos* transcription in PC12 cells induced with sodium butyrated may be the lack of the trans-repressor function of the Fos protein.

DISCUSSION

Sodium butyrate has been shown to be a potent differentiation inducing agent in a wide variety of cells. In Friend erythroleukemia cells (42), Manin-Darby canine kidney cells (43), rat pancreatic islet tumor cells (44) or NCB-20 cells (a hybrid resulting from fusion of neuroblastoma N18TG2 and Chinese hamster 18-day embryonic brain cell) (45), sodium butyrate has profound effects on cell division, morphology and expression of specific target genes. It has been hypothesized that sodium butyrate modifies chromatin structure by increasing the degree of histone acetylation (46) since it has been shown to inhibit histone deacetylases (47). The exact mechanism by which sodium butyrate stops PC12 pheochromocytoma cell division and gives rise to the endocrine-chromaffin phenotype is not yet known (4, 5).

Differentiation of PC12 pheochromocytoma cells along the endocrine pathway resulted in a stable high level of c-*fos* and c-*jun* mRNAs which correlates with the constitutively high levels of both proto-oncogene products observed in cultures of chromaffin cells from bovine adrenal medulla or in adrenal medulla from adult rat (J.R.N. unpublished results). Whether a stable high level of c-*jun* gene expression is a consequence of high levels of c-Fos protein or is an independent effect of sodium butyrate remains to be established. A prolonged activation of c-*jun* gene (at least six hours) with levels of c-Fos protein only transiently increased, has been reported in human fibroblasts after the addition of tumor necrosis factor-α (48).

Differentiation of PC12 pheochromocytoma cells along the neuronal pathway has been associated with a transient induction of c-*fos*, c-*jun* and *jun* B genes (19−24, 28), and to a minor extent of the c-*myc* gene (20−23). Stable high expression of some non-nuclear proto-oncogenes such as c-*src* (49) and c-Ha-*ras* (24, 50−52) induces the neuronal phenotype may be through the activation of similar molecular mechanisms triggered by NGF (24).

The late induction of c-*jun* differs from the effects observed with NGF treatment of PC12 cells (24, 41). On the other hand, *jun* D, which shows a very weak response to serum stimulation (17), is slightly induced after sodium butyrate differentiation (see fig. 1A). The fact that sodium butyrate affects the expression of *jun* related genes in such a different way than NGF may point to distinctive regulation and functions within this group of early response genes. This is supported by the previous observation that c-*jun* and *jun*-B induction can be dissociated in PC12 pheochromocytoma cells after membrane depolarization (28).

A novel conclusion from our studies is that the induction of high levels of c-*fos* mRNA and c-*jun* mRNA by sodium butyrate correlates with a distinctive post-translational processing of the c-Fos protein, which is still capable of efficient binding to the TRE sequence. Similar characteristics in the maturation of c-Fos protein have been described in PC12 cells after c-*fos* induction by depolarizing stimuli (25, 27). Results from Curran and Morgan (27) indicate that a group of c-Fos immunoreactive bands in the range 50 to 62 kDa followed an accumulation of c-*fos* mRNA shortly after the depolarization. However, these effects were more transient than those observed after sodium butyrate treatment. Furthermore, differently from the results obtained with sodium butyrate, the increase in both c-*fos* mRNA and c-Fos immunoreactivity after depolarization is sensitive to calmodulin inhibitor W7 (27). These differences suggest that a distinct mechanism contributes to butyrate- induced changes in proto-oncogene expression with respect to those induced by NGF or depolarizing agents.

Interestingly, when gel shift analysis was applied to study the ability of the immature forms of c-Fos protein to form a nucleoprotein complex with Jun/AP-1, no obvious change in binding capacity to a TRE was observed. However, butyrate induced-binding showed a lower molecular form of the complex formed as compared to HeLa cell nuclear extracts (data not shown) or to untreated PC12 cells; this is likely to be explained by the Western blot results which showed an unusually modified Fos protein (fig. 2A). Thus, apparently, the Fos protein obtained after sodium butyrate induction is able to participate in the formation of a stable complex and consequently its ability to dimerize with Jun/AP-1 appears unaffected.

The Fos product is known to have pleiotropic regulatory functions (8−13, 31). Particularly, it has been shown that $p55^{fos}$ is involved in the negative regulation of the c-*fos* promoter after serum induction (31). Because of the sustained elevation of *fos* mRNA after sodium butyrate induction and the unusual modifications which the Fos protein undergoes (fig. 2A), we reasoned that the lack of down-regulation of the *fos* mRNA level might be due to loss of the autoregulatory function by the Fos protein. From the transfection experiment performed (fig. 4) it appears that $p55^{fos}$ modifications induced by NGF or sodium butyrate treatments have distinct effect on the autoregulatory activity. The Fos product induced by sodium butyrate is unable to trans-regulate the c-*fos* promoter, strongly suggesting that this is one reason for the sustained *fos* mRNA level. It is tempting

to speculate that the modifications which the Fos protein undergoes are directly affecting its *trans*-regulatory function. Since it has been shown that the carboxy-terminus of the Fos protein is involved in the autoregulation of the gene transcription (ref. 31 and fig. 4), it is conceivable that sodium butyrate might affect that domain of the Fos protein. In the carboxy terminus of Fos are located clusters of serine residues that are known sites of phosphorylation (39). Thus, it could be that sodium butyrate treatment might affect Fos protein phosphorylation. This hypothesis is currently under investigation. These results also indicate that sodium butyrate-induced changes in c-*fos* gene expression are likely to be independent of conformational modifications in chromatin structure. Alternatively, sodium butyrate could activate another factor which inhibits Fos autoregulatory function.

It has also been suggested that *fos* mRNA is unstable, because of the presence of specific AUUUA sequences in the 3'-untranslated region of the messenger (53). It would be interesting to analyze the mechanism of *fos* mRNA stability in sodium butyrate induced PC12 cells, which clearly differs from NGF treated cells (22, 24). It could be argued that the Fos protein might participate in the molecular processes involved in the *fos* mRNA instability. The unusual protein modifications which occurs during sodium butyrate induction would then prevent this putative action of the Fos product.

The PC12 cell line provides an excellent model for the study of neuroendocrine differentiation. Because of the putative role played by proto-oncogenes in cellular proliferation and physiology, it is likely that their study will help us understand the molecular mechanisms involved. The use of PC12 cells induced to differentiate by sodium butyrate also appears to constitute an useful tool for the study of *fos* function. It is conceivable, indeed, that the accurate analysis of Fos protein modifications upon sodium butyrate treatment will contribute to the characterization of the Fos protein domains responsible of the gene autoregulation function.

ACKNOWLEDGEMENTS

We thank T.J. Bos and P. Vogt for Pep-1 anti-Jun antibody; M.G. Iadarola for M-Fos antibody; R. Bravo and M. Yaniv for the *jun* B and *jun* D cDNAs; A. Harel-Bellan for the mutated c-*fos* expression vector. C. Werlé, B. Boulay, F. Haenel and I. Martin for help in the preparation of the manuscript. We are grateful to Dr. Joaquin Del Rio for continuous support. B.M. is supported by a fellowship from the Spanish M.E.C. J.A. was supported by a short-term EMBO fellowship and was on leave from LEGENDO, Leuven, Belgium. This work was partially funded by 'Fundacion Ramon Areces' (J.R.N.) and by the CNRS, INSERM and Association pour la Recherche sur le Cancer (P.S.C.).

REFERENCES

1. Patterson, P.H. (1987) Ann. New York Acad. Sci. *493*, 20–26.
2. Anderson, D.J. & Axel R. (1986) Cell *47*, 1079–1090.
3. Greene, L.A. & Tischler, A.S. (1976) Proc. Natl. Acad. Sci. USA *73*, 2424–2428.
4. Byrd, J.C. & Alho, H. (1987) Dev. Brain Res. *31*, 151–156.
5. Byrd, J.C., Naranjo, J.R. & Lindberg, I. (1987) Endocrinology *121*, 1299–1305.
6. Tocco, M.D., Contreras, M.L., Koizumi, S., Dickens, G. & Guroff, G. (1988) J. Neurosci. Res. *20*, 411–419.
7. Almendral, J.M., Sommer, D., MacDonald-Bravo, H., Burckhardt, J., Perera, J. & Bravo, R. (1988) Mol. Cell. Biol. *8*, 2140–2148.
8. Sassone-Corsi, P., Lamph, W.W., Kamps, M. & Verma, I.M. (1988a) Cell *54*, 553–560.
9. Sassone-Corsi, P., Ransone, L.J., Lamph, W.W. & Verma, I.M. (1988b) Nature *336*, 692–695.
10. Kouzarides, T. & Ziff, E. (1988) Nature *336*, 646–651.
11. Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E. & Leder, P. (1988) Cell *55*, 917–924.
12. Turner, R. & Tjian, R. (1989) Science *243*, 1689–1694.
13. Gentz, R., Rauscher III, F.J., Abate, C. & Curran, T. (1989) Science *243*, 1695–1699.
14. Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K. & Tjian, R. (1987) Science *238*, 1386–1392.
15. Angel, P., Allegretto, E., Okino, S., Hattori, K., Boyle, W., Hunter, T. & Karin, M. (1988a) Nature *332*, 166–171.
16. Ryder, K., Lau, L.F. & Nathans, D. (1988) Proc. Natl. Acad. Sci. USA *85*, 1487–1491.
17. Hirai, S.I., Ryseck, R.P., Mechta, F., Bravo, R. & Yaniv, M. (1989) EMBO J. *8*, 1433–1439.
18. Nakabeppu, Y., Ryder, K. & Nathans, D. (1988) Cell *55*, 907–915.
19. Boonstra, J., Moolenaar, W.H., Harrison, P.H., Moed, P., van der Saag, P.T. & de Laat, S.W. (1983) J. Cell. Biol. *97*, 92–98.
20. Curran, T. & Morgan, J.I. (1985) Science *229*, 1265–1268.
21. Greenberg, M.E., Greene, L.A. & Ziff, E.B. (1985) J. Biol. Chem. *260*, 14101–14110.
22. Kruijer, W., Schubert, D. & Verma, I.M. (1985) Proc. Natl. Acad. Sci. USA *82*, 7330–7334.
23. Milbrandt, J. (1986) Proc. Natl. Acad. Sci. USA *83*, 4789–4793.
24. Sassone-Corsi, P., Der, C.J. & Verma, I.M. (1989) Mol. Cell. Biol. *9*, 3174–3183.
25. Morgan, J.I. & Curran, T. (1986) Nature *322*, 552–555.
26. Greenberg, M.E., Ziff, E.B. & Greene, L.A. (1986) Science *234*, 80–83.
27. Curran, T. & Morgan, J.I. (1986) Proc. Natl. Acad. Sci. USA *83*, 8521–8524.
28. Bartel, D.P., Sheng, M., Lau, L.F. & Greenberg, M.E. (1989) Genes & Development *3*, 304–313.
29. Visvader, J., Sassone-Corsi, P. & Verma, I.M. (1988) Proc. Natl. Acad. Sci. USA *85*, 9474–9478.
30. Dignam, J.D., Lebowitz, R.M. & Roeder, R. (1983) Nucl. Acids Res. *11*, 1475–1489.
31. Sassone-Corsi, P., Sisson, J.C. & Verma, I.M. (1988c) Nature *334*, 314–319.
32. Curran, T., Van Beveren, C., Ling, N. & Verma, I.M. (1985) Mol. Cell. Biol. *5*, 167–172.
33. Angel, P., Hattori, K., Smeal, T. & Karin, M. (1988b) Cell *55*, 875–885.
34. Verma, I.M. & Sassone-Corsi, P. (1987) Cell *51*, 513–514.
35. Greenberg, M.E. & Ziff, E.B. (1984) Nature *311*, 433–438.
36. Kruijer, W., Cooper, J.A., Hunter, T. & Verma, I.M. (1984) Nature *312*, 711–716.
37. Müller, R., Bravo, R., Burckhardt, J. & Curran, T. (1984) Nature *312*, 716–720.
38. Cohen, D.R. & Curran, T. (1988) Mol. Cell. Biol. *8*, 2063–2069.
39. Barber, J. & Verma, I.M. (1987) Mol. Cell. Biol. *7*, 2201–2211.
40. Zerial, M., Toschi, L., Ryseck, R.P., Schuermann, M., Müller, R. & Bravo, R. (1989) EMBO J. *8*, 805–813.
41. Wu, B.-Y., Fodor, E., Edwards, R. & Rutter, W.J. (1989) J. Biol. Chem. *264*, 9000–9003.
42. Reeves, R. & Cserjesi, P. (1979) J. Biol. Chem. *254*, 4283–4294.
43. Lever, J.E. (1979) Proc. Natl. Acad. Sci. USA *16*, 1323–1327.
44. Green, R. & Shields, D. (1984) Endocrinology *114*, 1990–1994.
45. Zhu, X.Z. & Chuang, D.M. (1988) J. Neurochem. *50*, 17–26.
46. Loidl, P. (1988) FEBS LETT. *227*, 91–95.
47. Sealy, L. & Chalkley, R. (1978) Cell *14*, 115–121.
48. Brenner, D.A., O'Hara, M., Angel, P., Chojkier, M. & Karin, M. (1989) Nature *337*, 661–663.
49. Alema, S., Casalbore, P., Agostini, E. & Tato, F. (1985) Nature *316*, 557–560.
50. Noda, M., Ko, M., Ogura, D., Liu, D., Amano, T., Takano, T. & Ikawa, I. (1985) Nature *318*, 73–75.
51. Bar-Sagi, D. & Feramisco, J.R. (1985) Cell *42*, 841–848.
52. Hagag, N., Halegoua, S. & Viola, M. (1986) Nature *319*, 680–682.
53. Raymond, V., Atwater, J.A. & Verma, I.M. (1989) Oncogene Res. *5*, 1–12.