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The Role of Jun Transcription Factor Expression and Phosphorylation in Neuronal Differentiation, Neuronal Cell Death, and Plastic Adaptations *in Vivo*

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SUMMARY

1. To investigate the role of the Jun transcription factors in neuronal differentiation, programmed neuronal cell death, and neuronal plasticity, we used phosphorothioate oligodeoxynucleotides (S-ODN) to inhibit selectively the expression of c-Jun, JunB, and JunD.

2. We have shown previously that in contrast to c-Jun, the JunB and JunD transcription factors are negative regulators of cell growth in various cell lines. Here we confirm this finding in primary human fibroblasts.

3. c-Jun and JunB are counterplayers not only with respect to proliferation, but also in cell differentiation. Since JunB expression is essential for neuronal differentiation, we analyzed possible posttranslational modifications of JunB after induction of PC-12 cell differentiation by nerve growth factor (NGF).

4. JunB was strongly phosphorylated after induction of PC-12 cell

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differentiation with NGF but not after stimulation of cell proliferation with serum. Thus, while cell proliferation is associated with c-Jun phosphorylation, cell differentiation is correlated with JunB phosphorylation. This supports the finding that c-Jun and JunB play antagonistic roles in both proliferation and differentiation.

5. The JunB transcription factor together with the c-Fos transcription factor is also induced *in vivo* in the suprachiasmatic nucleus (SCN) of rat brain after a light stimulus that induces resetting of the circadian clock.

6. Using antisense oligonucleotides injected into the third ventricle, we selectively cosuppressed the two transcription factors *in vivo* as shown by immunohistochemistry. Expression of c-Jun, JunD, and FosB was not affected. Inhibition of JunB and c-Fos expression prevented the light-induced phase shift of the circadian rhythm. In contrast, rats injected with a randomized control oligonucleotide showed the same phase shift as untreated animals.

7. In primary rat hippocampal cultures, anti-c-jun S-ODN selectively inhibited neuronal cell death and promoted neuronal survival. This indicates a causal role of c-Jun in programmed neuronal cell death.

8. These findings demonstrate the essential role of inducible transcription factors in the reprogramming of cells to a different functional state. Jun transcription factors play an essential role not only in fundamental processes such as cell proliferation, differentiation, and programmed neuronal cell death, but also in such complex processes as plastic adaptations in the mature brain. The inhibition of neuronal cell death by anti-c-jun S-ODN shows the great therapeutic potential of selective antisense oligonucleotides.

INTRODUCTION

How do cells alter their genetic program to allow long-term changes in function and structure? Which genes reprogram the cell to induce, e.g., proliferation after quiescence or to initiate differentiation of proliferating cells? Do such regulatory genes also play a role in programmed neuronal cell death and in neuronal plasticity? Inducible transcription factors such as members of the Jun protein family are prime candidates to orchestrate these changes at the level of gene expression.

Reprogramming of cell function and structure requires the coordinated induction of previously silent genes and the repression of certain currently active genes. Rapid induction and/or posttranslational modification of transcription factors can be hypothesized to play an essential role during this process. Here we test this hypothesis by blocking the expression of different transcription factors using the antisense technique.

Two inducible transcription factors, which are rapidly expressed in response to various extracellular stimuli, are c-Jun and JunB (reviewed by Schlingensiepen *et al.*, 1994). Together with JunD they form a protein family that shares high-sequence homology in several functionally important domains (reviewed by Vogt and Bos, 1990). Both *c-jun and jun*B belong to the "classical" immediate

early genes (IEGs), the most rapidly inducible genes in the cell, which show a large and transient increase after stimulation of cells with various stimuli (reviewed by Sheng and Greenberg, 1990; Morgan and Curran, 1991). The *jun*D gene, in contrast, is not an IEG but is constitutively expressed in considerable amounts in many cell types and tissues. However, like the other two family members, JunD is a transcription factor that binds specific DNA elements and thus regulates transcription of the respective target genes. All three Jun proteins form either homodimers or heterodimers with other leucine zipper-type proteins, including members of the Fos family.

The transactivation properties and binding affinities of the three Jun proteins differ from each other (Schütte *et al.*, 1989; Chiu *et al.*, 1989; Ryseck and Bravo, 1991). However, until recently, little was known about the function, e.g., of JunB and JunD in cell proliferation and of all three Jun transcription factors in cell differentiation. Moreover, hardly anything is known about their role in complex processes such as plastic adaptations in th brain, albeit their expression during these processes was frequently shown.

The c-*jun* gene is a protooncogene that promotes cell growth. In contrast, experiments with antisense oligonucleotides recently showed that *jun*B and *jun*D are inhibitors of cell proliferation (Schlingensiepen and Brysch, 1992). The inhibition of cell growth by *jun*D was recently confirmed by overexpression experiments (Pfarr *et al.*, 1994). JunB not only is a negative regulator of proliferation, but also plays an important role in cell differentiation. JunB expression is required both for commitment to differentiation of PC-12 pheochromocytoma cells (Schlingensiepen *et al.*, 1993) and for differentiation of hippocampal neurons in the post commitment stage (Schlingensiepen *et al.*, 1994). The c-Jun protein, in contrast is a counterplayer with respect to these functions.

Since the findings of a growth inhibitory role of JunB and JunD were obtained in cell lines, they need to be confirmed in primary cultures of proliferating cells. Here we analyze the role of the three Jun proteins in the proliferation of primary cultures of human fibroblasts.

Because of the essential role of JunB in cell differentiation, we studied possible posttranslational modifications of the JunB transcription factor during cell differentiation. We found specific phosphorylation of the JunB protein during nerve growth factor (NGF)-induced differentiation of PC-12 cells.

In vivo, JunB and c-Fos are the predominantly expressed AP-1 transcription factor components in the suprachiasmatic nucleus (SCN) of rat brain after light stimulation that induces resetting of the circadian clock (Kornhauser *et al.*, 1992). Such a phase shift of the circadian rhythm can be induced in rats housed in darkness by a light pulse during the subjective night phase (reviewed by Wollnik, 1992). To determine the functional relevance of the JunB/c-Fos coexpression in this paradigm, we interferred with their expression using the antisense technique.

Cosuppression of the c-Fos and JunB transcription factors in rat SCN with antisense oligonucleotides completely prevented the light-induced phase shift of the circadian rhythm, while injection of randomized control oligonucleotide had no effect.

The inducible transcription factors c-Jun and JunB are antagonists not only

with respect to their role in cell differentiation and proliferation, but also with respect to neuronal cell death and survival. Following inhibition of JunB expression with antisense S-ODN in cultured rat hippocampal neurons, we found a reduction in neuronal survival by two-thirds. In contrast, hippocampal neurons treated with anti-c-*jun* oligonucleotide displayed strongly increased survival compared to untreated and control oligonucleotide treated neurons.

Taken together, these findings show that the Jun transcription factors play a fundamental role in such diverse processes as cell proliferation, cell differentiation, programmed cell death, and neuronal plasticity.

MATERIALS AND METHODS

Antisense Phosphorothioate Oligodeoxynucleotide (S-ODN) Synthesis

S-ODN were synthesized and highly purified as described previously (Schlingensiepen *et al.*, 1993; Brysch *et al.*, 1994). Briefly, oligonucleotides were synthesized on an Applied Biosystems Model 394 DNA synthesizer using β-cyanoethyl phosphoramidite chemistry and a 30-min sulfurization step with 0.5 g elemental sulfur/4.8 ml CS2/4.8 ml pyridine/0.4 ml triethylamine. The sequences of c-*jun*, *jun*B, c-*fos*, p53, and randomized control oligonucleotides were as described previously (Schlingensiepen *et al.*, 1993; Brysch *et al.*, 1994; Gillardon *et al.*, 1994). The *jun*D antisense sequences were TTCGCGTAGACAGG for human *jun*D and TCCTCGCCATAGAA for rat *jun*D. Oligodeoxynucleotides were purified by reverse-phase HPLC on a Waters Delta-Pak C18 column and detritylated with 80% acetic acid. Subsequently, S-ODN were extracted with diethyl ether, lyophilized, and resuspended in water. Finally, S-ODN were purified by anion-exchange chromatography on a Waters Protein-Pak DEAE 8HR column as follows: gradient—10–100? B, linear over 45 min, hold for 10 min; buffer A—25 m*M* Tris/Cl, 1 m*M* EDTA, pH 8.0, 10% acetonitrile; buffer B—2.5 *M* NaCl in A. S-ODN eluted around 90% of buffer B. S-ODN were precipitated from the eluate with 2 × vol 100% EtOH, resuspended in sterile water and dialyzed against 0.1 *M* Tris, pH 8.0.

Fluorescein isothiocyanate (FITC) labeling was performed using a C3-amino-linker (Glen Research). For the labeling reaction, 100 nmol of 5′ C3-amino-modified oligonucleotide was diluted in 400 μl of 1 *M* carbonate-bicarbonate buffer (pH 9), to which 5 mg of fluorescein isothiocyanate was added, dissolved in 400 μl of 50% dimethylformamide. The labeling reaction was carried out at 37°C for 2 hr in the dark. Labelled oligonucleotides were separated from unincorporated product by reverse-phase HPLC on a C18 column and the product peak was lyophilized and purified as described previously (Tischmeyer *et al.*, 1994).

Cell Proliferation Experiments

Primary human skin fibroblasts were established and grown for five passages in RPMI medium (GIBCO) supplemented with 10% fetal calf serum (FCS). For thymidine incorporation experiments, cells were then plated into 96 well flat-bottom microplates at a density of 2500 cells/well in RPMI medium with 5%

FCS. S-ODN were added at a 2 μM concentration 1 hr after plating. Before harvesting, cells were incubated with 0.15 μCi ^{3}H-thymidine per well for 6 hr. Cells were lysed by freezing, spotted onto glass filters, and washed and the amount of incorporated tritium was determined by liquid scintillation counting.

PC-12 Cell Differentiation

PC-12 pheochromocytoma cells were plated into poly-L-lysine-coated 96-well flat-bottom microplates at a density of 2500 cells/well in Dulbecco's modified Eagle's medum (DMEM), supplemented with 5% FCS. S-ODN were added at a 2 μM concentration 6 hr after plating. Twenty-four-hours after plating cells were incubated with 10 ng/ml NGF (Sigma) for 10 days. Neuronal differentiation was assessed independently by two different observers in a blinded fashion.

Primary Rat Hippocampal Cultures

Hippocampi from the brains of embryonal day 18/19 rats were dissociated by mild trypsinization and plated into 96-well plates at a density of 6.4×10^5/ml in a serum free mixture of HM medium (Honegger) and N2 medium (Boddenstein-Sato) (1:1, v:v), conditioned by primary rat astrocytes. Oligonucleotides were added 4 hr after plating at a concentration of 1 μM. Neuronal survival was assayed using the fluorescein diacetate/ethidium bromide double-staining method as described previously (Schlingensiepen *et al.*, 1994). For oligonucleotide uptake studies FITC-labeled S-ODN were added to the culture medium at a 1 μM concentration 4 days after plating of neurons.

Western Blotting

PC-12 cells were plated into poly-L-lysine-coated 260 ml culture flasks at a density of 4×10^6 cells in 30 ml of DMEM, supplemented with 5% FCS. After 24 hr, cells were serum starved for 24 hr by incubation in DMEM with 1% FCS. On the next day, either cells were stimulated with 40 ng/ml NGF to induce neuronal-like differentiation or they were stimulated to proliferate by the addition of 10% FCS to the culture flask. At the time points 4, 8, 24, 48, and 72 hr after treatment with NGF or FCS, cells were harvested and processed for western blotting.

At the time points indicated cells were lysed by the addition of lysis buffer consisting of 0.1 *M* Tris buffer (pH 6.8), 20% glycerin, 2% sodium dodecyl sulfate (SDS). For dephosphorylation experiments, lysates were incubated with bovine intestinal alkaline phosphatase type XXX-L (Sigma) at 0.3 U/μg lysate for 30 min at 37°C. Proteins were separated by electrophoresis on a 10% SDS–polyacrylamide gel (SDS-PAGE) and blotted onto Immobilon-P membrane (Millipore). Blots were probed with either rabbit polyclonal anti-c-Jun antibody (Oncogene Science, PC 07), rabbit polyclonal anti-JunB antibody (Santa Cruz, N-17), or a second rabbit polyclonal anti-JunB antibody (Santa Cruz, N-210). The two anti-JunB antibodies are directed against different epitopes of the JunB protein. For antibody detection, goat anti-rabbit IgG–alkaline phosphatase

conjugate (Boehringer Mannheim) was used as the second antibody. Detection was performed by chemiluminescence with CSPD (Tropix) according to manufacturer's instructions.

Antisense Experiments *in Vivo*

Anesthetized adult male rats (100 mg/kg ketamine hydrochloride and 10 mg xylazine) were implanted stereotaxically with a microinjection guide cannula, targeted to the bottom of the third ventricle at the level of the ventral SCN. Two weeks later, the animals were placed in cages equipped with a running wheel, connected to an online monitoring computer system as described previously (Wollnik, 1991). Rats were housed under continuous darkness for 16 weeks.

Every 4 weeks rats were exposed to a light pulse (300 lux for 1 hr) at subjective circadian time 15 hr (CT 15). At this time point of the circadian cycle, light pulses induce maximal phase shift of circadian activity rhythms. Randomized control S-ODN (1 m*M* in 4 μl) or a mixture of anti-c-*fos* and anti-*jun*B S-ODN (1 m*M* in 2 μl each), diluted in physiological saline, was injected 6 hr prior to light exposure.

Phase shifts in circadian rhythmic activity were determined according to Daan and Pittendrigh (1976). Differences between experimental groups were analyzed by univariate one-way analysis of variance (Statistica, StatSoft, Tulsa, OK). Sheffé's multiple *t* test was used for individual mean comparisons.

Immunohistochemistry

To determine protein suppression in rat SCN after treatment with either antisense or control oligonucleotides, a subset of animals was sacrificed 4 hr after light exposure. Following transcardial perfusion with 4% paraformaldehyde under anesthesia, brains were removed, cryoprotected in 30% sucrose, and sectioned on a cryostat (35 μm). Free-floating sections were processed for immunocytochemistry. Primary antisera (Kovary and Bravo, 1991) were diluted 1:30.000 for c-Jun and 1:4.000 for JunB. Detection was performed as previously described using the avidin–biotin protocol (Herdegen *et al.*, 1991).

RESULTS

Proliferation of Human Fibroblasts

After 24 hr of S-ODN treatment, there was no significant difference in thymidine incorporation between untreated cells and those treated with randomized control S-ODN. Treatment with anti-JunB S-ODN as well as with anti-JunD S-ODN, however, strongly increased thymidine uptake to more than threefold. These values are similar to those obtained after suppression of the

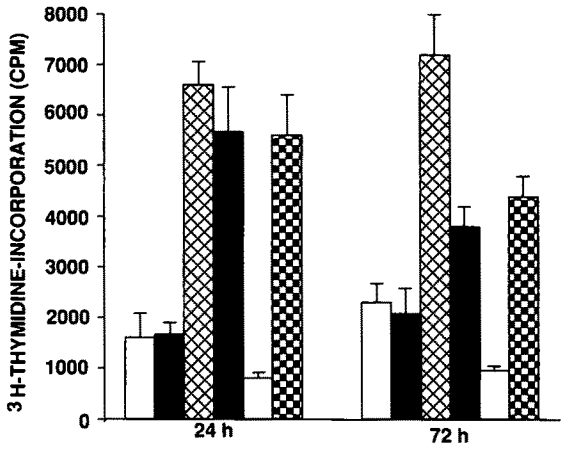

Fig. 1. Proliferation of primary human fibroblasts after inhibition of JunB, c-Jun, or JunD expression. ^{3}H-Thymidine incorporation rates in human fibroblasts after treatment with 2 μM concentrations of either control S-ODN, anti-*jun*B S-ODN, anti-c-*jun* S-ODN, anti-*jun*D S-ODN, or anti-p53 S-ODN. Light gray bars: Control cells without S-ODN addition. Dark gray bars: Cells treated with randomized control S-ODN. Cross-hatched bars: Treatment with anti-*jun*D S-ODN. Black bars: Treatment with anti-*jun*B S-ODN. White bars: Treatment with anti-c-*jun* S-ODN. Dotted bars: Treatment with anti-p53 S-ODN.

known growth inhibitory tumor suppressor protein p53 (Fig. 1). In contrast, inhibition of c-Jun expression reduced thymidine incorporation by more than 50%. At 72 hr after a single addition of S-ODNs, proliferation was still increased by 50% in anti-JunB-treated cells and to more than threefold in anti-JunD-treated cells.

PC-12 Cell Differentiation

While the effects of JunB and JunD suppression were similar with respect to cell proliferation, they were different in PC-12 cell differentiation. Inhibition of JunB completely prevented neurite outgrowth and led to increased cell proliferation. In contrast, suppression of c-Jun protein enhanced morphological differentiation, while suppression of JunD protein did not alter the morphological differentiation pattern. Similarly, p53 antisense S-ODN-treated cells did not significantly differ from control cells with respect to neurite outgrowth (Fig. 2). Thus, suppression of the rapidly inducible transcription factors c-Jun and JunB showed opposing functions in cell differentiation. But suppression of neither the more constitutively expressed JunD transcription factor nor the p53 tumor suppressor protein altered morphological differentiation of neuronal differentiation of PC-12 cells.

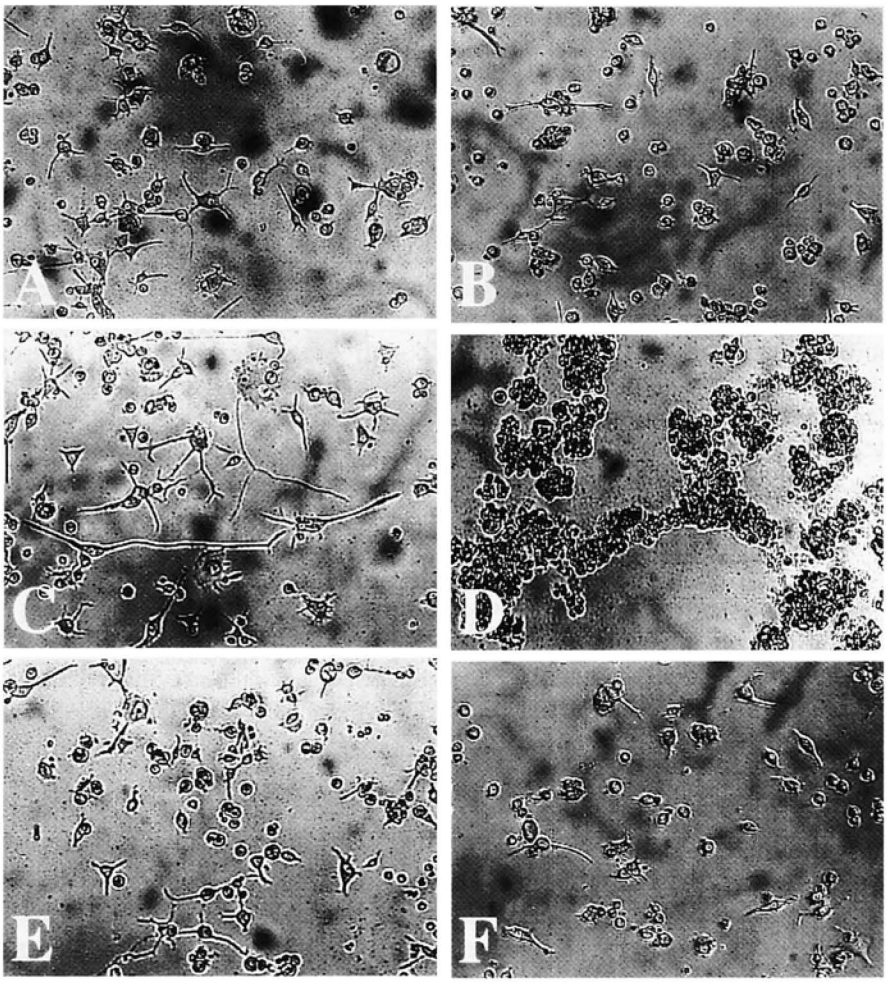

Fig. 2. Differentiation of PC-12 cells after inhibition of JunB, c-Jun, JunD, or p53 expression. Morphological differentiation of PC-12 cells after treatment with 2 μM concentrations of different S-ODN. (A) Untreated control cells. (B) Treatment with randomized control S-ODN. (C) Cells treated with anti-c-*jun* S-ODN. (D) Cells treated with anti-*jun*B S-ODN. (E) Treatment with anti-*jun*D S-ODN. (F) Treatment with anti-p53 S-ODN. Morphological differentiation of PC-12 cells was completely prevented by anti-*jun*B S-ODN. In contrast, enhancement of neurite outgrowth was seen after treatment with anti-c-*jun* S-ODN. Inhibition of expression of the two proteins JunD and p53 did not show significant differences from control conditions.

JunB Phosphorylation During PC-12 Cell Differentiation

The dependence of PC-12 cell differentiation on JunB expression, as revealed by the antisense experiments, prompted us to determine whether posttranslational modifications of JunB might also be involved in the differentiation process. Four hours after treatment of serum-starved PC-12 cells with either NGF or FCS, western blotting revealed no difference in JunB expression. However, after 8 hr JunB expression had almost disappeared in FCS treated cells, while it was still

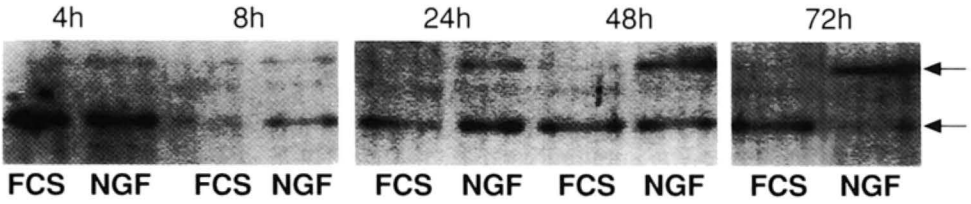

Fig. 3. Expression of a high molecular weight form of JunB in differentiating PC-12 cells. Western blot of PC-12 cell lysates after induction of differentiation with NGF or of proliferation with FCS, probed with an anti-JunB antibody. JunB expression was induced by both stimuli (lower arrow). At 8 and 24 hr after stimulation, JunB expression is more intense in NGF-treated cells, compared to FCS stimulation. Beginning at 24 hr after stimulation, a second higher molecular weight band begins to appear in NGF-treated cells (upper arrow). After 72 hr following NGF treatment, almost all of the protein detected by the anti-JunB antibody is present in the high molecular weight form.

present—albeit at a lower level—in NGF-treated cells. After 24 hr not only was expression of JunB more pronounced in NGF-treated cells, but also, a second, higher molecular weight band—only faintly observed at the early time points—became clearly visible. After 48 hr of NGF stimulation about half of the JunB protein was already present in this high molecular weight form. In contrast, this band was almost undetectable in FCS-treated cells. At 72 hr after NGF addition nearly all the JunB protein was present in the high molecular weight form (Fig. 3). In contrast, western blots probed with an anti-c-Jun antibody revealed no increase in the apparent molecular weight of c-Jun after NGF stimulation. To determine whether the higher molecular weight form of JunB in differentiating PC-12 cells was the result of phosphorylation, an aliquot of the cell lysates was treated with alkaline phosphatase. Phosphatase treatment led to complete disappearance of the high molecular weight band in NGF treated cells, while the low molecular weight band reappeared (Data not shown). To confirm further that the upper molecular band represents a form of JunB protein, a second anti-JunB antibody, raised against a different epitope of JunB, was used. Again, the same higher molecular weight band was detected in NGF-treated cells (Fig. 4). In summary, induction of PC-12 cell differentiation by NGF leads to phosphorylation of JunB. In contrast, stimulation of cell proliferation with FCS does not induce JunB phosphorylation.

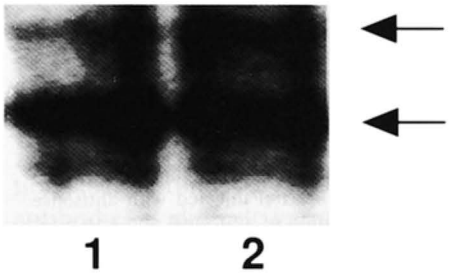

Fig. 4. Direction of phosphorylated JunB with a second antibody. Western blot of PC-12 cell lysates after induction of differentiation with NGF. The blot was probed with a second anti-JunB antibody raised against a different epitope of the protein. This antibody again detects the phosphorylated form of JunB. Lane 1, 24 hr after NGF treatment; Lane 2 48 hr after NGF treatment.

Selectivity of Gene Suppressionin *in Vivo* by Anti-*junB* S-ODN

A 1-hr light pulse at subjective circadian time 15 hr (CT 15) induced a distinct immunoreactivity for c-Fos and JunB and, to a lower extent, for c-Jun and FosB. Suppression of c-Fos immunoreactivity in the CNS *in vivo* by the anti-c-*fos* S-ODN that has been shown previously (Gillardon *et al.*, 1994) was confirmed in the antisense-injected animals. The number of c-Fos-immunoreactive nuclei was reduced by more than 50% and the intensity of staining was markedly reduced in the remaining cells (data not shown). similarly, in antisense-treated animals the number of JunB-reactive cells was reduced to less than half compared to those injected with control S-ODN. Furthermore, the intensity of staining was strongly reduced in the remaining cells (Figs. 5A and B). In contrast, the number and staining intensity of c-Jun-immunoreactive nuclei were not altered by antisense treatment (Figs. 5C and D). Equally the immunoreactivity of FosB and JunD was not changed (data not shown), confirming *in vivo* the selectivity of JunB suppression by the anti-*junB* S-ODN previously demonstrated in cell culture (Schlingensiepen *et al.*, 1994).

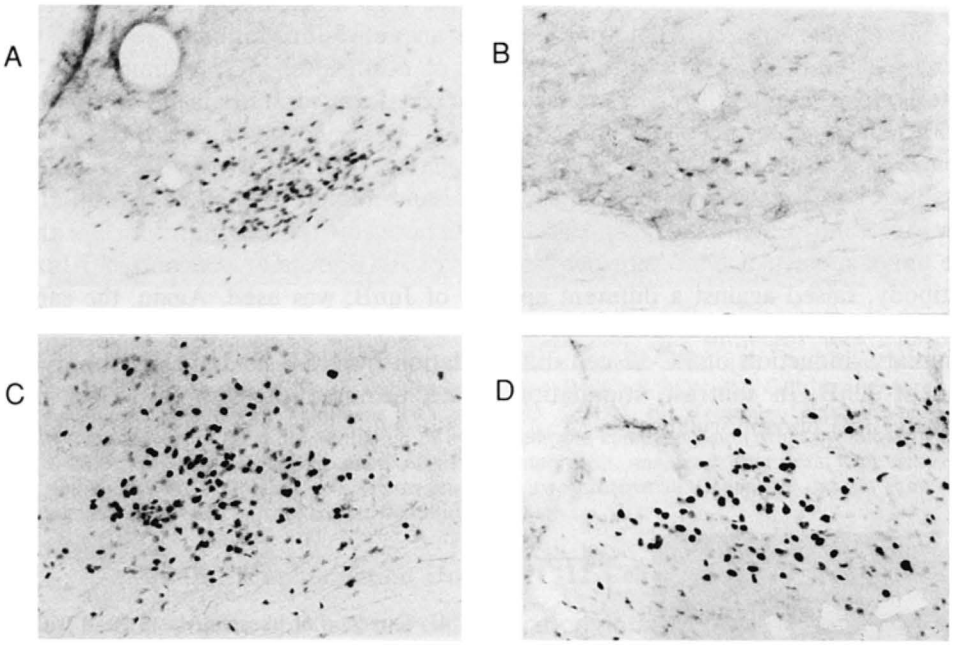

Fig. 5. Selectivity of antisense-mediated protein suppression *in vivo*. Immunohistochemical detection of JunB and c-Jun expression in SCN after treatment of rats with a light pulse. (A, B) JunB expression; (C, D) c-Jun expression. A and C are from animals not injected with antisense oligonucleotide. B and D are from animals injected with a combination of anti-junB and anti-c-fos oligonucleotide prior to the light pulse. Note the selective reduction of JunB but not of c-Jun expression.

Inhibition of Circadian Rhythm Shift by *junB* and c-*fos* Cosuppression in the Rat

Rats treated with a 1-hr light pulse at CT 15 displayed a robust phase shift of more than 2 hr in circadian rhythmic activity (-125 ± 15 min). Animals injected with nonsense control oligonucleotide prior to the light pulse showed a similar phase shift by more than 2 hr (-127 ± 20 min). In contrast, in animals injected with a combination of anti-*junB* S-ODN and anti-c-*fos* S-ODN, the phase shift was prevented (-10 min $\pm$ 16 min) (Fig. 6). To confirm these results in individual rats, animals were injected with control S-ODN at one time point and with antisense S-ODNs 4 weeks later. Figure 7 shows activity of a control animal, not injected with S-ODNs, subjected to a light pulse (LP) on day 36 of the experiment and a second time 4 weeks later. Both light pulse treatments result in a phase shift of 100 and 130 min, respectively. Figure 8 shows the activity record from an animal that received antisense oligonucleotides (ASO) the first time and nonsense control S-ODN (NSO) 4 weeks later. Antisense S-ODN treatment 6 hr before the light pulse prevented light-induced circadian phase shift, while the same animal, injected with randomized nonsense control S-ODN (NSO) 4 weeks later, showed a phase shift of 130 min in circadian rhythmic activity. This finding was confirmed in another animal, yielding similar results. Phase shift after control oligo (NSO) injection prior to light pulse treatment was 160 min in this animal, while antisense treatment again prevented the phase shift (Fig. 9).

Effects of Anti-c-*jun* and Anti-*junB* S-ODN on Neuronal Survival

Following treatment with anti-*junB* S-ODN, neuronal survival was reduced to 61% of controls on day 7 and to 27% on day 15. In contrast, in anti c-*jun*-treated cells, neuronal survival was increased by 54% on day 15 compared to cells treated with randomized control oligonucleotide and by 51% compared to untreated control neurons (Fig. 10).

The uptake of FITC-labeled S-ODN was very efficient in neurons. Strong labeling of neurons was observed after 72 hr of FITC–S-ODN incubation. FITC labeling was not confined to neuronal cell bodies, but was also seen in the neurites (Fig. 11). To control for possible uptake of FITC that might have resulted from degradation of the FITC–S-ODN complex, uncoupled FITC was added to neuronal cultures. No significant labeling of neurons was observed in these control cells.

DISCUSSION

Inhibition of Jun Transcription Factor Expression with Antisense Molecules

The antisense technology is particularly suited for the analysis of transcription factor function. Since these are intracellular proteins, inactivation by antibodies is difficult *in vitro* and impossible *in vivo*. A further approach, more commonly used, is the overexpression of genes to study the physiological

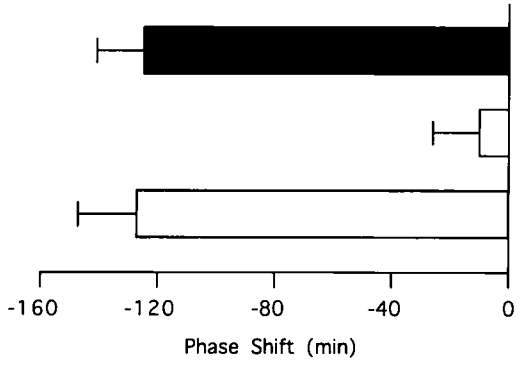

Fig. 6. Phase shift in rat activity after light pulse treatment. Black bar: Animals treated with light pulse alone. White bar: Animals injected with a combination of anti-junB and anti-c-fos oligonucleotide prior to the light pulse. Gray bar: Animals injected with randomized control oligonucleotide before light pulse treatment.

function of the encoded proteins. However, massive overexpression of a transcription factor will alter its concentration-dependent DNA binding properties and enable binding to low-affinity sites. Furthermore, a transcription factor may activate a gene at a low concentration and repress it at a high concentration (Sauer and Jäckle, 1993).

Whereas generation of transgenic "knowkout" animals is a valuable tool for developmental studies, it is often of limited value for functional analysis in the adult animal, since it may lead to serve abnormalities during development. Thus, the antisense approach offers a valuable alternative to these techniques. Phosphorothioate oligodeoxynucleotides (S-ODN) are nuclease resistant and allow the selective inhibition of the synthesis of single proteins. The resulting loss of function permits conclusions on the natural function of the suppressed proteins and/or their role in disease processes *in vitro* and *in vivo* (Patinkin *et al.*, 1990; Schlingensiepen and Brysch, 1992, Jachimczak *et al.*, 1993; Behl *et al.*, 1993; Schlingensiepen *et al.*, 1993; Stein and Cheng, 1993, Lev-Lehmann *et al.*, 1994, Tischmeyer *et al.*, 1994).

In our cell proliferation experiments, inhibition of the three Jun transcription factors in primary human fibroblasts revealed very different roles in cell growth. Both JunD and JunB proved to be inhibitors of cell proliferation, similar to the findings obtained in permanent cell lines (Schlingensiepen and Brysch, 1992; Schlingensiepen *et al.*, 1993; Pfarr *et al.*, 1994). This functional antagonism of JunB and JunD to the protooncogene c-Jun in cell proliferation is initially surprising but may be an important mechanism for fine-tuning of cellular responses to extracellular stimuli.

In vivo, a large number of signal molecules converge on every cell at the same time, e.g., growth factors, hormones, differentiation factors, and cytokines. All of these signals can induce certain cellular programs like differentiation,

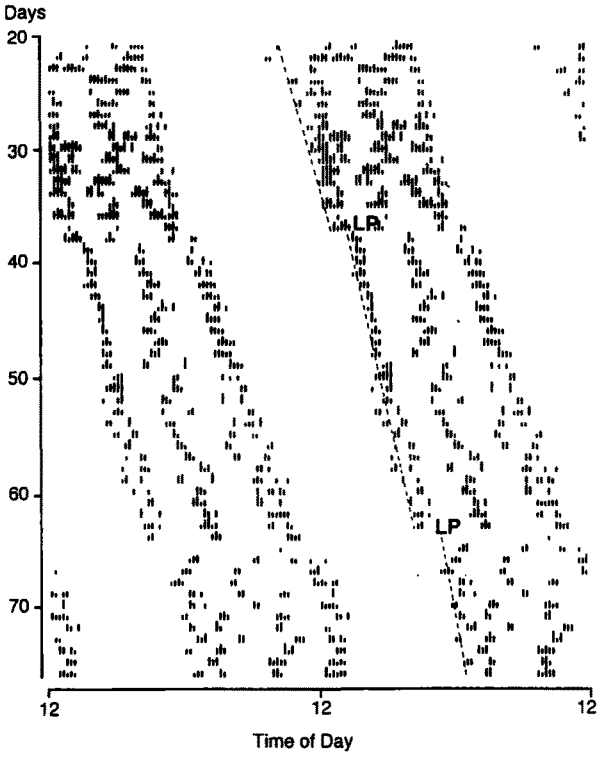

Fig. 7. Circadian rhythm shift after light pulse treatment. Left: The activity of an individual rat as measured by online monitoring. Each bar represents 20 min of running wheel activity. Right: The same activity plot, with a dotted line connecting the beginning of activity according to Daan and Pittendrigh (1976). Also, on the right the times of light pulse treatment as indicated (LP).

changes in cell structure, cell proliferation, or programmed cell death (apoptosis), to name just a few. Most of these signals induce one or several of the Jun proteins, often in combination with other transcription factors. The fact that the different Jun proteins can act in an opposing manner but are often coexpressed suggests that their relative amounts may play a significant role in determining which of the above mentioned cellular programs will actually be activated (Gass *et al.*, 1993).

This would require varying intensity of relative expression of the different *jun* genes in changing physiological situations. Indeed, alterations in the time course and magnitude of *jun* gene expression are observed during organ development. c-*jun* expression predominates during the proliferation phase, while *jun*B expression is predominant during the differentiation stage (Dungy *et al.*, 1991; Wilkinson *et al.*, 1989). This expression pattern fits well with the functional analysis in our PC-12 cell differentiation experiments, showing that c-Jun inhibits and JunB promotes differentiation of PC-12 cells. Initially both *jun* genes are coexpressed after stimulation with NGF (Schlingensiepen *et al.*, 1993). This

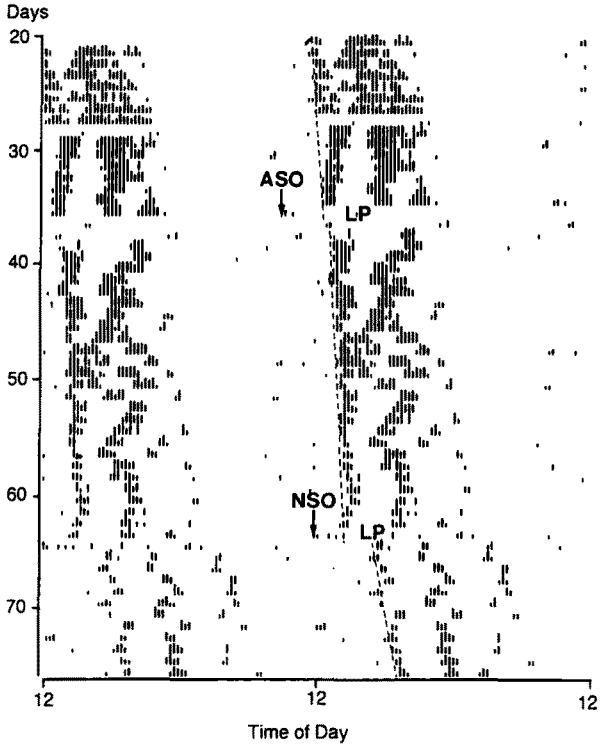

Fig. 8. Difference in phase shift after specific or nonsense S-ODN injection. Activity plot from a rat injected with a mix of anti-junB and anti-c-fos antisense oligonucleotides (ASO) prior to light pulse (LP) treatment on day 36. No phase shift is induced. In contrast, injection with control nonsense oligonucleotide (NSO) on day 64 prior to the 1 hr light pulse does not prevent the phase shift: The phase shift is 130 min.

coincides with a transient stimulation of cell growth. The phase of morphological differentiation correlates with the time, when c-*jun* mRNA expression has decreased, while *jun*B mRNA is still expressed at high levels. Interestingly, inhibition of the less regulated JunD protein does not show a measurable effect on morphological differentiation in the antisense experiments. Possibly JunD expression plays a role for the fine tuning of other processes in the CNS, e.g., nerve regeneration after injury (Herdegen *et al.*, 1993).

Phosphorylation of JunB in Cell Differentiation

Apart from alteration in expression levels, posttranslational modifications offer another mechanism for functional modulation of cell programs via transcription factors. Thus, Ha-Ras overexpression augments c-Jun activity by stimulating phosphorylation of its transactivation domain. This phosphorylation of c-Jun is in fact required for the cooperation of c-Jun and Ha-Ras in oncogenic cell transformation (Binetruy *et al.*, 1991; Smeal *et al.*, 1991).

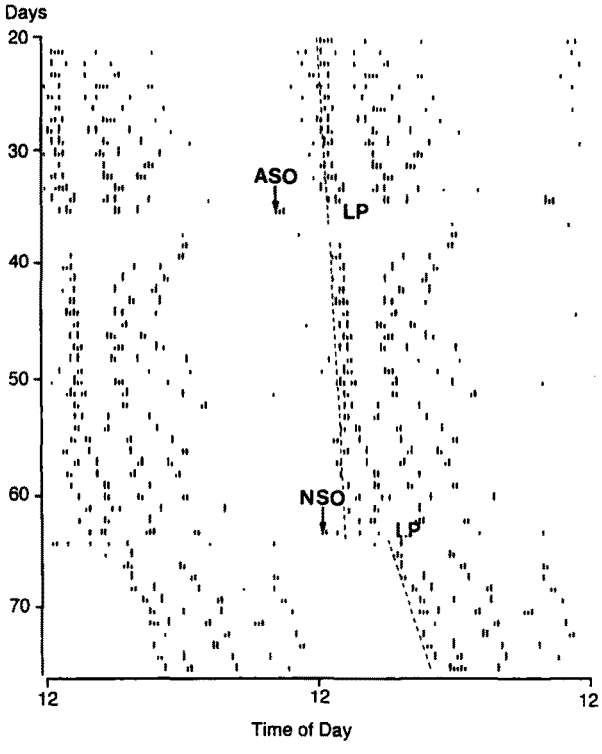

Fig. 9. Differences in phase shift in a rat with low basic activity. Activity plot from a rat with lower basic activity compared to that in Fig. 8. Injection with antisense oligonucleotides (ASO) again prevents circadian rhythm phase shift after a light pulse (LP). Injection with control nonsense oligonucleotide (NSO) leads to a normal phase shift of 160 min.

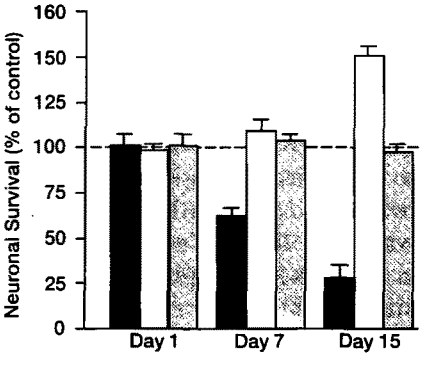

Fig. 10. c-jun antisense oligo prevents neuronal cell death. Survival of developing hippocampal neurons after treatment with different antisense oligonucleotides. Anti-c-jun oligonucleotide strongly increases neuronal survival by more than 50% by day 15 and inhibits the cell death occurring under both control conditions. Values are expressed as a percentage of the untreated controls. Neurons were incubated with the following oligonucleotides: gray bars, control S-ODN; black bars, anti-junB S-ODN; white bars, anti-c-jun S-ODN. The dotted line marks the value of untreated control neurons, set at 100%.

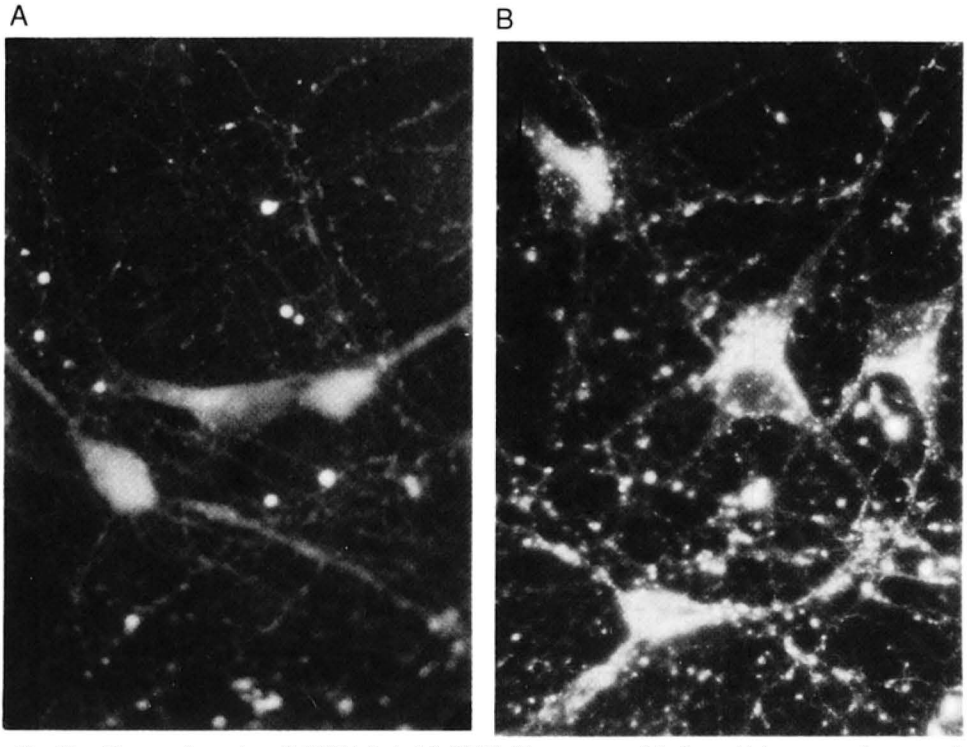

Fig. 11. Neuronal uptake of FITC-labeled S-ODN. Fluorescence labeling of hippocampal neuronal cultures after (A) 2 hr of incubation with FITC-labeled S-ODN: (B) 72 hr of FITC-S-ODN incubation.

Here we provide data that show specific induction of JunB phosphorylation in response to induction of cell differentiation. This may suggest that phosphorylation of JunB plays an equally important role in cell differentiation. Thus, it is conceivable that while c-Jun phosphorylation is required to transmit the mitogenic and oncogenic signaling of Ha-Ras, phosphorylation of JunB is required to mediate the differentiation signal. Mapping of the phosphorylation sites of JunB will be required in the future to determine where they are located, e.g., in the activation domain or in the DNA-binding domain of the JunB protein and to allow site specific mutagenesis for further characterization of phosphorylation effects.

In any case, the opposite phosphorylation patterns in differentiation and proliferation of c-Jun and JunB not only show that they act in an opposing functional manner, but also suggest that their activity is regulated by different kinases. Thus, not only their expression, but also their posttranslational modification is regulated by different intracellular pathways. Phosphorylation of c-Jun is part of the proliferation-inducing cascade activated by Ha-Ras overexpression, but not of NGF-induced cell differentiation. In contrast, JunB phosphorylation occurs in response to differentiation-inducing stimuli, but not after induction of proliferation.

Taken together, the results of the proliferation experiments and the results on JunB phosphorylation during differentiation may suggest that expression of unphosphorylated JunB is sufficient to inhibit cell growth, while phosphorylation of JunB is required for the actual differentiation process.

Cosuppression of JunB and c-Fos *in Vivo*

The functional significance of transcription factor induction in the brain is an area of intense research that is only just emerging. Models of neuronal plasticity have to be combined with functional analysis of transcription factor action. Resetting of the circadian rhythm offers a well-defined model of neuronal plasticity, in which a circumscribed structure, the suprachiasmatic nucleus (SCN), is the predominant pacemaker (reviewed by Takahashi, 1993). Light-induced phase shifts are correlated with immediate early gene (IEG) expression, including *jun*B, c-*jun*, *fos*B, and c-*fos*. The c-Fos and JunB transcription factors exert the main inducible transcription activity of AP-1 proteins *in vivo* following light pulse stimulation (Kornhauser *et al.*, 1992). Their induction in the SCN by light occurs only during that circadian time, when light is capable of shifting the circadian rhythm and occurs mainly in the ventrolateral part, which received input from retinal afferents (reviewed by Takahashi, 1993). Our antisense experiments demonstrate that this correlation of JunB and c-Fos expression with circadian rhythm shift is of causal relevance.

Long-term alterations in circadian rhythmic activity are prevented by cosuppression of JunB and c-Fos. This confirms the hypothesis that reprogramming of cellular function in a complex model of neuronal plasticity is dependent on the expression of inducible Jun and Fos transcription factors.

Finally, the opposite role of JunB and c-Jun in neuronal survival and cell death in hippocampal cultures suggests that both survival and death of these neurons can be regulated by Jun transcription factor expression. The increase in survival in anti c-*jun*-treated neurons becomes apparent after day 7 in culture, the time of intense formation of synaptic contacts in hippocampal cultures (Seifert *et al.*, 1983). Thus, competition for synaptic contacts may act via the c-Jun transcription factor to regulate the number of neurons by programmed cell death.

The role of c-Jun in cell proliferation and our results on its role in neuronal cell death are reminiscent of the bivalent regulatory role of c-*myc* that can either lead to cell proliferation or to apoptosis, depending on the supply with growth factors and trophic factors (reviewed by Wyllie, 1993).

Furthermore, the increase in neuronal survival after treatment of cells with antisense c-*jun* oligonucleotides highlights the therapeutic potential of antisense oligonucleotides for treatment of diseases involving programmed neuronal cell death, including such prevalent diseases as delayed neuronal cell death after hypoxic brain injury and Alzheimer type neuronal degeneration.

In conclusion, the expression of inducible transcription factors is essential for the realization of alterations in cell function and structure as required by changing physiological demands. Thus, their suppression with selective antisense oligonucleotides allows intervention with such vital and complex processes as

programmed neuronal cell death and resetting of the circadian pacemaker which represents a specific form of a memory engram.

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