

# Increase of *c-jun* mRNA upon hypo-osmotic cell swelling of rat hepatoma cells

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## Abstract

*c-jun* mRNA levels were increased in rat hepatoma cells (H4-II-E-C3) when exposed to hypotonic medium (205 mosmol/l) with a maximal induction observed after 1 h of hypotonic exposure. At this time point an approximate 5-fold increase in *c-jun* expression could be detected in relation to normotonic control incubations (305 mosmol/l). Hypertonic exposure (405 mosmol/l) had only a slight effect on *c-jun* expression. In contrast to the increased *c-jun* mRNA levels under hypotonic conditions, expression of the *c-fos* proto-oncogene was unaffected by changes in the osmolarity. The hypotonicity-induced increase in *c-jun* expression was also detectable in the presence of a protein kinase C (PKC) inhibitor. This indicates that PKC is not involved in the signal transduction pathway leading to *c-jun* expression upon hypotonic cell swelling in these cells.

**Key words:** *c-jun*; *c-fos*; Proto-oncogene; Cell swelling; Hypotonic; Hypertonic; Protein kinase C; Hepatoma cell

## 1. Introduction

Cell swelling as it occurs following hypotonic exposure, cumulative substrate uptake or the influence of insulin, modulates metabolic liver cell function (for reviews see [1,2]). Recent studies have shown that hypotonic cell swelling also has an effect on the expression of the  $\beta$ -actin gene in the perfused rat liver [3] and in isolated rat hepatocytes [4], indicating that cell volume changes not only modulate metabolic functions but may also regulate gene expression. The Jun protein is a member of the AP-1 transcription factor family [5,6]. It dimerizes with itself or with the product of the *c-fos* proto-oncogene, c-Fos [7]. These dimers bind to specific enhancer DNA sequences and regulate transcription positively or negatively. The promoter of the *c-jun* proto-oncogene contains an AP-1 binding site and is positively autoregulated by its product [6]. Here we report an increase in *c-jun* mRNA levels in response to hypotonic cell swelling, whereas the hypotonicity-induced cell swelling had no effects on *c-fos* mRNA levels in a rat hepatoma cell line (H4-II-E-C3).

## 2. Materials and methods

### 2.1. Chemicals

Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma (Deisenhofen, Germany).  $\alpha$ -D-Raffinose was from Serva (Heidelberg, Germany). The PKC inhibitor, Gö 6850 [8], was kindly provided by Dr. Schächtele (Gödecke AG, Freiburg, Germany). Cell culture media and fetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany). 100 mm cell culture plates were obtained from Costar (Cambridge, USA). [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, Germany).

### 2.2. Cell culture

H4-II-E-C3-cells were grown in DMEM/F12 (low glucose) medium, supplemented with 10% fetal calf serum (FCS) in a 37°C, 5% CO<sub>2</sub> atmosphere. After the cells were grown to confluence, the medium was changed to DMEM/F12 without FCS for 24 h to induce quiescence. The medium was changed to hypotonic (205 mosmol/l), normotonic (305 mosmol/l) or hypertonic (405 mosmol/l) DMEM/F12 medium without FCS for the indicated time points: the osmolarity was varied by changing the NaCl concentration. After stimulation, the cells were washed twice with 5 ml of phosphate-buffered saline and harvested for RNA extraction.

### 2.3. Northern blot analysis

Total RNA from confluent cells was prepared using the guanidinium thiocyanate method [9]. 15  $\mu$ g of RNA was electrophoresed in 0.9% agarose/3% formaldehyde gels and blotted onto Hybond-N nylon membranes with 20  $\times$  SSC (1  $\times$  SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). After crosslinking with a UV-crosslinker (UV-stratalinker 1800; Stratagene), blots were subjected to prehybridization at 43°C in 50% formamide, 0.25 M NaHPO<sub>4</sub> (pH 7.2), 0.25 M NaCl, 1 mM EDTA, 100  $\mu$ g/ml salmon sperm DNA and 7% SDS for 4 h. Hybridization was carried out in the same solution containing 10<sup>6</sup> cpm/ml denatured [<sup>32</sup>P]dCTP-labeled random primed DNA probes. The probes used for hybridization were the 1.8 kb *EcoRI*–*PstI* mouse *c-jun* cDNA fragment [10], the 1.3 kb *BstxI* mouse *c-fos* genomic fragment of pc-fos-3 [11] and the 1.1 kb cDNA fragment of the human glyceraldehyde 3-phosphate dehydrogenase (Clontech). Membranes were washed 3 times with 2  $\times$  SSC, 0.1% SDS for 10 min, twice with 25 mM NaHPO<sub>4</sub> (pH 7.2), 0.1% SDS, 1 mM EDTA and twice with 25

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**Abbreviations:** PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

mM NaHPO<sub>4</sub> (pH 7.2), 1% SDS, 1 mM EDTA for 20 min at 53°C. The filters were then exposed to Kodak AR X-OMAT X-ray films at -70°C with intensifying screens. Autoradiograms were analyzed using a pdi scanner (Pharmacia, Freiburg).

### 3. Results and discussion

In order to examine the induction of *c-jun* mRNAs in H4-II-E-C3-cells in response to cell volume changes, the cells were serum-starved for 24 h prior to exposure to normotonic (305 mosmol/l), hypotonic (205 mosmol/l) or hypertonic (405 mosmol/l) medium. Total RNA was collected at the indicated times and analyzed by Northern blot analysis (Fig. 1). There were two species of *c-jun* mRNAs of 3.2 kb and 2.7 kb, as previously reported [10,12]. Hypotonic exposure gave rise to a rapid increase in *c-jun* mRNA levels in relation to normotonic control incubations, with maximal induction observed at about 1 h (Fig. 1, compare lanes 1 and 4). At this time point a 5-fold increase in *c-jun* expression in relation to normotonic control incubation could be observed. *c-jun* mRNA levels rapidly decreased thereafter and were reduced to control levels after 3 h. In contrast to the hypotonicity-induced cell swelling, the hypertonicity-induced cell shrinkage had only a slight effect (about 1.7-fold) on the expression of the *c-jun* proto-oncogene in these cells at

the 30 min time point, but not at other time points (Fig. 1, compare lane 7 with lanes 8–12). To investigate, whether the hypotonicity-induced increase in *c-jun* mRNA level is due to the decrease in osmolarity or due to a decrease in Na<sup>+</sup> activity, 50 mM NaCl was substituted for 100 mM raffinose, thereby maintaining normotonicity (305 mosmol/l) (Fig. 1). Under these conditions no increase in the expression of the *c-jun* proto-oncogene could be detected (Fig. 1; compare lane 13 with lanes 14 and 15), indicating that the effects on *c-jun* mRNA expression are due to the osmolarity decrease and not to the change of extracellular Na<sup>+</sup> or Cl<sup>-</sup> activity. It is known that both Jun homodimers and the Jun-Fos heterodimer complex possess DNA-binding properties, although the Jun-Fos heterodimer has much greater affinity for the AP-1-binding site and is a more potent transcriptional activator than the Jun homodimers [13–15]. Therefore we examined whether hypotonicity also leads to an increase in the expression of the *c-fos* proto-oncogene. As shown in Fig. 2, the hypotonic exposure of serum-starved cells for 0.5 and 1 h, where a strong induction of *c-jun* could be detected (Fig. 1), did not increase *c-fos* expression (Fig. 2, compare lane 1 with lanes 2 and 3), whereas the phorbol ester, PMA, which is a well-known inducer of *c-fos* expression [16–18], led to a strong induction of *c-fos* expression in H4-II-E-C3

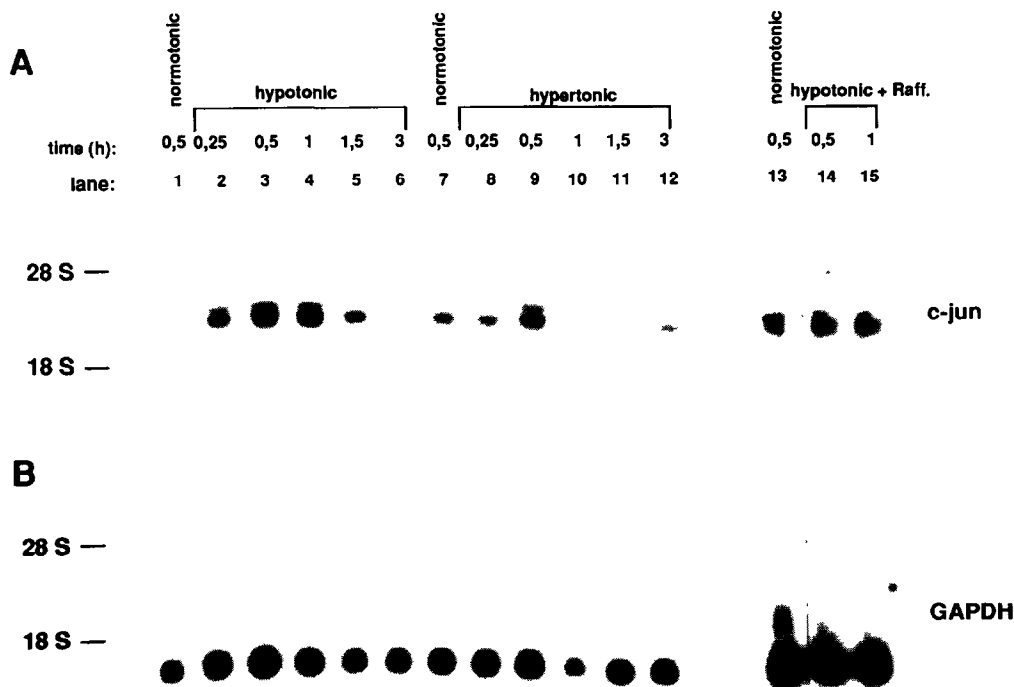


Fig. 1. Induction of *c-jun* expression in H4-II-E-C3 cells. The cells were grown to confluence in medium containing 10% FCS and were then incubated for 24 h in medium without FCS to induce quiescence. Medium was then changed to normotonic (305 mosmol/l) (lanes 1 and 7), hypotonic (205 mosmol/l) (lanes 2–6) and hypertonic (405 mosmol/l) (lanes 8–12) medium without FCS. After the indicated time points (in hours) the cells were harvested for RNA extraction. In lanes 14 and 15 RNA was loaded from cells which were incubated for 0.5 h (lane 14) and 1 h (lane 15) in normotonic medium in which 50 mM NaCl was substituted against 100 mM raffinose. 15 µg of total RNA were loaded in each lane. The filters were probed with the 1.8 kb *EcoRI-PstI* mouse *c-jun* cDNA fragment [10] (A) and the 1.1 kb cDNA fragment of the human glyceraldehyde 3-phosphate dehydrogenase (Clontech) (B). The RNA bands 28 S and 18 S are indicated on the left.

cells (Fig. 2, compare lanes 1 and 4). Equal amounts of RNA were loaded, as shown by hybridization with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) complementary DNA (Fig. 2B). Exposing the cells to hypertonic medium for different time points also did not induce significant alterations in *c-fos* expression in comparison to normotonic exposure (data not shown). In order to examine the role of protein kinase C activity in the hypotonicity-induced expression of the *c-jun* gene, the cells were treated with the PKC inhibitor, Gö 6850 [8], which is identical to GF 109203 X [19]. This inhibitor is highly specific for PKC and inhibits the  $\text{Ca}^{2+}$ -dependent, as well as the  $\text{Ca}^{2+}$ -independent isoforms of PKC [8]. In the absence of the PKC inhibitor, hypotonic exposure for 1 h, as well as the PKC activator, PMA, induced the expression of *c-jun* mRNA (Fig. 3, compare lanes 1, 2 and 3). In the presence of this PKC inhibitor, stimulation with PMA did not result in an increase of *c-jun* mRNA (Fig. 3, compare lanes 4 and 6), whereas the hypotonicity-induced expression of the *c-jun* gene was unaffected by the PKC-inhibitor (Fig. 3, compare lanes 4 and 5). Equal loading of the gel was confirmed by hybridization with the glyceraldehyde 3-phosphate dehydrogenase cDNA (Fig. 3B). The hypotonicity-induced increase in *c-jun* mRNA expression in the presence of the PKC inhibitor suggests that protein kinase C is not a necessary

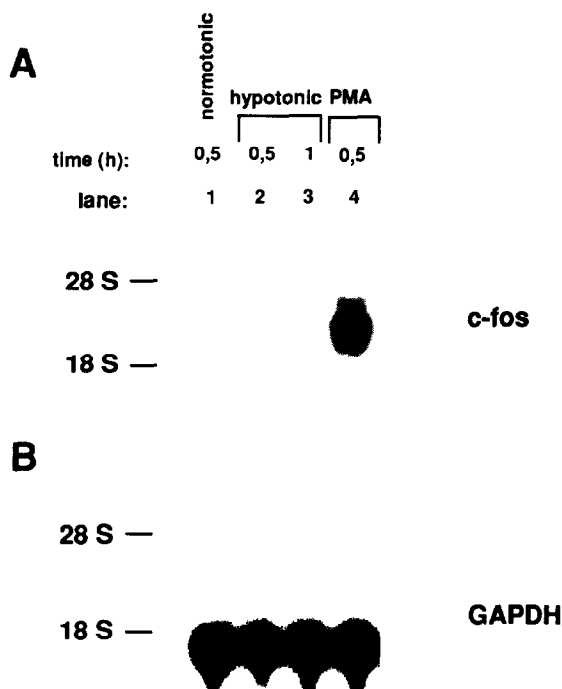


Fig. 2. Expression of *c-fos* mRNA in H4-II-E-C3 cells. After serum starvation for 24 h, medium was changed to normotonic medium (lanes 1 and 4) or hypotonic medium (lanes 2 and 3) for the indicated time points. *c-fos* expression was induced by 1  $\mu\text{g}/\text{ml}$  PMA for 30 min (lane 4). 15  $\mu\text{g}$  of total RNA/lane were analyzed by Northern blotting. The filter was probed with the 1.3 kb BstX-1 mouse *c-fos* genomic fragment of pc-fos-3 [11] (A) and the 1.1 kb glyceraldehyde 3-phosphate dehydrogenase cDNA fragment (Clontech) (B).

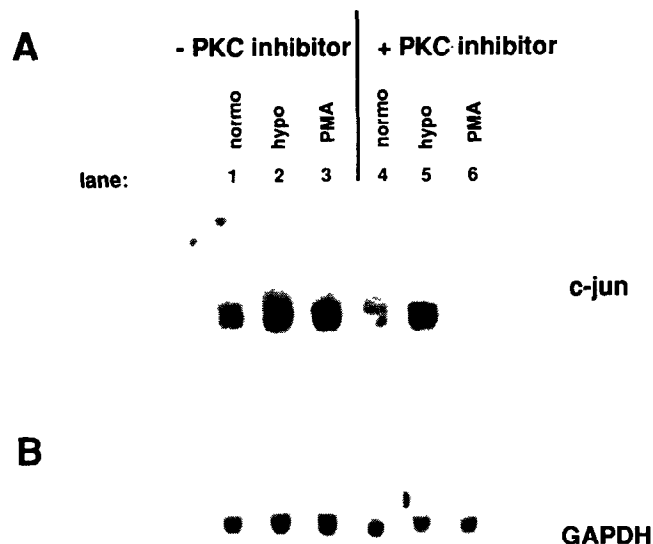


Fig. 3. Effect of the PKC inhibitor, Gö 6850, on *c-jun* expression. The cells were serum-starved for 24 h. The medium then was changed to normotonic (lanes 1, 3, 4, 6) and hypotonic (lanes 2 and 5) medium without FCS for 30 and 60 min, respectively. Cells were treated with 1  $\mu\text{g}/\text{ml}$  PMA for 30 min (lanes 3 and 6). Cells were grown in the absence of the PKC inhibitor (lanes 1–3) or in the presence of 1  $\mu\text{M}$  of the PKC inhibitor, Gö 6850 (lanes 4–6). The filters were probed with the cDNAs for *c-jun* and glyceraldehyde 3-phosphate dehydrogenase as described for Fig. 1.

signal transduction component for the hypotonicity-induced *c-jun* expression in these cells. PKC plays an important role in the PMA-induced expression of the *c-jun* gene and the *c-jun* expression is stimulated by several growth factors. On the other hand, there are a few examples where growth factor or hormone-induced expression of the *c-jun* proto-oncogene is PKC-independent, such as the EGF and insulin induction of *c-jun* in rat fibroblasts [20] and SV 40-transformed murine 3T3 T cells [21], respectively. Taken together, these results show that hypotonicity-induced cell swelling leads to alterations in the expression of the *c-jun* proto-oncogene. It remains to be determined which signal transduction pathway is responsible for this effect.

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