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# Expression of c-*jun*/AP-1 during myogenic differentiation in mouse $C_2C_{12}$ myoblasts

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Mitogen withdrawal triggers myogenic differentiation in skeletal myoblasts in culture. We have examined the expression of the proto-oncogene c-*jun* during this process in mouse $C_2C_{12}$ myoblasts. c-*jun* belongs to a family of immediate early genes whose expression is activated in cultured cells in response to the addition of serum growth factors. Interestingly, expression of c-*jun* was maintained in mouse $C_2C_{12}$ and rat L6 myoblasts undergoing myogenic differentiation under low-serum conditions. Previously it has been reported that expression of c-*jun* is downregulated during differentiation of C2 cells. However, our results using $C_2C_{12}$ cells, a subclone of the C2 line, show that c-*jun* mRNA, protein and the activator-protein 1 (AP-1) DNA-binding activity were easily detected in proliferating myoblasts and differentiated myotubes. Although overexpression of c-*jun* has been shown to block myogenic differentiation in C2 cells, results presented here suggest that expression of c-*jun* at physiological levels may not interfere with skeletal myogenesis.

AP-1; c-*jun*; Muscle differentiation; MyoD; Myogenin

## 1. INTRODUCTION

Skeletal myoblasts grown in culture differentiate in response to depletion of exogenous growth factors. Upon mitogen withdrawal, myoblasts exit from the cell cycle, express various muscle-specific genes, and fuse together to from multinucleate myotubes. Since many events associated with cell commitment and terminal differentiation are well characterized in skeletal myoblasts, these cells offer an excellent model for understanding the role of proto-oncogenes during cellular proliferation and differentiation. In the recent years various studies have shown that the proto-oncogene c-*jun* plays an important role in cell growth and differentiation (reviewed in [1]).

The product of the proto-oncogene c-*jun* is a major component of the AP-1 complex [2,3]. In addition to homodimers, c-Jun forms heterodimers with Jun-B and Jun-D which are members of the *jun* family, and c-Fos, Fra-1, Fra-2 and Fos-B which are members of the *fos* family of proteins (reviewed in [4]). These dimers regulate expression of target genes containing the AP-1 binding site (TGACTCA). Expression of c-*jun* is regulated both at transcriptional and post-translational levels. Transcription of c-*jun* is regulated by various serum growth factors, interleukin-1, phorbol esters, cyclic AMP and other agents (reviewed in [1]). An AP-1 site present in the c-*jun* promoter mediates positive autoregulation by AP-1 [5]. The DNA-binding activity of c-Jun is modulated by site-specific phosphorylation and dephosphorylation [6,7], and protein–protein interactions [1,8].

Using in situ hybridization techniques, it has been previously shown that c-*jun* mRNA is expressed in 14.5- and 17.5-day mouse embryo skeletal muscle [9]. Transcripts for the muscle regulatory factors MyoD and myogenin were also found to be expressed at this time [10]. However, it is not known whether c-*jun* expression plays any role in skeletal myogenesis in vivo. To address whether c-*jun* is expressed during terminal differentiation of skeletal muscle cells in culture we have previously examined the expression of c-*jun* in rat skeletal L6 myoblasts. Withdrawal of serum mitogen triggers terminal differentiation of skeletal myoblasts in culture. We have found that expression of c-*jun*/AP-1 was maintained during myogenic differentiation of L6 cells. Notably, expression of c-*jun* was not markedly affected by addition of serum or depletion of the AP-1 binding activity by inhibiting protein synthesis in rat L6 cells (G. Thinakaran and J. Bag, unpublished results).

The uncharacteristic expression of c-*jun* in rat L6 cells maintained in low-serum conditions prompted us to study its expression in another well characterized muscle cell line namely the mouse $C_2C_{12}$. Our results show that mouse $C_2C_{12}$ myoblasts also express appreciable amounts of c-*jun* mRNA and c-Jun protein during differentiation. Gel retardation assays detected AP-1 bind-

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ing activity in the nuclear extracts prepared from $C_2C_{12}$ cells. The finding that c-*jun* and various contractile protein mRNAs were co-expressed in $C_2C_{12}$ cultures demonstrate that expression of c-*jun* is compatible with myogenic differentiation of these cells.

## 2. MATERIALS AND METHODS

### 2.1. *Cell culture*

Mouse skeletal $C_2C_{12}$ cells (ATCC CRL 1772) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO). Rat skeletal L6 cells were grown in Alpha modified minimal essential medium supplemented with 10% fetal calf serum. Myotube formation was induced by the addition of medium containing 2.5% horse serum (differentiation medium) to subconfluent cultures. Approximately 80–90% of the nuclei were present in myotubes 3–4 days after the shift to the differentiation medium.

### 2.2. *RNA analysis*

Total cellular RNA was isolated by the acid guanidinium thiocyanate method [11]. Twenty micrograms of RNA was fractionated by formaldehyde-agarose gel electrophoresis and transferred to Zetabind membrane (Cuno, Inc.). For dot blot analysis, RNA contained in 100 $\mu$l of water was mixed with 150 $\mu$l of formaldehyde solution (37%, w/w) and 150 $\mu$l of SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), incubated at 65°C for 15 min and loaded onto Zetabind membrane using a Hybri-dot 96-well filtration manifold (BRL).

Northern blotting was performed as described previously [12]. DNA probes specific to c-*jun* (an *Eco*RI fragment of pJac7) [13], MyoD (an *Eco*RI fragment of pVZCIIα) [14], myogenin (an *Eco*RI fragment of pUC65-20) [15], actin (linearized plasmid p AC 269) [16] and glyceraldehyde-3-phosphate dehydrogenase (linearized plasmid pRGAPDH13) [17] were used for hybridizations. Synthetic oligodeoxynucleotides were used for fast troponin C (5′-CTCAGCCTGTTGGTCCGT-3′) and fast troponin T (5′-TTCCTCGTCAGACAT-3′) hybridizations. Autoradiographic signals from appropriately exposed films were quantified using a video image processing system (GelPrint, BioPhotonics Corp.). The signal intensities were normalized using the amount of 28 S rRNA, quantified by ethidium bromide staining of the RNA in the gel prior to transfer.

### 2.3. *Measurement of the c-Jun polypeptide levels*

Nuclear extracts were prepared as described previously [18]. The protein concentrations were determined using the BioRad protein assay kit. Western blotting was performed using a polyclonal c-Jun antibody (c-Jun/AP-1 Ab-1, Oncogene Science, Inc.) using an alkaline phosphatase system (ProtoBlot, Promega Corp.) according to the manufacturer's instructions.

### 2.4. *Gel mobility shift assay*

Electrophoretic mobility shift assay was performed as described previously [19] using synthetic oligodeoxynucleotides containing the consensus AP-1 site (TGACTCA) [20] or a mutated AP-1 site (TGCCTCA). Binding was carried out using 10 $\mu$g of nuclear extract and $^{32}$P-labelled oligodeoxynucleotides (20 fmol, ≈15,000 cpm) in 20 $\mu$l of binding buffer at room temperature for 20 min. DNA–protein complexes were resolved on a 5% polyacrylamide gel. The gels were vacuum dried and exposed to Kodak XAR film.

## 3. RESULTS

Rat L6 cells express c-*jun* mRNA and protein during myogenic differentiation in low-mitogen medium (G. Thinakaran and J. Bag, unpublished results). To test whether expression of c-*jun* in low-serum conditions is

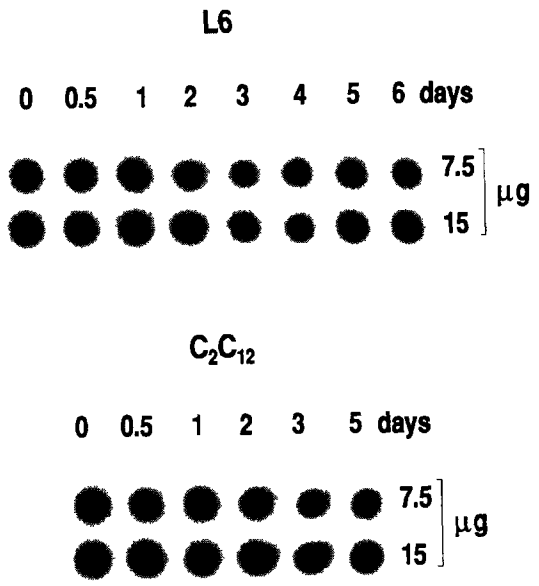


Fig. 1. Comparison of c-*jun* mRNA expression in L6 and $C_2C_{12}$ cells. Subconfluent L6 and $C_2C_{12}$ cultures were allowed to differentiate in the differentiation medium. RNA isolated from proliferating myoblasts (0) and differentiating cells maintained in the differentiation medium for the indicated time were spotted onto nylon membrane and hybridized with radiolabeled c-*jun* probe as described in section 2.

characteristic of all skeletal myoblasts, we examined the steady-state level of c-*jun* mRNA during differentiation in mouse $C_2C_{12}$ cells. As shown in Fig. 1, the level of c-*jun* mRNA expressed during proliferation and terminal differentiation was comparable in L6 and $C_2C_{12}$ cells. When this work was in progress, it was reported that constitutive expression of c-*jun* blocked myogenic differentiation of C2 myoblasts [21]. Expression of c-*jun* mRNA was abolished during myogenic differentiation of the particular population of C2 cells used in that study.

The $C_2C_{12}$ cells used in this study (obtained from the American Type Culture Collection) were derived from the original mouse C2 line established by Yaffe and Saxel [22,23]. Variations in the expression of proto-oncogenes between different subclones of a skeletal myoblast line have been reported previously [24]. Therefore, we decided to further investigate the expression of c-*jun* during myogenic differentiation of $C_2C_{12}$ cells. As shown in Fig. 2, the steady-state level of c-*jun* mRNA declined slightly during differentiation but cells maintained in the differentiation medium for 15 days still contained detectable amounts of c-*jun* mRNA. The same membrane was used to measure the expression of MyoD and myogenin mRNAs. MyoD and myogenin belong to the Helix-Loop-Helix family of myogenic factors [25]. MyoD mRNA was expressed at a low level during proliferation and differentiation of these cells (Fig. 2). Expression of myogenin was induced 12 h after

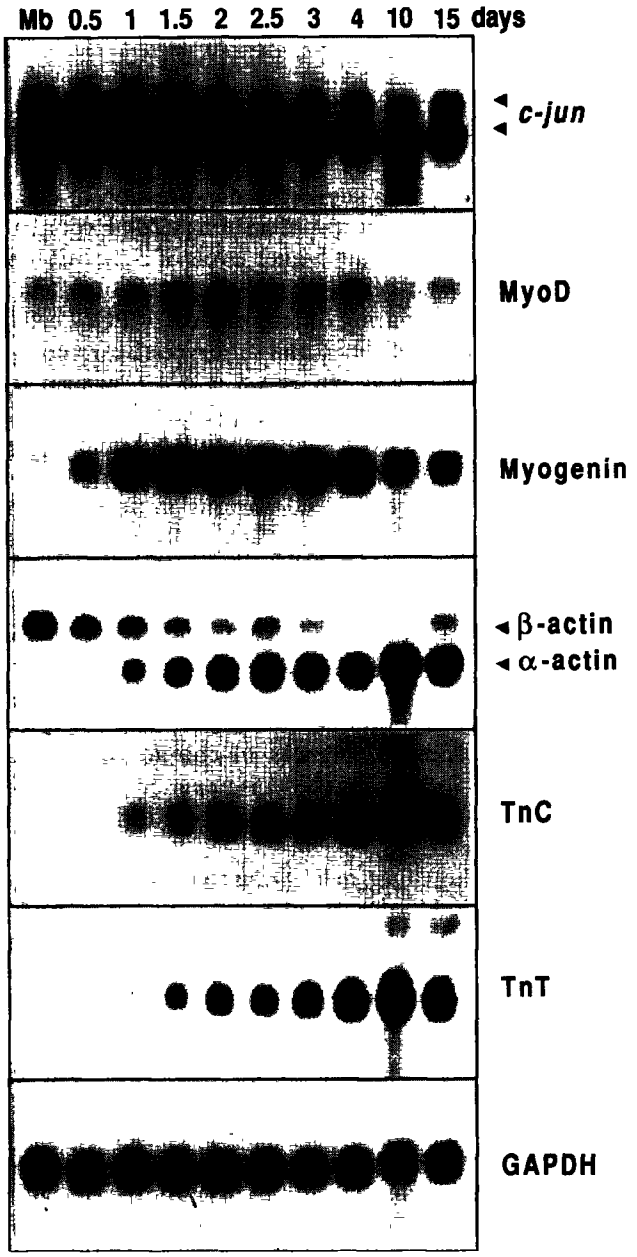


Fig. 2. Expression of c-*jun* and muscle-specific mRNAs during $C_2C_{12}$ myoblast differentiation. Subconfluent $C_2C_{12}$ cultures were allowed to differentiate in the differentiation medium. Twenty micrograms of total RNA isolated at indicated times after addition of the differentiation medium was fractionated by formaldehyde-agarose gel electrophoresis and blotted to a nylon filter. The same membrane was sequentially hybridized with various probes. The Mb lane represents RNA from proliferating $C_2C_{12}$ myoblasts.

the shift to the differentiation medium indicating that myogenic differentiation was induced in these cultures. In addition to this early differentiation marker, expression of muscle-specific genes such as actin, fast troponin C, and fast troponin T were also examined (Fig. 2). Results of these studies demonstrated that mRNAs encoding various muscle-specific proteins and c-Jun were co-expressed during differentiation. It should be noted that significant levels of muscle mRNAs were expressed within two days in differentiation medium. As judged by the presence of multinucleated myotubes, nearly 90% of the cells were differentiated within three to four days under these conditions. Therefore, these results indicate that c-*jun* mRNA is expressed in differentiated $C_2C_{12}$ muscle cells.

The c-*jun* mRNA has been reported to be inefficiently translated in HeLa cells [5]. This is probably due to the presence of a long 5′ untranslated region measuring roughly 1 kb [26]. Since c-Jun protein has a half-life of about 90 min in various cell lines [27], measurement of the steady-state level by Western blot is a good estimate of its synthesis. We have therefore performed Western blots to determine whether the c-*jun* mRNA was translated into its protein product in $C_2C_{12}$ cells. Nuclear extracts prepared from proliferating and differentiating $C_2C_{12}$ cells were reacted with a polyclonal anti-c-Jun antibody. As shown in Fig. 3, the steady-state level of c-Jun polypeptide did not decline in $C_2C_{12}$ cells maintained in the differentiation medium during the first 2 days when expression of muscle specific genes and myoblast fusion occurred. Although the level of c-Jun decreased later, it could be easily detected even after 10 days in the differentiation medium.

Transient transfection assays performed in F9 cells indicated that MyoD could repress the transcriptional activity of c-Jun [21]. Therefore, although c-Jun polypeptide was present in $C_2C_{12}$ cells during differentiation, it is possible that the AP-1 activity may be suppressed by MyoD and myogenin. To test this possibility, we performed gel retardation assays with a double-stranded oligodeoxynucleotide containing an AP-1

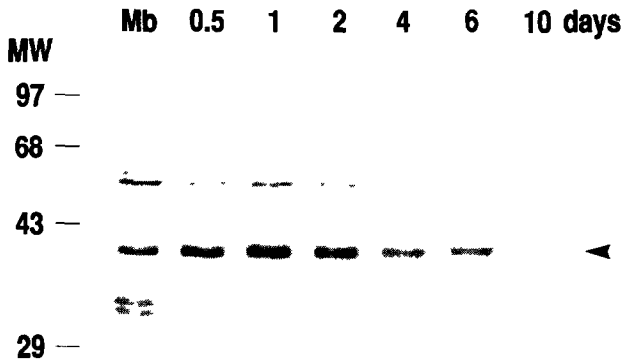


Fig. 3. Western blot analysis of c-Jun expression. Nuclear extracts (10 μg/lane) isolated from proliferating $C_2C_{12}$ myoblasts (Mb) or cells maintained in the differentiation medium for the indicated time were subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transfer to nylon membranes, c-Jun protein was detected using a polyclonal c-Jun antibody as described in section 2. The band corresponding to c-Jun is marked with an arrowhead. Position of the migration of molecular weight markers (MW, in kDa) is also marked.

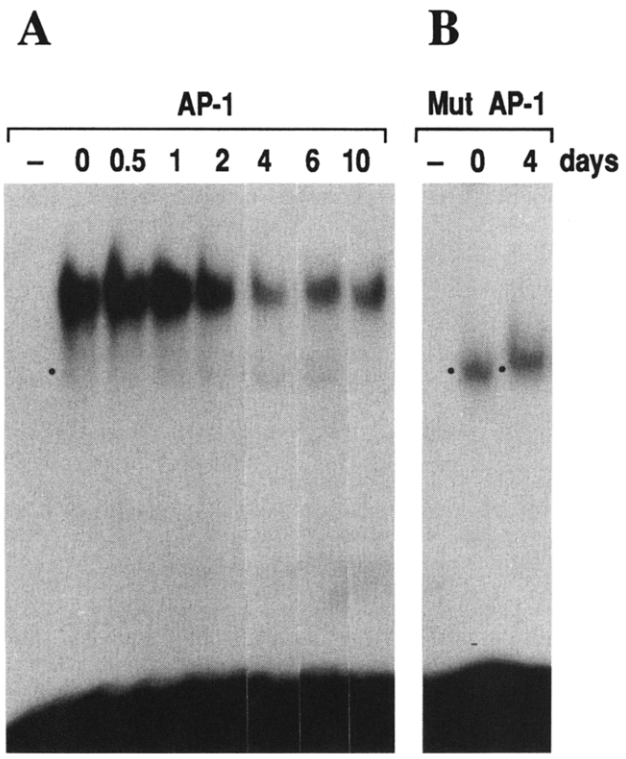


Fig. 4. Analysis of the AP-1 binding activity in differentiating $C_2C_{12}$ cells. Nuclear extracts were prepared from subconfluent myoblasts (0) and cells undergoing myogenic differentiation. A synthetic AP-1 binding site was used to assay the presence of the AP-1 binding proteins in 10 $\mu$g of each of the nuclear extracts. (B) A 1-bp change in the AP-1 binding site abolished specific complex but increased the formation of the nonspecific faster-migrating complex (marked with a dot).

binding site. As shown in Fig. 4, the AP-1 oligomer readily formed one major complex with the $C_2C_{12}$ nuclear proteins. Myoblasts and $C_2C_{12}$ cells maintained in the differentiation medium for up to 2 days contained similar amounts of AP-1 binding activity. Later this activity decreased but did not completely disappear even after 10 days. Experiments performed with an oligomer containing a 1-bp change at the AP-1 binding site (TGCCTCA) ascertained the specificity of the mobility-retarded complex (Fig. 4B). These results suggest that nuclear proteins of differentiating $C_2C_{12}$ cells expressing MyoD and myogenin could still form specific AP-1 protein complexes in vitro (compare Figs. 2, 3 and 4). However, AP-1 activity gradually decreased when the differentiated cells were maintained in the low-serum medium for several days after differentiation. Since maximal expression of myogenin and MyoD was observed within 2 days and 90% of the cells were in a multinucleated state within 3–4 days in the differentiation medium, it is not clear whether this decrease in AP-1 activity is directly related to differentiation. It is interesting to note that the AP-1 activity in these cells did not correspond to the c-Jun polypeptide levels (Figs. 3 and 4). Thus, the slow decrease in AP-1 activity may be due to post-translational regulation of c-Jun function in differentiated cells.

## 4. DISCUSSION

Several observations suggest that expression of c-*jun* is associated with cellular proliferation [1,28]. Exponentially growing cells contain higher levels of c-*jun* mRNA than serum starved cells [5]. However, the high level expression of c-*jun* during differentiation of PC12 cells, myeloid leukemia cell lines and embryonal carcinoma cells indicate that c-*jun* expression is not restricted to proliferating cells [29–32]. Results presented here show that $C_2C_{12}$ cells also expressed c-*jun* mRNA and c-Jun protein during proliferation and differentiation.

Co-expression of c-*jun* and various muscle-specific genes during differentiation of $C_2C_{12}$ cells is consistent with similar results obtained in rat L6 myoblasts (unpublished results). In contrast to these results, a recent report showed that c-*jun* mRNA was undetectable during differentiation of C2 myoblasts [21]. However, expression of c-Jun protein and the AP-1 binding activity were not measured in differentiating C2 cells. It is not clear whether some differences in culture conditions could account for the marked discrepancy in the observed steady-state level of c-*jun* mRNA during differentiation of these cells.

Our results show that, in addition to c-*jun*, the pattern of expression of MyoD was also different between C2 and $C_2C_{12}$ cells. MyoD mRNA increased 5-fold during differentiation of C2 cells [21], whereas there was no marked difference in the steady-state level of MyoD mRNA in proliferating and differentiating $C_2C_{12}$ cells used in our study (Fig. 2). This is not unusual because expression of MyoD has been previously found to be upregulated, maintained, or downregulated during terminal differentiation in different populations of C2 and $C_2C_{12}$ myoblasts [14,33,34]. In our studies, we have not seen a decrease in c-*jun* expression until the cells were fully differentiated (Figs. 2 and 3) which occurred between days two and four in differentiation medium. Following differentiation there was a gradual decline of both c-*jun* expression and AP-1 activity. It is possible that the precise mechanism and temporal order of regulating c-*jun* expression and function might vary in different cell lines. The molecular mechanism of this regulation may depend on the level and mode of regulation of a repertoire of regulatory factors during myogenesis.

It is not known whether the differences observed between C2 and $C_2C_{12}$ cells in the expression of c-*jun* and MyoD genes are related. Expression of MyoD inhibited transactivation of an AP-1-dependent reporter gene by c-Jun in F9 cells [21]. Interestingly, c-Jun also repressed transcriptional activation by MyoD and Myogenin in primary chicken muscle cells and 10T1/2 cells [21,35]. These reports concluded that the mutual repression of

Jun and MyoD depends on the ratio of these proteins present in the cells. Transcription of both c-*jun* and MyoD genes are positively autoregulated by their protein products [5,36]. Therefore, small increases in the relative amounts of either protein could result in the amplification of its own expression and suppression of the other gene. It is possible that the increased expression of MyoD during differentiation of C2 cells was responsible for abolishing c-*jun* expression by blocking the autoregulation of c-*jun* promoter by c-Jun/AP-1.

Using transient expression of c-*jun* in C2 myoblasts, it was shown that its overproduction could suppress MyoD expression and myogenic differentiation supporting the theory that an increase in the relative amount of one of these transcription factors suppresses the expression of the other gene [21]. Whether the continued expression of c-*jun* during differentiation of $C_2C_{12}$ cells is the cause or result of the lack of an increase in MyoD expression remains to be determined. Despite the continued presence of c-*jun*/AP-1, expression of myogenin and various contractile protein genes like $\alpha$-actin, fast troponin C and fast troponin T (Fig. 2), and myotube formation was normally induced in $C_2C_{12}$ cells upon mitogen withdrawal. Although c-Jun and MyoD could suppress each other's activities in transient expression assays [21], expression of c-*jun* at physiological levels does not appear to interfere with terminal differentiation of $C_2C_{12}$ cells. This is not surprising because it has been previously reported that only 4–8% of MyoD present in myoblasts is subject to negative regulation by interaction with c-Jun [21]. It is also possible that $C_2C_{12}$ cells utilize a c-*jun*/MyoD independent pathway for establishing skeletal lineage commitment and terminal differentiation.

In addition to MyoD, commitment to skeletal muscle lineage is regulated by myogenin [15,39], *myf*-5 [40], MRF-4, also called herculin and *myf*-6 [38,41,42], and possibly other genes that are not yet identified. During embryogenesis the various myogenic factors exhibit differences in the spatial and temporal pattern of expression [43,44]. Furthermore, established skeletal muscle cell lines express only subsets of the myogenic regulatory factors indicating the presence of multiple pathways for myogenic commitment and differentiation. Therefore, analysis of c-*jun* expression in different established skeletal myoblasts might lead to a better understanding of its possible role in terminal differentiation of skeletal muscle cells.

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