Differentiation Modulates the Balance of Positive and Negative Jun/AP-1 DNA Binding Activities to Regulate Cellular Proliferative Potential: Different Effects in Nontransformed and Transformed Cells

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Abstract. Differentiation of 3T3T cells into adipocytes results in the progressive repression of growth factor responsiveness. This is associated with the transcriptional repression of the inducibility of *c-jun* and *junB* expression by serum. In contrast, differentiation of SV-40 large T antigen–transformed 3T3T cells (CSV3-1) does not repress growth factor responsiveness nor *c-jun* or *junB* inducibility even though CSV3-1 cells can differentiate into adipocytes. To better explain these observations, we have studied compositional changes in AP-1 DNA binding activity attributed to c-Jun, JunB, and JunD during the differentiation process in 3T3T and CSV3-1 cells. The results show that in nontransformed 3T3T cells, differentiation represses AP-1 DNA binding activity via a proportionate downregulation of c-Jun, JunB, and JunD. In contrast, in CSV3-1 cells, AP-1 DNA binding activity increases twofold during differentiation, which is accounted for by an increase in JunD with no change in c-Jun and JunB. If c-Jun and JunB serve as positive regulators and JunD serves as a negative regulator for cell proliferation as suggested by previous studies, the repression of JunD expression in differentiating CSV3-1 cells should be mitogenic because decreasing JunD/AP-1 DNA binding activity would allow c-Jun/AP-1 and JunB/AP-1 DNA binding activities to be dominant. The results confirm this prediction showing that antisense *junD* oligodeoxyribonucleotides are mitogenic for differentiating CSV3-1 cells whereas antisense *c-jun* and *junB* inhibit mitogenesis. These data support the conclusion that differentiation can regulate cellular proliferative potential by modulating the balance of positive and negative Jun/AP-1 DNA binding activities in distinct ways in nontransformed and transformed cells.

THE processes of proliferation and differentiation are integrally controlled in that differentiation can progressively repress cellular proliferative potential in nontransformed cells. One of the best studied in vitro examples of this involves murine 3T3T mesenchymal stem cells that show a predilection to undergo the multistep process of adipocyte differentiation (Scott et al., 1982*b*; Wang et al., 1994). The first step involves arrest of proliferation at a distinct G_1 cell cycle restriction point (Scott et al., 1982*a*). At this predifferentiation growth arrest state, cells possess the potentiality to undergo adipocyte differentiation if they are maintained in differentiation-inducing medium or to reinitiate proliferation if they are exposed to appropriate growth factors. The next step is designated nonterminal differentiation where cells express an adipocyte morphology and also show a marked decrease in their growth factor responsiveness (Hoerl and Scott, 1989). Nonterminally differentiated cells do however retain their proliferative potential and can be stimulated to reenter the cell cycle when treated with very high concentrations of FBS (Hoerl and Scott, 1989). Cells at the nonterminally differentiated state can also be induced to undergo terminal differentiation wherein they irreversibly lose their proliferative potential and become unresponsive to all tested growth factors (Wier and Scott, 1986*a*).

Many neoplastically transformed cells show defects in their proliferation and differentiation control mechanisms (Wille and Scott, 1986; Estervig et al., 1990). Transformed cells typically show a markedly reduced serum requirement for growth, decreased requirement for substrate attachment, and aberrant differentiation characteristics. Some transformed cells may, however, nonterminally differentiate even though they cannot adequately repress proliferative potential to cause irreversible terminal differentiation.

To enhance our understanding of the biology that un-

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derlies the defective control of proliferation and differentiation in transformed cells, we have developed a highly useful model cell system during the past several years. This system includes the cell line designated CSV3-1, which was derived from 3T3T cells via transformation by SV-40 large T antigen (Estervig et al., 1990). Unlike other SV-40 T antigen–transformed cells, CSV3-1 and several related cell lines retain the ability to differentiate into adipocytes and can undergo predifferentiation growth arrest and nonterminal differentiation as efficiently as nontransformed 3T3T cells. However, the progressive reduction in growth factor responsiveness associated with differentiation does not occur in CSV3-1 cells and the terminal step of differentiation that is associated with the irreversible loss of proliferative potential is blocked. For example, under the conditions that induce terminal differentiation in 3T3T cells, CSV3-1 adipocytes can reinitiate DNA synthesis and dedifferentiate after treatment with serum at concentrations of as low as 5% (Estervig et al., 1990). In addition, CSV3-1 cells at the predifferentiation growth arrest and nonterminal differentiation states show an increased mitogenic responsiveness to certain atypical mitogens including vanadate and insulin, whereas 3T3T cells show no response to these agents (Wang and Scott, 1991; Wang et al., 1991).

The molecular mechanisms that mediate the progressive loss of cellular proliferative potential during adipocyte differentiation have not been fully explained, but numerous changes in gene expression are known to occur (Wier and Scott, 1986*a*, 1987; Minoo et al., 1989; Sadowski et al., 1992; Spiegelman et al., 1993; Vasseur-Cognet and Lane, 1993). In this regard, we recently demonstrated that two immediate early growth factor response protooncogenes, *c-jun* and *junB*, are critically important in mediating the control of proliferation in both nontransformed and transformed cells. When nontransformed 3T3T cells undergo nonterminal adipocyte differentiation, the reduction of growth factor responsiveness is associated with a selective repression in the inducibility of *c-jun* and *junB* by serum growth factors. The repression occurs at the transcription level and can be reversed by treatment with protein synthesis inhibitors or higher serum concentrations. This effect is specific because the serum inducibility of other immediate early response genes, such as c-*fos* and c-*myc*, is not affected by differentiation (Wang and Scott, 1994). On the other hand, the increased mitogenic responsiveness of SV-40 T antigen–transformed CSV3-1 cells to vanadate, which occurs during the differentiation process, is associated with a selective induction of *c-jun* and *junB* without inducing c-*fos* and c-*myc* (Wang et al., 1991).

Both *c-jun* and *junB* are members of the *jun* protooncogene family that also includes *junD*. The products of these genes contain a leucine zipper domain and a basic region that mediate protein–protein interactions and DNA binding (Angel and Karin, 1991). It is well established that all three Jun proteins possess indistinguishable abilities to form either homodimers among themselves or heterodimers with Fos proteins including c-Fos, FosB, Fra-1, and Fra-2 (Nakabeppu et al., 1988). Both Jun–Fos heterodimers and Jun–Jun homodimers, known as AP-1 transcription factors, bind to DNA at AP-1 sites and regulate the transcriptional activity of AP-1–dependent genes that are important in the control of cell growth and differentiation (Angel and Karin, 1991). Compared with Jun–Jun homodimers, Jun–Fos heterodimers bind DNA more tightly and are more potent regulators of transcription (Abel and Maniatis, 1989). Fos proteins, on the other hand, cannot form homodimers and thus cannot independently bind DNA (Angel and Karin, 1991). From this perspective, Jun proteins are more important than Fos proteins in regulating gene expression because they are absolutely required for AP-1 functioning.

To investigate further the molecular mechanisms by which adipocyte differentiation represses growth factor responsiveness in nontransformed cells but not in transformed cells, the AP-1 DNA binding activity mediated by c-Jun, JunB, and JunD and the levels of expression of these Jun proteins at the different biological states during the process of adipocyte differentiation were examined. The effects on cell proliferation of inhibiting the expression of individual Jun proteins using antisense oligodeoxyribonucleotides were also investigated. The results indicate that cellular proliferative potential is differently regulated by differentiation in nontransformed and transformed cells through a changing balance between positive and negative Jun/AP-1 DNA binding activities.

Materials and Methods

Cell Lines and Culture

Murine 3T3T mesenchymal stem cells and CSV3-1 cells were used in these experiments. 3T3T cells (Diamond et al., 1977) are nontransformed and can undergo all the defined steps in the adipocyte differentiation process (Scott et al., 1982*b*). CSV3-1 cells are SV-40 large T antigen–transformed 3T3T cells that can undergo predifferentiation growth arrest and nonterminal adipocyte differentiation but not terminal differentiation (Estervig et al., 1989, 1990). Growing cells were routinely cultured at 37°C in 5% CO_2/95% air in DME (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (HyClone, Logan, UT).

Induction of Predifferentiation Growth Arrest and Adipocyte Differentiation

To induce predifferentiation growth arrest at the G_1 phase cell cycle restriction point that is a prerequisite for adipocyte differentiation (Scott et al., 1982*a*, *b*), 3T3T or CSV3-1 cells were directly passaged onto tissue culture plates or onto ethylene oxide–sterilized bacteriological petri dishes at 2.5×10^3 cells/cm^2 in heparinized DME containing 25% human plasma and 10^{-5} M biotin, a medium that induces adipocyte differentiation (Wang et al., 1994). Most cells arrested their growth within 4 d in this differentiation-promoting medium before expressing the adipocyte phenotype. Growth arrest was documented by demonstration of a marked decrease in DNA synthesis as determined by [*methyl*-^{3}H]thymidine incorporation (Wang and Scott, 1991).

To induce nonterminal adipocyte differentiation, cells were passaged onto 100-mm ethylene oxide–sterilized bacteriological petri dishes at 5×10^3 cells/cm^2 in heparinized DME containing 25% CEPH. As described previously, CEPH is a defined fraction of human plasma that is deficient in aproliferin, the protein that induces the terminal loss of cellular proliferative potential during differentiation (Wier and Scott, 1986*b*; Wang et al., 1994). Adipocyte differentiation of 3T3T cells induced by CEPH has been shown to be nonterminal because such adipocytes retain the ability to undergo DNA synthesis when stimulated with 30% FBS (Scott et al., 1982*b*). In this medium, both 3T3T and CSV3-1 cells express an adipocyte phenotype at d 6–8.

Preparation of Nuclear Protein Extraction

Nuclear extracts were prepared as previously described (Ye and Samuels, 1987). Briefly, cell monolayers were rinsed twice with 4°C PBS (pH 7.4)

and were harvested in 4°C PBS with a cell scraper. After centrifugation at 500 *g* for 5 min at 4°C, cell pellets were resuspended in 4 ml of 4°C STM buffer that contains 20 mM Tris-HCl (pH 7.85), 250 mM sucrose, 1.1 mM $MgCl_2$, and 0.2% Triton X-100 and incubated on ice for 5 min. The cell pellets were then washed once with STM buffer containing 0.2% Triton X-100 and once with STM buffer not containing Triton X-100. The isolated nuclei were then resuspended in STM buffer (without Triton X-100) that contains 0.4 M KCl and 5 mM β-mercaptoethanol and incubated on ice for 10 min to extract nuclear proteins. After centrifugation at 2,000 *g* for 10 min at 4°C, the supernatant was collected, aliquoted, and frozen at −80°C before use. When needed, the protein concentrations were determined by using a protein assay kit (Bio Rad Laboratories, Richmond, VA) according to the supplier's instructions.

Electrophoretic Mobility Shift Assay and Gel Supershift Assay

The double-stranded oligonucleotides used in these experiments included 5′-CGCTTGATGACTCAGCCGGAA-3′ which contains a consensus AP-1 binding site that is underlined (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). These oligonucleotides were radioactively end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) and T4 polynucleotide kinase (Promega Corp., Madison, WI). For mobility shift assays, 0.035 pmol ^{32}P-labeled oligonucleotides (~30,000 cpm) and 10 μg nuclear protein (in 2 μl) was incubated in a total vol. of 25 μl in the presence of 2 mM Tris-HCl (pH 7.5), 8 mM NaCl, 0.2 mM EDTA, 0.2 mM β-mercaptoethanol, 0.8% glycerol, and 1 μg poly(dI-dC). The binding reactions were allowed to proceed at room temperature for 20 min. Thereafter, 2 μl of bromophenol blue (0.1% in water) was added and protein–DNA complexes were resolved by electrophoresis on nondenaturing 5% polyacrylamide gels and visualized by autoradiography.

For gel supershift assays, 1 μg (in 1 μl) of TransCruz™ supershift antibody was added to the reaction mixture and incubated for additional 30 min at room temperature. The antibodies used in these experiments included rabbit polyclonal IgG raised against either c-Jun, JunB, or JunD, and a polyclonal antibody raised against all Fos proteins including c-Fos, FosB, Fra-1, and Fra-2. All these gel supershift antibodies were purchased from Santa Cruz Biotechnology, Inc.

Western Immunoblotting Analysis

10 μg of nuclear protein used for gel mobility shift assays were mixed with SDS sample buffer, boiled for 5 min, and then subjected to electrophoresis on 10% SDS–polyacrylamide gels. Western immunoblotting procedures were performed as described (Otter et al., 1987). After the transfer of the proteins onto nitrocellulose filters, immunological evaluation on c-Jun, JunB, and JunD expression was performed by using ECL Western blotting analysis kit (Amersham Corp.) using polyclonal antibodies that had been used for gel supershift assays as described above.

Oligodeoxyribonucleotide Design, Cell Treatment, and [^{3}H]Thymidine Incorporation Assay

Phosphothionate-modified antisense and sense oligodeoxyribonucleotides corresponding to codons 1-6 of murine *c-jun*, *junB*, and *junD* were synthesized on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) at St. Jude Children's Research Hospital and purified by ethanol precipitation and multiple washes in 70% ethanol. They were lyophilized to dryness and redissolved in DME at a concentration of 2 μg/μl. The sequences of these antisense and sense oligodeoxyribonucleotides are: *c-jun* antisense: 5′-TTCCATCTTTGCAGTCAT-3′; *c-jun* sense: 5′-ATGACTGCAAAGATGGAA-3′; *junB* antisense: 5′-TTCCATTTTCGTGCACAT-3′; *junB* sense: 5′-ATGTGCACGAAAATGGAA-3′; *junD* antisense: 5′-ATAGAAGGGCGTTTCCAT-3′; *junD* sense: 5′-ATGGAAACGCCCTTCTAT-3′.

For these assays, cells were cultured in 12-well tissue culture plates for 4 d in DME containing 25% human plasma and 10^{-5} M biotin to induce predifferentiation growth arrest. Thereafter, two approaches were employed to determine if antisense oligodeoxyribonucleotides could affect cell growth: (1) Cells were cultured with either antisense or sense oligodeoxyribonucleotides in differentiation-promoting medium at the concentrations between 0 and 80 μg/ml for 24 to 30 h in the presence of 2.5 μCi/ml of [*methyl*-^{3}H]thymidine (70–85 Ci/mmol; Amersham Corp.). The ability of cells to synthesize DNA was measured by [^{3}H]thymidine incorporation as determined by scintillation counting. (2) Cells at the predifferentiation growth arrest state were rinsed twice with DME and refed with DME buffered with 15 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid. Antisense or sense oligodeoxyribonucleotides were added at appropriate concentrations. After 2 h incubation, FBS was added to reach a final concentration of 10%. Cells were further incubated for 24 to 30 h in the presence of 2.5 μCi/ml of [*methyl*-^{3}H]thymidine. Thymidine incorporation into DNA was then determined as above. For both approaches, the effects of various oligodeoxyribonucleotides on the expression of each Jun protein were analyzed by Western blotting as described above.

Results

Correlation of AP-1 DNA Binding Activity with Proliferative Potential during Differentiation

Previous studies have shown that during the adipocyte differentiation process, nontransformed 3T3T cells progressively lose their growth factor responsiveness, whereas transformed CSV3-1 cells retain their proliferative potential. CSV3-1 cells even show an increased mitogenic responsiveness to several unconventional mitogens, such as insulin and vanadate (Hoerl and Scott, 1989; Estervig et al., 1990; Wang et al., 1991; Wang and Scott, 1991, 1994, 1995). These differences in mitogenic responsiveness are summarized in Table I.

Since the reduction in growth factor responsiveness during differentiation in 3T3T cells is associated with a selective repression in *c-jun* and *junB* inducibility and since the increased mitogenic responsiveness to vanadate in CSV3-1 cells is associated with a selective induction of *c-jun* and *junB* (Wang et al., 1991; Wang and Scott, 1994), experiments were designed to investigate changes in AP-1 DNA binding activity during differentiation. Nuclear extracts were prepared from 3T3T and CSV3-1 cells at growing, predifferentiation growth arrest, and nonterminal differentiation states and electrophoretic mobility shift assays were performed using ^{32}P-labeled oligonucleotides containing a single AP-1 binding site. Fig. 1 shows that at the growing state, no significant difference in AP-1 binding activity between 3T3T and CSV3-1 cells is evident, which is consistent with previous observations that SV-40 large T antigen does not activate AP-1 (Wasylyk et al., 1988). However, when 3T3T and CSV3-1 cells were compared at the various stages of the differentiation process, marked differences were observed. As 3T3T cells underwent adipocyte differentiation, the AP-1 DNA binding activity decreased dramatically. At the predifferentiation growth arrest state AP-1 DNA binding activity decreased by ~80% and at the nonterminal differentiation state by >90%,

Table I. Comparison of Mitogenic Responsiveness of 3T3T and CSV3-1 Cells at the Predifferentiation Growth Arrest and Nonterminal Differentiation States

	3T3T cells		CSV3-1 cells	
Mitogens	PGA	NTD	PGA	NTD
FBS, 30%	+	+	+	+
FBS, 10%	+	−	+	+
Insulin, 1 μg/ml	−	−	+	+
Vanadate, 5 μM	−	−	+	ND

Cells at the predifferentiation growth arrest (PGA) or nonterminal differentiation (NTD) states were treated with either 30% FBS, 10% FBS, 1 μg/ml insulin, or 5 μM vanadate for 24–48 h and mitogenic responsiveness was assayed by [^{3}H]thymidine incorporation. +, responsive to the mitogen; −, not responsive to the mitogen.

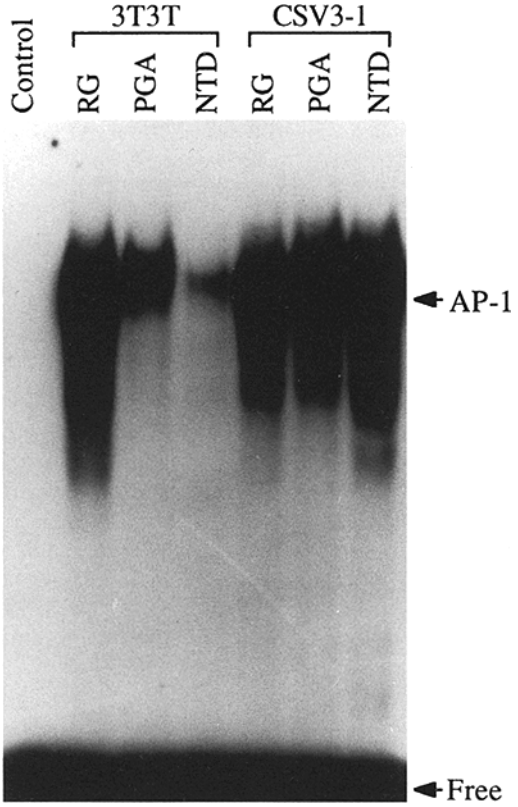

Figure 1. Comparison of the changes in AP-1 DNA binding activity of 3T3T and CSV3-1 cells during the process of adipocyte differentiation. Nuclear extracts were prepared from growing, predifferentiation growth-arrested, and differentiated 3T3T and CSV3-1 cells, and electrophoretic mobility shift assays were performed using 10 μg of nuclear protein and 0.035 pmol of ^{32}P-end labeled oligonucleotides containing a single AP-1 binding site. Positions of the specifically bound DNA–protein complex and the freely migrating probes are indicated. Control lane represents the binding reaction mixture without nuclear extracts. Four experiments were performed that showed consistent results. *RG*, rapidly growing; *PGA*, predifferentiation growth-arrested; *NTD*, nonterminally differentiated.

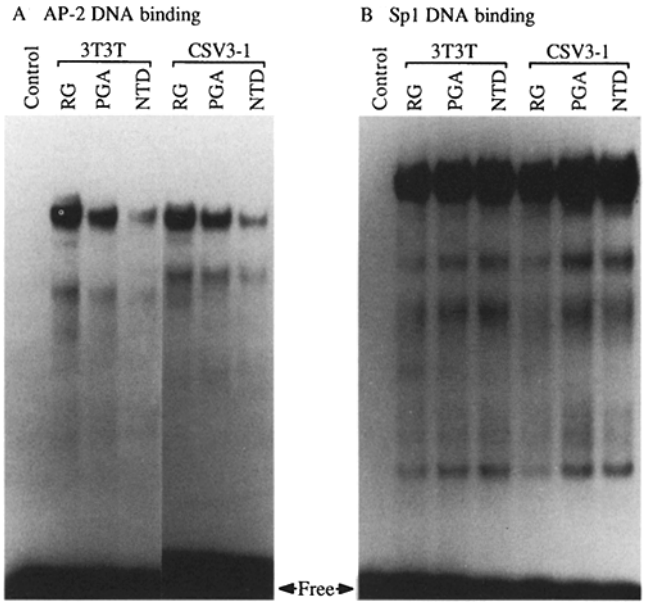

Figure 2. Comparison of the changes in AP-2 (*A*) and Sp1 (*B*) DNA binding activity between 3T3T and CSV3-1 cells during the process of adipocyte differentiation. Nuclear extracts were prepared from growing, predifferentiation growth-arrested, and differentiated 3T3T and CSV3-1 cells, and electrophoretic mobility shift assays were performed using 10 μg of nuclear protein and 0.035 pmol of ^{32}P-end labeled oligonucleotides containing either a single AP-2 binding site or a single Sp1 binding site. The position of freely migrating probes is indicated. Control lane represents the binding reaction mixture without nuclear extracts. *RG*, rapidly growing; *PGA*, predifferentiation growth-arrested; *NTD*, nonterminally differentiated.

compared to that at the growing state. In contrast, adipocyte differentiation induced AP-1 DNA binding activity in CSV3-1 cells. As shown in Fig. 1, an ~1.5-fold and an ~2-fold increase in AP-1 binding activity is observed in predifferentiation growth-arrested and differentiated CSV3-1 cells, respectively.

The differences in AP-1 DNA binding activity observed during adipocyte differentiation in 3T3T and CSV3-1 cells are specific since other transcription factors, such as AP-2 and Sp1, showed no difference between nontransformed and transformed cells (Fig. 2). Fig. 2 *A* specifically shows that during the differentiation process, AP-2 DNA binding activity decreased in both 3T3T and CSV3-1 cells, indicating that the increased AP-1 DNA binding activity in growth arrested and differentiated CSV3-1 cells is distinct. On the other hand, Sp1 DNA binding activity slightly increased during differentiation in both cell types (Fig. 2 *B*), indicating that the decreased AP-1 DNA binding activity in growth-arrested and differentiated 3T3T cells is again unique. The AP-1 DNA binding specificity was also demonstrated by competition experiments using unlabeled oligonucleotides containing either an AP-1 binding site, or a mutated AP-1 binding site, or an AP-3 binding site (data not shown).

Therefore, there is a good correlation between proliferative potential and AP-1 DNA binding activity. That is, repression of the AP-1 DNA binding activity is associated with the progressive reduction in growth factor responsiveness that occurs during 3T3T cell differentiation. Conversely, increased AP-1 DNA binding activity in differentiated CSV3-1 cells is associated with retention of proliferative potential and even an increased mitogenic responsiveness.

Characterization of AP-1 Complexes in CSV3-1 and 3T3T Cells during the Process of Adipocyte Differentiation

Since AP-1 complexes are composed of the products of the *jun* and *fos* gene families, we next investigated the compositional changes of the AP-1 complexes that occur in CSV3-1 and 3T3T cells at the various stages of adipocyte differentiation. These studies were done by performing gel supershift assays using specific antibodies. Since Jun proteins are more critical than Fos proteins in determining dimerization and DNA binding (Angel and Karin, 1991) and since our previous studies have demonstrated that c-Jun and JunB are more important than c-Fos in controlling 3T3T and CSV3-1 cell proliferation (Wang et al., 1991; Wang and Scott, 1994), our experiments have focused

primarily on Jun components in the AP-1 complexes. The results of these studies are presented in Figs. 3 and 4.

Fig. 3 demonstrates that the AP-1 complexes present in growing CSV3-1 cells are predominantly Jun–Fos heterodimers because the AP-1 DNA binding activity can be essentially completely supershifted by an anti–pan-Fos antibody that recognizes all Fos proteins. However, as CSV3-1 cells undergo adipocyte differentiation, the AP-1 complexes that cannot be supershifted by the anti–pan-Fos antibody are increased somewhat. The nature of those AP-1 complexes remains to be determined. Furthermore, Fig. 3 shows that in CSV3-1 cells the Jun proteins that compose the AP-1 complexes are in a defined ratio. The AP-1 DNA binding activity attributed to JunB, JunD, and c-Jun in growing CSV3-1 cells is 46, 21, and 8% of the total AP-1 DNA binding activity, respectively. As CSV3-1 cells undergo predifferentiation growth arrest and adipocyte differentiation, the increase in AP-1 DNA binding activity is accounted for by a significant increase in JunD/AP-1 DNA binding activity while the AP-1 DNA binding activity attributed to JunB and c-Jun remains essentially unchanged. As a result, the ratio of c-Jun and JunB to the total AP-1 DNA binding activity decreases because of the increase in JunD.

Fig. 4 presents the results of gel supershift assays using nuclear extracts prepared from growing, predifferentiation growth arrested and nonterminally differentiated 3T3T cells. Again, at the growing state, the AP-1 composition shows no significant difference than that of growing CSV3-1 cells. However, at the predifferentiation growth arrest and nonterminal differentiation states in 3T3T cells, AP-1 DNA binding activity is markedly decreased and within the residual AP-1 complexes c-Jun and JunB are essentially undetectable. JunD levels are also decreased during differentiation, but still JunD represents the major constituent of the residual AP-1 DNA binding activity. Therefore, the decrease in AP-1 DNA binding activity that occurs during the process of 3T3T cell differentiation results from a decrease in all three Jun/AP-1 complexes, but primarily by a decrease in JunB and c-Jun.

Fig. 5 quantitates and summarizes the data presented in Figs. 3 and 4. The changes in AP-1 DNA binding activity attributed to individual Jun proteins during the process of adipocyte differentiation of 3T3T and CSV3-1 cells are quantitatively illustrated using the total AP-1 DNA binding activity at the growing state as a reference. Specifically, Fig. 5 shows that adipocyte differentiation differentially modulates Jun/AP-1 DNA binding activities in 3T3T and CSV3-1 cells. In 3T3T cells (Fig. 5 *A*), the marked decrease in AP-1 DNA binding activity during differentiation is attributed to a decrease in all three Jun/AP-1 complexes, especially c-Jun/AP-1 and JunB/AP-1. In contrast, in CSV3-1 cells (Fig. 5 *B*), the increase in AP-1 DNA binding activity during differentiation is primarily contributed by an increase in JunD/AP-1 while c-Jun/AP-1 and JunB/AP-1 DNA binding activity remains essentially unchanged. These data demonstrate that in both 3T3T and CSV3-1 cells, JunD/AP-1 binding activity is highest in quiescent and differentiated states.

To determine whether the changes in AP-1 DNA binding activity attributed to individual Jun proteins that occur during 3T3T and CSV3-1 cell differentiation correlate

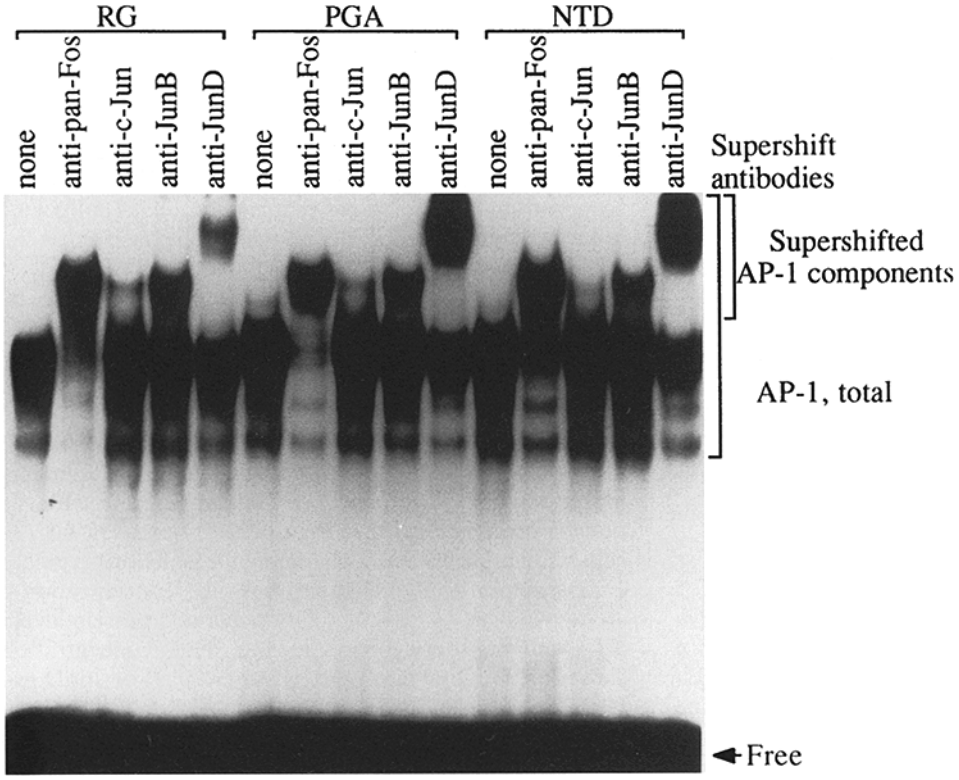

Figure 3. Analysis of the changes in AP-1 composition during CSV3-1 cell differentiation. Nuclear extracts were prepared from growing, predifferentiation growth-arrested, and differentiated CSV3-1 cells. Gel supershift assays were performed by initially incubating 10 μg of nuclear protein with 0.035 pmol of ^{32}P-end labeled oligonucleotides containing a single AP-1 binding site for 20 min at room temperature. Various antibodies (1 μg) against either c-Jun, JunB, JunD, or all Fos proteins (anti–pan-Fos) were then added to the binding reaction mixtures and the reaction was allowed to proceed for an additional 30 min at room temperature. The positions of supershifted AP-1 components, the total AP-1 DNA binding activity, and freely migrating probes are indicated. Four experiments were performed that showed similar results. *RG*, rapidly growing; *PGA*, predifferentiation growth arrested; *NTD*, nonterminally differentiated.

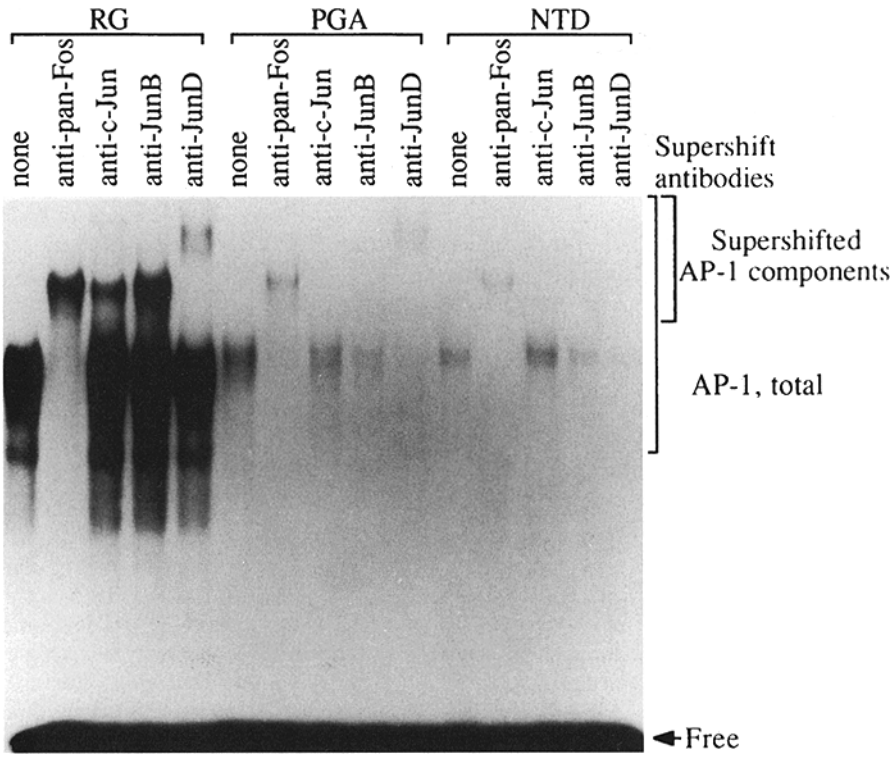

Figure 4. Analysis of the changes in AP-1 composition during 3T3T cell differentiation. Nuclear extracts were prepared from growing, predifferentiation growth-arrested, and differentiated 3T3T cells. Gel supershift assays were performed by initially incubating 10 μg of nuclear protein with 0.035 pmol of ^{32}P-end labeled oligonucleotides containing a single AP-1 binding site for 20 min at room temperature. Various antibodies (1 μg) against either c-Jun, JunB, JunD, or all Fos proteins (anti-pan-Fos) were then added to the binding reaction mixtures and the reaction was allowed to proceed for additional 30 min at room temperature. The positions of supershifted AP-1 components, the total AP-1 DNA binding activity and freely migrating probes are indicated. Two experiments were performed that showed similar results. *RG*, rapidly growing; *PGA*, predifferentiation growth-arrested; *NTD*, nonterminally differentiated.

with the changes in the level of expression of c-Jun, JunB, and JunD, Western blot analysis was performed using nuclear proteins comparable to those prepared for antibody supershift assays. The results of these studies are presented in Fig. 6 *A*. Generally, the AP-1 DNA binding activity changes associated with 3T3T and CSV3-1 cell differentiation (Figs. 3 and 4) are reflected by the changes in Jun protein expression levels (Fig. 6, *B* and *C*). However, several exceptions exist. For example, compared with growing state, the c-Jun protein level remains relatively high at the predifferentiation growth arrest state in 3T3T cells but the AP-1 DNA binding activity attributed to c-Jun is undetectable. In addition, the JunD protein level is actually increased at the predifferentiation growth arrest state even though the AP-1 DNA binding activity attributed to JunD remains low. Furthermore, in CSV3-1 cells, the JunB protein level is decreased during the differentiation process, but the AP-1 DNA binding activity attributed to JunB remains essentially unchanged. These exceptions suggest that functional regulation in Jun proteins, such as modulation in their phosphorylation status (Grover-Bardwick et al., 1994), also occurs during the process of adipocyte differentiation in addition to regulation at the expression level to regulate AP-1 DNA binding activities.

Effects of Inhibiting Jun Protein Expression on Cell Proliferation

The results presented above suggest that cell proliferative potential is modulated by the changes in the balance of different Jun/AP-1 DNA binding activities in our cell system. Experiments therefore were carried out to determine if inhibition of the expression of individual Jun proteins by antisense oligodeoxyribonucleotides could affect cell proliferation. These studies were performed by using phosphothionate-modified antisense and sense oligomers since it has been demonstrated that with this modification the oligomers remain stable in cells for up to 24 h (Brennscheidt et al., 1994). For these studies, differentiating cells at the predifferentiation growth arrest state were used.

We first tested the prediction that decreasing JunD levels in differentiating CSV3-1 cells should allow c-Jun and JunB to act as the dominant activating AP-1 factors and thereby to promote cell proliferation. We specifically treated predifferentiation growth-arrested CSV3-1 cells with antisense oligomers to *junD* for 6 to 24 h while the cells were maintained in differentiation-inducing medium. Cell proliferation was then assayed by [^{3}H]thymidine incorporation. Fig. 7 *A* shows that antisense *junD* oligomers induce a highly significant mitogenic responsiveness in a dosage-dependent manner in quiescent CSV3-1 cells in the absence of any other added growth factors. More specifically, antisense *junD* oligomers at a concentration of 5 μg/ml induce an ~10% maximum mitogenic response (maximum is defined as the mitogenic response when cells are stimulated with 10% FBS which typically induces >90% of the cells to grow). An ~60% maximum mitogenic response is achieved at a concentration of 40 μg/ml and higher dosages show no increased mitogenic effects. Fig. 7 *B* further demonstrates that treatment of quiescent CSV3-1 cells with antisense *junD* oligomers for 24 h downregulates JunD protein level significantly. At the concentration of 40 μg/ml, a >80% downregulation at JunD protein level is achieved. Sense oligomers at the same concentrations show only minimal inhibitory effect (<20%) on JunD protein expression.

To reproduceably detect repression of JunD expression by JunD antisense treatment of cells requires analysis of

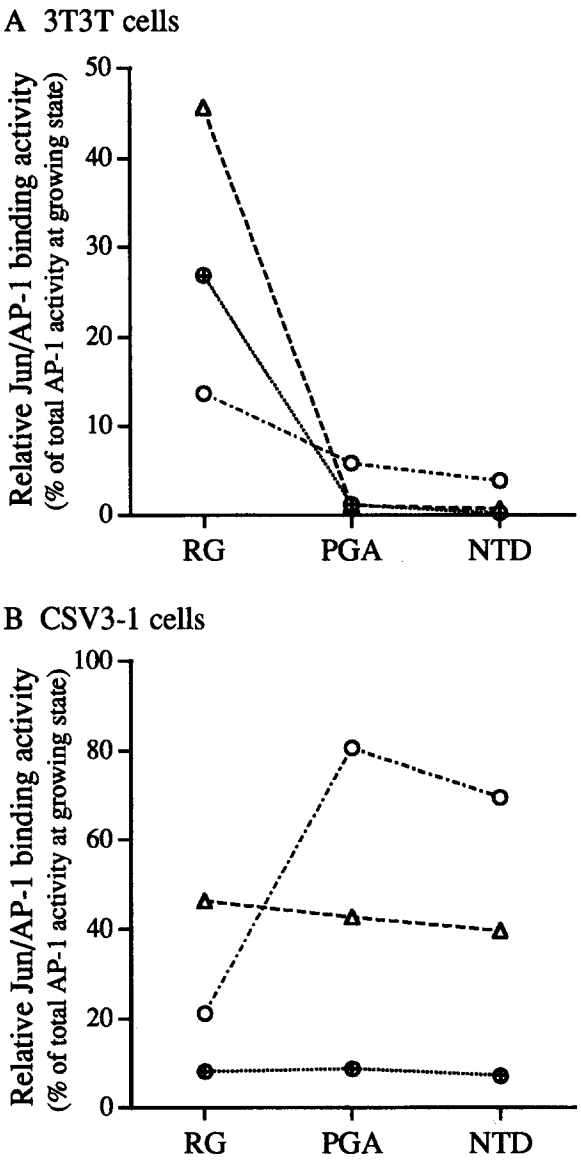

Figure 5. Comparison of the relative changes in Jun/AP-1 DNA binding activity between 3T3T (*A*) and CSV3-1(*B*) cells during the process of adipocyte differentiation. Data presented in Figs. 3 and 4 were quantitated by densitometric analysis and the relative changes in AP-1 DNA binding activity attributed to c-Jun, JunB, or JunD determined by supershift assays are reported as the percentages relative to the total AP-1 DNA binding activity of growing 3T3T or CSV3-1 cells which is represented by the first lanes of Figs. 3 and 4. *RG*, rapidly growing; *PGA*, predifferentiation growth-arrested; *NTD*, nonterminally differentiated. ···⊕···, c-Jun/AP-1; ···△···, JunB/AP-1; ···○···, JunD/AP-1.

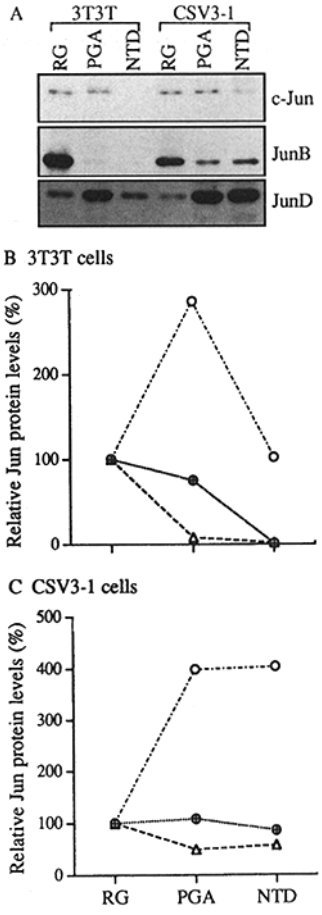

Figure 6. Western blot analysis of the Jun protein levels in the nuclear extracts comparable to those used for gel supershift assays. (*A*) 10 μg of nuclear protein prepared from growing, predifferentiation growth-arrested, and differentiated 3T3T and CSV3-1 cells was subjected to electrophoresis on 10% SDS–polyacrylamide gels and Western blotting. c-Jun, JunB, and JunD were identified by probing the nitrocellulose membranes with specific antibodies used for gel supershift assays. Two experiments were performed that showed similar results. The changes in Jun protein expression levels during 3T3T (*B*) and CSV3-1 (*C*) cell differentiation were quantitated by densitometric analysis and are reported as the percentages relative to those of rapidly growing cells where the c-Jun, JunB, and JunD protein levels are defined as 100%. *RG*, rapidly growing; *PGA*, predifferentiation growth-arrested; *NTD*, nonterminally differentiated. ···⊕···, c-Jun; ···△···, JunB; ···○···, JunD.

cells treated for different time intervals between 12 to 24 h, because of the apparent variable concentration of nucleases in various lots of human plasma and serum.

Since differentiating 3T3T cells contain essentially no c-Jun/AP-1 and JunB/AP-1 DNA binding activities, we predicted that decreasing JunD/AP-1 should have no effect on these cells. To test this, antisense *junD* oligomers were added to differentiating 3T3T cells as above and no mitogenic effect could be detected (data not shown). Furthermore, sense *junD*, antisense and sense *c-jun*, and antisense and sense *junB* oligomers were not mitogenic in either quiescent differentiating CSV3-1 or 3T3T cells (data not shown).

We next examined the effects of antisense *c-jun*, *junB*, and *junD* oligomers on the induction of DNA synthesis when predifferentiation growth-arrested cells were stimulated with 10% FBS. These experiments were specifically performed to determine which of the Juns are required for the induction of DNA synthesis. Differentiating predifferentiation growth-arrested cells were pretreated with oligomers for 2 h and then exposed to 10% FBS for an additional 24 to 30 h in the presence of the oligomers. DNA synthesis was assayed by [^{3}H]thymidine incorporation.

Fig. 8 *A* shows that antisense oligomers to *c-jun* at a concentration of 5 μg/ml significantly blocked the FBS-induced DNA synthesis in predifferentiation growth-arrested CSV3-1 cells (~10% inhibition). At the concentration of 40 μg/ml, ~75% inhibition was observed. Higher concentrations showed no increased inhibitory effect. Sense *c-jun* oligomers at the same concentrations however showed no significant inhibitory effects. Fig. 8 *B* further shows that antisense *c-jun* oligomers inhibited FBS-induced c-Jun expression, whereas sense oligomers did not. When quiescent CSV3-1 cells were similarly treated with antisense *junB* oligomers, similar results were obtained. Fig. 9 *A* specifically shows that at a concentration of 5 μg/ml, antisense *junB* oligomers inhibited FBS-induced DNA synthesis by ~20%. The maximum inhibition was achieved at the concentra-

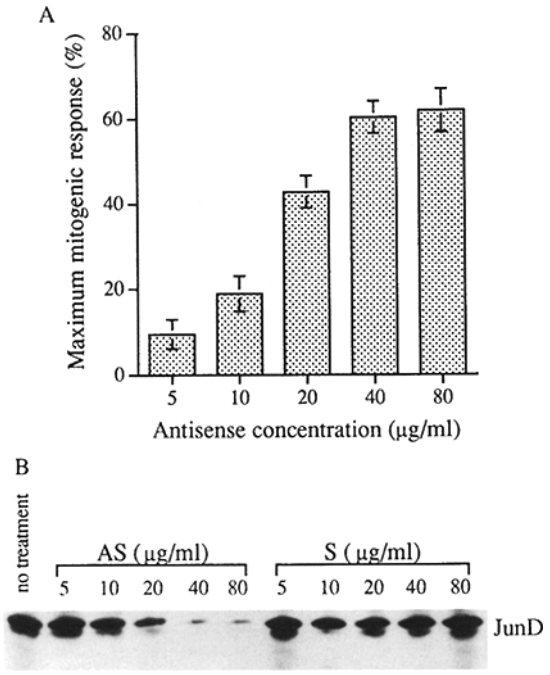

Figure 7. Effects of antisense *junD* oligodeoxyribonucleotides on the induction of DNA synthesis in CSV3-1 cells and the effects of antisense and sense *junD* oligomers on the expression of JunD. (*A*) Predifferentiation growth-arrested CSV3-1 cells were exposed to antisense *junD* oligomers at the indicated concentrations for 24 to 30 h without the addition of serum or any other growth factors. DNA synthesis was assayed by [^{3}H]thymidine incorporation and is reported as the percentage of maximum mitogenic response where 100% maximum is defined as the mitogenic effect of 10% FBS. Data are expressed as the mean ± SD of three separate experiments (each in triplicate) minus background. (*B*) Predifferentiation growth-arrested CSV3-1 cells were treated with antisense (*AS*) or sense (*S*) *junD* oligomers at the indicated concentrations for 6 to 24 h. In each experiment the optimum treatment time to observe the effect of JunD antisense had to be determined due to the apparent different stability of the oligonucleotide in various lots of human plasma and serum. 100 μg of cellular protein was subjected to electrophoresis on 10% SDS–polyacrylamide gel and Western blot analysis. JunD (~40 kD) is identified by probing with a specific antibody described in Materials and Methods.

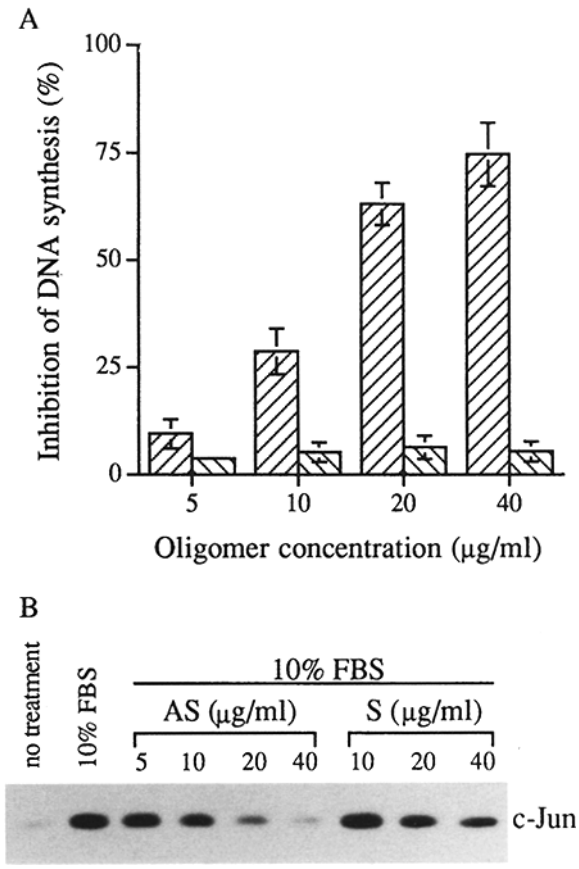

Figure 8. Effects of antisense and sense *c-jun* oligodeoxyribonucleotides on the induction of DNA synthesis and on the induction of c-Jun expression by serum. (*A*) Predifferentiation growth-arrested CSV3-1 cells were pretreated with antisense or sense *c-jun* oligomers at the indicated concentrations for 2 h followed by exposure to 10% FBS for an additional 24 to 30 h in the presence of the oligomers. DNA synthesis was measured by [^{3}H]thymidine incorporation and is reported as the percentage of inhibition where zero percent inhibition represents the mitogenic effect of 10% FBS in the absence of antisense or sense oligomers. Data are expressed as the mean ± SD of three separate experiments (each in triplicate) minus background. (*B*) Predifferentiation growth-arrested CSV3-1 cells were treated with antisense (*AS*) or sense (*S*) *c-jun* oligomers as described above at the indicated concentrations. 100 μg of cellular protein was subjected to electrophoresis on 10% SDS–polyacrylamide gel and Western blot analysis. c-Jun (~40 kD) is identified by probing with a specific antibody described in Materials and Methods. ▨, antisense; ▧, sense.

tion of 40 μg/ml which blocked DNA synthesis by ~80%. Sense oligomers showed no significant inhibitory effects. Again, Fig. 9 *B* demonstrates that antisense *junB* oligomers inhibited FBS-induced JunB expression, whereas sense oligomers showed no significant effects. In addition, antisense (but not sense) *c-jun* and *junB* oligomers showed similar inhibitory effects on FBS-induced DNA synthesis in predifferentiation growth-arrested 3T3T cells (data not shown). In contrast, neither antisense nor sense *junD* oligomers inhibited FBS-induced DNA synthesis in differentiating CSV3-1 and 3T3T cells (data not shown).

Discussion

Regulation of the AP-1 transcription factors has been proposed to be critically important in the control of cell proliferation and neoplastic transformation (Angel and Karin, 1991). Numerous studies have demonstrated that *c-jun* and *junB* function as immediate early growth factor response genes and activation of these two genes (especially *c-jun*) is required for cell cycle progression (Ryder et al., 1988; Ryder and Nathans, 1988; Ryseck et al., 1988; Herschman, 1991). It was also reported that cells overexpressing *c-jun* showed a decreased serum requirement for their growth (Castellazzi et al., 1991). In Friend erythroleukemia cells, inhibition of *c-jun* expression by antisense RNA or oligomers caused reversible proliferative arrest and withdrawal from the cell cycle (Smith and Prochownik, 1992) and promoted dimethyl sulfoxide–induced erythroid differentiation (Francastel et al., 1994). Overexpression of

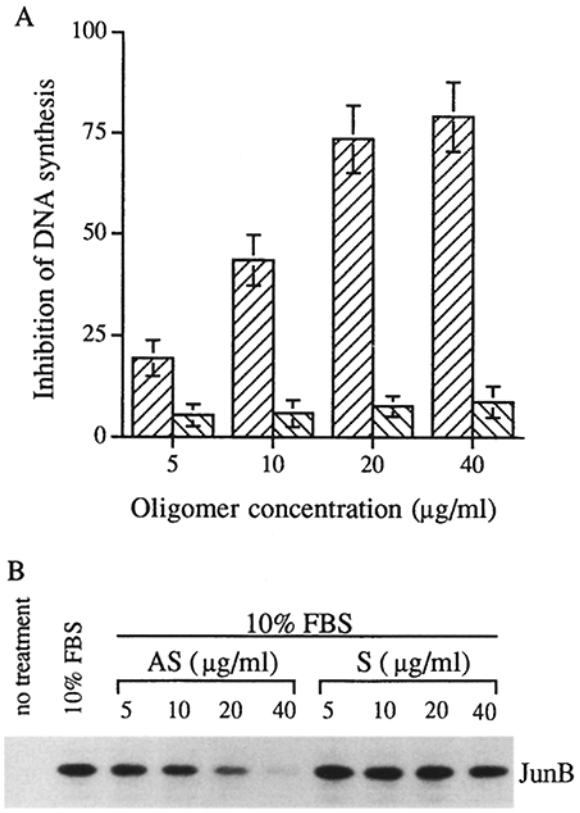

Figure 9. Effects of antisense and sense *junB* oligodeoxyribonucleotides on the induction of DNA synthesis and on the induction of JunB expression by serum. (*A*) Predifferentiation growth-arrested CSV3-1 cells were pretreated with antisense or sense *junB* oligomers at the indicated concentrations for 2 h followed by exposure to 10% FBS for an additional 24 to 30 h in the presence of the oligomers. DNA synthesis was measured by [^{3}H]thymidine incorporation and is reported as the percentage of inhibition where zero percent inhibition represents the mitogenic effect of 10% FBS in the absence of antisense or sense oligomers. Data are expressed as the mean ± SD of three separate experiments (each in triplicate) minus background. (*B*) Predifferentiation growth-arrested CSV3-1 cells were treated with antisense (*AS*) or sense (*S*) *junB* oligomers as described above at the indicated concentrations. 100 μg of cellular protein was subjected to electrophoresis on 10% SDS–polyacrylamide gel and Western blot analysis. JunB (~40 kD) is identified by probing with a specific antibody described in Materials and Methods. ▨, antisense; ▧, sense.

c-jun in these cells, on the other hand, resulted in a decreased ability to undergo differentiation (Francastel et al., 1994). The mitogenic response to tumor necrosis factor–α in human fibroblasts also requires c-Jun since these cells fail to proliferate if cultured in the presence of antisense *c-jun* oligodeoxyribonucleotides (Brach et al., 1993). In addition, the AP-1 DNA binding activity is decreased substantially during the aging process of human fibroblasts (Riabowol et al., 1992) and selective inhibition of AP-1 DNA activity with retinoids effectively inhibits the proliferation of several tumor cell lines (Fanjul et al., 1994). In murine 3T3T cells, the decreased growth factor responsiveness is specifically associated with a decreased serum inducibility of *c-jun* and *junB* (Wang and Scott, 1994).

Numerous viral oncogenes and carcinogenic agents have been shown to activate AP-1 DNA binding activity. Examples include v-*src*, v-*mos*, polyoma middle T antigen, Ha-*ras*, *neu*, v-*raf*, v-*yes*, v-*fps*, dimethylbenzanthracene, 4-nitroquinolineoxide, ionizing radiation, ultraviolet light, and asbestos (Sherman et al., 1990; Angel and Karin, 1991; Heintz et al., 1993; Suzuki et al., 1994). Among these, ionizing radiation and asbestos were reported to specifically induce *c-jun* expression (Sherman et al., 1990; Heintz et al., 1993). In fact, overexpression of *c-jun* alone will lead to cell transformation (Castellazzi et al., 1991; Hughes et al., 1992; Watts et al., 1995). Further evidence indicates that the transformation induced by *c-jun* overexpression is sufficiently mediated by Jun homodimers since heterodimerization with Fos proteins is not required (Hughes et al., 1992). In JB6 mouse epidermal cells, progressive elevation of AP-1 DNA binding activity, which was primarily attributed to an increase in *c-jun* expression, was demonstrated during the process of preneoplastic-to-neoplastic progression (Dong et al., 1995*b*). Inhibition of AP-1 DNA binding activity by AP-1 inhibitors such as retinoic acid, fluocinolone acetonide, or forskolin, or by transfection of a dominant negative *c-jun* mutant encoding a transcriptionally inactive product reversed the transformation phenotype or blocked transformation induced by tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate or EGF (Dong et al., 1994, 1995*a*; Li et al., 1996). Using spontaneously reverting cell lines, it was also found that *c-jun* was overexpressed in highly tumorigenic clones and the reversion to a nontumorigenic phenotype resulted in a dramatic decrease in *c-jun* expression (Lavrovsky et al., 1994). Compared with *c-jun*, *junB* appears to be less potent in transforming cells (Castellazzi et al., 1991). However, elevated expression of *junB* seems essential for v-*fos*–induced transformation of Rat-1 cells (Van Amsterdam et al., 1994).

Unlike *c-jun* and *junB*, *junD* is usually constitutively expressed and is not inducible by growth factors (Hirai et al., 1989; Ryder et al., 1989). Overexpression of *junD* does not promote cell proliferation nor transformation (Castellazzi et al., 1991). Therefore, JunD appears to have different functions than c-Jun and JunB in regulating cell growth. Recently, it was reported that overexpression of *junD* actually slowed cell growth, which resulted in an increase in the percentage of cells in G_0/G_1 phase of the cell cycle, and partially antagonized *ras*-induced transformation (Pfarr et al., 1994). Thus, JunD may have a function opposed to c-Jun and JunB in regulating cell proliferation, that is, JunD promotes quiescence. Pfarr et al. (1994), in fact, correlated high JunD and low c-Jun levels with growth arrest and low JunD and high c-Jun levels with proliferation. A high ratio of c-Jun to JunD also correlated with the severity of the transformed phenotype (Pfarr et al., 1994). Similar changes have also been observed in papillomavirus-transformed mouse cell lines in which an increase in the c-Jun and JunB to JunD ratio became evident as cells progressed from fibromatosis to fibrosarcoma (Bossy-Wetzel et al., 1992). Therefore, Jun/AP-1 transcription factors can be dissected into two parts: c-Jun/AP-1 and JunB/AP-1 positively regulate cell growth, and JunD/AP-1 negatively regulates cell growth. It has however not been determined

whether the changes in the balance between the positive and negative Jun/AP-1 transcription factors modulate cellular proliferative potential.

To investigate if adipocyte differentiation modulates the balance of positive and negative Jun/AP-1 to regulate proliferative potential, we have used the well-characterized 3T3T and CSV3-1 cell systems. We demonstrate that in SV-40 T antigen–transformed CSV3-1 cells, total AP-1 DNA binding activity increases 1.5- to 2-fold at the predifferentiation growth arrest and nonterminal differentiation states, respectively. This increase is primarily attributed to an increase in the expression of JunD/AP-1. The c-Jun/AP-1 and JunB/AP-1 binding activities in contrast remain constant and relatively high in cells at these states. In contrast, in nontransformed 3T3T cells, total AP-1 DNA binding activity decreases progressively during the differentiation process. The residual AP-1 DNA binding activity that exists in differentiated cells is primarily contributed by JunD even though JunD/AP-1 DNA binding activity is also repressed markedly in these cells. The c-Jun/AP-1 and JunB/AP-1 levels are essentially undetectable in differentiated 3T3T cells since their expression is transcriptionally repressed (Wang and Scott, 1994).

These observations support the hypothesis that the reason CSV3-1 cells do not lose their growth factor responsiveness even though they are fully differentiated is because they retain relatively high levels of c-Jun/AP-1 and JunB/AP-1. Furthermore, the hypothesis suggests that the fact that these cells have a JunD/AP-1 DNA binding activity higher than c-Jun/AP-1 and JunB/AP-1 DNA binding activities explains why they can growth arrest and differentiate. On the other hand, 3T3T cells show a reduced growth factor responsiveness once they are differentiated because their c-Jun/AP-1 and JunB/AP-1 DNA binding activities are so low and because of that they require much less JunD/AP-1 to antagonize c-Jun and JunB to maintain a differentiated state. This hypothesis predicted that mitogenesis should result in quiescent CSV3-1 cells if the level of JunD/AP-1 was reduced so that c-Jun/AP-1 and JunB/AP-1 could function maximally to promote proliferation. The results indeed show that treatment of differentiating CSV3-1 cells with antisense *junD* oligomers effectively promote mitogenesis, which is not observed in differentiating 3T3T cells. This is so because in CSV3-1 cells, repression of JunD expression changes the balance between c-Jun and JunB versus JunD. Since c-Jun/AP-1 and JunB/AP-1 are already present in CSV3-1 cells at a relatively high level, they do not need to be further induced to drive cells into the cell cycle. In 3T3T cells, however, repression of JunD expression itself is not sufficient to enhance cell proliferation because c-Jun/AP-1 and JunB/AP-1 levels are too low and would have to be induced for proliferation to occur. This hypothesis is further supported by findings that antisense *c-jun* and antisense *junB* oligomers both effectively block serum-induced DNA synthesis in CSV3-1 and 3T3T cells, indicating that both c-Jun and JunB are indeed functioning as positive regulators for proliferation in our cell systems. These data together strongly suggest that the balance between positive and negative Jun/AP-1 transcription factors is critical to regulating a cell's proliferative potential during differentiation. To the best of our knowledge, this is the first report showing that differential modulation of the ratio of c-Jun/AP-1 and JunB/AP-1 to JunD/AP-1 by a biological process such as differentiation in transformed and nontransformed cells can determine whether cells retain or lose their growth factor responsiveness.

The results presented here raise a series of interesting questions for future studies. The first question concerns why adipocyte differentiation selectively represses *c-jun* and *junB* inducibility by serum in nontransformed 3T3T cells but not in transformed CSV3-1 cells. Since *c-jun* and *junB* repression in 3T3T adipocytes occurs at the transcription level (Wang and Scott, 1994) and since differentiation does not induce significant changes in the expression of growth factor receptors and associated signaling pathways (Wang, H., Z. Xie, and R.E. Scott, manuscript in preparation), we hypothesize that differentiation may preferentially activate specific negative regulators for *c-jun* and *junB* transcription in nontransformed cells and that transformation blocks this effect of differentiation. In this regard, it is important to emphasize that the data presented in this paper do not show a significant difference in the ratio of c-Jun/AP-1 and JunB/AP-1 to JunD/AP-1 between growing undifferentiated transformed CSV3-1 and nontransformed 3T3T cells, even though data from other cell systems have shown some differences (Bossy-Wetzel et al., 1992; Pfarr et al., 1994). One explanation is that SV-40 T antigen–induced transformation does not require AP-1 participation (Wasylyk et al., 1988). On the other hand, transformation induced by agents such as *ras* or papillomavirus (Bossy-Wetzel et al., 1992; Pfarr et al., 1994) may use an AP-1–dependent pathway (Angel and Karin, 1991; Suzuki et al., 1994). Therefore, the changing balance between positive and negative Jun/AP-1 DNA binding activities observed in differentiating CSV3-1 cells are probably the result of a combination of effects of cellular transformation and differentiation rather than being directly caused by the expression of SV-40 T antigen in those cells. In fact, it has been demonstrated that T antigen expression is transcriptionally repressed once CSV3-1 cells differentiate into adipocytes (Estervig et al., 1989; Scott et al., 1989). Future studies will use other DNA tumor virus transforming proteins, such as adenovirus E1A or human papillomavirus E7, that have similar functions as SV-40 large T antigen, to investigate whether adipocyte differentiation induces similar compositional changes in Jun/AP-1 transcription factors in E1A- or E7-transformed cells as in CSV3-1 cells.

The second question concerns the molecular mechanisms by which *junD* is overexpressed in differentiating CSV3-1 cells. It is not clear at present whether the increased JunD protein level that leads to increased JunD/AP-1 DNA binding activity is due to an increased transcription rate, stabilization of the mRNA, an increased translation rate, or a decreased JunD degradation. Although the *junD* mRNA level shows no significant changes after short-term serum stimulation in quiescent NIH 3T3 cells (Hirai et al., 1989), the possibility that growing and differentiated CSV3-1 cells may show a difference at *junD* mRNA level deserves further investigation. It has been reported that the rate of incorporation of labeled amino acids into JunD protein in quiescent cells is much lower than in growing cells (Pfarr et al., 1994), suggesting that an increased protein stability may be also involved.

The third question concerns the interrelationship between c-Jun, JunB, and JunD AP-1 DNA activities. At growth-arrested and differentiated states, elevated JunD/AP-1 binding activity can function to prevent cells from entering the cell cycle. However, the exact mechanisms concerning how JunD/AP-1 functions in this regard is not known. Since all AP-1 complexes in quiescent and differentiated CSV3-1 and 3T3T cells are predominantly Fos–Jun heterodimers, the possibility that JunD may dimerize with c-Jun or JunB to suppress their functions is low. Other possibilities include that JunD–Fos might compete with c-Jun–Fos or JunB–Fos for DNA binding or that JunD–Fos may regulate genes that are primarily involved in negatively controlling cell proliferation, whereas c-Jun–Fos and JunB–Fos may regulate genes that facilitate proliferation. How c-Jun and JunB cooperate in positively controlling cell proliferation is also an important question. Although JunB is more abundant than c-Jun in CSV3-1 cells, the antisense data show that both are equally important for cell growth, suggesting that c-Jun and JunB may regulate different sets of growth-related genes.

The fourth question concerns the possibility that in differentiating CSV3-1 cells the AP-1 complexes may recruit new protein(s) which modulates the AP-1 activity. This possibility is supported by observations that a small portion of the AP-1 complexes presented in differentiating CSV3-1 cells cannot be supershifted by antibodies against either pan-Fos or three Jun proteins, whereas the AP-1 complexes in growing CSV3-1 cells can be supershifted completely by anti–pan-Fos antibodies. Understanding the nature of this portion of the AP-1 complexes may help to explain further the molecular mechanisms by which differentiating CSV3-1 cells show an increased growth factor responsiveness. The possibility that adipocyte differentiation may modulate the phosphorylation status of Jun proteins is also worth further investigation since the DNA binding affinity and the transactivation activity of AP-1 can be altered significantly by changes in Jun protein phosphorylation (Boyle et al., 1991).

The final question that needs to be studied is whether adipocyte differentiation differentially regulates the expression and activity of various Fos proteins in 3T3T and CSV3-1 cells. Although our previous studies have demonstrated that the differentiation process does not affect c-*fos* expression in 3T3T cells (Wang and Scott, 1994), the expression and/or function of other *fos* genes, such as *fosB*, *fra-1*, and *fra-2*, may be affected. The possibility exists that different Fos proteins may preferentially dimerize with different Jun proteins at different biological states. These possible changes in Fos proteins during differentiation, together with the changes in Jun proteins, which lead to different combinations of Fos–Jun in differentiated cells, may be more effective in influencing a cell's proliferative potential.

In summary, the current studies have clearly established that differentiation can modulate a cell's proliferative potential by changing the balance of positive and negative Jun/AP-1 in different ways in nontransformed and transformed cells.

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