Inhibition of c-jun Causes Reversible Proliferative Arrest and Withdrawal From the Cell Cycle

By Michael J. Smith and Edward V. Prochownik

We studied the effect of c-jun depletion in Friend murine erythroleukemia (F-MEL) cells stably transfected with a plasmid that allowed for the glucocorticoid-mediated conditional expression of c-jun antisense sequences. The c-jun cDNA used for the construction of the vector was modified so as to prevent the nonspecific targeting of junB and junD transcripts. High level and rapid induction of c-jun antisense transcripts was achieved with as little as 10⁻¹⁷ mol/L dexamethasone (DEX) and resulted in a 80% to 90% reduction in c-jun protein levels. The continuous exposure of the transfected cells to DEX inhibited growth by greater than 85% over a 5-day period, whereas DEX had no effect on the growth rate of control F-MEL cells. This proliferative block was associated with a reversible accumulation of cells with a 2n DNA content. When these cells were recultured in the absence of DEX, c-jun protein rapidly reappeared and the immediate early response genes egr-1, junB, and c-myc were transiently expressed. Thus, inhibition of c-jun protein causes logarithmically growing cells to leave the cell cycle and to enter a state closely resembling, if not identical to, G₀. These results underscore the importance of c-jun in maintaining cellular proliferation and provide additional evidence for the participation of proto-oncogenes in cell cycle control.

© 1992 by The American Society of Hematology.

PROTO-ONCOGENES whose encoded proteins are localized to the nucleus have recently been shown to be involved in many aspects of cellular differentiation. For example, numerous reports have shown that the chemically induced differentiation of Friend murine erythroleukemia (F-MEL), F9 teratocarcinoma, and 3T3/L1 preadipocyte cell lines can be prevented by the deregulated expression of c-myc. Conversely, c-myc antisense transcripts or synthetic antisense oligonucleotides can accelerate or induce differentiation of F-MEL and HL-60 cells, respectively. In vivo studies have shown that mice expressing Ig promoter–c-myc chimeric transgenes in their lymphoid tissues possess an expanded pool of non-neoplastic early pre-B cells with high proliferative capacity and an apparent block to differentiation. Overexpression of the c-myb nuclear proto-oncogene also leads to inhibition of F-MEL differentiation, although apparently through c-myc–independent pathways. Mice rendered homozygous for a c-myb null mutation, achieved through homologous recombination in embryonal stem cells, show a severe impairment of adult-type erythropoiesis.

In addition to their effects on development and differentiation, proto-oncogenes are also thought to play important roles in cellular proliferation. In part, this stems from the observation that the nuclear proto-oncogenes c-myc, c-fos, and c-jun are a subset of the so-called “immediate early response” repertoire of genes expressed within minutes after a mitogenic stimulus of quiescent cells. Evidence for a role of these genes in proliferation has come from experiments in which some of these proto-oncogene products were selectively inhibited. Thus, the inhibition of c-fos expression blocks both the G₁-G₀ transition as well as the proliferation of logarithmically growing cells. Antisense c-myb oligonucleotides have been shown to impair proliferation and antisense c-myc oligonucleotides inhibit proliferation by preventing progression past the G₁-S boundary. c-jun is a nuclear phosphoprotein that encodes a major component of the AP-1 transcription complex that includes the c-fos protein. In vitro studies have shown that c-jun and c-fos can form heterodimers and that c-jun can form homodimers. DNA binding of dimers occurs through a consensus sequence known as the AP-1 recognition site or TPA-response element (TRE), although the binding of heterodimers is 25-fold more efficient than that of homodimers. c-jun is closely related to at least two other genes, jun-B and jun-D, all of whose products can dimerize with one another or with c-fos. This combinatorial variety, coupled with distinct differences in the modes of expression and control of the jun and fos families, provides great potential for transcriptional diversity and versatility.

To explore the possible relationship between c-jun expression and cell cycle events, we have expressed inducible c-jun–specific antisense transcripts in F-MEL cells. We chose this cell line for three reasons: (1) F-MEL cells express low levels of endogenous c-jun transcripts, thus maximizing the likelihood of effective antisense inhibition; (2) plasmids containing dihydrofolate reductase transcription units can be amplified to high levels in F-MEL cells, allowing for antisense expression to be maximized even further; and (3) c-jun, like c-myc and c-myb, appears to be important for the process of F-MEL differentiation. In this report, we provide evidence that the selective inhibition of c-jun causes exit from the cell cycle and the reversible entry into a state indistinguishable from G₀. c-jun thus appears to be an integral component of a mechanism that actively operates to maintain cells in a proliferative state.

MATERIALS AND METHODS

Construction of a glucocorticoid-inducible murine c-jun antisense vector. The pMSG plasmid was obtained from Pharmacia (Piscataway, NJ). The pMSG vector contains the pMSG packaging signal, fused to the human IgG leader sequence and the chicken β-globin polyadenylation signal, upstream of the hCMV immediate early promoter, which drives transcription of the downstream c-jun antisense sequence.

From the Division of Hematology/Oncology and the Committee on Cellular and Molecular Biology, University of Michigan School of Medicine, Ann Arbor, MI.


Supported by Grant No. ROI HL33741 from The National Institutes of Health. E.V.P. is an Established Investigator of the American Heart Association.

Address reprint requests to Edward V. Prochownik, MD, PhD, Section of Hematology/Oncology, Department of Pediatrics, MSRB I, Room A514, Box 0624, University of Michigan School of Medicine, Ann Arbor, MI 48109.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

0006-4971/92/7908-0025$3.00/0
away, NJ). It contains a glucocorticoid-inducible murine mammary tumor virus (MMTV) promoter upstream of a polylinker cloning site (Fig 1). Downstream of the polylinker site is a SV40 early splice and poly(A) region. An SV40-gpt transcription unit in the original plasmid was excised with BamHI. A 2.8-kb transcription unit for a mutant murine dihydrofolate reductase (dhfr) was then cloned into the unique EcoRI site of the resultant plasmid to give the plasmid pMS-Gdhfr. A 2.6-kb murine c-jun cDNA in pUC19 was a kind gift from Dr Rodrigo Bravo (Bristol-Myers Squibb Institute for Pharmaceutical Research, Princeton, NJ). c-jun protein coding sequences were removed by digestions with BstHII and Hpa I followed by repair with the Klenow fragment of Escherichia coli DNA polymerase I (Bethesda Research Labs, Gaithersburg, MD) and self-ligation. The resultant construct contained 287 bp of c-jun 5' untranslated sequence, 929 bp of 3' untranslated sequence, and 48 bp of coding sequence and bore no homology to junB or junD cDNAs as determined by computer-assisted alignment of published DNA sequences. c-jun cDNA fragment was excised from the plasmid with EcoRI, blunt-ended with the Klenow fragment, and blunt-end ligated into the unique Xho I site in the pMS-Gdhfr polylinker. An antisense construct (pMS-G c-jun AS dhfr) was identified by restriction mapping (Fig 1).

Cell culture and transfections. F-MEL cells were cultured in Dulbecco's modified Eagle's minimal essential medium (MEM) containing 10% supplemented calf serum (Hyclone, Logan, UT), 200 mmol/L glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin. For transfections, 2 x 10^6 cells were pelleted at 500 x g for 10 minutes, washed twice in phosphate-buffered saline (PBS), and resuspended in 0.5 mL of PBS. Forty micrograms of BamHI-linearized pMS-G c-jun (AS) dhfr DNA plus 4 μg of Nde I-linearized pSVneo plasmid DNA in a total volume of 50 μL of sterile water was added to the cell suspension. Transfection was accomplished by electroporation with a Biorad Gene-Pulsar (Richmond, CA) at settings of 1.0 kV and 25 μF with a resultant pulse time of 0.8 milliseconds. The cells were grown for 48 hours in MEM at which time G-418 (GIBCO, Grand Island, NY) was added to a final concentration of 0.47 mg/mL (actual concentration). G-418-resistant clones were detected within 6 to 10 days. Pooled G-418-resistant clones were then selected in MEM containing 10% dialyzed supplemented calf serum and methotrexate (MTX; Lederle, Indianapolis, IN) at a final concentration of 50 nmol/L. We have previously shown that this sequential selection procedure ensures that nearly every MTX-resistant clone contains highly amplified and unarranged plasmid sequences. In the studies reported here, MTX-resistant cells were pooled so as to eliminate clonal variability as an explanation for our results. Synchronous populations of F-MEL cells were obtained by resuspending log phase cells in MEM + 0.5% serum at a concentration of 2 x 10^6 cells/mL for 48 hours. In general, this resulted in 50% to 70% of the cells being in the G2/M state as opposed to 30% to 35% of cells from a log phase culture.

Flow cytometric analysis. F-MEL cells were pelleted by centrifugation as described above, washed twice in PBS, and, finally, resuspended in 1 mL of 10 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 0.1% NP-40 detergent, 7 x 10^-3 mol/L propidium iodide (Sigma, St Louis, MO) and 10 μg/mL DNase-free RNase A. Nuclei were analyzed on an Epics C flow cytometer (Coulter Instruments, Hialeah, FL) as described previously and forward angle light scatter was used to gate out cellular debris. The percent of nuclei in G0/G1, S, and G2/M were determined with the PARA 1 program. Approximately 10,000 nuclei were enumerated for each sample.

Nucleic acid analyses. Total cellular RNAs were extracted by the guanidine hydrochloride method as previously described. In some cases, nuclei and cytoplasmic compartments were first separated and RNAs were extracted from each fraction. S1 nuclease analyses were performed as previously described. Digested products were resolved on 2% agarose gels, which were then dried and exposed to x-ray film (X-OMAT; Kodak, Rochester, NY) using a single calcium tungstate intensifying screen. For the analysis of c-jun antisense transcripts, a 1.13-kb Cla-Sal I restriction fragment from the pMS-G c-jun (AS) dhfr plasmid, end-labeled with polyribonucleotide kinase at the Sal I site, was used. For the analysis of c-myc transcripts, a 0.94-kb Nde I-Xho I restriction fragment from the plasmid pSV junB-dhfr was labeled at the Xho I site and used as previously described. For the analysis of junB transcripts, we used a 1.1-kb Nde-Nhe I restriction fragment from the plasmid pSV junB-dhfr. The fragment was end-labeled at the Nhe I site. In the analysis of egr-1 transcripts, we used a 0.87-kb Nde-Bgl II restriction fragment from the plasmid pSV egr-1 dhfr. The fragment was end-labeled at the Bgl II site. junB and egr-1 cDNAs were generous gifts from Drs D. Nathans (Johns Hopkins University) and V. Sukhatme (University of Chicago), respectively.

Preparation of a c-jun fusion protein and detection of c-jun protein in F-MEL cells. A 570-bp Smal 1-Sca I fragment, encoding the C-terminal 155 amino acids of murine c-jun, was cloned into the path23 procarboxylic expression vector and expressed as an approximately 55-kD TrpE-c-jun fusion protein in E coli strain DH5α cells. Insoluble material from indoleacrylic acid-induced cells was solubilized with sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) and electrophoresed through a 7.5% linear SDS-polyacrylamide gel. The fusion protein accounted for 30% to 50% of the total protein in such preparations. The fusion protein (approximately 200 μg) was visualized with fluorescamine (Sigma), excited, solubilized in complete Freund’s adjuvant, and used for immunizations of rabbits. Additional booster injections consisting of 200 μg...
of fusion protein in incomplete Freund’s adjuvant were administered every 3 weeks. Antibody production was monitored by assessing its ability to precipitate 3S-methionine–labeled in vitro-translated c-jun protein from rabbit reticulocyte lysates (Promega Biotech, Madison, WI).

To detect c-jun protein, F-MEL cells were pelleted by centrifugation, washed twice in PBS, and lysed in standard RIPA buffer (0.15 mol/L NaCl; 50 mmol/L Tris-HCl, pH 8.0; 0.1% SDS; 1% NP-40; 0.5% deoxycholate) containing 100 μg/mL phenylmethysulfonyl fluoride (PMSF); and 1 μg/mL each of pepstatin, leupeptin, and aprotinin. Lysates were then sonicated briefly to reduce viscosity. One hundred micrograms of total protein was electrophoresed through a linear 7.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose in TBS buffer (0.15 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.6). The filter was blocked as previously described for 4 to 6 hours at room temperature in a 5% suspension of non-fat dry milk prepared in TBS buffer.3 Anti-c-jun antibody was then added to a final dilution of 1:100 and the incubation was continued for an additional 2 to 3 hours. The blot was washed against several changes of TBS containing 0.5% Tween-20. Bound anti-c-jun antibody was detected by enhanced chemiluminescence (New England Nuclear, Boston, MA) using conditions recommended by the supplier.

RESULTS

Conditional expression of c-jun antisense transcripts. A 1.26-kb cDNA fragment containing only 48 bp of c-jun coding plus 5′ and 3′ untranslated sequences was cloned in antisense orientation into the pMS-Gdhfr vector (Fig 1). The virtual absence of c-jun coding sequences ensured that no regions homologous to junB or junD would be present and that only c-jun message would be targeted by the antisense transcripts. This vector contains a glucocorticoid-inducible MMTV promoter as well as a transcription unit for a mutant dhfr molecule43 (Fig 1). Plasmid DNA was linearized in the procaryotic vector backbone and cotransfected into F-MEL cells along with linearized pSV2neo. G-418-resistant clones were further selected in 50 nmol/L MTX. We have previously shown that this sequential selection protocol ensures that virtually all surviving MTX-resistant cells contain highly amplified copies of unarranged plasmid sequences (generally 50 to 200 copies/cell).3,12,41,42 To avoid potential artifacts that might arise as a result of clonal variation, all studies reported here were performed with a pooled population of MTX-resistant F-MEL cells (hereafter referred to as c-junAS cells). We estimate that this population consists of at least several hundred independent transfectants.

We first examined the expression of c-jun antisense transcripts in response to varying concentrations of glucocorticoid hormone. Logarithmically growing c-junAS cells were exposed to different concentrations of dexamethasone (DEX) for 4 hours. RNAs were then extracted and hybridized with 108 dpm of an end-labeled 1.13-kb Cla I-Sal I restriction fragment from the pMS-GjunAS dhfr. The S1 nuclease-protected fragment at 283 nt represents c-jun antisense transcripts. Control hybridizations containing RNA from untransfected F-MEL cells showed no protected fragments (not shown).

To detect c-jun protein, F-MEL cells were pelleted by centrifugation, washed twice in PBS, and lysed in standard RIPA buffer (0.15 mol/L NaCl; 50 mmol/L Tris-HCl, pH 8.0; 0.1% SDS; 1% NP-40; 0.5% deoxycholate) containing 100 μg/mL phenylmethysulfonyl fluoride (PMSF); and 1 μg/mL each of pepstatin, leupeptin, and aprotinin. Lysates were then sonicated briefly to reduce viscosity. One hundred micrograms of total protein was electrophoresed through a linear 7.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose in TBS buffer (0.15 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.6). The filter was blocked as previously described for 4 to 6 hours at room temperature in a 5% suspension of non-fat dry milk prepared in TBS buffer. Anti-c-jun antibody was then added to a final dilution of 1:100 and the incubation was continued for an additional 2 to 3 hours. The blot was washed against several changes of TBS containing 0.5% Tween-20. Bound anti-c-jun antibody was detected by enhanced chemiluminescence (New England Nuclear, Boston, MA) using conditions recommended by the supplier.

RESULTS

Conditional expression of c-jun antisense transcripts. A 1.26-kb cDNA fragment containing only 48 bp of c-jun coding plus 5′ and 3′ untranslated sequences was cloned in antisense orientation into the pMS-Gdhfr vector (Fig 1). The virtual absence of c-jun coding sequences ensured that no regions homologous to junB or junD would be present and that only c-jun message would be targeted by the antisense transcripts. This vector contains a glucocorticoid-inducible MMTV promoter as well as a transcription unit for a mutant dhfr molecule43 (Fig 1). Plasmid DNA was linearized in the procaryotic vector backbone and cotransfected into F-MEL cells along with linearized pSV2neo. G-418-resistant clones were further selected in 50 nmol/L MTX. We have previously shown that this sequential selection protocol ensures that virtually all surviving MTX-resistant cells contain highly amplified copies of unarranged plasmid sequences (generally 50 to 200 copies/cell).3,12,41,42 To avoid potential artifacts that might arise as a result of clonal variation, all studies reported here were performed with a pooled population of MTX-resistant F-MEL cells (hereafter referred to as c-junAS cells). We estimate that this population consists of at least several hundred independent transfectants.

We first examined the expression of c-jun antisense transcripts in response to varying concentrations of glucocorticoid hormone. Logarithmically growing c-junAS cells were exposed to different concentrations of dexamethasone (DEX) for 4 hours. RNAs were then extracted and hybridized with a single-stranded probe designed to detect the presence of c-jun antisense transcripts in an S1 nuclease protection assay. As seen in Fig 2, very low levels of antisense transcript were seen in c-junAS cells in the absence of DEX and probably represent the intrinsic “leakiness” of the MMTV promoter. On the other hand, high levels of transcript were seen upon exposure to DEX concentrations between 10−9 and 10−6 mol/L. At concentra-
Inhibition of c-jun protein after the induction of c-jun antisense transcripts. pMS-Gdhfr cells or c-junAS cells were treated (+) with 10^{-7} mol/L DEX for 24 hours, washed, and lysed in RIPA buffer. As controls, duplicate plates of non-DEX-treated cells were included (−). One hundred micrograms of protein was electrophoresed, Western blotted, and incubated with a polyclonal rabbit anti-c-jun antibody. The c-jun-specific band was then visualized by enhanced chemiluminescence.

Fig 3. Inhibition of c-jun protein after the induction of c-jun antisense transcripts. pMS-Gdhfr cells or c-junAS cells were treated (+) with 10^{-7} mol/L DEX for 24 hours, washed, and lysed in RIPA buffer. As controls, duplicate plates of non-DEX-treated cells were included (−). One hundred micrograms of protein was electrophoresed, Western blotted, and incubated with a polyclonal rabbit anti-c-jun antibody. The c-jun-specific band was then visualized by enhanced chemiluminescence.

pMS-Gdhfr cells and in c-junAS cells in the absence of DEX. That this band corresponded to c-jun was confirmed in several ways: (1) F-MEL cells transfected with an SV40 promoter-driven c-jun sense construct expressed about 10-fold more of an identically sized protein; (2) the 39-Kd band could be competed by adding an excess of soluble TrpE–c-jun fusion protein to the blot but not by adding an excess of TrpE–c-myc fusion protein; and (3) the addition of glucocorticoid did not affect the intensity of the band in pMS-Gdhfr cells, although it did result in a 80% to 90% reduction of its level in c-junAS cells. Thus, highly amplified expression of c-jun antisense transcripts was an efficient means of reducing the levels of c-jun protein.

Reversible inhibition of c-junAS cell growth. To determine the effect that reduced levels of c-jun protein might have on cell growth, c-junAS cells were cultured continuously in two different concentrations of DEX. For controls, we used pMS-Gdhfr cells or pooled clones of F-MEL cells that had been transfected with the pMS-Gdhfr vector containing a full-length c-jun cDNA in sense orientation.

Daily cell counts were performed for a total of 5 days. In several independent experiments, DEX had little effect on cell growth in either of the two control groups (Fig 4A and B). In contrast, c-junAS cells were inhibited by greater than 85% at a DEX concentration of 10^{-7} mol/L and by 50% to 75% at a concentration of 10^{-8} mol/L. In all groups, cell viability remained at greater than 95% for the duration of the experiment.

We next asked whether the growth inhibition of c-junAS cells might be associated with their accumulation at a particular point in the cell cycle. c-junAS cells were incubated in 10^{-7} mol/L DEX for various periods of time. Nuclei were then purified, stained with propidium iodide, and subjected to flow cytometric analysis to assess DNA content. Table 1 shows that, typically, 30% to 35% of nuclei from logarithmically growing c-junAS cells contained a 2n DNA content, indicative of cells in the G_{0} or G_{1} state. This proportion was the same as that seen with untransfected F-MEL cells or with F-MEL cells transfected with control vector sequences alone. After the addition of DEX to c-junAS cells, we noted a gradual accumulation of nuclei with a 2n DNA content that reached maximal levels by about 36 hours. This degree of synchrony is comparable to or better than that attained when F-MEL cells are density arrested and serum deprived, for 48 hours (45% to 50% of nuclei with a G_{0}/G_{1} DNA content). Incubation of control pMS-Gdhfr cells in 10^{-7} mol/L DEX had no effect on the profiles of nuclear DNA content (Table 1).

To determine whether the effect on nuclear DNA content was reversible, we treated c-junAS cells for 60 hours with 10^{-7} mol/L DEX. At the end of this time, flow cytometric analysis of propidium iodide-stained nuclei indicated that 60% of the cells had a 2n DNA content (Table 2). The cells were then washed in fresh medium and incubated in the absence of DEX for an additional 24 hours. Aliquots of cells were taken at timed intervals and once again subjected to flow cytometric analysis. We observed that the percentage of cells in G_{0}/G_{1} progressively decreased until about 19 hours after DEX removal. The

Fig 4. Proliferation of c-junAS cells is inhibited by DEX. F-MEL cells were transfected with the pMS-Gdhfr plasmid alone (A), or containing murine c-jun sequences in sense (B) or antisense (C) orientation relative to the MMTV promoter. Pooled MTX-resistant clones were cultured continuously in 50 nmol/L MTX. For growth studies, 35-mm plastic plates were seeded with 1.5 × 10^5 cells in a total volume of 3 mL. The plates contained no DEX (○) or DEX at a final concentration of 10^{-7} mol/L (△) or 10^{-8} mol/L (□). Viable cell counts were performed daily. In all cases, viability remained greater than 95%.
which cells were washed but resuspended in fresh medium containing DEX were removed at the times indicated and subjected to flow cytometric analysis to determine DNA content. In control experiments (not shown) in which DEX-treated c-junAS cells were actually blocked in GI or whether they had left the cell cycle and entered the Go-like state. From the foregoing experiments, it was not possible to ascertain whether DEX-treated c-junAS cells were actually blocked in G0 or whether they had left the cell cycle and entered the quiescent G0 state. In the former case, genes such as c-myc, which are expressed equally at all points in the cell cycle, should not show any change in expression after DEX removal. In the latter case, however, removal of DEX and reversal of the antiproliferative block might be expected to result in the transient induction of immediate early response genes commonly associated with the Go-G1 transition.

To distinguish between these two possibilities, logarithmically growing c-junAS cells were cultured in the presence of 10^{-7} mol/L DEX for 60 hours, at which time 72% of the cells were in the G0/G1 state (not shown). This contrasts with 33% in G0/G1 from a parallel culture simultaneously propagated in DEX-free medium. The DEX-treated cells were washed free of the drug and then recultured in its absence. At various times thereafter, portions of the culture were removed and total RNA was extracted. Individual RNAs were then hybridized with single-stranded, end-labeled DNA probes specific for the immediate early response transcripts egr-1, c-myc, and junB. Each RNA was also examined for the expression of c-jun antisense transcripts. All three immediate early response gene endogenous transcripts were induced with kinetics that resembled those seen after growth factor stimulation of quiescent cells with the same immediate-early response gene probes described above. The results of these studies are also presented in Fig 5, in which it can be seen that the kinetics of induction of these transcripts was virtually identical to that seen in c-junAS cells after removal of DEX. The sole exception to this occurred in the case of c-jun antisense transcripts. As expected, none were detected in control F-MEL cells. Because endogenous c-jun transcripts are expressed at low levels in F-MEL cells and because high levels of antisense transcripts may interfere with the ability to reliably detect endogenous transcripts, we measured the kinetics of c-jun protein reappearance in c-junAS cells within 30 to 60 minutes of DEX removal despite the persistence of c-jun antisense transcripts (Fig 5). Thus, based on the expression of several immediate-early response gene transcripts, as well as on the reappearance of c-jun protein, we conclude that inhibition of c-jun protein in c-junAS cells causes them to reversibly enter a state closely resembling, if not identical to, Go.

Because endogenous c-jun transcripts are expressed at low levels in F-MEL cells and because high levels of antisense transcripts may interfere with the ability to reliably detect endogenous transcripts, we measured the kinetics of c-jun protein reappearance in c-junAS cells within 30 to 60 minutes of DEX removal despite the persistence of c-jun antisense transcripts (Fig 5). Thus, based on the expression of several immediate-early response gene transcripts, as well as on the reappearance of c-jun protein, we conclude that inhibition of c-jun protein in c-junAS cells causes them to reversibly enter a state closely resembling, if not identical to, Go.

The rapid reappearance of c-jun protein (Fig 6A) in the face of persistently elevated levels of c-jun antisense transcripts (Fig 5) was initially puzzling and suggested that antisense inhibition was somehow less effective after the decrease was associated with a concurrent increase of cells in the S and G2/M phases. After 19 hours there again was a progressive reaccumulation of cells in G2/M, as is seen when a synchronized population of cells re-enters G1. Thus, the inhibitory effect of lowered c-jun levels on the proliferation of c-junAS cells was reversible and their re-entry into the cell cycle strongly resembled that seen when serum-deprived and density-arrested F-MEL cells are provided with a strong mitogenic stimulus.

<table>
<thead>
<tr>
<th>Time After Addition of DEX (h)</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (log phase)</td>
<td>31</td>
<td>45</td>
<td>24</td>
</tr>
<tr>
<td>12</td>
<td>33</td>
<td>43</td>
<td>24</td>
</tr>
<tr>
<td>24</td>
<td>33</td>
<td>44</td>
<td>14</td>
</tr>
<tr>
<td>36</td>
<td>47</td>
<td>39</td>
<td>14</td>
</tr>
<tr>
<td>48</td>
<td>50</td>
<td>37</td>
<td>13</td>
</tr>
<tr>
<td>60</td>
<td>50</td>
<td>36</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 2. Reversibility of DEX-Mediated Growth Inhibition of c-junAS Cells

<table>
<thead>
<tr>
<th>Time After Removal of DEX (h)</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>47</td>
<td>32</td>
<td>21</td>
</tr>
<tr>
<td>11</td>
<td>41</td>
<td>35</td>
<td>24</td>
</tr>
<tr>
<td>13</td>
<td>40</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>16</td>
<td>33</td>
<td>40</td>
<td>27</td>
</tr>
<tr>
<td>17</td>
<td>30</td>
<td>39</td>
<td>26</td>
</tr>
<tr>
<td>19</td>
<td>25</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td>21</td>
<td>31</td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td>24</td>
<td>30</td>
<td>31</td>
<td>30</td>
</tr>
</tbody>
</table>

Logarithmically growing c-junAS cells (31% G0/G1, DNA content, not shown) were treated for 60 hours with 10^{-7} mol/L DEX, at which time 60% of cells had a 2n G0/G1 DNA content (top line). The cells were washed twice in DEX-free MEM and resuspended at a concentration of 10^6 cells/mL. Aliquots of cells were withdrawn at the times indicated for determinations of DNA content. In control experiments (not shown) in which cells were washed but resuspended in fresh medium containing DEX, no cell cycle progression was observed.
were removed at the times indicated and total RNAs were extracted.

Ten micrograms of each RNA was hybridized with 10^6 dpm of a single-stranded DNA probe designed to detect c-jun antisense transcripts or endogenous transcripts for egr-1, c-myc, or junB (see Materials and Methods). In control experiments, F-MEL cells were synchronized for 48 hours by density arrest and serum deprivation, and diluted into fresh medium containing 10% serum at time 0, and sampled thereafter at the same times as the c-junAS cell population. RNAs from these cells were hybridized with the same S1 probes and electrophoresed in parallel (side B). The sizes of the S1 nuclease-protected fragments are indicated next to each autoradiogram.

Fig 5. c-junAS cells are in a G0-like state after DEX exposure. Logarithmic-phase c-junAS cells (31% of cells in G0/G1) were grown for 60 hours in MEM containing 10^-4 mol/L DEX, at which time 72% of cells were in G0/G1. The cells were washed free of DEX and resuspended in fresh, DEX-free MEM at time 0 (side A). Aliquots of cells were removed at the times indicated and total RNAs were extracted. Ten micromolars of each RNA was hybridized with 10^6 dpm of a single-stranded DNA probe designed to detect c-jun antisense transcripts or endogenous transcripts for egr-1, c-myc, or junB (see Materials and Methods). In control experiments, F-MEL cells were synchronized for 48 hours by density arrest and serum deprivation, diluted into fresh medium containing 10% serum at time 0, and sampled thereafter at the same times as the c-junAS cell population. RNAs from these cells were hybridized with the same S1 probes and electrophoresed in parallel (side B). The sizes of the S1 nuclease-protected fragments are indicated next to each autoradiogram.

DISCUSSION

Evidence to support a role for nuclear proto-oncogenes in cell cycle progression has recently begun to emerge from a number of different studies. Among the earliest of these consisted of observations that many proto-oncogenes (c-fos, c-myc, c-jun, junB) were rapidly induced by mitogenic stimuli. These studies suggested that the products of these genes might be required by quiescent cells to re-enter the cell cycle. This is a particularly attractive hypothesis given the fact that most of the immediate early response gene transcripts described to date encode proven or putative transcription factors. More direct evidence to support this notion has come from studies demonstrating that the unscheduled expression of c-myc in serum-deprived NIH/3T3 cells is sufficient to allow for at least a dampened mitogenic response in the absence of any added growth factor. Others have shown that the induction of c-myc from a heat shock protein promoter also leads to the expression of other proteins usually affiliated with the G0/G1 transition. Additional studies have shown that antisense-mediated inhibition of c-myc in normal peripheral blood lymphocytes does not prevent the G0/G1 transition per se but does prevent entry into S-phase from G0. A role for c-fos in NIH/3T3 cell proliferation has been postulated by at least three groups using either conditional expression of antisense RNA or microinjection of specific antibodies. These studies indicated that expression of c-fos is required for the G0/G1 transition as well as for the maintenance of an actively proliferative state. The actual point(s) in the cell cycle at which c-fos is required, however, was not addressed in these studies.

c-jun is also an immediate early response gene that shares both structural and functional homologies with at least two other genes, junB and junD. c-jun dimerizes with c-fos and forms part of the AP-1 transcription complex that binds to a specific consensus sequence found in a number of genes. It has been shown that junB and junD proteins can substitute for c-jun in regard to both dimerization with c-fos and AP-1 site binding. However, differences in the biologic functions of these various complexes have been suggested. This is consistent with a recent report demonstrating that c-jun homodimers and c-jun:c-fos heterodimers induce DNA bends in opposite orientations after their binding to AP-1 sites. More recently, the potential transcriptional targets for the fos/jun family have been expanded with the observation that its members may also dimerize with members of the ATF/CREB family of transcription factors and bind uniquely to consensus DNA sites. This suggests that members of the fos/jun and ATF/CREB family may comprise a “superfamily” of transcription factors with a more global role in gene regulation than previously appreciated.

The previous findings indicating a role for c-fos in cell proliferation imply that at least one of the jun family members might serve a similar role. In this work, we have provided evidence that this is indeed the case for c-jun. We have determined not only that c-jun expression is necessary for continuous F-MEL proliferation but that the inhibition of c-jun is associated with a reversible entry into a state indistinguishable from G0. Because junB and junD transcripts are also expressed in F-MEL cells, our findings imply that these gene products are unable to substitute for...
c-jun in maintaining proliferation. Our results also indicate that cells that are in the S and G/M phases of the cell cycle at the time of DEX addition complete their passage through these states before entering G0, and, eventually, G1. Recently, using microinjection of specific jun member antibodies into Swiss 3T3 cells, Kovary and Brad' were able to inhibit either asynchronously growing cells or the entrance of serum-stimulated quiescent cells into S-phase. Although their results are entirely consistent with those presented here, it was not possible to determine in that study whether, in the former case, the microinjected cells entered a G0-like state.

All jun family member cDNAs are highly homologous to one another, particularly in certain portions of the 3' ends of their coding regions. Thus, it was important to ensure that only c-jun mRNAs were being targeted by our antisense vector. This was achieved by designing a vector that did not contain c-jun sequences whose transcripts would have the potential to form double-stranded hybrids with junB or junD mRNAs.

The ability of c-junAS cells to re-express c-jun protein within minutes of DEX removal was initially somewhat surprising in light of the persistence of c-jun antisense transcripts during this time (Fig 5). We believe this can be explained in part by the rapid transcriptional silencing of the MMTV promoter after DEX withdrawal. This would result in the immediate processing and transport of c-jun antisense transcripts from the nucleus to the cytoplasm, where they would persist with the relatively long half-life we have observed (Fig 5). The depletion of nuclear antisense transcripts would then allow for the efficient transport of newly synthesized endogenous c-jun transcripts to the cytoplasm, where they might be expected to be less prone to double-stranded formation. That such a scenario is likely is suggested by the experiments presented in Fig 6 showing the rapid induction of c-jun protein in c-junAS cells after DEX removal and the abrupt redistribution of c-jun antisense transcripts.

Although our experiments indicate a significant antisense-mediated reduction of c-jun protein, the observed phenotype may not be solely attributable to this effect. Several groups have reported that the glucocorticoid receptor can suppress the activity of AP-1 responsive genes. This appears to be mediated through a direct physical association between the glucocorticoid receptor and either DNA-bound or unbound AP-1. Thus, the reduction in c-jun protein observed in the current work may well underestimate the true extent of the inhibitory effect as any residual c-jun protein could well be rendered inactive through its interaction with functional glucocorticoid receptor. Conversely, the reactivation of glucocorticoid receptor bound c-jun protein after DEX withdrawal, together with the well-documented positive autoregulation of the c-jun gene may contribute significantly to the rapid reappearance of c-jun protein seen in Fig 6A.

Whatever the role for c-jun, it is likely that it may exert other, even diametrically opposed effects on cellular proliferation. For example, whereas many studies have now demonstrated the transcriptional activation of c-jun after mitogenic stimuli, others have shown the induction of c-jun...
in response to antimitogenic signals such as transforming growth factor-β and tumor necrosis factor-α. Thus, c-jun may well be a generalized transcription factor whose ultimate targets depend not only on the nature of the stimulus but on the particular cell type and its proliferative state. From the results reported here, it is clear that c-jun also plays an important role in promoting cell cycle progression as well as the G1/G0 transition.

ACKNOWLEDGMENT

We thank Michael Long for critical review of the manuscript and Ruth Douglas for expert secretarial assistance.

REFERENCES

5. Freytag SO: Enforced expression of the c-myc oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G1/G0. Mol Cell Biol 8:1614, 1988
36. Turner R, Tjian R: Leucine repeats and an adjacent DNA
binding domain mediate the formation of functional cFos-cJun heterodimers. Science 243:1689, 1989
Inhibition of c-jun causes reversible proliferative arrest and withdrawal from the cell cycle

MJ Smith and EV Prochownik