Amplified Expression of Three *jun* Family Members Inhibits Erythroleukemia Differentiation

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Several different proto-oncogenes have been shown to influence cellular differentiation. One of the most widely studied model systems has been the Friend murine erythroleukemia cell (F-MELC) line, which can be induced to undergo erythroid differentiation by a variety of chemical agents. Constitutive overexpression of either the c-myc or c-myb proto-oncogenes has been previously shown to inhibit F-MELC differentiation, whereas c-myc antisense sequences accelerate the process. To investigate the potential involvement of other proto-oncogenes and immediate early response genes in F-MELC differentiation, we studied the expression of the three known members of the *jun* family as well as another gene, *egr*-1, which, like the *jun* family members, is expressed as an immediate early response gene in growth factor–stimulated quiescent cells. All four genes were expressed in F-MELC, although the levels of expression and modes of regulation differed. Transfection with amplifiable c-jun, junB, or junD expression plasmids inhibited differentiation, whereas transfection with an *egr*-1 expression plasmid was without effect. These results indicate that *jun* family members play a role in mediating F-MELC differentiation. The known inhibitory effect of phorbol ester tumor promoters on F-MELC differentiation may be the result of their known stimulation of *jun* expression.

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**MATERIALS AND METHODS**

**Construction of recombinant plasmids.** The starting vector for all expression constructs was pSV<sub>dhfr</sub>, which was derived from the plasmid pSV<sub>neo</sub><sup>18</sup> by removing neo coding sequences with HindIII and Smal and inserting a Smal linker. A 2.8-kb PstI-SalI fragment from the plasmid pFR400<sup>19</sup> containing a complete transcription unit for a mutant murine diphosphatase reductase (dhfr) enzyme, was modified by the addition of EcoRI linkers and cloned into the unique EcoRI site of the modified pSV<sub>neo</sub> plasmid. A full-length murine c-jun cDNA was kindly provided by Dr R. Bravo<sup>20</sup> (European Molecular Biology Laboratory, Heidelberg).

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full-length murine junB and junD cDNAs (gifts from Dr D. Nathans, Johns Hopkins University, Baltimore, MD) and murine egr-1 cDNA (gift from Dr V. Sukhatme, University of Chicago) were excised from their plasmid vectors as 1.5-kb, 1.7-kb, and 3.2-kb EcoRI fragments, respectively, blunt-ended with Klenow DNA polymerase, and ligated into SmaI-digested pSV,dhfr as described above (Fig 1). For DNA transfections into F-MELC, the above DNAs were first linearized with NdeI or BglI, which recognize unique restriction sites within the pBR322 plasmid backbone. Forty micrograms of linearized DNA was combined with 2 to 3 µg of EcoRI-linearized pSV2neo in 50 µL of sterile water for electroporation into F-MELC.

Cell culture and DNA transfections. A subclone of the F-MELC clone 745, designated clone 25, was used for all DNA transfections. This clone and derived subclones routinely gave greater than 80% benzidine positive cells during a 5-day exposure to 1.5% dimethyl sulfoxide (DMSO). Cells were grown in Dulbecco's modified minimal essential medium (MEM) containing 10% supplemented calf serum (HyClone, Logan, UT), 100 U/mL penicillin G, 100 µg/mL streptomycin, and 2 mmol/L glutamine. Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere. For DNA transfections, 2 × 10⁷ logarithmically growing F-MELCs were washed in phosphate-buffered saline (PBS), and resuspended in 0.5 mL of ice cold PBS. F-MELCs were then electroporated as previously described. After a 10-minute incubation on ice, cells were plated into 30 mL of MEM in a 150-mm plastic Petri dish. Two days later, G-418 (Geneticin, GIBCO, Grand Island, NY) was added to a final absolute concentration of 0.5 mg/mL. G-418-resistant clones were subsequently subjected to a second round of selection in 0.25-µmol/L methotrexate (MTX, Lederle Laboratories, Wayne, NJ). MTX-resistant survivors were cloned by limited dilution in 96-well microtiter plates.

Benzidine staining. Logarithmically growing cells were seeded at densities of approximately 5 × 10⁴/mL in 35-mm tissue culture dishes. DMSO was added to a final concentration of 1.5%. Cultures were fed with fresh DMSO-containing medium on day 3. One hundred-microliter aliquots of cells were removed daily to assess benzidine positivity as previously described. A minimum of 200 cells were counted for each determination.

Nucleic acid analyses. RNA was prepared by the guanidinium-HCl method as previously described. All samples were examined on agarose formaldehyde gels to ensure equality of concentrations and intactness. S1 nuclease protection assays were performed as previously described using end-labeled probes specific for each expression plasmid (Fig 1 through 4). Each probe was end labeled with [³²P]-y-ATP (Amersham, Arlington Heights, IL) and polynucleotide kinase to specific activities of 2 to 5 × 10⁶ dpm/µg. Each hybridization reaction contained 10 µg of total RNA and 10⁵ dpm of single-stranded probe. Nuclease S1 concentrations in all cases were 200 U/mL.
Cellular DNAs were prepared from individual clones as previously described. Southern blot analyses were performed after digestion of 10 μg of each DNA with appropriately chosen enzymes that allowed for a clear distinction of transfected from endogenous sequences and enabled us to establish clonal uniqueness. Probes for Southern blots were the EcoR1 cDNA fragments described above, each labeled by random priming with 32P-α-dCTP to specific activities of 7 to 9 × 10⁶ dpm/μg.

RESULTS

Expression of egr-1 and jun family members in differentiating F-MELC. We first studied the expression of c-jun, junB, junD, and egr-1 transcripts in F-MELC induced to differentiate into mature erythroid precursors by DMSO. Total RNAs were purified at different points during the 5-day induction period and used in nuclease protection assays.

Fig 3. Kinetics of junB, junD, and egr-1 transcripts in differentiating F-MELC. Logarithmically growing F-MELC were treated for the periods of time indicated with 1.5% DMSO as described previously. Total RNAs were purified and hybridized with the 32P-end-labeled nuclease S1 probes indicated below each panel. The same RNA samples were used with each probe. The diagrams indicate the sizes of S1 nuclease fragments protected by transcripts originating from the endogenous genes as well as those originating from transfected plasmids. Only the former are expressed here.

Fig 4. Expression of transfected jun and egr-1 sequences in individual F-MELC clones. Several randomly selected clones from each group were examined for the expression of c-jun, junB, junD, and egr-1 transcripts using the end-labeled S1 nuclease probes depicted in Figs 2 and 3. Protected fragments corresponding to endogenous and transfected exogenous transcripts are indicated (see Figs 2 and 3). In the case of c-jun, endogenous transcripts cannot be seen (see Fig 2). Numbers above each lane indicate individual clones. Control lanes (c) contained RNAs from an uncloned population of F-MELC transfected with pSV2dhfr vector sequences only.
with specific end-labeled DNA probes. Transcripts from each of the four genes were detected at different levels and under different circumstances.

In the case of c-jun, we were unable to detect transcripts at any of the times chosen in logarithmically growing DMSO-treated F-MELC (not shown). However, we could detect c-jun transcripts if F-MELC was first rendered quiescent and then stimulated to re-enter the cell cycle. Under these conditions, c-jun transcripts were detected as early as 15 to 30 minutes following stimulation and persisted for greater than 2 hours (Fig 2). We were also able to detect c-jun transcripts in logarithmically growing F-MELCs if the cells were treated for 4 hours with cycloheximide (CHX), an inhibitor of protein synthesis, which in combination with growth factors has been reported to increase the rate of c-jun gene transcription as well as to stabilize pre-existing c-jun mRNAs. Thus, under conditions of normal logarithmic growth and during DMSO-induced differentiation, c-jun transcripts are expressed at extremely low levels. Low levels of c-jun expression have also been reported in F9 murine teratocarcinoma cells.

In contrast to c-jun, transcripts for junB, junD, and egr-1 were easily detected in differentiating F-MELC using specific nucleic protection assays (Fig 3). Of note was that endogenous junB transcripts were modulated over the 5-day course of the experiment, whereas transcripts for junD and egr-1 remained constant. JunB transcripts were expressed at approximately equivalent levels until 8 to 24 hours after DMSO addition, when they decreased approximately 10- to 20-fold. They then increased gradually, reaching levels somewhat higher than those in uninduced F-MELC by 5 days. Similar levels of junB expression were observed when F-MELCs were stimulated to differentiate with the structurally unrelated agent hypoxanthine (unpublished observations, January 1990).

In summary, the expression and regulation of jun family member transcripts in DMSO-treated F-MELC differ despite the overall similarity in structure and function of the gene products. c-jun transcripts are undetectable in logarithmically growing F-MELC unless superinduced in the presence of CHX. JunB and junD transcripts on the other hand, are easily detected in F-MELCs. JunD transcripts are not regulated during differentiation, whereas those for junB show a transient decline between 8 and 24 hours after DMSO addition.

Molecular analysis of individual jun-transfected F-MELC clones. F-MELCs were transfected with linearized DNA from each of the expression vectors shown in Fig 1. As controls, F-MELCs were also transfected with pSV, dhfr vector sequences only. Stable transfecants were generated by sequential selection in G-418 and MTX, and 10 to 20 individual clones from each group were obtained by limited dilution. Randomly selected clones from each group were examined for the integration and expression of transfected sequences. In the case of pSV,c-jun-dhfr-transfected clones, eight of eight clones examined contained unrearranged c-jun sequences. Seven of seven expressed high levels of transfected c-jun sequences (Fig 4A and unpublished observations, January 1990). Due to the low levels of endogenous c-jun transcripts in F-MELCs (Fig 2), it was not possible to determine the ratios of endogenous to exogenous transcripts. However, exogenous levels were, on average, at least 10 to 20 times higher than the endogenous superinduced levels seen in CHX-treated logarithmically growing F-MELCs (Fig 2B).

In the case of pSV,junB-dhfr-transfected cells, 9 of the 10 clones examined contained unrearranged junB sequences (not shown). Eight of these nine clones were examined for the expression of junB transcripts. In all eight cases, transfected junB sequences were expressed at levels 1 to 20 times those of endogenous junB transcripts (Fig 4B).

Nine of nine pSV,junD-dhfr transfected clones examined contained unrearranged plasmid sequences (not shown). Seven clones were analyzed for exogenous junD sequences. All seven expressed these sequences at levels approximately 1 to 30 times those of endogenous levels (Fig 4C).

Similar analyses were performed on pSV, egr-1-dhfr-transfected F-MELCs. Seven of seven expressed levels of exogenous egr-1 transcripts that were 5- to 20-fold higher than endogenous levels (Fig 4D).

In summary, our results confirm that the G-418/MTX sequential selection procedure is highly efficient as previously described. In the present study, 34 of 35 clones examined contained unrearranged plasmid sequences and generally expressed them at levels that were significantly higher than that of the respective endogenous gene. It should be noted that even those clones expressing the highest levels of jun transcripts grew at rates that were indistinguishable from control F-MELCs (not shown).

Transfected jun sequences are unperturbed during DMSO-induced differentiation. We have previously demonstrated that transfected c-myc and c-myc sequences are not regulated in response to DMSO in differentiating F-MELC. This contrasts with the expression of transcripts originating from the endogenous gene, which continue to be regulated in a manner very much like that seen in untransfected F-MELCs. The loss of normal regulation of the transfected sequences presumably reflects the absence of critical promoter and intronic elements that have been shown to be critical for the expression of the normal gene.

To determine whether transfected sequences were regulated in response to DMSO, one clone each of c-jun, junB, junD, and egr-1-transfected F-MELCs were treated with 1.5% DMSO. RNAs were extracted at different times and simultaneously examined for the expression of endogenous and transfected sequences. As seen in Fig 5, DMSO treatment had little if any effect on the expression of transfected c-jun sequences over the 5-day course of the experiment. Even with this high level of exogenous expression, we were unable to demonstrate endogenous c-jun sequences as might have been expected given the observation that c-jun is capable of upregulating its own expression.

Similar observations were made in the case of pSV,junB-dhfr and pSV,junD-dhfr-transfected F-MELC clones. Interestingly, in the presence of high-level constitutive expression of transfected junB sequences, endogenous
junB sequences were not regulated. This may be a general property of differentiation-defective F-MELCs because no regulation of endogenous junB sequences has been observed in c-myc- or c-myb-transfected F-MELC clones either (unpublished observations, January 1990).

In keeping with the above observations, transfected pSV.egr-1dhfr sequences were also not modulated during DMSO treatment.

**Constitutive jun expression inhibits F-MELC differentiation.** To determine whether the enforced expression of jun sequences exerted any effect on F-MELC differentiation, clones described above along with several other randomly selected ones were treated with DMSO for 5 days and examined by benzidine staining for terminal differentiation. Figure 6 shows the results of this study. Neither transfected pSV.dhfr vector sequences alone nor egr-1 sequences had any significant effect on DMSO-induced erythroid differentiation. On the other hand, clones transfected with any one of the three jun expression vectors showed a substantial loss of differentiation capacity. All three jun members were about equally effective in the suppression of erythroid differentiation. The scatter seen among the various jun-transfected clones presented in Fig 6 has been previously reported in c-myc- and c-myb-transfected F-MELC lines13,16,19,24,25 and is likely due to several factors, including clonal variability and the level of expression of transfected jun sequences. Indeed, we have observed a general, although not absolute, inverse correlation between jun transcript levels and differentiation. For example, in Fig 4, pSV.c-jundhfr-transfected clones 1, 6, and 7 expressed the lowest levels of c-jun transcripts. After DMSO treatment, 60%, 60%, and 38% of cells from these clones were scored as benzidine positive, respectively. On the other hand, clones 2, 3, and 4, which expressed the highest levels of c-jun transcripts, gave rise to 10%, 26%, and 4% benzidine-positive cells, respectively. In the case of pSV.junBdhfr-transfected F-MELC, clones 5, 6,
and 7 gave rise to 6% to 40% benzidine-positive cells, whereas clones 1 through 4 gave rise to 30% to 88% benzidine-positive cells. Finally, in the case of pSV_junDdhfr-transfected F-MELC (Fig 4C), clones 3 through 5 gave rise to 8% to 40% benzidine-positive cells, whereas clones 1 and 6 showed 42% and 60% benzidine positivity.

**DISCUSSION**

The process by which F-MELCs become irreversibly committed to the pathway of terminal differentiation involves several distinct and sequential steps. At the earliest stage, it is necessary that the specific inducing agent be kept in contact with the undifferentiated cells for a minimal period of 12 to 16 hours after which time F-MELC are capable of autonomous differentiation. Numerical and molecular changes are associated with this reversible precommitment phase, which culminates in commitment and an irreversible progression to terminal differentiation without the requirement for continued exposure to the inducing agent. True commitment is associated with a finite number of additional cell doublings (usually four to eight) and the simultaneous acquisition of the differentiated phenotype.

Whereas much of the biochemical basis for the triggering of the precommitment and commitment phases remains to be elucidated, it is clear that cellular proto-oncogenes play a significant role. Initial observations that c-myc and c-myb gene transcripts were modulated during F-MELC differentiation led to experiments in which these genes, driven by foreign promoters, were reintroduced into the cells so as to override the intrinsic regulation of the endogenous gene. In both cases, the result was a loss of inducible F-MELC differentiation. Additional evidence has suggested that these genes operate at distinct levels, with c-myb serving to suppress differentiation at a more proximal step.

In the current study, we have investigated the role of the three known members of the jun proto-oncogene family on F-MELC differentiation. In each case, overexpression of these genes drastically changed the sensitivity of F-MELC to the inducer. Because the basal levels of c-jun transcripts in logarithmically growing F-MELCs are so low, we cannot be certain as to whether they are regulated as in the case of junB transcripts. We have also investigated the regulation of an additional early response gene, egr-1. Like c-myc, c-fos, and jun family members, egr-1 is a member of the so-called immediate early response gene repertoire, induced in quiescent cells by serum or purified growth factors. egr-1 encodes a nuclear protein with three putative DNA binding zinc fingers and is likely to function as a transcription factor. Like junD, the levels of egr-1 transcripts do not change during F-MELC differentiation (unpublished observation, January 1990). egr-1 is different from jun family members, however, in that its overexpression is without apparent effect in F-MELC. Thus, the ability to inhibit F-MELC differentiation is a biologic property that is not necessarily shared by all immediate early response genes.

That all three jun members can inhibit F-MELC differentiation does not prove a direct role for each in this process. Rather, it is possible that this is the result of common physical or biochemical attributes shared by these three proteins. The ability to form homodimers and heterodimers with the c-fos gene product coupled with the ability of these dimers to bind to the same DNA elements, suggests that the jun members may also share common biological properties. The recent finding that L-myc can substitute for c-myc in inhibiting F-MELC differentiation, even though L-myc expression is not normally seen in these cells, provides some evidence for the functional overlap of related proto-oncogene products in this system.

The relative levels of jun expression required to achieve inhibition of F-MELC differentiation are quite high in comparison with c-myc and c-myb. Several non–mutually exclusive models, which take known properties of jun proteins into account, might account for this. (1) It is known that jun-fos heterodimerization is favored over jun-jun homodimerization and that the DNA binding of heterodimers is approximately 25-fold more efficient than that of homodimers. However, in the presence of large excesses of jun protein, increased jun-jun homodimerization would be favored and, at the high constitutive levels seen here, might even predominate. This could result in the binding of the homodimer to DNA sites that are not normally occupied or in the displacement of jun-fos heterodimers or other transcription factors from normally occupied sites. (2) Excessive amounts of jun gene products may act as dominant negative mutants, depriving another jun member product from an integral association with c-fos or a foslike gene product. (3) c-jun has recently been shown to possess a negative regulatory domain near its N-terminus. This region is highly conserved among all three jun proteins and its deletion in c-jun enhances the in vitro transcriptional activity of bacterially expressed protein. Thus, transfected jun members may be less efficient transcriptionally than c-myc or c-myb, although formal comparisons have not been made.

Phorbol ester tumor promoters such as TPA activate protein kinase C (PKC) and increase levels of c-jun mRNA by stimulating transcription of the c-jun gene and by stabilizing pre-existing mRNA transcripts. Phorbol esters are also potent inhibitors of F-MELC differentiation. We have previously shown that some DMSO-resistant F-MELC lines have alterations in the overall cellular levels and subcellular distribution of PKC that mimic those seen in TPA-treated cells. The present findings suggest that an additional mechanism through which TPA might operate to inhibit F-MELC differentiation is through the chronic activation of jun family member genes.

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