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Growth and differentiation of embryonic stem cells that lack an intact c-*fos* gene

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ABSTRACT **The c-*fos* protooncogene encodes a transcription factor that is thought to play a critical role in proliferation and differentiation as well as in the physiological response of mature cells to their environment. To test directly the role of c-*fos* in growth and differentiation, we generated mouse embryonic stem cell lines in which both copies of the c-*fos* gene were specifically disrupted by homologous recombination. Remarkably, the disruption of both copies of c-*fos* in these cells has no detectable effect on embryonic stem cell viability, growth rate, or differentiation potential. Embryonic stem cells lacking c-*fos* can differentiate into a wide range of cell types in tissue culture and also in chimeric mice. We conclude that despite a large body of literature suggesting an important role for c-*fos* in cell growth and differentiation, in at least some cell types this gene is not essential for these processes.**

Transcription of the c-*fos* protooncogene is rapidly induced in many cell types upon stimulation with growth or differentiation factors (reviewed in ref. 1). Once synthesized, the c-Fos protein can regulate gene expression by a number of mechanisms (1–4), including the formation of transcription factor complexes with members of the Jun family (reviewed in ref. 5). The control of gene expression by c-Fos is believed to play a critical role in the cellular response to growth factors (5, 6). Supporting this view is the observation that introduction of anti-Fos antibodies (7, 8) or c-*fos* antisense RNA (9, 10) into fibroblasts inhibits their proliferation and their ability to reenter the cell cycle from quiescence. Further evidence for the role of c-*fos* in the control of cell growth is suggested by the finding that alterations in the normal pattern of c-Fos expression can lead to oncogenesis (11, 12). These studies strongly implicate c-*fos* as playing a critical role in cellular proliferative and developmental responses to extracellular stimuli. To more directly investigate the importance of c-Fos during cell growth and differentiation, embryonic stem (ES) cell lines were generated in which both copies of the c-*fos* gene were specifically disrupted. These cell lines were used to examine the requirement for c-*fos* in processes of growth and differentiation.

MATERIALS AND METHODS

Construction of c-*fos* Gene Replacement Vectors. The c-*fos–neo* vector (Fig. 1*A*) has been described (13). A 6.7-kb *Hin*dIII fragment subcloned from the mouse c-*fos* genomic clone pc-fos-3 (15) was used to construct the c-*fos–hyg* targeting vector (Fig. 1*A*). The first intron of the c-*fos* DNA was made distinguishable from the wild-type intron by filling in the *Xho* I site of pc-fos-3 to produce a *Pvu* I site. The c-*fos* gene was disrupted by inserting the hygromycin-resistance gene PGK-*hyg* (14) into the *Bgl* I site in the first exon of c-*fos*. The *Hin*dIII fragment of the disrupted c-*fos* gene was cloned into the *Hin*dIII site of a pBluescript SK vector (Stratagene) that already contained the herpes simplex virus 1 thymidine kinase gene cloned into the *Nae* I site (13). Before transfection the c-*fos–hyg* targeting vector was linearized with *Not* I.

Isolation of Cell Lines with c-*fos* Gene Replacements. ES cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, and 1% conditioned medium from Chinese hamster ovary cells overexpressing leukemia inhibitory factor (LIF; Genetics Institute, Cambridge, MA). The c-*fos–hyg* gene replacement vector was electroporated into cell line F16 (in which one copy of c-*fos* had been specifically disrupted by the *neo* gene; ref. 13). Ten micrograms of linearized c-*fos–hyg* DNA was electroporated into 10^7 F16 cells in 0.80 ml of complete medium by using settings on the Bio-Rad Gene Pulser of 450 V and 25 μF. Electroporated cells (2.5×10^6) were grown under selection with hygromycin B (200 μg/ml) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine (FIAU, 200 nM) (16). Thirty-three of the 79 colonies surviving double selection were screened with the polymerase chain reaction, and 10 were found to have undergone homologous recombination (data not shown). A separate experiment using a slightly different c-*fos–hyg* targeting vector yielded similar recombination frequencies.

Southern Analysis. DNA was isolated from ES cells by a standard protocol (17), digested with *Sac* I (or other restriction enzymes), and fractionated by electrophoresis through a 0.9% agarose gel. The DNA was transferred to GeneScreen (NEN) and hybridized to a probe prepared from a 0.3-kb *Sac* I–*Hin*dIII fragment of c-*fos* genomic DNA (pc-fos-3), according to standard methods (18).

Northern Analysis. Cells were serum-starved for 24 hr and then stimulated with serum for 60 min, and RNA was isolated (19). RNA was electrophoresed in a 1% agarose/formaldehyde gel, transferred to GeneScreen, crosslinked to the filter by UV irradiation, and hybridized according to the GeneScreen instructions. A 1.6-kb *Pst* I fragment of the mouse c-*fos* cDNA plasmid pGEMfos3 (J. G. Belasco and M.E.G., unpublished data) was used to prepare a hybridization probe by random primed labeling (Boehringer Mannheim kit). As a control for the amount and integrity of the RNA in each lane, the filter was subsequently stripped in 1% SDS/10 mM Tris·HCl, pH 7.5/1 mM EDTA for 30 min at 90°C and rehybridized to a probe prepared from a 1.3-kb *Pst* I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase cDNA (20).

Immunoprecipitations. Cells were serum-starved for 24 hr, serum-stimulated for 40 min, and then switched to methio-

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Abbreviations: ES, embryonic stem; LIF, leukemia inhibitory factor.

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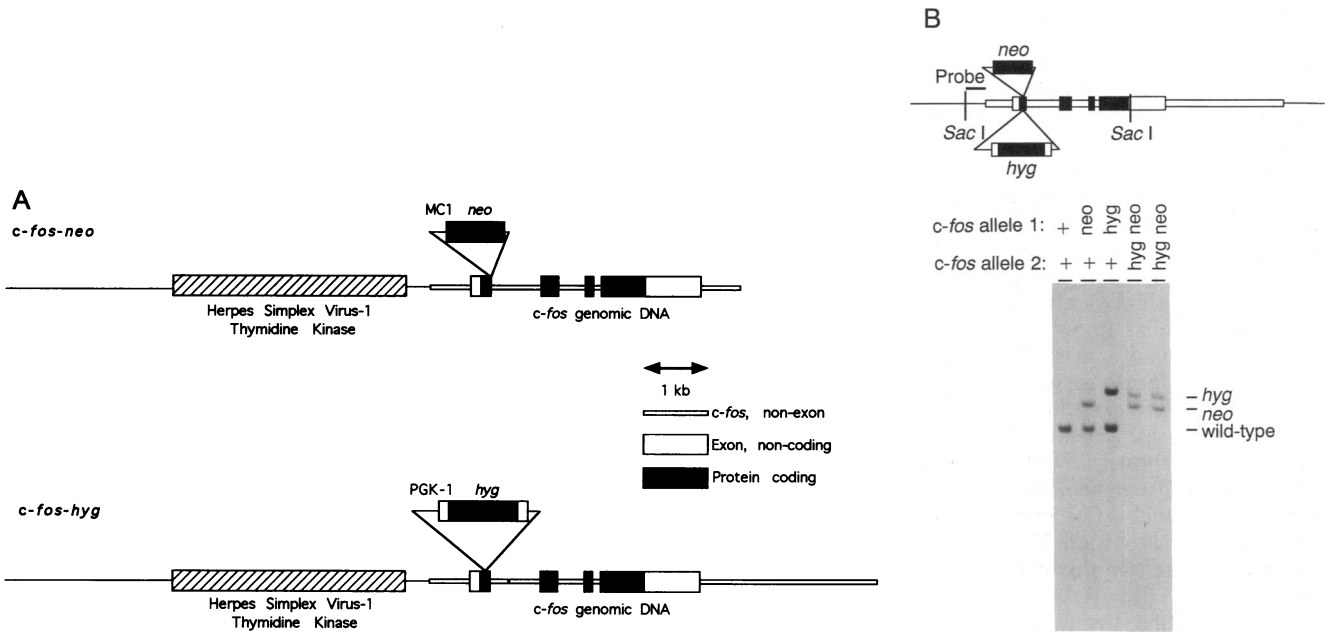

FIG. 1. Targeted disruption of both copies of the c-*fos* gene. (*A*) Structure of the c-*fos* replacement vectors. The c-*fos–neo* vector has been described (13). The c-*fos–hyg* vector contains the hygromycin-resistance gene PGK-*hyg* (14) inserted into the first exon of mouse c-*fos* genomic DNA. Upstream of the disrupted c-*fos* genomic DNA is the herpes simplex virus 1 thymidine kinase gene. kb, Kilobase. (*B*) Southern analysis confirming the doubly disrupted genotype. DNA isolated from candidate cell lines was digested with *Sac* I and analyzed by Southern blotting. Wild-type (+/+) cells exhibit a single band of 3.5 kb. F16 (*neo*/+) cells exhibit the 3.5-kb band and a 4.7-kb band of equal intensity, corresponding to the *neo*-disrupted c-*fos* locus. A cell line in which *hyg* has been swapped for *neo* (*hyg*/+) exhibits the 3.5-kb band and a 5.3-kb band of equal intensity, corresponding to the *hyg*-disrupted c-*fos* locus. Two doubly disrupted (*neo*/*hyg*) cell lines exhibit 4.7-kb and 5.3-kb bands of equal intensity, and no wild-type 3.5-kb band. Additional restriction digests and the use of a probe internal to the targeting vectors confirmed the results shown here and further showed that only one copy of each gene replacement vector was present in each cell line (data not shown).

nine-free medium plus 500 μCi of [^{35}S]methionine (1100 Ci/mmol; 1 Ci = 37 GBq). Fifty minutes later the cells were lysed with boiling 0.5% SDS/50 mM Tris·HCl, pH 7.4, and immunoprecipitation was carried out in radioimmunoprecipitation assay buffer, as described (21). Amounts of lysate yielding equal trichloroacetic acid-precipitable ^{35}S radioactivity were used in each immunoprecipitation. Antiserum "1" used for immunoprecipitation was raised to an *Escherichia coli*-produced TrpE/c-Fos fusion protein (containing c-Fos amino acids 74–151; ref. 21). Antiserum "2" (kindly provided by R. Bravo) specifically recognizes c-Fos and not other Fos-related proteins (22).

Growth Curves and Cell Doubling Times. ES cells were plated at low density as a single-cell suspension to form ≈30 colonies per 10-cm dish. Cells were trypsinized and counted after 90, 130, 190, 235, 285, or 330 hr. The number of colonies was counted at 235, 285, and 330 hr and used to estimate the number of colonies at earlier time points. Since each colony arose from a single cell, the number of cell doublings is given by $\log_2$ (number of cells/number of colonies).

Doubling times were assessed by measuring the number of cell doublings 9 days after plating a single-cell suspension to yield ≈100 colonies per 10-cm dish. Cell doubling time is equal to the elapsed time since plating divided by the number of cell doublings.

Exponentially growing ES cells were growth-inhibited (cell number ceased to increase) by changing to medium lacking serum or LIF for 2 days. Exponential growth was restored when the ES cells were subsequently refed with complete medium. The number of cells was counted on identically treated cultures harvested on various days during the experiment.

Differentiation of ES Cells. ES cells were plated at low density on bacterial Petri dishes in complete medium lacking LIF. Within a few days floating embryoid bodies formed, which subsequently attached to the surface of the dish. Cells were allowed to grow and differentiate for 3–4 weeks. A range of cell types, including neuron-like, muscle-like, and adipocyte-like cells, could be seen on any given dish.

RESULTS

To disrupt both copies of the c-*fos* gene, two consecutive rounds (23) of the positive/negative selection strategy (24) were used to isolate cell lines in which homologous recombination had replaced both of the c-*fos* genomic loci with disrupted c-*fos* gene constructs. The first round produced an ES cell line (termed F16) heterozygous for a disruption at the c-*fos* locus, as described (13). For the second round, a homologous recombination replacement vector (Fig. 1*A*) was employed in which 6.7 kb of c-*fos* genomic DNA was disrupted within the first exon by insertion of a positive selectable marker conferring resistance to hygromycin B. The c-*fos–hyg* gene replacement vector was introduced into F16 ES cells by electroporation, and the cells were selected for resistance to hygromycin B (positive selection for *hyg*) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine (FIAU) (16) (negative selection against thymidine kinase). Thirty-three of the doubly resistant colonies were screened by a polymerase chain reaction protocol that distinguishes between the *hyg*-recombinant and wild-type c-*fos* genes (data not shown). In seven clones the wild-type c-*fos* gene had been replaced by the *hyg*-disrupted construct, producing cells that were "doubly disrupted" in c-*fos*. In three additional clones the previously *neo*-disrupted c-*fos* gene had been replaced by the *hyg*-disrupted construct (effectively swapping a *hyg* gene for a *neo* gene). The structure of the c-*fos* loci in these cell lines was confirmed by Southern blot analysis (Fig. 1*B*).

The disrupted c-*fos* loci should be unable to produce functional c-*fos* mRNA. This prediction was tested by Northern blot analysis of RNA isolated from doubly disrupted ES cell lines (Fig. 2*A*). To facilitate detection of mRNAs transcribed from the c-*fos* locus, ES cell lines were serum-starved and then reexposed to serum, a procedure known to greatly increase c-*fos* mRNA production in a variety of cell types (1). We found that serum stimulation of starved wild-type ES cells led to a dramatic increase in c-*fos* mRNA levels within 60 min, making the message easy to detect by Northern analysis (Fig. 2*A*). With the same stimulation protocol, the 2.2-kb wild-type c-*fos* mRNA was undetectable in doubly disrupted ES cells. Long exposures of the Northern blots allowed detection of low levels of additional serum-inducible mRNAs that hybridized with the c-*fos* probe. These transcripts most likely resulted from transcription that read through the *neo* or *hyg* gene and then into the rest of the c-*fos* gene (data not shown). While these rare hybrid transcripts contain some c-*fos* sequences, they would not be expected to produce functional c-Fos protein since the gene disruption interrupts c-*fos* at codon 21, leaving the bulk of the c-*fos* protein-coding region downstream of the translation termination site.

To confirm that the doubly disrupted ES cells do not synthesize wild-type c-Fos protein or a c-Fos/Neo or c-Fos/Hyg fusion protein, immunoprecipitation experiments were performed using antisera that recognize a region of c-Fos downstream of the point of disruption (Fig. 2*B*). In these experiments, wild-type or doubly disrupted ES cells were serum-starved and then labeled with [^{35}S]methionine during serum stimulation. Cell lysates made under denaturing conditions were immunoprecipitated with either of two antisera that recognize the c-Fos protein. Antiserum raised to a bacterially expressed TrpE/c-Fos fusion protein (21) immunoprecipitated a 53-kDa protein from serum-stimulated wild-type ES cells. The intensity of this band was greatly reduced in unstimulated cells or when the antiserum was preincubated with TrpE/c-Fos protein. Furthermore, a second antiserum, with specificity for c-Fos [and not other known Fos-related proteins (22)], recognized the same protein. Thus, we conclude that this protein is c-Fos. Neither the 53-kDa c-Fos protein nor new truncation or fusion proteins was detected in immunoprecipitates from doubly disrupted ES cells with either antiserum. Hence, the doubly disrupted ES cells do not contain functional c-*fos* mRNA or detectable c-Fos protein and thus, to the limits of detection, are c-*fos*-null. Remarkably, these ES cells clearly are viable.

The ability of the doubly disrupted ES cell lines to grow and differentiate was examined. As shown in Fig. 3*A*, a doubly disrupted ES cell line and wild-type ES cells had comparable growth curves and were still growing exponentially 9 days after plating at low density. We took advantage of this fact to assess more accurately the doubling times of various singly or doubly disrupted ES cell lines by measuring the number of cell doublings over 9 days. All cell lines had indistinguishable doubling times of ≈15.4 hr (Fig. 3*B*). When grown at a range of densities the wild-type and doubly disrupted ES cells maintained the same doubling time and continued to grow even when cultured in low concentrations of serum (data not shown). A number of studies have suggested that c-*fos* plays

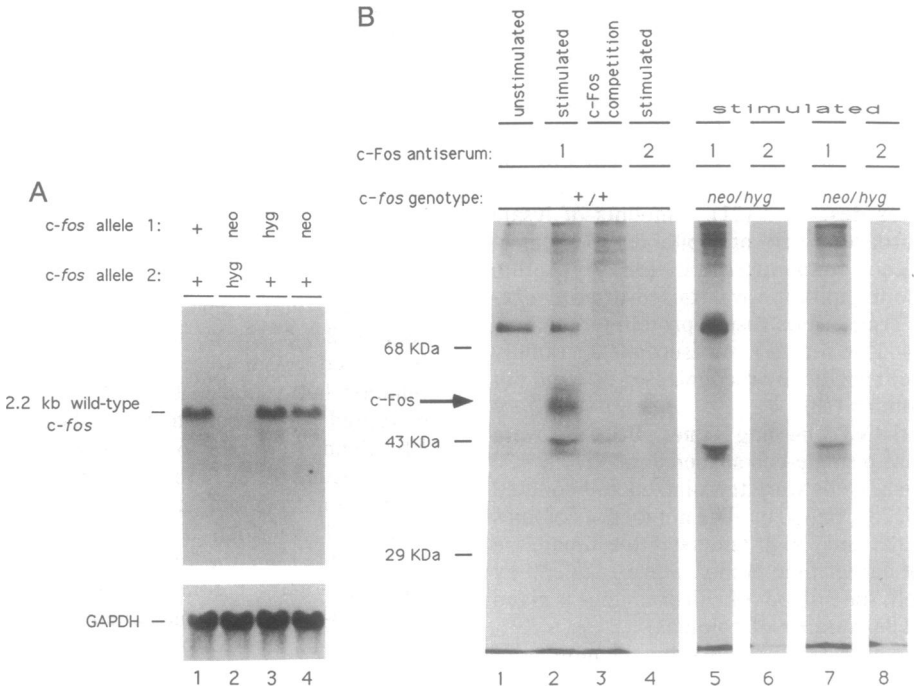

FIG. 2. c-*fos* mRNA and protein are undetectable in c-*fos* doubly disrupted ES cells. (*A*) Northern hybridization analysis using a c-*fos* probe fails to detect wild-type c-*fos* mRNA in the doubly targeted cells. Cells were grown in the absence of serum or LIF for 24 hr, and then stimulated to induce c-*fos* transcription by changing to fresh medium with 20% fetal bovine serum. Sixty minutes later, total RNA was harvested from wild-type (CC1.2, lane 1), doubly disrupted (lane 2), *hyg*-swapped-for-*neo* (lane 3), or *neo*-disrupted (F16, lane 4) cell lines and used for Northern hybridization analysis. The lower panel shows an autoradiogram of the same blot after it was stripped and rehybridized with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. In a separate experiment, a Northern blot examining the expression of c-*fos* mRNA in actively growing cells revealed the same bands, albeit much weaker (data not shown). (*B*) Immunoprecipitations with antisera that recognize c-Fos fail to precipitate c-Fos from doubly targeted cells. Cells were starved and stimulated as in *A*, and 40 min after stimulation (or no stimulation), cells were metabolically labeled with [^{35}S]methionine. Fifty minutes later cells were lysed and immunoprecipitation was carried out. Lysates were made from wild-type cells (CC1.2, lanes 1–4) or either of two doubly disrupted cell lines (lanes 5–8) either after serum starvation with no stimulation (lane 1) or after serum starvation followed by serum stimulation (lanes 2–8). Antiserum raised to an *E. coli*-expressed TrpE/c-Fos fusion protein (antiserum 1) was preincubated with lysates of TrpE-expressing *E. coli* (lanes 1, 2, 5, and 7) or TrpE/c-Fos-expressing *E. coli* (lane 3) before being used for immunoprecipitation. Antiserum 2 (used for lanes 4, 6, and 8) specifically recognizes c-Fos and not other Fos-related proteins.

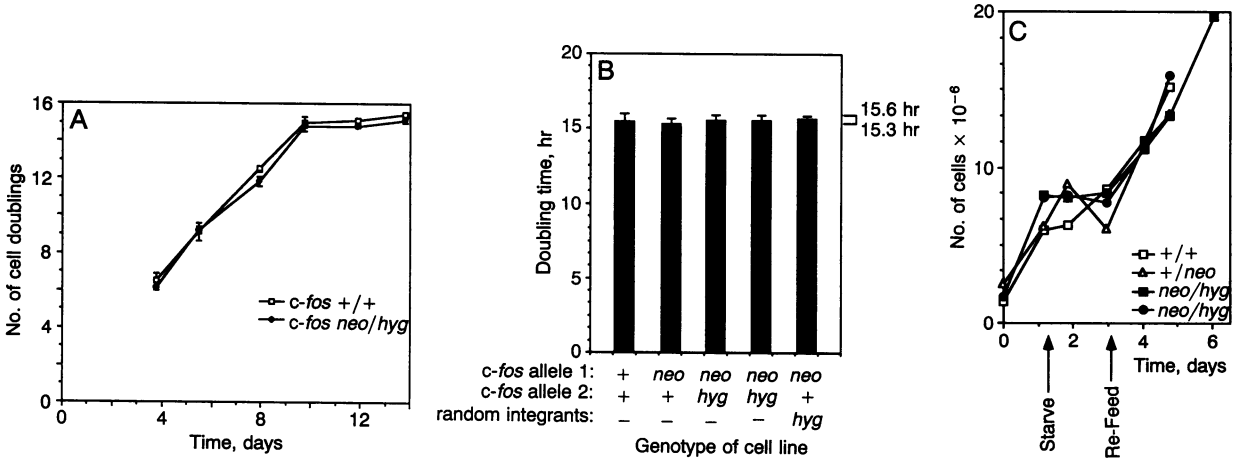

FIG. 3. Growth rates of wild-type and c-*fos* doubly disrupted ES cell lines. (*A*) Growth curves for wild-type (CC1.2) or doubly disrupted cells. The experiment was performed in duplicate. Each point indicates the mean with error bars representing the two measured values. (*B*) Doubling times for several cell lines are compared. For each cell line, doubling time shown is the mean. Error bars represent 95% confidence limits for the mean, with $n = 4$ (bars 2, 4, and 5, from left) or $n = 6$ (bars 1 and 3). (*C*) Ability of various ES cell lines to arrest and then resume growth. Exponentially growing cells were starved for growth factors, to arrest their proliferation, and two days later were refed with complete medium to allow resumption of growth. Values for days 2, 3, and 4 represent the mean for duplicate measurements.

a critical role during the transition of growth-arrested cells from G_0 back into the cell cycle upon treatment with growth factors (7–10). When doubly disrupted (or wild-type) ES cells were starved of growth factors they ceased to increase their cell number, indicating growth arrest. However, when subsequently refed with complete medium containing 20% fetal bovine serum, the doubly disrupted ES cells could resume growth as effectively as wild-type cells (Fig. 3*C*). Thus, the lack of c-Fos had no detectable effect on the ability of ES cells to proliferate under a variety of conditions.

Wild-type ES cells are capable of spontaneously differentiating into a wide variety of cell types *in vitro*. They first form cystic embryoid bodies (25) and subsequently produce a range of morphologically distinguishable cell types, including contractile muscle (26, 27), pigmented erythroid cells (26, 28), adipocyte-like cells with refractile lipid droplets, and neuron-like cells (25, 29). All of these cell types were also observed in differentiated cultures of doubly disrupted cells (Fig. 4), indicating that an intact c-*fos* gene is not essential for ES cells to differentiate into a variety of cell types.

Injection of cells from either of two doubly disrupted clones into mouse blastocysts demonstrated that doubly disrupted cells are also capable of differentiating *in vivo*. Coat color and isoenzyme markers (30) examined in 12 chimeras

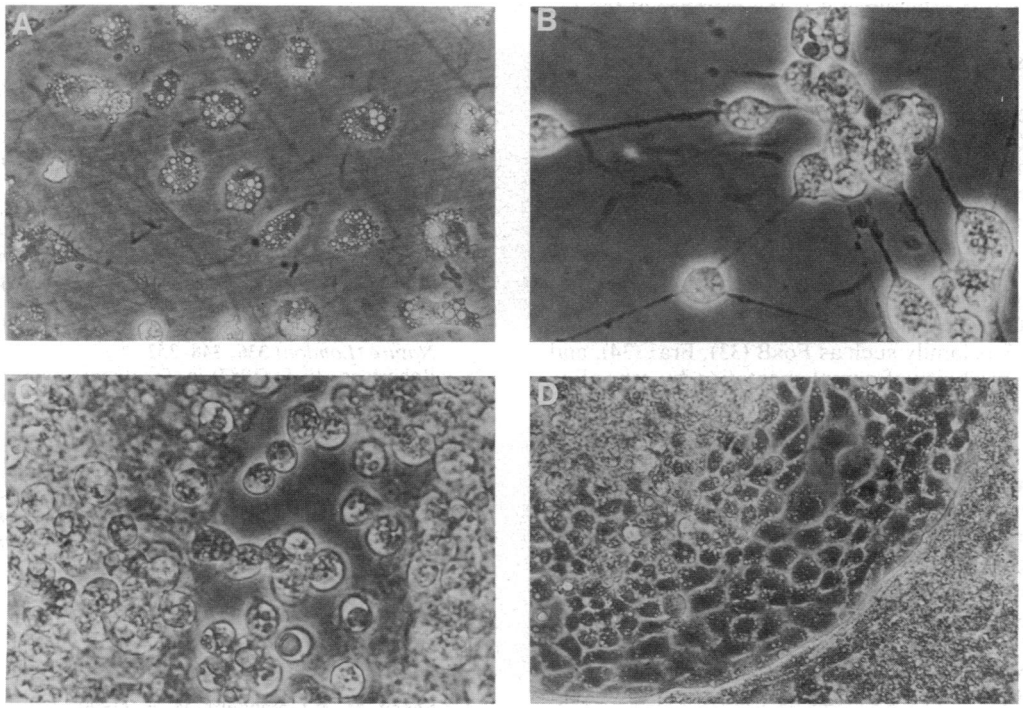

FIG. 4. Examples of the varied morphologies exhibited by c-*fos* doubly disrupted ES cells differentiated *in vitro*. Cells were allowed to grow and differentiate for 3–4 weeks. (*A*) Adipocyte-like cells. (*B*) Neuron-like cells [identified on the basis of cell morphology and staining with a neuron-specific antibody to the 200-kDa neurofilament protein (data not shown)]. (*C* and *D*) Unknown cell types with other distinct morphologies. Other cell types observed included contractile muscle [identified by spontaneous contractility (data not shown)] and pigmented erythrocytes [identified by pigmentation and by the presence of hemoglobin as determined by benzidine staining (data not shown)].

showed up to 25% contributions of doubly disrupted ES cells to a number of tissues, including hair follicles, skin, lung, and brain (data not shown). We conclude that an intact c-*fos* gene is not required for ES cell viability, growth, or differentiation into a number of distinct cell types.

DISCUSSION

The finding that c-*fos* is not essential for ES cell growth and differentiation raises the possibility that c-*fos* actually plays no role in these processes. However, the large body of evidence that has implicated c-*fos* in signal transduction pathways for growth and differentiation (reviewed in refs. 31 and 32) would suggest the alternative hypothesis that other proteins in the doubly disrupted ES cells may compensate for the absence of c-Fos. Such an effect could be due to the inadvertent selection for a compensating mutation or due to a preexisting or newly up-regulated protein with compensatory function. Selection for a compensating extragenic mutation seems unlikely, since the frequency of recovery of homologous recombinants in this study is quite high compared with other reported rates (13, 14). Moreover, because the frequency of homologous recombination into the wild-type c-*fos* locus (rendering the cell doubly disrupted) is similar to the frequency of homologous recombination into the *neo*-disrupted c-*fos* locus (swapping *hyg* for *neo*), a second-site mutation is probably not required for cell viability in the absence of c-*fos*. Likewise, the ability of doubly disrupted ES cells to undergo simple differentiation is not dependent on a second-site mutation since doubly disrupted ES cells can form cystic embryoid bodies at the same frequency as wild-type ES cells (data not shown).

While an extragenic compensatory mutation seems an unlikely explanation for the ability of ES cells to survive in the absence of c-*fos*, it remains possible that a secondary consequence of the loss of c-*fos* is the up-regulation of a gene that has a complementary function. The gene *fosB* could potentially serve this role, since it is the member of the *fos* gene family whose activation and repression upon serum stimulation most closely parallel those of c-*fos* (33). However, we found that *fosB* mRNA levels were unchanged in serum-stimulated c-*fos* doubly disrupted ES cell lines compared with wild-type ES cells. Likewise, the induction of c-*jun*, *junD*, and c-*myc* mRNAs occurred normally when doubly disrupted cells were serum-stimulated (unpublished results). Thus, at least these immediate early genes are not up-regulated to compensate for the loss of c-*fos*.

It is possible that a protein that complements c-Fos function may always be present in ES cells at levels sufficient to replace c-Fos. Good candidates for such proteins are other members of the Fos family such as FosB (33), Fra1 (34), and Fra2 (35). The possibility of complementation by other Fos family members is supported by the results of a recent study (8) in which the microinjection of a combination of antibodies specific for c-Fos, FosB, or Fra-1 into 3T3 fibroblasts was found to be more efficient at preventing cell cycle progression than microinjection of each antibody alone.

In summary, this study has shown that despite a large body of research suggesting otherwise, c-*fos* is not essential for cell viability, proliferation, or differentiation of at least some cell types. This may reflect either an ability of related genes to compensate for c-*fos* function or a lack of a role for c-*fos* in these processes. Nevertheless, the evolutionary conservation of the c-*fos* gene strongly suggests that there are likely to be some functions unique to c-*fos*. Further analysis of the role of c-*fos* in animals bearing mutations in the c-*fos* gene will be useful in determining the nature of these functions and their relationship to the ability of c-Fos to regulate gene expression.

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