MOLECULAR AND CELLULAR BIOLOGY, Dec. 1995, p. 6864–6874 Vol. 15, No. 12
0270-7306/95/$04.00+0

E2F-1:DP-1 Induces p53 and Overrides Survival Factors To Trigger Apoptosis

SCOTT W. HIEBERT,[1] GRAHAM PACKHAM,[2]† DAVID K. STROM,[1] REBECCA HAFFNER,[3] MOSHE OREN,[3] GERARD ZAMBETTI,[2] AND JOHN L. CLEVELAND[2,4]*

Departments of Tumor Cell Biology[1] and Biochemistry,[2] St. Jude Children's Research Hospital, Memphis, Tennessee 38105; Department of Chemical Immunology, Weizmann Institute of Science, Rehovot, Israel[3]; and Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38163[4]

Received 10 July 1995/Returned for modification 23 August 1995/Accepted 30 August 1995

The E2F DNA binding activity consists of a heterodimer between E2F and DP family proteins, and these interactions are required for association of E2F proteins with pRb and the pRb-related proteins p107 and p130, which modulate E2F transcriptional activities. E2F-1 expression is sufficient to release fibroblasts from G_0 and induce entry into S phase, yet it also initiates apoptosis. To investigate the mechanisms of E2F-induced apoptosis, we utilized interleukin-3 (IL-3)-dependent 32D.3 myeloid cells, a model of hematopoietic progenitor programmed cell death. In the absence of IL-3, E2F-1 alone was sufficient to induce apoptosis, and p53 levels were diminished. DP-1 alone was not sufficient to induce cell cycle progression or alter rates of death following IL-3 withdrawal. However, overexpression of both E2F-1 and DP-1 led to the rapid death of cells even in the presence of survival factors. In the presence of IL-3, levels of endogenous wild-type p53 increased in response to E2F-1, and coexpression of DP-1 further augmented p53 levels. These results provide evidence that E2F is a functional link between the tumor suppressors p53 and pRb. However, induction of p53 alone was not sufficient to trigger apoptosis, suggesting that the ability of E2F to override survival factors involves additional effectors.

Members of the E2F family of transcription factors are thought to regulate cell cycle progression by activating the transcription of a set of genes necessary for the induction of S phase (30, 53). E2F DNA binding activities are dependent on growth factors (52), and their function as transcription factors is temporally regulated throughout the cell cycle by complex formation with the tumor suppressor protein pRb and the pRb-related proteins p107 and p130 (9, 10, 13, 43, 69). E2F is activated by adenovirus E1A binding to pRb and its related proteins, and release of E2F from pRb is critical for transformation induced by both E1A (18, 61) and pRb inactivation (29, 31, 57, 58).

The DNA binding activity originally termed free E2F (3) is now recognized to be a heterodimer containing the product of an E2F gene family member (*E2F1* to *E2F5*) and a DP family member (5, 21–23, 27, 34, 38, 44, 72, 76). E2F can bind DNA in vitro, whereas DP proteins bind DNA only weakly (23). Dimerization of DP proteins with E2F proteins increases the transcriptional activity of E2F and is required for association of E2F with pRb or pRb-related proteins (4, 5, 21, 28, 41). Furthermore, enforced DP-1 expression augments E2F-mediated transformation of primary rat embryo fibroblast cells in cooperation with an activated *ras* oncogene (5, 21, 35).

Microinjection of serum-starved fibroblasts with an E2F-1 expression plasmid (37) or glutathione *S*-transferase–E2F-1 fusion protein (17), or activation of E2F-1 expression in transfected cell lines (59, 67), is sufficient to drive quiescent cells into S phase. Inappropriate entry of these cells into S phase, in the absence of survival factors, is associated with the activation of apoptosis (59, 67). E2F-1-induced apoptosis in fibroblasts is potentiated by high levels of exogenous wild-type p53 (59, 73), a known trigger of programmed cell death (47, 75). In addition, E2F-1-induced apoptosis in serum-deprived fibroblasts is partially inhibited by overexpression of mutant p53 (40, 59). Conversely, p53-induced apoptosis can be blocked by overexpression of functional pRb (26, 55).

The physiological relevance of E2F-1-induced apoptosis in fibroblasts is unclear. In contrast to hematopoietic cells, fibroblasts enter a quiescent state, rather than die, in response to growth factor withdrawal. Committed hematopoietic progenitors are programmed to undergo apoptosis unless continually provided with hemopoietins which promote cell survival both in vivo and in vitro (63, 71). We have utilized diploid 32D.3 myeloid cells which continuously require interleukin-3 (IL-3) for their growth and survival as a physiological model of programmed cell death. Like normal myeloid progenitors, 32D.3 cells transiently accumulate in G_1 when deprived of IL-3, before ultimately succumbing to apoptosis (1). However, this program can be dramatically altered by enforced expression of oncogenes, including c-*myc* (1), *Bcl-2* (2), or v-*raf* (12).

Here we report that overexpression of E2F has unusual biological consequences. High levels of E2F activity override survival factors to trigger apoptosis. Furthermore, p53 levels increase in response to high levels of E2F. However, E2F-induced apoptosis can be uncoupled from p53 accumulation.

MATERIALS AND METHODS

Establishment and maintenance of cell lines. 32D.3 cells were maintained in RPMI 1640–10% fetal calf serum–1% L-glutamine medium supplemented with 20 U of IL-3 per ml as previously described (12). Parental 32D.3 cells were electroporated as previously described (1) with 10 μg of linearized expression plasmids. All expression plasmids were derived from the dexamethasone-inducible pMAM-Neo plasmid (Clontech). The E2F-1 expression plasmid contains the human E2F-1 cDNA (38) (the cDNA was a gift from William Kaelin, Dana-

* Corresponding author. Phone: (901) 495-2398. Fax: (901) 525-8025.
† Present address: Ludwig Institute for Cancer Research, St. Mary's Hospital Medical School, London, Great Britain.

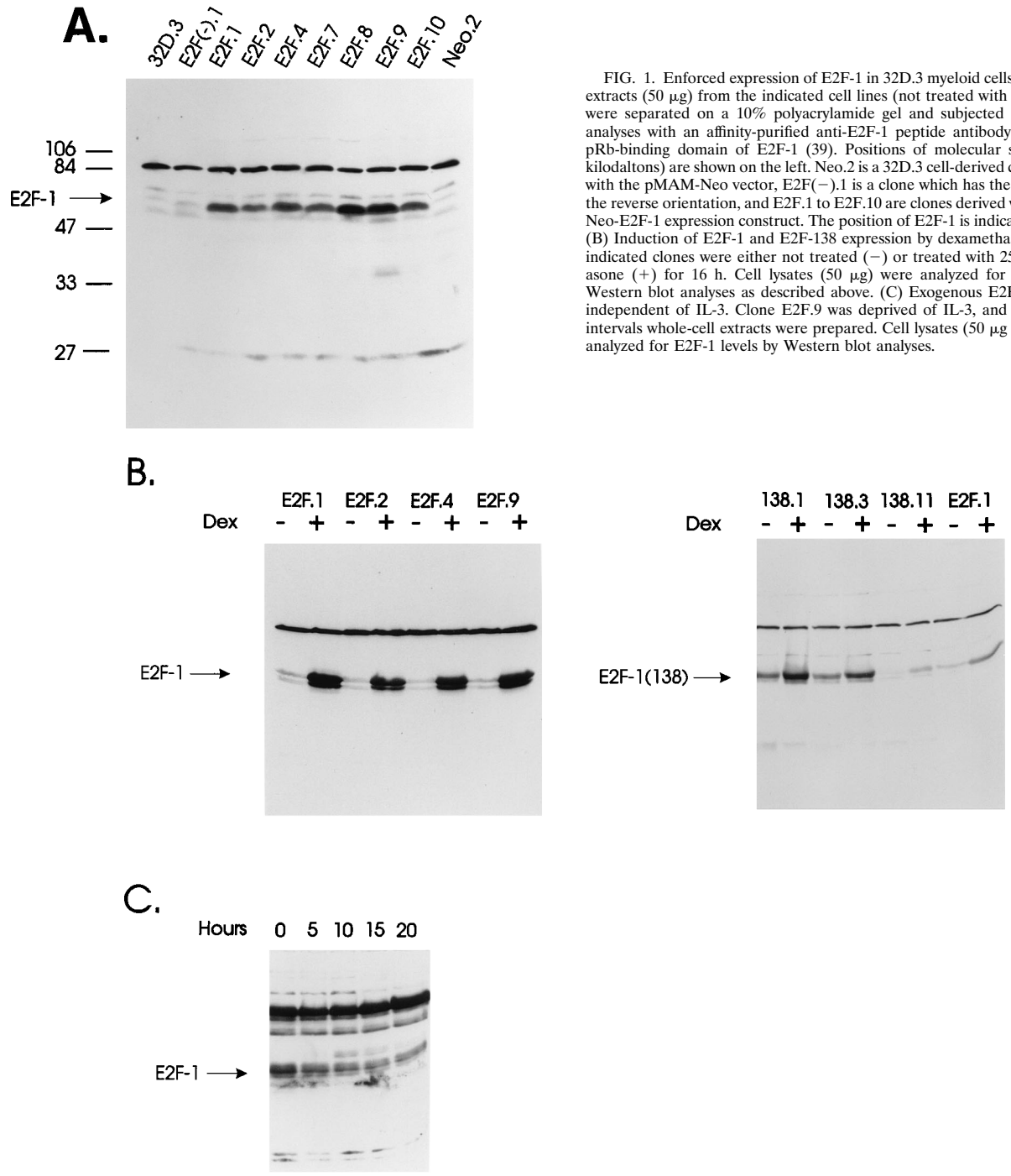


FIG. 1. Enforced expression of E2F-1 in 32D.3 myeloid cells. (A) Whole-cell extracts (50 μg) from the indicated cell lines (not treated with dexamethasone) were separated on a 10% polyacrylamide gel and subjected to Western blot analyses with an affinity-purified anti-E2F-1 peptide antibody directed to the pRb-binding domain of E2F-1 (39). Positions of molecular size markers (in kilodaltons) are shown on the left. Neo.2 is a 32D.3 cell-derived clone transfected with the pMAM-Neo vector, E2F(−).1 is a clone which has the E2F-1 cDNA in the reverse orientation, and E2F.1 to E2F.10 are clones derived with the pMAM-Neo-E2F-1 expression construct. The position of E2F-1 is indicated by an arrow. (B) Induction of E2F-1 and E2F-138 expression by dexamethasone (Dex). The indicated clones were either not treated (−) or treated with 25 μM dexamethasone (+) for 16 h. Cell lysates (50 μg) were analyzed for E2F-1 levels by Western blot analyses as described above. (C) Exogenous E2F-1 expression is independent of IL-3. Clone E2F.9 was deprived of IL-3, and at the indicated intervals whole-cell extracts were prepared. Cell lysates (50 μg of protein) were analyzed for E2F-1 levels by Western blot analyses.

Farber Cancer Institute). The E2F-1 mutant expression plasmid, here designated E2F-138, contains an *Eco*RI linker inserted at human E2F-1 amino acid 138, which abolishes DNA binding (14) (the cDNA was a gift from Joseph Nevins, Duke University Medical Center). The human DP-1 pMAM-Neo expression plasmid contains the cDNA insert of DP-1 fused at its amino terminus with an influenza virus hemagglutinin (HA) epitope tag (HA-DP-1; the cDNA was a gift from Ed Harlow, Massachusetts General Hospital Cancer Center) (28). Transfected cells were selected in medium containing G418, and single-cell clones (10 from each transfection) were derived by cloning in methylcellulose containing IL-3 and G418 as previously described (1). To generate E2F-1 clones which coexpressed DP-1, two independent E2F-1 clones (E2F.1 and E2F.9) were cotransfected with pMAM-Neo HA-DP-1 plasmid DNA and the hygromycin resistance expression plasmid pRMM (kindly provided by Celeste Simon, University of Chicago). For controls, drug-resistant colonies were also derived from transfections of pMAM-Neo (Neo clones) and plasmids containing the E2F-1 and DP-1 cDNAs in the reverse orientation [pMAM-Neo-E2F-1(−) and pMAM-Neo-HA-DP-1(−), respectively].

To ensure that all clones were analyzed at equivalent exponential growth phases, cells were set twice at 0.5×10^6 cells per ml on consecutive days before analysis. To induce high levels of E2F-1 and DP-1 expression, cells were treated with 25 μM dexamethasone (Sigma). To investigate the effects of enforced E2F-1 or DP-1 expression upon viability, cells in IL-3 were treated with dexamethasone for 16 h, washed twice in RPMI 1640 medium lacking IL-3, and resuspended at 10^6 cells per ml in RPMI 1640 medium. Alternatively, the viability of cells in IL-3 was assessed in medium either lacking or containing 25 μM dexamethasone. Cell viability was determined with a hemocytometer and trypan blue dye exclusion as an indicator of cell membrane integrity.

Determination of cell cycle distributions. Cells were collected by centrifuga-

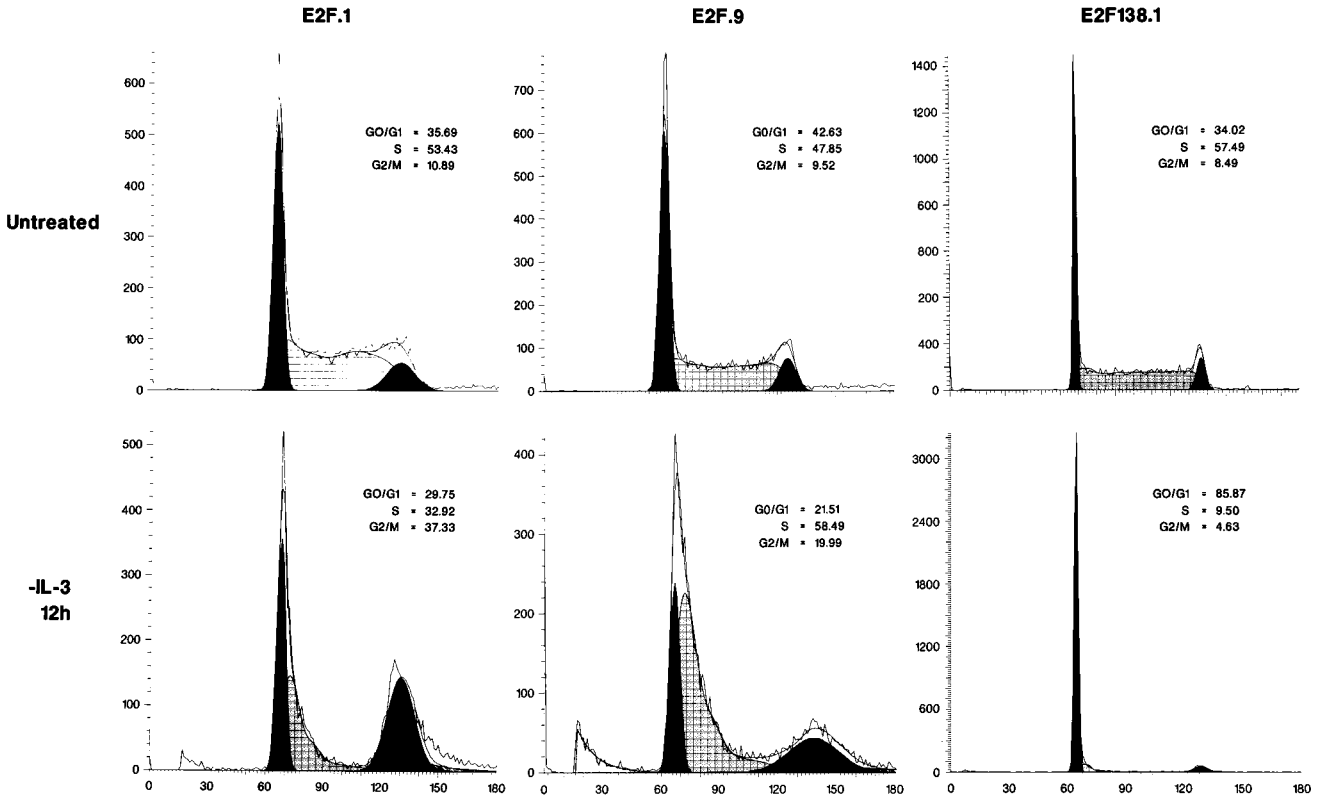


FIG. 2. Enforced E2F-1 expression suppresses G_1 arrest and promotes S-phase entry in the absence of IL-3. Cells were divided on consecutive days to ensure they were at similar phases of growth and then were treated with dexamethasone for 16 h prior to the experiment to superinduce E2F levels. Representative histograms from the indicated cell lines are shown. The cell cycle distribution of cells from exponentially growing cultures (untreated) is compared with that of cells 12 h after IL-3 withdrawal. The percentages of cells in the G_1, S, and G_2/M phases of the cell cycle are indicated. Note that the distribution of cells within S phase for E2F-1 clones indicates efficient entry of these cells into S phase.

tion and resuspended in 0.1% sodium citrate containing 50 μg of propidium iodide per ml. Fluorescence of propidium iodide-stained cells was measured as previously described (1) with a FACSCAN flow cytometer (Becton Dickinson). The percentages of cells within the G_0/G_1, S, and G_2/M phases of the cell cycle were determined as previously described (1).

Apoptosis assays. Cells at exponential growth phase were washed twice in medium lacking IL-3 and resuspended at 10^6 cells per ml in medium lacking IL-3. Cell morphology was analyzed following cytospin and Wright-Giemsa staining, and genomic DNA was isolated from 10^6 cells and analyzed on a 2% agarose gel as previously described (1).

Gel mobility shift assays. Whole-cell extracts from cells cultured in IL-3 with or without 25 μM dexamethasone were prepared by the sonication method of Scholer et al. (65). Gel mobility shift assays were performed with 5 to 10 μg of whole-cell extract and 0.25 ng of a ^{32}P-end-labeled double-stranded oligonucleotide containing the E2F site from the c-*myc* promoter as described previously (64).

Antibodies and protein analysis. Anti-p107 serum was a kind gift from Joseph R. Nevins (Duke University Medical Center). The E2F-1 antibody, directed against the E2F-1 pRb-binding domain, has been characterized previously (39, 44) and detects all E2F family members expressed in SAOS-2 cells (39) and 32D.3 cells. The anti-HA monoclonal antibody 12CA5 was purchased from Babco (Berkeley, Calif.). The pRb antibody (Rb-1) was purchased from Oncogene Science. For Western blot (immunoblot) analysis, E2F-1 affinity-purified antibodies were used at a 1:100 dilution, and the anti-HA monoclonal antibody was used at a 1:1,000 dilution, in 5% nonfat dry milk in Tris-buffered saline.

32D.3 myeloid cells are wild type for p53 (24). For analysis of p53 levels, cells were split twice on consecutive days at 0.5×10^6 cells per ml and treated with 25 μM dexamethasone to induce high-level E2F-1 and HA-DP-1 expression. Cells were harvested and lysates were prepared as previously described (20). Equal amounts of protein were immunoprecipitated with the anti-p53 monoclonal antibody PAb421 (20). Immunoprecipitated proteins were resolved by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and transferred to nitrocellulose, and p53 protein was detected by using CM1 p53 antibody (Nova Costra) with an ECL detection kit (Amersham).

To analyze p53 levels following IL-3 withdrawal, cells were treated with dexamethasone overnight, washed to remove steroid and IL-3, and resuspended in medium with or without IL-3 (and lacking dexamethasone). After 8 h, the cells were harvested, equal amounts of protein were immunoprecipitated with p53 antibody, and p53 levels were determined by Western blot analysis. For negative controls, lysates of the murine myeloid leukemia M1 cell line, which is devoid of endogenous p53, were immunoprecipitated with PAb421.

RESULTS

Enforced E2F-1 overexpression in 32D.3 myeloid cells promotes S-phase entry and induces apoptosis following IL-3 withdrawal. To investigate whether enforced E2F-1 expression would influence the G_1-to-S-phase transition and induce apoptosis in a physiological setting, we generated individual 32D.3 clones overexpressing E2F-1 (clones E2F.1 and E2F.2, etc.) and examined these clones for levels of E2F-1 protein. The E2F-1 gene was cloned downstream of the dexamethasone-inducible mouse mammary tumor virus promoter in the pMAM-Neo expression vector. Of 10 G418-resistant clones tested, 7 displayed levels of E2F-1 5- to 10-fold higher than those present in parental 32D.3 cells, in clones containing vector alone (Neo.2), or in clones transfected with the E2F-1 cDNA in the reverse orientation, even in the absence of dexamethasone [E2F(−).1; Fig. 1A]. Therefore, in contrast to the case with fibroblasts or keratinocytes (49, 59, 67, 73), E2F-1 overexpression is tolerated in these myeloid cells. We also generated clones expressing an E2F-1 mutant, E2F-138, which has a linker insertion within the DNA binding domain of E2F-1 which abolishes DNA binding (14). These clones expressed levels of E2F-1 comparable to or exceeding those present in clones expressing wild-type E2F-1 (Fig. 1B). The

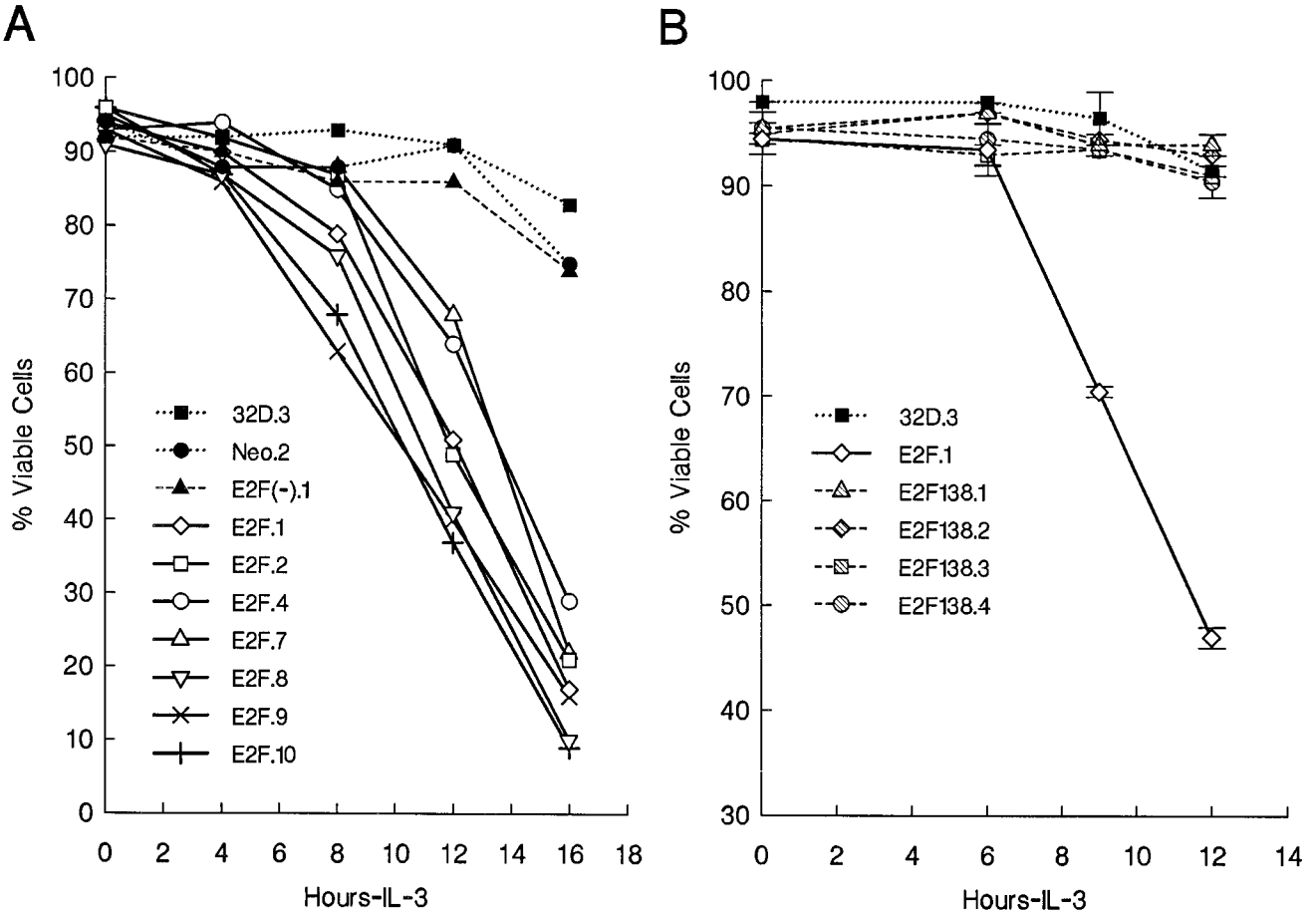


FIG. 3. Enforced expression of E2F-1 accelerates cell death following IL-3 withdrawal. The indicated cell lines were thoroughly washed to remove IL-3 and resuspended in medium without IL-3. Cell viability was determined by trypan blue dye exclusion at the indicated intervals following IL-3 withdrawal. (A) Rates of cell death of E2F-1 clones compared with those of a control Neo clone (Neo.2), a clone with the E2F-1 cDNA in the reverse orientation [E2F(−).1], and parental 32D.3 cells. (B) Comparison of the rates of death of E2F-1 and E2F-138 clones (in duplicate). Results are representative of more than 10 separate experiments with E2F-1 clones and 3 experiments with E2F-138 clones. The error bars in panel B are derived from averages of viability determinations for duplicate cultures of cells. Similar results were obtained in the presence or absence of dexamethasone.

addition of dexamethasone to the culture medium markedly increased (another 5- to 10-fold) levels of the 58-kDa E2F-1 protein in both wild-type and mutant E2F-138 clones (Fig. 1B), confirming that this represented exogenous E2F-1 expression. E2F-1 overexpression was maintained in E2F-1 clones deprived of IL-3 (Fig. 1C), demonstrating that activity of the expression construct was independent of IL-3.

When cells were grown in IL-3, overexpression of E2F-1 did not result in a reduction of cell doubling time (data not shown), and the cell cycle distributions of E2F-1 overexpressers were similar to those of parental cells and to those of clones expressing mutant E2F-1 (Fig. 2 and data not shown). However, E2F-1 overexpression can induce serum-deprived fibroblasts to exit from a quiescent G_0 state and progress through G_1 into S phase (17, 37, 59, 67). Therefore, we deprived cells overexpressing E2F-1 and E2F-138 of IL-3 and measured changes in their cell cycle distribution. E2F-1 clones were markedly deregulated and continued to enter S phase in the absence of IL-3 (Fig. 2). By contrast, cells expressing the E2F-138 mutant protein behaved like Neo clones and parental 32D.3 cells (1) and accumulated in G_1 following IL-3 withdrawal (Fig. 2). Therefore, E2F-1 overexpression is sufficient to drive cells into S phase in the absence of IL-3.

The ability of enforced E2F-1 expression to override signals in 32D.3 cells to withdraw from the cell cycle following removal of IL-3 was similar to that observed with clones constitutively expressing c-*myc*, which also undergo rapid apoptosis (1). To test whether enforced overexpression of E2F-1 was similarly associated with accelerated apoptosis, E2F-1, E2F-138, and pMAM-Neo clones and clones which expressed E2F-1 in the reverse orientation [E2F(−) clones] were deprived of IL-3, and their viabilities were assessed by trypan blue dye exclusion. All E2F-1 clones displayed an accelerated loss of viability following IL-3 withdrawal, whereas the maintenance of viability of E2F-138 clones was indistinguishable from those of parental 32D.3 cells, Neo clones, and E2F-1(−) clones (compare Fig. 3A and B).

To verify that the accelerated death of E2F-1 clones was due to the induction of apoptosis, E2F-1 clones were characterized for their morphology and the integrity of their genomic DNA following IL-3 withdrawal. The morphologies of parental 32D.3 cells and E2F-1 clones grown in IL-3 were virtually identical. However, following IL-3 withdrawal, E2F-1 clones displayed characteristics typical of apoptosis, including the formation of micronuclei (Fig. 4A). Furthermore, in contrast to parental 32D.3 cells and E2F-1(−) and E2F-1-138 clones, all

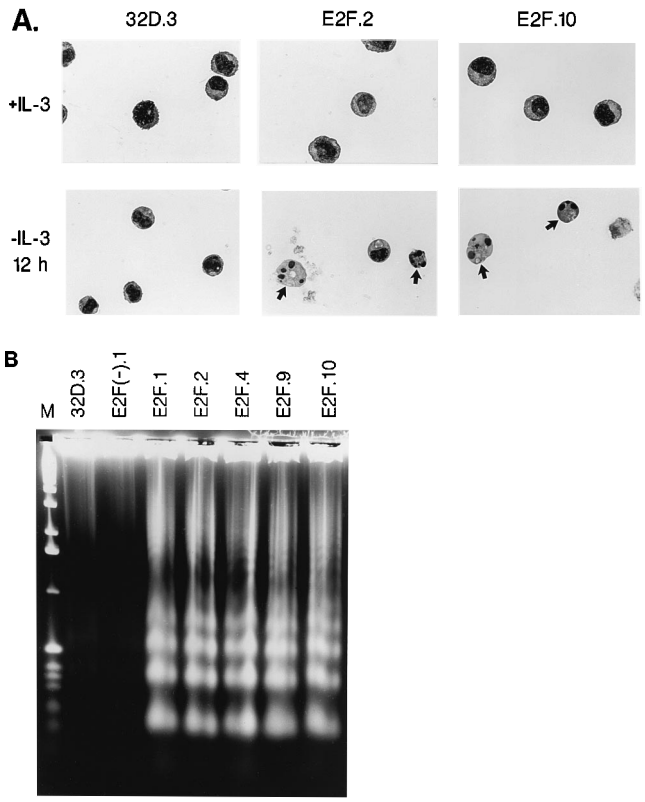


FIG. 4. E2F-1 induces apoptosis following IL-3 withdrawal. (A) Morphologies of 32D.3 cells and E2F-1 clones grown in IL-3 and following IL-3 withdrawal. Parental 32D.3 and E2F-1 clones were set at 0.5×10^6 cells per ml on the day before the experiment. On the following day the cells were washed thoroughly and resuspended in growth medium without IL-3. Cytospins were prepared from cells grown in IL-3 and 12 h after removal of IL-3, and cells were stained with Wright-Giemsa stain. Arrows indicate cells undergoing apoptosis. (B) Analysis of genomic DNAs of E2F-1 clones following IL-3 withdrawal. Parental 32D.3 cells, an E2F-1(−) clone, and E2F-1 clones were set at 0.5×10^6 cells per ml, and on the following day the cells were washed thoroughly and resuspended in growth medium without IL-3. Genomic DNA was analyzed at 9 h following IL-3 withdrawal. Lane M, DNA from the 1-kbp DNA ladder (Gibco).

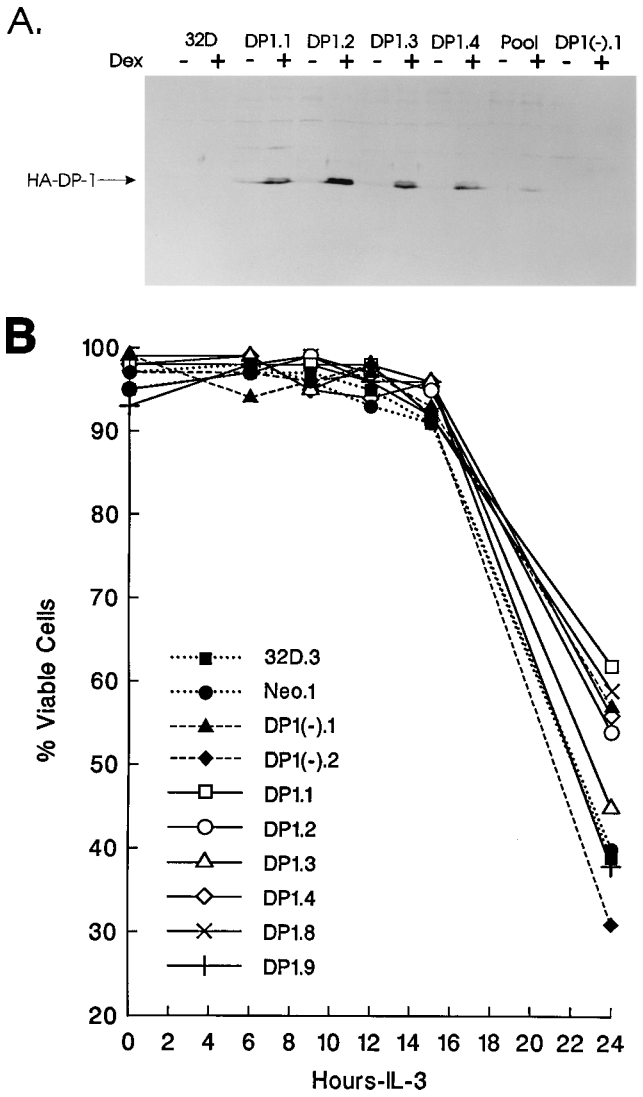


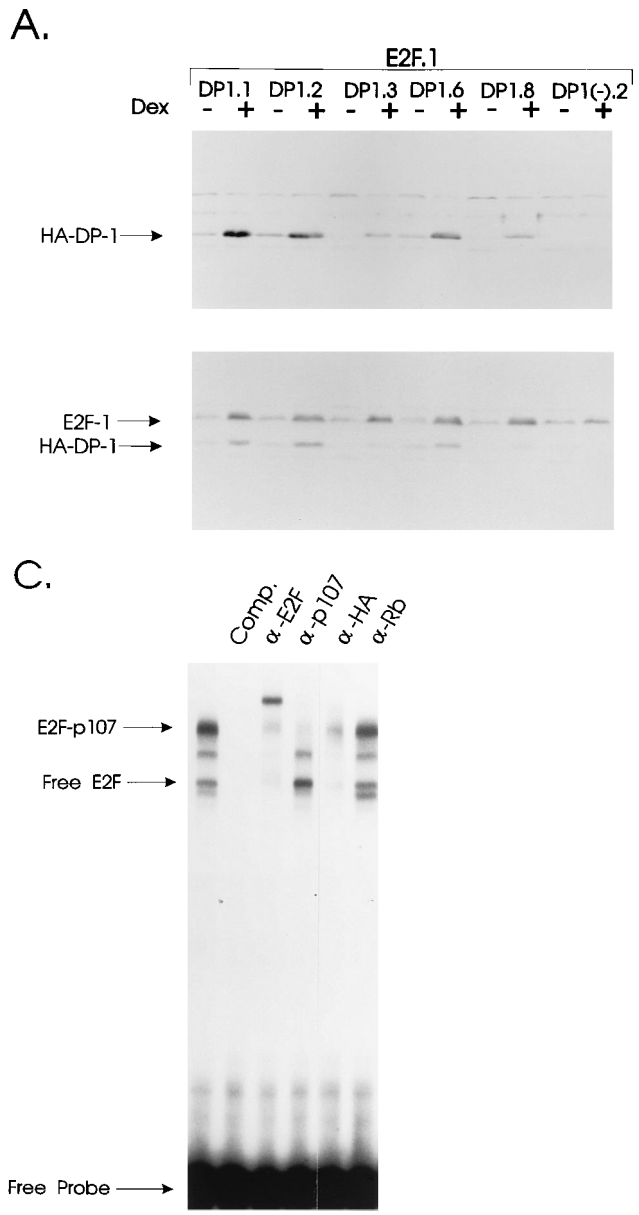
FIG. 5. Enforced DP-1 expression is not sufficient to induce apoptosis. (A) Western blot analysis of cells expressing HA-DP-1 in the absence of dexamethasone (Dex) (−) or after treatment with dexamethasone for 16 h (+). Whole-cell extracts (50 μg) from the indicated clones and parental 32D.3 cells were separated on a 10% polyacrylamide gel and subjected to Western blot analysis with the anti-HA monoclonal antibody 12CA5. The position of migration of HA-DP-1 is indicated by an arrow. Clone DP(−).1 is derived from cells transfected with the HA-DP-1 cDNA in the reverse orientation. (B) Overexpression of DP-1 does not accelerate cell death. The indicated clones were deprived of IL-3, and their viabilities were determined at the indicated intervals. The results shown are representative of four independent experiments.

E2F-1 clones displayed a rapid degradation of genomic DNA into oligonucleosomal ladders typical of apoptosis after 9 h of IL-3 deprivation (Fig. 4B). Enforced E2F-1 expression is therefore sufficient to promote inappropriate S-phase entry and to accelerate apoptosis. The DNA binding activity of E2F-1 is required for the induction of apoptosis, as inferred from the results obtained with the E2F-138 mutant.

Enforced DP-1 expression fails to alter cell cycle progression or to induce apoptosis. DP-1 protein interacts with E2F-1 and is considered to be required for E2F-1 function (4, 28, 41). To test the effects of DP-1 expression, we generated clones which expressed human DP-1 fused to an antigenic epitope of influenza virus HA (HA-DP-1). Individual clones harboring the HA-DP-1 construct in the sense, but not reverse, orientation expressed a dexamethasone-inducible protein of the predicted size (50 kDa) for HA-DP-1 as detected by Western blot assays with the anti-HA monoclonal antibody (Fig. 5A).

To address whether DP-1 altered cell cycle regulation or survival of 32D.3 cells, DP-1 clones were deprived of IL-3, and their cell cycle distributions and viability were determined. In contrast to the case for E2F-1, starved DP-1 clones displayed cell cycle distributions typical of control Neo clones and accumulated in G_1 (data not shown). Augmented DP-1 levels were not associated with changes in the rates of cell death (Fig. 5B). Therefore, DP-1 does not alter cell cycle regulatory controls or induce apoptosis in 32D.3 myeloid progenitor cells.

DP-1 augments DNA binding activity in E2F-1-overexpressing cells. DP-1 enhances E2F-1 DNA binding in vitro, suggesting that the combined overexpression of E2F-1 plus DP-1 may increase E2F binding in cells (28, 35, 41). Subclones of two E2F-1 clones which also overexpressed HA-DP-1 (Fig. 6A) were generated, and extracts were tested for changes in DNA binding activity following induction with dexamethasone. Similar to the findings of others (35, 59), overexpression (approximately 10- to 50-fold) of E2F-1 alone (Fig. 1B) had modest effects on E2F-1 DNA binding activity (Fig. 6B). Similar re-



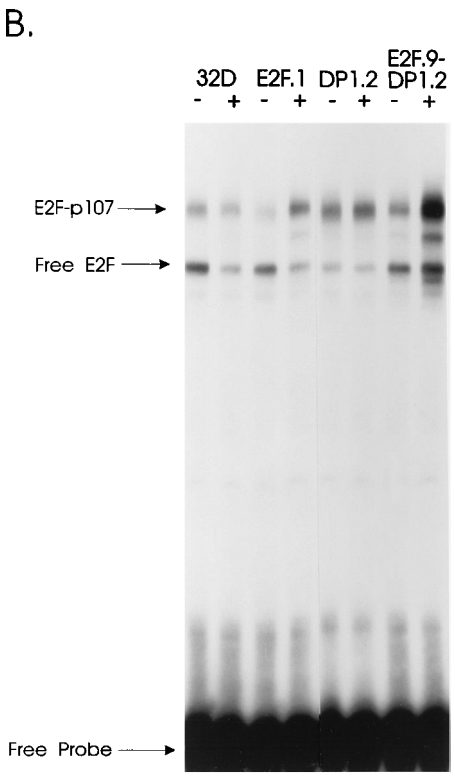


FIG. 6. DP-1 modulates E2F-1-induced DNA binding activity. (A) Western blot analysis of cells expressing both HA-DP-1 and E2F-1, first probed with anti-HA (top panel) and then incubated with anti-E2F-1 antibodies (bottom panel [some HA-DP-1 is still visible in this panel]). The cells were incubated with (+) or without (−) dexamethasone (Dex). (B) DNA binding activities of whole-cell extracts (10 μg) from clones expressing E2F-1, HA-DP-1, or both, in the absence (−) or presence (+) of dexamethasone, were analyzed by electrophoretic mobility shift assay. (C) Identification of E2F-containing complexes. Whole-cell extracts (10 μg) from an E2F-1- and HA-DP-1-expressing clone grown in IL-3 were supershifted with the indicated antiserum. Comp., E2F binding site competitor DNA was added. α-, anti-.

sults were obtained with HA-DP-1. However, overexpression of both E2F-1 and HA-DP-1 increased the abundances of all detectable E2F binding complexes (Fig. 6B).

To confirm the composition of the E2F-containing complexes, antibodies specific for E2F and E2F-binding proteins were included in gel mobility shift assays of extracts from E2F-1- and HA-DP-1-overexpressing cells (Fig. 6C). Addition of anti-p107, but not anti-pRb, antibodies disrupted the upper complex (labeled E2F-p107 in Fig. 6C), whereas anti-E2F antibodies affected all complexes (Fig. 6C). Importantly, anti-HA antibody supershifted all complexes (the lower complex likely shifts into the upper complex in this experiment [Fig. 6C]), demonstrating that expression of exogenous HA-DP-1 was sufficient to replace nearly all of the endogenous DP-1 present in this binding complex. Thus, as expected (44), enforced overexpression of both E2F-1 and HA-DP-1 augments E2F-p107 complex formation (Fig. 6C). The intermediate complex detected in induced E2F-1 and E2F-1–HA-DP-1 extracts was not affected by pRb antibodies and migrates with a mobility consistent with an E2F-p130 complex (Fig. 6B and C) (13). Although pRb appears to be wild type in these cells (69a), no E2F-pRb complex was detected, consistent with observations with other murine cells (51, 66).

An elevated level of E2F-1 plus DP-1 overrides survival functions provided by IL-3. Since overexpression of both E2F-1 and DP-1 was required to augment DNA binding activity, we examined the consequences of high levels of E2F-1 and DP-1 for cell survival in the presence and absence of IL-3. Surprisingly, induction of E2F activity was deleterious to survival, as cells expressing both E2F-1 and HA-DP-1, but not cells expressing E2F-1 alone, died in the presence of IL-3 (10 to 20% viable cells by 40 h [Fig. 7A]). This cell death was due to apoptosis as judged by typical changes in morphology (data not shown). Measurements of cell cycle distributions revealed no obvious differences in dexamethasone-treated cells expressing both E2F-1 and HA-DP-1 (data not shown). Therefore, death due to elevated E2F activity was not due to an apparent imbalance in the cell cycle. Withdrawal of IL-3 from cells expressing both E2F-1 and DP-1 (after 16 h in dexamethasone) further accelerated their death compared with the case for E2F-1 clones (Fig. 7B).

p53 accumulates in response to elevated E2F levels. Because p53 is stabilized in response to E1A (16, 46), we induced E2F-1

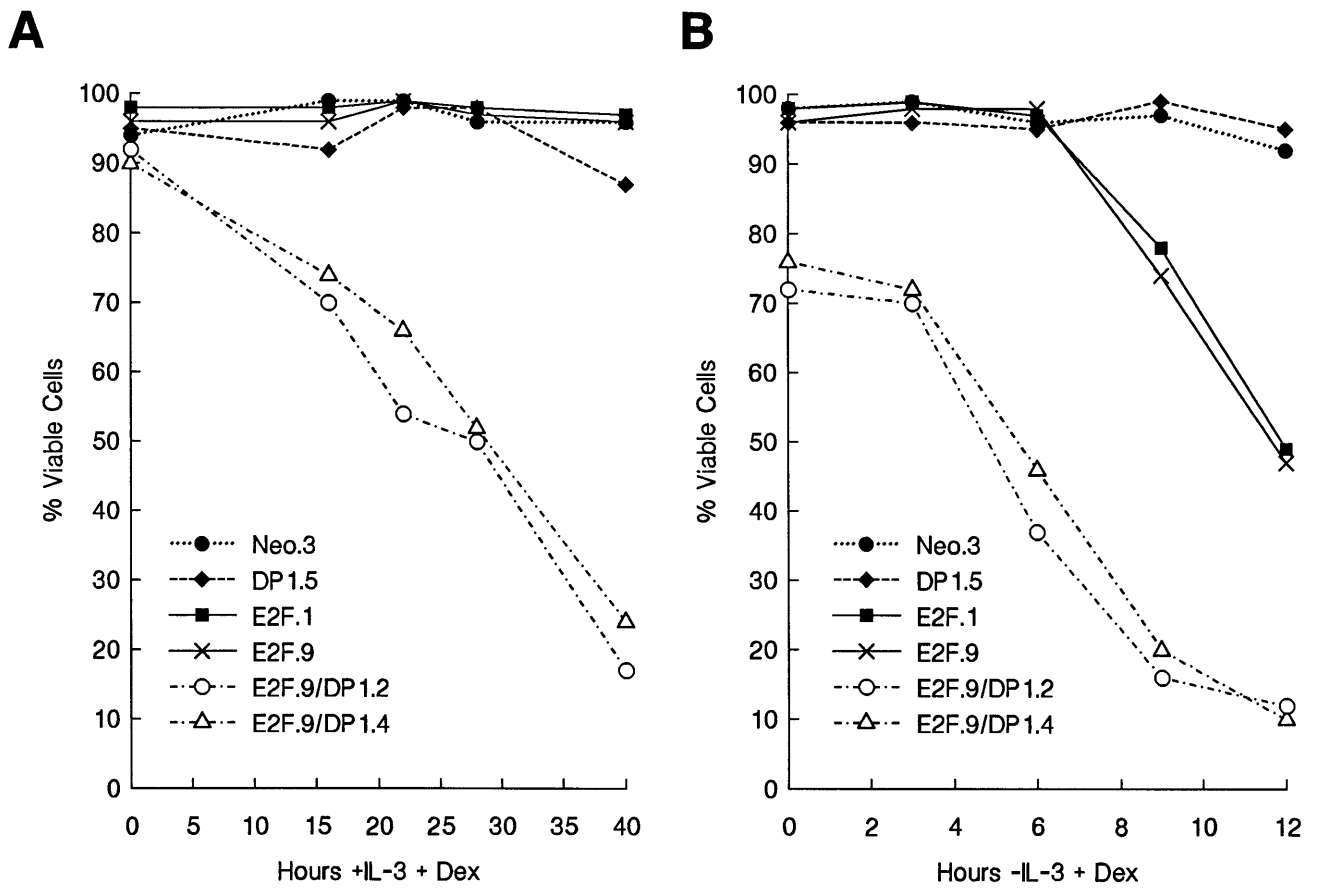


FIG. 7. DP-1 plus E2F-1 overrides survival factor to induce apoptosis. (A) High levels of E2F-1 plus DP-1 induce apoptosis even in the presence of IL-3 and serum. The indicated clones were treated with dexamethasone (Dex), and their viabilities were determined at the indicated intervals. (B) DP-1 accelerates E2F-1-induced cell death in the absence of IL-3. The rates of death of HA-DP-1 subclones of clone E2F.9 were compared with those of E2F-1, DP-1, and Neo clones. The results shown in each panel are representative of five independent experiments, and similar findings were observed with HA-DP-1 subclones of clone E2F.1.

and DP-1 and examined the effects on the steady-state levels of p53. After 4 h of dexamethasone treatment, E2F-1 and HA-DP-1 reached maximum levels in all clones (Fig. 8A). Wild-type p53 levels were increased in E2F-1 clones after 8 h of hormone treatment (approximately fourfold; Fig. 8B and data not shown) but not in vector control clones or in cells expressing only HA-DP-1 (Fig. 8B and data not shown). By contrast, p53 levels were induced to a greater extent, and continued to accumulate, in cells expressing both E2F-1 and HA-DP-1. The induction of p53 occurred at a posttranscriptional level, as p53 mRNA levels were not induced (data not shown).

Accumulation of p53 can be uncoupled from the induction of apoptosis. Since overexpression of E2F-1 alone was sufficient to induce accelerated death when 32D.3 cells were deprived of IL-3, we investigated whether these effects were also associated with increases in p53. Therefore, cells cultured in IL-3 were treated with dexamethasone for 16 h, washed to remove the ligand and steroid, and cultured in medium with or without IL-3 for 8 h. Removal of dexamethasone from E2F-1-expressing cells reduced their p53 levels, whereas in those cells overexpressing both E2F-1 and DP-1, p53 remained elevated (Fig. 9). However, in the absence of IL-3, p53 levels did not increase but were significantly decreased in all cells (Fig. 9). Therefore, p53 accumulation is not necessarily associated with E2F-induced apoptosis.

To confirm that high levels of p53 do not induce death in 32D.3 cells, we measured the effects of gamma irradiation on p53 levels and cell viability in the presence of IL-3. Although cells rapidly accumulated very high levels of p53 (approximately 20-fold induction within 2 h; Fig. 10A), no appreciable effects on survival were observed (Fig. 10B). Therefore, stabilization of p53 alone is not sufficient to induce apoptosis.

DISCUSSION

We have demonstrated that increasing levels of E2F beyond a specific threshold induces apoptosis and overrides the ability of survival factors to suppress cell death. Interestingly, E2F-1 overexpression also induces the accumulation of p53, and indirect evidence has suggested that E2F-induced apoptosis in vivo may be mediated, at least in part, through p53. For example, inactivation of pRb in vivo by human papillomavirus E7 transgenes induces inappropriate apoptosis (32, 56), and human papillomavirus E6, which targets p53 for degradation, partially rescues these cells from cell death (56). However, human papillomavirus E6 fails to protect keratinocytes from cytotoxic effects of E2F-1 (49), and mutant p53 only partially protects fibroblasts overexpressing E2F-1 from apoptosis in low serum concentrations (59). We have demonstrated that the E2F-1-induced apoptosis observed following withdrawal of survival factors is likely independent of p53 function, as p53 levels diminish. Therefore, the increase in p53 observed when

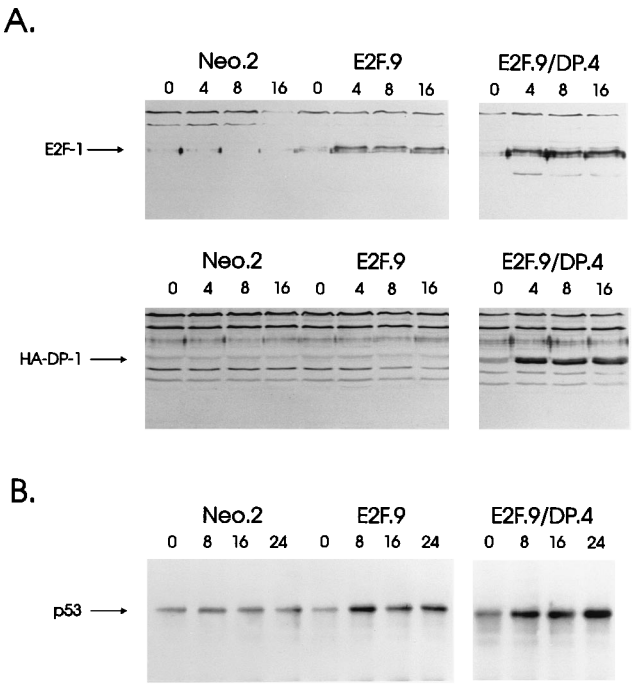


FIG. 8. Overexpression of E2F-1:DP-1 induces p53. (A) Analysis of E2F-1 and HA-DP-1. The indicated cell clones were induced with dexamethasone for 0, 4, 8, or 16 h prior to Western blot analysis first with E2F-1 antiserum (top panel) and then with HA antiserum to detect HA-DP-1 (bottom panel). (B) p53 is induced in response to E2F. Extracts from parallel cultures of the cells used for panel A were immunoprecipitated with the p53-specific monoclonal antibody PAb421, and p53 protein was detected by Western blot analysis with the polyclonal CM1 antibody.

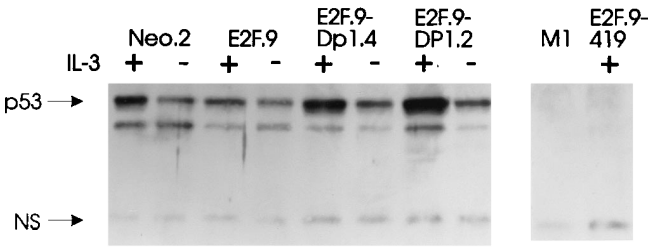


FIG. 9. p53 does not accumulate in cells overexpressing E2F in the absence of IL-3. The indicated cell lines were treated with dexamethasone overnight, washed to remove steroid and IL-3, and resuspended in medium with (+) or without (−) IL-3 (and lacking dexamethasone). After 8 h, the cells were harvested, equal amounts of protein were immunoprecipitated with p53 antibody, and levels were determined by Western blot analysis. Lane M1, immunoprecipitation of lysates of the murine myeloid leukemia M1 cell line, which is devoid of endogenous p53, with PAb421. Lane E2F.9-419, immunoprecipitation with an irrelevant (T-antigen) antibody. NS, nonspecific band.

E2F is induced may be a response of p53 to E2F-mediated proliferative signals. The introduction of the dominant-negative p53 miniprotein DD (68) into 32D.3 cells resulted in predictable defects in ploidy (15), making a definitive analysis of the requirements for p53 function for apoptosis in 32D.3 cells uninterpretable. However, our results suggest that other mediators of E2F-induced apoptosis likely exist, which may explain the partial protection observed after p53 inactivation in other cell systems.

Previous results have suggested a connection between pRb, p53, and E2F-1. However, our results showing that endogenous p53 protein is induced as a function of E2F activity is the first direct evidence that the pRb and p53 pathways are physiologically linked. This work defines an order for the pathway from pRb to E2F to p53, as pRb constrains E2F functions and release from this control leads to p53 accumulation in proportion to E2F activity.

p53 accumulates in response to E2F. The function of p53 as a tumor suppressor has been linked to its response to DNA damage (42). However, p53 has also been linked to proliferation responses, and its levels oscillate during the cell cycle (6, 62), implying an intrinsic cell cycle regulatory role. One interpretation of our results is that E2F overexpression directly leads to DNA damage and that the subsequent induction of p53 is indirect. Alternatively, E2F may induce inappropriate cell cycle progression, and p53 may function as a checkpoint to balance these signals. Finally, p53 may respond to E2F by cooperating with other effectors to trigger apoptosis (see below). Importantly, the response of p53 to E2F appears to be specific, as other inappropriate proliferative signals, such as those invoked by enforced c-Myc expression, do not, at least in these cells, result in p53 accumulation (11). Therefore, either directly or indirectly, p53 functions as a sensor of E2F activity.

E1A-mediated apoptosis of fibroblast cells has been associated with p53 stabilization (16, 46). E1A has pleiotropic effects, targeting multiple cellular pathways (50). Our observations indicate that, at least in part, E1A-mediated induction of p53 utilizes an E2F-dependent pathway. However, accumulation of p53 alone is clearly not a trigger for death in these myeloid cells. First, overexpression of wild-type p53 in 32D.3 cells is not deleterious to survival in IL-3 (7). Second, both IL-3 and IL-6 suppress p53-mediated death induced by overexpression or by the induction of DNA damage (8, 75). Finally, apoptosis induced by E2F-1, in the absence of IL-3, is not associated with increases in p53 levels (Fig. 9). Therefore, other modulators of apoptosis are likely to be influenced by overexpression of E2F.

If p53 functions as a monitor of E2F-mediated cell cycle progression, deletion or inactivation of p53 would be predicted to alter normal cell cycle regulatory controls. In agreement with this concept, mouse embryonic fibroblasts derived from $p53^{-/-}$ mice have marked alterations in their cell cycle following growth factor withdrawal (45). It is also of note that during the proliferative response (62), and in the normal cell cycle, the peak p53 protein level (6) temporally follows the peak of E2F transcriptional activity (33, 36, 52). Finally, it has recently been demonstrated that the p53-interacting protein MDM-2 can also bind and inactivate pRb and functions as an E2F coactivator (48, 74). Thus, it is possible that the accumulation of p53 in response to E2F overexpression is an attempt to inhibit E2F function by titrating MDM-2. Therefore, several lines of evidence suggest that p53 may perform a checkpoint function during proliferation by monitoring E2F activity.

Elevation of E2F activity overrides survival functions provided by growth factors. Survival factors are continuously required to suppress apoptosis (60, 71) and are dominant to the effects of oncogenes which induce death (1, 19, 25, 75). However, in the absence of survival factors, oncogenes can suppress (e.g., Bcl-2 [54, 70] and Raf [12]) or accelerate (e.g., p53 and c-Myc [1, 7, 19, 75]) the rate at which these events occur. To date only E2F activity has been demonstrated to override the functions of survival factors (Fig. 7). In particular, the effects of E2F are in contrast to those of c-Myc overexpression, which is not deleterious to exponentially growing cells provided that they are supplemented with ample survival factors (1, 19, 25). Our results demonstrate that increasing E2F activity beyond a threshold overrides the survival signals provided by IL-3 and serum. One prediction would be that removing the survival

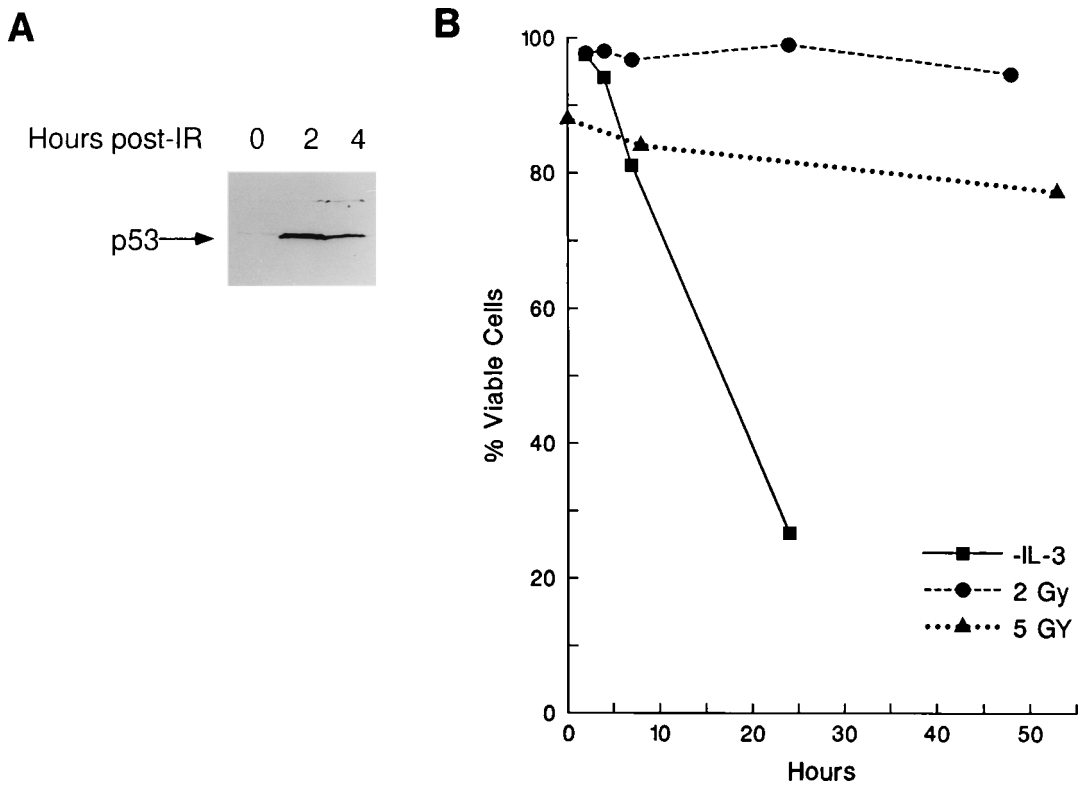


FIG. 10. Induction of p53 by DNA damage is not sufficient to trigger apoptosis. (A) Cells were grown in the presence of IL-3 to a density of 0.5×10^6/ml, exposed to 5 Gy of gamma radiation, and harvested in protein sample buffer at the indicated intervals (hours postirradiation [post-IR]). Extracts were loaded directly onto the gel, and p53 was detected by Western blot analysis with the PAb421 antibody. Each lane represents 1.5×10^6 cells. (B) Cell viability following DNA damage. Cells were grown as described above and at 0 h were exposed to either 2 or 5 Gy of gamma radiation in the presence of IL-3 or were deprived of IL-3 (-IL-3). Viability was determined by propidium iodide staining of nonfixed cells followed by fluorescence-activated cell sorter analysis, as detailed by Gottlieb et al. (24). The data for 5 Gy are from a separate experiment.

factor would lower the threshold required to induce death (25). Several of our observations are consistent with this concept. First, low levels of E2F activity are tolerated in IL-3 but induce death when E2F-1 clones are deprived of IL-3. Second, rates of apoptosis induced by E2F in the absence of IL-3 are dose dependent, as higher levels of E2F activity induced more rapid death (Fig. 7B). Finally, the rates of death induced by high levels of E2F activity are significantly decreased in IL-3 relative to those observed following removal of ligand (Fig. 7). Therefore, the ability of survival factors to suppress apoptosis is a function of specific thresholds of signals.

ACKNOWLEDGMENTS

We are grateful to Hui Yang and Elsie White for their technical expertise, to Kathy Fry for secretarial assistance, and to James Ihle, Charles Sherr, and Robert Rooney for critical reading of the manuscript. We thank William Kaelin for providing the E2F-1 cDNA clone, Ed Harlow for the HA-DP-1 cDNA clone, Charles Sherr for anti-cdk2 and anti-pRb, and Joseph R. Nevins for the anti-p107 antiserum and the E2F-138 mutant cDNA.

This work was supported by grant CB-39 from the American Cancer Society and NIH grant RO1-CA64140 (to S.W.H.), by NIDDK grant DK44158 (to J.L.C.), by a grant from the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities (to M.O.), by Cancer Center Support CORE grant 5 P30 CA 21765, and by the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital. G.P. is the recipient of the Martin Morrison Endowed Fellowship.

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