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Transformed Cells Require Continuous Activity of RNA Polymerase II To Resist Oncogene-Induced Apoptosis

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Studies have indicated that deregulated oncogene expression can result in either programmed cell death or proliferation, depending on the cellular microenvironment. However, little is known about whether oncogenic signals in themselves are able to activate a cellular apoptotic program. We have tested the hypothesis that oncogenic signals in the absence of gene expression are sufficient to induce cell death, which would indicate that constitutive expression of antiapoptotic genes is necessary for maintenance of the transformed state. Using two highly specific RNA polymerase (RNAP) II inhibitors, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and α-amanitin, which inhibit RNAP II function by two distinct mechanisms, we found that inhibition of gene expression substantially increased apoptosis in a time- and dose-dependent manner in $p53^{+/+}$- and $p53^{-/-}$-transformed mouse embryonic fibroblasts and in HeLa cells, demonstrating that this type of apoptosis does not require wild-type p53. Engineered expression of an α-amanitin resistance RNAP II gene rendered cells resistant to induction of apoptosis by α-amanitin without affecting their sensitivity to DRB, indicating that α-amanitin induces apoptosis solely by inhibiting RNAP II function and not by a nonspecific mechanism. DRB-induced apoptosis was independent of the cell cycle or ongoing DNA replication, since DRB induced similar levels of apoptosis in asynchronous cells and cells synchronized by collection at mitosis. Inhibition of RNAP II in untransformed cells like Rat-1 or human AG1522 fibroblasts resulted not in apoptosis but in growth arrest. In contrast, deregulated expression of c-Myc in Rat-1 cells dramatically increased their sensitivity to DRB, directly demonstrating that apoptosis following inhibition of RNAP II function is greatly enhanced by oncogenic expression. The requirement for RNAP II function to prevent oncogene-induced apoptosis implies the need for the constitutive expression of an antiapoptotic gene(s) to maintain the transformed state. The differential sensitivities of untransformed and transformed cells to induction of apoptosis by transcriptional inhibition, coupled with the finding that this type of apoptosis is independent of p53 status, suggest that inhibition of RNAP II may be exploited therapeutically for the design of successful antitumor agents.

Apoptosis, or programmed cell death, is a process by which a cell initiates an endogenous program of self-destruction which results in the disintegration of its genetic and physical architecture in response to genotoxic damage or changes in its microenvironment (19, 49, 52). An important modulator of the cellular response to stress is the product of the tumor suppressor gene p53. The loss of functional p53 can have profound implications for tumorigenesis, tumor promotion, and the response to chemotherapy (17, 26, 31, 36). In the past, the ability of inhibitors of macromolecular synthesis to block some forms of stress-induced apoptosis (38) led to the hypothesis that apoptosis is an active, energy-dependent process of self-destruction. Though data to support this hypothesis exist, it has also been shown that in certain cases, inhibitors of macromolecular synthesis have no effect on the induction of apoptosis by stress (5, 39, 47). In fact, some studies have suggested that inhibition of macromolecular synthesis may itself actually trigger the apoptotic induction (9, 18, 39). However, little attention has been paid to the genotypic changes in the cell that predispose it to this form of death.

The consequences of deregulated oncogene expression are highly dependent on the cellular microenvironment. For example, under physiological conditions, cells expressing the c-*myc*, adenovirus early region 1A (*E1A*), or human papillomavirus (HPV) E6/E7 oncogenes exhibit increased proliferation rates, but when they are exposed to serum deprivation or low-oxygen conditions they readily undergo apoptosis (10, 15, 19). The theory that has arisen from these findings is that exogenous stress is necessary to initiate the apoptotic program in oncogenically transformed cells and that proapoptotic gene products like p53 modulate the apoptotic sensitivity of transformed cells to stress. We wished to investigate whether, and under which conditions, oncogenic activity could signal for apoptosis in the absence of exogenous stress (19, 31, 37). The basic premise underlying these studies is that some forms of oncogenic expression require the constitutive expression of antiapoptotic genes to direct the cell towards the proliferative state instead of the apoptotic state. If this hypothesis is correct, then inhibition of gene expression by inhibition of RNA polymerase (RNAP) II function should be sufficient to signal for apoptosis in oncogenically transformed cells while having little effect on untransformed cells.

A crucial drawback of studies using inhibitors of macromolecular synthesis is the lack of specificity and the resulting pleiotropic effects of these agents on cellular function, which may mask or compound their effects on macromolecular synthesis (39). For example, actinomycin D, which has been used as an inhibitor of RNA synthesis in some studies, is not a bona fide transcription inhibitor but rather a DNA-damaging agent

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which inhibits RNA synthesis by intercalating itself into the bases of DNA and creating a physical block to the movement of RNAPs I, II, and III. In fact, because of these properties, actinomycin D has been widely used as a DNA-damaging agent to study the cellular response to genotoxic stress (2, 27, 29). Therefore, it is possible that actinomycin D may elicit apoptosis primarily through its DNA-damaging properties and not by inhibiting transcription.

Since we wished to study the effects of RNAP II inhibition without significant additional effects elicited by DNA damage or extensive inhibition of the other two RNAPs (RNAP I and RNAP III), we decided to use two nongenotoxic inhibitors, α-amanitin and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), that exhibit the highest possible specificity for RNAP II. The use of two agents which inhibit RNAP II function by two distinct mechanisms (see below) further minimized the possibility that the observed effects on cell viability would be due to a nonspecific action rather than to RNAP II inhibition. Moreover, the existence of an α-amanitin resistance RNAP II gene, RPII 215, provided us with a unique genetic tool for investigating the specificity of the effects of RNAP II inhibition on the cellular apoptotic program. Recent studies have also linked the inhibition of RNAP II with the induction of the p53 protein (35, 54), suggesting that inhibition of RNA synthesis may lead to apoptosis via a p53-dependent pathway. To investigate this possibility, we examined the effects of RNAP II inhibition in cells expressing wild-type (wt) p53 and in cells lacking functional p53. These experiments investigated the requirement of ongoing gene expression to prevent cell death and promote proliferation in these different types of oncogenically transformed cells.

MATERIALS AND METHODS

Cell lines. Murine embryonic fibroblasts (MEFs) with two intact p53 alleles ($p53^{+/+}$) or two homozygous deleted p53 alleles ($p53^{-/-}$), transfected with the activated *Ha-ras* and adenovirus *E1A* genes, have been previously described (19, 37). MEFs were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum, 100 μg of G418 (Sigma Chemicals, St. Louis, Mo.) ml^{-1}, and 20 μg of hygromycin (Sigma) ml^{-1}. Rat-1 fibroblasts transfected with the plasmid pBABEpuro c-mycER, which encodes a Myc/ER chimera protein that is activated by 200 nM 4-hydroxytamoxifen (4-HT) (19, 33), were cultured in DMEM with 10% fetal calf serum containing 1.5 μg of puromycin ml^{-1}. HeLa cells (American Type Culture Collection) and untransformed Rat-1 fibroblasts (a gift from Susan Knox, Stanford University) were maintained in DMEM supplemented with 10% fetal calf serum. All cells were seeded in 60-mm dishes at densities of 2×10^5 to 5×10^5 cells/plate the day before treatment.

DRB and α-amanitin treatments. DRB (Sigma) was first dissolved in dimethyl sulfoxide (DMSO) and then in culture medium to the desired final concentration in 0.1% (vol/vol) DMSO in medium. DMSO treatment alone (0.1% [vol/vol]) did not have any significant effect on cell viability or on [^{3}H]uridine incorporation. α-Amanitin (Sigma) was dissolved in phosphate-buffered saline (PBS). All incubations with drugs were performed in a 37°C cell incubator.

Measurement of apoptosis. Following treatment, cells with apoptotic morphology were identified by staining with Hoechst dye 33342 (blue) (5 μg ml^{-1}) for changes in nuclear characteristics and with propidium iodide (pink) (5 μg ml^{-1}) for loss of membrane integrity or by the terminal deoxytransferase-mediated deoxyuridine nick end-labeling (TUNEL) assay (Oncor, Gaithersburg, Md.) for in situ detection of fragmented chromatin. The TUNEL assay was performed according to the manufacturer's specifications. The percent apoptotic cells relative to the total number of cells in each field was calculated and represents the average of at least four randomly selected fields per 60-mm dish.

Effect of DRB on RNA and DNA synthesis. To assess the effect of DRB on [^{3}H]uridine incorporation, cells were treated with DRB for 3 h as described above, after which [^{3}H]uridine (2 μCi ml^{-1}) was added to the medium. After 1 h of labeling at 37°C, cells were washed three times with ice-cold PBS, collected in PBS, and centrifuged at $5,000 \times g$. Cells were resuspended in PBS containing 2 mM EDTA and lysed by three rounds of freeze-thawing. Trichloroacetic acid (TCA) precipitation of macromolecules and scintillation counting were performed as described previously (41). The percent [^{3}H]uridine incorporation in treated cells relative to that in untreated cells was graphed. Due to the inhibitory effect of DRB on [^{3}H]uridine uptake by the cells (45), a correction factor was used for measuring [^{3}H]uridine incorporation. In this procedure, the ratio of experimental to control TCA-precipitable radioactivity was divided by the ratio of experimental to control total radioactivity of the samples. For measurement of incorporation of [^{3}H]uridine into mRNA, cells were incubated with [^{3}H]uridine as described above, and poly(A)$^+$ RNA was isolated with an oligo(dT) column with the Micro-FasTrack kit (Invitrogen, San Diego, Calif.) according to the manufacturer's protocol. The isolated poly(A)$^+$ RNA was resuspended in 60 μl of Tris-HCl buffer, and 20 μl was used for scintillation counting of radioactivity. To assess the effect of DRB on DNA synthesis, TCA precipitation of macromolecules and scintillation counting were performed after incubation with [^{3}H]thymidine (1 μCi ml^{-1}) for 30 min. We did not observe any inhibitory effect of DRB on thymidine uptake. The percent [^{3}H]thymidine incorporation in treated cells relative to that in untreated cells was graphed.

Cell transfections. $p53^{-/-}$ MEFs were transfected with 10 μg of RPII 215 DNA, and HeLa cells were cotransfected with 10 μg of RPII 215 DNA and 1 μg of a neomycin resistance gene vector (pMC1neo Poly A; Stratagene). All transfections were performed with the cationic lipid Lipofectamine (Gibco-BRL) according to the manufacturer's protocol. Control cells received no DNA or were transfected with the neomycin resistance gene alone. $p53^{-/-}$ MEFs were grown for 24 h before α-amanitin (2 μg ml^{-1}) was added to the medium for an additional 14 days. HeLa cells were grown for 24 h before Geneticin (500 μg ml^{-1}) (Sigma) was added to the medium for an additional 18 days. Selected clones were individually tested for α-amanitin and DRB sensitivity.

Immunoblotting and immunocytochemistry. For immunoblotting following treatment, cells were rinsed three times with PBS and lysed in lysis buffer (PBS containing 2 μg each of aprotinin, pepstatin, leupeptin, and antipain ml^{-1} and 1 mM phenylmethylsulfonyl fluoride). For analysis of RNAP II phosphorylation status, the lysis buffer also contained EDTA (2 mM) and NaF (1 mM). Whole-cell extracts were prepared by three rounds of freeze-thawing, and protein estimation was performed with the bicinchoninic acid method (Pierce, Rockford, Ill.). Extracts (25 μg of protein) were subjected to denaturing gel electrophoresis and immunoblotting. After transfer, the nitrocellulose membrane was first incubated either with a mouse monoclonal antibody (12CA5; Berkeley Antibody Company) which recognizes the 9-amino-acid hemagglutinin epitope of the RPII 215 gene product or with a mouse monoclonal antibody (8WG16; a gift from Nancy Thompson, University of Wisconsin) which recognizes the carboxy-terminal domain (CTD) of the largest RNAP II subunit; then the membrane was incubated with a goat anti-mouse antibody, followed by enhanced chemiluminescence treatment (ECL kit; Amersham).

For immunocytological detection of RPII 215 expression, cells plated on chamber slides were washed with PBS and fixed with methanol (100% [vol/vol], −20°C). After blocking with goat serum (10% [vol/vol]), cells were incubated with antihemagglutinin antibody for 1 h, rinsed three times with PBS, and incubated with a fluorescein-labeled anti-mouse secondary antibody for 20 min. To determine cells with apoptotic morphology, cells were also counterstained with Hoechst dye 33342 (200 μg ml^{-1}) and visualized by fluorescent microscopy.

RESULTS

DRB induces apoptosis in transformed MEFs and HeLa cells. MEFs expressing wt p53 ($p53^{+/+}$ MEFs) and MEFs from p53-deficient mice ($p53^{-/-}$ MEFs) that express the adenovirus *E1A* gene and activated *Ha-ras* oncogenes have been well characterized in terms of their apoptotic response to a variety of stress stimuli (19, 37). The *E1A* oncogene binds to and inactivates the retinoblastoma (Rb) protein, a key modulator of cell cycle regulation (10). This type of oncogenic expression, in conjunction with enhanced ras activity, dramatically lowers the threshold of these cells' sensitivity to apoptotic stimuli and provides a useful system for the study of the influence of p53 status on apoptotic response.

The ribonucleoside analog DRB is a potent and reversible inhibitor of transcriptional elongation by RNAP II (45, 57) through a mechanism that appears to involve inhibition of a TFIIH-associated kinase activity and subsequent dephosphorylation of the CTD of RNAP II (13, 55). DRB specifically inhibits RNAP II-dependent heterogeneous nuclear RNA synthesis without significantly affecting RNAP I- or RNAP III-dependent RNA synthesis or altering the physical integrity of the DNA (45, 57).

DRB was found to induce substantial apoptosis in both $p53^{+/+}$- and $p53^{-/-}$-treated cells compared to untreated control cells when the former were assayed for changes in nuclear morphology and membrane integrity (Fig. 1a to d and 2a). The effect of DRB was not restricted to MEFs, as treatment of human HeLa cells, in which p53 protein expression is abrogated by the HPV E6 gene product, also induced significant

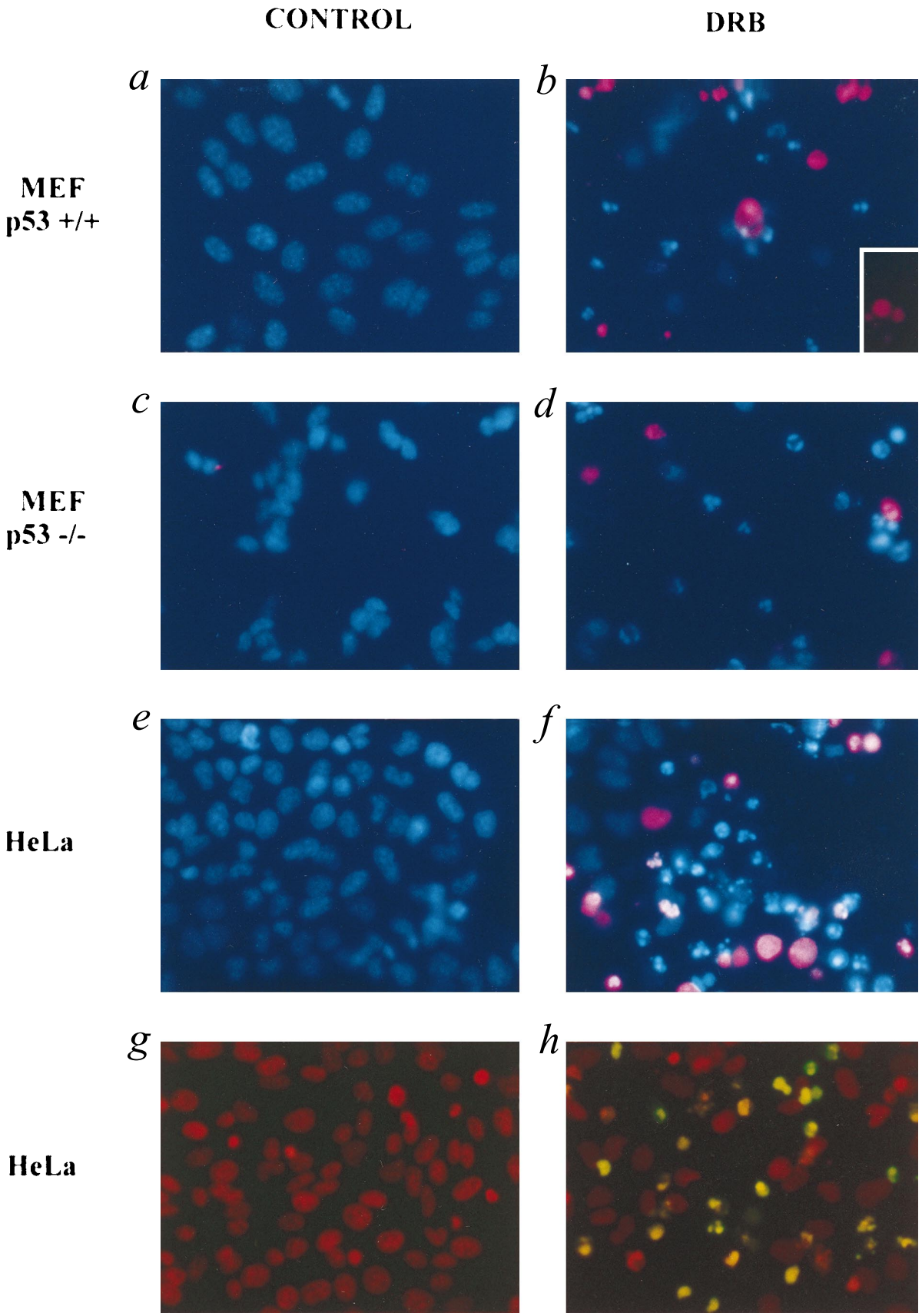

FIG. 1. DRB induces apoptosis in oncogenically transformed cells independent of their p53 status. Cells were treated with DMSO alone (a, c, e, and g) or with DRB (100 μM) in DMSO (b, d, f, and h) for 12 h. Following treatment, cells with apoptotic morphology were identified by staining with Hoechst dye 33342 (blue) for changes in nuclear characteristics and with propidium iodide (pink) for loss of membrane integrity (a to f) or by the TUNEL assay (yellow-green fluorescence) for in situ detection of fragmented chromatin (g and h) at a ×200 magnification. The inset in panel b shows a larger magnification (×600) of the characteristic nuclear condensation and fragmentation observed in many cells undergoing apoptosis.

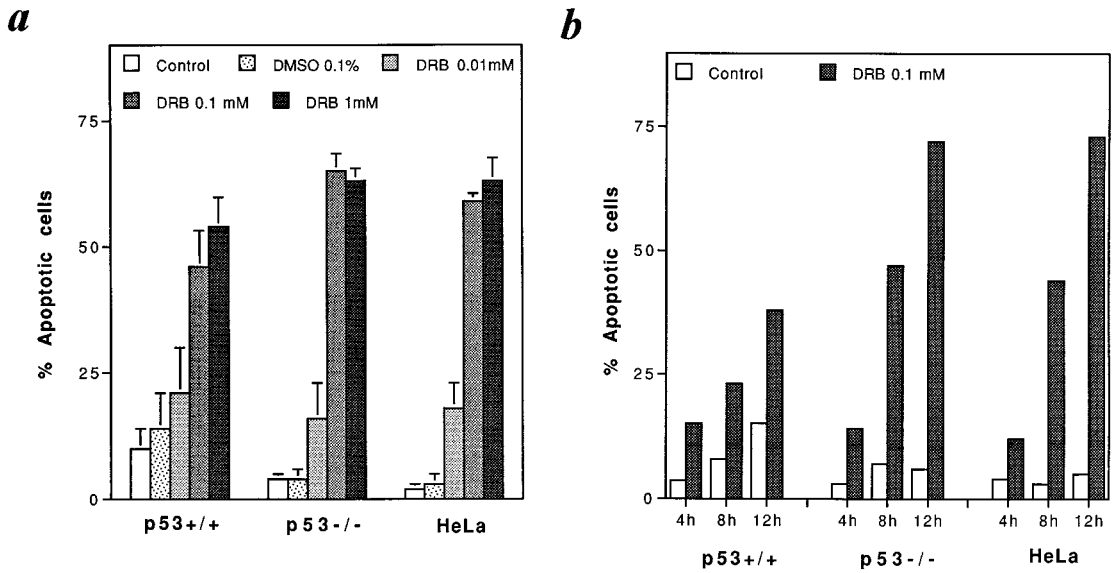

FIG. 2. (a) Quantification of the apoptotic responses of $p53^{+/+}$, $p53^{-/-}$, and HeLa cells to various concentrations of DRB after 12 h of treatment. Data are the averages ± standard deviations of three independent experiments. (b) Quantification of DRB-induced apoptosis after treatment of $p53^{+/+}$, $p53^{-/-}$, and HeLa cells with DRB (100 μM) for various times.

apoptosis, as determined by assessing morphological changes (Fig. 1e and f and 2a) and by assessing DNA fragmentation with the TUNEL assay (Fig. 1g and h).

DRB-induced apoptosis was evident as early as 4 h after incubation with drugs and reached 50 to 72% by 12 h after (Fig. 2a and b). Prolonged (24-h) incubation of MEFs or HeLa cells with DRB resulted in the appearance of apoptotic morphology in >90% of the treated cell populations (data not shown). The induction of apoptosis by DRB correlated with its inhibitory effect on transcription, as determined by measuring the inhibition of [^{3}H]uridine incorporation into total RNA (TCA-precipitable material) (Fig. 3a). DRB induced significant apoptosis and inhibition of RNA synthesis at concentrations between 0.01 and 0.1 mM, which have previously been demonstrated to inhibit transcription in vivo (13) and have also been shown to inhibit phosphorylation of the RNAP II CTD by TFIIH kinase in vitro (55). We investigated whether DRB induced dephosphorylation of the CTD of RNAP II in vivo in cells that were apoptotically sensitive to this drug. MEFs and HeLa cells were treated with DRB (0.1 mM) for 2 h, and whole-cell extracts were subjected to gel electrophoresis and immunoblotting with an anti-CTD antibody. The immunoreactivity of a series of bands extending from ~220 to 240 kDa which represent RNAP II with phosphorylated CTD was substantially decreased in extracts from cells treated with DRB (Fig. 3b), suggesting that DRB induces dephosphorylation of the RNAP II CTD.

α-Amanitin also induces apoptosis in transformed MEFs and HeLa cells. Although DRB is a highly specific inhibitor of RNAP II, there still exists the possibility that induction of apoptosis following DRB treatment is due to a nonspecific effect of DRB on a cellular target other than RNAP II. To further investigate the role of RNAP II inhibition in signaling for apoptosis, we treated $p53^{+/+}$ and $p53^{-/-}$ MEFs as well as HeLa cells with a second RNAP II inhibitor, α-amanitin, which directly and specifically binds to the largest (210-kDa) subunit of RNAP II and prevents the elongation of nascent heterogeneous nuclear RNA chains (8, 11). α-Amanitin is the most specific inhibitor of RNAP II known, with a K_i ranging between 3×10^{-8} and 3×10^{-9} M in vitro (50). RNAP III, which is responsible for the synthesis of tRNAs and low-molecular-weight RNAs, is 10^3 to 10^4 times less sensitive, while RNAP I, which synthesizes rRNAs, is not sensitive to α-amanitin, even at high drug concentrations (50). α-Amanitin also induced substantial apoptosis in $p53^{+/+}$ and $p53^{-/-}$ MEFs and HeLa cells in a dose-dependent manner (Fig. 4). Apoptosis induced by α-amanitin had kinetics similar to those of DRB-induced apoptosis (data not shown) and reached 47 to 52% after a 16-h treatment.

An α-amanitin resistance RNAP II gene construct (RPII 215) confers resistance to α-amanitin-induced apoptosis and transcriptional inhibition when transfected into $p53^{-/-}$ MEFs and HeLa cells. To further investigate whether α-amanitin-induced apoptosis results from direct inhibition of RNAP II, we transfected $p53^{-/-}$ MEFs and HeLa cells with an α-amanitin resistance RNAP II gene construct (RPII 215) (Fig. 5a). The RPII 215 gene product carries a single amino acid substitution at the α-amanitin binding pocket, which results in a 500-fold increase in its resistance to α-amanitin (1) (see Materials and Methods). The construct also contains a hemagglutinin epitope for monitoring its expression (16). $p53^{-/-}$ MEF clones were transfected with the RPII 215 construct and selected by long-term exposure to low doses of α-amanitin. HeLa cells were cotransfected with the RPII 215 gene and a neomycin resistance gene, and clones were selected by resistance to the antibiotic G418 (Geneticin). Untransfected cells and cells transfected only with the neomycin resistance gene did not form any colonies after selection with α-amanitin. Immunoblot analysis of α-amanitin-resistant $p53^{-/-}$ MEFs and HeLa clones with an antihemagglutinin monoclonal antibody revealed that only transfected cells expressed the hemagglutinin epitope, while RNAP II immunoreactivity was evident in both nontransfected and transfected clones (Fig. 5b). Seven α-amanitin-resistant $p53^{-/-}$ clones (MRPr) and five α-amanitin-resistant HeLa clones (HRPr) were subsequently tested for apoptotic induction after exposure to α-amanitin and DRB. All clones were highly resistant to apoptosis when challenged with high doses of α-amanitin (20 μg ml^{-1}) for 16 h (Fig. 5c

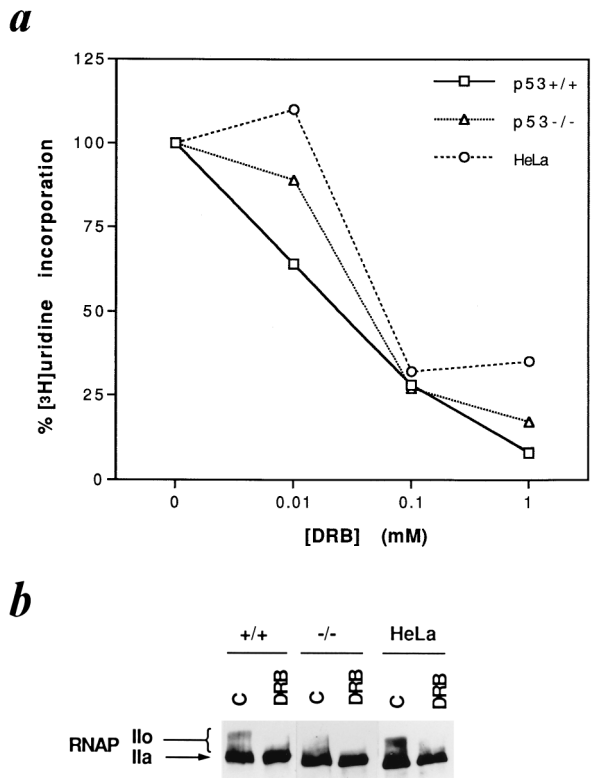

FIG. 3. (a) Dose-dependent inhibition of [^{3}H]uridine incorporation by DRB in p53$^{+/+}$, p53$^{-/-}$, and HeLa cells after a 3-h treatment. Cells were preincubated with [^{3}H]uridine for 15 min, after which DRB at various concentrations was added to the medium and labeling was continued for 3 h. TCA-precipitable counts were measured and normalized against total cell counts. (b) DRB inhibits the phosphorylation of the CTD of RNAP II. Cells were treated with DRB (100 mM) for 2 h, after which they were lysed and whole-cell extracts were subjected to sodium dodecyl sulfate electrophoresis and immunoblotting with an anti-CTD monoclonal antibody (8GW15). The hyperphosphorylated (IIo) and unphosphorylated (IIa) forms of RNAP II are indicated. C, control.

and d), unlike their wild-type counterparts. All the α-amanitin-resistant p53$^{-/-}$ and HeLa clones were found to have retained their sensitivity to DRB-induced apoptosis. These results indicate that α-amanitin induces apoptosis in these cells by inhibition of RNAP II function and not through a secondary, nonspecific mechanism.

To more directly examine the effect of RPII 215 expression on α-amanitin sensitivity, untransfected HeLa cells were mixed with HeLa-RPII 215 cells (clone HRPr.4) at a 1:1 ratio, plated on chamber slides, and exposed to α-amanitin. Following treatment, the cells were fixed, immunostained with the antihemagglutinin antibody, and stained with Hoechst dye 33342. Morphological features characteristic of apoptosis were evident in the majority of the cells that were not immunoreactive (i.e., untransfected HeLa), while none of the immunoreactive cells (i.e., those expressing the epitope-tagged RPII 215 gene) showed any morphological signs of apoptosis (Fig. 6a to c). When the treatment period was 48 h, all surviving cells were positively stained for the epitope-tagged RPII 215 gene (not shown). These results more directly demonstrate that RNAP II inhibition by α-amanitin is the signal for apoptosis in α-amanitin-treated cells.

To examine whether the protection against α-amanitin-induced apoptosis conferred by the α-amanitin resistance gene correlated with a resistance against inhibition of mRNA synthesis, p53$^{-/-}$ MEFs and p53$^{-/-}$ MEFs transfected with the RPII 215 gene (clone MRPr.4) were incubated with [^{3}H]uridine and then treated with α-amanitin. Poly(A)$^+$ RNA was isolated, and TCA-precipitable counts were measured. In p53$^{-/-}$ MEFs, α-amanitin reduced mRNA synthesis to 41% ± 9% (standard error of the mean; $n = 3$) of control values 4 h after treatment and to 23% ± 14% of control values 8 h after treatment. Beyond this incubation period, extensive apoptosis in the p53$^{-/-}$ MEFs did not allow for an accurate estimation of [^{3}H]uridine incorporation into poly(A)$^+$ RNA. In contrast, α-amanitin had no significant effect on mRNA synthesis 4 h after treatment (106% ± 23% of control values) and only a modest effect (73% ± 15% of control values) at 8 h after treatment with the MRPr.4 clone. This level of inhibition remained constant even after 16 h of incubation with α-amanitin (71% ± 12% of control values). These results confirm that the RPII 215 gene confers resistance to α-amanitin-induced apoptosis by continuing to synthesize mRNA in these cells in the presence of this agent.

DRB-induced apoptosis occurs independently of the cell cycle or ongoing DNA replication. It has been proposed that progression of cells in S phase under conditions which inhibit the propagation of the replication fork (e.g., limiting ribonucleotide pools) may lead to DNA damage and increased chromosomal breakage (32, 51). Since DRB also affects the rate of DNA replication (see below), the possibility existed that DRB could be inducing apoptosis by inhibiting DNA replication in cells traversing the S phase of the cell cycle and by inducing DNA damage by such an inhibitory mechanism. To investigate this possibility, we collected HeLa cells in M phase by mitotic shake-off and studied the effects of DRB on the synchronized cell population at several times after shake-off. The progression of synchronized cells through G_1 phase and into S phase was monitored by the degree of [^{3}H]thymidine incorporation into TCA-precipitable material. We found that DRB induced very similar levels of apoptosis at all times in an asynchronous cell population and in synchronous cell populations derived by mitotic shake-off (Fig. 7). More significantly, DRB induced apoptosis in the synchronized cell population well before the cells entered S phase, an event that occurred between 8 and

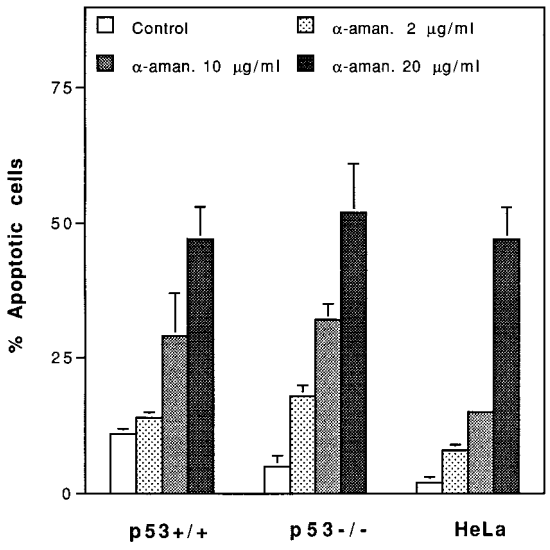

FIG. 4. α-Amanitin induces apoptosis in oncogenically transformed cells. The apoptotic responses of p53$^{+/+}$, p53$^{-/-}$, and HeLa cells to various concentrations of α-amanitin (α-aman.) after 16 h of treatment were quantified. Data are the averages ± standard deviations of two independent experiments.

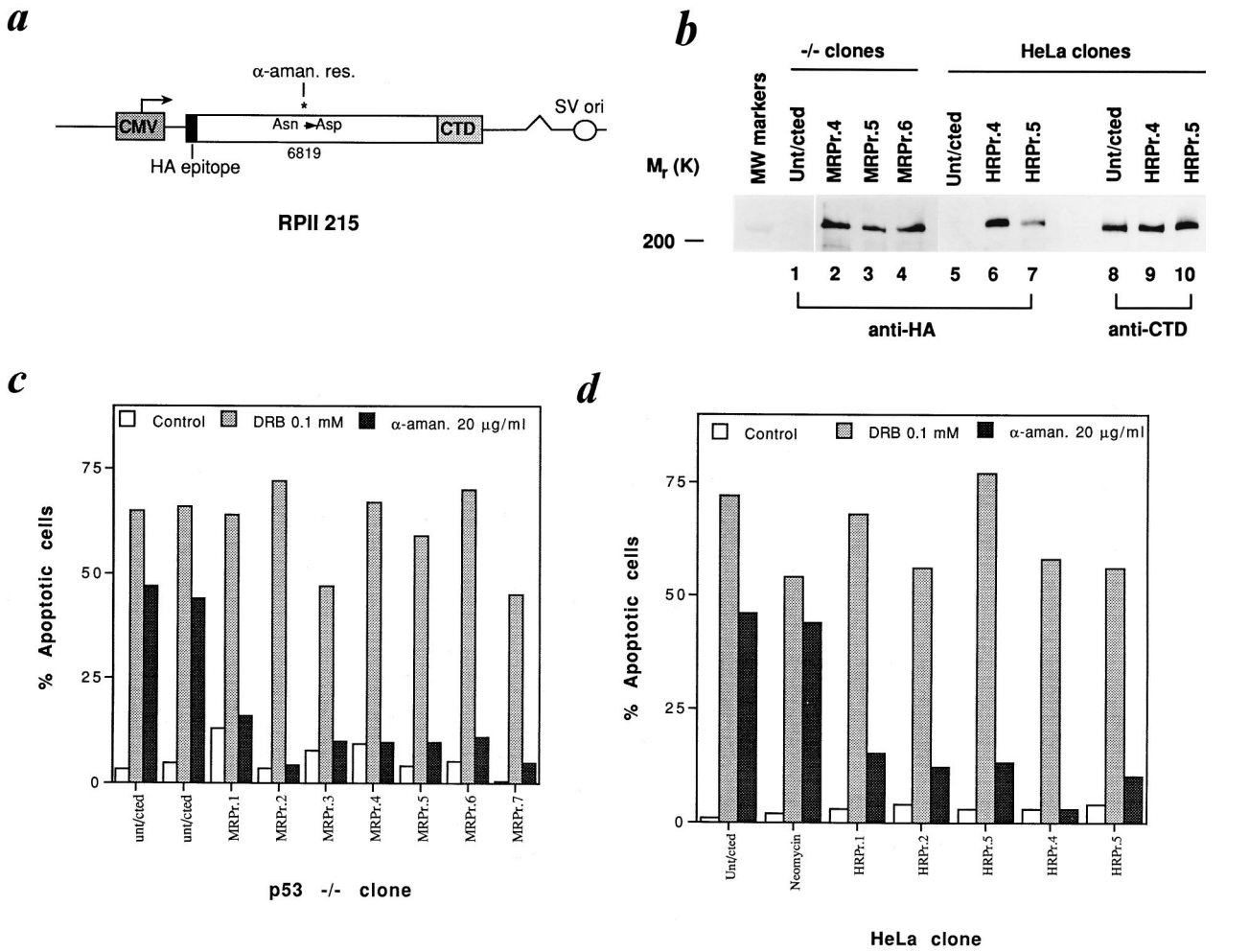

FIG. 5. Transfection of cells with an α-amanitin resistance gene (RPII 215) rescues cells from α-amanitin-induced apoptosis. (a) Diagram of the expression vector containing RPII 215 that was used in generating stable transfections of $p53^{-/-}$ and HeLa cells. The construct contains RPII 215 genomic DNA cloned into expression vector pSTC driven by the cytomegalovirus (CMV) promoter (15). This RNAP II gene carries a point mutation at position 6819, which translates into an asparagine-to-aspartate change at the α-amanitin binding region, resulting in a 500-fold increase in its resistance to α-amanitin (α-aman. res.) (1). The construct also contains nine amino acids forming the hemagglutinin (HA) epitope at the N terminus of the protein. SV ori, simian virus origin. (b) Expression of the RPII 215 protein in MEFs and HeLa clones. Immunoblot with an anti-HA antibody (lanes 1 to 7) shows that only the transfected HeLa clones express the RPII 215 protein, while immunoblotting with an anti-RNAP II antibody (lanes 7 to 10) shows that both untransfected (unt/cted) and transfected cells express native RNAP II protein. Because the expression of the RPII 215 subunit and not its phosphorylation status needed to be determined here, phosphatase inhibitors were not included in the lysis buffer, and thus only the IIa form of the largest RNAP II subunit is seen in these immunoblots. MW, molecular weight. (c) Apoptotic response of untransfected and RPII 215-transfected $p53^{-/-}$ MEFs to DRB and α-amanitin. Cells were transfected with RPII 215 DNA, and stably transfected clones were selected by incubation in a low concentration of α-amanitin. Cells were then treated with α-amanitin (α-aman.) or DRB as described in Materials and Methods. (d) Apoptotic response of untransfected and RPII 215-transfected HeLa cells to DRB and α-amanitin. Cells were transfected with RPII 215 DNA and a plasmid containing the neomycin resistance gene at a ratio of 10:1, and stably transfected clones were selected with G418 (Geneticin; Gibco-BRL). Cells were then treated with α-amanitin (α-aman.) or DRB as described in Materials and Methods.

12 h after mitotic shake-off, as was evident by [^{3}H]thymidine incorporation (Fig. 7). These results indicate that DRB induces similar levels of apoptosis in both asynchronous and synchronous cells in G_1 phase and that apoptosis can occur in the absence of any significant DNA replication. Therefore, it seems highly unlikely that DRB induces apoptosis by inducing DNA damage during replication. This is further supported by the fact that treatment of HeLa cells with very high levels of ionizing irradiation (50 Gy), which results in extensive DNA damage, fails to induce apoptosis within the first 24 h after treatment (30a).

Untransformed cells do not undergo apoptosis after RNAP II inhibition but undergo growth arrest instead. Oncogenic transformation has been shown to lower the threshold of cell sensitivity to apoptotic stimuli (7, 10, 20). The results shown above raise the question of whether the apoptotic effect of RNAP II inhibition is specific for transformed cells or whether it is more general, extending to untransformed cell types as well. To investigate the effects of RNAP II inhibition on untransformed cells, we treated untransformed AG1522 human fibroblasts with DRB. AG1522 cells failed to undergo apoptosis after a 12-h exposure to DRB (Fig. 8a). Only after 24 h of continuous exposure to DRB was there a small detectable level of apoptosis in these cells. To investigate what happens to AG1522 cells when mRNA synthesis is blocked, we measured the rate of DNA synthesis after treatment with DRB. As shown in Fig. 8b, DRB dramatically reduced the incorporation of [^{3}H]thymidine into TCA-precipitable material after 4 h of treatment. DMSO, the carrier for DRB, had only a small effect on [^{3}H]thymidine incorporation. The same treatment with

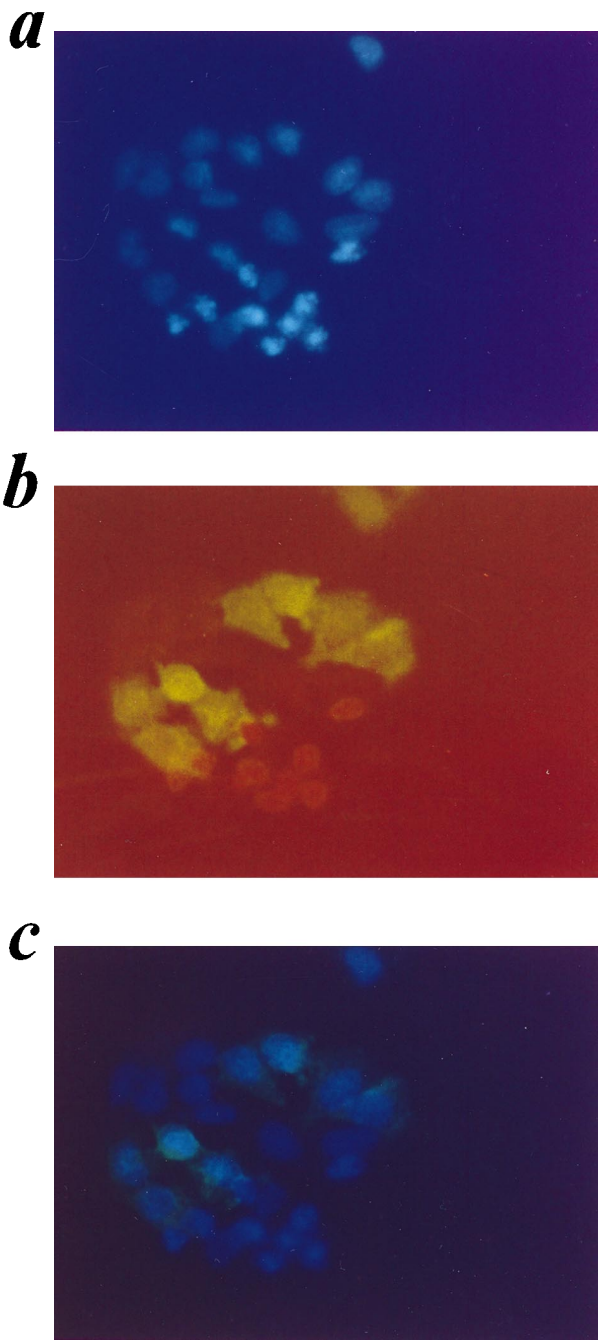

FIG. 6. Expression of RPII 215 protein protects HeLa cells from apoptosis. Untransfected and RPII 215-transfected HeLa cells were mixed at a 1:1 ratio, plated on chamber slides, and exposed to α-amanitin. After 16 h of treatment, cells were fixed and immunostained with the antihemagglutinin primary antibody and a fluorescein-labeled secondary antibody. Cell nuclei were counterstained with Hoechst dye 33342. (a) Photograph of cells whose nuclei were stained with Hoechst dye 33342. Apoptotic cells exhibit distinct nuclear-fragmentation and condensation patterns. (b) Photograph of the same group of cells stained for hemagglutinin epitope (RPII 215 construct) expression (green fluorescence) obtained by using a different single-band-pass filter (560 nm). (c) Composite double-exposure photograph of the same group of cells, showing both Hoechst dye 33342 staining and fluorescence labeling. Magnification (a through c), ×200.

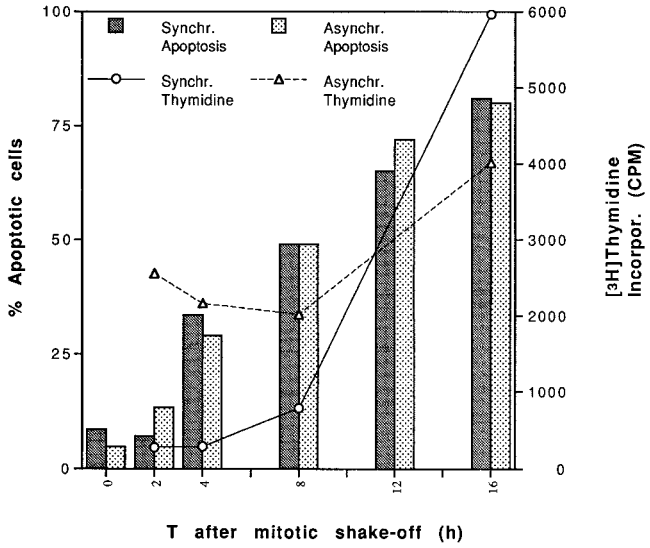

FIG. 7. HeLa cells in G_1 undergo apoptosis when exposed to DRB. Cells synchronized in M phase by mitotic shake-off and an equal number of cells from an asynchronous culture were plated in 100-mm dishes. One set of synchronous and asynchronous cells was exposed to DRB (0.1 mM) for the indicated durations and then examined for apoptotic morphology by staining with propidium iodide and Hoechst 33342 dye. Another set was not treated with DRB but was pulsed with [^{3}H]thymidine for the last 60 min of the treatment periods to monitor progression into G_1 and S phases. As shown by the rate of [^{3}H]thymidine incorporation into TCA-precipitable material, S phase in the synchronous population occurs well after 8 h following shake-off, while apoptosis in the DRB-treated population is evident as early as 4 h following treatment.

DRB also inhibited [^{3}H]uridine incorporation by 72% (data not shown), an inhibition similar to that observed for transformed cells. Thus, inhibition of RNAP II function and subsequently of mRNA synthesis causes untransformed cells to arrest DNA synthesis, presumably because of a lack of one or more critical components for DNA replication. However, arrest of DNA synthesis per se cannot be the underlying cause of resistance against apoptosis in untransformed cells, because we found that DRB had a similar inhibitory effect on DNA synthesis in transformed cells (results not shown).

Induced expression of the c-Myc oncogene product sensitizes Rat-1 fibroblasts to DRB-induced apoptosis. To more directly investigate the effect of oncogenic transformation on the apoptotic response of cells to transcription inhibitors, we used Rat-1 fibroblasts constitutively expressing a c-Myc–estrogen receptor chimera protein (Myc/ER) that is activated by the addition of 4-HT to the medium (19, 33). Increased expression of the proto-oncogene c-*myc* increases the apoptotic potential of cells under certain conditions of stress (15, 19, 22), while the proto-oncogene product bcl-2 has been shown to protect c-Myc-upregulated cells from apoptosis (22). When untransformed Rat-1 fibroblasts were treated with DRB, there was no significant induction of apoptosis, in accordance with our previous finding that untransformed AG1522 fibroblasts are resistant to DRB-induced apoptosis. Treatment of Rat-1 cells with 4-HT or with 4-HT followed by DRB also had no effect on cell viability (Fig. 8c). Similarly, Myc/ER-expressing fibroblasts were insensitive to treatments with 4-HT alone or DRB alone. However, when Myc activity was increased by pretreatment of cells with 4-HT for 12 h, subsequent treatment with DRB dramatically increased the percentage of apoptotic cells. Expression of the bcl-2 gene product significantly reduced DRB-induced apoptosis in Rat-1 Myc/ER cells in which Myc activity had been increased by 4-HT. Taken together, these data indicate that oncogenic transformation by upregulation of c-Myc expression directly sensitizes cells to apoptotic induction sig-

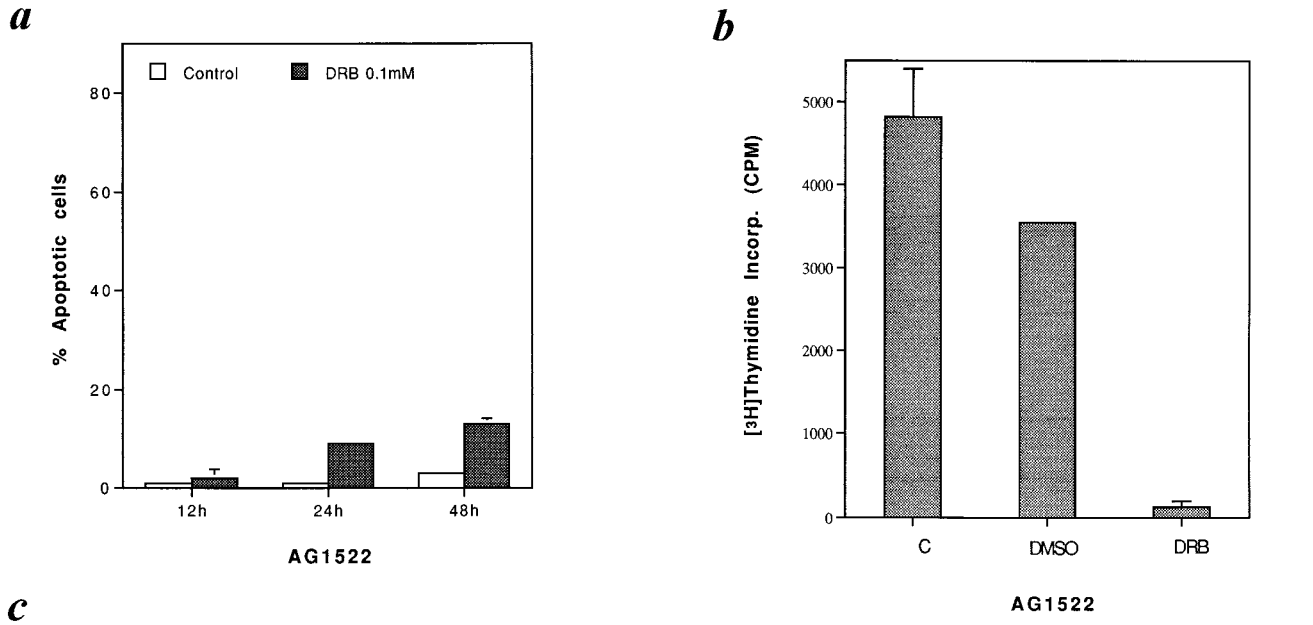

FIG. 8. Effect of oncogenic transformation on DRB-induced apoptosis. (a) Quantification of the apoptotic response of AG1522 fibroblasts to DRB (0.1 mM) after 12, 24, and 48 h of treatment. (b) Treatment of AG1522 fibroblasts with DRB results in inhibition of DNA synthesis. Cells were treated with DRB for 4 h. During the last 30 min of the treatment, [^{3}H]thymidine was added to the media. Following treatment, TCA-precipitable counts were measured and normalized to total cell counts. Control cells (c) received neither DRB or DMSO. DMSO was found to have only a minimal effect on DNA synthesis. (c) Deregulated c-Myc expression sensitizes Rat-1 cells to DRB-induced apoptosis. Untransformed Rat-1 cells, Rat-1 cells transfected with a Myc/ER chimeric construct, and Rat-1 cells transfected with the Myc/ER construct and bcl-2 were treated with DMSO alone, 4-HT (100 nM), or DRB alone (100 mM), or with DRB after a 12-h pretreatment with 4-HT. After a 12-h treatment with DRB, cells were stained with Hoechst 33342 and propidium iodide, and the number of cells with apoptotic morphology and the total number of cells were determined.

naled by inhibition of RNAP II and that bcl-2 acts downstream of this apoptotic signal.

DISCUSSION

In this study we have provided both biochemical and genetic evidence indicating that the specific inhibition of RNAP II leads to rapid and extensive induction of apoptosis in oncogenically transformed cells, while it results in minimal levels of cell death and growth arrest in untransformed cells. Furthermore, we have shown that this type of apoptotic induction can occur in the absence of ongoing DNA replication and that it does not require the participation of p53, since cells that completely lack functional p53 undergo apoptosis with kinetics similar to those of wt-p53-expressing cells.

Previous studies have also suggested that inhibitors of macromolecular synthesis can induce apoptosis in certain cell lines (9, 18). Our results allow a more detailed and definitive analysis of this generalized observation because of the following limitations of previous studies. (i) The lack of specificity of the inhibitors used in previous studies complicated the identification of the cellular target for apoptosis. We sought to overcome this problem by using two inhibitors which are highly specific for RNAP II and which do not exhibit any documented DNA-damaging activity. By demonstrating that DRB-induced apoptosis can occur at the G_1 phase of the cell cycle, we have also shown that this type of apoptosis can be uncoupled from ongoing DNA replication and that possible indirect DNA damage can be induced by inhibition of DNA replication. (ii) The range of the cell types evaluated was limited. Previous studies have focused on only one cell line or on cells from the same tissue of origin (18, 38). In this study, we have shown that RNAP II inhibition results in apoptosis in MEFs, human cervical epithelial cells, and Rat-1 fibroblasts which are transformed by *E1A*/*ras*, HPV, or *myc* oncogenes, while two untransformed cell lines (Rat-1 and AG1522 human fibroblasts) are insensitive to apoptotic induction by transcriptional inhibition. (iii) Analysis of the role of p53 in the induction of apoptosis by macromolecular-synthesis inhibitors was not performed. Although cell lines with mutant p53 have been used occasionally in such studies, it is not clear whether the particular p53 mutation which abrogated the function of p53 as a cell cycle regulator also abrogated its apoptotic effect. Evidence that the two functions of p53 are distinct and that p53 mutations that inactivate one of the functions may have no effect on the other exists (42). To obviate such problems, we have used cell lines that completely lack p53. In the $p53^{-/-}$ MEFs, p53 has been genetically ablated from the cells, while HeLa cells constitutively express the product of the HPV E6 oncogene,

which binds to p53 protein and targets it for ubiquitination and rapid degradation. (iv) The comparison of the effects of macromolecular-synthesis inhibitors on oncogenically transformed and untransformed cells in the same study was incomplete. In this study, we show that inhibition of RNAP II cells results in vastly different responses in transformed and untransformed cells. This is more directly demonstrated by the ability of a single oncogene product, c-Myc, to lower the threshold of apoptotic sensitivity to DRB when overexpressed. The difference in the response to transcription inhibitors between transformed and untransformed cells indicates that oncogenic activity, whether by deregulated expression of c-Myc, E1A/Ras, or HPV E6/E7, sensitizes cells to apoptotic induction signaled by the inhibition of RNAP II.

The mechanism(s) by which p53 induces apoptosis is still unclear. In contrast to its role in cell cycle arrest after genotoxic damage, where the transactivation properties of p53 appear to be essential (14, 28, 44), the transcriptional activity of p53 may not always be required for its participation in the apoptotic program (3, 6, 23). Recent studies even suggest that the transrepression activity of p53 may be important for the induction of apoptosis in certain cell types (31, 40). A similar mechanism has been proposed for glucocorticoid-induced apoptosis in leukemic cell lines (24), where the transrepression of survival genes rather than the transactivation properties of the glucocorticoid receptor are responsible for induction of apoptosis in these cells. A potential transrepression component in the p53-dependent apoptotic pathway would also imply the inactivation of such survival genes by p53 and would be consistent with findings which suggest that most cells appear to constitutively express all the proteins necessary for programmed cell death (25, 53).

A model which encompasses the inactivation of survival genes by proapoptotic signals can also provide a possible mechanism for apoptotic induction after RNAP II inhibition by α-amanitin or DRB. Such a model is depicted in Fig. 9. According to this model, some forms of oncogenic transformation result in an increase in the sensitivity of the cell to apoptotic stimuli. However, the proapoptotic sensitivity is antagonized by the activity of a cellular antiapoptotic gene product(s), or inhibitor(s), thereby allowing the cell to survive and proliferate. The activity level of the inhibitor(s) is strongly dependent on continuous gene expression, and inhibition of RNAP II alters the stoichiometry of the activator/inhibitor ratio, thereby shifting the balance back to a proapoptotic pathway. It is hypothesized that exposure of the cell to conditions of stress (e.g., low concentration of serum, UV or ionizing radiation, and hypoxia) would also disrupt the activator/inhibitor ratio and would have similar consequences for cell survival. Though the actual survival genes were not identified in this study, a few candidate genes that could fill this role exist. One possibility is that a member of the expanding bcl-2 family may protect against oncogene-induced apoptosis. Our data imply that the survival gene must have a relatively short half-life (i.e., 4 to 6 h). However, the very long half-life of the bcl-2 protein (about 14 h) (30) suggests that a different and perhaps yet-unidentified member of this family or an entirely different family of apoptotic suppressors may be primarily responsible for maintaining the suppression of apoptosis in oncogenically transformed cells. An alternative candidate for a cell survival gene that has recently emerged is the transcription factor NF-κB. Activity of the NF-κB protein has been shown to protect cells against stress-induced apoptosis, including treatments with tumor necrosis factor, UV, or ionizing radiation (4, 34, 46, 48). Since NF-κB controls the expression of numerous downstream genes via transcriptional activation, treatment with potent transcription inhibitors such as DRB and α-amanitin would dramatically affect its antiapoptotic activity. If so, this would imply that NF-κB activity protects cells not only against stress-induced apoptosis but also against oncogene-induced apoptosis. We are currently examining this possibility.

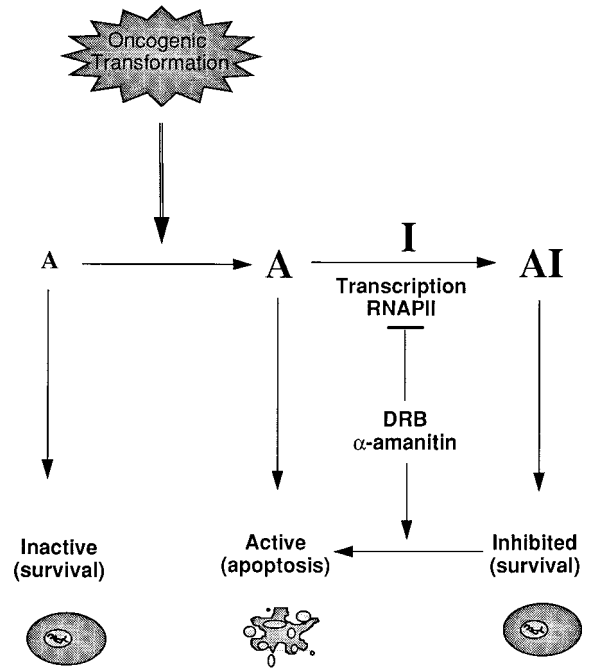

FIG. 9. Model for the role of RNAP II activity in preventing oncogenically transformed cells from undergoing apoptosis. An untransformed cell normally has very low sensitivity to apoptotic stimuli, and exposure to RNAP II inhibitors or exogenous stress results in either a cell cycle arrest or mitotic death. Oncogenic transformation of the cell results in an increase in its sensitivity to apoptotic stimuli (A). However, this apoptotic sensitivity is counteracted by the presence of one or more survival genes, or apoptotic inhibitors (I), whose presence and/or activity is tightly dependent on ongoing RNAP II activity. Exposure of this cell to RNAP II inhibitors (and possibly to exogenous stress like hypoxia, low concentration of serum, or UV or ionizing irradiation) results in an inhibition of the activity or cellular levels of the apoptotic inhibitors, which subsequently leads to a shift towards the apoptotic state.

However, we cannot discount the possibility that other mechanisms in addition to inhibition of a survival gene may also contribute to the induction of apoptosis after inhibition of RNAP II. A well-documented effect of inhibition of RNAP II is the induction of changes in chromatin conformation which include the dissociation and dispersion of the nucleolus into the nucleoplasm (12, 21, 43), and some studies have suggested that changes in chromatin structure make the chromatin more susceptible to endonuclease degradation (56). Whether treatment of oncogenically transformed cells with DRB or α-amanitin induces chromatin rearrangements and whether these changes result in an increased susceptibility of chromatin to exogenously added nuclease remain to be proven.

Our results demonstrate that oncogenically transformed and untransformed cells differ dramatically in their apoptotic responses to inhibitors of RNAP II function in vivo. Therefore, agents that target RNAP II function may constitute a new class of anticancer agents that are able to overcome a major obstacle in anticancer therapies, the loss of wt-p53 function, and provide a selective means of killing transformed tumor cells while sparing normal tissue.

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REFERENCES

1. **Bartolomei, M. S., and J. L. Corden.** 1987. Localization of an α-amanitin resistance mutation in the gene encoding the largest subunit of mouse RNA polymerase II. Mol. Cell. Biol. **7:**586–594.
2. **Bates, S., S. Rowan, and K. H. Vousden.** 1996. Characterisation of human cyclin G1 and G2: DNA damage inducible genes. Oncogene **13:**1103–1109.
3. **Bates, S., and K. H. Vousden.** 1996. p53 in signaling checkpoint arrest or apoptosis. Curr. Opin. Genet. Dev. **6:**12–18.
4. **Beg, A. A., and D. Baltimore.** 1996. An essential role for NF-κB in preventing TNF-alpha-induced cell death. Science **274:**782–784.
5. **Borner, M. M., C. E. Myers, O. Sartor, Y. Sei, T. Toko, J. B. Trepel, and E. Schneider.** 1995. Drug-induced apoptosis is not necessarily dependent on macromolecular synthesis or proliferation in the p53-negative human prostate cancer cell line PC-3. Cancer Res. **55:**2122–2128.
6. **Caelles, C., A. Helmberg, and M. Karin.** 1994. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature **370:** 220–223.
7. **Canman, C. E., and M. B. Kastan.** 1995. Induction of apoptosis by tumor suppressor genes and oncogenes. Semin. Cancer Biol. **6:**17–25.
8. **Cochet-Meilhac, M., and P. Chambon.** 1974. Animal DNA-dependent RNA polymerases. Biochim. Biophys. Acta **353:**160–184.
9. **Cotter, T. G., J. M. Glynn, F. Echeverri, and D. R. Green.** 1992. The induction of apoptosis by chemotherapeutic agents occurs in all phases of the cell cycle. Anticancer Res. **12:**773–779.
10. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A which is inhibited by E1B. Genes Dev. **7:**546–554.
11. **De Mercoyrol, L., C. Job, and D. Job.** 1989. Studies on the inhibition by alpha-amanitin of single-step addition reactions and productive RNA synthesis catalysed by wheat-germ RNA polymerase II. Biochem. J. **258:**165–169.
12. **Desnoyers, S., S. H. Kaufman, and G. G. Poirier.** 1996. Alteration of the nucleolar localization of poly(ADP-ribose) polymerase upon treatment with transcription inhibitors. Exp. Cell Res. **227:**146–153.
13. **Dubois, M.-F., V. T. Nguyen, S. Bellier, and O. Bensaude.** 1994. Inhibitors of transcription such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and isoquinoline sulfonamide derivatives (H-8 and H-7) promote dephosphorylation of the carboxy-terminal domain of RNA polymerase II largest subunit. J. Biol. Chem. **269:**13331–13336.
14. **El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
15. **Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. C. Hancock.** 1992. Induction of apoptosis in fibroblasts by c-myc protein. Cell **69:**119–128.
16. **Gerber, H. P., M. Hagmann, K. Seipel, O. Georgiev, M. A. West, Y. Litingtung, W. Schaffner, and J. L. Corden.** 1995. RNA polymerase II C-terminal domain required for enhancer-driven transcription. Nature **374:**660–662.
17. **Giaccia, A.** 1996. Hypoxic stress proteins: survival of the fittest. Semin. Radiat. Oncol. **6:**46–58.
18. **Gong, J., X. Li, and Z. Darzynkiewicz.** 1993. Different patterns of apoptosis of HL-60 cells induced by cycloheximide and camptothecin. J. Cell. Physiol. **157:**263–270.
19. **Graeber, T. G., C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe, and A. J. Giaccia.** 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. Nature **379:**88–91.
20. **Graeber, T. G., J. F. Peterson, M. Tsai, K. Monica, A. J. Fornace, Jr., and A. J. Giaccia.** 1994. Hypoxia induces accumulation of p53 protein, but activation of a G_1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol. Cell. Biol. **14:**6264–6277.
21. **Haaf, T., and D. C. Ward.** 1996. Inhibition of RNA polymerase II transcription causes chromatin decondensation, loss of nucleolar structure, and dispersion of chromosomal domains. Exp. Cell Res. **224:**163–173.
22. **Harrington, E. A., A. Fanidi, and G. I. Evan.** 1994. Oncogenes and cell death. Curr. Opin. Genet. Dev. **4:**120–129.
23. **Haupt, Y., Y. Barak, and M. Oren.** 1996. Cell type-specific inhibition of p53-mediated apoptosis by mdm2. EMBO J. **15:**1596.
24. **Helmberg, A., N. Auphan, C. Caelles, and M. Karin.** 1995. Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. EMBO J. **14:**452–460.
25. **Ishizaki, Y., L. Cheng, A. W. Mudge, and M. C. Raff.** 1995. Programmed cell death by default in embryonic cells, fibroblasts and cancer cells. Mol. Biol. Cell **6:**1443–1458.
26. **Kastan, M. B., C. E. Canman, and C. J. Leonard.** 1995. p53, cell cycle control and apoptosis: implications for cancer. Cancer Metastasis Rev. **14:**3–15.
27. **Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig.** 1991. Participation of p53 in the cellular response to DNA damage. Cancer Res. **51:**6304–6311.
28. **Kastan, M. B., Q. Zhan, W. El-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein, and A. J. Fornace, Jr.** 1992. A mammalian cell-cycle checkpoint pathway utilizing p53 and *GADD45* is defective in ataxia-telangiectasia. Cell **71:**587–597.
29. **Kessis, T. D., R. J. Slebos, W. G. Nelson, M. B. Kastan, B. S. Plunkett, S. M. Han, A. T. Lorincz, L. Hedrick, and K. R. Cho.** 1993. Human papillomavirus 16 E6 expression disrupts the p53-mediated response to DNA damage. Proc. Natl. Acad. Sci. USA **90:**3988–3992.
30. **Kitada, S., T. Miyashita, S. Tanaka, and J. C. Reed.** 1993. Investigations of antisense oligonucleotides targeted against bcl-2 RNAs. Antisense Res. Dev. **3:**157–169.

30a. **Koumenis, C., et al.** Unpublished data.

31. **Levine, A. J.** 1997. p53, the cellular gatekeeper for growth and division. Cell **88:**323–331.
32. **Linke, S. P., K. C. Clarkin, A. D. Di Leonardo, A. Tsou, and G. M. Wahl.** 1996. A reversible, p53-dependent G0-G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. Genes Dev. **10:**934–947.
33. **Littlewood, T. D., D. C. Hancock, P. S. Danielian, M. G. Parker, and G. I. Evan.** 1995. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. **23:**1686–1690.
34. **Liu, Z. G., H. Hsu, D. V. Goeddel, and M. Karin.** 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell **87:**565–576.
35. **Ljungman, M., and F. Zhang.** 1996. Blockage of RNA polymerase as a possible trigger for U.V. light-induced apoptosis. Oncogene **13:**823–831.
36. **Lowe, S. W., S. Bodis, A. McClatchey, L. Remington, H. E. Ruley, D. E. Fisher, D. E. Housman, and T. Jacks.** 1994. p53 status and the efficacy of cancer therapy in vivo. Science **266:**807–810.
37. **Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman.** 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957–967.
38. **Martin, D. P., R. E. Schmidt, P. S. DiStefano, O. H. Lowry, J. G. Carter, and E. M. J. Johnson.** 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J. Cell Biol. **106:**829–844.
39. **Martin, S. J., and T. G. Cotter.** 1994. Apoptosis of human leukemia: induction, morphology, and molecular mechanisms, p. 185–229. *In* L. D. Tomei and F. O. Cope (ed.), Apoptosis two: the molecular basis of apoptosis in disease. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
40. **Murphy, M., A. Hinman, and A. J. Levine.** 1996. Wild-type p53 negatively regulates the expression of a microtubule-associated protein. Genes Dev. **10:**2971–2980.
41. **Raju, U., C. Koumenis, M. Nunez-Regueiro, and A. Eskin.** 1991. Alteration of the phase and period of a circadian oscillator by a reversible transcription inhibitor. Science **253:**673–675.
42. **Rowan, S., R. L. Ludwig, Y. Haupt, S. Bates, X. Lu, M. Oren, and K. H. Vousden.** 1996. Specific loss of apoptotic but not cell-cycle arrest function in a tumor derived p53 mutant. EMBO J. **15:**827–838.
43. **Scheer, U., B. Hugle, R. Hazan, and K. M. Rose.** 1984. Drug-induced dispersal of transcribed rRNA genes and transcriptional products: immunolocalization and silver staining of different nucleolar components in rat cells treated with 5,6-dichloro-β-D-ribofuranosylbenzimidazole. J. Cell Biol. **99:** 672–679.
44. **Smith, M. L., I. T. Chen, Q. Zhan, I. Bae, C. Y. Chen, T. M. Gilmer, M. B. Kastan, P. M. O'Connor, and A. J. J. Fornace.** 1994. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science **266:**1376–1380.
45. **Tamm, I., R. Hand, and L. A. Caliguri.** 1976. Action of dichlorobenzimidazole riboside on RNA synthesis in L-929 and HeLa cells. J. Cell Biol. **69:**229–240.
46. **Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma.** 1996. Suppression of TNF-alpha-induced apoptosis by NF-κB. Science **274:**787–789.
47. **Vaux, D. L., and I. L. Weissman.** 1993. Neither macromolecular synthesis nor myc is required for cell death via the mechanism that can be controlled by Bcl-2. Mol. Cell. Biol. **13:**7000–7005.
48. **Wang, C. Y., M. W. Mayo, and A. S. Baldwin, Jr.** 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science **274:**784–787.
49. **White, E.** 1996. Life, death, and the pursuit of apoptosis. Genes Dev. **10:**1–15.
50. **Wieland, T., and H. Faulstich.** 1991. Fifty years of amanitin. Experientia **47:**1186–1193.
51. **Windle, B., B. W. Draper, Y. X. Yin, S. O'Gorman, and G. M. Wahl.** 1991. A

central role for chromosome breakage in gene amplification, deletion formation and amplicon integration. Genes Dev. **5:**160–174.
52. **Wyllie, A. H.** 1994. Death from inside out: an overview. Philos. Trans. R. Soc. Lond. B. **345:**237–241.
53. **Wyllie, A. H.** 1995. The genetic regulation of apoptosis. Curr. Opin. Genet. Dev. **5:**97–104.
54. **Yamaizumi, M., and T. Sugano.** 1994. UV-induced nuclear accumulation of p53 is evoked through DNA damage of actively transcribed genes independent of the cell cycle. Oncogene **9:**2775–2784.
55. **Yankoulov, K., K. Yamashita, R. Roy, J.-M. Egly, and D. L. Bentley.** 1995. The transcriptional elongation inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole inhibits transcription factor IIH-associated protein kinase. J. Biol. Chem. **41:**23922–23925.
56. **Yoon, Y. S., J. W. Kim, K. W. Kang, Y. S. Kim, K. H. Choi, and C. O. Joe.** 1996. Poly(ADP-ribosyl)ation of histone H1 correlates with internucleosomal DNA fragmentation during apoptosis. J. Biol. Chem. **271:**9129–9134.
57. **Zandomeni, R., B. Mittleman, D. Bunick, S. Ackerman, and R. Weinmann.** 1982. Mechanism of action of dichloro-β-D-ribofuranosylbenzimidazole: effect on in vitro transcription. Proc. Natl. Acad. Sci. USA **79:** 3167–3170.