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c-*myc* and *bcl-2* modulate p53 function by altering p53 subcellular trafficking during the cell cycle

(oncogenes/apoptosis)

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ABSTRACT **We have studied the ability of c-*myc* and *bcl-2* oncogenes to modulate p53 function. Our studies show that coincident expression of human Bcl-2 protein with p53 prolongs survival of murine erythroleukemia cells. This effect was associated with a loss of the G_1 specificity of p53-mediated cell cycle arrest. Furthermore, we found that the c-*myc* and *bcl-2* genes cooperate to inhibit p53 functions. Coexpression of *bcl-2* and c-*myc* can totally overcome p53-induced apoptosis and cell cycle arrest by altering the subcellular trafficking of p53 during the cell cycle: the p53 remains in the cytoplasm of the cotransfected cells during a critical period in G_1. This finding suggests a mechanism by which normal hematopoietic progenitors can survive and proliferate despite p53 expression and by which the inappropriate expression of *bcl-2* and c-*myc* can cooperate in transformation.**

Alteration of the p53 tumor-suppressor gene is probably the most common abnormality in human malignancies (1). However, the fact that not all tumors harbor p53 mutations indicates that this is not essential for transformation. Expression of p53 in many tumor cell lines, including those of hematopoietic origin such as murine erythroleukemia (MEL) cells, results in G_1 arrest and subsequent programmed cell death (apoptosis) (2, 3). We and others have shown that p53-induced apoptosis in hematopoietic cells occurs only in G_1 and appears to be a separate function from p53-induced cell cycle arrest (3). Paradoxically, normal hematopoietic progenitor cells are viable despite expression of significant levels of p53 (4).

Inappropriate expression of one of the members of the *myc* gene family is also one of the most common events occurring in tumors. Expression of c-*myc* is obligate for G_1 transit (5), and inappropriate expression of c-*myc* can lead to apoptosis (6). *myc* cooperates with Ha-*ras* to transform normal cells, and, in contrast to cells transformed only by Ha-*ras*, cells transformed by Ha-*ras* and c-*myc* do not require a p53 mutation for transformation (7). Despite this implication of an interaction of c-*myc* with p53, Myc was unable to overcome p53-induced growth arrest (8).

The *bcl-2* protooncogene encodes an intracellular membrane-associated protein that has been localized to the mitochondria, endoplasmic reticulum, and perinuclear regions and is expressed in early lymphoid and myeloid progenitor cells (9, 10). Hematopoietic cells transfected with *bcl-2* are resistant to apoptosis resulting from growth factor withdrawal (11). However, *bcl-2* does not universally inhibit apoptosis (12). For example, *bcl-2* fails to inhibit the deletion of autoreactive lymphocytes *in vivo* (13). Although *bcl-2* alone does not stimulate cell proliferation or cause transformation, it can cooperate with c-*myc* to transform cells (11).

We have now investigated whether *bcl-2* and/or c-*myc* might modulate the function of p53 in MEL cells. Coincident expression of human Bcl-2 protein with p53 prolongs survival of MEL cells. Cells that coexpressed *bcl-2* and c-*myc* can overcome completely both the growth inhibitory and the cytotoxic effects of p53. Such cells are shown to alter the subcellular localization of p53 during the cell cycle, suggesting that c-*myc* and *bcl-2* can overcome p53-induced growth inhibition through exclusion of p53 from the nucleus during a critical period in G_1. These data demonstrate a mechanism by which tumors can arise without mutation of the p53 gene and suggest a potential mechanism by which normal hematopoietic progenitor cells, which coexpress p53, *bcl-2*, and c-*myc*, remain viable and proliferate.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. The construction of $p53^{ts}$neo has been described (3). $p53^{ts}$hygro was derived from $p53^{ts}$neo by deletion of the *Bam*HI/*Hin*dIII fragment containing the neomycin resistance gene and insertion of a 2.3-kb *Bam*HI/*Hin*dIII fragment containing the hygromycin resistance gene from p65 hygro. pSV_2mycS-dhfr and pSFFVbcl-2neo have been described (9, 14). DP16-1 cells (15) were transfected by electroporation as described (3). Cell viability was monitored by trypan blue staining.

RNA and Protein Analysis. Northern blots analyzing p53 expression were done as described (3). p53 immunoprecipitations were as described (3). A mixture of PAb421 (Oncogene Science), a pan-specific monoclonal antibody (mAb) against p53, and PAb248, a murine specific mAb against p53, was used.

Detection of p53 protein by flow cytometry was done as follows: 2×10^6 cells were washed in FACS buffer (3) and fixed by the dropwise addition of 70% (vol/vol) methanol. After incubation at −20°C for 5 min PAb421 was added for 1 hr, followed by incubation for 1 hr with $F(ab')_2$-biotin goat anti-mouse IgG (Southern Biotechnology Associates). The cells were then incubated with streptavidin–RED670 (GIBCO/BRL) for 1 hr, resuspended in 1 ml of phosphate-buffered saline, and analyzed using a FACScan (Becton Dickinson) equipped with LYSIS II software. Immunohistochemical staining of transfected MEL cells for p53 was done as described (3).

Exogenous c-Myc transcripts were detected by S1 nuclease analysis as described (14). To detect Myc protein, a murine specific anti-Myc peptide antiserum (16) was used.

Bcl-2 immunoblots were done as described (12). Flow cytometry was done as for p53, except cells were fixed with

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Abbreviation: mAb, monoclonal antibody.

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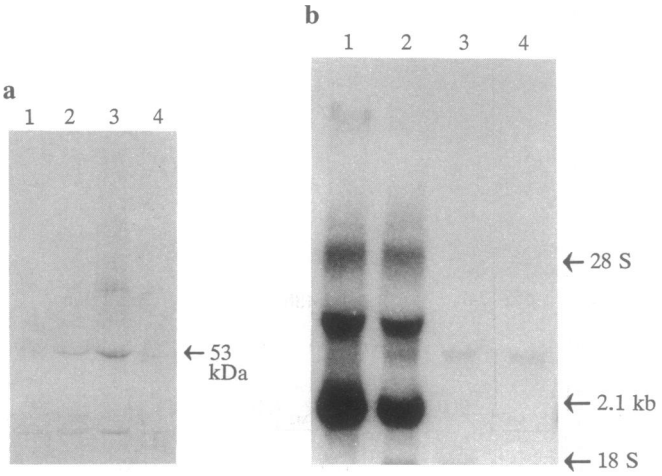

FIG. 1. (*a*) Immunoprecipitation of p53 using mAbs PAb421 and PAb248. Lanes: 1, parental line DP16-1; 2, cells transfected with p53^{ts}neo; 3, cells cotransfected with p53^{ts}hygro and pSFFVbcl-2neo; and 4, cells transfected with p53^{ts}hygro. (*b*) Northern blot demonstrates p53 expression. Lanes: 1, cells transfected with p53^{ts}hygro and pSFFVbcl-2neo; 2, cells transfected with p53^{ts}hygro; 3, cells transfected with pSFFVbcl-2neo; and 4, untransfected parental line DP16-1.

1% paraformaldehyde and then incubated with 3F11 mAb, a hamster IgG mAb directed to murine Bcl-2 (17), 6C8 mAb [hamster IgG anti-human Bcl-2 (9)], or control hamster IgG anti-trinitrophenyl mAb (PharMingen).

Cell Cycle Analysis. Transfected MEL cells in various phases of the cell cycle were purified by centrifugal elutriation. Elutriated fractions were analyzed for DNA content (3) and then used for p53 immunohistochemistry and quantitation of p53 protein by flow cytometry.

RESULTS

***bcl-2* Delays p53-Induced Apoptosis.** To study the interaction of *bcl-2* and p53 in the control of apoptosis in MEL cells, we singly transfected and cotransfected pSFFVbcl-2neo, a plasmid that expresses human *bcl-2* and geneticin resistance genes, and p53^{ts}hygro, a plasmid that expresses a temperature-sensitive mutant of murine p53 (2) and a hygromycin resistance gene, into DP16-1 cells; DP16-1 is a MEL cell line with no endogenous expression of p53 (15). DP16-1 cells transfected with p53^{ts}hygro, but not the parental cell line, expressed p53 protein (Fig. 1*a*) and mRNA (Fig. 1*b*). Human Bcl-2 protein was detected in the transfected cells by immunoblot (Fig. 2*a*). Antibodies specific for either mouse or human Bcl-2 proteins were used to detect expression of endogenous or exogenous Bcl-2 by flow cytometry. Fig. 2*b* shows that transfected cells expressed a low level of endogenous murine Bcl-2. In addition, transfected cells stained homogeneously for a high level of human Bcl-2.

The p53^{ts} protein encoded by p53^{ts}hygro is in the mutant conformation when cells are grown at 37°C but assumes a wild-type conformation when cells are grown at 32.5°C (2, 3). In contrast to cells expressing only p53, cells expressing both p53 and *bcl-2* had prolonged survival at 32.5°C (Fig. 3*a*). However, cell number did not appreciably change in the p53/*bcl-2*-expressing cells during culture at 32.5°C for 4 days (Fig. 3*b*), and thymidine incorporation studies showed that little or no DNA synthesis occurred (data not shown). In comparison, DP16-1 cells expressing only *bcl-2* continued to proliferate at 32.5°C and exhibited a high rate of DNA synthesis (data not shown). Growth arrest also occurred rapidly in cells expressing only p53; by 15 hr at 32.5°C thymidine incorporation had diminished to barely detectable levels (data not shown).

Bcl-2 Alters the Cell Cycle-Regulatory Function of p53. To assess the mechanism by which p53 and *bcl-2* interact to influence MEL cell viability and growth, the cell cycle status of these cell populations was examined by DNA content analysis. DP16-1 cells expressing wild-type p53 arrested in the G_1 phase of the cell cycle; by 24 hr at 32.5°C <10% of cells were found in S phase (Fig. 3*c*). Although viability remained high at this time, cell death rapidly ensued after this G_1 arrest (Fig. 3*a*). This arrest was largely irreversible, as cells become committed to undergoing apoptosis after only a brief exposure to wild-type p53 (3). In contrast, DP16-1 cells coexpressing p53 and human *bcl-2* showed a different cell cycle profile when cultured at 32.5°C. A specific G_1 arrest was not

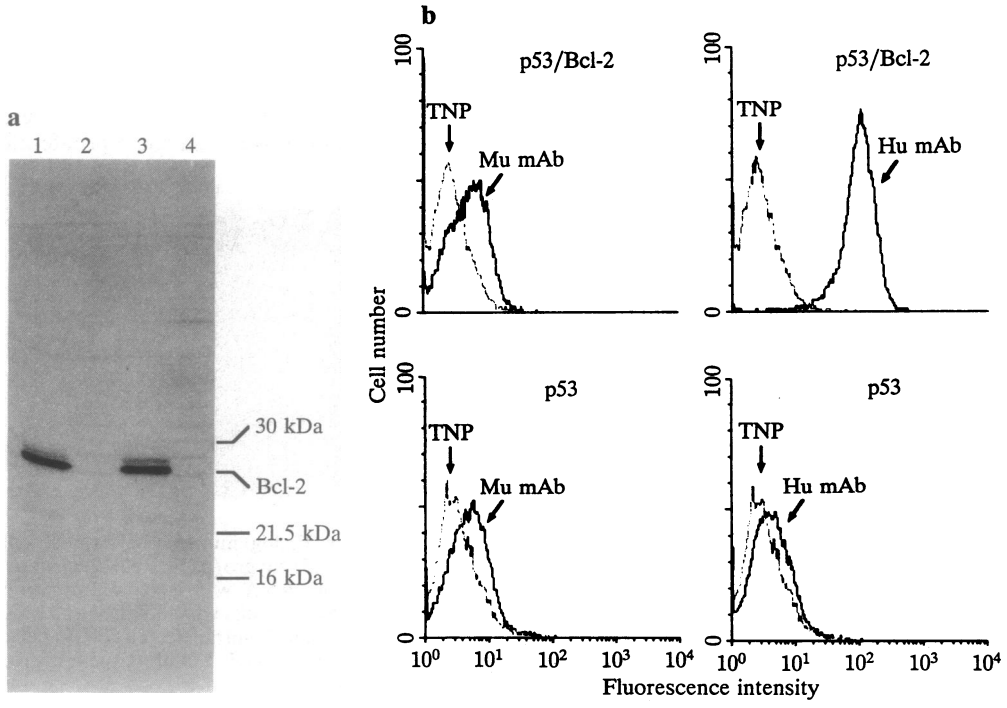

FIG. 2. (*a*) Immunoblot of pSFFVbcl-2neo-transfected cells using 6C8 mAb. Lanes: 1, cells transfected with pSFFVbcl-2neo; 2, untransfected DP16-1 cells; 3, cells cotransfected with pSFFVbcl-2neo and p53^{ts}hygro; and 4, cells transfected with p53^{ts}hygro. (*b*) Human and murine Bcl-2 expression in transfected MEL cells was measured by flow cytometric analysis. Cells cotransfected with pSFFVbcl-2neo and p53^{ts}hygro (p53/Bcl-2) stained with 3F11 mAb (Mu) and with 6C8 mAb (Hu) are shown. Also shown are cells transfected with p53^{ts}hygro alone (p53) stained with the same Bcl-2-specific mAbs. All samples are also stained with a control mAb (anti-trinitrophenyl; TNP).

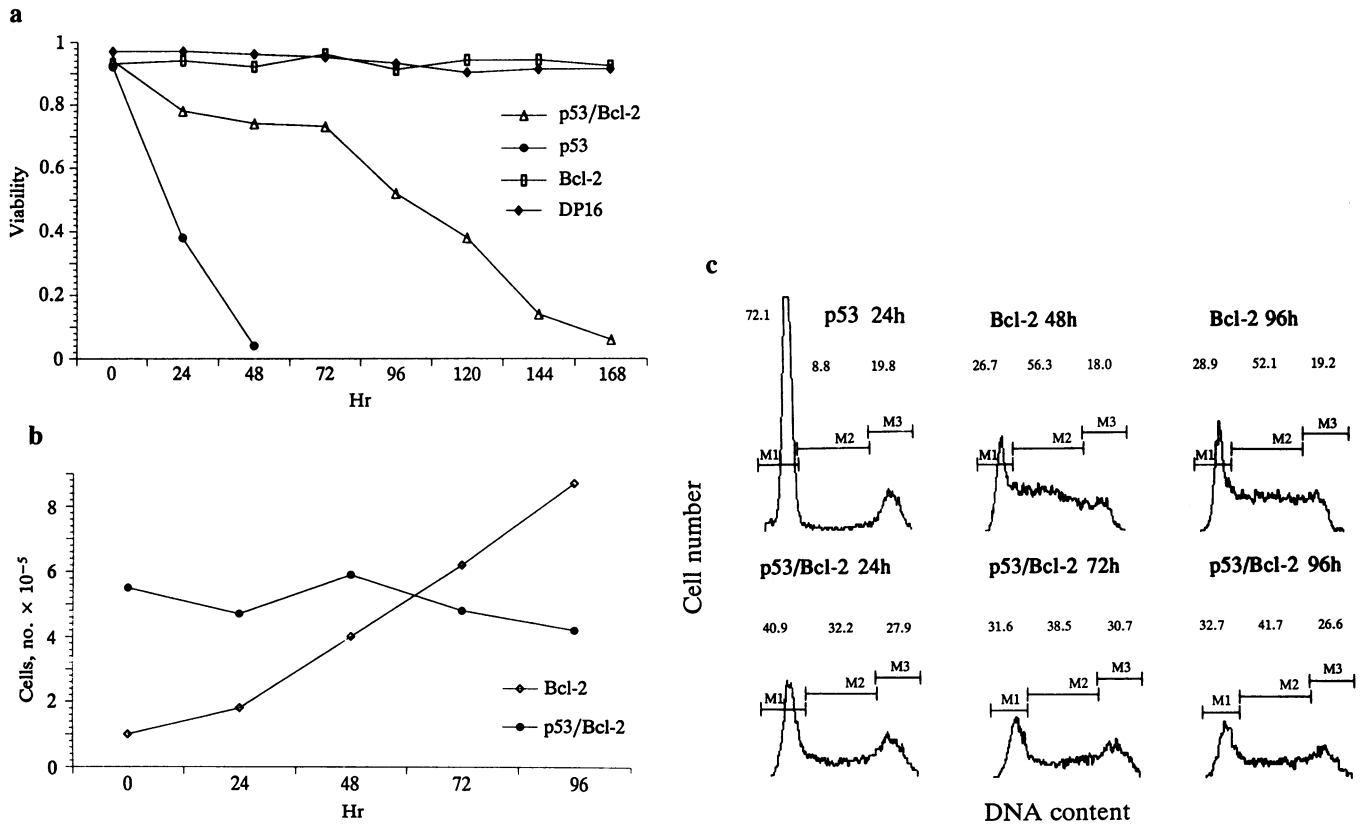

FIG. 3. (*a*) Transfected DP16-1 cells were cultured at 32.5°C (0 hr), and viability was monitored. Although cells expressing only p53 lose viability rapidly, coexpression of Bcl-2 allows prolonged survival. (*b*) During culture at 32.5°C (0 hr), total cell numbers were monitored over several days. Cells expressing only Bcl-2 continue to proliferate; cells coexpressing p53 fail to increase in number. (*c*) DP16-1 cells expressing the indicated proteins were cultured at 32.5°C for the specified time period, and DNA content was measured by flow cytometry. The percentage of cells in G_1 (M_1), S (M_2), and G_2/M (M_3) is shown for each sample. A $G_1 \rightarrow S$ block is seen in cells expressing only p53. This result is not seen in cells expressing Bcl-2 alone or coexpressing p53 and Bcl-2.

seen; rather, the cells exhibited a DNA content profile consistent with cells in multiple phases of the cell cycle (Fig. 3*c*). This profile did not change over 4 days of culture at 32.5°C, and the cells that coexpressed p53 and *bcl-2* remained viable for several days (Fig. 3*a*). Together with the observations that cell number remains constant over this time period and that thymidine incorporation was low, these data imply that cells expressing both p53 and *bcl-2* were arrested at random points throughout the cell cycle. The growth arrest induced at 32.5°C in p53/*bcl-2*-expressing cells was reversible, as cells returned to 37°C after culture for 48 or 72 hr at 32.5°C resumed logarithmic growth after a short delay (data not shown).

Expression of Wild-Type but Not Mutant p53 Induces Down-Regulation of c-Myc RNA. A plausible explanation for the cell death induced by $p53^{ts}$neo in DP16-1 MEL cells would be the continued expression of endogenous c-*myc* after p53-mediated growth arrest. Therefore, RNA was isolated from $p53^{ts}$neo-transfected DP16-1 cells that had been placed at either 37°C or 32.5°C for 6 hr. In four separate experiments, the expression of wild-type, but not mutant, p53 resulted in the rapid down-regulation of c-Myc mRNA (Fig. 4). These results show that endogenous c-*myc* expression was suppressed coincident with commitment to p53-mediated apoptosis; this suggests that p53-associated cell death is not secondarily caused by inappropriate c-*myc* expression. Constitutive overexpression of *bcl-2* did not affect the down-regulation of c-Myc mRNA levels by p53 (Fig. 4).

Coexpression of c-*myc* and *bcl-2* with p53 Completely Overcomes p53 Effects. Because the expression of c-*myc* is obligate for the G_1–S transition in hematopoietic cells and *bcl-2* only partially overcomes p53 effects, we determined whether c-*myc* might cooperate with *bcl-2* to rescue cells from p53-induced growth arrest. MEL cells were cotransfected with pSV_2mycS-dhfr, pSFFVbcl-2neo, and $p53^{ts}$hygro (T3 cells). Cotransfected cells expressed p53 (Fig. 5*a*) and Bcl-2 (Fig. 5*b*), and an S1 nuclease analysis showed that the cells expressed high levels of exogenous c-Myc mRNA (Fig. 5*c*). An equivalent amount of Myc protein was present in the c-*myc*-transfected cells grown at 37°C and 32.5°C (Fig. 5*d*).

To determine the effect of coexpression of *bcl-2* and c-*myc* on p53 function, we analyzed the growth of the cells at 32.5°C. Although they proliferated at a slower rate, these cells retained viability (data not shown) and could proliferate

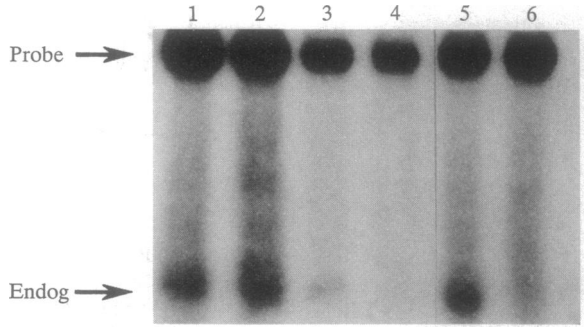

FIG. 4. Levels of endogenous (Endog) murine c-Myc transcripts were measured in $p53^{ts}$neo and $p53^{ts}$hygro/pSFFVbcl-2neo transfectants by S1 nuclease analysis. RNA was isolated from cells growing at 37°C (lanes 1, 3, and 5), and cells were cultured at 32.5°C for 6 hr (lanes 2, 4, and 6). Lanes: 1 and 2, untransfected DP16-1 cells; 3 and 4, $p53^{ts}$neo-transfected cells; and 5 and 6, $p53^{ts}$hygro/pSFFVbcl-2neo-transfected cells.

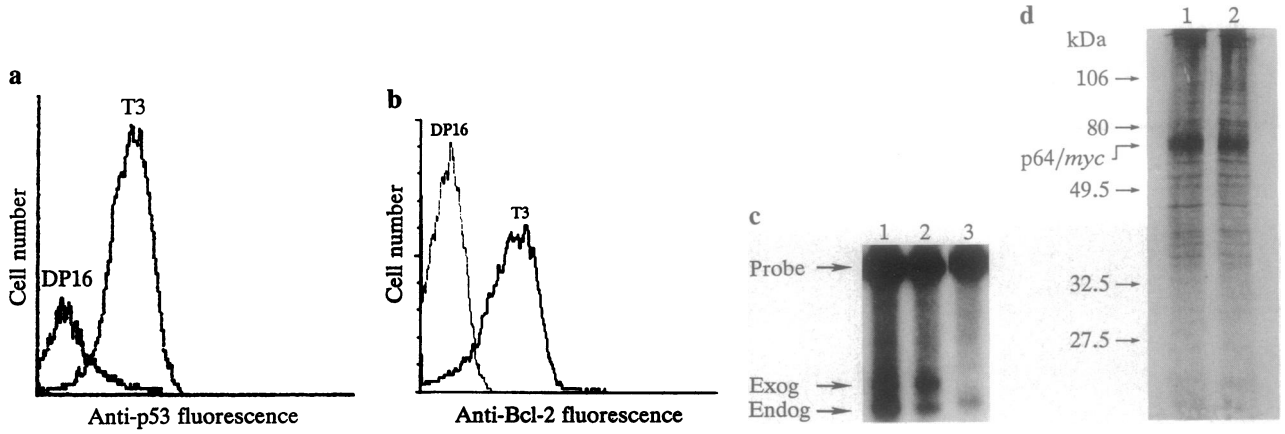

FIG. 5. T3 cells were analyzed for expression of the relevant mRNA and proteins by flow cytometry, S1 nuclease analysis, and immunoprecipitation. (*a*) Transfected cells and untransfected DP16-1 cells were stained for p53 expression using PAb421. (*b*) Human Bcl-2 expression was detected by using 6C8 mAb. Untransfected DP16-1 cells were used as a control. (*c*) S1 nuclease analysis of c-Myc expression was done as described (8). Arrows indicate the S1 nuclease-protected exogenous (Exog) transcript and the endogenous (Endog) c-Myc transcript. Lanes: 1, T3 cells grown at 37°C; 2, T3 cells grown at 32.5°C; 3, DP16-1 parental cells grown at 37°C. (*d*) Myc protein was immunoprecipitated from T3 cells grown at 37°C (lane 1) and 32.5°C (lane 2).

despite the expression of p53 (Fig. 6). In contrast, cells expressing wild-type p53 and c-*myc* undergo growth arrest and apoptosis.

c-*myc* and *bcl-2* Affect p53 Subcellular Localization. MEL cells transfected with p53^{ts}neo alone translocate p53 to the nucleus, arrest in G_1, and undergo apoptosis when cultured at 32.5°C (3). Because Bcl-2 is located in intracellular membranes, including the nuclear membrane, we determined whether c-*myc* and *bcl-2* block the transit of p53 into the nucleus. The enforced expression of either c-*myc* or *bcl-2* alone did not appear to alter the p53 nuclear localization (data not shown). However, in T3 cells at 32.5°C, the p53 was located both in the cytoplasm and in the nucleus (data not shown).

Because p53 effects are cell-cycle specific we examined p53 localization during the cell cycle in T3 cells. Cells in different parts of the cell cycle were isolated by centrifugal elutriation. The DNA content was analyzed by flow cytometry, and p53 cellular localization was determined by immunohistochemistry. The trafficking of p53 was altered in the cells that constitutively express c-*myc* and *bcl-2*. During G_1, the p53 was located in the cytoplasm (Fig. 7). During S phase, when MEL cells are not susceptible to p53-induced cell death, the p53 translocated to the nucleus. p53 was again found in the cytoplasm in cells in G_2/M (Fig. 7). The relative amounts of p53 protein did not significantly change during the cell cycle (data not shown).

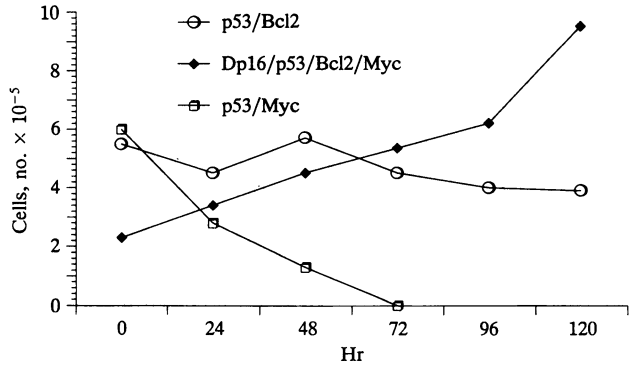

FIG. 6. DP16-1 cells transfected with p53^{ts}neo and pSV$_2$mycS-dhfr, p53^{ts}hygro and pSFFVbcl-2neo, or T3 cells were placed at 32.5°C (0 hr), and total cell numbers were monitored for several days.

DISCUSSION

Wild-type p53 expression induces a G_1 growth arrest in many tumor cell lines, although some rat embryo fibroblast lines arrest during multiple phases of the cell cycle (18). A transient G_1 arrest associated with p53 expression is also seen in many normal cells after DNA damage, by agents such as UV irradiation (19). This result has led to the proposal that p53 serves as an important checkpoint in the cell cycle before entering S phase. This hypothesis is supported by recent findings that p53 is essential for apoptosis in normal thymocytes after treatment with DNA-damaging agents (20). Our data demonstrate that the specificity of p53-mediated growth arrest can be altered in MEL cells by *bcl-2* expression. They also suggest that p53 may be functionally altered in cells in which the apoptosis pathway is inhibited. The ability of *bcl-2* to block p53-induced apoptosis, however, may not depend on the loss of p53-associated G_1 arrest. p53-mediated growth arrest and apoptosis are separable functions (3). *bcl-2* also inhibits apoptosis in MEL cells due to factors other than wild-type p53 expression (J.J.R. and M.F.C., unpublished observations).

bcl-2 is expressed in early hematopoietic and early epithelial progenitor cells (9), as well as in a percentage of lymphoid and epithelial neoplasms (21). When growth factors are withdrawn from hematopoietic progenitor cells, they rapidly undergo programmed cell death (22), and recent evidence suggests that p53 is central to this process (23). Our findings suggest that *bcl-2* plays a role in protecting normal progenitors from p53-induced apoptosis, and c-*myc* overcomes p53-induced cell cycle arrest. This suggests another model of hematopoiesis, in which coordinate regulation of c-*myc*, *bcl-2*, and p53 expression through signal-transduction pathways may be involved in control of hematopoietic progenitor cell fate.

Low-grade (follicular) lymphomas often have deregulated *bcl-2* expression (24). Our results suggest that *bcl-2* overexpression may contribute to carcinogenesis by protecting cells from p53-induced apoptosis. The transformation of low-grade follicular lymphomas to aggressive diffuse lymphomas is associated with either c-*myc* overexpression or p53 mutation (25, 26). Mutations that lead to aberrant expression of both *bcl-2* and c-*myc* may allow tumors to arise without p53 mutation. *bcl-2* has been found to cooperate with c-*myc* in tumor induction or progression (11) and to antagonize c-*myc*-induced apoptosis (6). Our results suggest that the ability of

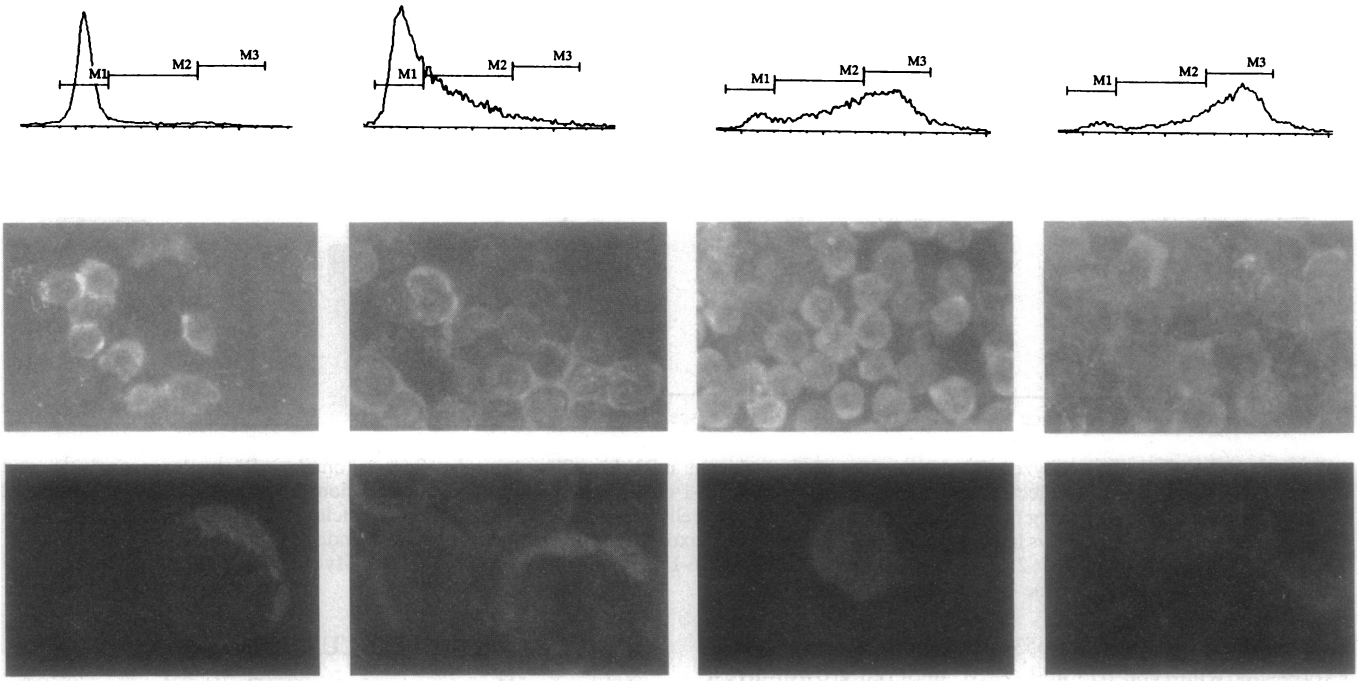

FIG. 7. T3 cells growing at 32.5°C were fractionated by centrifugal elutriation, and p53 was subcellularly localized by immunohistochemical staining with PAb421 mAb. Cells in G_1 and G_2/M had a predominantly cytoplasmic location of p53; in mid-to-late S phase p53 localized almost exclusively to the nuclear compartment. (Upper row, ×400; Lower row, ×1000.)

bcl-2 to cooperate in transformation with c-*myc* may also result from the functional inactivation of wild-type p53.

The subcellular location of p53 is thought to modulate its actions. However, in some tumors that express wild-type p53, the p53 is located in the cytoplasm (27). In many cancer cell lines with absent or mutant p53, wild-type protein, expressed by transfection, becomes localized in the nucleus, and the cells undergo either G_1 arrest or apoptosis. Our results suggest that coincident expression of c-*myc* and *bcl-2* affects the subcellular localization of p53 during the cell cycle and may be a mechanism to explain these apparently contradictory observations. This finding is compatible with the potential localization of Bcl-2 near the pore structures of the nuclear membrane and a possible role of Bcl-2 in regulation of protein transport across membranes (10).

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