Growth Suppression of Friend Virus-Transformed Erythroleukemia Cells by p53 Protein Is Accompanied by Hemoglobin Production and Is Sensitive to Erythropoietin

PENNY JOHNSON, STEPHEN CHUNG, AND SAM BENCHIMOL*

*Department of Medical Biophysics, University of Toronto, and Ontario Cancer Institute,
500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9*

Received 8 May 1992/Returned for modification 29 July 1992/Accepted 17 December 1992

The murine allele temperature-sensitive (ts) p53^{Val-135} encodes a ts p53 protein that behaves as a mutant polypeptide at 37°C and as a wild-type polypeptide at 32°C. This ts allele was introduced into the p53 nonproducer Friend erythroleukemia cell line DP16-1. The DP16-1 cell line was derived from the spleen cells of a mouse infected with the polycythemia strain of Friend virus, and like other erythroleukemia cell lines transformed by this virus, it grows independently of erythropoietin, likely because of expression of the viral gp55 protein which binds to and activates the erythropoietin receptor. When incubated at 32°C, DP16-1 cells expressing ts p53^{Val-135} protein, arrested in the G₀/G₁ phase of the cell cycle, rapidly lost viability and expressed hemoglobin, a marker of erythroid differentiation. Erythropoietin had a striking effect on p53^{Val-135}-expressing cells at 32°C by prolonging their survival and diminishing the extent of hemoglobin production. This response to erythropoietin was not accompanied by down-regulation of viral gp55 protein.

The p53 tumor suppressor gene is a common target for mutation in diverse human and animal tumors, and its loss or inactivation is, therefore, believed to play an important role in oncogenesis (reviewed in references 3, 18, and 29). A number of studies have examined the consequences of expressing a foreign wild-type p53 gene in transformed cells containing mutated endogenous p53 alleles. One common finding is that wild-type p53 protein has antiproliferative activity. While expression of a mutated p53 allele is tolerated in transformed cells, overexpression of wild-type p53 is not compatible with the continued growth of these cells and blocks cells in the G₁ phase of the cell cycle (4, 6, 20, 36-38, 50).

Michalovitz et al. (38) have described a temperature-sensitive (ts) mutant murine p53 allele in which the predicted amino acid at position 135 is changed from alanine to valine. This allele was previously shown to cooperate with an activated *ras* gene to transform rat embryo fibroblasts at 37 but not at 32°C (38). At 37°C, cells expressing *ras* and the ts p53^{Val-135} allele were morphologically transformed. p53 protein was mainly cytoplasmic and bound to hsc70 protein, and it was predominantly in a conformation that was recognized by PAb240 (a monoclonal antibody that preferentially recognizes mutant p53 protein) but not by PAb246 (a monoclonal antibody that preferentially recognizes wild-type p53 protein). At 32°C, p53 protein underwent a conformational change that rendered it more reactive with PAb246 than with PAb240. Moreover, binding to hsc70 protein was diminished, and p53 molecules localized to the nucleus. These striking changes in p53 protein were associated with growth arrest of the cells at the G₁/S border (13, 35). Cells already in the S or G₂/M phase at the time of the temperature shift continued to traverse the cell cycle until they entered G₁ and became blocked. Cells could be maintained at the low temperature in a viable but noncycling, G₀/G₁-arrested state for several days (35). In addition, the ts p53^{Val-135} allele,

when expressed at the low temperature, mimicked wild-type p53 in its ability to suppress rat embryo fibroblast transformation by other pairs of oncogenes (38). Thus, the p53 protein encoded by the ts p53 allele behaves as a mutant polypeptide at 37°C and as a wild-type polypeptide at 32°C.

Chen et al. (4) showed that the expression of wild-type human p53 protein suppressed the neoplastic phenotype of human osteosarcoma cells (SAOS-2) lacking endogenous p53 protein. Cells expressing wild-type p53 protein became larger and flatter, and they had a prolonged doubling time in culture. These cells lost their abilities to grow in soft agar and to form tumors in nude mice. In contrast to results of previous studies, these cells continued to proliferate, albeit more slowly, while they expressed wild-type p53 protein.

Recently, wild-type p53 expression was reconstituted in the p53-nonproducer, pre-B-cell line L12. p53 expression resulted in partial differentiation of these cells which was reflected by the synthesis of the cytoplasmic immunoglobulin (Ig) μ heavy chain and by that of B220, a B-cell-specific surface antigen. p53-expressing clones had reduced tumorigenicity and continued to proliferate in culture with a prolonged doubling time (46). Thus, the reduced tumorigenicity and reduced self-renewal seen with certain reconstituted cells may reflect an involvement of p53 in promoting cell differentiation. However, it is clear that not all cell types respond to p53 reconstitution in the same way. In the murine myeloid cell line M1, expression of the ts p53 allele at the low temperature resulted in growth arrest and a loss in viability that was associated with apoptosis. However, these cells, unlike the reconstituted L12 cells, did not undergo differentiation (50). The inclusion of interleukin-6, an inducer of monocytic differentiation of M1 cells, in the growth medium of these cells prolonged their survival.

Previously, we reported that mutated but not wild-type p53 genes could be expressed stably in p53-negative, Friend virus-transformed murine erythroleukemia cell clones (20). In this study, we examined the effects of p53 expression directed by the ts p53^{Val-135} allele in the p53-negative Friend cell line DP16-1. At 37°C, the growth of clones expressing

* Corresponding author.

the ts p53^{Val-135} protein was similar to that of the parental DP16-1 cells. At the low temperature, however, the cessation of cell growth and a loss of viability were seen only with clones expressing the ts p53 protein. These changes were accompanied by a block in the G₁ phase of the cell cycle and by an increase in the number of differentiated, hemoglobin-synthesizing cells. Furthermore, these effects were diminished by the addition of recombinant human erythropoietin (Epo) to the growth medium. Thus, unlike parental DP16-1 cells, which grow independently of Epo, cells expressing ts p53^{Val-135} protein at the low temperature exhibited responsiveness to Epo.

MATERIALS AND METHODS

Cell lines. All cell lines were maintained at 37°C in α -minimal essential medium (α -MEM) supplemented with 10% fetal calf serum.

The p53-negative murine erythroleukemia cell line DP16-1 (16, 41) was derived from the spleen cells of a DBA/2J mouse infected with the polycythemia strain of Friend murine erythroleukemia virus complex. This cell line grows in culture independently of supplementary exogenous Epo. The following cell lines were derived from DP16-1 by coelectroporation of plasmids containing murine p53 alleles and pSV2neo, a plasmid encoding a selectable marker conferring resistance to the drug analog G418, as described previously (20). DP16-1pro193 was obtained by coelectroporation of pSV2neo and a mutant murine p53 allele, Mp53pro193, in which a mutation at codon 193 converts arginine to proline (42). A single G418-resistant colony growing on methylcellulose was isolated and expanded in liquid culture to give rise to the DP16-1pro193 cell line. The cell populations, val135-5, -15, and -17, were independently derived from coelectroporation of pSV2neo and a plasmid carrying a mutant murine p53 allele, ts p53^{Val-135}, in which a mutation at codon 135 converts alanine to valine (8, 9). Twenty-four hours after being electroporated, cells were distributed into 24-well tissue culture dishes at a concentration of 5×10^4 cells per ml in 1 ml of α -MEM containing 10% serum and 0.4 mg of G418 (GIBCO) per ml and incubated at 37°C for about 10 days. At this cell density, all wells contained G418-resistant cells. val135-5, -15, and -17 were derived from cells growing in three independent wells, and they are likely to be oligoclonal. Clones were subsequently obtained from these cells by plating in methylcellulose (lacking G418), picking individual colonies, and expanding these in suspension culture.

DP16-1pro193, val135-5, val135-15, and val135-17 cells were, on alternate passages, grown in 0.4 mg of G418 per ml to maintain their drug resistance phenotypes. However, the drug was removed during the experimental protocols. Cells were supplemented with purified recombinant human Epo (1 U/ml) where indicated.

p53 protein labelling and immunoprecipitation. Cells were labelled for 1 h at 37 or 32°C with 0.2 mCi of [³⁵S]methionine in 1 ml of α -MEM lacking methionine. Cells were lysed for 30 min on ice in 0.3 to 0.6 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris [pH 8.0], 0.5 mM phenylmethylsulfonyl fluoride). The cell debris was removed by centrifugation, and the remaining supernatant was pre-cleared by incubation overnight with 5 μ g of nonspecific IgG2a murine monoclonal antibody followed by the addition of 0.5 ml of a 10% suspension of Formalin-treated *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring) which were then removed by centrifugation. The supernatant was immunoprecipitated with appropriate antibodies:

PAb419, a control monoclonal antibody against simian virus 40 large T antigen (15); PAb421, a panspecific monoclonal antibody against p53 (15); PAb246, a murine p53-specific monoclonal antibody that recognizes an epitope present on wild-type p53 but not generally on mutant forms of p53 (49); and PAb240, a p53-specific monoclonal antibody that recognizes an epitope present on denatured and mutant forms of p53 protein (11). Immune complexes were collected with 50 μ l of *S. aureus* cells, washed twice in NET-gel buffer (150 mM NaCl, 5 mM EDTA [pH 8.0], 50 mM Tris [pH 7.4], 0.05% Nonidet P-40, 0.02% sodium azide, 0.25% gelatin), and then eluted into 30 μ l of sample buffer (2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol, 25 mM Tris [pH 6.8], 0.1 M dithiothreitol) by heating at 70°C for 10 min. Samples were loaded onto a 10% polyacrylamide gel in the presence of SDS. Electrophoresis was at 45 mA. Dried gels were exposed to Amersham Hyperfilm-MP.

gp55 protein labelling and immunoprecipitation. Cells were labelled for 1 h at 37 or 32°C with [³⁵S]methionine, and lysates were prepared as described above. Lysates were immunoprecipitated with goat anti-Rauscher virus gp69/71 antibody (Quality Biotech Inc.), which recognizes the spleen focus-forming virus (SFFV) gp55 *env* gene product, or with the control antibody, goat anti-mouse IgM. The immune complexes were collected with Omnisorb (Calbiochem, Inc.) and processed for SDS-polyacrylamide gel electrophoresis as described above.

Cell viability determination. Cell viability was determined by flow cytometry following propidium iodide (PI) staining of unfixed cells. Cells were placed in α -MEM supplemented with 10% serum at a final concentration of 5×10^5 cells per ml and grown overnight (18 h) at 37°C. The cultures were then shifted to 32°C, and aliquots were removed at designated intervals. The cells were collected by low-speed centrifugation and immediately resuspended in 0.5 ml of phosphate-buffered saline (PBS) and 1 μ l of PI (5 mg/ml). Viability was determined with a FACS analyzer (Becton Dickinson Corp.) to detect forward-angle light scatter and fluorescence intensity due to PI at 488 nm. Cells with low-level PI staining and high forward-angle light scatter were scored as viable.

Hemoglobin staining. The number of cells expressing hemoglobin was determined by staining with 2,7-diaminofluorene (DAF) (Sigma Corp.) (21). One milliliter of DAF stock solution (10 mg/ml in 90% acetic acid) was mixed first with 0.1 ml of 30% hydrogen peroxide (Fisher Corp.) and then 10 ml of 0.2 M Tris-HCl, pH 7.0, to make the working solution. A 0.1-ml volume of the working solution was then added to an equal volume of cells in α -MEM containing 10% serum, mixed, and allowed to stand at room temperature for 5 min. A 10- μ l aliquot of the stained cells was then counted with a hemocytometer, and the proportion of blue-stained cells was determined (number of stained cells per total cell number).

Flow cytometric analysis of DNA content. At designated intervals, 4-ml aliquots were removed from the cultures growing at 32°C. The cells were pelleted by centrifugation and were resuspended in 0.1 ml of FACS buffer (1% bovine serum albumin, 0.5% Tween 20, 6 mM EDTA in PBS minus Mg and Ca). The cells were then fixed in 1 ml of 70% cold methanol on ice for a minimum of 30 min. Once all aliquots had been collected, the cell samples were washed with FACS buffer and resuspended in 1 ml of FACS buffer–100 μ l of RNase A (10 mg/ml) (Sigma Corp.)–10 μ l of PI (5 mg/ml) per 10^6 cells. Detection of fluorescence due to PI (DNA content) at 488 nm was determined by flow cytometry. The

FIG. 1. Stable expression of p53^{Pro-193} and p53^{Val-135} proteins in DP16-1 cells. (A) DP16-1 cells were coelectroporated with plasmids encoding mutant p53^{Pro-193} protein or ts p53^{Val-135} protein and pSV2neo as a selectable marker. Independent G418-resistant populations were labelled metabolically with [³⁵S]methionine for 1 h. Lysates were prepared, and volumes representing equal amounts of trichloroacetic acid-insoluble radioactivity (10⁷ cpm) were immunoprecipitated with PAb419 (lanes 1, 5, and 9), a monoclonal antibody specific for simian virus 40 large T antigen that was used here as a control; PAb421 (lanes 2, 6, and 10), a panspecific monoclonal antibody for p53; PAb240 (lanes 3, 7, and 11), a monoclonal antibody recognizing mutant forms of p53 protein; and PAb246 (lanes 4, 8, and 12), an antibody recognizing a conformation-dependent epitope normally present only on wild-type p53 protein. DP16-1 (lanes 1 to 4), DP16-1pro193 (lanes 5 to 8), and val135-5 (lanes 9 to 12) cells were used. (B) Cells growing at 37°C or cultured for 18 h at 32°C were labelled metabolically with [³⁵S]methionine for 1 h. Lysates were prepared, and volumes representing equal amounts of trichloroacetic acid-insoluble radioactivity (10⁷ cpm) were immunoprecipitated with mouse IgG1 antibody as a control (lanes 1, 3, 5, 7, 9, and 11) or with PAb246 (lanes 2, 4, 6, 8, 10, and 12). DP16-1pro193 (lanes 1, 2, 7, and 8), val135-5 (lanes 3, 4, 9, and 10), and val135-15 (lanes 5, 6, 11, and 12) cells were used.

DNA content profiles were analyzed with the Multicycle program (Coulter Corp.).

RESULTS

Characterization of p53 proteins expressed at 37 and 32°C. Michalovitz et al. (38) have shown that the mutant murine p53 polypeptide containing valine rather than alanine at position 135 behaves as a mutant polypeptide at 37°C and as a wild-type polypeptide at 32°C. The p53-nonproducing, Friend virus-transformed erythroleukemia cell line DP16-1 (16, 41) was electroporated with pSV2neo and with the mutant ts p53^{Val-135} allele and selected at 37°C in suspension culture containing G418 as described in Materials and Methods. Oligoclonal G418-resistant cells were analyzed for p53 protein expression at 37°C by metabolic labelling with [³⁵S]methionine (Fig. 1A). Three G418-resistant populations (val135-5, -15, and -17) that expressed abundant levels of p53 protein recognized by PAb246, as well as the mutant-specific monoclonal antibody PAb240, were selected for further analysis. The reactivity of the p53^{Val-135} protein with PAb240 and PAb246 was observed previously and may reflect an equilibrium between two conformational states of p53 protein (35). DP16-1 cells expressing a non-ts missense mutant p53 allele were obtained after electroporation with pSV2neo and p53^{Pro-193} DNA (42). One representative clone, DP16-1pro193, isolated as a single colony from a methylcellulose culture dish and expanded in suspension culture, was chosen for further study, and it provides a useful comparison with p53^{Val-135}-expressing cells. DP16-1pro193 cells express PAb240-positive, PAb246-negative p53 protein (Fig. 1A). Both p53^{Val-135} and p53^{Pro-193} proteins coimmunoprecipitated with a cellular protein having the mobility of the hsc70 protein, which has been shown to bind to mutant forms of p53 protein.

A similar metabolic labelling experiment was performed after the cells had been growing at 32°C for 18 h. p53^{Pro-193} protein remained PAb240 positive and PAb246 negative, while there was a notable increase in the reactivity of p53^{Val-135} protein with PAb246 in two of the three ts Val-135-expressing cell lines (Fig. 1B).

Proliferation and viability of p53-expressing cells. val135-5, -15, and -17 cells grew well at 37°C, with a doubling time that was similar to those of the parental cell line DP16-1 and DP16-1pro193 (data not shown). At 32°C, val135-5, -15, and -17 cells ceased to grow (Fig. 2A) and showed a decrease in viability over a period of 72 h (Fig. 2B). These changes were specific to p53^{Val-135}-expressing cells and were not seen with parental DP16-1 cells and with p53^{Pro-193}-expressing cells.

Cells such as DP16-1 which have been transformed by the polycythemia-inducing strain of the Friend virus complex (FVP) grow independently of Epo in tissue culture. The target cells for FVP infection are believed to be Epo-responsive erythroid progenitor cells. Conversion to Epo independence is believed to be mediated by expression of the SFFV *env* gene product, also known as gp55, that mimics the action of Epo by binding intracellularly to the Epo receptor and delivering a mitogenic signal to infected cells (30, 44, 51). In the next series of experiments, we wished to determine whether the inclusion of Epo in the culture medium had any effect on the survival of p53-expressing Friend cells. Figure 2C and D shows a typical time course experiment in which cell number and viability were measured for the three ts Val-135-expressing cell populations and the two control cell lines over a period of 72 h at 32°C in the presence of 1 U of recombinant human Epo per ml. Epo was added once at the start of the 32°C incubation period. The addition of this concentration of Epo to cultures of val135-5, -15, and -17 cells prolonged their survival at 32°C without promoting cell proliferation. The control cultures were not affected by the addition of Epo.

Differentiation of p53-expressing cells. We next wished to address the possibility that the inhibition of cell proliferation and the loss of viability resulting from p53^{Val-135} expression at 32°C were associated with the differentiation of p53-expressing cells. It is well established that Friend virus-transformed erythroleukemia cell lines are responsive to a variety of chemical agents that induce an approximation of terminal differentiation along the erythroid lineage (reviewed in reference 34). Hemoglobin, which can be readily measured in cells by a colorimetric assay based on DAF conversion (21), provides a convenient marker to assess the differ-

FIG. 2. Growth and viability of DP16-1 and DP16-1-derived cells at 32°C. Cells growing in α -MEM at 37°C were transferred to 32°C. Aliquots were removed at the indicated time points after the temperature shift, and the cell number (A and C) and percent viability (B and D) were determined. The proportion of viable cells was determined by flow cytometry after PI staining of unfixed cells. Viable cells were detected by high forward-angle light scatter and low PI staining. Epo was added at a concentration of 1 U per ml of medium at the beginning of the experiment shown in panels C and D. $\circ$, DP16-1; $\square$, DP16-1pro193; $\bullet$, val135-5; $\blacksquare$, val135-15; $\blacktriangle$, val135-17.

entiation status of erythroleukemia cells. Accordingly, the number of cells expressing hemoglobin was determined for DP16-1, DP16-1pro193, and val135-5, -15, and -17 cultures after they were incubated for different periods at 32°C in the presence or absence of Epo. As shown in Fig. 3, hemoglobin was detected in the cell populations expressing p53^{Val-135} at 32°C but not in the control DP16-1 and DP16-1pro193 cells. In the absence of Epo, the number of hemoglobin-producing cells increased with prolonged incubation at 32°C. In the

presence of Epo, however, fewer hemoglobin-positive cells were detected. The addition of Epo had no effect on the proliferation, viability (Fig. 2), or differentiation (Fig. 3A and B) of the control cell lines.

To address the issue of clonal variation within each of the three ts^{Val-135} cell populations, independent clones were isolated as single colonies growing in methylcellulose culture medium and were expanded in suspension culture. All of the clones tested (two clones derived from val135-5 cells, three clones derived from val135-15 cells, and four clones derived from val135-17 cells) expressed p53 protein by metabolic labelling and immunoprecipitation analysis (data not shown). Two clones from each population were subsequently tested for cell viability, expression of hemoglobin, and response to Epo. We found that the single-cell clones responded to incubation at 32°C in a manner similar to that of the cell populations from which they were derived. The bar graph in Fig. 4 shows the proportion of hemoglobin-positive cells detected in parental cell cultures and in derivative clones that were incubated for 48 h at 32°C in the presence or absence of Epo.

The levels of β -globin mRNA in ts Val-135-expressing cells at 37 and 32°C were measured by Northern (RNA) blotting. As shown in Fig. 5, the levels are elevated in cells after incubation for 18 h at 32°C.

Cell cycle profiles of cells grown at 32°C. To determine whether the loss in viability at 32°C was associated with a block in cell cycle progression, the ts Val-135-expressing cells and the control cell lines, DP16-1 and DP16-1pro193, were sampled at various time points after the incubation temperature was shifted from 37 to 32°C. The cells were fixed, stained with PI, and assessed for DNA content by flow cytometry as described in Materials and Methods.

All cell lines displayed a normal cell cycle profile at 37°C. The consecutive cell cycle profiles of val135-5 and -17 cells incubated at 32°C showed a block at the G₁/S boundary that was evident by 8 h, when the first sample was collected for analysis (Fig. 6). A progressive loss of cells in the S and G₂/M phases was observed; cells already in S or G₂/M at the time of the temperature shift continued to traverse the cell cycle until they entered G₁ and became blocked from progressing further. This was seen as a widening gap between the G₁ and G₂/M phases of the cycle. By 24 h, there were

FIG. 3. Presence of hemoglobin in DP16-1 and DP16-1-derived cells at 32°C. Cells (5×10^5 per ml) were incubated at 32°C in the absence (—) or presence (---) of 1 U of Epo per ml, and the percentage of cells expressing detectable hemoglobin was determined at the indicated time points after cells were stained with DAF.

FIG. 4. Proportion of ts p53^{Val-135}-expressing cells that produce hemoglobin at 32°C. Parental cell cultures (val135-5, val135-15, and val135-17) and two clones obtained from each cell population (ts5.2 and ts5.3, ts15.1 and ts15.2, and ts17.1 and ts17.5) were incubated for 48 h at 32°C in α -MEM plus 10% serum with or without Epo. Hemoglobin-positive cells were detected by staining with DAF. The values shown for val135-5, val135-15, and val135-17 represent the means $\pm$ standard errors of three different experiments. The clones were each tested once.

virtually no cells in the S phase, and by 36 h the number of cells in G₂/M was also markedly reduced. As expected for a G₁ block, the proportion of val135-5, -15, and -17 cells in G₁ increased during the first 24 h at 32°C (Fig. 7). The number of cells arrested in G₁ declined thereafter, and a new population of cells characterized by a DNA content less than that seen in G₁ began to appear after 24 h. These likely represent nonviable cells in which DNA degradation had occurred. In a separate study, Friend virus-transformed cells were shown to undergo apoptosis following p53 gene expression (49). The control cell lines, DP16-1 and DP16-1pro193, displayed normal cell cycle profiles at 32°C (Fig. 6), did not accumulate at any point in the cell cycle (Fig. 7), and continued to grow throughout the course of the experiment (Fig. 2A).

Parallel cultures were incubated at 32°C in the presence of Epo (1 U/ml) and subjected to cell cycle analysis by flow cytometry. The cell cycle profiles of ts Val-135 cells incubated in the presence of Epo were different from those of cells incubated in the absence of Epo (Fig. 6). The population of cells with a DNA content less than that of G₁ cells was diminished in the presence of Epo, and an increase in the proportion of cells in G₁ and G₂/M was observed. However, the number of cells in the S phase appeared to be diminished relative to those in control cultures, consistent

FIG. 5. Expression of β -globin mRNA in p53^{Val-135}-expressing cells. RNA was prepared from val135-15 cells incubated at 37°C or after they had been incubated at 32°C for 18 h and examined by Northern blot analysis with a ³²P-labelled cDNA probe specific for β -globin. The same filter was stripped and reprobed with ³²P-labelled tubulin cDNA.

with the inability of val135-5, -15, and -17 cells to proliferate at 32°C (Fig. 2C).

Expression of gp55 protein. One way in which FVP-transformed erythroleukemia cells, which normally grow independently of Epo, might acquire responsiveness to Epo is through down-regulation of viral gp55 expression. The recent demonstration that wild-type p53 protein can repress transcription from various cellular promoters in cotransfection experiments (12, 45) prompted us to examine whether viral gp55 expression was down-regulated in val135-5, -15, and -17 cultures at 32°C. We examined the levels of expression of gp55 protein in the ts Val-135 cells and control cells at 37°C and after incubation at 32°C for 18 h by metabolic labelling and immunoprecipitation with antibodies that recognize gp55 protein. Figure 8 shows the results for val135-15 cells and DP16-1pro193 control cells. There did not appear to be down-regulation of gp55 expression in either of these types of cells at 32°C. Similar results were seen with val135-5 and val135-17 cells (data not shown). Thus, p53 protein does not interfere with SFFV gp55 expression.

DISCUSSION

Friend virus-transformed erythroleukemia cells are derived from erythroid progenitor cells infected with the polycythemia- or anemia-inducing strains of the Friend virus complexes known as FVP and FVA, respectively. These complexes consist of two viruses, a replication-defective SFFV and a replication-competent Friend murine leukemia virus. Friend murine leukemia virus alone has also been shown to induce erythroleukemia in certain strains of newborn mice (reviewed in references 43 and 47). Soon after infection, the number of erythroid progenitors increases. These cells have limited self-renewal capacities, and they retain the ability to differentiate terminally into mature erythrocytes (reviewed in reference 34). In contrast to normal erythroid progenitors, those present in the spleens of FVP-infected mice become independent of Epo early after infection. The SFFV *env* gene product, gp55, was previously shown to interact with the Epo receptor (30, 51). SFFV infection of cells expressing the Epo receptor results in cells that grow in the absence of Epo (30, 44). These and more recent data showing elevated amounts of Epo receptor-associated protein kinase activity in cells expressing the Epo receptor and gp55 (31) provide strong support for the hypothesis that interaction of gp55 with the Epo receptor leads to receptor activation and cell proliferation in the absence of Epo. Late after Friend virus infection, one or a few malignant clones with extensive self-renewal capacities emerge in the spleen. The leukemic cells can be grown in methylcellulose or in suspension cultures, and they give rise to established cell lines (Friend cell lines) in the absence of Epo (33). The transition from polyclonal nonleukemic proliferation to malignant disease is likely to involve additional genetic changes.

A common feature of Friend cell lines is recessive mutation of the cellular p53 tumor suppressor gene. Different types of mutations, including deletion, proviral insertion, and missense mutations, all of which result in a loss of wild-type p53 expression, have been detected (reviewed in references 1 and 19). We have previously demonstrated that these mutations occur in vivo in the spleen cells of Friend virus-infected mice (41) and have suggested that the loss of wild-type p53 expression is an obligatory event in the development of Friend virus-induced erythroleukemia. In addition to p53 gene inactivation, activation of the cellular

FIG. 6. Flow cytometric analysis of cells grown at 32°C in the presence or absence of Epo. Cells expressing ts p53^{Val-135} and the control cell lines, DP16-1 and DP16-1pro193, were sampled at the indicated time points after being incubated at 32°C. Cells were fixed and stained with PI as described in the text. Histograms plotted on a linear scale of DNA fluorescence from 10⁴ cells are shown. Cells incubated with Epo received 1 U/ml at the start of incubation at 32°C.

Spi-1/PU.1 (40) or *Fli-1* (2) gene as the result of provirus insertion is commonly seen in murine erythroleukemias induced by the Friend virus complexes or the Friend murine leukemia virus, respectively. Both *Spi-1/PU.1* and *Fli-1* are members of the *ets* oncogene family (2, 23). The *Spi-1/PU.1* protein is a transcriptional regulator with specific DNA binding activity.

In this study, the ts mutant p53 allele (p53^{Val-135}) was introduced and expressed in the FVP-transformed cell line DP16-1, which does not express endogenous p53 protein. At 32°C, the p53 protein encoded by this allele has the properties of wild-type p53, whereas at 37°C, it behaves as a mutant polypeptide (13, 35). Two findings were presented. First, at

32°C, p53^{Val-135}-expressing cells arrested in the G₀/G₁ phase of the cell cycle and failed to enter the S phase. Cell cycle arrest was accompanied by loss of viability (assessed by flow cytometric analysis on the basis of PI exclusion in unfixed cells and reduced forward-angle light scatter) and expression of β -globin mRNA and hemoglobin, markers of erythroid maturation.

It is pertinent to consider which of these changes is influenced most directly by p53 expression. Numerous reports have indicated that wild-type but not mutant p53 protein expression results in the interruption of cell cycle progression at the G₁/S boundary (4, 6, 13, 20, 35–38, 50). In addition, Kastan et al. (22), Kuerbitz et al. (27), and Lane (28) have proposed a model in which wild-type p53 protein acts as a cell cycle checkpoint determinant to restrict cell growth in the G₁ phase of the cell cycle under adverse

FIG. 7. Percentage of total cells in the G₁ phase. Data gathered from analysis of DNA content and percent viabilities of the cells by FACS were further analyzed by the Multicycle program (Coulter Corp.) to determine the percentage of cells in G₁ at the indicated time points.

FIG. 8. Expression of viral protein gp55 at 37 and 32°C. Cells grown at 37°C (lanes 1 to 4) or cultured for 18 h at 32°C (lanes 5 to 8) were labelled metabolically for 1 h with [³⁵S]methionine at 37°C (lanes 1 to 4) or at 32°C (lanes 5 to 8). Lysates were prepared, and proteins were immunoprecipitated with a goat anti-mouse IgM antibody as a control (lanes 1, 3, 5, and 7) or with an antibody recognizing Rauscher virus gp69/71 protein which also recognizes SFFU gp55 (lanes 2, 4, 6, and 8). Extracts prepared from DP16-1pro193 cells were immunoprecipitated in lanes 1, 2, 5, and 6; extracts prepared from val135-15 cells were immunoprecipitated in lanes 3, 4, 7, and 8. Lysates containing equal amounts of trichloroacetic acid-insoluble radioactivity (10⁷ cpm) were immunoprecipitated in each lane.

conditions. It is possible that the differentiation seen in this study is a consequence of p53-mediated G₁ arrest and is not a direct result of p53 protein function. Prolonged residency of Friend cells in the G₁ phase of the cell cycle may promote entry into the erythroid maturation pathway. Previous studies of Friend cell differentiation with dimethyl sulfoxide indicated that this was a stochastic process (14) and that it was associated with a transient arrest of cells in the G₁ phase of the cell cycle (10, 48).

In light of the importance of p53 in the evolution of Friend erythroleukemia, our observations suggest that disruption of p53 function may be necessary to interrupt the maturation program of infected erythroid cells leading to erythroleukemia. A role for p53 in normal erythroid differentiation has not been established. Indeed, it is not required, since mice lacking p53 have normal erythropoiesis, even though they display increased susceptibilities to various malignancies, including leukemia (7). In addition, Friend cell lines lacking p53 can be induced to differentiate with a variety of chemical inducers (reviewed in reference 34). Thus, erythropoiesis can occur in the absence of p53 protein. Note, however, that p53 RNA is abundantly expressed in normal murine erythroid precursors (2a), which could imply a role for p53 in normal erythroid development.

The second important finding of this study is that p53^{Val-135}-expressing Friend cells displayed responsiveness to Epo at 32°C. This was demonstrated in several ways. First, loss of viability was markedly reduced in the presence of Epo (Fig. 2), second, flow cytometric analysis of DNA content revealed less cell cycle perturbation and less DNA degradation in the presence of Epo (Fig. 6), and third, the proportion of cells producing hemoglobin was diminished in the presence of Epo (Fig. 3 and 4). Parental DP16-1 cells or cells expressing p53^{Pro-193} were able to grow at 32°C in the presence or absence of Epo, indicating that the requirement for Epo is not merely the result of cell growth at a low temperature.

Although we have chosen to report these two findings together, we have no evidence that they are connected. We demonstrate first that p53 expression is associated with G₁ arrest and hemoglobin production in DP16-1 cells and second that these cells respond to Epo. We have no evidence that the response to Epo is controlled by p53.

Possible modes of Epo action include promotion of cell proliferation, maintenance of cell viability, and initiation of erythroid differentiation (reviewed in references 5 and 26). In the clonogenic assays that have been developed to examine hemopoietic precursors, Epo is a necessary component for the development of erythroid colonies. Erythroid colonies result from cells undergoing proliferation and differentiation, and so it has been difficult to assign a specific role to Epo in this process. Our data indicate that Epo prolongs the survival of Friend cells expressing wild-type p53 protein and argue against a role for Epo in initiating the differentiation process in Friend cells. Koury and Bondurant (25) demonstrated that programmed cell death (apoptosis) is a component of erythropoiesis in Epo-dependent FVA-infected cells. Moreover, Epo was shown to retard DNA cleavage (a marker of apoptosis) without blocking differentiation into reticulocytes. They suggested that the prolonged survival of CFU-E (CFU-erythroid) and their immediate descendants by Epo facilitated completion of the erythroid differentiation program (24, 25).

The importance of Epo receptor signalling in the development of murine erythroleukemia is highlighted by studies in which infection of mice with recombinant retroviral vectors carrying genes encoding either Epo or a constitutively

activated Epo receptor developed splenomegaly and polycythemia, which are similar to the early stages of Friend disease induced by FVP (17, 32). Epo-independent, proerythroblast cell lines established from the spleens of mice infected with the activated Epo receptor virus had rearranged and inactivated both p53 alleles (32).

We have shown that gp55 is expressed in the presence of p53^{Val-135} protein at both 32 and 37°C. Thus, down-regulation of gp55 expression does not provide a plausible mechanism to account for the inhibition of cell proliferation that is observed with p53^{Val-135}-expressing cells at 32°C. Moreover, it is unclear why these cells respond to Epo even while they continue to express gp55. Expression of gp55 in FVP-infected cells has been shown to cause a reduction in the amount of Epo receptor on the cell surface, presumably because of retention of the Epo receptor within the endoplasmic reticulum (44, 51). Migliaccio et al. (39) have suggested that the major regulatory step determining the erythroid-specific response to Epo is the efficiency of Epo receptor protein translocation to the cell surface and not activation of the Epo receptor gene. It is possible, therefore, that Friend cells that have been growth arrested by wild-type p53 express more Epo receptor molecules on their cell surfaces. An increased number of receptor molecules on these cells could explain their responsiveness to Epo. This possibility is being investigated.

ACKNOWLEDGMENTS

We thank Moshe Oren (Wiezmann Institute) for providing the ts p53^{Val-135} allele and Norman Iscove and Deborah Hyam (Ontario Cancer Institute) for supplying Epo. We are grateful to Michael Clarke (University of Michigan) and Norman Iscove and Ged Brady (Ontario Cancer Institute) for sharing their results with us prior to publication. We also thank Norman Iscove, Richard Gronostajski, and Tak Mak (Ontario Cancer Institute) for reading the manuscript.

P.J. is supported by a Stephen Fonyo Studentship from the National Cancer Institute of Canada. This work was supported by the National Cancer Institute of Canada and the Medical Research Council of Canada.

REFERENCES

1. Ben-David, Y., and A. Bernstein. 1991. Friend virus-induced erythroleukemia and the multistage nature of cancer. *Cell* 66:831-834.
2. Ben-David, Y., E. Giddens, and A. Bernstein. 1990. Identification and mapping of a common proviral integration site Fli-1 in erythroleukemia cells induced by Friend murine leukemia virus. *Proc. Natl. Acad. Sci. USA* 87:1332-1336.
- 2a. Brady, G., and N. Iscove. Personal communication.
3. Caron de Fromental, C., and T. Soussi. 1992. TP53 tumor suppressor gene: a model for investigating human mutagenesis. *Genes Chromosomes Cancer* 4:1-15.
4. Chen, P.-L., Y. Chen, R. Bookstein, and W.-H. Lee. 1990. Genetic mechanism of tumor suppression by the human p53 gene. *Science* 250:1576-1580.
- 4a. Clarke, M. Personal communication.
5. D'Andrea, D. A., and L. I. Zon. 1990. Erythropoietin receptor. *J. Clin. Invest.* 86:681-687.
6. Diller, L., J. Kassel, C. E. Nelson, M. A. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. J. Baker, B. Vogelstein, and S. H. Friend. 1990. p53 functions as a cell cycle control protein in osteosarcoma. *Mol. Cell. Biol.* 10:5772-5781.
7. Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, J. R. Montgomery, J. S. Butel, and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature (London)* 356:215-221.
8. Eliyahu, D., N. Goldfinger, O. Pinhasi-Kimhi, G. Shaulsky, Y. Skurnik, N. Arai, V. Rotter, and M. Oren. 1988. Meth A fibrosarcoma cells express two transforming mutant p53 species. *Oncogene* 3:313-321.

9. Finlay, C. A., P. W. Hinds, T.-H. Tan, D. Eliyahu, M. Oren, and A. J. Levine. 1988. Activating mutations for transformation by p53 produce a product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell. Biol.* **8**:531-539.
10. Friedman, E. A., and C. L. Schildkraut. 1978. Lengthening of the G1 phase is not strictly correlated with differentiation in Friend erythroleukemia cells. *Proc. Natl. Acad. Sci. USA* **75**:3813-3817.
11. Gannon, J. V., R. Greaves, R. Iggo, and D. P. Lane. 1990. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.* **9**:1595-1602.
12. Ginsberg, D., F. Mechta, M. Yaniv, and M. Oren. 1991. Wild-type p53 can down-modulate the activity of various promoters. *Proc. Natl. Acad. Sci. USA* **88**:9979-9983.
13. Ginsberg, D., D. Michael-Michalovitz, D. Ginsberg, and M. Oren. 1976. Induction of growth arrest by a temperature-sensitive p53 mutant is correlated with increased nuclear localization and decreased stability of the protein. *Mol. Cell. Biol.* **11**:582-585.
14. Gusella, J., R. Geller, B. Clarke, V. Weeks, and D. Housman. 1991. Commitment to erythroid differentiation by Friend erythroleukemia cells: a stochastic analysis. *Cell* **9**:221-229.
15. Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson. 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.* **39**:861-869.
16. Hicks, G., and M. Mowat. 1988. Integration of Friend murine leukemia virus into both alleles of the p53 oncogene in an erythroleukemic cell line. *J. Virol.* **62**:4752-4755.
17. Hoatlin, M. E., S. L. Kozak, F. Lilly, A. Chakraborti, C. A. Kozak, and D. Kabat. 1990. Activation of erythropoietin receptors by Friend viral gp55 and by erythropoietin and down-modulation by the murine Fv-2r resistance gene. *Proc. Natl. Acad. Sci. USA* **87**:9985-9989.
18. Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris. 1991. p53 mutations in human cancers. *Science* **253**:49-53.
19. Johnson, P., and S. Benchimol. 1992. Friend virus induced murine erythroleukemia: the p53 locus. *Cancer Surv.* **12**:137-151.
20. Johnson, P., D. Gray, M. Mowat, and S. Benchimol. 1991. Expression of wild-type p53 is not compatible with continued growth of p53-negative tumor cells. *Mol. Cell. Biol.* **11**:1-11.
21. Kaiho, S.-I., and K. Mizuno. 1985. Sensitive assay systems for detection of hemoglobin with 2,7-diaminofluorene: histochemistry and colorimetry for erythrodifferentiation. *Anal. Biochem.* **149**:117-120.
22. Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig. 1991. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **51**:6304-6311.
23. Klemsz, M. J., S. R. McKercher, A. Celada, C. van Beveren, and R. A. Maki. 1990. The macrophage and B cell-specific transcription factor PU.1 is related to the *ets* oncogene. *Cell* **61**:113-124.
24. Koury, M. J., and M. C. Bondurant. 1988. Maintenance by erythropoietin of viability and maturation of murine erythroid precursor cells. *J. Cell. Physiol.* **137**:65-74.
25. Koury, M. J., and M. C. Bondurant. 1990. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* **248**:378-381.
26. Krantz, S. B. 1991. Erythropoietin. *Blood* **77**:419-434.
27. Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh, and M. B. Kastan. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA* **89**:7491-7495.
28. Lane, D. P. 1992. p53, guardian of the genome. *Nature (London)* **358**:15-16.
29. Lane, D. P., and S. Benchimol. 1990. p53: oncogene or anti-oncogene? *Genes Dev.* **4**:1-8.
30. Li, J.-P., A. D. D'Andrea, H. F. Lodish, and D. Baltimore. 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. *Nature (London)* **343**:762-764.
31. Linnekin, D., G. A. Evans, A. D'Andrea, and W. L. Farrar. 1992. Association of the erythropoietin receptor with protein tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA* **89**:6237-6241.
32. Longmore, G. D., and H. F. Lodish. 1991. An activating mutation in murine erythropoietin receptor induces erythroleukemia in mice: a cytokine receptor superfamily oncogene. *Cell* **67**:1089-1102.
33. Mager, D. L., T. W. Mak, and A. Bernstein. 1981. Quantitative colony method for tumorigenic cells transformed by two distinct strains of Friend leukemia virus. *Proc. Natl. Acad. Sci. USA* **78**:1703-1707.
34. Marks, P. A., and R. A. Rifkind. 1978. Erythroleukemic differentiation. *Annu. Rev. Biochem.* **47**:419-448.
35. Martinez, J., I. Georgoff, J. Martinez, and A. J. Levine. 1991. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. *Genes Dev.* **5**:151-159.
36. Mercer, W. E., M. Amin, G. J. Sauve, E. Appella, S. J. Ullrich, and J. W. Romano. 1990. Wild-type human p53 is antiproliferative in SV40-transformed hamster cells. *Oncogene* **5**:973-980.
37. Mercer, W. E., M. T. Shields, M. Amin, G. J. Sauve, E. Appella, J. W. Romano, and S. J. Ullrich. 1990. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA* **87**:6166-6170.
38. Michalovitz, D., O. Halevy, and M. Oren. 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell* **62**:671-680.
39. Migliaccio, A. R., G. Migliaccio, D. A. D'Andrea, M. Baiocchi, S. Rotta, S. Nicolis, S. Ottolenghi, and J. W. Adamson. 1991. Response to erythropoietin in erythroid subclones of the factor-dependent cell line 32D is determined by translocation of the erythropoietin receptor to the cell surface. *Proc. Natl. Acad. Sci. USA* **88**:11086-11090.
40. Moreau-Gachelin, F., A. Tavittian, and P. Tambourin. 1988. Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. *Nature (London)* **331**:277-280.
41. Mowat, M., A. Cheng, N. Kimura, A. Bernstein, and S. Benchimol. 1985. Rearrangements of the cellular p53 gene in erythroleukemic cells transformed by Friend virus. *Nature (London)* **314**:633-636.
42. Munroe, D. G., J. Peacock, and S. Benchimol. 1990. Inactivation of the cellular p53 gene is a common feature of Friend erythroleukemia: relation to dominant transforming alleles. *Mol. Cell. Biol.* **10**:3307-3313.
43. Ruscetti, S., and L. Wolff. 1984. Spleen focus-forming virus: relationship of an altered envelope gene to the development of a rapid erythroleukemia. *Curr. Top. Microbiol.* **112**:21-44.
44. Ruscetti, S. K., N. J. Janesch, A. Chakraborti, S. T. Sawyer, and D. Hanksaw. 1990. Friend spleen focus-forming virus induces factor independence in an erythropoietin-dependent erythroleukemia cell line. *J. Virol.* **63**:1057-1062.
45. Santhanam, U., A. Ray, and P. B. Sehgal. 1991. Repression of the interleukin-6 gene promoter by p53 and the retinoblastoma susceptibility gene product. *Proc. Natl. Acad. Sci. USA* **88**:7605-7609.
46. Shaulsky, G., N. Goldfinger, A. Peled, and V. Rotter. 1991. Involvement of wild-type p53 in pre-B-cell differentiation in vitro. *Proc. Natl. Acad. Sci. USA* **88**:8982-8986.
47. Tambourin, P. E., F. Wendling, C. Jasmin, and F. Smadja-Joffe. 1979. The physiopathology of Friend leukemia. *Leuk. Res.* **3**:117-129.
48. Terada, M., J. Fried, U. Nudel, R. A. Rifkind, and P. A. Marks. 1977. Transient inhibition of initiation of S-phase associated with dimethyl sulfoxide induction of murine erythroleukemia cells to erythroid differentiation. *Proc. Natl. Acad. Sci. USA* **74**:248-252.
49. Yewdell, J. W., J. V. Gannon, and D. P. Lane. 1986. Monoclonal antibody analysis of p53 expression in normal and transformed cells. *J. Virol.* **59**:444-452.
50. Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren. 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature (London)* **352**:345-347.
51. Yoshimura, J. W., A. D. D'Andrea, and H. F. Lodish. 1990. Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. *Proc. Natl. Acad. Sci. USA* **87**:4139-4143.