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Mutation of *Tp53* Contributes to the Malignant Phenotype of Abelson Virus-Transformed Lymphoid Cells

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Abelson murine leukemia virus transforms pre-B cells in vitro and induces rapid-onset pre-B-cell lymphoma in vivo. Expression of an active v-Abl protein tyrosine kinase is required for the oncogenic functions of the virus. Despite the strong growth-stimulatory signal provided by v-Abl, the virus-induced tumors are clonal or oligoclonal, and changes in the growth and oncogenic potential of in vitro transformants occur during the derivation of the cell lines. Both of these features suggest that v-Abl expression must be complemented by changes in expression of one or more cellular genes for cells to acquire a fully malignant phenotype. Such genes could include other oncogenes or tumor suppressor genes. Among the latter is *Tp53*, a gene mutated in many spontaneous cancers. To determine if mutation of the *Tp53* tumor suppressor gene plays a role in Abelson virus transformation, conformation-specific monoclonal antibodies were used to examine p53 expression in a panel of Abelson virus-transformed pre-B cells. Expression of mutant forms of p53 was detected in over 40% of the isolates. Sequence analysis revealed the presence of point mutations affecting the highly conserved central portion of the protein. These mutations interfered with the ability of p53 to activate transcription from a promoter containing p53-responsive elements and to induce apoptosis in response to DNA damage. In addition, cells expressing mutant forms of p53 induced a higher frequency of tumors with a more rapid course compared to transformants expressing wild-type p53. These data suggest that *Tp53* is one important cellular gene involved in malignant transformation by Abelson virus.

Abelson murine leukemia virus (Ab-MLV) is a highly oncogenic, transforming retrovirus that expresses the v-Abl protein tyrosine kinase (reviewed in references 42 and 43). An active v-Abl protein is required to induce pre-B-cell lymphomas in vivo and to transform pre-B cells in vitro. Despite the presence of a strongly transforming oncogene, Ab-MLV-induced tumors are usually clonal or oligoclonal (20, 21), suggesting that events in addition to expression of the v-Abl protein are required for tumor formation. In addition, in vitro-derived lymphoid transformants undergo a series of changes during culture before they acquire a fully malignant phenotype (55, 56). Thus, Ab-MLV-induced transformation, like tumor induction by retroviruses lacking oncogenes and spontaneous tumor induction, is a multistep process in which expression of the v-Abl protein is complemented by changes in the expression of other, currently ill-defined cellular genes.

Multistep carcinogenesis usually involves an interplay between dominant-acting, growth-stimulatory signals and negative growth-regulatory elements encoded by tumor suppressor genes (reviewed in references 28, 31, and 46). The role of tumor suppressor genes in retroviral oncogenesis has received limited attention (4–6, 9, 52, 53). However, experiments with an Ab-MLV-transformed cell line have suggested that mutation of the *Tp53* gene may be one important event in Ab-MLV-induced transformation (59). This cell line, L1-2, lacks expression of p53 because one copy of the gene has been lost and the second copy contains a proviral integration in the *Tp53* gene (60). Unlike most established Ab-MLV-transformed cells, L1-2 fails to induce tumors in syngeneic mice (57, 59). However, cells that express a mutant *Tp53* allele form aggressive tumors rapidly (59). Other studies suggest that at least two other Ab-MLV transformants express mutant forms of p53 protein (58).

An extensive literature documents the frequent occurrence of *Tp53* mutation in many different tumor systems (reviewed in references 32 and 34). Over 50% of human tumors including a significant number of *BCR/ABL*-induced leukemias, contain *Tp53* mutations (reviewed in references 2, 29, and 40), and several different types of oncogenic viruses encode proteins which interfere with p53 expression. For example, simian virus 40 T antigen binds p53 and sequesters the molecule in the cytoplasm (reviewed in references 36 and 37), papillomavirus E6 protein targets p53 for rapid degradation (reviewed in references 17 and 51), and adenovirus E1b binds p53, masking the p53 transactivation domain (reviewed in references 38 and 54). In all of these instances, altered p53 function appears to contribute to tumorigenesis by interfering with its multiple roles in cell cycle control and apoptosis.

To examine the possibility that *Tp53* mutation plays a role in Ab-MLV-induced tumorigenesis, we determined the frequency with which transformants acquire *Tp53* mutations and their effects on p53 function and on tumorigenesis. Mutations in *Tp53* were found in about 40% of all in vitro-derived transformed pre-B-cell lines. These mutations are detectable as soon as 1 month postinfection and correlate with an increased ability of the cells to form tumors in syngeneic mice. Cells expressing mutant forms of p53 protein fail to activate expression of a promoter containing p53-responsive elements and do not respond to γ-irradiation-induced DNA damage by undergoing apoptosis. Because the mutations that occur in the Ab-MLV system resemble those observed in many spontaneous tumors, this viral transformation system provides an excellent model with which to examine the ways in which these mutations are selected during oncogenesis.

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MATERIALS AND METHODS

Cells and mice. Ab-MLV-transformed bone marrow cells were derived as described previously (44), using the Ab-MLV-P160 strain (16). These cells were maintained in RPMI 1640 medium supplemented to contain 10% heat-inactivated fetal calf serum (HyClone) and 50 μM 2-mercaptoethanol. F9 embryonal carcinoma cells (7) were maintained in low-glucose Dulbecco's modified Eagle's medium supplemented to contain 10% newborn calf serum (GIBCO), 0.375% sodium bicarbonate, 2 mM L-glutamine, streptomycin (50 μg/ml), and penicillin (50 U/ml). To facilitate adherence of the cells, tissue culture plates were coated with 0.3% gelatin (Fisher Scientific) prior to addition of the cells. Tumorigenicity studies were conducted with BALB/cByJ mice bred in the Tufts University School of Medicine animal facility. Adult mice were injected subcutaneously and monitored for evidence of tumor formation such as visible tumor, general ill health, or palpable nodules at the injection site. Animals were sacrificed and autopsied when tumors larger than 1 cm^3 were evident or 70 days postinjection if the animals remained healthy.

Protein analysis. Immunoprecipitation experiments were performed as described elsewhere (16). Briefly, exponentially growing cells were labeled with 100 μCi of [^{35}S]methionine (New England Nuclear) for 1 h, and the cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). After a 30-min incubation on ice, the lysates were clarified by ultracentrifugation. The p53 proteins were immunoprecipitated with 1 μg of anti-p53 monoclonal antibody (MAb) PAb421, PAb240, or PAb246 (Oncogene Science). The immune complexes were recovered by using Sepharose CL-4B beads (Pharmacia) and analyzed by electrophoresis through sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. The processed gels were exposed to Kodak XAR film at −70°C. For Western analysis, cell lysates were prepared and analyzed as described elsewhere (39). Briefly, the cells were washed once in phosphate-buffered saline (PBS) and treated with lysis buffer (10 mM Tris [pH 7.5], 1% SDS, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM Na_3VO_4). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride membranes (Millipore), and probed with MAb PAb240 directed against p53 or antibody Ab-5 directed against WAF1 (Oncogene Science). The blots were developed by using a chemiluminescence kit (Tropix) according to the manufacturer's instructions.

Sequence analysis. *Tp53* cDNA was prepared by using reverse transcriptase (RT) and PCR. For each reaction, 1 μg of total cellular RNA (11) was added to a 20-μl reaction mixture containing 1× RT buffer, 10 mM dithiothreitol 0.5 mM each dGTP, dATP, dTTP, and dCTP, RT (10 U/μl; GIBCO), RNasin (2 U/μl), and p53 3′ primer (5′ GGAATTCAGCCCTGAAGTCATAAGA 3′; 20 ng/μl). Reactions containing no RNA and no RT were used as controls. The tubes were placed in a Programmable Thermal Controller (MJ Research, Inc.) and incubated for 10 min at 25°C, 60 min at 42°C, and then 10 min at 95°C. PCR amplification was carried out in reactions containing a portion of the RT product, 1× PCR buffer (Perkin-Elmer Cetus), 300 μM each dGTP, dATP, dTTP, and dCTP (Pharmacia), and 0.5 μM each primer 5′ GGAATTCAGG CCCTCATCCT 3′ and p53 3′ primers. After a 90-s incubation at 94°C, 0.5 μl of *Taq* polymerase (Perkin-Elmer Cetus) was added, and the samples were subjected to 35 cycles of amplification (1 min at 95°C, 2 min at 56°C, and 3 min at 72°C) and a final extension of 7 min at 72°C. For amplification of genomic DNA, the reaction cocktail contained the DNA, 1× PCR buffer, 200 μM each dGTP, dATP, dTTP, and dCTP, 0.25 μM each primers 5′ CTCTCCTCCCC TCAATAAGC 3′ and 5′ TGGTGGTATACTCAGAGCCG 3′, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus). Reactions were amplified for 30 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C. After the last cycle, the samples were incubated for 5 min at 72°C. Amplified products were gel purified and ligated directly into the TA cloning vector (Invitrogen) exactly as described by the manufacturer. The sequence of each cloned fragment was determined by using a Sequenase 2.0 kit as described by the manufacturer. In all cases, at least two clones from two independent amplifications were examined.

Expression assays. The p21P, p21PΔ1.1, and p21PSmaΔ1 luciferase expression plasmids (see Fig. 3A) (14, 15) and pSVβ-galactosidase (Promega) were introduced into cells by electroporation (960 μF, 300 V). Prior to electroporation, cells were washed twice with unsupplemented RPMI medium, mixed with plasmids, and incubated at room temperature for 10 min. After electroporation, the cells were incubated on ice for an additional 10 min, plated in RPMI growth medium, and incubated for 18 h at 37°C and 6% CO_2. Lysates were prepared and analyzed for luciferase activity by using the Luciferase assay system (Promega) according to the manufacturer's instructions. Levels of β-galactosidase expression were monitored by using a Galacto-Light Plus chemiluminescent reporter kit (Tropix), and the values obtained were used to control for the efficiency of the electroporation. Each transfection was done in triplicate, and each cell line was assayed three to four times.

Apoptosis analysis. Cells were treated with γ irradiation by using a cesium 137-Gammacell-1000 source and incubated for various time intervals in growth medium. The cells were washed once in PBS and incubated in Hoechst 33342 dye (1 μg/ml in PBS; Molecular Probes) for 15 min at 37°C (50). After an additional 15 min on ice, the cells were stained with propidium iodide (10 μg/ml in H_2O) as described previously (10). The cells were analyzed immediately by flow cytometry using a FACstar Plus (Becton Dickinson) with dual laser capacity. The data were analyzed by using WinLit 2.01 software (Verity Software House).

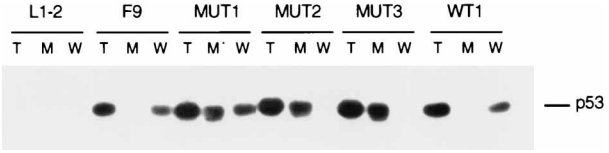

FIG. 1. Expression of wild-type and mutant p53 by lymphoid transformants. Lysates from [^{35}S]methionine-labeled Ab-MLV-transformed pre-B-cell lines were immunoprecipitated with PAb421 directed against total p53 (lanes T), PAb240 directed against mutant p53 (lanes M), or PAb246 directed against wild-type p53 (lanes W). The immune complexes were recovered and analyzed by SDS-PAGE. L1-2 is a p53-null cell line (60); F9 is an embryonal carcinoma cell line documented to express wild-type p53 (18); MUT1, MUT2, and MUT3 are 143-2M, 1-21, and 1-22, respectively; WT1 is 1-20.

RESULTS

Many Ab-MLV-transformed pre-B cells express mutant p53 protein. To assess whether *Tp53* mutation is a common feature of Ab-MLV-transformed pre-B cells, a panel of transformants was screened by immunoprecipitation with antibodies that recognize different conformational epitopes commonly present on mutant or wild-type p53 (25, 34). Cells were labeled with [^{35}S]methionine, and p53 was immunoprecipitated with anti-p53 MAbs PAb421 (pan-specific), PAb240 (mutant specific), and PAb246 (wild-type specific) (Fig. 1). F9 embryonal carcinoma cells, previously demonstrated to express wild-type p53 (18) and the Ab-MLV-transformed pre-B-cell line L1-2, which carries only a single nonfunctional *Tp53* allele and does not synthesize p53 (59, 60), served as controls.

All cells except L1-2 contained a p53 protein that reacted with the pan-specific antibody. However, some expressed p53 that reacted only with the wild-type-specific antibody (Fig. 1, lanes WT1), while others expressed p53 that reacted only with the mutant-specific antibody (lanes MUT2 and MUT3). A few cell lines, such as MUT1, expressed p53 that reacted with all antibodies tested. Analyses of 10 subclones from one such cell line revealed that all of the subclones had the parental pattern of reactivity, suggesting most or possibly all cells in the population express both mutant and wild-type forms of p53 (data not shown). Similar results have been obtained for other cell lines (3, 30). Evaluation of a larger panel of transformants revealed that 9 of 22, or 42%, of the cell lines harbored mutant forms of p53 (data not shown). Thus, expression of mutant forms of the p53 is a common event in Ab-MLV-transformed pre-B cells.

Mutant p53 can be detected early in in vitro culture. The panel of cell lines analyzed above had been passaged in vitro for periods ranging from several months to years. In many tumor systems, *Tp53* mutation is a late-stage event (2, 31). To determine when mutant p53 became detectable in Ab-MLV-transformed pre-B cells, time course assays were performed. Cells were infected and plated in soft agar (44); after 10 days, 48 colonies were isolated and placed in liquid culture. At intervals, p53 status was assayed by immunoprecipitation with conformation-specific MAbs. The earliest time point that could be evaluated was 18 h after isolation from soft agar. All 13 samples evaluated 18 h after the cells were removed from agar expressed only wild-type protein (Fig. 2A), suggesting that mutant p53 protein is not readily detectable during the initial steps of pre-B-cell transformation.

The cells were passaged in vitro for 18 weeks and assayed for p53 expression at multiple time points. A total of 21 cell lines were derived from the 48 colonies. Mutant p53 protein was detected in 10 of 21, or 48%, of the cell lines (Fig. 2B). Eight of these cell lines expressed both mutant and wild-type p53 protein, while two expressed only mutant p53 protein (data not

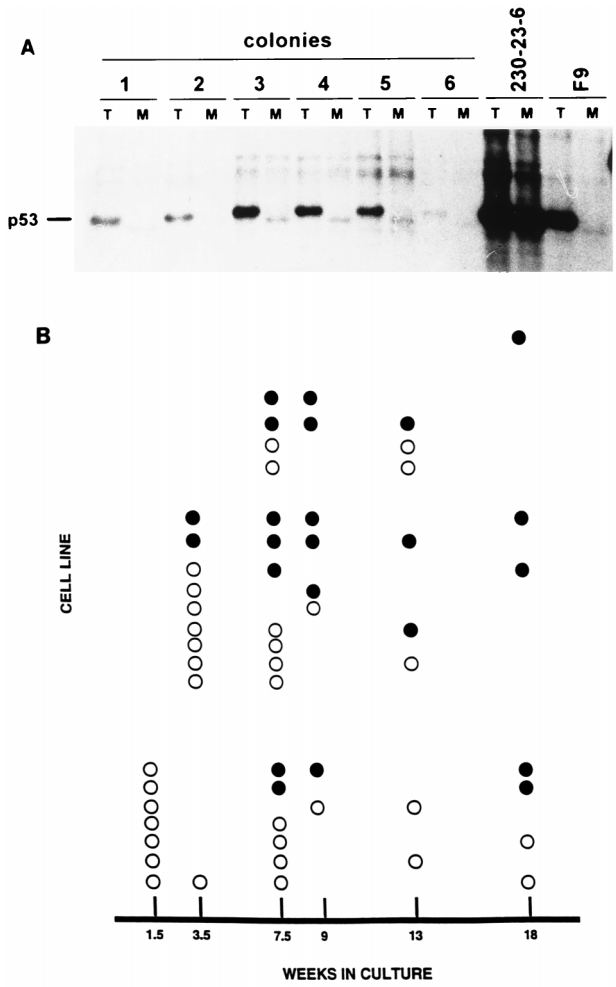

FIG. 2. (A) Transformed pre-B-cell colonies were harvested from agar 10 days after infection, and the cells were labeled with [^{35}S]methionine. The lysates were immunoprecipitated with PAb421 directed against total p53 (lanes T) or PAb240 directed against mutant p53 (lanes M) and analyzed by SDS-PAGE. Representative analysis of six different colonies is shown. 230-23-6 is an Ab-MLV transformant that expresses mutant p53; F9 is an embryonal carcinoma cell line that expresses wild-type p53 (18). (B) Colonies similar to those shown in panel A were expanded in vitro and analyzed for p53 expression at multiple time points. Each row of dots represents analysis of a single colony. Open circles represent samples in which only wild-type p53 was detected; filled circles represent samples in which mutant p53 was detected.

shown). The percentage of cells that expressed mutant forms of p53 at any time point was not assessed; however, the earliest time point at which mutant p53 protein could be detected was 3.5 weeks after isolation from soft agar. Eight of the cell lines expressed mutant p53 during the first 9 weeks. Despite careful observation, no correlation could be established between the appearance of mutant p53 protein and an altered growth pattern. Similar data were obtained when colonies from a second, independent infection were analyzed (data not shown). These results suggest that *Tp53* mutations accumulate within a short period of time in a significant frequency of Ab-MLV-transformed pre-B cells.

Types of *Tp53* mutations. To determine the types of mutations present in *Tp53*, as well as to confirm the results obtained from the immunoprecipitation assays, RT-PCR assay was used to amplify the entire coding sequence of *Tp53* from several cell lines. The products were cloned and sequenced from at least two independent RT-PCR reactions, and sequence changes were considered significant only if they were found in the products from both amplifications.

TABLE 1. *Tp53* mutations in Ab-MLV-transformed pre-B-cell lines[a]

Cell line	Mutation	Effect
3-52	CCA→GCA	Pro^{146}→Ala
	AGA→AAA	Arg^{274}→Lys
1-21	AGC→AGG	Ser^{149}→Arg
	T insertion	Frameshift
3-55	GTG→ATG	Val^{167}→Met
3-67	GCG→CCG	Ala^{132}→Pro
143-2M	TGC→TGG	Cys^{170}→Trp

[a] For 3-52, 1-21, and 3-55, RT-PCR was used to amplify the entire coding sequence of *Tp53*, and the amplified material was cloned and sequenced. For 3-67 and 143-2M, *Tp53* genomic DNA including exons 5 to 7 was amplified by PCR, cloned, and sequenced. In all cases, results were confirmed by examination of at least two clones from two independent PCRs.

Clones recovered from 1-21 cells, cells that expressed only mutant p53, contained one of two mutations (Table 1). One generates a Ser-to-Arg change at amino acid 149, a position that is affected in several human tumors (26). The other causes a frameshift in exon 6, resulting in a p53 protein in which the 29 amino acids predicted to follow His^{187} are not found in the wild-type protein. This protein does not contain the epitopes recognized by either the pan-specific or the mutant-specific anti-p53 MAb, making its expression difficult to confirm. Analyses of PCR products from two cell lines that expressed both wild-type and mutant protein forms revealed clones containing mutant sequence and other clones containing wild-type sequence (Table 1). The mutations identified in both of these cell lines have been observed in various human tumors (26). PCR products spanning the five evolutionarily conserved domains (47) were sequenced from two other cell lines that express only wild-type p53. These were found to contain wild-type sequence. The remaining region, coding for the carboxyl-terminal 100 amino acids, was not analyzed. However, a search of 2,573 documented human *Tp53* mutations yielded only 98 instances of alterations affecting this C-terminal region (26). Therefore, the likelihood that these cell lines contain *Tp53* mutations is small.

Three of the four missense mutations found in the Ab-MLV transformants occurred in a 67-base region in exon 5. This exon contains sequences encoding portions of evolutionarily conserved regions II and III (32, 34, 47). A limited spectrum of *Tp53* mutations has been noted in other tumor systems (1, 8). To determine if exon 5 mutations are a common feature of Ab-MLV-transformed pre-B cells, a 795-bp fragment containing exons 5 and 6 was amplified from genomic DNA of five more cell lines that express mutant p53. Sequencing revealed that two of these cell lines contained mutations affecting exon 5 (Table 1). Both of these mutations have been documented in human tumors (26). Presumably the mutations affecting p53 in the other cell lines are located elsewhere in the gene. Nonetheless, the observation that five of eight cell lines examined contained mutations clustered within a 117-bp segment suggests that alterations in this region are extremely common among Ab-MLV transformants.

Wild-type p53 functions as a transcription factor in Ab-MLV transformants. Although Ab-MLV-transformed cells can be classified as expressing wild-type or mutant p53, the presence of a mutation does not guarantee disrupted function. In addition, cells transformed by other viruses such as simian

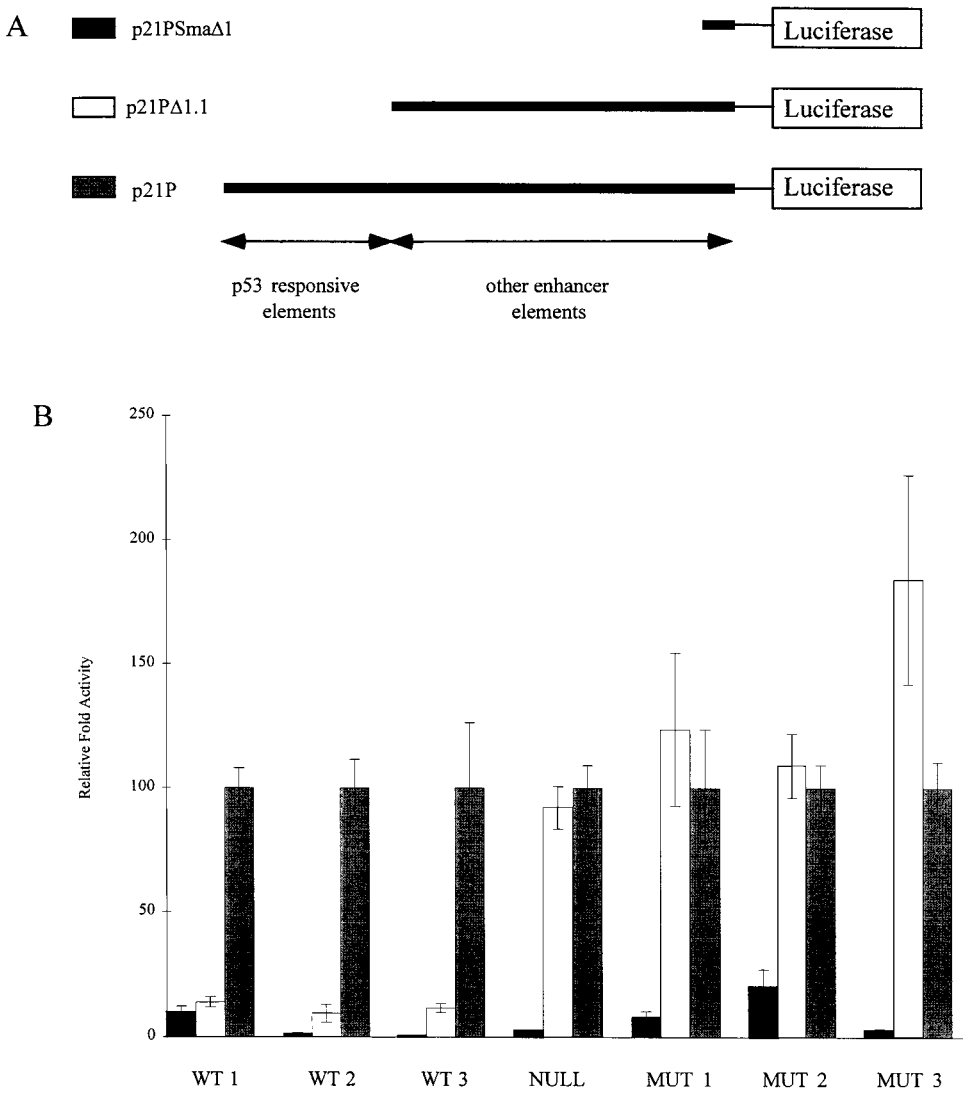

FIG. 3. Transformants expressing wild-type p53 stimulate expression from a promoter containing p53-responsive elements. (A) Diagrams of the expression constructs used in these experiments. (B) Cells were cotransfected with the luciferase expression constructs and a control β-galactosidase expression construct, and levels of luciferase activity were standardized on the basis of β-galactosidase expression. Each cell line shown was assayed in triplicate at least three times. In the graph, the level of activity for the p21P construct for each cell line is set at 100%; the actual average luciferase units obtained for p21PSmaΔ1 are as follows: WT1 (298-18), 137; WT2 (204-3-1), 140; WT3 (38B9), 891; NULL (L1-2), 1,368; MUT1 (379-10), 352; MUT2 (143-2M), 99; and MUT3 (300-31), 269.

virus 40, adenovirus, and papillomavirus express wild-type p53 but use diverse strategies to interfere with p53 function (17, 36–38, 51, 54). One such function is the ability to activate transcription of a variety of genes (reviewed in references 32 and 34), including *p21*, the gene encoding WAF1 (CIP1), an inhibitor of cyclin-dependent kinases (15, 23, 24, 61).

To determine if the p53 status of the transformants correlated with the ability to activate transcription of a promoter containing p53-responsive elements, reporter constructs in which a luciferase gene was placed downstream of a minimal promoter and enhancer elements from the *p21* gene were used (14, 15). One construct, p21P (Fig. 3A), contains both p53-responsive elements and other enhancer elements upstream of the minimal promoter; another, p21PΔ1.1, lacks the p53-responsive elements but contains other enhancer sequences. The p21PSmaΔ1 construct contains only the minimal promoter. This set of constructs has been used by others to assess the transcriptional function of p53 (14). When these constructs were introduced into cell lines that expressed wild-type p53, expression of p21P exceeded that of p21PΔ1.1 8- to 10-fold, indicating the presence of a functional p53 protein. In contrast, cell lines expressing a mutant form of p53, or the L1-2 cell line, which expresses no p53 protein (59, 60), did not show increased expression of p21P, indicating that the mutant form of p53 does not activate transcription from these p53-responsive elements. Indeed, MUT3 expressed the full-length construct at a reduced level compared to p21PΔ1.1. The mechanism involved in this response requires further study. However, a similar pattern was not observed in any of the other cell lines expressing mutant p53 (data not shown).

DNA damage-induced apoptosis occurs in transformants expressing wild-type p53. Another function of p53 is induction of apoptosis following DNA damage (reviewed in reference 34). Under these circumstances, wild-type p53 accumulates and many types of cells, including lymphocytes, undergo rapid apoptosis (12, 35, 62). To determine if p53 status correlated

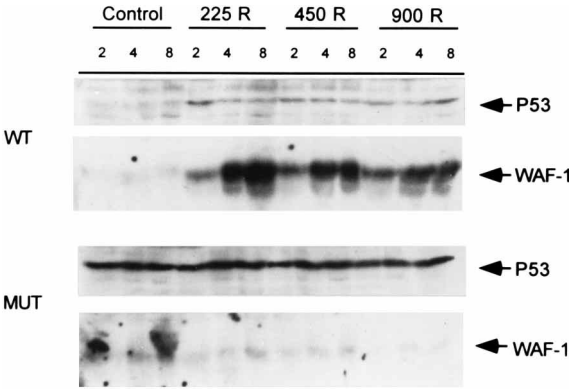

FIG. 4. p53 and p21^{WAF-1} accumulate in transformants expressing wild-type p53 following γ irradiation. Representative transformants expressing wild-type (WT; 298-18) or mutant (MUT; 300-31) p53 were either sham treated (Control) or exposed to 225, 450, or 900 rads of γ irradiation and cultured. Lysates were prepared from the cells 2, 4, and 8 h after irradiation and analyzed by Western blotting using PAb240 for p53 and Ab-5 for p21^{WAF-1}. Note that PAb240 is specific for mutant p53 only when the protein retains its conformation; this antibody detects both mutant and wild-type forms of p53 protein on Western blots.

with changes in p53 and p21^{WAF-1} levels following γ irradiation, cells were treated with several different doses of irradiation and the levels of p53 were examined by Western blotting. Within 2 h of treatment, cells which express wild-type p53 displayed increased levels of the protein, even at the lowest dose of irradiation (Fig. 4). In contrast, the level of p53 did not change in cells expressing mutant p53, even at the highest dose of irradiation used. The higher steady-state levels of p53 observed in the cells expressing mutant p53 reflect the increased stability of the mutant p53 proteins (data not shown). Similar results were obtained with two other wild-type and two other mutant cell lines (data not shown). As expected (59, 60), no p53 could be detected in the L1-2 cells under any conditions (data not shown). Consistent with these patterns and the ability of p53 to affect expression of *p21*, increased levels of p21^{WAF-1} were observed when wild-type p53 protein accumulated (Fig. 4).

To determine if cells expressing wild-type p53 responded to γ irradiation by undergoing rapid apoptosis, irradiated cells were doubly stained with Hoechst 33342 and propidium iodide and analyzed by flow cytometry. Cells undergoing apoptosis are much more permeable to Hoechst dye than live cells; cells that have died do not retain the Hoechst dye but are highly stained with propidium iodide (50). These studies revealed that transformants which expressed wild-type p53 showed a characteristic apoptotic peak by 4 h postirradiation; by 8 h, more than 90% of the Ab-MLV-infected pre-B cells wild type for p53 were dead (Fig. 5). In contrast, the staining pattern of cells expressing mutant p53 or cells which lack p53 did not change during the course of the experiment. Similar results were obtained with two other wild-type and two other mutant cell lines (data not shown). These results confirm that a second function of p53, induction of apoptosis following irradiation-induced DNA damage, is retained in transformants that express wild-type protein but not in those that express mutant forms of p53.

***Tp53* mutations confer enhanced oncogenic potential to the transformants.** To determine if the presence of *Tp53* mutations affected the ability of the transformants to induce tumors in vivo, the tumorigenicity of three independently derived cell lines was examined in syngeneic mice. Each clone was tested when only wild-type p53 was detectable and later when mutant p53 was present. When tumors developed, animals were sacrificed and DNA prepared from the tumor was analyzed to confirm the presence of the Ab-MLV integration that marked the injected clone (data not shown). In addition, several of the tumors that arose in mice injected with cells expressing wild-type p53 were analyzed for the presence of mutant p53 by immunoprecipitation with the epitope-specific antibodies. No mutant forms were detected, revealing that *Tp53* mutation is not absolutely required for tumor formation. This result is not unexpected because less than half of all established cell lines express mutant p53 even though almost all of them are highly tumorigenic (56, 57). Despite this, in all three cases, more animals injected with cells expressing wild-type p53 survived, and those that did succumb often developed tumors after a longer latent period (Fig. 6). Analysis of these data by using a Cox proportional hazard model (13) revealed that animals receiving cells expressing mutant p53 were about 17 times more likely to develop tumors than those receiving wild-type cells ($P = 0.0001$). Thus, the presence of a *Tp53* mutation contributes to the oncogenic potential of the transformant.

DISCUSSION

Mutation of *Tp53* is a frequent occurrence in Ab-MLV-transformed pre-B-cell lines; our survey detected altered forms of the protein in over 40% of the transformants. This estimate reflects the minimum frequency because the immunoprecipitation assay used for screening detects mutations that affect the central domain of the protein (48, 49). However, these data correlate closely with the sequence analysis and the results of the expression and irradiation experiments. Thus, large numbers of mutations have probably not escaped detection. Our results extend earlier reports showing that p53 expression correlates with tumorigenic potential in one Ab-MLV transformant (59). However, deletion of *Tp53*, documented in the L1-2 cell line (60), and in many cases of Friend virus-induced erythroleukemia (5) appears to be a rare occurrence in Ab-MLV transformants. Indeed, point mutations similar to those observed in other types of tumors were detected in most of the cases examined (26).

Although the total number of mutations identified at the sequence level is small, five of eight clustered between codons 132 and 172 of *Tp53*. Use of the antibody screening approach biases toward detection of mutations that affect the central, DNA binding domain of the protein, but more precise clustering is usually not evident. However, the mechanism of action of particular carcinogens is thought to restrict their effects to a single or a few codons (22). In addition, mutations affecting different amino acids may result in p53 proteins with subtly different properties (22). Interestingly, one study has reported that over 50% of the *Tp53* mutations in Burkitt's lymphoma cluster in a region between codons 213 and 248 of the human gene and that 20% of the mutations fall within the region that appears prominent in the Ab-MLV transformants (8). Further studies to determine if this region is preferentially targeted in B-cell lymphoid malignancies appear to be warranted.

Mutations in *Tp53* are not required for initiation of Ab-MLV-induced transformation because the majority of cells in the transformed colonies do not express mutant p53. However, mutant p53 protein was present as soon as 3.5 weeks after the primary transformants were isolated. The transformants arise from single cells and double approximately every 18 h (10, 19, 44), suggesting that the mutations can occur and be selected in fewer than 50 doublings. This rapid selection suggests that cells expressing mutant p53 have a strong growth advantage in vitro.

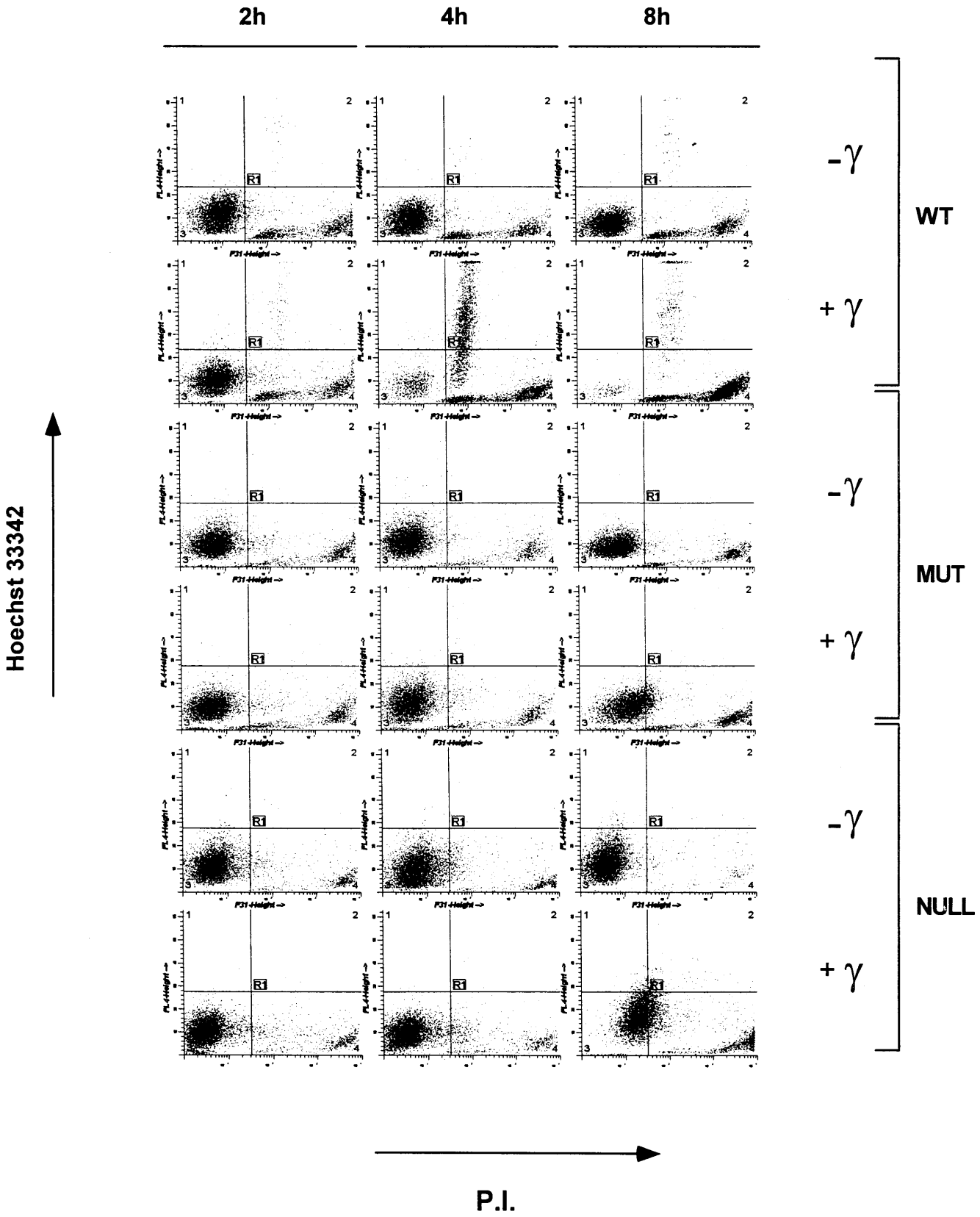

FIG. 5. Transformants expressing wild-type p53 are sensitive to γ-irradiation-induced apoptosis. Representative transformants expressing wild-type (WT; 204-3-1) or mutant (MUT; 379-10) p53 or the p53-null transformant L1-2 were sham treated (−γ) or exposed to 900 rads of γ irradiation (+γ) and stained with Hoechst 33342 and propidium iodide 2, 4, and 8 h later. The samples were analyzed with a FACstar Plus; the data were analyzed by using WinLit 2.01 software.

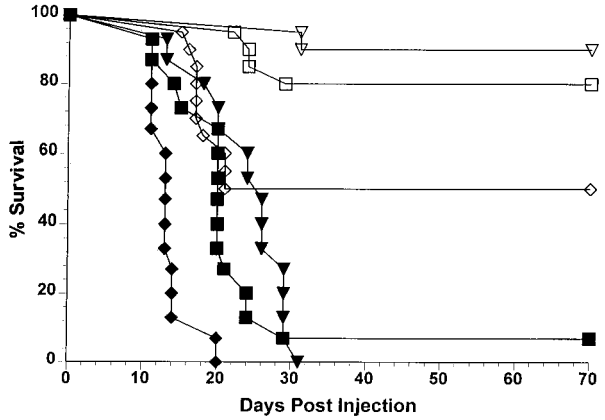

FIG. 6. Transformants expressing mutant p53 form tumors more readily than those expressing wild-type p53. Three independently derived clonal cell lines (3-52, 3-55, and 2-36) were injected into syngeneic mice when the cells expressed wild-type p53 (open symbols) and when the cells expressed mutant p53 (filled symbols). The 3-55 cells contained one mutant and one wild-type copy of *Tp53* sequences; in 3-52 cells, both copies of *Tp53* contained mutations; the mutation(s) present in 2-36 cells were not characterized, but both wild-type and mutant p53 proteins are recovered from these cells. Mice received either 10^5, 10^3, or 10^2 cells (diamonds, squares, and triangles, respectively). The data from the three mutant and the three wild-type cell lines are pooled and plotted to show the percentage of mice that did not develop tumors during the experiment; in all cases, cells expressing mutant p53 formed tumors more rapidly and in a higher proportion of mice.

In an avian erythroblastosis transformation system, selection for immortalized cells that escape senescence correlates well with p53 loss (52). However, this type of selection is probably not involved in the Ab-MLV system because murine pre-B cells do not senesce in vitro if provided with adequate growth factors (41). Several other murine retroviruses can transform pre-B cells that are phenotypically similar to Ab-MLV transformants (27); analysis of the *Tp53* status in such cells would reveal if *Tp53* mutation is a common feature of pre-B-cell transformants or reflects a change more specific to the Ab-MLV system.

At least two p53-mediated functions, induction of apoptosis following DNA damage and transcriptional activation of a promoter containing p53-responsive elements, are impaired in transformants expressing mutant p53. Apoptotic cell death is prominent during the initial expansion of most primary transformants (our unpublished data and references 44 and 55), and escape from cell death can facilitate the inheritance of mutations. In addition, expression of mutant forms of p53 can alter cell cycle progression because several genes which encode key cell cycle-regulatory proteins contain p53-responsive elements (reviewed in references 32 and 34). Altered apoptotic responses and changes in cell cycle progression are found in many types of cells that express mutant p53, and their loss almost certainly plays an important role in oncogenesis. However, p53 is a multifunctional protein, and loss of these particular functions may not be providing the selective advantage in the Ab-MLV system.

Ab-MLV transformants acquire increasing malignant potential during the early phases of in vitro culture (55, 56), and the kinetics with which the *Tp53* mutations arise, coupled with the tumorigenicity studies, suggests that the mutations may correlate with progression to a fully tumorigenic phenotype. Consistent with this idea, direct comparison of the oncogenic potential of clonal cell isolates before and after *Tp53* mutation revealed that cells carrying a mutation were more likely to form tumors than those which did not. Interestingly, a similar correlation has been observed in some cases of chronic myelogenous leukemia, a disease initiated by the formation of a *BCR/ABL* gene fusion (reviewed in references 33 and 45). About 30% of patients express mutant forms of *Tp53* after the transition from the chronic phase to the fully malignant, blast crisis stage (2, 29, 40).

Despite the correlation between oncogenic potential and *Tp53* mutation, cells that lack these can form tumors which continue to express wild-type p53 (data not shown). These data are consistent with the fact that virtually all Ab-MLV transformants are tumorigenic in vivo after several months of in vitro culture (56, 57), even though fewer than half of the transformants that we surveyed had a *Tp53* mutation. Clearly, *Tp53* mutation is not required for tumor formation by transformed cells. Further studies are required to assess the possible role of such mutations in primary virus-induced tumors. Analysis of cell lines derived from a limited number of primary virus-induced tumors reveals the presence of mutant p53 (50a). However, the frequency and timing with which mutations arise in such tumors must be analyzed in detail before a clear picture can emerge.

Despite the strong selection for cells carrying *Tp53* mutation in vitro, the absence of these mutations in all transformants suggests that changes in other genes may contribute to the development of a fully transformed phenotype in the Ab-MLV system. Some tumors expressing wild-type p53 express elevated levels of the p53-regulated *Mdm2* gene (29). However, analyses of steady-state levels of *Mdm2* RNAs in a small panel of pre-B-cell transformants suggests that increased expression of *Mdm2* is not a common feature of the cells studied here (our unpublished data). Our recent observation that many Ab-MLV-transformed pre-B cells have lost expression of $p16^{INK4a}$ suggests that this tumor suppressor may also be involved (40a). p16 affects cell cycle progression during G_1 by binding cdk4 and cdk6 and suppressing their ability to phosphorylate D cyclins (reviewed in reference 46). Although p53 affects other pathways, it also plays key regulatory roles during G_1. Because an active v-Abl protein is required for transit of the early phase of G_1 (10), alterations affecting genes which regulate the G_1-to-S transition may complement v-Abl-mediated signals and foster continued growth, leading to the emergence of a fully transformed phenotype.

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The first two authors contributed equally to this work.

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