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Deletion of 5′-Coding Sequences of the Cellular p53 Gene in Mouse Erythroleukemia: a Novel Mechanism of Oncogene Regulation

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The p53 gene is rearranged in an erythroleukemic cell line (DP15-2) transformed by Friend retrovirus. Here, we characterize the mutation and identify a deletion of ≈3.0 kilobases that removes exon 2 coding sequences. The gene is expressed in DP15-2 cells and results in synthesis of a 44,000-dalton protein that is missing the N-terminal amino acid residues of p53. The truncated protein is unusually stable and accumulates to high levels intracellularly. Moreover, it appears to have undergone a change in conformation as revealed by epitope mapping studies. This study represents the first description of an altered p53 gene product arising by mutation during neoplastic progression and identifies a region in the p53 protein molecule that plays a role in determining p53 stability in vivo.

There is now good evidence that the cellularly encoded nuclear phosphoprotein, p53, is involved in the transformation process (for reviews, see references 8, 20, and 35). p53 protein levels are elevated in a variety of transformed cells from different species, including cells transformed by viruses (22, 23, 26, 32, 43, 45) and chemical agents (9). Recently, it was reported that expression of the murine p53 gene can immortalize early-passage rodent cells in culture (17) and that the p53 gene can replace *myc* in a *myc-ras* immortalization-transformation assay in rat embryo fibroblasts (12, 17, 37). Furthermore, overproduction of p53 protein in certain cells has been shown to confer an enhanced tumorigenic phenotype (11, 19, 32, 53). Hence, the p53 gene appears to have oncogenic potential.

Several studies indicate that p53 expression is correlated with cell cycling and may play a role in the proliferation of normal cells (27–30, 40). p53 expression increases before DNA synthesis when resting cells are stimulated to divide by mitogen (30) or by serum (40). In addition, microinjection of p53-specific monoclonal antibodies into the nuclei of quiescent mouse cells blocks their proliferative response to serum stimulation (27, 29). Finally, primary cultures of early mouse embryos synthesize p53 (31).

Regulation of p53 expression in cells can occur at the level of mRNA abundancy or p53 protein stability (40, 41). Occurrence of the first mechanism was demonstrated in embryonal carcinoma cells (F9) which have nearly 20-fold-higher levels of p53 mRNA than their differentiated progeny (41) and in cells induced to proliferate (30, 39, 40). In general, however, p53 protein levels are not correlated with the amount of p53 mRNA, indicating that the amount of p53 protein is regulated at the posttranscriptional level, perhaps through changes in protein stability (25, 36). p53 protein can be stabilized by binding to other proteins. In cells transformed by simian virus 40 (SV40) or adenovirus type 5, p53 protein is physically complexed with the virus-encoded tumor antigens that are required for transformation, namely the SV40 large T antigen and the adenovirus E1B 58K protein (22–24, 26, 47). As a consequence of this association, the stability and steady-state levels of the p53 protein are considerably increased (36, 41). Specific interaction between p53 and the major heat shock proteins HSP68 and HSP70 has also been reported (38).

DNA rearrangements have been shown to alter the expression of the p53 gene, leading to complete gene inactivation (32, 54, 55) and expression of truncated proteins antigenically related to p53 (32). Previously, we demonstrated the presence of rearrangements in the p53 gene which occurred in vivo during the progression of Friend-virus-induced erythroleukemia (32). One rearrangement of the p53 gene resulted in expression of a truncated polypeptide of 44,000 daltons (p44). In this communication, we describe some features of the p44 protein and present a detailed characterization of the genomic rearrangement responsible for its synthesis.

MATERIALS AND METHODS

Cells. Cell lines DP15-1 and DP15-2 were established from two independent methylcellulose colonies derived from a single spleen of a Friend-virus-infected mouse as previously described (32). Cells were maintained in alpha minimum essential medium supplemented with 10% fetal calf serum (BDH) at 37°C.

Monoclonal antibodies. The following monoclonal antibodies were used in this study. PAb421 (14) and PAb419 (14) were obtained from E. Harlow (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). PAb242, PAb246, and PAb248 (57) were provided by D. Lane (Imperial Cancer Research Fund, United Kingdom). RA3-2C2 (7, 44) was obtained from the American Type Culture Collection.

Metabolic labeling and immunoprecipitation. Approximately 10^7 cells were labeled for 1 h at 37°C with 0.2 mCi of [^{35}S]methionine in 0.5 ml of alpha minimum essential medium lacking methionine or for 3 h at 37°C with 1.0 mCi of $^{32}P_i$ in 2.0 ml of alpha minimum essential medium lacking phosphate. Cells were then washed with phosphate-buffered saline and lysed for 30 min on ice with 0.3 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris [pH 8.0], 0.5 mM phenylmethylsulfonylfluoride). Lysates were cleared by centrifugation for 1 min and by the addition of 0.5 ml of a

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10% suspension of Formalin-treated *Staphylococcus aureus* Cowan 1 cells (Pansorbin; Calbiochem-Behring) for 15 min on ice, followed by centrifugation and retention of the supernatant. For [^{35}S]methionine-labeled cells, lysate volumes corresponding to equal numbers of trichloroacetic acid-insoluble counts (10^7 cpm) were immunoprecipitated overnight at 4°C with antibody in 400 μl of 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). Immune complexes were collected on 50 μl of *S. aureus* cells as described above, washed twice in NET/gel buffer, and then eluted into 30 μl of sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1% bromophenol blue, 25 mM Tris [pH 6.8]) by heating at 70°C for 10 min. *S. aureus* cells were removed by centrifugation, and samples were loaded onto a 12.5% polyacrylamide gel in the presence of SDS. Electrophoresis was at 35 mA until the blue dye front reached the bottom of the gel. Gels were fixed in 7.5% acetic acid–25% methanol for 30 min before drying and exposure to Eastman Kodak XAR-5 film.

For the pulse-chase analysis, cells were labeled with [^{35}S]methionine as described above and then chased for various times in alpha minimum essential medium supplemented with 10% fetal calf serum and 20 mM L-methionine.

Radioimmunoassay. Monoclonal antibody PAb421 (1 μg in 40 μl of 10 mM phosphate buffer [pH 7.0]) was bound to the wells of a polyvinyl chloride microtiter plate overnight at room temperature in a humid chamber. The wells were then washed with NET/gel buffer to remove unbound antibody and to saturate the binding capacity of the plastic. Various concentrations of cell extract diluted in NET/BSA buffer (NET/gel containing bovine serum albumin, 2 mg/ml) were then added to the wells in 20-μl portions. After 3 h at room temperature, the extracts were removed and replaced with a dilution of hybridoma supernatant containing monoclonal antibody RA3-2C2. After a further 3 h at room temperature, the wells were washed with NET/gel, and ^{125}I-labeled anti-rat immunoglobulin (Amersham Corp.) was added and left on the plate for 18 h. The amount of radioactivity bound is related to the input of p53 (2).

Southern blot analysis. High-molecular-weight DNA was isolated as described by Blin and Stafford (6). DNA samples (20 μg) were digested to completion with the appropriate restriction enzymes (Boehringer Mannheim Biochemicals) under conditions recommended by the suppliers and fractionated on a 0.8% agarose gel (Sigma Chemical Co.) in Tris-borate-EDTA buffer. Gels were soaked at room temperature twice in 12.3 M HCl for 15 min, twice in 0.5 N NaOH–1.5 M NaCl for 30 min, and then twice for 30 min in 0.5 M Tris (pH 8.0)–1.5 M NaCl. DNA was then transferred to nitrocellulose filters according to the method of Southern (48). After transfer, filters were rinsed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), air dried, and baked for 2 h at 80°C in vacuo. Prehybridization was done overnight at 42°C in a solution containing 50% formamide, 5× SSPE (1× SSPE is 0.15 M NaCl plus 0.01 M $NaH_2PO_4 \cdot H_2O$ [pH 7.4] plus 0.5 mM EDTA [pH 8.0]), 5× Denhardt solution (1× Denhardt solution is 0.2 mg of Ficoll per ml, 0.2 mg of polyvinylpyrrolidone per ml, 0.2 mg of bovine serum albumin per ml), and denatured salmon sperm DNA (200 μg/ml). The filters were then hybridized for 16 h at 42°C in a similar mixture modified with 2× Denhardt solution and the addition of ≈10^6 cpm of nick-translated (42) heat-denatured p53 cDNA plasmid p27.1a (18). Hybridized blots were washed twice for 5 min at room temperature in 2× SSC–0.1% SDS, twice in 2× SSC–0.1% sodium pyrophosphate for 30 min at 50°C, and then twice for 30 min in 0.1× SSC–0.1% SDS–0.1% sodium pyrophosphate at 50°C. Autoradiography was then performed at −70°C on Kodak XAR-5 film in the presence of an intensifying screen (Cronex Lighting-Plus EC; Du Pont Co.).

Molecular cloning. DP15-2 DNA was isolated, digested to completion with *Hin*dIII and *Bam*HI, and fractionated on a 0.8% low-melting-point agarose gel (Bethesda Research Laboratories, Inc.). A region of the gel containing DNA fragments ranging in size between 2 and 4 kilobases (kb) was removed, melted at 68°C, and diluted with an equal volume of solution containing 20 mM Tris (pH 8.0), 200 mM NaCl, and 2 mM EDTA. DNA was extracted with phenol and phenol-chloroform, precipitated with ethanol, and redissolved in 10 mM Tris (pH 8.0)–1 mM EDTA.

Size-fractionated DNA was ligated to the purified arms of λNM1151 DNA (33) previously digested with *Hin*dIII and *Bam*HI. The resulting recombinants were packaged in vitro (1, 15) and plated on *Escherichia coli* C600. The λ library was screened with the mouse p53 cDNA clone p27.1a (18).

DNA from a Friend-virus-transformed erythroleukemic cell line (CB7) expressing p53 protein (32) was digested to completion with *Eco*RI and fractionated by agarose gel electrophoresis. DNA in the region of the gel corresponding in size to ≈16 kb was extracted and ligated to the purified arms of λ Charon 4A DNA. The resulting recombinants were packaged and screened as described above.

Oligonucleotide synthesis and DNA sequencing. Synthetic oligonucleotides complementary to sequences in exon 3 and exon 4 of the p53 gene were prepared in an automated Applied Biosystems 380A DNA synthesizer and used as sequencing primers. Nucleotide sequences were determined by the dideoxy chain termination method of Sanger et al. (46) after subcloning of appropriate restriction fragments into phage M13 (34, 56).

RESULTS

Expression of the p53 gene in erythroleukemic cell lines. The Friend-virus-transformed erythroleukemic cell line DP15-2 has a rearrangement in the p53 gene resulting in expression of a truncated polypeptide of 44,000 daltons (p44). We have previously demonstrated that this rearrangement occurred in vivo during the natural progression of Friend-virus-induced erythroleukemia and that it was accompanied by loss of the normal homologous allele (32).

In the studies described here, we made use of an independent cell line, DP15-1, established from the same infected spleen as the cell line DP15-2. Although these cells have different Friend virus integration sites in their genome and, hence, are not clonally related (V. Chow et al., unpublished data), they represent very closely matched cells for the comparative studies described in this communication.

DP15-1 and DP15-2 cells were labeled metabolically with $^{32}P_i$, and cellular extracts were prepared; lysates were immunoprecipitated with PAb421 (14), a monoclonal antibody against p53, and the resulting immune complexes were subjected to electrophoresis in SDS-polyacrylamide gels. The autoradiogram shown in Fig. 1 demonstrates that DP15-2 cells synthesize p44, whereas DP15-1 cells synthesize p53. Figure 1 also shows that both polypeptides are phosphorylated in vivo.

Epitope mapping. To identify which region of the p53 molecule was affected by the mutation giving rise to p44, we made use of several monoclonal antibodies that recognize distinct epitopes on p53. DP15-1 and DP15-2 cells were

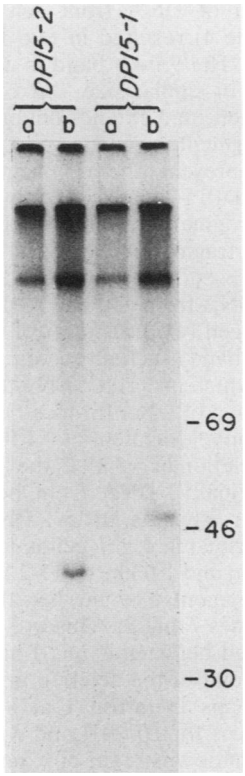

FIG. 1. Expression of p53 gene products in DP15-1 and DP15-2 erythroleukemic cells. Cells were labeled metabolically with $^{32}P_i$ for 3 h. Extracts were prepared and immunoprecipitated with control monoclonal antibody (PAb419; lanes a) and monoclonal antibody against p53 (PAb421; lanes b). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. Numbers at right indicate molecular weight (10^3).

labeled metabolically with [^{35}S]methionine and subjected to immunoprecipitation analysis. The data shown in Table 1 indicate that amino acid residues at the extreme amino terminus of p53 are missing (or masked) in p44, since p44 was not recognized by PAb242. In addition, p44 was recognized by PAb246, a monoclonal antibody that did not recognize p53 in a number of erythroleukemic cells (data not shown) including DP15-1 cells (Table 1). However, PAb246 did recognize p53 in SV40-transformed cells (Table 1). It has been suggested that PAb246 recognizes a conformation-dependent epitope that is not exposed on p53 in all cells (57).

Steady-state level of p53 gene products. The steady-state level of p53 and p44 in DP15-1 and DP15-2 cells, respectively, was estimated by using a quantitative radioimmunological solid-phase assay (2). The assay relies on binding of monoclonal antibody PAb421 (14) to the wells of plastic microtiter plates and adsorption to p53 or p44 from cell extracts, followed by binding of monoclonal antibody RA3-2C2 (7, 43). These two antibodies bound both p53 and p44 (Table 1). Finally, the antibody RA3-2C2, a rat immunoglobulin, was detected by binding ^{125}I-labeled antibody directed against rat immunoglobulin. Bound radioactivity was counted and plotted against the amount of protein extract applied to each well. The data are shown in Fig. 2. From the slopes of the curves, calculated by linear regression, the amount of p44 in DP15-2 cells was about 100-fold higher than the amount of p53 in DP15-1 cells. The lower limit of the assay was determined by using a cell extract prepared from DP20-2 cells, a Friend-virus-transformed erythroleukemic cell line that does not synthesize p53 (unpublished data) (Fig. 2). Similar estimates of p53 and p44 protein levels were obtained by Western blotting (data not shown).

TABLE 1. Comparison of antigenic sites on p53 and p44 by epitope mapping

Cell line	Recognition of epitope by monoclonal antibody[a]				
	PAb242	PAb246	PAb248	RA3-2C2	PAb421
DP15-1[b]	+	−	+	+	+
DP15-2[c]	−	+	+	+	+
3T3 (SV40)	+	+	+	+	+

[a] Amino acid residues containing the epitope recognized by PAb242, 9 to 25; PAb246, 88 to 109; PAb248, 157 to 192; RA3-2C2, 157 to 192; PAb421, 370 to 378 (51).
[b] DP15-1 cells express p53 protein.
[c] DP15-2 cells express p44 protein.

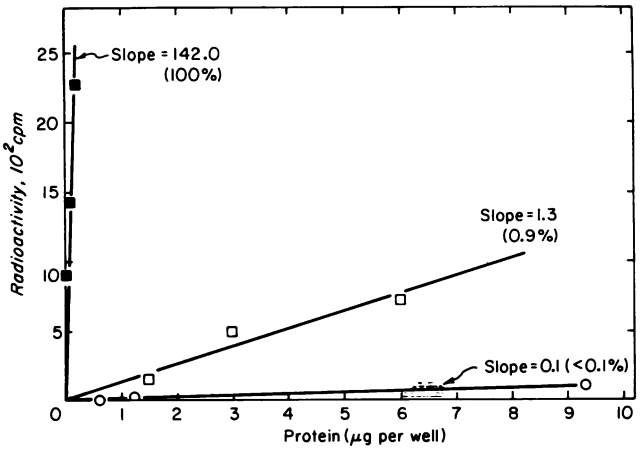

FIG. 2. Steady-state levels of p53 gene products in Friend-virus-transformed erythroleukemic cells. Increasing amounts of cell extracts prepared from DP15-1 (□), DP15-2 (■), and DP20-2 (○) cells were used to determine p53 protein content in a radioimmunoassay as described in the text. In each case, only the first few points on the curve are shown, with the initial slope and the relative levels of p53.

The high level of p44 protein in DP15-2 cells could be the result of increased mRNA levels or increased protein stability. However, mRNA levels for p44 and p53 were similar in DP15-2 and DP15-1 cells, respectively (data not shown).

p53 protein stability. The stability of p44 was compared with that of p53 by pulse-chase analysis. DP15-1 and DP15-2 cells were labeled for 1 h with [^{35}S]methionine and then chased for various times in medium containing an excess of unlabeled methionine. Extracts were prepared, and protein was immunoprecipitated with monoclonal antibody PAb421, followed by resolution of the immune complexes by SDS-polyacrylamide gel electrophoresis. The removal of [^{35}S]methionine from the cells was effective, and the rate of total protein degradation in the two cell lines was identical (Fig. 3A). p53 has a half-life ($t_{1/2}$) of about 2 h (Fig. 3B). This is consistent with the $t_{1/2}$ of p53 measured in other Friend-virus-transformed erythroleukemic cells (3, 10). In contrast, p44 has a $t_{1/2}$ of >9.5 h. Hence, the increased stability of p44 may account for the elevated steady-state level of this protein in DP15-2 cells.

Southern blot analysis. Cell line DP15-2 was shown previously to contain a rearrangement in the p53 gene and to have lost the homologous p53 allele (32). To characterize this

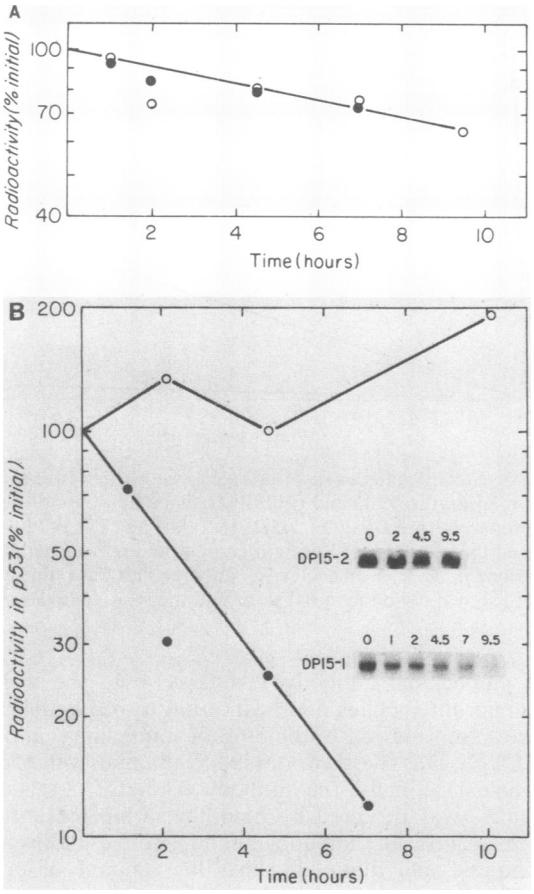

FIG. 3. Pulse-chase analysis of p53 gene products. (A) Rate of total protein degradation in the DP15-1 and DP15-2 cell lines. The cells were labeled metabolically with [^{35}S]methionine for 1 h. The cells were then washed and incubated in nonradioactive medium. At the designated times, cell extracts were prepared as described in Materials and Methods. A sample was removed, precipitated with trichloroacetic acid, and collected by filtration on Whatman GF/C filters. The level of radioactivity present in each sample was determined by liquid scintillation counting. Symbols: ●, DP15-1; ○, DP15-2. (B) Rate of degradation of p53 and p44. Samples of the extracts used in panel A, containing the same amount of trichloroacetic acid-insoluble radioactivity (10^7 cpm per sample), were immunoprecipitated with monoclonal antibodies against p53 (PAb421) and analyzed by gel electrophoresis. The data shown in the inset were quantitated by excising the region of the dried polyacrylamide gel corresponding to p53 or p44. The radioactivity in the excised gel was eluted and determined by liquid scintillation counting. Symbols: ●, p53 in DP15-1 cells; ○, p44 in DP15-2 cells.

genomic rearrangement in more detail, DNA from this cell line and from DP15-1 cells was analyzed by the Southern blot technique. The probe used was a partial cDNA clone (p27.1a [18]) containing mouse p53-specific nucleotide sequences extending from exon 4 to exon 11 (see Fig. 5A).

Digestion of genomic DNA from the p53-producing cell line DP15-1 with *Eco*RI (Fig. 4, lane 2) generated two DNA fragments of ≈16.0 and ≈3.3 kb, which have previously been shown to contain the entire functional p53 gene (4, 58) and a p53 pseudogene, respectively (54, 58). *Eco*RI digestion of DNA from cell line DP15-2 (Fig. 4, lane 1), however, resulted in a DNA fragment of ≈13.0 kb, indicating that the p53 gene was rearranged in DP15-2. The 3.3-kb *Eco*RI fragment from the p53 pseudogene remained intact in this cell line. Digestion of DNA from cell line DP15-1 with *Bam*HI (Fig. 4, lane 4) resulted in two DNA fragments of ≈9.0 and ≈6.0 kb. The 9.0-kb band is a composite of two genomic fragments of similar size, one of which belongs to the functional p53 gene and extends beyond the 3′ end of the 16.0-kb *Eco*RI fragment (see Fig. 5A); the other 9.0-kb *Bam*HI fragment represents the p53 pseudogene (54). When DNA from cell line DP15-2 was digested with *Bam*HI (Fig. 4, lane 3), the 6.0-kb fragment from the functional p53 gene was replaced by a novel fragment of ≈3.0 kb. The 9.0-kb *Bam*HI fragment from the pseudogene remained intact in this cell line. Digestion of DNA from cell line DP15-1 (Fig. 4, lane 6) with *Hin*dIII produced two fragments of ≈7.0 and ≈2.0 kb. The 7.0-kb *Hin*dIII band is actually a composite of two DNA fragments, one of which is derived from the p53 pseudogene (54). *Hin*dIII digestion of DNA from cell line DP15-2 (Fig. 4, lane 5) produced a novel fragment of ≈4.0 kb. These data are consistent with a deletion of ≈3 kb in the functional p53 gene of DP15-2 cells. Finally, DNA from both cell lines was digested with *Kpn*I. Whereas DP15-1 DNA (Fig. 4, lane 8) contains three hybridizing *Kpn*I genomic fragments of approximately 5.0, 2.0, and 1.0 kb, DP15-2 DNA contains only two *Kpn*I DNA fragments, having lost the 5.0-kb genomic fragment (Fig. 4, lanes 7 and 8). This loss reflects the loss of the *Kpn*I site located between exons 1 and 2 (see Fig. 5B). Hence, the 5′ endpoint of the deletion in the p53 gene from cell line DP15-2 maps in intron 1 at a site between the recognition sequences for *Hin*dIII and *Kpn*I. The 3′ end of the deletion must lie downstream of exon 2.

Isolation of DP15-2 genomic sequences. To define the limits of the deletion in the p53 gene of DP15-2 cells precisely, the *Hin*dIII-*Bam*HI DNA fragment shown in Fig. 5 was isolated from DP15-2 genomic DNA by using the bacteriophage lambda cloning vector λNM1151 (33). The cloned DNA

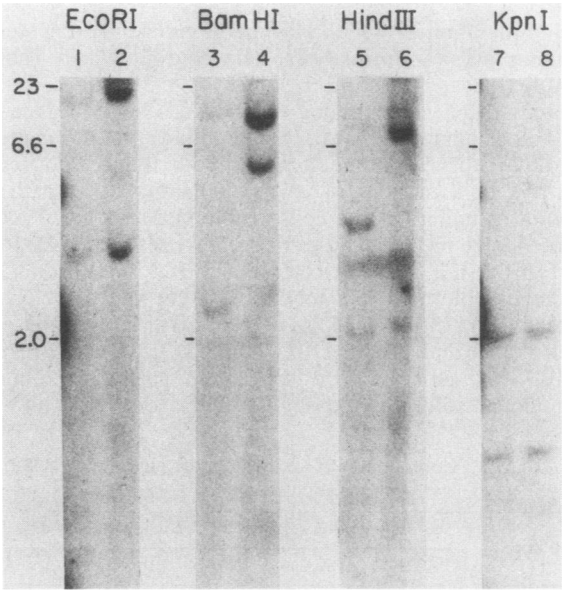

FIG. 4. Southern blot analysis of Friend-virus-transformed cell lines DP15-2 and DP15-1. Genomic DNA from cell lines DP15-2 (lanes 1, 3, 5, and 7) and DP15-1 (lanes 2, 4, 6, and 8) was digested with the following enzymes: *Eco*RI (lanes 1 and 2); *Bam*HI (lanes 3 and 4); *Hin*dIII (lanes 5 and 6); *Kpn*I (lanes 7 and 8). The DNA blots that were obtained were hybridized to 10^6 cpm of nick-translated p53 cDNA plasmid p27.1a (18). The markers used were *Hin*dIII-digested fragments of lambda DNA (Bethesda Research Laboratories).

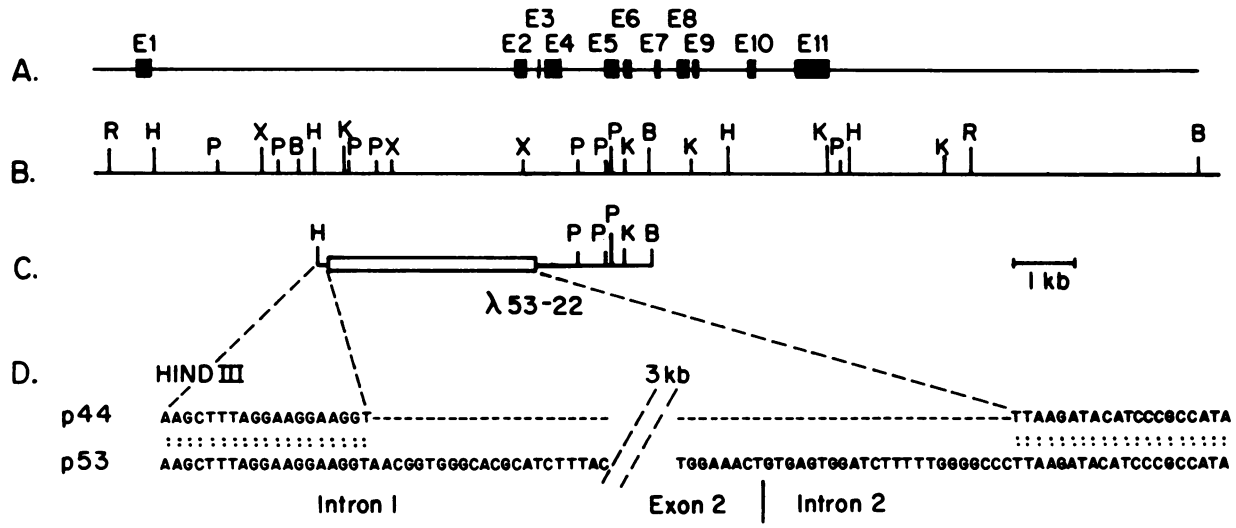

FIG. 5. Physical map of the mouse p53 gene (A), taken from the published data of Bienz et al. (4) and Wolf and Rotter (54). The approximate positions and extent of exons as determined by Bienz et al. (4) are depicted as solid boxes. The cleavage sites for the enzymes *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Kpn*I (K), *Pvu*II (P), and *Xho*I (X) are indicated in panel B. The genomic clone, λ53-22, which contains nucleotide sequences from the p53 gene in DP15-2 cells, is shown in panel C. The cloned *Hin*dIII-*Bam*HI insert is ≈2.3 kb long. The open box in λ53-22 represents the extent of the genomic deletion of the p53 gene in DP15-2 cells. Nucleotide sequences from the intact p53 gene and from the deleted gene encoding p44 are aligned and presented in panel D.

fragment called λ53-22 has a size of ≈2.3 kb, consistent with a ≈3.0-kb deletion in the p53 gene. Comparison of DP15-2 genomic Southern blots with λ53-22 DNA indicated that no rearrangements occurred during cloning. Sequence determination from within exon 4 to the *Hin*dIII site at the 5′ end of λ53-22 insert DNA revealed that exon 4, intron 3, and all of exon 3 sequences were present, including the splice acceptor sites. However, exon 2 nucleotide sequences were entirely missing.

To define the precise endpoints of the deletion at the nucleotide level, a 16-kb *Eco*RI DNA fragment containing the intact p53 gene from a Friend-virus-transformed erythroleukemic cell line (CB7; 32) was cloned in Charon 4A. The *Hin*dIII-*Xho*I fragment contained within intron 1 of the intact gene (Fig. 5B) was subcloned into M13, and its nucleotide sequence was determined. This fragment contains nucleotide sequences that define the 5′ end of the deletion. To define the 3′ end of the deletion, the *Xho*I-*Pvu*II fragment of the intact gene, containing sequences from exon 2 to intron 4 (Fig. 5B), was subcloned into M13, and its nucleotide sequence was determined. Partial nucleotide sequences from the intact gene (CB7) and from the deleted gene (DP15-2) are aligned and presented in Fig. 5D.

DISCUSSION

We have previously demonstrated that the Friend-virus-transformed erythroleukemic cell line DP15-2 has a rearrangement in the p53 gene resulting in expression of a truncated polypeptide of 44,000 daltons (p44). This rearrangement occurred in vivo during the natural progression of Friend-virus-induced erythroleukemia and was accompanied by loss of the normal homologous allele (32). In this communication, we describe some features of the p44 protein and present a detailed characterization of the genomic rearrangement responsible for its synthesis.

The data shown in Fig. 1 and 2 demonstrate that p44 is phosphorylated in vivo and is present at much higher levels than the p53 from other murine erythroleukemic cells such as the DP15-1 cell line. When the stability of p44 was compared with that of p53 by pulse-chase analysis, the $t_{1/2}$ of p44 was found to be greater than 9.5 h, whereas the $t_{1/2}$ of p53 was ≈2 h (Fig. 3). This suggests that the increased stability of p44 may account for the elevated steady-state level of this protein in DP15-2 cells (Fig. 2).

The epitope mapping studies summarized in Table 1 indicate that p44 is not recognized by monoclonal antibody PAb242. Since this antibody recognizes an epitope at the extreme amino terminus of p53 (49, 51, 57), our data suggest that amino acid residues in this region of the p53 molecule are missing (or masked) in p44. Southern blot hybridization analysis (Fig. 4) and genomic cloning (Fig. 5) reveal that DP15-2 cells contain a ≈3-kb deletion of nucleotide sequences in the p53 gene spanning a region extending from intron 1 to intron 2. The untranslated exon 1 is left intact. Nucleotide sequence determination of a genomic fragment that contains the entire deletion indicated that exon 3 coding sequences, including the splice acceptor site, were intact. However, exon 2 coding sequences, which contain the two potential ATG initiation codons, were deleted. As a result, we predict that the next available ATG codon in exon 4 (Met 41; 4, 18) acts as the initiator methionine for p44. This is consistent with the change in molecular weight of the truncated protein. The region around this methionine, -ACUGCAUGG-, shares only certain features with the consensus sequence suggested by Kozak (21), -CC$^{A}_{G}$-CCAUGG-, for the initiation of translation. However, methionine residues farther downstream are unlikely potential initiation sites because of the epitope mapping data (Table 1) and the observed molecular weight of p44.

Cellular proto-oncogenes may become activated into oncogenes by genetic alterations that result in either deregulation of expression of the normal gene or alteration in the structure of the encoded protein (5, 50, 52). The products of certain oncogenes such as *myc*, N-*myc*, *myb*, and p53 are found in the nucleus and share the ability to immortalize

early-passage cultures of normal cells. Nuclear oncogenes can be activated to exert their full oncogenic potential by several mechanisms, including gene amplification, insertional activation, and chromosomal translocation. In general, activation of nuclear oncogenes is associated with deregulation of expression. Because the encoded proteins have short $t_{1/2}$s (20 to 120 min), deregulation often results in increased levels of the gene products.

The mechanism responsible for p53 gene activation in vivo is not known. Whereas p53 protein in normal cells is metabolically labile, many transformed cells have a stable form of p53 that accumulates to high steady-state levels (8, 35). Hence, quantitative changes in p53 are believed to be important in oncogene activation. Here, we describe an erythroleukemic cell line, DP15-2, that expresses a stable form of p53 protein apparently as a result of a mutation in the p53 gene. Both qualitative and quantitative changes in the gene product are associated with this mutation, which occurred in vivo during the natural progression of Friend-virus-induced erythroleukemia. Thus, mutation, specifically deletion of 5′-coding sequences in the p53 gene, provides a novel mechanism for increasing p53 protein levels.

The p53 gene can be activated experimentally by fusion with a strong promoter (12, 16, 37). In addition, Jenkins et al. (16) have shown that the p53 gene can be activated in vitro by deletion of 5′-coding sequences. The resulting protein is metabolically stable with a $t_{1/2}$ of about 24 h. Comparison of the amino acid residues predicted to be absent in p44 (amino acids 1 to 40; 18) with those missing in the in vitro cDNA construct of Jenkins et al. (amino acids 14 to 66; 18) allows us to identify a sequence of 27 amino acids (residues 14 to 40) that may play a critical role in determining p53 protein stability. It is conceivable that some of these sequences constitute a specific recognition site for proteases that are involved in p53 protein degradation or a specific binding site for other cellular factors that regulate p53 levels in vivo. One such factor might be ubiquitin, which plays an essential role in ATP-dependent protein degradation (13).

It is also possible that loss of 5′-coding sequences results in a conformational change of the p53 molecule leading to increased stability. In this model, there is no need to postulate the loss of recognition elements, only their concealment. The epitope mapping studies indicate that a conformational change may have occurred, since the epitope on p53 recognized by PAb246 is masked in a number of erythroleukemic cell lines but is exposed on p44. The epitope recognized by PAb246 appears to be conformation dependent (57). In this regard, it is intriguing that p44 resembles p53 that is bound to the SV40 large T antigen but not free p53 (Table 1). Exposure of amino acid residues recognized by PAb246 may reflect a conformational state of p53 that is associated with increased stability (see also references 49 and 57).

In a previous study (32), we demonstrated that expression of p53 in erythroleukemic cells was associated with tumorigenicity. It is interesting that DP15-2 cells, unlike other erythroleukemic cell lines with rearranged p53 genes that do not express p53, are highly tumorigenic and indistinguishable from p53-producing cells (32). This observation and the similarity in structure and stability between p44 and the experimentally derived p53-related polypeptide described by Jenkins et al. (16), which was shown to be functionally active in extending cellular lifespan, lead us to infer that the p53 gene may have been functionally activated in DP15-2 cells by deletion of 5′-coding sequences during neoplastic progression.

We also examined the cellular location of p53 and p44 by immunofluorescence (unpublished results) and saw no difference in their nuclear staining. Hence, nuclear targeting of p53 does not appear to be affected by loss of exon 2 coding sequences.

Regulation of p53 expression in cells can occur at the level of mRNA abundancy or p53 protein stability (40, 41). p53 protein can be stabilized in at least two ways in transformed cells: (i) physical association with other proteins such as the SV40 large T antigen or the adenovirus E1B 58K protein (36, 41), and (ii) deletion of 5′-coding sequences (16; this work). Both mechanisms result in increased levels of p53 protein in the cell, a feature that is common to many, but not all, transformed cells. In summary, we identified a region on the p53 protein molecule that plays a role in determining p53 stability. Furthermore, we demonstrate that mutations in the p53 gene can occur in vivo during the progression of Friend-virus-induced erythroleukemia, resulting in the synthesis of a stable p53-related protein that accumulates to high steady-state levels.

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