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Multistage Friend Erythroleukemia: Independent Origin of Tumor Clones with Normal or Rearranged p53 Cellular Oncogenes

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The erythroleukemia induced by Friend virus complex in adult mice is a multistage malignancy characterized by the emergence, late in the disease, of tumorigenic cell clones. We have previously shown that a significant proportion of these clones have unique rearrangements in their cellular p53 oncogene. The clonal relationships among Friend tumor cells isolated in the late stages of Friend erythroleukemia were analyzed by examining the unique integration site of Friend murine leukemia virus and the unique rearrangement in their cellular p53 oncogene. The majority of clones isolated from individual mice infected with Friend virus were clonally related as judged by the site of Friend murine leukemia virus integration. However, Southern gel analysis of DNA from individual Friend cell clones indicated that all of the clones with a normal p53 gene from the same mice were clonally related, but were unrelated to the Friend cell lines with a rearranged p53 gene. These results suggest that Friend tumor cells with rearrangements in their p53 gene arise as the result of a unique transformation event, rather than by progression from already existing tumor cells with a normal p53 gene. They also suggest that such rearrangements in the p53 gene confer a strong selective advantage to these cells in vivo.

The leukemia induced by Friend leukemia virus is a multistage malignancy (for reviews see references 2, 24, 27, and 28). The early stage of the disease is associated with a marked increase in the number of erythroid cells in the bone marrow and spleen (27). These cells retain the ability to differentiate terminally to mature erythrocytes, have little or no self-renewal capability, are nontumorigenic in vivo, and cannot give rise to cell lines in culture. The late stage of the disease, beginning approximately 3 to 4 weeks after infection, is characterized by the appearance of cells that are tumorigenic in vivo and capable of growing as permanent cell lines in culture (7, 30). These cells can be detected and characterized by their ability to give rise to spleen colonies in $S1/S1^d$ recipient mice (13) and to form macroscopic colonies in vitro in semisolid culture medium (14).

The original isolate of Friend virus (FV-A) causes anemia and a rapid enlargement of the spleen (6). A subsequent isolate of Friend virus (FV-P), derived from a stock of FV-A, differs from FV-A in that it causes an increase in the number of mature erythrocytes (polycythemia; 15). Both FV-A and FV-P are complexes of a replication-defective spleen focus-forming virus ($SFFV_A$ and $SFFV_P$, respectively) and a replication-competent Friend murine leukemia virus (F-MuLV). Molecular and biological studies have shown that the SFFV components of FV-A and FV-P are responsible for the induction of spleen foci, erythroid proliferation, and splenomegaly associated with the early stages of Friend leukemia in adult mice (reviewed in references 24, 27, and 28) and, furthermore, that many of the properties of the late stages of the disease are specified by SFFV and not its helper virus (12).

In an attempt to understand the molecular events that underlie the progression from the early to the late stages of Friend leukemia, we have examined the structure and expression of a number of cellular proto-oncogenes in malignant cell clones derived from the spleens of Friend virus-infected mice. These studies demonstrated that the p53 gene, a cellular gene implicated in the transformation process (for review, see reference 4), underwent major genomic rearrangements in a high proportion of Friend leukemia cell lines. These rearrangements in the cellular p53 gene were associated with either the loss of p53 synthesis or the synthesis of truncated p53 proteins with lower molecular weight (19, 23). Furthermore, these rearrangements appear to take place in vivo during the evolution of Friend leukemia before the isolation of malignant clones in culture (19).

During the course of these studies, it became apparent that it was possible to isolate, from the same mouse, malignant clones that either had a normal p53 gene or contained a rearrangement in their p53 gene. This result, together with observations suggesting that the late stages of Friend leukemia are clonal (17), raised the possibility that the clones with rearrangements in their p53 gene were derived from the clones with unrearranged p53 genes by tumor progression in vivo. Alternatively, it is possible that the two populations of tumor cells in Friend leukemia, distinguishable on the basis of the status of their p53 gene, arise as the result of two (or more) unrelated transformation events. To decide between these two hypotheses, we have examined the clonal origins of the tumor cells in Friend spleens by using the random integration sites of F-MuLV as a unique clonal marker. These studies support previous results indicating that the late stages of Friend leukemia result from a small number of malignant transformation events and, furthermore, that tumor cells with rearrangements in their p53 gene are clonally independent from cells with a normal p53 gene derived from the same mouse.

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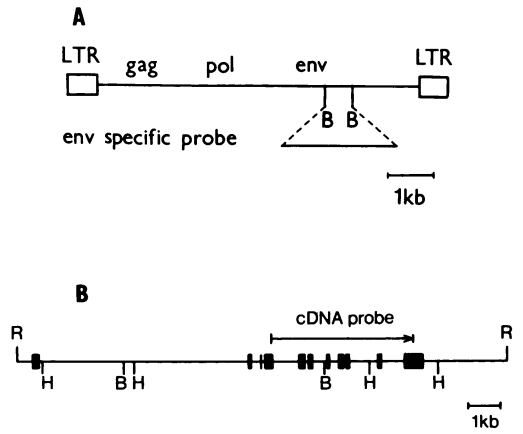

FIG. 1. Restriction endonuclease map of the probes used to analyze the genomic arrangements of the p53 gene and the integration site of F-MuLV. (A) The *env*-specific probe is an 830-bp *Bam*HI fragment derived from F-MuLV (21); (B) the p53 probe is a 900-bp *Bgl*II-*Pst*I fragment of p53 cDNA from the mouse cDNA clone 27.1a (10). Exons are shown as solid boxes and introns as lines. Endonucleases: B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI.

MATERIALS AND METHODS

Viruses and cell lines. Friend virus stocks containing SFFV-A or SFFV-P and F-MuLV were obtained by harvesting the filtered supernatant from virus-producing cell lines as previously described (19). The helper virus F-MuLV was originally obtained by transfecting DNA clone 57 of F-MuLV (21) into NIH 3T3 cells. Cell lines were derived from methylcellulose (0.9%) colonies of spleen cells derived from DBA/2J adult mice infected with either FV-A or FV-P, as described previously (14). The series of cell lines DP20 and DA22 were isolated in this manner. Cell lines DP15-1, DP15-2, and DP15-3, and DP16-1, DP16-2, and DP16-3 have been previously described (19).

DNA isolation and molecular hybridization. High-molecular-weight DNA was isolated by modification of the proteinase K–phenol-chloroform method of Gross-Bellard et al. (8) as previously described (18) or from guanidinium-thiocyanate CsCl gradients (3) as previously described (19). DNA was digested with restriction enzymes and run on agarose gels (18). The DNA was acid depurinated (29) before denaturation and transferred to nitrocellulose or nylon (AMF Corp.) filters (26). The filters were hybridized with 10^6 cpm of nick-translated probe (22) per ml of hybridization mix which contained 50% formamide and 10% dextran sulfate at 42°C (29). The final wash of the filters was 0.1× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 50°C. Hybridized probe was removed from the filters by twice adding boiling distilled water to the filters and allowing them to cool to room temperature.

Probes. The F-MuLV envelope probe was a 830-base-pair (bp) *Bam*HI fragment derived from plasmid pHC6 (20), which contains a 2.4-kbp *Hin*dIII-*Cla*I fragment from clone 57 of F-MuLV (kindly provided by A. Oliff). The p53 probe was a 900-bp *Bgl*II-*Pst*I fragment from mouse p53 cDNA clone 27.1a (10). The DNA probes were purified from plasmid sequences by preparative agarose gel electrophoresis followed by electroelution from excised gel fragments and passage over Elutip columns (Schleicher and Schuell). Restriction endonuclease maps of both probes are shown in Fig. 1.

Determination of p53 protein. The presence of p53 protein in cell lines was determined by metabolic labeling of cells with [^{35}S]methionine followed by immunoprecipitation and polyacrylamide gel electrophoresis (as previously described [19]) using p53-specific monoclonal antibodies (9).

RESULTS

Analysis of the p53 protein in newly isolated Friend cell lines. To examine the clonal origins of Friend cell lines with different p53 genotypes and phenotypes, we isolated a large number of cell lines from individual mice infected with either FV-A or FV-P. In mouse DP20, infected with the polycythemia-inducing virus FV-P, 14 cell lines were isolated from colonies derived by plating spleen cells in methylcellulose. One line, DP20-1, did not synthesize detectable levels of p53 protein (Fig. 2A, lane 2), whereas another cell line, DP20-2, from the same mouse expressed high levels of p53 protein (Fig. 2A, lane 4). The other cell lines isolated from this mouse were also positive for p53 expression (data not shown). Eight cell lines were isolated from a second mouse, DA22, infected with the anemia-inducing strain of Friend virus (FV-A). Three cell lines isolated from this mouse showed altered p53 expression. These three cell lines synthesized a 46-kilodalton protein that was precipitated with the anti-p53 antibody (Fig. 2B, lane 2). The remaining five cell lines isolated from mouse DA22 synthesized a normal-sized p53 protein (Fig. 2B, lane 4).

Analysis of the p53 gene in the newly isolated Friend cell lines. We next asked whether the altered p53 expression

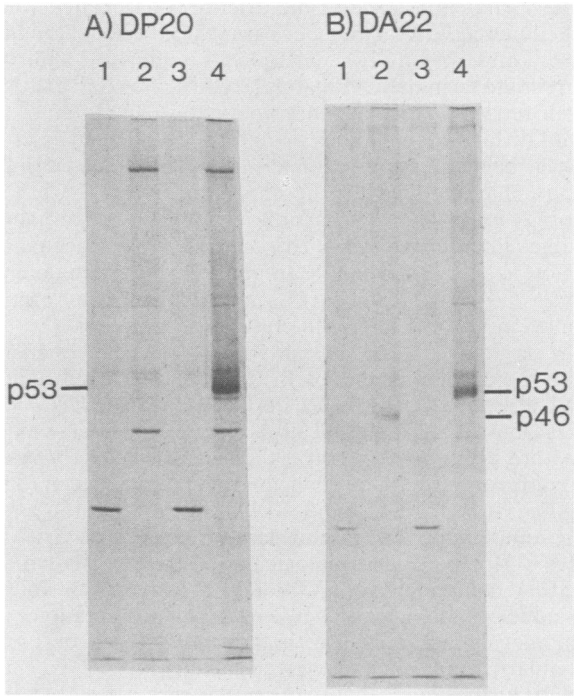

FIG. 2. Analysis of p53 synthesis in Friend cell lines. Friend cell lines were cloned from spleens of mice infected with either FV-P (DP20) or FV-A (DA22), labeled with [^{35}S]methionine, and immunoprecipitated as described in reference 19. Lanes 1 and 3, PAb419 (control monoclonal antibody); lanes 2 and 4, PAb421 (anti-p53 monoclonal antibody). (A) Lanes 1 and 2, DP20-1; lanes 3 and 4, DP20-2. (B) Lanes 1 and 2, DA22-1; lanes 3 and 4, DA22-4.

observed in the cell lines described above was due to structural alterations in the p53 gene. This was determined by Southern blot analysis using a p53 cDNA clone as the probe (10). The normal p53 gene was detected as a 16-kilobase (kb) *Eco*RI fragment, as 2.0- and 7.6-kb *Hin*dIII fragments, and as 9- and 6.0-kb *Bam*HI fragments on Southern blots (Fig. 3, lanes 6). The inactive p53 pseudogene gave rise to a 3.3-kb *Eco*RI fragment, an 8-kb *Hin*dIII fragment, and a 9-kb *Bam*HI fragment (32) (Fig. 3, lanes 6).

Of the 14 transformed cell lines cloned from mouse DP20, the cell line DP20-1, which synthesizes no detectable p53, showed new p53-hybridizing bands of 24 kb (Fig. 4A, lane 1), 12 kb (Fig. 4B, lane 1), and 11 and 2.8 kb (Fig. 4C, lane 1) when cellular DNA was analyzed by the restriction enzymes *Eco*RI, *Hin*dIII, and *Bam*HI, respectively. From mouse DA22, three cell lines were isolated (DA22-1, DA22-2, and DA22-3) that had identical genomic alterations in the p53 gene (Fig. 3). By *Hin*dIII restriction analyses, a new p53-specific band of 5.8 kb was detected in all three cell lines (Fig. 3B, lanes 1 to 3). Upon *Bam*HI analysis, a 12-kb band common to all three cell lines was detected (Fig. 3C, lanes 1 to 3). There was no apparent change in size in the *Eco*RI restriction fragments of the p53 gene in these three cell lines (Fig. 3A, lanes 1 to 3). All of the cell lines that synthesized p53 from mice DP20 and DA22 appeared to have a normal p53 gene as judged by Southern blot analysis with the enzymes *Eco*RI, *Hin*dIII, and *Bam*HI (Fig. 3, lanes 4 and 5, and Fig. 4, lanes 2 and 3).

In addition to these Friend cell lines, we have previously reported seven other cell lines displaying aberrant p53 expression (19). Six of these seven cell lines were found to contain a rearrangement in their p53 gene. Thus, from a total of 12 mice analyzed, 6 mice have given rise to cell lines with altered p53 synthesis. In the 10 cell lines (CB3, CB5, DP15-2, DP15-3, DP16-1, DP16-2, DP20-1, DA22-1, DA22-2, and DA22-3) that showed rearrangements in the p53 gene, 9 contained only the rearranged allele. Interestingly, the cell line DP20-1 described above appears to have both a normal as well as a rearranged p53 gene (Fig. 4, lanes 1). However, the Southern gel analysis does not rule out the possibility that other changes had occurred in the apparently normal allele, such as point mutations, which were not detected at this level of resolution. To exclude the possibility that the presence of an apparently normal p53 gene was due to contamination with another cell line during the cloning process, the cell line DP20-1 was recloned in methylcellulose, and individual colonies were picked and again analyzed by Southern gel hybridization. Both the rearranged and apparently normal p53 alleles were detected in all the clones examined (results not shown).

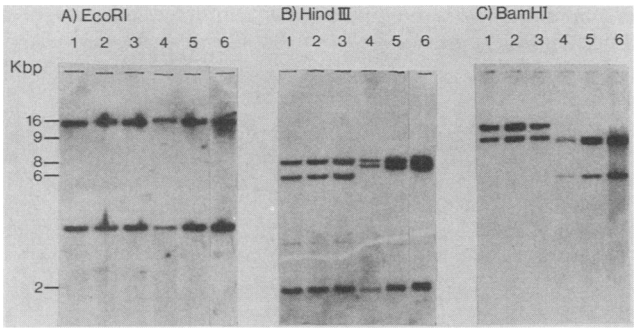

FIG. 3. Southern gel analysis of the p53 gene in Friend cell lines from mouse DA22. Genomic DNA from cell lines DA22-1, DA22-2, DA22-3, DA22-4, and DA22-5 (lanes 1 through 5, respectively) was digested with *Eco*RI (A), *Hin*dIII (B), or *Bam*HI (C), fractionated by electrophoresis in 0.6% agarose gels, transferred to nitrocellulose paper, and probed with nick-translated murine p53 cDNA 27.1a (10). Lanes 6 contain liver DNA from a normal DBA/2J mouse.

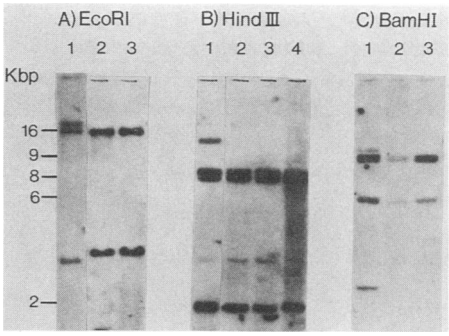

FIG. 4. Southern gel analysis of the p53 gene in Friend cell lines derived from mouse DP20. The analysis was done as described in the legend of Fig. 3. Genomic DNA extracted from cell lines DP20-1, DP20-2, and DP20-3 (lanes 1, 2, and 3, respectively) was digested with *Eco*RI (A), *Hin*dIII (B), or *Bam*HI (C). Lane 4 of panel B contains *Hin*dIII-digested liver DNA from a normal DBA/2J mouse.

In the five mice (CB, DP15, DP16, DP20, and DA22) in which rearrangements in the p53 gene were detected, only one rearrangement in the p53 gene per mouse was observed. Because the molecular rearrangements appear to be unique to each mouse, this observation suggests that the cell lines with p53 rearrangements all derived from a single clone in vivo. This possibility was tested experimentally, as described below.

Clonal origins of Friend cell lines. We were interested in determining whether the Friend cell lines with rearrangements within the p53 gene were derived from the p53-positive cell lines isolated from the same mouse. To establish the clonal relationships among these cell lines, genomic DNA from the Friend cell lines described above was analyzed for the integration sites of the infecting F-MuLV. Since retroviruses integrate randomly into the host genome, their sites of integration may be used as a marker of clonality (5), provided the site of integration does not itself contribute to the tumorigenic phenotype. Restriction enzymes (*Eco*RI and *Hin*dIII) were used to generate junction fragments containing partial proviral genomes contiguous to host cell DNA. An isolated 830-bp fragment of the envelope gene of F-MuLV (20) was used to detect these junction fragments (Fig. 5). DBA/2J mice (the strain used in these experiments) harbor an endogenous ecotropic provirus in their genome which is detected as a 6.4-kb fragment in *Hin*dIII-digested DNA (11). In the results presented here, this 6.4-kb fragment was detected in the DNA digests of normal and infected mouse spleen (Fig. 5C, lane 5) and in all the cloned cell lines of mice DP15, DP16, DP20, and DP22 (Fig. 5).

The cell line DP20-1, which had a rearranged p53 gene, also contained a unique F-MuLV *Hin*dIII junction fragment of molecular size greater than 23 kb (Fig. 5C, lane 1) not detected in cell lines from the same mouse which showed normal arrangements of the p53 gene (Fig. 5C, lanes 2, 3, and 4). A similar finding was also observed in the cell lines from mice DP15 (Fig. 5A), DP16 (Fig. 5B), and DA22 (Fig. 5D). Restriction analyses using *Eco*RI (data not shown) yielded results in agreement with the above observations. These results suggest that the cell lines with rearranged p53 genes

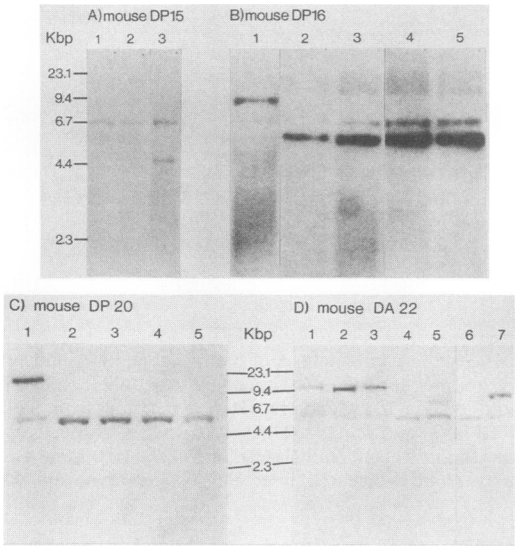

FIG. 5. Analysis of the sites of F-MuLV integration in the genome of Friend cell lines. Genomic DNA was digested with *Hin*dIII and analyzed as described for Fig. 3. The probe used was the nick-translated *env*-specific restriction fragment of F-MuLV as described in Materials and Methods and the legend of Fig. 1. (A) Lanes 1, 2, and 3, DNA from cell lines DP15-2, DP15-3, and DP15-1, respectively. (B) Lanes 1 through 5, Cell lines DP16-1, DP16-3, DP16-4, DP16-5, and DP16-6, respectively. (C) Lanes 1 through 4, Cell lines DP20-1, DP20-2, DP20-3, and DP20-4, respectively. Lane 5, Liver DNA from an uninfected DBA/2J mouse. (D) Lanes 1 through 7, cell lines DA22-1, DA22-2, DA22-3, DA22-4, DA22-5, DA22-6, and DA22-7, respectively.

are clonally unrelated to cell lines with an unrearranged p53 gene from the same mouse.

DISCUSSION

Previous studies on the nature of Friend virus-induced erythroleukemia have suggested that the disease evolves from a polyclonal stage involving many SFFV-infected cells to a clonal disease characterized by the emergence of one or a small number of tumorigenic cells. Although many hematopoietic cells are infected and express gp55 in the early stages of the disease, tumorigenic cells can only be detected 1 to 2 months later. Berger et al. (1) reported that helper-free stocks of Friend $SFFV_P$ were able to induce the early stage of Friend leukemia but not the late stage associated with the emergence of tumor cell clones. These findings suggest that expression of the SFFV-encoded glycoprotein gp55 is not sufficient to induce the late stages of Friend leukemia. Rather, some second event, in addition to infection by SFFV, appears to be necessary to generate complete Friend leukemia. Moreau-Gachelin et al. (16, 17) analyzed the integration sites of SFFV in premalignant and malignant cells transformed by the Friend virus complex. Their results also indicated that the events leading to malignant transformation resulted in the emergence of one or a small number of tumor cell clones. Sola et al. (25) analyzed the integration sites of F-MuLV in myeloblastic cell lines that were transformed by F-MuLV alone and also concluded a monoclonal origin of these cells.

In the present study, we have used the integration site of F-MuLV and the unique rearrangements associated with the p53 gene to analyze clonal relationships in the late stage of Friend leukemia. The results indicate that the transformed cells that emerge during the late stages of Friend leukemia are in general monoclonal in origin. However, an important exception was observed in our studies. In those mice containing tumor cells with rearranged p53 genes, at least two tumor cell populations could be detected: those that contained a normal p53 gene and those that contained rearranged p53 genes. These two cell populations were apparently clonally distinct, as judged by the unique F-MuLV integration site in their genome. These results are not consistent with the possibility that the cells with rearranged p53 genes evolve from the tumorigenic and clonogenic cells with normal p53 genes. Rather, they suggest that these abnormal clones arise as the result of an independent transformation event. It clearly will be of interest to determine whether similar or distinct molecular events are contributing to the tumorigenic phenotype in clones distinguishable on the basis of the status of their p53 gene.

The high frequency of rearrangement in the p53 gene in Friend tumor cells could suggest either that such alterations confer a selective advantage to these cells or that the structure of the p53 gene is particularly labile in Friend cells. Two observations argue against the latter possibility. First, molecular cloning and characterization of rearranged p53 genes in three independent Friend cell lines suggest that distinct molecular events, including deletions (23; unpublished data) and insertions (unpublished data), are responsible for the observed alterations in the p53 gene in these cells. Second, with the possible exception of the cell line DP20-1, all of the cell lines with one rearranged p53 allele have also lost the wild-type p53 gene, indicating that at least two events involving this gene have taken place in these cells. These observations argue against any simple model involving hot spots for genomic rearrangements within the p53 gene.

Thus, these results, together with the high frequency with which Friend cell clones with rearranged p53 genes can be detected, suggest that the molecular rearrangements in the p53 gene confer a strong selective advantage on erythroid cells during the course of Friend leukemia in vivo. The nature of this selective advantage is unclear. Like an Abelson cell line carrying an inactivated p53 gene (31), the Friend cell lines that we have examined appear to be less, not more, tumorgenic than their p53-positive counterparts when injected subcutaneously into syngeneic recipients (19). It will be of interest to determine whether there are additional biological differences between these cells to explain the apparent selective advantage conferred by loss of a functional p53 gene.

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