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The *tsA58* simian virus 40 large tumor antigen disrupts megakaryocyte differentiation in transgenic mice

(thrombocytopenia/platelet/leukemia)

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ABSTRACT **Thrombocytopenia is a condition of multiple etiologies affecting the megakaryocyte lineage. To perturb this lineage in transgenic mice, the *tsA58* mutation of the simian virus 40 large tumor antigen was targeted to megakaryocytes using the platelet factor 4 promoter. Ten of 17 transgenic lines generated exhibited low platelet levels, each line displaying a distinct, heritable level of thrombocytopenia. Within a line, the degree of the platelet reduction correlated directly with transgene zygosity. The platelet level could be further reduced by the inactivation of one copy of the endogenous retinoblastoma gene. Western blot analysis detected large tumor antigen protein in the most severely affected lines; less affected lines were below the level of detection. Platelets and megakaryocytes from thrombocytopenic mice exhibited morphological abnormalities. Mice with either normal or reduced platelet levels developed megakaryocytic malignancies with a mean age of onset of about 8 months. There was no correlation between severity of thrombocytopenia and onset of malignancy. These mice provide a defined genetic model for thrombocytopenia, and for megakaryocytic neoplasia, and implicate the retinoblastoma protein in the process of megakaryocyte differentiation.**

Thrombopoiesis is the process of proliferation and differentiation of megakaryocytes leading to the formation of blood platelets (1, 2). Several known factors or serum components are able to affect either proliferation of megakaryocyte progenitors or maturation of megakaryocytes, operationally dividing the process into at least two distinct levels of regulation. A recently identified factor, megakaryocyte growth and differentiation factor or thrombopoietin, appears to act at both the early and late phases of thrombopoiesis (3, 4). Disruptions in either compartment of thrombopoiesis can lead to thrombocytopenia as manifested in a number of human clinical conditions (5). Progress in the treatment of these conditions is ultimately linked to a basic understanding of megakaryocytopoiesis and the ability to manipulate platelet development.

To address the molecular mechanisms involved in megakaryocyte differentiation we have generated transgenic mice containing the gene for a temperature-sensitive simian virus 40 (SV40) large tumor antigen (T antigen) (6) under the control of the platelet factor 4 (PF4) promoter (7). Expression of this promoter in hematopoietic cells is limited primarily, if not exclusively, to postmitotic megakaryocytes. The SV40 T antigen is known to interact with proteins involved in cell cycle regulation (8) and can lead to cell proliferation and tumor development in transgenic mice (9). In some cell types it has also been shown to inhibit differentiation or cause cell death (10–12). T antigen appears to exert its effects through a number of cellular proteins, including the retinoblastoma (Rb) gene product. Rb is highly expressed in mature megakaryocytes (13) but is not detectable in leukemic megakaryoblasts from patients with chronic myelogenous leukemia (14). These observations suggest a role for Rb in thrombopoiesis and led us to propose that expression of T antigen in megakaryocytes may provide insight into the differentiative function of Rb or other T antigen target proteins.

The present study shows that T antigen disrupts megakaryocyte development resulting in thrombocytopenia, apparently acting in a dose-dependent manner. Furthermore, mice exhibiting both normal and affected phenotypes develop T antigen-related megakaryocytic neoplasia.

MATERIALS AND METHODS

Gene Construct and Generation of Transgenic Mice. A 1.1-kb region of the rat PF4 promoter was PCR-amplified from Sprague–Dawley rat genomic DNA using oligonucleotide primers 5′-GCTTGAATTCCTTTACTCTGCG and 3′-GGAATTCAAGCTTGATATCCAAGGGCTACCTCGG designed from published sequence (15). A *Kpn* I to *Bam*HI fragment of the SV40 early region containing the *tsA58* mutation was cloned into the same sites of the pBluescript II KS(+) vector, and the PF4 promoter fragment was inserted into a unique *Avr* II site 5′ to the SV40 T antigen coding region. The resulting construct was isolated from vector sequences as a 3.8-kb *Not* I/*Eco*RI fragment. Transgenic mice were generated as described (16).

Transgene Identification. The sequences of the oligonucleotide primers used for T antigen PCR are 5′-CAACCTGACTTTGGAGGCTTCand3′-ACACTCTATGCCTGTGTGGAG positioned to span the small T intron to differentiate between DNA (750-bp product) and RNA (406-bp product). The wild-type and the inactivated Rb alleles were identified by PCR using primers described previously (17). Zygosity was determined by an RNA/DNA solution hybridization assay performed on tail DNA from each mouse using both T antigen and mouse stem cell factor (SCF) ^{35}S-labeled RNA probes (18). The ratio between the T antigen signal and the SCF signal was used to determined the zygosity of the mice.

Western Blot. Bone marrow cells (1×10^6) from each mouse were sonicated in 10 μl of sample buffer containing 1% SDS, 50 mM Tris (pH 6.8), 0.1 mM EDTA, and 10% glycerol. The samples were boiled for 5 min, electrophoresed on a 6% polyacrylamide gel, blotted onto a nitrocellulose membrane, and then incubated with T antigen antibody (1:500) dilution as described (19). The T antigen reactivity was visualized using an ECL Western blot analysis kit (Amersham). The expected

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; PF4, platelet factor 4; Rb, retinablastoma; AchE, acetylcholinesterase.

position of T antigen protein was confirmed by Western blots of cells from the VA13 T antigen transformed cell line.

Blood Analysis. Blood samples were collected from the lateral tail vein into EDTA-containing tubes and 20 μl was immediately diluted into manufacturer's diluent for the Sysmex Cell analyzer (TOA Medical Electronics, Kobe, Japan). Samples were analyzed using the Sysmex Cell analyzer within 30 min of collection.

Acetylcholinesterase (AchE) Assay. The number of AchE-positive cells in the bone marrow was determined by suspending marrow in Levine's Catch medium at 1×10^6 nucleated cells per ml. One hundred microliters of each cell suspension was aliquoted to triplicate wells of a flat-bottomed 96-well microtiter plate. AchE staining was performed as described (20) and the mean number of AchE-positive cells per 100,000 cells in the triplicate wells was determined. Additional criteria based on size and morphology were used to confirm that the AchE-positive cells were megakaryocytes.

Immunohistochemistry. Tissues were removed, fixed in 10% neutral buffered formalin, and embedded in paraffin. Three-micron serial sections were cut on a microtome and sequential sections were allowed to react with specific antibodies and visualized with peroxidase staining. Sections were counterstained with hematoxylin. Antibody was incubated at 1:75 dilution, followed by incubation with a biotinylated anti-rabbit or anti-mouse immunoglobulin and then by streptavidin-conjugated horseradish peroxidase, and finally visualized with 3,3'-diaminobenzidine. Antibodies were either an SV40 T antigen monoclonal antibody (Oncogene Science) or polyclonal antibody serum produced against mouse platelets.

Ploidy Analysis. The relative DNA content of normal and transgenic megakaryocytes was determined by Feulgen microdensitometry using an Olympus (New Hyde Park, NY) Cue Series image analysis system (densitometry program). Whole marrow was treated with rabbit anti-platelet antiserum and then stained with a β-galactose/5-bromo-4-chloro-3-indolyl β-D-galactoside system (Kirkegaard & Perry Laboratories). Cells were then cytospun onto slides, acid hydrolyzed for 7 min, and then stained with Feulgen dye. Diploid (2*N*) standards (granulocytes, $n \geq 20$) were determined for each slide and megakaryocytes were identified by cytoplasmic blue reaction product from the immunostaining procedure. The coefficient of variation values for the signals in the diploid cells and the megakaryocytes ranged from 10% to 20%.

RESULTS

Expression of PF4/SV40 Transgene. Of 109 mice born from the injections, 20 contained the transgene as assayed by specific PCR amplification of genomic tail DNAs. Seventeen transgenic lines were established. The copy number of the transgene insertion was assayed by RNA/DNA solution hybridization for transgenic lines PS2, PS7, PS22, and PS29. No correlation between copy number and platelet phenotype was observed. Transgene expression was determined by reverse transcriptase/PCR of bone marrow RNA prepared from F_1 and F_2 generation mice of lines PS2, PS7, PS14, PS22, PS25, PS29, and PS77. A 406-bp PCR product corresponding to the spliced T antigen message was observed in all lines assayed.

Thrombocytopenia in Transgenic Mice. Two founder mice died unexpectedly after tail biopsy apparently from excessive bleeding. The remaining 18 mice were bled by tail vein nick and samples were analyzed for blood cell content on a Sysmex cell counter. All blood cell parameters examined were within the range of the nontransgenic littermates except for the platelet counts, which varied among the founders. The platelet counts of the F_1 generation mice (Fig. 1*A*) fell in a range similar to those of their respective founders, demonstrating that the degree of thrombocytopenia is heritable within lines. The thrombocytopenia was observed as early as 3 weeks of age (the earliest age assayed) and remained stable throughout the life of the animal. Sysmex readings of blood from lines PS2, PS7, and PS14 indicated a mean platelet volume (±SD) of 6.8 ± 0.42 fl, 7.8 ± 0.47 fl, and 8.0 ± 0.32

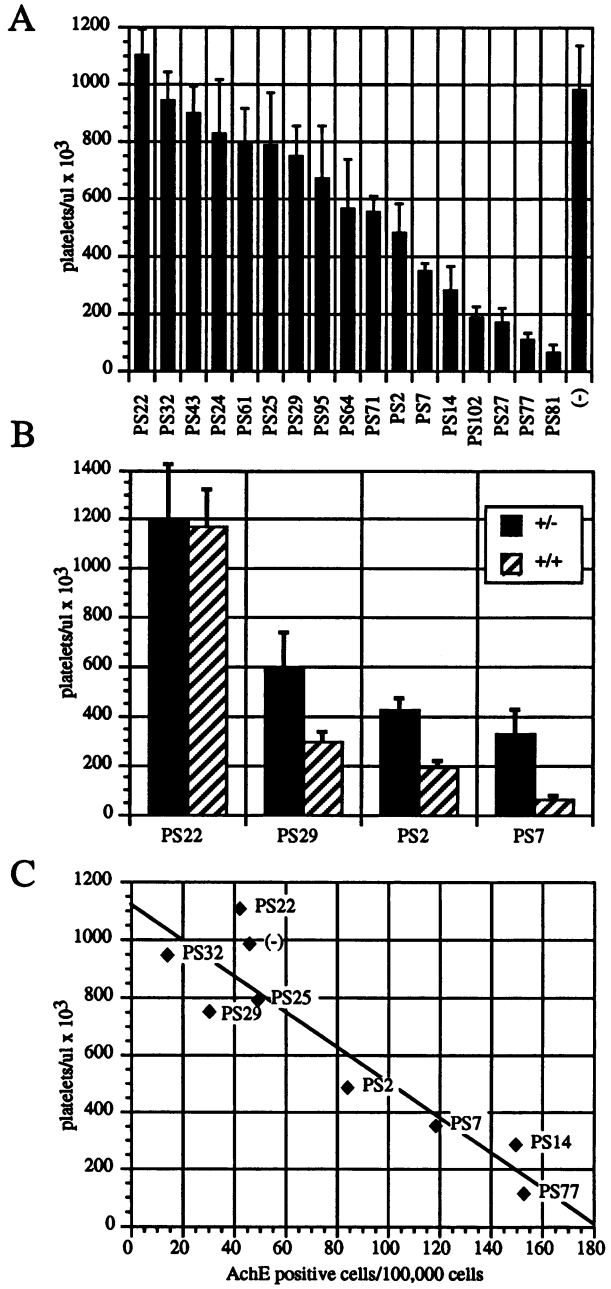

FIG. 1. Platelet counts in peripheral blood and megakaryocytes in bone marrow of transgenic mice. (*A*) Platelet counts of hemizygous F_1 generation transgenic mice. Platelet counts are shown ± SD, $n \geq 4$. (*B*) Comparison of platelet counts in hemizygous (+/−) and homozygous (+/+) mice. F_1 mice from the lines indicated were bred and the litters were assayed for platelet counts. Hemizygous (solid) and homozygous (hatched) mice platelet levels (±SD) are presented for each of the four lines. The *P* values for the difference in platelet count between (+/+) and (+/−) mice are <0.05 for PS29, PS2, and PS7. (*C*) Correlation between platelet level in peripheral blood and AchE-positive cells in bone marrow of transgenic mice. Platelet counts are presented as the mean of individual determinations. The data are presented as the mean, $n \geq 2$.

fl, respectively. Nontransgenic littermates had a mean value of 6.2 ± 0.22 fl. Examination of peripheral blood smears from the affected lines confirmed the thrombocytopenia. Splenectomies performed on 9 mice from line PS7 did not result in any change in platelet count, suggesting that the thrombocytopenia is not due to sequestration of platelets in the spleen. Injection of 100 μl of serum from PS7 mice into nontransgenic littermates did not lower platelet counts, suggesting that the thrombocytopenia is not due to anti-platelet antibodies.

Because each transgenic line exhibited a different, heritable level of thrombocytopenia, the relationship between transgene expression level and degree of platelet reduction was examined. Many transgenes are regulated at the transcriptional level, so the quantitative effect of transgene expression on the thrombocytopenia was determined genetically by comparing hemizygous and homozygous mice from F_2 crosses within each of the lines PS2, PS7, PS22, and PS29 (Fig. 1*B*). Homozygous mice exhibited a more severe phenotype than hemizygous mice in each case except PS22, which remained normal. The correlation between transgene dosage and platelet phenotype within the same line suggests that the level of T antigen expression determines the degree of platelet reduction. To further address the correlation between transgene expression and the severity of the platelet phenotype, Western blot analysis was performed on bone marrow cells from lines PS2, PS7, PS77+/−, PS77+/+, and PS81 (Fig. 2). A band corresponding to T antigen protein was observed in PS81 and PS77+/+ bone marrow cell samples, indicating that the most affected lines have the highest levels of T antigen protein.

Rb Gene Copy Number Affects Platelet Level. To address the potential role of Rb protein in the observed thrombocytopenia, line PS7 was mated to a SV129 mouse line heterozygous for an inactivated Rb gene. Pups from this mating were bled for platelet counts and then characterized for the presence of the transgene and the inactivated Rb allele (Table 1). Mice that did not receive the SV40 transgene had normal platelet levels regardless of whether they received one or two copies of the Rb gene. Mice receiving the transgene and two copies of the Rb gene had a mean platelet level of 268,000 ± 60,000 per μl of blood (±SD) whereas mice receiving the transgene and only a single copy of the Rb gene had mean platelet levels of 162,000 ± 16,000 per μl of blood ($P < 0.01$).

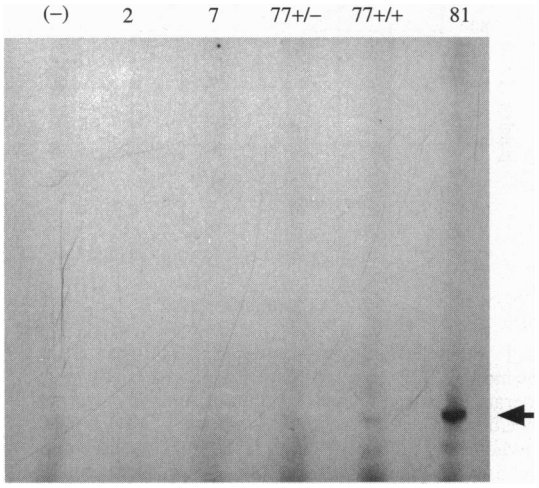

FIG. 2. SV40 T antigen Western blot of bone marrow cells of transgenic mice. Bone marrow cells (1×10^6) from the indicated transgenic lines were loaded per lane, blotted, incubated with a monoclonal antibody directed against SV40 T antigen, and developed using a chemiluminescent indicator. The band corresponding to the T antigen protein is indicated (arrow).

Table 1. Effect of Rb copy number on platelet count

Mouse	Rb copy no.	Platelets per μl	*n*
(−)	2	951,000 ± 46,000	5
(−)	1	926,000 ± 50,000	5
PS7	2	268,000 ± 60,000	7
PS7	1	162,000 ± 16,000	8

Platelet level is expressed as mean ± SD.

Megakaryocyte Abnormalities. Megakaryocytes were analyzed from a selection of the observed phenotypes. Bone marrow from lines PS2, PS7, PS14, PS22, PS25, PS29, PS32, and PS77 were stained for AchE and with modified Giemsa stain to identify and quantitate the megakaryocytes. This examination revealed an inverse correlation between platelet count in blood and megakaryocytes in bone marrow (Fig. 1*C*), with the most severely thrombocytopenic line examined (PS77) containing three times the normal number of bone marrow megakaryocytes. The ploidy of megakaryocytes from transgenic lines PS22 and PS77 was analyzed by Feulgen microdensitometry (Table 2). Although the average ploidy for PS22 was slightly lower than that of nontransgenic controls, PS77 megakaryocytes show higher average ploidy. The PS77 data are consistent with published reports describing the effects of acute thrombocytopenia on megakaryocyte ploidy (21).

Cytologic examination of bone marrow cytospin preparations from the thrombocytopenic lines revealed the presence of many atypical megakaryocytes with different lines exhibiting different morphological characteristics. Megakaryocytes typical of lines PS7, PS22, and PS81 are shown in Fig. 3. In general, megakaryocytes were dramatically increased in size and contained large, irregularly shaped, multilobed nuclei. In many cells, the modified Giemsa staining showed unusual patterns. Hyperchromatic nuclei and an apparent asynchrony between nuclear and cytoplasmic maturation made many cells difficult to type by standard criteria, exemplified by the highly disorganized megakaryocytes of line PS81 (Fig. 3*D*). Emperipolesis, the presence of marrow cells within the cytoplasm of the megakaryocyte, was also observed in many of the atypical megakaryocytes. In contrast, megakaryocytes from PS22 mice, a line with normal platelet levels, appeared normal (Fig. 3*B*).

Megakaryocytic Neoplasia in Transgenic Mice. At 6–12 months of age many of the mice from each of the lines became moribund and/or died. The mean age at which the mice succumbed to the neoplasia was calculated for lines PS7, PS14, PS22, PS24, PS29, PS77, PS81, and PS102 (Table 3). No correlation between platelet phenotype and tumor incidence was observed. Postmortem examination of the affected animals revealed grossly enlarged spleens and mottled discoloration of the livers. A representative mouse from line PS22 was analyzed further. This animal had massive infiltrates of poorly differentiated neoplastic cells in the splenic red pulp and throughout the liver. Additionally, focal neoplastic infiltrates were present in the stomach, small intestine, and a mesenteric lymph node. This neoplastic infiltrate included large numbers of bizarre, atypical megakaryocytes, some of which exhibited emperipolesis (Fig. 4). Many of the

Table 2. Megakaryocyte ploidy in transgenic mice

	Ploidy, % in each class						
Mouse	2*N*	4*N*	8*N*	16*N*	32*N*	64*N*	128*N*
Control	0	20	26	40	28	2	0
PS22	1	17	33	29	16	3	0
PS77	0	2	13	19	37	28	2

n values were as follows: control, 321 cells; PS22, 72 cells; PS77, 224 cells.

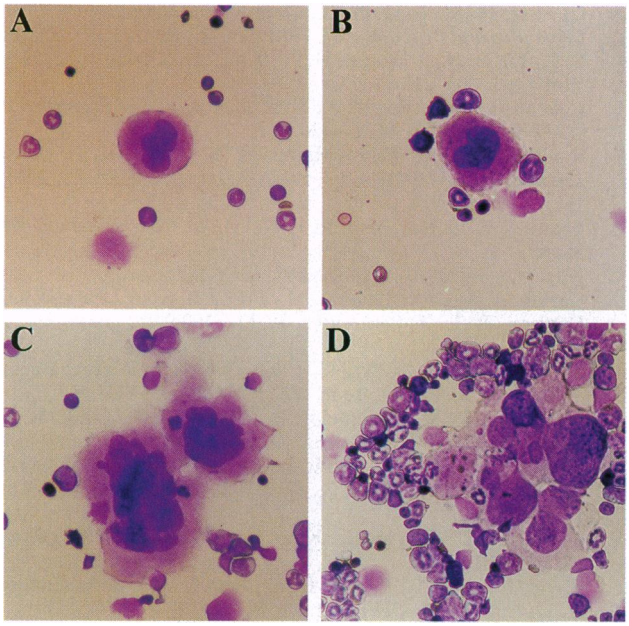

FIG. 3. Megakaryocytes in transgenic and nontransgenic bone marrow. Bone marrow suspensions were cytospun onto glass slides, air dried, and stained with modified Giemsa stain. Each panel shows megakaryocytes typical of the indicated line. (*A*) Nontransgenic. (*B*) PS22. (*C*) PS7. (*D*) PS81. (×160.)

neoplastic cells, including most of the atypical megakaryocytes, stained positively with a polyclonal antibody directed against mouse platelets (Fig. 4*A*). Nuclei of nearly all infiltrating cells expressed T antigen protein as determined by immunoperoxidase staining (Fig. 4*B*). These immunostaining results indicate that the neoplastic cells most likely derived from a T antigen transformed megakaryocyte precursor. Megakaryocytic neoplasia in mice with normal platelet levels (PS22, PS24) indicates that although the thrombocytopenia and malignancy share the same underlying cause, each can occur independently. Additionally, the lack of correlation between the severity of the thrombocytopenia and the onset of neoplasia suggests that these two phenotypes are separable.

DISCUSSION

The *in vivo* phenotype described here shows that the SV40 T antigen is interfering with the ability of megakaryocytes to form normal platelets. Circulating platelets are reduced in number and some exhibit increased size. Each transgenic line stably maintains a distinct level of thrombocytopenia, suggesting a dose-dependent response to the transgene. Megakaryocytes are morphologically altered and appear to be differentiating abnormally. The increased number, size, and ploidy of AchE-positive megakaryocytes in the bone marrow

Table 3. Rate of tumor development in transgenic mouse lines

Transgenic line	Mean survival, days
PS22	207 ± 30
PS24	230 ± 19
PS29	222 ± 44
PS7	251 ± 4
PS14	212 ± 37
PS102	334 ± 28
PS77	275 ± 87
PS81	175 ± 61

Transgenic lines are presented in order of decreasing platelet count. Survival time is expressed as mean ± SD.

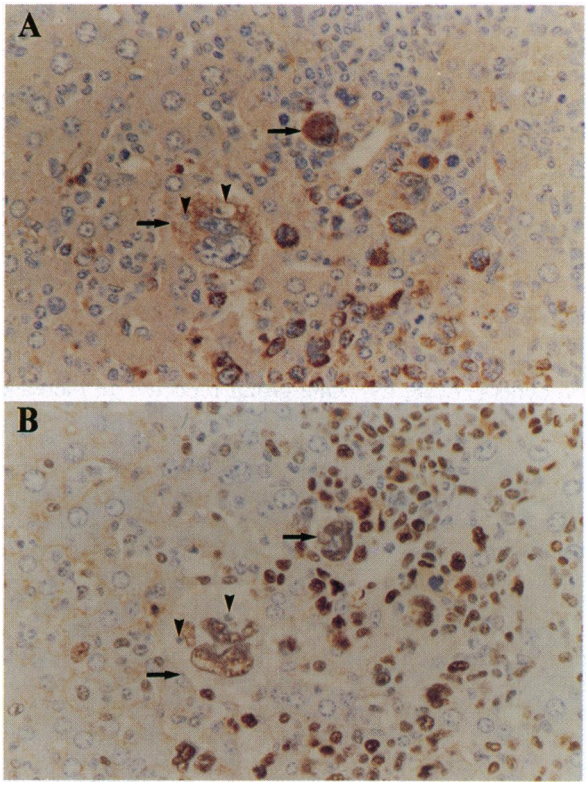

FIG. 4. Immunoperoxidase staining of PS22 liver containing neoplastic infiltrate. Tissues were removed, fixed in 10% neutral buffered formalin, and embedded in paraffin. Three-micron serial sections were cut on a microtome and sequential sections were allowed to react with specific antibodies and visualized with peroxidase staining. Sections were counterstained with hematoxylin. (*A*) Immunostaining with rabbit anti-mouse platelet antiserum. Immunoreactivity is indicated by orange/brown staining. (*B*) SV40 T antigen immunoreactivity using an SV40 T antigen monoclonal antibody. (×200.) Neoplastic megakaryocytic cells are indicated by arrows. Emperipoletic cells are indicated by arrowheads.

are correlated with the degree of platelet decrease. This correlation has been observed in experimentally induced thrombocytopenia (22) and suggests the presence of a physiological compensatory reaction to low platelet mass. It is also possible that the increased megakaryocyte numbers are the result of deregulated proliferation caused by T antigen. This latter possibility seems less likely, as the megakaryocyte number correlates with the degree of thrombocytopenia and not with the propensity for tumor development. However, both mechanisms could be contributing to the increase in cell number. These animals are thrombocytopenic despite the presence of compensatory mechanisms that serve to maintain platelet homeostasis. Treatment of these animals with factors known to affect platelet levels, such as interleukin 6, interleukin 11, or thrombopoietin, may enable the identification of the pathways by which these factors stimulate platelet formation.

Other experiments have shown that T antigen expression can interfere with terminal differentiation (10). The molecular mechanism of this disruption may provide insight into the role of T antigen target proteins in differentiation. T antigen has been shown to bind and presumably inactivate at least four proteins in mammalian cells: the Rb gene product (23), p53 (24), p107 (25), and p130 (26). Mice lacking the p53 gene (27) are normal with respect to hematopoiesis, and mice lacking the p107 gene (E. Harlow, personal communication) display no notable hematopoietic defects, indicating that neither gene is essential for platelet formation. Furthermore, the *tsA58* mutation, which resides in the p53 binding domain

of T antigen, does not bind p53 at nonpermissive temperatures but does retain Rb binding activity (Peter Tegtmeyer and Sanjit Ray, personal communication).

Several experiments provide evidence implicating Rb in megakaryocyte differentiation. Immunocytochemical analyses demonstrate a high level of the Rb protein in the nuclei of mature megakaryocytes (13). Chronic myelogenous leukemia patients with megakaryocyte blast crisis have been shown to lack detectable Rb protein in leukemic cells (14), suggesting that the deletion of Rb may be required for neoplastic transformation of this lineage. Furthermore, mice lacking the Rb gene die at day 13–15 of gestation, apparently due to hypoxia stemming from a defect in terminal differentiation of erythrocytes, cells that share a common lineage with megakaryocytes (17, 28, 29). One group reported an increase in megakaryocyte number in the mutant mice, indicating the possibility of a megakaryocyte defect.

Accumulating evidence suggests that Rb is involved in the initiation and maintenance of the terminally differentiated state of a number of cell types. Rb is known to bind and sequester the E2F transcription factor (30). Rb levels are high in cells undergoing terminal differentiation (13), Rb is required for terminal differentiation of erythrocytes and some neuronal cell types in the developing mouse (17, 28, 29), and Rb appears to be important for the terminal differentiation of muscle cells *in vitro* (31). Therefore, a plausible explanation for the thrombocytopenia observed in the PF4/T antigen mice rests on the interaction of T antigen with Rb or another related protein. We postulate that Rb or related proteins normally contribute to the process of megakaryocyte differentiation and that T antigen can act to block one or more of the target protein's functions. The transgene zygosity experiments and T antigen Western blot indicate a correlation between T antigen level and severity of phenotype, suggesting that T antigen can interfere with the differentiative function of its target(s) in a dose-dependent manner. Further reduction of platelet levels by reducing the copy number of the Rb gene suggests that the level of Rb is important for proper differentiation of megakaryocytes. Further analysis of this transgene in genetically modified mice expressing variable levels of Rb or other T antigen targets will be useful in evaluating the roles of these proteins in megakaryocyte development.

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1. Hoffman, R. (1989) *Blood* **74,** 1196–1212.
2. Gewirtz, A. M. & Poncz, M. (1991) in *Hematology: Basic Principles and Practice*, eds. Hoffman, R., Benz, E. J., Shattil, S. J., Furie, B. & Cohen, H. J. (Churchill Livingstone, New York), pp. 1148–1157.
3. Bartley, T. D., Bogenberger, J., Hunt, P., Li, Y.-S., Lu, H. S., *et al.* (1994) *Cell* **77,** 1117–1124.
4. Lok, S., Kaushansky, K., Holly, R. D., Kuijper, J. L., Lofton-Day, C. E., *et al.* (1994) *Nature (London)* **369,** 565–568.
5. Gewirtz, A. M. & Hoffman, R. (1991) in *Hematology: Basic Principles and Practice*, eds. Hoffman, R., Benz, E. J., Shattil, S. J., Furie, B. & Cohen, H. J., (Churchill Livingstone, New York), pp. 205–210.
6. Loeber, G., Tevethia, M. J., Schwedes, J. F. & Tegtmeyer, P. (1989) *J. Virol.* **63,** 4426–4430.
7. Ravid, K., Beeler, D. L., Rabin, M. S., Ruley, H. E. & Rosenberg, R. D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1521–1525.
8. Fanning, E. & Knippers, R. (1992) *Annu. Rev. Biochem.* **61,** 55–85.
9. Hanahan, D. (1989) *Science* **246,** 1265–1275.
10. Garvin, A. M., Abraham, K. M., Forbush, K. A., Farr, A. G., Davison, B. L. & Perlmutter, R. M. (1990) *Int. Immunol.* **2,** 173–180.
11. al-Ubaidi, M. R., Hollyfield, J. G., Overbeek, P. A. & Baehr, W. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1194–1198.
12. Feddersen, R. M., Ehlenfeldt, R., Yunis, W. S., Clark, H. B. & Orr, H. T. (1992) *Neuron* **9,** 955–966.
13. Szekely, L., Jiang, W., Bulic-Jakus, F., Rosen, A., Ringertz, N., Klein, G. & Wiman, K. G. (1992) *Cell Growth Differ.* **3,** 149–156.
14. Towatari, M., Adachi, K., Kato, H. & Saito, H. (1991) *Blood* **78,** 2178–2181.
15. Ravid, K., Doi, T., Beeler, D. L., Kuter, D. J. & Rosenberg, R. D. (1991) *Mol. Cell. Biol.* **11,** 6116–6127.
16. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 152–182.
17. Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A. & Weinberg, R. A. (1992) *Nature (London)* **359,** 295–298.
18. Hunt, P., Zsebo, K. M., Hokom, M. M., Hornkohl, A., Birkett, N. C., del Castillo, J. C. & Martin, F. (1992) *Blood* **80,** 904–911.
19. Towbin, H. & Gordon, J. (1984) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
20. Burstein, S. A., Boyd, C. N. & Dale, G. L. (1985) *J. Cell. Physiol.* **122,** 159–165.
21. Corash, L., Chen, H. Y., Levin, J., Baker, G., Lu, H. & Mok, Y. (1987) *Blood* **70,** 177–185.
22. Mazur, E. M. (1987) *Exp. Hematol.* **15,** 340–350.
23. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J., Huang, C., Lee, W., Marsilio, E., Paucha, E. & Livingston, D. M. (1988) *Cell* **54,** 275–283.
24. Lane, D. P. & Crawford, L. V. (1979) *Nature (London)* **278,** 261–263.
25. Ewen, M. E., Xing, Y., Lawrence, J. B. & Livingston, D. M. (1991) *Cell* **66,** 1155–1164.
26. Hannon, G. J., Demetrick, D. & Beach, D. (1993) *Genes Dev.* **7,** 2378–2391.
27. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Butel, J. S. & Bradley, A. (1992) *Nature (London)* **356,** 215–221.
28. Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M. T., van der Valk, M., Hooper, M. L., Berns, A. & te Riele, H. (1992) *Nature (London)* **359,** 328–330.
29. Lee, Y.-H. P., Chang, C.-Y., Hu, N., Wang, Y.-C. J., Lai, C.-C., Herrup, K., Lee W.-H. & Bradley, A. (1992) *Nature (London)* **359,** 288–294.
30. Nevins, J. R. (1992) *Science* **258,** 424–429.
31. Gu, W., Schneider, J. W., Condorelli, G., Kaushai, S., Mahdavi, V. & Nadal-Ginard, B. (1993) *Cell* **72,** 309–324.