# Targeted Expression of a Conditional Oncogene in Hematopoietic Cells of Transgenic Mice

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*Abstract.* We have produced two lines of transgenic mice in which the expression of temperature-sensitive SV-40 large T antigen is targeted to bone marrow megakaryocytes via the platelet factor 4 (PF4) tissue-specific promoter. The progeny of these transgenic mice were observed for about 3 mo, and no malignancies were detected over this period of time. The offspring of these transgenic mice, 6- to 12-wk of age, served as a source of bone marrow cells, which upon in vitro cultivation at the permissive temperature yielded immortalized cell lines (MegT). At the permissive temperature, MegT cells exhibit the characteristics of early 2N and 4N megakaryocytes which include the presence of specific gene products such as PF4, glycoprotein IIb, acetylcholinesterase, and CD45 as well as the absence of molecular markers of other cell lineages such as the macrophage marker Mac-1, the T helper cell marker CD4, the mast cell marker IgE, the T cell marker CD2 or the erythroid cell marker α-globin. The inactivation of the oncogene by a shift of temperature from 34° to 39.5°C produces a reduction in the frequency of the 2N cells, in conjunction with the appearance of 8N and 16N cells, consisting of 27 and 3% of total cells, respectively. Thus, we have generated hematopoietic cell lines that are trapped in the early stages of megakaryocyte commitment, but able to undergo part of the normal program of terminal differentiation.

PLURIPOTENT bone marrow stem cells give rise to 2N megakaryoblasts which are subsequently converted to mature polyploid megakaryocytes as a result of DNA replication in the absence of mitosis and the development of α-granules which contain specific gene products (Hawiger et al., 1987). The mature megakaryocytes then fragment into small anucleate platelets which circulate within the blood. Past studies of megakaryocyte biochemistry and differentiation have been hampered because these cells constitute only 0.05-0.1% of the nucleated cells of bone marrow. The currently available cell lines, all derived from patients with leukemia, exhibit multilineage properties and/or low ploidy states (Martin and Papayannopoulou, 1982; Tabilio et al., 1983; Tabilio et al., 1984; Ogura et al., 1985; Sledge et al., 1986; Greenberg et al., 1988). Therefore, the use of a molecular biological approach to generate megakaryocytic cell lines which maintain lineage fidelity and become polyploid would constitute a significant advance in this field of research.

We have used tissue-specific expression of a conditional oncogene in transgenic mice to produce immortalized megakaryocytic cell lines. In prior studies, transient expression experiments as well as transgenic mice were used to define the tissue-specific elements of the platelet factor 4 (PF4)[1] gene and the promoter region which allows selective expression in megakaryocytes and platelets (Ravid et al., 1991a,b). We now describe the generation of transgenic mice expressing the temperature-sensitive SV-40 large T antigen driven by the above tissue-specific regulatory domain (Meg-T mice). This conditional oncogene has previously been linked to retroviral vectors to immortalize in vitro precursors of neural cells (Frederiksen et al., 1988) and myogenic cells (Iujuidin et al., 1990). The offspring of the Meg-T transgenic mice serve as a source of primary bone marrow cells which yield immortalized 2N or 4N megakaryocytes when cultured at the permissive temperature and subsequently transform to higher ploidy states when cultured at the nonpermissive temperature. Thus, our investigations demonstrate that hematopoietic cells can be immortalized at a specific stage of commitment with a lineage specific–promoter linked to a conditional oncogene.

## Materials and Methods

### Experimental Procedures

*Plasmid Construction and Generation of Transgenic Mice.* The plasmid

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1. *Abbreviations used in this paper:* GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; IMDM, Iscove's modification of Dulbecco's medium; PF4, platelet factor 4; vWF, von-Willebrand Factor.

PF4SVtsA58 was constructed by using pPF4GH (Ravid et al., 1991b) which contains the rat 1.1-kb PF4 promoter linked to the HGH gene. This construct has a unique EcoRI site at the 3' end of the HGH gene and a unique BanII site 20-bp downstream of the transcriptional start. A unique KpNI site was introduced at the 5' end of the PF4 promoter in pPF4GH, via linkers, to produce pPF4GH$_{KB}$. This later plasmid was digested with BanII and EcoRI to remove the HGH gene and a BglIII/EcoRI SVtsA58 fragment (Frederiksen et al., 1988) was introduced, via BanII/BglII linkers, to generate pPF4SVtsA58. The orientation of insertion of the SVtsA58 gene was confirmed by DNA sequencing. The PF4 promoter/SVtsA58 gene of 3.6 kb was obtained by cutting pPF4SVtsA58 with KpNI and EcoRI and purifying the appropriate fragment by agarose gel electrophoresis. This fragment was used to produce transgenic mice as described previously (Ravid et al., 1991a). Foster mice females were of ICR strain (Harland, Frederick, MD) and the microinjected eggs were of FVB strain (Taconic Farms, Germantown, NY). Mice were screened for transgene integration by Southern blot analysis of tail DNA (Hogan et al., 1986) and transgene expression was detected by Northern blot analyses (Ravid et al., 1991a).

*Cultured Cells.* Mouse bone marrow cells were isolated and cultured as described previously (Ravid et al., 1991b). The cells were grown in 5% $CO_2$ at 34°C in a liquid culture under conditions that were shown before to support maturation and ploidy of primary megakaryocytes (Kuter et al., 1989). To this end, the cells were grown in the presence of Iscove's modification of Dulbecco's medium (IMDM) supplemented with penicillin (2,000 U/ml), streptomycin (200 µg/ml), L-glutamine (0.592 mg/ml), horse serum (20%), and the hemopoietic growth factors erythropoietin (1 U/ml), Interleukin (IL)-3 (5 ng/ml), IL-6 (5 ng/ml), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (5 ng/ml). The immortal cells derived were cloned by seeding 200-500 cells/10 ml IMDM supplemented as above in a 100-mm-diam culture dish. Individual colonies were isolated with a glass cloning ring, removed from the plate by trypsinization and grown in a 35 mm dish. To induce cell differentiation, 0.5-1 × 10^5 cells were seeded into a 25 cm² culture flask in the presence of 5 ml of the above medium and incubated in 5% $CO_2$ at 39.5°C for 4 to 5 d. Cells were counted by hemocytometer and cell death was followed by staining with Trypan blue.

*Immunofluorescent Staining.* Cell lines were grown on glass cover slides for 3 to 4 d, bone marrow cells were spun onto a polylysine-treated slide in a Cytospin 2 (Shannon, Pittsburgh, PA) and both cell types were subjected to immunofluorescence staining as described in (Ravid et al., 1993) except that fixation was carried out with 100% methanol for 2 min at room temperature.

*Flow Cytometric Analysis and Fluorescence Activated Cell Sorting (FACS).* For analysis of DNA content per cell, adhering cell lines were trypsinized and then washed with culture media whereas bone marrow cells were harvested in CATCH (Ravid et al., 1991b) and both cell types were stained with Hoechst dye (Ravid et al., 1991a). Flow cytometric analysis and FACS were carried out on a FACStarplus flow cytometer (a registered trademark of Becton Dickinson, San Jose, CA) (Kuter et al., 1989; Ravid et al., 1991a) and DNA histograms were analyzed by a cell cycle analysis program (Modfit Verity Software House, Inc., Topsham, ME). The ploidy analysis of mouse bone marrow cells, using a rat platelet antibody, was done as described (Kuter et al., 1989). A coefficient of variation of the 2N peak was maintained at 2.2 to 3.0% by alignment of the optical system.

*Northern Blot Analyses and Polymerase Chain Reaction.* Total RNA was prepared from cells grown at 34° or 39.5°C and subjected to Northern blot analyses as described in (Ravid et al., 1991a). The different probes used were cDNAs of GPIIb (gift of Dr. M. Poncz), vWF (gift of Dr. D. Lynch), α-globin (gift of Dr. L. Gehrke), PF4 (Doi et al., 1987), and the gene coding for T antigen (Frederiksen et al., 1988). Low levels of messenger RNA were detected by the PCR as described in (Ravid et al., 1991b). The PCR primers used to detect CD2 (Diamond et al., 1988) were: 5'GGTCGGTGCAGG-AGG3' (sense) and 5'CGATGGCCAGGTGTC3' (antisense) which extend 267 bp.

*Materials.* The materials used and their sources were as follows: tissue culture media and horse serum (GIBCO-BRL, Gaithersburg, MD); hematopoietic growth factors (R&D Systems, Minneapolis, MN); Hoechst dye, polylysine, and diisopropylfluorophosphate (Sigma Immunochemicals, St. Louis, MO) PCR reagents (Perkin-Elmer Cetus, Norwalk, CT); PKH2-GL and PKH26-GL dyes (Zynaxis Cell Science, Inc., Malvern, PA); M-MLV reverse transcriptase (GIBCO-BRL); mAbs against CD45R (GIBCO-BRL), Mac-1 (Boehringer Mannheim, Indianapolis, IN), and L3T4 antibody against CD4 (Becton Dickinson Immunocytometry System, San Jose, CA); antibody against IgE (Nordic Immunological Lab, Tilburg, The Netherlands); antibody to large T antigen (Oncogene Science, Manhasset, NY); [α-³²P]ATP and [α-³⁵S]ATP (New England Nuclear Research Products, Boston, MA); and oligonucleotides were synthesized with β-cyanoethylphosphamidates by the Biopolymers Laboratory at M.I.T.

# Results

## Generation of Transgenic Mice Containing the PF4 Promoter Linked to the Temperature-sensitive Mutant of SV-40 Large T Antigen

The construct used to generate transgenic mice contains 1,104 bp of the 5' upstream region of the rat PF4 gene linked to the tsA58 mutant SV-40 large T antigen (PF4SVtsA58) (Fig. 1 a). The oncogene used encodes a mutant gene product which is rapidly degraded by raising the temperature from 34° to 39°C (Tegtemeyer 1975a,b). Given that the mouse body temperature is about 38°C (Kaplan et al., 1983), large T protein should be at very low concentrations or absent under in vivo conditions. The above segment of the PF4 promoter was selected because our previous transgenic studies demonstrated that the critical tissue-specific regulatory domain is located within this region (Ravid et al., 1991a). PF4SVtsA58 was microinjected into pronuclei of fertilized mouse eggs and the injected embryos were implanted into pseudopregnant outbred females. The offspring were screened for transgene integration by Southern blot analyses of tail DNA digested with PstI, using the SVtsA58 gene as a probe (Fig. 1 b). Five founder mice were identified, of 33 mice produced, exhibiting 1 to 10 copies of PF4SVtsA57 as determined by comparison with diluted linearized control DNA. While not very low, the percentage of transgenic mice obtained is lower than our usual results with other constructs. We do not rule out the possibility that this is related to an effect of SV-40 large T antigen in early stage embryos. The transgene was integrated into a single chromosomal site in a head-to-tail fashion, as judged by the 2.5- and 1.1-kb DNA fragments obtained after digestion with PstI. The offspring of the founders were tested for T antigen expression in bone marrow cells and other tissues by Northern blot analyses (Fig. 1 c). The results indicated that the bone marrow cells of the offspring of founders 10 and 18 possessed a low level of oncogene mRNA. However, the liver, skeletal muscle, heart, brain, kidney, adrenal, and lung of offspring from the above founders were negative for transgene expression. This observation confirms the tissue-specific nature of the PF4 promoter used.

## Analyses of the Hematopoietic System of Transgenic Mice

We have carefully monitored alterations in the transgenic mice colony over a period of about three months. No abnormalities were recorded except for the sudden death of founder 10 at the age of 11 wk. Unfortunately, it was not possible to carry out an autopsy on this animal. The platelets and megakaryocytes from the blood and bone marrow of transgenic mice (ages 6-12 wk) were examined by immunohistochemical methods to determine whether large T antigen was present. The results showed that these cell types possessed no detectable level of oncogene protein under in vivo conditions (not shown). Platelets were also counted in whole-blood samples collected from age-matched transgenic and nontransgenic mice. The mean platelet counts, as determined by hemocytometer (Kuter et al., 1989), were 680 ± 102 × 10³/µl (n = 3) in transgenic mice and 730 ± 90 × 10³/µl (n = 3) in non-transgenic mice. The extent of bone marrow megakaryocyte ploidy was also evaluated in two transgenic (offspring of founder 10) and non-transgenic
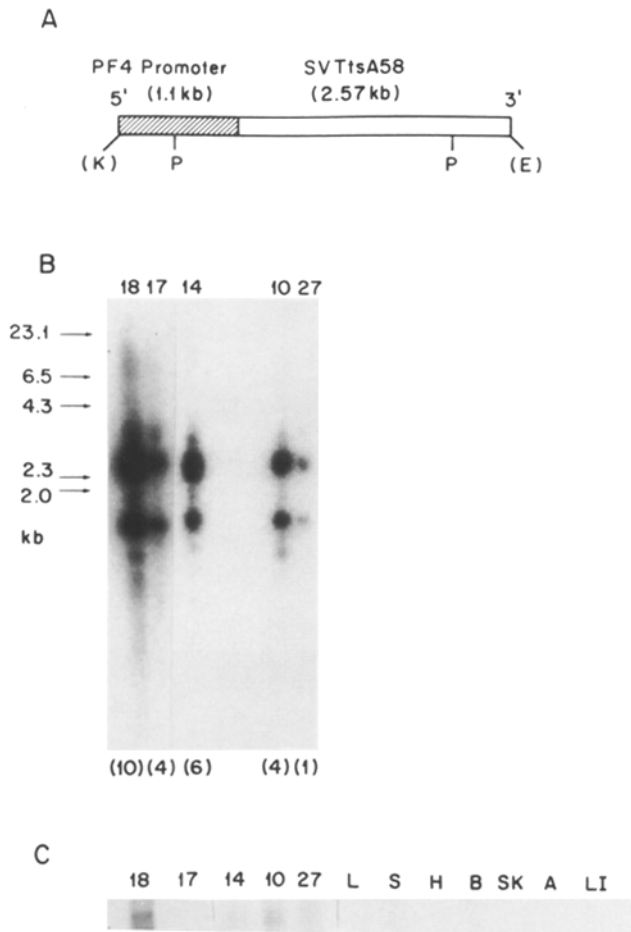
A

PF4 Promoter    SV TtsA58
5'  (1.1 kb)      (2.57 kb)              3'

(K)    P                          P    (E)

B

18 17 14      10 27

23.1 →
6.5 →
4.3 →

2.3 →
2.0 →
kb

(10)(4)(6)      (4)(1)

C

        18  17  14  10 27  L  S  H  B  SK  A  LI

*Figure 1.* (A) The PF4SVtsA58 construct used for production of transgenic mice. The construct contains 1.1 kb of the 5' upstream region of the rat PF4 promoter linked to 2.57 kb of STtsA58 gene. *K*, Kpn I cohesive ends; *E*, EcoRI cohesive ends; and *P*, Pst I site. (*B*) Southern blot analyses of 10 µg of DNA isolated from different founders, digested with PstI, and fractionated by 1.2% agarose gel electrophoresis. DNA concentrations were determined by absorbancy at 260 nm before loading on the agarose gel. The figure in parenthesis indicates the number of copies of DNA integrated into a single chromosomal site as determined with a linearized control DNA (data not shown). Lambda DNA HindIII markers are indicated by arrows. (*C*) Northern blot analysis of 20 µg of total RNA prepared from bone marrow cells of the offspring of different founders (*18, 17, 14, 10,* and *27*) as well as the lung (*L*), spleen (*S*), heart (*H*), brain (*B*), skeletal muscle (*SK*), adrenal (*A*), and liver (*LI*) of the offspring of founder 18. The blots were probed with the T antigen gene fragment and the bands shown correspond to the T antigen message. Equal loading of RNA was confirmed by ethidium bromide staining of the ribosomal bands 28S (5 kb) and 18S (2.5 kb). The experimental details are provided in Materials and Methods.

mice. The above parameter was determined by identifying megakaryocytes by cell surface labeling with antibody to rat platelets and then estimating the amount of DNA per cell by flow cytometry as described previously (Kuter et al., 1989). A non-transgenic mouse exhibited a megakaryocyte ploidy distribution of 39.2, 17.7, 5.6, and 10.4% for 2N, 4N, 8N, and 16N or higher, respectively, whereas a transgenic mouse possessed a megakaryocyte ploidy distribution of 40.8, 16.8,

4.87, and 10.2% for 2N, 4N, 8N, and 16N or higher, respectively (the results are from a representative experiment). The relatively high percentage of mouse 2N megakaryocytes detected as compared to that reported in rats and mice (Corash et al., 1987; Kuter et al., 1989) may have been due to low level nonspecific binding of antibody to nonmegakaryocytic mouse bone marrow cells which comprise 99.9% of cells analyzed.

## Establishment of Bone Marrow Cell Lines

Bone marrow cells were derived from the offspring of all transgenic mice and cultured at the permissive temperature of 34°C in a liquid culture as described in Materials and Methods. Centrifugation was used to replace 50% of the media every 4 d and resuspended cells were returned to the original dish. After 4 wk of culture, the nonadhering and adhering cells originating from the offspring of all founders, except 10 and 18, died. Within days, colonies of adhering cells derived from the offspring of founders 10 and 18 filled the dishes. These cells were cloned by the ring cloning technique, expanded to obtain stocks and subsequently frozen. Clone 37 derived from offspring 37 of founder 10 (designated as MegT37) and clones 1 and 8 from founder 18 (designated as MegT1 and MegT8) were further characterized. At 34°C, cells were spindle shaped and adherent and showed no change in morphology up to ∼30 passages. At 39.5°C, cells attached with an efficiency of 50–80%, and then gradually detached and rounded up over 4 to 5 d in culture after which cell death was observed. About 30% of the cells were identified as dead at day six in culture at 39.5°C. It should be pointed out that immunofluorescence staining with an antibody to large T antigen revealed that the oncogene product was degraded only after about 2 d in culture at 39.5°C, after which the cell number did not increase (not shown). The cells MegT37 and MegT1 grew at 34°C with a doubling time of 22 h while MegT8 grew with a doubling time of 30 h. However, upon prolonged culturing MegT8 displayed a doubling time of 24 h. When the immortal cells were cultured at 37°C the cells remained adhering to the dish and continued cycling, albeit, with a doubling time of 42 h. This later observation correlates with our finding that at 37°C the cultured cells still possess the oncogene product (not shown).

## Lineage Properties of the Cell Lines

The identity of the cell lines was established by documenting the presence of lineage specific markers with immunohistochemical and Northern blot analyses. MegT37 exhibited a strong positive staining with an antibody to rat PF4 (Fig. 2, *a* and *b*). As controls, all mouse bone marrow cells with the exception of megakaryocytes were negative (Fig. 2, *c* and *d*). MegT37 cells also showed weak positive staining with an antibody to CD45 antigen, but were otherwise negative with antibodies to the macrophage-specific antigen Mac-1, T helper cell specific antigen CD4 and mast cell specific antigen IgE (not shown) (Spangrude et al., 1988). MegT37 also possessed the megakaryocytic marker acetylcholine esterase (Jackson, 1973) (Fig. 2, *e* and *f*). Similar results were obtained with the other cell lines (not shown). Northern blot analyses were used to detect expression of
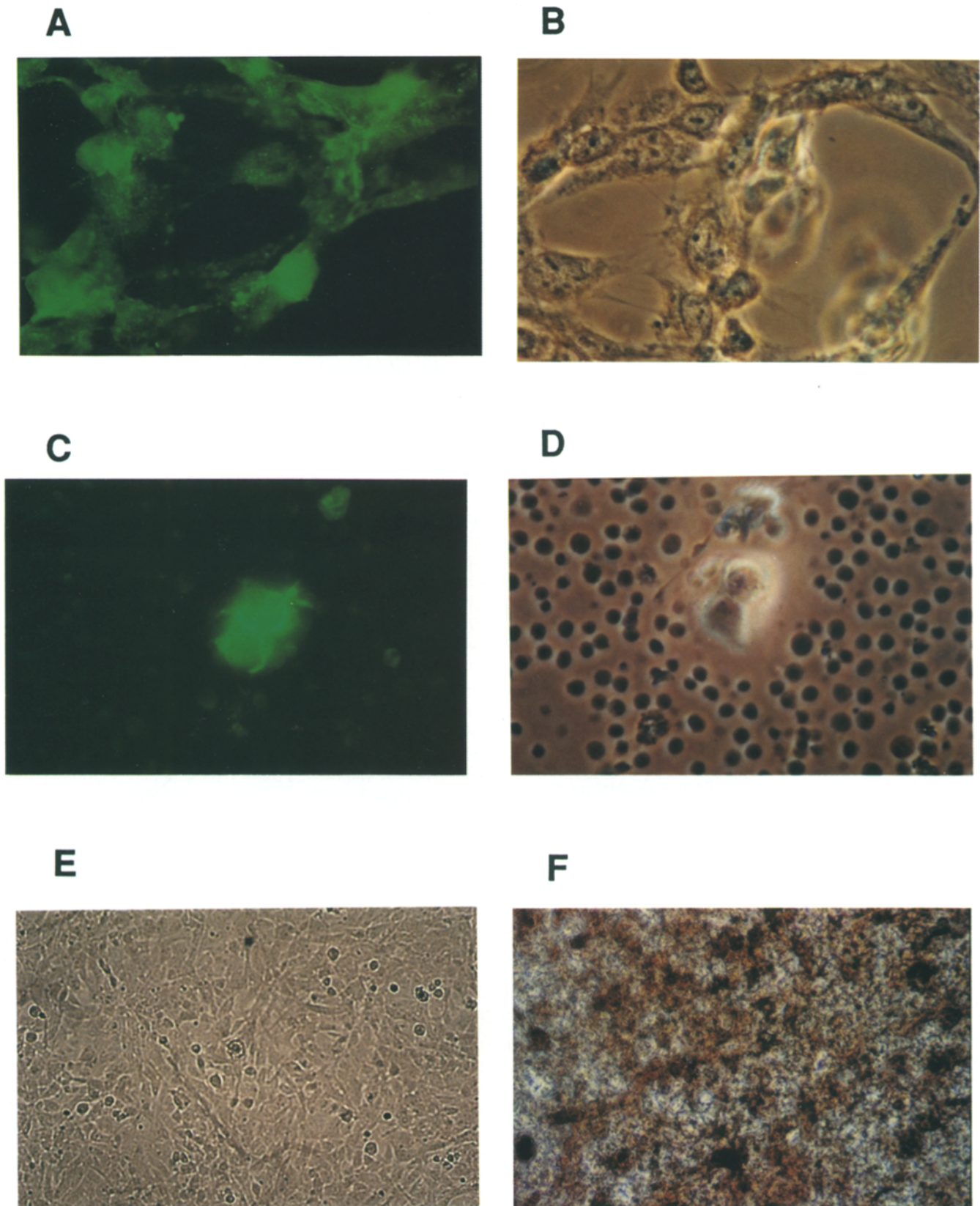
*Figure 2.* The presence of lineage-specific markers in MegT37. Immunofluorescence photomicrographs of MegT37 cells cultured at 34°C (*A*) or of normal mouse bone marrow cells (*C*) stained with an antibody to rat PF4 (Doi et al., 1987). Phase–contrast photomicrographs of the same MegT37 cells cultured at 34°C (*B*) or the same normal mouse bone marrow cells (*D*). Staining for acetylcholine esterase for six-seven hours in the absence of (*E*) or presence of (*F*) an enzyme inhibitor (0.5 mM diisopropylfluorophosphate) as described in (Jackson, 1973). Original magnification: ×400 (*A–D*), or ×100 (*E* and *F*). The experimental details are provided in Materials and Methods.

markers on the cell lines to which antibodies to rodent antigens are not available and to compare the levels of the T antigen message in the different cell lines. A significant amount of the large T antigen message was noticed in all cell lines with the order MegT1 > MegT37 > MegT8 (Fig. 3). The mRNA for von-Willebrand Factor (vWF) was not detected in the cells (not shown) nor was the erythroid marker $\alpha$-globin (Nishioka and Leder, 1979) while the messages for the megakaryocytic markers GPIIb and the PF4 were detectable either at 34° or at 39.5°C (Fig. 3). As also seen in Fig. 3, the level of the PF4 message in all the cell lines cultured at

A



B

GPIIb

PF4

$\alpha$-globin

Tag

Figure 3. The determination of lineage-specific markers in different cell lines. 30 $\mu$g of total RNA from mouse bone marrow cells (lane 1), MegT1 (lanes 2 and 3), MegT8 (lanes 4 and 5) and MegT37 (lanes 6 and 7) cultured at 34°C (lanes 2, 4, and 6) or 39.5°C (lanes 3, 5, and 7), was electrophoresed (1% agarose) per lane; equal loading was confirmed by ethidium bromide staining of the ribosomal bands 28S (5 kb) and 18S (2.5 kb) (A). The gel from A was subjected to Northern blot analysis and probed with $\alpha$-globin cDNA, PF4 cDNA, and GPIIb cDNA. RNA was also prepared from cells grown at 34°C, subjected to Northern blot analysis and probed with the T antigen gene (Tag) (B). The experimental details are provided in Materials and Methods. The blots were either subjected to autoradiography or scanned by Betascope (Betagen Co., Waltham, MA).

the permissive conditions was similar to the level detected when the cells were cultured at 39.5°C while the level of the GPIIb message increased by about two fold upon shifting the cells to 39.5°C. The existence of the PF4 and GPIIb messages in the cell lines was also confirmed by amplifying reverse transcribed mRNA by the polymerase chain reaction (data not shown). This method was also used to exclude the presence of mRNA for the T cell surface marker CD2 (Diamond et al., 1988) to which rodent cDNA was not available to us. The results shown in Figs. 2 and 3 and in the text are summarized in Table I which lists all the lineage markers tested and indicates their presence or absence in our MegT cell lines.
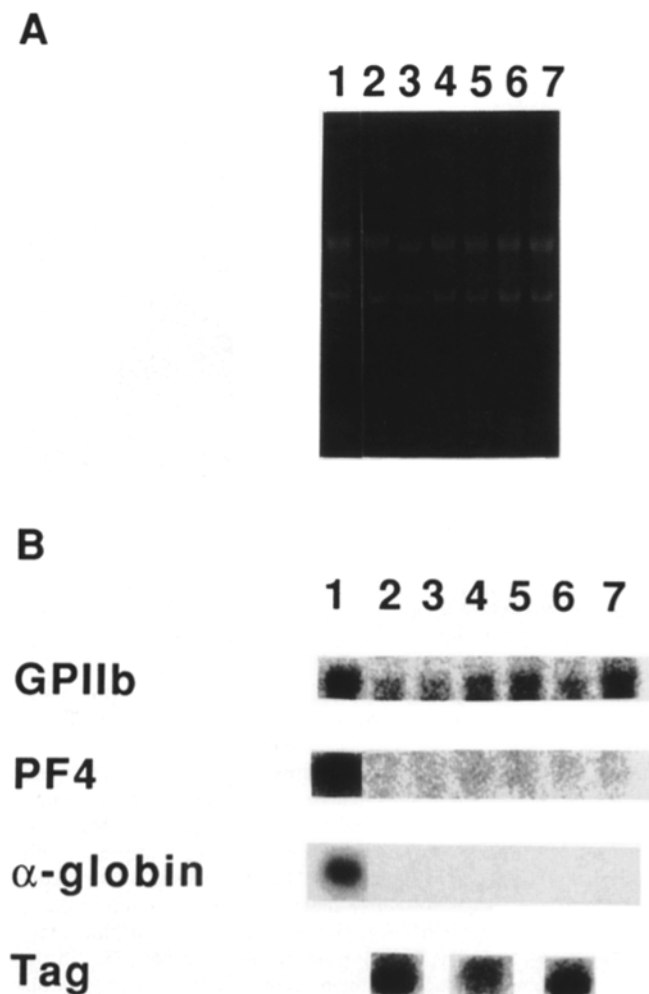
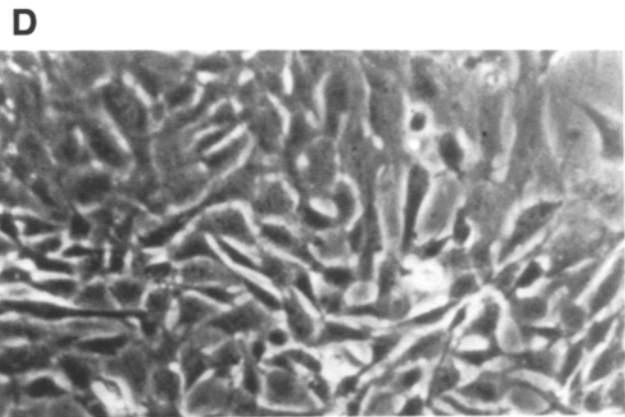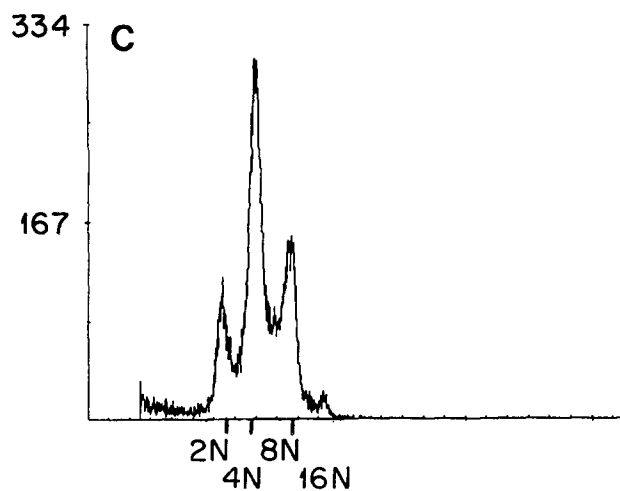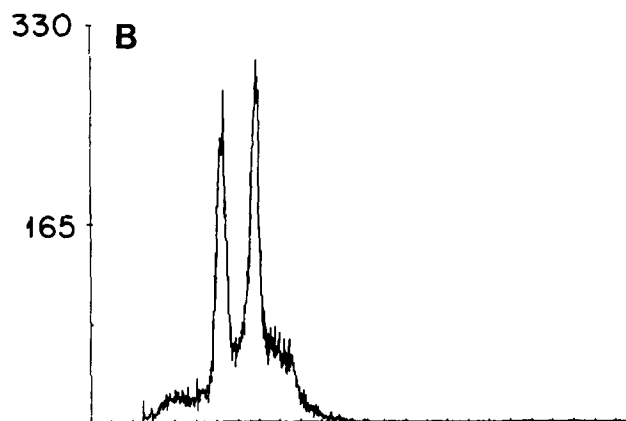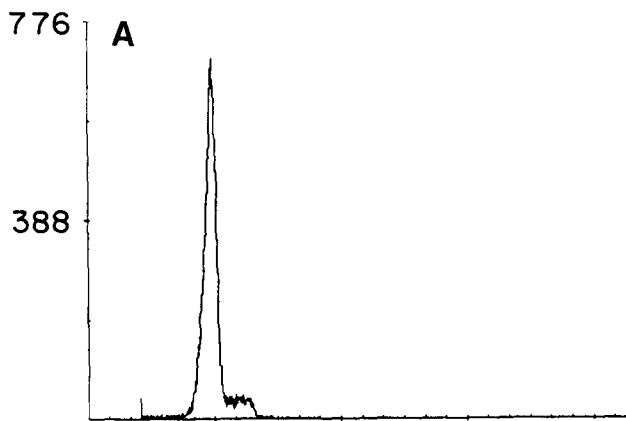## In Vitro Ploidy of the Megakaryocytic Cell Lines

To ascertain whether inactivation of large T antigen would allow MegT37 to become polyploid, the cell line was analyzed for DNA content per cell by flow cytometry as outlined in Materials and Methods. The localization of the 2N peak was confirmed by using mouse bone marrow cells as control (Fig. 4 a). When grown at 34°C, the majority of MegT37 (95%) exhibited 2N or 4N nuclei, with very few cells possessing 8N nuclei (Fig. 4 b). When grown at 39.5°C, the frequency of MegT37 with 2N nuclei decreased to about 25%, and those with 8N nuclei increased to about 30%, with some cells possessing 16N nuclei (Fig. 4 c). Phase-contrast photomicrographs of the cells grown at 34°C (Fig. 4 d) or at 39.5°C (Fig. 4 e) were taken before flow cytometry analyses. At 34°C, cells were spindle shaped and adherent while at 39.5°C a large fraction of the cells detached from the plate. All of these non-adhering cells appeared oval or round and about 70% of them had a diameter larger than that of a 2N cell (>10–15 $\mu$m), large nuclei and multiple nucleoli. To exclude the possibility that cell fusion might have generated MegT37 with nuclei greater than 4N, two pools of cells were separately labeled with red fluorescence dye PKH2-GL or green fluorescence dye PKH26-GL before cultivation. The labeled cells were mixed in equal numbers, grown at 34° or 39.5°C for 4 to 5 d, stained with Hoechst dye, and analyzed by flow cytometry. We then determined the single and double

Table I. Lineage Properties of MegT Cells

| Hematopoietic cells | Lineage markers | MegT cells |
|---|---|---|
| Megakaryocytes | PF4 | + |
| | Acethycholine esterase | + |
| | GPIIb | + |
| | vWF | − |
| Myelomonocytes | Mac1 | − |
| T cells | CD4 | − |
| | CD2 | − |
| Mast cells | IgE | − |
| Erythrocytes | $\alpha$-Globin | − |
| Leukocytes | CD45 | +* |

The table indicates the presence (+) or absence (−) of markers of different hematopoietic lineages in MegT cells, as concluded from the experiments presented in Figs. 2 and 3 and in the text. The distribution of the various markers was identical at 34° and at 39.5°C.
* A weak positive staining with an antibody to CD45 was observed in MegT cells, however, a reaction of similar intensity was also observed in primary bone marrow megakaryocytes of low ploidy class (Kuter, D. J., unpublished observation).

**776**

**388**

**A**

**330**

**165**

**B**

**334**

**167**

**C**

2N  8N
4N  16N

**D**

**E**

*Figure 4.* The flow cytometric and electron microscopic examination of MegT37. Flow cytometric analysis of normal mouse bone marrow cells (*A*), or MegT37 cells cultured for 4 d at 34°C (*B*), or at 39.5°C (*C*), respectively. The abscissa shows the DNA content on a logarithmic scale, determined based on fluorescence due to Hoechst staining, and the ordinate reflects the number of cells at each DNA value (linear scale). Two times repetition of this experiment yielded identical results. The results shown are from a representative assay. The experimental details are provided in Materials and Methods. Phase–contrast photomicrographs of samples of cells grown at 34°C (*D*), or at 39.5°C (*E*). Original magnification ×200. Cells grown at 39.5°C were also subjected to electron microscopic analysis (*F*) as described before (Ravid et al., 1993). Original magnification: ×5538. The arrows point to the different lobes in the large multilobulated nucleus which occupies a large portion of the cytoplasma, thus resulting with a high nuclear-cytoplasmic ratio. This morphology is characteristic of polyploid megakaryocytes.

labeled cells in the total population and within each ploidy class. Within the total cell population, 7–8% of the cells were double labeled at 34°C and 10–11% at 39.5°C. The generation of double labeled cells is most likely to occur because of cell fusion but a small contribution from dye transfer can not be excluded. The percentage of cells undergoing fusion in a cell population should be equivalent to three times the percentage of double labeled cells (*red, green*; *green, green*; *red, red*). Therefore, cell fusion could generate a maximum

of ∼30% of the polyploid megakaryocytes. Similar results were obtained when each ploidy class was separately analyzed (data not shown). Based on these analyses, we conclude that at least 70% of the polyploid MegT37 cells must have arisen from 2N megakaryocytes by DNA replication without cell division. Similar ploidy analyses carried out with MegT1 and MegT8 cells revealed a minimal number of ≥8N cells (not shown).

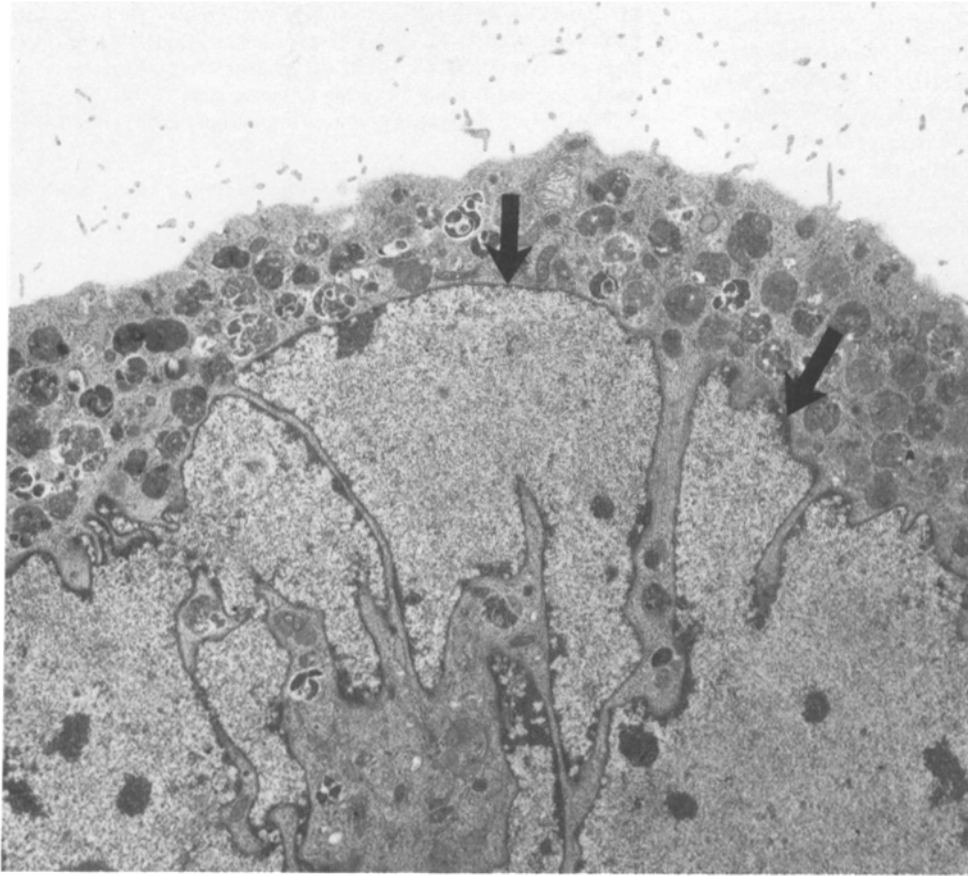The electron microscopic analyses of MegT37 grown at

**F**



*Figure 4.*

39.5°C confirmed the presence of a large multilobulated nucleus and a high nuclear–cytoplasmic ratio which is characteristic of polyploid megakaryocytes. However, typical α-granules were absent while lysosomes were readily apparent (Fig. 4 *f*). The large nucleus and lysosomes were not present in cells grown at 34°C (not shown).

Several attempts were made to induce MegT37 to convert to cells with 16N and 32N nuclei at frequencies observed in normal murine bone marrow (Corash et al., 1987; Kuter et al., 1989). These manipulations included addition of hemopoietic growth factors (see Methods) and phorbol ester or dimethylsulfoxide, known to induce differentiation in erythroleukemia cell lines (Greenberg et al., 1988), as well as co-culture of MegT37 (prelabeled with fluorescent dye) with normal mouse bone marrow. No augmentation in the extent of ploidy of MegT37 was observed. It should also be noted that differentiation of the cell line in agar cultures at 39.5°C under colony forming unit conditions (Chatelain et al., 1983) was unsuccessful because of the poor stability of agar (1–2%).

## Discussion

Several different oncogenes encode nuclear proteins which are able to immortalize somatic cells in vitro. In some cases,

oncogene expression induces the formation of tumor cells which synthesize many of the major differentiation products of normal cells from which they are derived (Amsterdam et al. 1988; Garcia et al., 1986; Moura Neto et al., 1986; Muller and Wagner, 1984). In other situations, oncogene-mediated cell immortalization inhibits expression of the terminally differentiated state (Beug et al., 1987; Cherington et al., 1986; Dmitrovsky et al., 1986; Falcone et al., 1985). The availability of conditional oncogenes makes it potentially possible to establish cell lines which cycle at the permissive conditions, and differentiate under nonpermissive conditions in the presence of appropriate growth factors. Indeed, retroviral vectors driving the expression of conditional oncogenes such as SV-40 large T or *myc* have been used in vitro to produce cell lines having the potential to differentiate (Frederiksen et al., 1988; Iujvidin et al., 1990; Eilers et al., 1989). Viral and cellular non-conditional oncogenes have been also used in vivo to produce transgenic mice (Leder et al., 1986; Andres et al., 1987; Bender and Pfeifer, 1987; Mahon et al., 1987; Efrat et al., 1988; Harris et al., 1988; Reynolds et al., 1988). In each case, tumors were noted in tissues that are targets for high levels of oncogene expression which frequently caused the death of the transgenic mice.

In the present study, we attempted to generate hematopoietic cell lines that could provide models for lineage program-

ming decisions. The retroviral-mediated introduction of oncogenes into pluripotent mouse hematopoietic cells has previously been reported (Williams et al., 1984). However, we used the PF4 promoter linked to a temperature-sensitive SV-40 large T antigen to target oncogene expression in transgenic mice to a specific hematopoietic cell lineage. The use of this conditional oncogene allowed the large T antigen to be maintained in an inactive state in transgenic mice, which have a body temperature higher than the permissive one. This later feature permitted the mice to exhibit normal hematopoietic cell function for at least three months and prevented the emergence of hematopoietic tumors during this period of time. However, it is possible that the death of founder 10 was due to the leaky nature of the conditional oncogene in conjunction with slight variations in body temperature. Given that the focus of our work was to generate immortalized megakaryocytes, we did not study potential changes in these mice with aging. The bone marrow cells obtained from the young transgenic mice were cultured at 34°C which yielded immortalized cell lines expressing megakaryocytic characteristics, e.g., PF4, GPIIb, and acetylcholinesterase. In contrast to bone marrow megakaryocytes, the immortalized cell lines do not contain vWF, contain relatively low levels of PF4 mRNA, and consist mainly of cells with 2N and 4N nuclei. It may be that vWF is expressed in mature megakaryocytes of high ploidy class and thus will not be detected in our cell lines. It is possible that the low extent of PF4 expression is due to competition for trans-acting factors, the later resulting from the high copy number of the transgene. Alternatively, the low level of PF4 message may be typical of low ploidy bone marrow megakaryocytes. However, the PF4 protein was definitely detectable by antibody staining.

The successful production of immortalized cell lines which exhibit lineage fidelity depends on the ability to induce cell differentiation in vitro. Our cell lines respond to a rise in temperature with inactivation of the oncogene and changes in cell behavior and morphology. The MegT37 cells initiate and progress along a differentiation pathway including arrest of cellular proliferation, enlargement of cell size, frequent detachment from the plate, increase in the nuclei/cytosol ratio, and appearance of cells with 8N and 16N nuclei. Although a cocktail of hemopoietic growth factors was used, we were unable to stimulate MegT cells to become polyploid to the same extent as normal mouse bone marrow megakaryocytes nor to form α-granules. In normal megakaryocyte differentiation, CD34 positive cells initially exhibit platelet specific membrane glycoproteins and only later develop α-granules and full ploidy (Debili, N., C. Issaad, J. M. Masse, J. Guichard, A. Katz, J. Berton Gorious, and W. Vainchenker. 1992. Blood. 80:126a). It is possible that the liquid culture system used at the high temperature lacks cellular interactions and/or an unknown growth factor that are critical for formation of high ploidy cells and for α-granule formation in cells that have been subjected to immortalization. Thus, MegT cells might undergo normal maturation if transplanted into the bone marrow of irradiated mice. Based upon the above data, we believe that these cell lines constitute a new tool for investigating the biology of early megakaryocytes as well as of polyploid cells. We also believe that our molecular biological approach used to generate megakaryocytic cell lines should assist in future studies on oncogene-mediated immortalization of megakaryocytic cells.

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