Deregulated Expression of c-myc in Megakaryocytes of Transgenic Mice Increases Megakaryopoiesis and Decreases Polyploidization*

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Platelets, essential for vascular integrity and hemostasis, fragment from polyploid megakaryocytes, characterized by their endomitotic cell cycle. We studied the influence of overexpression of c-myc oncogene on megakaryopoiesis and endomitosis in vivo, using transgenic mice carrying c-myc fused to the estrogen receptor under the control of the platelet factor 4 (PF4) megakaryocyte-specific promoter. The rationale behind this strategy was to obtain controlled overexpression of an active c-Myc, depending on the estrogen level in the mouse circulation. Analysis of these transgenic mice revealed that the bone marrow of female megakaryocytes or of estrogen-injected male transgenic mice, but not of age-matched transgenic males nor nontransgenic females, contained frequent immature myeloid cells and an increased number of megakaryocytes. Deregulated expression of c-Myc shifted the normal ploidy profile of megakaryocytes due to a significant increase in proliferating megakaryocytes and a decrease in the fraction of polyploidizing cells. These transgenic mice represent a novel in vivo model for a Myc-induced myeloproliferative disorder which can be controlled.

Platelet precursors, the megakaryocytes, undergo an endomitotic cell cycle whereby they replicate DNA but do not undergo cytokinesis. This unique cell cycle leads to the formation of polyploid cells which mature and subdivide their cytoplasm into platelets (reviewed by Mazur (1987)). A normal cell cycle in eukaryotic cells consists of a tightly regulated sequence of phases including, gap (G1), DNA synthesis (S) followed by a gap (G2), and mitosis (M). Progression through the cell cycle is regulated by cyclin-dependent protein kinases (reviewed by Kelly and Siebenlist (1986)). Cdk2 associates with the G1 phase cyclin, cyclin D3 (Wang et al., 1995; Odell et al., 1968). However, little is known about the role of specific genes in megakaryocyte polyploidization. In a recent study we found that the endomitotic cell cycle in this cell type is associated with a reduced level of cyclin B1 and high level of the G1 phase cyclin, cyclin D3 (Wang et al., 1995; Zhang et al., 1996).

One oncogene whose protein product plays a well-established role in the regulation of the cell cycle, cell growth, and differentiation is c-myc (reviewed by Kelly and Siebenlist (1986)). Myc expression is sufficient to induce cells to enter the S phase of the cell cycle (reviewed by Cory (1986)). Expression of Myc is induced following mitogenic stimulation, is shut off during entry into quiescence, and its expression is deregulated in various neoplasias (Cole, 1986; Marcu et al., 1992). These effects of Myc were attributed to its ability to bind growth suppressors such as the retinoblastoma gene product (Rustgi et al., 1991), to act as a sequence-specific transcripational regulator (Amin et al., 1993), and to induce DNA replication (Clasen et al., 1987). Recently, c-Myc was also connected to apoptosis, as overexpression of Myc in cells deprived of serum-induced programmed cell death (Evan et al., 1992).

Earlier studies measured c-Myc mRNA levels in isolated megakaryocytes and in hematopoietic cell lines that can be induced to express megakaryocytic features (Dorn et al., 1994; Eckhardt et al., 1994; Gewirtz and Shen, 1990). However, the effects of altered c-Myc expression on megakaryopoiesis and platelet production in vivo have not been described. Since megakaryocyte precursor proliferation occurs prior to megakaryocyte polyploidization, and the cell undergoing polyploidization is more differentiated than the cell undergoing cell division, we hypothesized that c-Myc overexpression in early megakaryocytes may increase precursor proliferation and, thus, decrease the fraction of polyploid cells. We have tested this hypothesis by using a transgenic model with constitutive overexpression of c-Myc in the megakaryocytic lineage in vivo. The model was constructed by targeting the conditionally active c-Myc estrogen receptor chimeric protein (Eilers et al., 1987) to megakaryocytes and platelets via the platelet factor 4 (PF4) megakaryocyte-specific promoter (Ravid et al., 1991a). This approach was taken in order to establish an in vivo model with controlled activation of c-Myc, depending on estrogen level in the mouse. Our results support the hypothesis.

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1 The abbreviations used are: PF4, platelet factor 4; kb, kilobase(s); HGH, human growth hormone; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.
that c-Myc overexpression induces megakaryocyte precursor proliferation at the expense of polyploidization.

**MATERIALS AND METHODS**

**Plasmids**—The plasmid PF4MERGH was constructed by using gene fragments from the following FUC based plasmids: pPF4GH (Ravid et al., 1991b) which contains the rat 1.1-kb promoter linked to the growth hormone gene (HGH); PMV-7MER (Eilers et al., 1989) which contains exons 2 and 3 of human myc fused to the estrogen receptor (MER). pPF4GH has a unique NdeI site at the 5′ end of the PF4 promoter, a unique EcoRI site at the 3′ end of the HGH gene and a unique BanII site 20 bp downstream of the transcriptional start. The plasmid containing EcoRI and EcoRI and XhoI sites was digested with BanII to release the HGH gene. The resulting 2.4-kb fragment (with EcoRI/BanII cohesive ends) was ligated to the 2.4-kb MER fusion gene fragment (Eilers et al., 1989), the latter obtained by digesting pMV-7MER (generous gift of Dr. Michael Bishop) with EcoRI. This ligation was done in the presence of the BanII/EcoRI linker TCGGTATA which destroys the BanII site. The orientation of insertion was tested by DNA sequencing (Ravid et al., 1991b) of the resulting plasmid, termed PF4MER. We next introduced a 442-basepair region of the HGH poly(A) tail starting from the stop codon of the HGH gene (Selden et al., 1986). The poly(A) fragment was generated by PCR of pPF4GH (Ravid et al., 1991b), with primers which also introduced a unique KpnI site at the 3′ end of the poly(A) tail. The plasmid containing the PF4 promoter, followed by MER and the HGH poly(A) tail, was termed PF4MERGH. This later plasmid also had unique SacI sites at both ends of the MER gene. The DNA sequence of the final construct pPF4MERGH was verified (Ravid et al., 1991b) and subsequently digested with NdeI and KpnI to free the genes from vector sequences. The resulting 3.9-kb fragment which contained the PF4 promoter followed by MER, followed by the HGH poly(A) tail, was purified as described before (Ravid et al., 1991a) and used for producing transgenic mice as described below.

**Generation of Transgenic Mice**—The DNA fragment was injected into one-cell embryos at a concentration of 3 μg/ml to produce transgenic mice, all as described previously (Ravid et al., 1991a). Foster mice females were of the CD1 strain (Charles River Breeding Laboratories), and the microinjected eggs were of the FVB strain (Taconic Farms, Germantown, NY). Mice were screened for transgenic integration by Southern blot analysis of tail DNA, using MER as a probe (Ravid et al., 1991a). Transgene expression was detected by reverse transcription-PCT. To this end, a HGH poly(A)-specific sense primer (TTCGGCGCCGGATTCCTGCACGGTGTTACATC) and an antisense primer consisting of 17 T residues were used to amplify DNA reverse-transcribed from the periphery of bone marrow of transgenic or normal mice, all as described before (Ravid et al., 1991a, 1991b).

**Estrogen Injection**—Injection of estrogen in the form of Premarin (Ayerst Laboratories, Philadelphia, PA) was performed as follows. A stock solution of Premarin phosphate-buffered saline of 200 μg/ml was prepared under sterile conditions. Male mice, wild type or transgenic, were anesthetized, and the large muscle of the hind limb was injected with 100 μl of the stock Premarin using a tuberculin syringe and a 30-gauge needle. Injections were given every other day, alternating from left to right hind limb, for up to 21 days.

**Bone Marrow Preparation and Assay for Acetycholine Esterase**—Bone marrow was harvested from the femurs of transgenic mice as described previously (Kuter et al., 1989; Ravid et al., 1991b). Megakaryocytes were identified by in situ staining for acetycholine esterase as described before (Jackson, 1973; Ravid et al., 1993).

**Platelet Count**—Blood was collected by cardiac puncture (Ravid et al., 1991a) into EDTA, and cells were counted in a Cell-Dyn 3500 automated blood analyzer, calibrated for rodent analyses using a Veterinary Package (Abbott Diagnostics, Abbott Park, IL).

**Immunohistochemistry and Flow Cytometric Analysis of Bone Marrow**—Bone marrow cells were stained with rat anti-mouse CD11b (MAC-1) monoclonal antibody at a concentration of 0.5 μg/106 cells/50 μl or with rat anti-mouse TER-119-erythroid cell monoclonal antibody at a concentration of 1.5 μg/106 cells/50 μl or with rat anti-mouse stem cell antigen monoclonal antibody at a concentration of 2 μg/106 cells/50 μl. All reactions with the first antibodies (Pharmingen, San Diego, CA) were done in the presence of phosphate-buffered saline supplemented with 2% fetal bovine serum, incubated at 4 ºC overnight. Fluorescein-conjugated anti-rat immunoglobulin (Sigma) was used as the second antibody at a concentration of 16.7 μg/ml at 4 ºC for 2 h. Cells were analyzed by flow cytometry on a FACScan system (Becton Dickinson, San Jose, CA). Data were collected and analyzed by Lysys program (Becton Dickinson). For immunohistochemistry, cytospun bone marrow cells were fixed in the presence of 2% formaldehyde in phosphate-buffered saline at 45 min at room temperature. Slides were washed with PBS and blocked with 2% fetal bovine serum and 0.1% bovine serum albumin in PBS, for 20 min at room temperature. This blocking solution was drained and the cells were incubated for 1 h at room temperature with 20 μg/ml mouse monoclonal antibody to human myc (Ab-1, Oncogene Science, Cambridge, MA). Cells were washed with PBS and reacted with a secondary antibody, anti-mouse IgG conjugated to FITC or FITC-isothiocyanate, and stained with mouse or human IgG (Bio-source International, Camarillo, CA), and diluted 125-fold. Both first and secondary antibodies were diluted in PBS supplemented with 0.1% bovine serum albumin. For peptide neutralization experiments, c-Myc antibody was reacted with a 10-fold (by weight) excess of Peptide-1 (Oncogene Science, Cambridge, MA) for 2 h at room temperature. In order to reduce background, the secondary-antibody complexes were centrifuged for 15 min at full speed in a microcentrifuge and the supernatant was discarded (according to the manufacturer's instructions). Immunohistochemistry was performed as described above, using c-Myc antibody neutralized with the peptide. Immunohistochemistry with anti-raft PA (generous gift of Robert D. Rosenberg) was performed as described before (Ravid et al., 1993).

**Histopathology**—Samples of sternum, femur, spleen, liver, heart, and lung were removed from the mouse following euthanasia. The samples were placed in a fixative (“OptimalFix,” American Histology, Lodi, CA) for shipment to the UCD Davis Transgenic Histopathology Laboratory. There, the samples were dehydrated, embedded in paraffin, sectioned at 10 microns, stained with hematoxylin and eosin, coverslipped, and presented for interpretation. The marrow contents of the femur and sternum were evaluated in three-step sections each. All the megakaryocytes in each compartment of each of the step sections were counted using a hand-held counter.

**Determination of DNA Content in Megakaryocytes**—Collection of bone marrow from femurs and tibias of transgenic and control mice was done as described before (Ravid et al., 1991a; Kuter et al., 1989). Bone marrow megakaryocytes were subjected to ploidy analyses, using a FACSscan flow cytofluorometer (Becton-Dickinson) as detailed elsewhere (Jackson et al., 1984; Shivdasani et al., 1995). To this end, megakaryocytes in bone marrow cell suspensions were labeled with 4A5 monoclonal antibody ascites (Burstein et al., 1992) (generous gift of Sam Burstein) and subsequently with fluorescein-conjugated goat anti-rat IgG(Fab′)2 (Tago, Inc.). DNA content of 4A5 antibody-positive cells stained with propidium iodide was determined and analyzed using a statistical package on a FACSscan flow cytometer (Becton-Dickinson).

**Detection of Apoptotic Cells**—We used the ApopTag Plus in situ Apoptosis Detection kit ( Oncor, Gaithersburg, MD) in order to determine apoptosis in individual bone marrow cells. The method which involves direct immunoperoxidase detection of endogenous DNA was used on cytospun bone marrow cells. Cells were treated with 2% H2O2 in phosphate-buffered saline in order to quench endogenous peroxidase activity and subjected to apoptosis assay, all as described by the manufacturer.

**RESULTS**

**Generation of Transgenic Mice Containing the PF4 Promoter Linked to the myc-Estragon Receptor Fusion Gene**—The construct used to generate transgenic mice, referred to as PF4MERGH, contains 1104 base pairs of the 5′ upstream region of the rat PF4 gene (promoter region) linked to human myc fused-in-frame to the hormone binding domain of the human estrogen receptor gene (together producing MER) tagged at 3′ with the poly(A) tail of the human growth hormone gene (HGH) (Fig. 1A). This fusion gene is expressed in cells constitutively, but remains in an inactive state. Exposure to estrogen deactivates the regulatory portion (hormone binding domain) of the fusion protein and thereby enables Myc to function (Eilers et al., 1989). Given that female mice contain high levels of estrogen, overexpressed Myc should be active in female, but not male, transgenic mice. 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). PF4MERGH was microinjected into promculie of fertilized mouse eggs, and the injected embryos were implanted into pseudopregnant outbred females. The offspring were screened for transgene inte-
myc Oncogene in Megakaryocyte Development

Fig. 1. Generation of transgenic mice expressing a PF4-driven myc-estrogen receptor fusion gene. A, the construct PF4MERGH used for generation of transgenic mice. The PF4 promoter was subcloned upstream to human c-myc linked to human estrogen receptor (HE-14) and to the poly(A) tail of the human growth hormone gene (HGH). N, NdeI; S, SacI; E, EcoRI; K, KpnI. Restriction sites indicated in parentheses were used to free the plasmid from vector sequences prior to microinjection. B, identification of transgenic mice. Southern blot analysis of tail genomic DNA digested with SacI were performed, using SacI fragment of the construct shown in A as a probe. Consequently, transgenic founders 33, 20, and 6 were identified. N, nontransgenic mouse; C, transgene expression in transgenic mice. Transgene expression was verified by reverse transcription of RNA prepared from bone marrow of F1 progeny of the transgenic mice or from nontransgenic ones, followed by the polymerase chain reaction (reverse transcriptase-PCR), using primers hybridizing to HGH sequences upstream to the poly(A) signal. The transgene-specific PCR product (130 bp) is abundant in various species. As shown in Table II, the number of megakaryocytes/compartment (30 to 35 megakaryocytes) from transgenic mice was increased by 24% ± 5% (n = 4) (p < 0.03) as compared to nontransgenic pregnant controls. The spleens in the female transgenic mice contained abundant zones of immature myeloid cells (Fig. 2), but the lymphoid follicles and red pulps are normal. A variety of other tissues tested were normal histologically (not shown). Histological analyses of marrow in all four compartments in the sternum indicated that the nontransgenic male or female mice as well as male transgenic mice contained a similar number of megakaryocytes/compartment (30 to 35 megakaryocytes). Female transgenic mice had 65/macrophage for the nonpregnant mice tested. A band identified as 110 base pairs was amplified nonspecifically in both the transgenic (lanes indicated by 6 or 20) and nontransgenic (lane N) mice. Bone marrow megakaryocytes from a male offspring of founder 6 (D and E) or female offspring of founder 6 (H and I) or from a nontransgenic mouse (F and G) were FITC-labeled with a mouse monoclonal antibody to human c-Myc (E and G). The arrows point to megakaryocytes from the transgenic mouse, strongly staining with the antibody, and to megakaryocytes from the nontransgenic mouse, displaying a background staining. In order to distinguish between specific antibody reaction and nonspecific effects, we performed a competitive inhibition experiment with Peptide-1 which binds to c-Myc antibody (see "Materials and Methods"). The fluorescence in cells derived from nontransgenic or transgenic mice was eliminated when Myc antibody was first reacted with Peptide-1 (not shown), indicating that this antibody has some cross-reactivity with mouse c-Myc by immunohistochemistry. Original magnification: × 400.
megakaryocytes in cytospun bone marrow cells was higher in transgenic mice as compared to nontransgenic mice. The majority of acetylcholine esterase-positive cells in the transgenic mice or nontransgenic mice were identified morphologically as large mature megakaryocytes. The majority of PF4-positive cells in transgenic mice, but not in nontransgenic mice, appeared as small and immature (not shown). It should be pointed out, however, that cells expressing low levels of PF4, as typical of early megakaryocytes (Vinci et al., 1984), could have been missed by the immunohistochemistry method used. These results indicated that PF4-driven expression of Myc induced proliferation of cells which did not reach the differentiation stage at which acetylcholine esterase is expressed. The phenotype in homozygous offspring of founder 6 was similar to that observed in heterozygote mice. All of the bone marrow analyses data are from transgenic offspring of founder 6; however, similar data also were obtained with founder 20, e.g. female transgenic mice derived from founder 20 and aged matched nontransgenic female mice had an average of 81 ± 9 (n = 3) and 30 ± 4 (n = 5) megakaryocytes per sternum compartment, respectively (p < 0.005).

In order to test if the megakaryocyte accumulation observed in the transgenic mice was due to increased survival or production, we determined the frequency of megakaryocytes undergoing apoptosis. Using the TUNEL method (Surh and Sprent, 1994) to detect DNA fragmentation, the presence of apoptosis was confirmed in some megakaryocytes of female offspring of line 6. Examination of three slides of cytospun bone marrow cells derived either from nontransgenic or transgenic pregnant mice revealed that apoptosis of megakaryocytes was more frequent in the transgenic mice, i.e. 4/53 megakaryocytes in transgenic mice and 1/77 megakaryocytes in nontransgenic mice underwent apoptosis, in accordance with a previous report (Guy et al., 1996).

The Erythroid Lineage and Stem Cell Population Are Not Altered in Transgenic Mice—The 1.1-kb 5′-noncoding region of the PF4 gene, used in this study, is a tissue-specific promoter which directs expression of transgenes in early megakaryocytes of transgenic mice (Ravid et al., 1991a, 1993). Since PF4-driven expression of Myc in our current transgenic mice resulted in an increased frequency of immature marrow cells (Fig. 3), we examined whether this transgene affected the other hematopoietic lineages. We thus evaluated the percentage of erythrocytes, myelomonocytes, and stem cells in bone mar-
Increased megakaryocytes in the sternums of transgenic mice

The megakaryocytes were identified on the basis of size and morphological characteristics. Blood platelet levels, expressed as the average ± S.D. for the number of mice indicated in parentheses, was determined as described under “Materials and Methods.” The data on megakaryocyte number represent an average of the number of megakaryocytes per compartment in the sternum ± S.D. for five determinations, except for the injected males which represent two experiments. F, female mouse; M, male mouse. The statistical difference between the treatment groups was derived from t tests. The number of megakaryocytes in female transgenic mice, either pregnant or not, was significantly higher than the number in nontransgenic female mice (p < 0.0001). Estrogen-injected male transgenic mice displayed an augmented number of megakaryocytes compared with the uninjected transgenic male mice (p < 0.001). p values for the statistical comparisons of platelet count in the different corresponding sets of transgenic and nontransgenic mice indicated that the differences were not statistically significant.

Table I

| Mouse       | Sex | Condition | Number of megakaryocytes | Platelet level |
|-------------|-----|-----------|--------------------------|----------------|
| Nontransgenic| F   | Pregnant  | 30 ± 4                   | 1.1 ± 0.2 (11) |
| Transgenic  | F   | Pregnant  | 65 ± 6                   | 1.2 ± 0.2 (12) |
| Nontransgenic| F   | Injected  | 43 ± 10                  | 0.9 ± 0.1 (5)  |
| Transgenic  | F   | Injected  | 76 ± 3                   | 1.1 ± 0.3 (13) |
| Nontransgenic| M   | 32 ± 5    | 1.3 ± 0.1 (4)            |                |
| Transgenic  | M   | Injected  | 35 ± 3                   | 1.2 ± 0.2 (9)  |
| Nontransgenic| M   | Injected  | 39 ± 10                  | 1.0 ± 0.1 (5)  |
| Transgenic  | M   | Injected  | 61 ± 12                  | 1.2 ± 0.1 (4)  |

* Mice at 1–2 weeks of gestation when estrogen level is maximal (Matsumura et al., 1984).
* Males injected with estrogen every other day for 2 weeks, as detailed under “Materials and Methods.”

Table II

The percentage of cells expressing acetylcholine esterase and PF4 in bone marrow of transgenic mice

Bone marrow cells harvested from the femurs of a nontransgenic pregnant mouse or a transgenic pregnant mouse were cytocentrifuged on a slide and subjected to in situ staining for acetylcholine esterase or to immunohistochemistry with an antibody to PF4, all as described under “Materials and Methods.” The results represent an average of two experiments each with two determinations.

| Mouse   | Acetylcholine esterase-positive cells | PF4-positive cells |
|---------|-------------------------------------|-------------------|
| Nontransgenic | 0.03                              | 0.04              |
| Transgenic  | 0.10                               | 0.95              |

Fig. 4. The distribution of erythroid, myeloid, and stem cells are not altered in the transgenic mice. Bone marrow cells, harvested from the femurs of a nontransgenic female mouse (T) and transgenic female mouse (T), were stained with an antibody (TER-119) identifying erythroid cells (A) or an antibody (Mac1) identifying myelomonocytes (B) or with an antibody recognizing stem cell antigen (C), and subjected to flow cytometric analysis. The results presented are of one experiment representative of three experiments in which the average percentage ± S.D. of erythroid, myelomonocytic, and stem cells in the nontransgenic mice was: 28.5 ± 0.5, 29.0 ± 1.0, and 5.6 ± 0.4, respectively. The corresponding values for transgenic mice were: 30.0 ± 0.6, 26.5 ± 2.8, and 5.0 ± 0.1.

Row of transgenic mice by flow cytometric analyses, using antibodies recognizing lineage-specific markers. Antibodies to Mac-1 were used to identify macrophages and granulocytes (Springer et al., 1979), erythroid cells were identified with TER-119 antibody (Ogawa et al., 1991), and primitive stem cells were identified by an antibody recognizing stem cell antigen (Müller-Sieberg, 1991). As shown in Fig. 4, the number of erythroid cells, myelomonocytes, and stem cells was not changed significantly in the transgenic female mice, as compared to nontransgenic mice. Similar results were obtained with pregnant transgenic mice (not shown). These analyses indicated that Myc overexpression in cells committed to the megakaryocytic lineage did not derail lineage development.

Ploidy and Size Analyses of Megakaryocytes in Transgenic Mice—We sought to determine the effect of deregulated expression of Myc on endomitosis in megakaryocytes, by evaluating the changes in ploidy distribution of megakaryocytes after staining with propidium iodide and identification by megakaryocyte-specific expression of the epitope defined by the 4A5 monoclonal antibody (Burstein et al., 1992). Of most interest was that deregulated expression of myc oncogene in megakaryocytes of female transgenic mice was associated with an increased frequency of low ploidy megakaryocytes and a significant decrease in the fraction of high ploidy cells (≥32N). As also shown in Table III, pregnancy in nontransgenic mice was associated with an increase in high ploidy cells (≥32N cells), as was also observed in rats (Jackson et al., 1992). However, overexpression of estrogen-driven Myc in pregnant transgenic mice reduced the fraction of ≥32N megakaryocytes.

In accordance, the average size of megakaryocytes of all ploidy classes was smaller in the female transgenic mice, particularly in pregnant ones (Table III). Although the size of a normal megakaryocyte correlates with its ploidy state (reviewed by Mazur (1987)), the observed decrease in the average size of megakaryocytes in the female transgenic mice was not solely due to a decrease in ploidy. We also observed a decrease in the size of 16N cells, from 329 ± 13 (n = 5) units of forward-angle scattering in nontransgenic female mice to 294 ± 6 (n = 6) in transgenic female mice (p < 0.03). This flow cytometry analysis provided additional evidence that the frequency of 2N and 4N megakaryocytes was significantly higher in female transgenic mice as compared to nontransgenic ones (p = 0.04) (Table III).
Bone marrow cells were harvested from the femurs and tibias, megakaryocytes were fluorescein isothiocyanate-labeled with an antibody to mouse platelets and their DNA were stained with propidium iodide for determination of megakaryocyte frequency and DNA content by two-color flow cytometric analyses. The results represent averages ± S.E. for the number of mice indicated in parentheses. Megakaryocyte size was assessed by forward-angle light scatter and expressed as mean channel number of the forward-angle light scatter distributions from the flow cytometer. The statistical difference between the treatment groups was derived from $t$ tests. When comparing the size of megakaryocytes in nontransgenic versus transgenic mice, nonpregnant or pregnant, the differences were statistically significant ($p < 0.025$). Similarly, the mean ploidy (2N to 128N) in transgenic female mice was significantly lower than the mean ploidy in nontransgenic mice ($p < 0.014$). The frequency of 2N and 4N megakaryocytes was significantly higher in female transgenic mice as compared to nontransgenic ones ($p = 0.04$).

### DISCUSSION

**Myc-Estrogen Receptor Fusion Protein Affects the Hematopoietic System of Female Transgenic Mice**—During lineage development, pluripotent stem cells give rise to immature megakaryoblasts which are not easily differentiated from cells of the myeloid lineage. The platelet factor 4 gene is expressed during early stages of megakaryocyte development (Vinci et al., 1991a). We reported previously that the promoter region of the platelet factor 4 promoter directed tissue-specific expression of a reporter gene in transgenic mice (Ravid et al., 1991a). In the current work we linked this promoter to a fusion gene consisting of c-myc and the binding site of the glucocorticoid receptor (Picard et al., 1988; Eilers et al., 1989) and used this resulting gene fragment to produce transgenic mice. The mechanism by which the hormone binding region of the glucocorticoid receptor (Kumar et al., 1986) controls the activity of the fused Myc protein has been studied before in an in vitro system (Yamamoto, 1985). These studies indicated that the glucocorticoid receptor is phosphorylated and forms a complex with the heat shock protein hsp90 (Howard and Distelhorst, 1988). It was suggested that the ligand-free receptor binds to hsp90 which in turn inhibits the ability of Myc to bind to DNA (Picard et al., 1988). The myc-estrogen receptor gene was used in vitro under the control of retroviral vectors in order to immortalize fibroblasts (Eilers et al., 1989). In our study, we created an in vivo model of conditional activation of overexpressed Myc, by which the phenotype could be induced depending on estrogen levels. We have shown that female mice, but not males, developed a myeloproliferative disorder which was manifested by a hypercellular marrow. However, the proliferation of these immature cells, induced by targeted expression of Myc by the PF4 promoter, was accompanied by induction of acetylcholine esterase, uniquely expressed in the rodent and cat megakaryocyte lineage (Jackson, 1973; Lepore et al., 1984). This is consistent with early expression of the PF4 gene in immature megakaryocytes (Vinci et al., 1984) prior to activation of the gene encoding acetylcholine esterase. All of the above described phenotypes seemed qualitatively similar in all transgenic lines we produced.

The Profile of Erythroid, Myeloid, and Stem Cell Lineage in the Transgenic Mice—The finding that hematopoietic cells with deregulated expression of Myc contained abundant immature myeloid cells, raised the question whether this oncogene induced more immature progenitors to develop in the macrophage, granulocyte, or erythroid lineages. Our flow cytometric analyses of bone marrow cells from transgenic mice indicated no significant change in the number of cells committed to these lineages as compared to normal mice. This suggested that PF4 promoter-gene targeting selected for cells committed to the megakaryocytic lineage, and, once committed, the cells did not express markers of other lineages.

**The Number of Megakaryocytes and Ploidy Distribution Is Altered in Transgenic Mice Overexpressing Estrogen-driven c-Myc**—Overexpression of c-Myc causes a variety of cell lines to enter the G$_1$/S phase and proliferate (reviewed by Kelly and Siebenlist (1986)). Inducible c-Myc activity in a Myc-estrogen receptor chimeric in vitro system increases cell proliferation, and the levels of the G$_1$ phase- and S phase-promoting cyclins, cyclins E and A, respectively (Hoang et al., 1994), and a transient increase in cyclin D1 (Daksis et al., 1994). Since the megakaryocytic cell cycle contains both the G$_1$ and S phases (Wang et al., 1995; Odell et al., 1968), we investigated whether overexpression of c-Myc in early megakaryocytes might have an influence on the number and ploidy distribution of cells committed to this lineage. We noted that female transgenic mice expressing myc-estrogen receptor fusion gene driven by the PF4 promoter, displayed increased megakaryopoiesis. Megakaryocyte accumulation was observed in the female transgenic mice despite the increased number of megakaryocytes undergoing apoptosis, suggesting that c-Myc accelerated megakaryocyte production. Overexpression of c-Myc was previously connected to induction of programmed cell death in other cell types (Evan et al., 1992).

Ploidy analyses indicated that deregulated expression of myc was associated with an increase in the ratio of 2N-8N to 16N-64N cells as well as a decrease in the average size of megakaryocytes. These results indicated that c-Myc overexpression accelerated the proliferation of megakaryocyte precursors and decreased the fraction of polyploid cells, resulting in a left-shifted ploidy profile. Since Myc accelerates entry into S phase in many cell types (Cory, 1986), and since both the mitotic cell cycle and endomitotic cell cycle include a round of DNA synthesis, one would not necessarily expect that megakaryocyte proliferation would be increased at the expense of polyploidization. This ploidy profile could, however, result if each megakaryocyte precursor had the potential for only a limited number of DNA replication cycles, as suggested by Arriaga et al. (1987). These authors found that high concentrations of a aplastic serum increased proliferation of megakaryocyte precursors which differentiated into low ploidy megakaryocytes. The augmentation in the number of megakaryocytes in our female transgenic mice, recognized morphologically by staining for acetylcholine esterase activity, was not proportional to the increase in the number of immature bone marrow cells expressing the PF4 protein. This further suggested that the majority of these proliferating immature
cells, although committed to the megakaryocytic lineage, were deleted from the pool of cells undergoing terminal differentiation and polyploidization.

The significant increase in the number of megakaryocytes in transgenic mice overexpressing an active c-Myc was accompanied by only a minute change in platelet count. This could be due to the fact that platelets fragment primarily from mature megakaryocytes characterized by high ploidy (reviewed by Mazur (1987)), while c-Myc overexpression caused a decrease in size and ploidy of megakaryocytes. Alternatively, Myc overexpression may produce some degree of ineffective thrombopoiesis. In a recent study by Guy et al. (1996), the PF4 promoter was used to drive the expression of the transcription factor E2F-1 specifically to megakaryocytes of transgenic mice. In this case, and in contrast to our Myc transgenic mice, increased megakaryopoiesis was accompanied by thrombocytopenia. Since Myc is activated by E2F-1 (Mudryj et al., 1990), it is possible that the common phenotypes in the Myc-ER and E2F-1 transgenic mice overexpressing an active c-Myc was accompanied by thrombocytopenia. This could be due to elevated c-Myc. E2F-1, however, must also act on a set of genes which are not affected by c-Myc and which are required for platelet fragmentation. The notion of a transcription factor-dependent regulation of platelet fragmentation was previously presented in mice engineered to lack the expression of NF-E2 (Shvidrasani et al., 1995). These latter mice displayed absolute thrombocytopenia despite the presence of megakaryocytes of high ploidy class. The analyses of our Myc transgenic mice as well as the E2F-1 transgenic and NF-E2 knockout mice, indicate that megakaryocyte polyploidization and platelet fragmentation are regulated by separate mechanisms.

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REFERENCES

Amin, C., Wagner, A. J., and Hay, N. (1993) Mol. Cell. Biol. 13, 383–389
Blow, J. J. and Nurse, P. (1990) Cell 62, 855–862
Burstein, S. A., Friess, P., Downs, T., and Mei, R.-L. (1992) Exp. Hematol. 20, 1170–1177
Classon, M., Henriksson, M., Sunegi, J., Klien, G., and Hammarkjold, M. L. (1987) Nature 320, 272–274
Cole, M. D. (1986) Annu. Rev. Genet. 20, 361–384
Cory, S. (1986) Adv. Cancer Res. 47, 189–234
Cross, F., Roberts, H., and Weintraub, H. (1989) Annu. Rev. Cell Biol. 5, 341–349
Dalziel, J. J., Lu, R. Y., Facchin, L. M., Martin, W. W., and Penn, L. J. Z. (1984) Oncogene 9, 3635–3645
Dorn, G. W., Davis, M. G., and D'Angelo, A. D. (1994) Am. J. Physiol. 266, C1231–C1239
Eckhardt, S. G., Dai, A., Davidson K. K., Forseth, B. J., Wahl, G. M., and Von Hoff, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6674–6678
Eilers, M., Picard, D., Yamamoto, K., and Bishop, M. J. (1989) Nature 330, 66–68
Evan, G., Wylie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992) Cell 99, 119–128
Gewirtz, A. M., and Sherr, Y. M. (1990) Exp. Hematol. 18, 945–952
Gould, K. L., Moreno, S., Owen, D. J., Sazer, S., and Nurse, P. (1991) EMBO J. 10, 3287–3292
Guy, C. T., Zhou, W., Kaufman, S., and Robinson, M. O. (1996) Mol. Cell. Biol. 16, 685–693
Huang, A. T., Cohen, K. Y., Barnett, J. P., Bergstrom, D. A., and Dang, C. V. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6875–6879
Howard, K. J., and Distelhorst, C. W. (1988) J. Biol. Chem. 263, 3474–3481
Jackson, C. W. (1986) Blood 68, 768–778
Jackson, C. W., Steward, S. A., Ashmun, R. A., and McDonald T. P. (1992) Blood 79, 1672–1678
Kelly, K., and Siebenlist, U. (1986) Annu. Rev. Immunol. 4, 317–338
Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. D., Franza, B. A., and Roberts, J. M. (1992) Science 257, 189–1893
Kumar, V., Green, S., Staub, A., and Chambon, P. (1986) EMBO J. 5, 2231–2236
Kuter, D. J., Greenberg, S. M., and Rosenberg, R. D. (1989) Blood 74, 1852–1857
Lepore, D. A., Harris, H. A., and Penington, D. G. (1984) Br. J. Haematol. 58, 473–481
Marcu, K. B., Bossone, S. A., and Patel, A. J. (1992) Annu. Rev. Biochem. 61, 809–860
Matsushima, H., Rousell, M. F., Ashmun, R. A., and Sherr, C. J. (1991) Cell 65, 701–713
Matsushima, H., Quelle, D. E., Shultzle, S. A., Shihuya, M., Sherr, C. J., and Kato, J. (1994) Mol. Cell. Biol. 14, 2066–2076
Mazur, E. M. (1987) Exp. Hematol. 15, 340–350
Mudryj, M., Hiesbert, S. W., and Nevins, R. J. (1990) EMBO J. 9, 2179–2184
Muller-Sieberg, C. (1991) J. Exp. Med. 174, 161–168
Nurse, P. (1990) Nature 344, 503–505
Odell, T. T., Jackson, C. W., Jr., and Reiter, R. S. (1988) Exp. Cell Res. 35, 321–328
Ogawa, M., Matsuzaki, T., Nishikawa, S., Hayash, S.-J., Konnada, T., Sode, T., Kira, T., Nakashiki, H., and Nishikawa, S.-I. (1993) J. Exp. Med. 174, 63–71
Picard, D., Salser, S. J., and Yamamoto, K. R. (1988) Cell 54, 1073–1080
Pines, J., and Hunter, T. (1987) EMBO J. 6, 2987–2991
Ravid, K., Beeler, D. L., Rabin, M. S., Ruley, H. E., and Rosenberg, R. D. (1991a) Proc. Natl. Acad. Sci. U. S. A. 88, 1521–1525
Ravid, K., Dai, T., Beeler, D. L., Kuter, D. J., and Rosenberg, R. D. (1991b) Mol. Cell. Biol. 11, 6116–6122
Ravid, K., Li, Y., Rayburn, H. B., and Rosenberg, R. D. (1993) J. Cell Biol. 123, 1545–1553
Rustgi, A., Dyson, N., and Bernarde, R. (1991) Nature 352, 541–544
Selden, R. F., Howie, K. B., Row, M. E., Goodman, H. M., and Moore, D. D. (1986) Mol. Cell. Biol. 6, 3173–3179
Shvidrasani, R. A., Rosenblat, M. F., Zucker-Franklin, D., Jackson, C. W., Hunt, P., Baris, C. J. M., and Orkin, S. H. (1995) Cell 81, 695–704
Springer, T., Galfre, G., Secher, D. J., and Milstein, C. (1979) Eur. J. Immunol. 9, 301–306
Surh, C. D., and Sprent, J. (1994) Nature 372, 100–103
Tao, L. H., Harlow, E., and Meyerson, M. (1991) Nature 353, 174–177
Vinci, G., Tabilio, A., Deschamps, J. F., Van Haecke, D., Henri, A., Guihard, J., Tetteroo, P., Lansdorp, P. M., Herecnd, T., Vainchenker, W., and Breton-Geirou, J. (1984) Br. J. Hematol. 56, 589–595
Wang, Z., Zhang, Y., Kanen, D., Lees, E., and Ravid, K. (1995) Blood 86, 3783–3788
Yamamoto, K. R. (1985) Annu. Rev. Genet. 19, 209–252
Zhang, Y., Wang, Z., and Ravid, K. (1996) J. Biol. Chem. 271, 4266–4272
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