STUDIES OF THE PU.1 TRANSCRIPTION FACTOR AND ITS ROLE IN FRIEND VIRAL ERYTHROLEUKEMOGENESIS

by

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ABSTRACT

Friend virus causes progressive erythroleukemia in susceptible mice. Friend erythroleukemogenesis is a multistep process that requires virally and cellularly encoded proteins. The replication-defective viral component, the spleen focus-forming virus (SFFV), encodes a membrane glycoprotein (gp55) that associates with erythropoietin receptors to cause proliferation and differentiation of infected erythroblasts. Most of these infected erythroblasts retain their commitment to terminally differentiate and have limited self-renewal capabilities. However, *in vivo* studies of Friend virus-induced disease have suggested that SFFV proviral integrations adjacent to the gene for the *ets*-related transcription factor PU.1 inhibit the commitment of erythroblasts to terminally differentiate and cause their immortality as indicated by a capability for indefinite transplantation (C. Spiro et al., J. Virol. **62**:4129-4135, 1988; R. Paul et al., J. Virol. **65**:464-467, 1991). To test the implications of these results, we produced polyclonal antiserum to bacterially synthesized PU.1, and we used it to analyze PU.1 expression throughout leukemic progression and during chemically induced differentiation of Friend erythroleukemia (F-MEL) cell lines. We also identified and studied biochemical modifications of PU.1. Although PU.1 proteins are abundant in F-MEL cells, they are absent or present in only trace amounts in normal erythroblasts and in differentiating erythroblasts from the preleukemic stage of Friend disease. Furthermore, chemicals (e.g. dimethyl sulfoxide and *N,N'*-hexamethylenebisacetamide) that overcome the blocked differentiation of F-MEL cells induced rapid declines of PU.1 mRNA and PU.1 proteins. The elimination of PU.1 proteins coincided with recommitment to the program of erythroid differentiation and with loss of immortality. To further characterize the specific cellular effects of ectopic PU.1 expression, PU.1 cDNA was ligated into a spleen focus-forming retroviral vector which was used to obtain infectious virus from cultured cells. Infection of fibroblasts with PU.1-encoding retrovirus resulted in PU.1 synthesis

followed by cell death. In contrast, infection of long-term bone marrow cultures caused specific immortalization of erythroblasts. The resultant cell lines contained PU.1, were morphologically mononuclear blasts, required erythropoietin and bone marrow stromal cells for survival and continuous proliferation, and spontaneously differentiated at low frequency to synthesize hemoglobin. Treatment of the immortalized erythroblast cell cultures with 1% dimethyl sulfoxide significantly increased the frequency of terminal erythroid differentiation. These results support the hypothesis that PU.1 interferes with the commitment of erythroblasts to differentiate. We propose that PU.1 perturbs the pathway(s) that control potential for indefinite proliferation, with opposite effects on fibroblasts (killing) and erythroblasts (immortalization).

I. INTRODUCTION

A. Overview of pathogenesis of leukemia.

All mature cellular elements of blood and lymph, with the exception of certain memory T and B cells, are short-lived and need to be constantly replenished. Hematopoiesis is the process whereby a continuous supply of blood cells are produced and maintained. Mature blood cells are ultimately derived from pluripotent precursor stem cells which have capacities for self-renewal (proliferation) and differentiation into all eight of the specific blood cell lineages (e.g. T lymphocytes; B lymphocytes; basophil and mast cells; monocytes and macrophages; megakaryocytes; neutrophilic granulocytes; eosinophils and erythrocytes; see figure 1) (reviewed in Dexter and Spooncer, 1987). Proliferation and differentiation of hematopoietic cells are complex processes involving cell-cell interactions and soluble growth factors and require a tightly regulated system to balance production of functional, mature cells to the needs of the organism (recently reviewed in Dexter et al., 1990). Different humoral factors in the appropriate environments can direct differentiation of pluripotent cells into distinct progenitors capable of maturation along restricted developmental pathways (Heyworth et al., 1990). The transition from a pluripotent state (potential to form different types of cells) to a unipotent state (single-lineage restricted capacity for development) is termed determination or commitment. As hematopoietic cells develop through sequential steps, the capability for self-renewal or proliferation progressively diminishes such that mature elements are incapable of replication. A single uncommitted stem cell, on the other hand, can entirely repopulate the radiation-ablated marrow of a mouse and maintain hematopoiesis over the life-time of an animal (Snodgrass and Keller, 1987; Jordan and Lemischka, 1990). Cellular amplification, which is key to hematopoietic reconstitution,

occurs as a result of proliferation of stem cells followed by commitment of progenitor cells to specific lineages and their subsequent development into mature components. Leukemia, simply viewed, is a consequence of the loss of coupled and regulated growth and differentiation control mechanisms of hematopoiesis (Sachs, 1978).

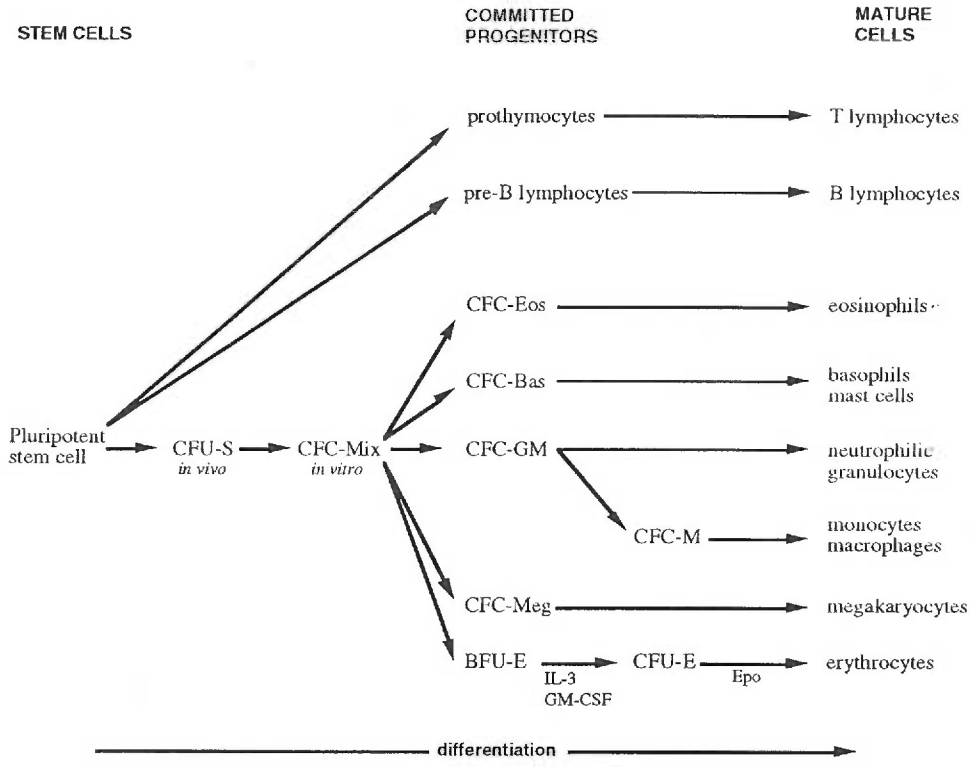


Figure 1. Schematic of hematopoiesis. The stem cell compartment contains pluripotent cells (bone marrow reconstituting cells), spleen colony-forming units (CFU-S) and mixed colony-forming cells (CFC-Mix). The lineage committed progenitors include eosinophil colony-forming cells (CFC-Eos), basophil colony-forming cells (CFC-Bas), granulocyte-macrophage colony-forming cells (CFC-GM), megakaryocyte colony-forming cells (CFC-Meg), and erythroid burst-forming units (BFU-E). The monocyte/macrophage colony-forming cells (CFC-M) and erythroid colony-forming units (CFU-E) are derived from earlier progenitors and exhibit less self-renewal capabilities than their earlier

progenitors. Interleukin-3 and granulocyte/ macrophage colony stimulating factor both stimulate proliferation and differentiation of early BFU-Es. Erythropoietin stimulates proliferation and differentiation of mature BFU-Es and CFU-Es. Self-renewal capacity declines progressively as cells become lineage restricted and differentiate. (Schematic fashioned after Dexter et al., 1990).

Leukemias are hematopoietic neoplasias that have been defined as 1) uncontrolled proliferation of hematopoietic cells that 2) do not retain the commitment to differentiate to mature functional blood cells (Sawyers et al., 1991). Both aspects of impaired cellular function are important because certain hematologic disorders display only one attribute of the full leukemic phenotype. Growth expansion is characteristic of myeloproliferative diseases such as polycythemia rubra vera or chronic myelogenous leukemia, and a block to differentiation appears to occur in myelodysplastic syndrome (Sawyers et al., 1991). However, many of these chronic disorders can progress to acute leukemia. Thus, these observations suggest that leukemogenesis is progressive and involves multiple steps or molecular alterations. Early studies of Friend disease helped define the multistage model of leukemogenesis (Wendling et al., 1981), and recent work has partially elucidated the molecular mechanisms responsible for transformation to a leukemic phenotype. Numerous examples of leukemias resulting from multiple distinct genetic changes have been found. The murine myelomonocytic leukemia WEHI-3B was caused by constitutive coexpression of the growth-promoting cytokine interleukin-3 and the homeobox Hox-2.4 protein (Ymer et al., 1985; Blatt et al., 1988; and Perkins et al., 1990), and other murine myeloid leukemias can be induced by expression of the *Evi*-1 zinc finger protein and the constitutively activated G-CSF receptor encoded by v-*fms* (Ihle and Askew, 1989). Activation of the G-CSF receptor stimulates proliferation and differentiation of granuolcyte precursors while *Evi*-1 blocks G-CSF induced differentiation (Morishita et al., 1992). The occurrence of a highly leukemogenic avian

retrovirus that contains two oncogenes (one with growth-promoting potential and one able to block normal differentiation) (Graf et al., 1978) further supports the multistep model of leukemogenesis. Avian erythroleukemia induced by the avian erythroblastosis virus is due to expression of v-*erb* B, which encodes an epidermal growth factor receptor homologue that mitogenically stimulates cells (Ullrich et al, 1984) and v-*erb* A, which encodes a mutant form of the thyroid hormone receptor and is able to block differentiation of erythrocytic cells (Gandrillon et al., 1989).

B. Initial description of Friend and Rauscher viral diseases.

In 1957, Charlotte Friend described a hematologic disease in adult Swiss mice that resembled leukemia and was caused by a cell-free filterable agent (Friend, 1957). The etiologic agent, which was later identified as a murine retroviral complex that contains a replication-competent helper and a replication-defective component, induced a progessive proliferative disease of the hematopoietic system when serially innoculated into mice. Grossly enlarged spleens could be detected within 2 to 3 weeks after injection of normal mice with a filtered spleen homogenate from a diseased animal. Infected mice, however, remained otherwise healthly in appearance until shortly before death 7 to 8 weeks later. In the terminal stage of the disease, marked proliferation of mononuclear blast-like cells resulted in invasion of spleens, livers, bone marrows, lungs and kidneys. Consequently, mice had elevated numbers of cirulating nucleated cells, were anemic and had hepatosplenomegaly (enlarged liver and spleen). Death was frequently due to intraperitoneal hemorrhage following rupture of the grossly swollen spleen or liver. This virally-induced, acutely progressive disease has been termed Friend disease.

Subsequently, Frank Rauscher identified and isolated a unique virus capable of inducing hematologic symptoms characteristic of Friend disease in BALB/c mice (Rauscher, 1962). Rauscher's isolate, like Friend virus, initially caused extreme enlargement of the spleen due to erythroblastosis. Later in the course of the disease,

leukemia emerged which was associated with anemia and the spleen, liver, thymus, lymph nodes and peripheral blood became infiltrated with large, basophilic mononuclear cells. Rauscher's and Friend's studies provided early evidence that leukemia in mice could be caused by viruses and furnished related models for the study of leukemogenesis.

C. General description of Friend and Rauscher leukemia viruses.

1. Friend and Rauscher leukemia viruses are retroviral complexes of a helper MuLV and a replication-defective spleen focus-forming virus (SFFV).

Different Friend and Rauscher viruses are very similar in structure and function. These viruses are complexes of a replication-competent murine leukemia virus (MuLV) and a replication-defective spleen focus-forming virus (SFFV) (Axelrad and Steeves, 1964; Pluznik and Sachs, 1964). The MuLV genome encodes the three essential genes, *gag*, *pol* and *env*, needed for successful retroviral replication within an infected cell; *gag* encodes the structural viral core proteins and the viral protease, *pol* encodes retroviral-specific enzymes required for reverse transcription of the viral single-stranded genomic RNA into double-stranded DNA and for integration of the DNA into the host cell's genome, and *env* encodes the viral envelope glycoproteins that mediate attachment of virions to cells via specific cell-surface receptors (reviewed in Coffin, 1982). Replication-competent retroviruses such as MuLV can act as helper viruses for packaging of replication-defective retroviral RNA genomes into functional virions. Thus, the replication-defective SFFV can be propagated in the presence of MuLV. The pathology of Friend disease has been principally attributed to the SFFV component because helper-free preparations of SFFV induce both the early erythroblastosis and the late erythroleukemia that are the essential characteristics of Friend disease (Wolff and Ruscetti, 1985; Wolff et al., 1986; Spiro et al., 1988a; also see below). MuLV alone does not cause Friend disease in adult mice. The molecular structure and pathologic functions of SFFV are described below (see section E).

2. Different strains of Friend SFFV exist.

Although the initial Friend and Rauscher virus isolates caused anemia (see above), several variants of Friend virus were subsequently isolated that cause an erythroleukemia associated with polycythemia (Mirand et al., 1961; Sassa et al., 1968; Mirand et al., 1968). These strain differences are caused by differences in the SFFV components (Ruscetti and Wolff, 1985; Chung et al., 1987), which are therefore called $SFFV_A$ for the anemia-inducing strains and $SFFV_P$ for the polycythemia-inducing strains (Mirand et al., 1968). As described in detail below, essential aspects of viral pathogenesis are nearly identical for the $SFFV_A$ and $SFFV_P$ variant strains. Basically, they both stimulate erythropoietin-independent proliferation and differentiation of infected erythroblasts. In the case of $SFFV_P$, differentiation proceeds to completion yielding reticulocytes and erythrocytes that enter the blood. On the other hand, the process of differentiation is aberrant in erythroblasts infected with $SFFV_A$, and it does not proceed to completion (Hankins and Troxler, 1980). This results in "ineffective erythropoiesis" with premature destruction of abnormal cells in the bone marrow and attendant anemia (Kabat, 1989). It is believed that both $SFFV_A$ and $SFFV_P$ strains cause disease by nearly identical mechanisms and that the pathogenic differences are secondary consequences of minor differences in viral gene products (see Kabat, 1989). Because these strain differences are probably not fundamental to the basic mechanisms of SFFV-induced erythroleukemogenesis, they will not be considered further. The subequent review concerns primarily the polycythemia-inducing strains of Friend virus, and all of my research was done using a molecularly cloned $SFFV_P$ strain of Lilly and Steeves (Linemeyer et al., 1980; Lilly and Steeves, 1973).

D. The multistage nature of Friend erythroleukemia.

1. Natural history of Friend disease: early events.

Infection of mice with the Friend viral complex causes an acutely progessive disease that primarily affects erythropoiesis (reviewed in Teich et al, 1982 and Kabat, 1989). *In vivo* and *in vitro* studies have strongly suggested that the pathogenic target cells in Friend disease are erythropoietin-responsive mature erythroid burst-forming units (mBFU-E) and erythroid colony-forming units (CFU-E) (see fig. 1) (Kost et al., 1981; Tambourin and Wendling, 1971).

As early as 30-40 hours after infection with SFFV$_P$, hyper-basophilic erythroid cells can be detected in the bone marrow and spleen (Tambourin and Wendling, 1971; Wendling et al., 1981). Extensive proliferation of these cells results in an exponential increase in the weight of the spleen starting about 30 hours after infection (Tambourin and Wendling, 1971), and within 9-10 days postinfection, macroscopic foci of proliferating erythroid cells in the spleen are clearly evident (Axelrod and Steeves, 1964). Reticulocytosis and polycythemia accompany splenic enlargement and result from erythroblastic maturation (differentiation) of hyperbasophilic cells, even in the absence of normal physiologic levels of erythropoietin (Tambourin and Wendling, 1971). Proliferation and differentiation of normal erythroid precursors is stimulated by and requires erythropoietin; on the contrary, erythroid cells that are infected by the polycythemia strain of Friend virus can proliferate and differentiate into erythrocytes *in vitro* in the absence of erythropoietin (Liao and Axelrod, 1975). Normal proliferating erythroblasts are committed to differentiate and have limited self-renewal capabilities. During the early course of Friend disease, SFFV$_P$-infected proliferating erythroid cells retain their commitment to terminally differentiate and are not transplantable to irradiated hosts suggesting that they have, like normal erythroblasts, a limited capacity for self-renewal and are not immortal (Wendling et al., 1981). Therefore, SFFV$_P$ acts as an erythropoietin mimic and stimulates proliferation of erythroblasts but does not abrogate their commitment to differentiate. Accordingly, in the majority of mice infected with helper-free preparations of the replication-defective SFFV$_P$, an initial erythroblastosis ,

that is due to polyclonal proliferation of infected erythroblasts, is followed by a wave of erythroid differentiation and recovery (Bestwick et al., 1985; Spiro et al., 1988a; Wolff et al., 1986).

2. Natural history of Friend disease: Late events.

Four to eight weeks after infection with Friend virus, tumorigenic cells resembling proerythroblasts begin accumulating in the spleen and are later found in the liver, bone marrow and peripheral blood (Friend and Haddad, 1960; Wendling et al., 1981). These cells can be transplanted to irradiated mice, and can be occasionally transplanted to nonirradiated mice. At 4 weeks postinfection with FV-P and 8 weeks postinfection with FV-A, cells can be isolated from spleens of infected mice that will establish colonies in methylcellulose in the absence of exogenous growth factors (Mager et al., 1981). These colonies are composed of erythroid cells that morphologically resemble tumorigenic proerythroblasts (Friend erythroleukemia cells), do not differentiate, and are transplantable to irradiated syngeneic mice. Careful study of spleens from Friend virus infected mice demonstrated that Friend erythroleukemia cells first appear in localized segments (Wendling et al., 1981). This finding suggested that emergence of erythroleukemia was due to an infrequent, selective malignant transformation event and potentially explained the lengthy delay between the early virus-induced erythroblastosis and the emergence of tumorigenic cells (Wendling et al., 1981). Subsequent studies using helper-free "tagged" SFFV have shown that the erythroleukemia stage is due to clonal outgrowth of a single infected cell that has apparently aquired growth characteristics not present in the vast majority of other infected cells (Spiro et al., 1988a). These Friend erythroleukemia cells are considered to be immortal based on their transplantability and their ability to establish permanent cell lines *in vitro*. Furthermore, they appear to be blocked in their ability to differentiate spontaneously or in the presence of erythropoietin. However, this impediment is not absolute since low concentrations of certain chemicals such as dimethyl sulfoxide

reinstate the committment to terminally differentiate, resulting in synthesis of hemoglobin and other erythroid-specific proteins (Friend et al., 1971; Marks and Rifkind, 1978). Chemically induced differentiation of Friend cells also correlates with loss of tumorigenicity (Friend et al., 1971). Thus, the later erythroleukemic stage of Friend disease is characterized by a predominance of primitive erythroblasts of clonal origin that have a greatly enhanced self-renewal capability and an abrogated commitment to differentiate but not a loss of the ability to terminally differentiate.

Interestingly, studies indicate that progression of the leukemic phenotype continues in the population of immortal proerythroblasts. Friend erythroleukemia cells isolated from spleens are incapable of direct growth as permanent cell lines in *in vitro* cultures and often require successive passage subcutaneously or intraperitoneally before suspension cell lines can be grown (Wendling et al., 1981; Spiro et al., 1988a). This suggests that further phenotypic change is necessary to give rise to *in vitro* cell lines. Genetically anemic *Sl /Sl*^{d} mice have a mutational loss of the Kit ligand cytokine which supports stem cell growth and erythroid precursor proliferation and differentiation (Huang et al., 1990). *Sl /Sl*^{d} mice are resistent to pathogenic effects of Friend virus and cannot support the growth of injected spleen colony forming units (CFU-S) or SFFV-infected proliferating erythroblasts from the early stage of Friend disease (Mager et al., 1980). However, 10 weeks or more after infection with FV-P, a small number of cells from spleens full of tumorigenic Friend cells can establish colonies in the spleens of irradiated *Sl /Sl*^{d} mice. Cells competent for growth in *Sl /Sl*^{d} mice arise substantially later than the emergence of Friend erythroleukemia cells at 4 weeks after infection. Erythroleukemic cells from the colonies in *Sl /Sl*^{d} mice are easily culturable *in vitro* (Mager et al., 1980) which strongly suggests that further progression of Friend disease is accompanied by autonomy from a specific cellular regulatory factor in the hematopoietic microenvironment.

3. Studies of Friend disease in long-term bone marrow culture.

In vitro analysis of the effects of Friend viral complex on hematopoiesis have substantially aided our understanding of Friend disease because the growth and differentiation characteristics of various hematopoietic lineages can be directly observed in long-term bone marrow cultures. In long-term bone marrow cultures, adherent bone marrow stroma consisting of endothelial cells, adipocytes, fibroblastic cells and macrophages form an appropriate environment for stem cell and precursor cell proliferation and differentiation (Dexter et al., 1977; Allen and Dexter, 1976). Erythropoiesis, in bone marrow culture, is induced and maintained by the the addition of erythropoietin (Eliason et al., 1979). When erythropoietically active bone marrow cultures were infected with FV-P, a progressive disease similar to Friend disease *in vivo* was observed (Dexter et al., 1981). One week after infection, an enormous number of CFU-Es were present, and the majority of these cells spontaneously differentiated in CFU-E assays in the absence of exogenous erythropoietin. In contrast, control cultures contained a thousand-fold fewer CFU-Es, and less than 10 percent of these differentiated in the absence of erythropoietin. The CFU-Es in the infected cultures produced virus that could transmit Friend disease when injected into mice Thus, erythroid progenitors appeared to aquire an independence of erythropoietin for growth and differentiation as a result of FV-P infection. Similar erythropoietin-independent erythropoiesis in erythroid burst colony-forming assays was observed following infection of bone marrow with either FV-P (Hankins et al., 1978) or SFFV (Hankins and Troxler, 1980).

After several more weeks, an emergence of morphologically more primitive cells in close association with the stromal layer was detected (Dexter et al., 1981). These cells were unable to undergo terminal differentiation, either in the presence or absence of erythropoietin, and produced tumors when injected into irradiated recipients. However, these tumorigenic cells rapidly died when they were removed from bone marrow stroma and passaged *in vitro* or cultured in soft agar. While the cells were able to grow in the absence of erythropoietin, they required a hematopoietic stromal cell environment for

survival. Following passage onto uninfected bone marrow stroma, a small number of cells eventually arose that grew in soft agar and established suspension cell cultures that resembled Friend erythroleukemia cell lines. These stroma-independent cells produced SFFV, were transplantable and differentiated when cultured in the presence of dimethyl sulfoxide. Alterations in growth and differentiation of other hematopoietic lineages following infection of bone marrow cultures with FV-P have not been consistently observed and can be attributed mostly to the MuLV helper virus component (Boettinger and Dexter, 1984). In summary, results from *in vitro* studies of Friend disease support conclusions drawn from analysis of Friend disease in mice and suggest that Friend erythroleukemogenesis is a multifactorial process involving growth factor independent proliferation, sustained self-renewal capacity and/or a block to differentiation, and growth independent of the hematopoietic microenvironment. The changes appear to be cumulative and to result from distinct molecular mechanisms (see below).

E. Molecular mechanisms of Friend pathogenesis.

1. The SFFV genome lacks a classical oncogene.

Helper-free SFFV was first cloned by limiting dilution into fibroblasts (Troxler et al., 1977c; Bernstein et al., 1977). SFFVs differ from all other retroviruses that cause rapidly developing neoplasms in that they lack classical, transduced oncogenes of the sort typified by the Rous sarcoma virus (Stehelin et al., 1976) and contain only retroviral-specific nucleic acid sequences that are related to *gag*, *pol* and *env* sequences of MuLVs. Isolates of Friend SFFV and Rauscher SFFV share highly homologous *env* genes and long terminal repeats (LTRs) but differ substantially in their *gag* and *pol* regions (reviewed in Teich et al., 1982; Ruscetti and Wolff, 1984; and recently reviewed in Kabat, 1989). The *gag* and *pol* regions of Friend SFFVs frequently contain deletions or rearrangements and only occasionally encode functional proteins (Teich et al, 1982; Clark and Mak, 1984; Ostertag et al., 1987; Spiro et al., 1988b). The above findings and other

mutational studies suggested that the *gag* and *pol* regions are nonessential for pathogenesis. Wolff and Ruscetti (1988) demonstrated that the *env* gene from $SFFV_A$, in the absence of other SFFV sequences, induced Friend disease in mice and transformed erythroid cells *in vitro*.

Replication competent MuLVs occur in four major different host-range classes called ecotropic, amphotropic, xenotropic and dualtropic. These MuLV classes use different host-cell receptors and have correspondingly distinct *env* genes. Thus, each class of *env* gene specifies a distinct glycoprotein envelope on the virion surface which binds to a corresponding receptor. Although mice inherit complete, potentially functional copies of ecotropic, amphotropic and xenotropic MuLVs that are expressed *in vivo*, the dualtropic *env* genes occur scattered throughout the murine genome as bits and pieces that do not occur as complete replication-competent MuLV structures (Risser et al., 1983; Chattopadhyay et al., 1982). Rather, dualtropic MuLVs (called "MCFs") are formed *de novo* by recombination when ecotropic MuLVs replicate in mice (Famulari, 1983; Koch et al., 1984; Evans and Malik, 1987). Thus, the MCFs that have been isolated are actually ecotropic-dualtropic hybrids that contain recombinant *env* genes (Famulari, 1983; Koch et al., 1984). MCF *env* glycoproteins consist of 5' amino terminal domains that are dualtropic-specific and carboxyl termini that are ecotropic-specific (Famulari, 1983; Koch et al., 1984). MuLV *env* genes encode M_r 90,000 precursors (gPr90) that are processed by glycosylation and partial proteolysis to form gp70-p15E complexes that occur on surfaces of infected cells and in virions that bud from the cells (Famulari et al., 1976; Naso et al., 1976; Leamnson et al., 1977; Pinter and Fleissner, 1977; Pinter et al., 1978).

SFFVs are structurally related to the MCF class of replication competent MuLVs (Troxler et al., 1977a; Troxler et al., 1977b;Clark and Mak, 1984). As mentioned above, SFFVs generally contain large deletions in their *gag* and *pol* gene regions. The only conserved protein-encoding element of all SFFV isolates is their *env* gene. The SFFV

env gene could have been derived from an MCF *env* gene by internal deletion of a 585 bp sequence and a single nucleotide insertion within the ecotropic-related domain (see figure 2) (Clark and Mak, 1984; reviewed in Kabat,1989; and Ruscetti and Wolff, 1984). The single base insertion results in a translational frameshift and premature termination of the polypeptide chain with loss of 33 amino acids at the carboxyl terminus. The internal deletion and single base insertion are required for pathogenic activity of SFFV (Amanuma et al., 1989; Watanabe et al., 1991). The SFFVs all encode Env-related glycoproteins with apparent M_r 55,000, called "gp55" (Racevskis and Koch, 1978; Dresler et al., 1979; Ruscetti et al., 1979; Ruta and Kabat, 1980). The $SFFV_A$ and $SFFV_P$ strains encode slightly different gp55 glycoproteins due to small differences in the carboxy terminal sequences (Ruscetti and Wolff, 1984; reviewed in Kabat, 1989). gp55 glycoproteins are not incorporated into budding virions (Ruta and Kabat, 1980). Substantial evidence suggests that gp55 causes specific mitogenic activation of erythroblasts, as described below.

2. SFFV Env protein (gp55) is an erythroblast mitogen.

Much evidence has accumulated that implicates the SFFV Env protein, gp55, as the factor responsible for erythroblastosis of Friend disease. Initial comparisons of pathogenic Friend and Rauscher SFFV variants revealed that the sequences with the highest degree of homology were in the *env* region and that the only consistently expressed protein was gp55 (reviewed in Ruscetti and Wolff, 1984). Furthermore, the only region unique to the pathogenic Rauscher SFFV compared to the non-pathogenic (for Friend disease) parent Rauscher MuLV is the *env* region encoding a gp55 protein which is highly related to Friend gp55 (Ruta and Kabat, 1980; Bestwick et al., 1983). Helper-virus-rescued sub-genomic fragments of $SFFV_A$ and $SFFV_P$ that retain only the long terminal repeats and the *env* gene induce formation of erythroid bursts in colony-forming assays and Friend disease *in vivo* (Kaminchik et al., 1982; Linemeyer et al., 1981; Ruscetti and Wolff, 1984). Moreover, expression of gp55 in the absence of other

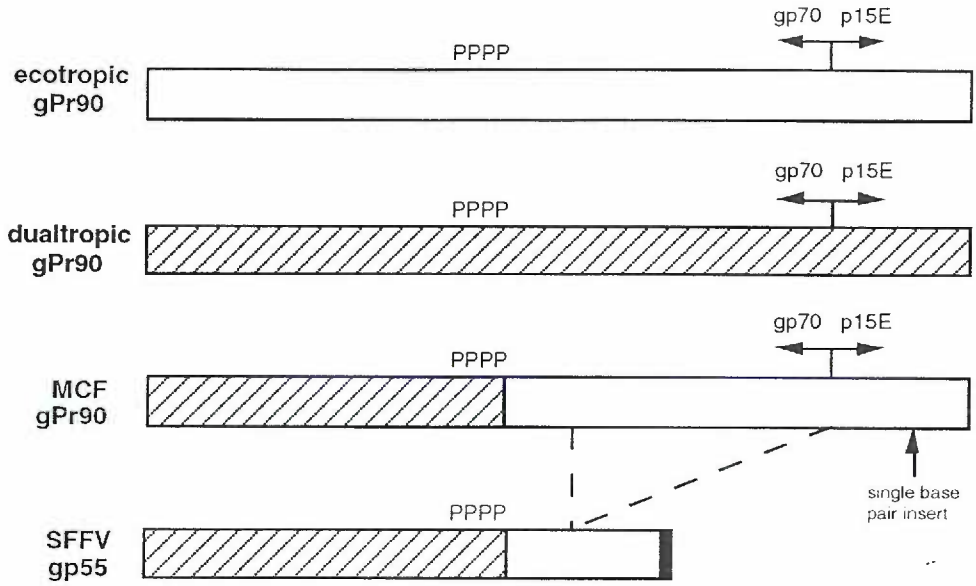


Figure 2. Diagram of the structures of SFFV-related *env* glycoproteins. The *env* glycoproteins of replication-competent murine retroviruses are synthesized as gPr90 precursors that are cleaved by partial proteolysis to form gp70 and p15E products (reviewed in Kabat, 1989). PPPP refers to a proline-rich hypervariable sequence that is approximately 50 amino acids long (Machida et al., 1985; Koch et al., 1984). Dualtropic *env* genes, which are endogenously inherited in mice, recombine with replicating ecotropic MuLVs *in vivo* to form MCFs. Formation of SFFV is believed to have involved a 585 base deletion and a single base insertion in an MCF *env* gene. The single base insert causes a frameshift and the resulting novel amino acid sequence is indicated in black. This novel sequence is in a highly hydrophobic region that serves as the site for membrane attachment of gp55 (Gliniak and Kabat, 1989). The frameshift also causes early termination of translation with loss of 33 amino acids from the carboxyl terminus.

SFFV sequences causes erythroblastosis and ultimately Friend disease in mice transgenic for *env* from SFFVP (Aizawa et al., 1990). More definitive studies in this laboratory suggested that the gp55 protein, versus the *env* gene sequence, is pathogenic and that gp55 must be processed to the plasma membrane to induce erythroblastosis. Transmissible Friend SFFVP mutants (Ruta et al., 1983) and Rauscher SFFV mutants (Machida et al., 1984) have been isolated that contain deletions or mutations in non-overlapping regions of the *env* gene and are non-pathogenic or exhibit substantially reduced abilities to induce erythroleukemia. All non-pathogenic mutants express altered *env* glycoproteins compared to wild-type SFFV, and these mutant glycoproteins are only found intracellularly and not on the plasma membrane. Numerous SFFVP in-frame *env* mutants have also been molecularly generated of which some are pathogenic (Li et al., 1987). Significantly, all pathogenic SFFVs encode *env* glycoproteins that are expressed on cell surfaces, whereas nonpathogenic *env* glycoproteins are exclusively intracellular (Li et al., 1987). In support of the above finding, pathogenic revertant mutants also express *env* glycoproteins on cell surfaces in contrast to the original nonpathogenic SFFVs (Li et al., 1986; Li et al., 1987).

Induction of erythropoietin-independent erythropoiesis by SFFV *env* protein suggests that gp55 activates erythropoietin receptors or circumvents the normal erythropoietin receptor pathway. Recent studies strongly imply that SFFV induces erythroblastosis via the first possibility, specifically at the cell surface. gp55 binds directly to erythropoietin receptors (Li et al., 1990). And quaternary complexes of a disulfide-bonded gp55 dimer, erythropoietin and erythropoietin receptor can be detected on the surfaces of Friend erythroleukemia cells by cross-linking and immuno-precipitation techniques (Casadevall et al., 1991; Ferro et al., 1992). The later studies suggest that gp55 dimers associate with the erythropoietin receptors at a site distinct from the erythropoietin binding site. Interleukin-3-dependent cells that express erythropoietin receptors (IL-3/EpoR cells) are capable of proliferating in the absence of IL-3 if

erythropoietin is either ectopically expressed or added to culture medium (Hoatlin et al., 1990; Li et al., 1990). Infection or transfection of IL-3/EpoR cells with SFFV renders these cells growth factor independent (Hoatlin et al., 1990; Li et al., 1990; Yoshimura et al., 1990). Moreover, pathogenic SFFV *env* mutants, that encode glycoproteins competent for transport to the cell surface, confer growth factor-independency to IL-3/EpoR cells, whereas nonpathogenic mutants have no effect on the growth factor requirements of IL-3/EpoR cells (Ferro et al., 1992). These results are entirely consistent with previous *in vivo* studies on pathogenesis of SFFV *env* mutants (see above) and support the conclusion that gp55 expression at the cell surface causes mitogenesis of infected erythroid cells in the absence of erythropoietin.

3. SFFV proviral integration in a common genomic site causes erythroblast immortalization.

Early studies demonstrated that helper-free preparations of SFFV were sufficient to cause progressive erythroleukemia in susceptible adult mice (Wolff and Ruscetti, 1985; Wolff et al., 1986); although, a majority of mice infected with SFFV developed only a transient erythroblastosis (Berger et al., 1985; Bestwick et al., 1985). Other studies implied that progression from erythroblastosis to malignant erythroleukemia involves selective outgrowth of a small number of cells exhibiting a leukemic phenotype (Wendling et al., 1981; Moreau-Gachelin et al., 1986). Often, these malignant cells have alterations in common loci such as p53 (Chow et al., 1987; Ben-David et al., 1988; Hicks and Mowat, 1988; Mowat et al., 1985). However, it has been difficult to analyze the clonal nature of pathogenesis of Friend disease because SFFV only contains MuLV-related nucleic acid sequences that occur in large numbers as inherited elements in the mouse genome (Chattopadyay et al., 1982; Moreau-Gachelin et al., 1986; Moreau-Gachelin et al., 1985) and because SFFV pathogenesis is restricted to mice (Dawson et al., 1966; Rowe and Brodsky, 1959; Steeves et al., 1971). Moreau-Gachelin and coworkers identified a small region of SFFV that hybridizes with only a few endogenous

sequences of the murine genome under stringent conditions. Using this small probe, they cloned four SFFV integration sites from an erythroleukemia that contained five SFFV and helper MuLV proviruses and subsequently screened each of these integration sites against independently derived erythroleukemias (Moreau-Gachelin et al., 1986). One site (*Spi*-1 for SFFV proviral integration-1) was rearranged in 95% of the advanced erythroleukemias that were examined (Moreau-Gachelin et al., 1988). However, the roles of *spi*-1 integration and of other MuLV and SFFV integrations in pathogenesis of Friend disease could not be determined from their analysis. Advanced erythroleukemias also frequently have amplified copies of the c-*myc* proto-oncogene (Gliniak and Kabat, unpublished results) alterations of p53 (Ben-David et al., 1988; Chow et al., 1987; Hicks and Mowat, 1988; Mowat et al., 1985) and various chromosomal rearrangements (Miller et al., 1979; Ostertag et al., 1972)

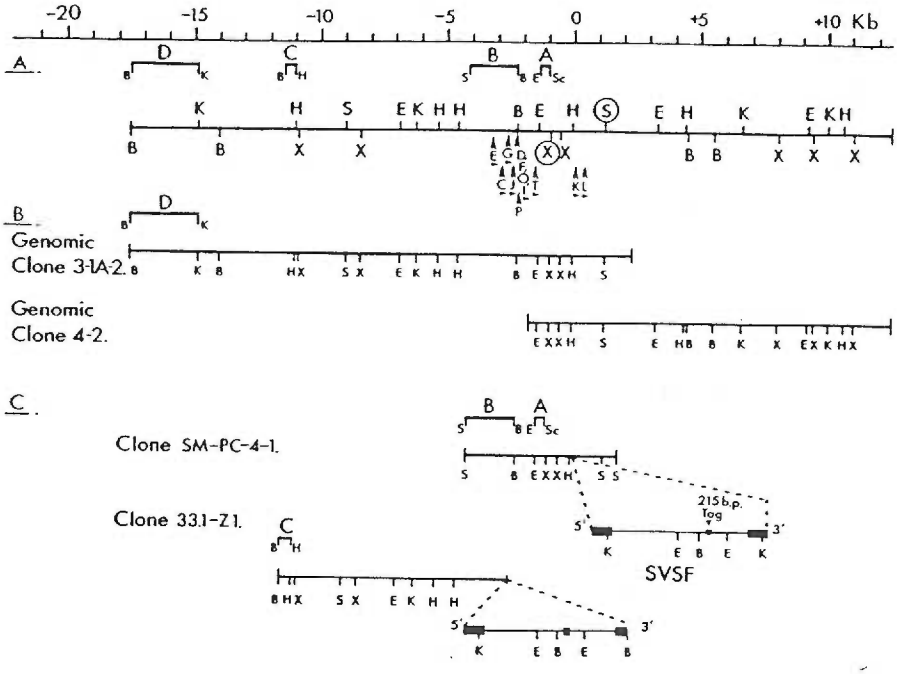
In an independent effort to clone SFFV integration sites, Spiro et al. (1988a) in this laboratory circumvented the problem encountered by Moreau-Gachelin et al. by inserting a 215 bp segment of simian virus 40 DNA into the nonfunctional *pol* gene region of SFFVp. This SV40 sequence is unrelated to murine sequences and can be used as a tag to identify specific proviral integrations within the murine genome. Injection of helper-free "tagged" SFFV caused polyclonal erythroblastosis of infected erythroblasts that resulted in transient mild splenomegaly and polycythemia. Although the great majority of infected erythroblasts differentiated and died out, rare clones grew extensively in 20-30% of the infected mice to form transplantable erythroleukemias by 26 to 33 days (Spiro et al., 1988a). All of the transplantable erythroleukemias contained a single tagged SFFV provirus, lacked helper virus and expressed the mitogenic gp55 *env* gene product. Two distinct genomic libraries were independently constructed in the EMBL-3 bacteriophage lambda vector by R. Paul and me using DNAs prepared from a leukemic spleen and an erythroleukemia cell line. Both erythroleukemias were derived from infections with helper-free tagged SFFV (Spiro et al., 1988a). Genomic fragments

containing tagged SFFV provirus were identified in the libraries using a plaque hybridization technique and radiolabeled SV40 DNA (Paul et al., 1989). Restriction maps of the isolated SFFV-host DNA junction fragment clones are presented in figure 3C. Suprisingly, both erythroleukemias contained an SFFV provirus in a common locus. Single copy genomic probes were prepared from these clones and were used to isolate additional adjacent host genomic sequence; two overlapping genomic clones spanned a 30 kilo base-pair region surrounding the sites of tagged SFFV proviral integration (see figure 3, panel B). By Southern-blotting, we then analyzed 18 independently derived Friend erythroleukemias with single-copy genomic probes to determine whether they also contained proviruses in this region. 78% (ca. 14/18) of the immortalized leukemias that were examined contained an SFFV provirus in this common locus termed *Sfpi*-1 (spleen focus-forming virus proviral integration-1) (Paul et al., 1989). The majority of the integrations in *Sfpi*-1 occurred in a tightly clustered region (see figure 3, panel A). This common locus for immortalizing integrations was subsequently found to be identical to the *spi*-1 integration site identified by Moreau-Gachelin and workers (Moreau-Gachelin et al., 1988; Moreau-Gachelin et al., 1990a). Our unique approach with helper-free, tagged SFFV, however, suggested that single integrations in *Sfpi*-1 contribute to immortalization of erythroblasts and that additional proviruses are unneccessary for leukemic progression.

4. The *Sfpi*-1 proviral integration site of Friend erythroleukemia is adjacent to the gene for the *ets*-related transcription factor PU.1.

To determine whether active gene expression occurred near the common SFFV proviral integration site, numerous restriction endonuclease fragments from the entire isolated *Sfpi*-1 locus were used as hybridization probes for related RNAs on northern blots of RNA extracted from different murine cell lines. Only probes 1 through 3 hybridized under stringent conditions to discrete RNAs (see figure 4). Probes 1 and 2 detected a 1.5 kb poly(A)-containing RNA approximately 15 kilobases upstream of the

Figure 3. (A) Map of the unrearranged *Sfpi*-1 genomic site showing locations and orientation of SFFV proviral integrations. Position zero is arbitrarily defined as the site of a tagged proviral integration in erythroleukemia cell line IP/IR (represented as integration K beneath the genomic map). The cleavage sites for *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Sal*I (S), *Xba*I (X), and *Sac*I (Sc) are indicated. Circled restriction sites identify some of the differences between the *Sfpi*-1 and *Spi*-1 (Moreau-Gachelin et al., 1988). ⇑, sites of proviral integration in erythroleukemias examined; ⇒, orientation of provirus 5' to 3'. Erythroleukemias C, D, E, F, G, I, and J are labeled as previously described (Spiro et al., 1988a). Proviral integrations in additional erythroleukemias are labeled as follows: L, FVT/A cells and FII cells; O, M1 cells; P, M4 cells; and T, M13 cells. Orientation of provirus P is not assigned because this DNA did not cut with *Eco*RI endonuclease. Fragments A through D (indicated by thick underline) were subcloned into the vector pGEM-3 (Promega Biotec) and used as probes for our DNA and RNA blots. Kb, kilobases. (B) Maps of overlapping genomic clones 3-1A-2 and 4-2. Abbreviations are described in legend for map A. (C) Maps of clones SM-PC-4-1 and 33.1-Z1 including tagged SFFV (called SVSF) proviral sequences present in each. Clones SM-PC-4-1 and 33.1-Z1 were obtained by hybridizing the 215 bp simian virus 40 probe to genomic libraries made with DNA from leukemias K and J, respectively. Single-copy probes A, B, and C were derived from fragments of these clones as indicated. Abbreviations are described in the legend for map A.


-20
-15
-10
-5
0
+5
+10 Kb
A.
D
C
B
A
B
Genomic
Clone 3-1A-2.
Genomic
Clone 4-2.
C.
Clone SM-PC-4-1.
Clone 33.1-Z.1.
215 b.p.
tag
SVSF

majority of integration sites in Friend erythroleukemia cells but not in a fibroblast cell line (see appendix 1, figure A) (Paul et al., 1991). The 1.5 kb RNA is transcribed in an opposite orientation to the proviruses and is expressed in SFFV-infected erythroleukemia cells whether or not they contain a provirus in *Sfpi*-1 (see appendix 1, figure A). Poly(A)-containing RNA from IP/IR cells was used by R. Paul to construct a cDNA library. IP/IR erythroleukemia cells contain an SFFV provirus in *Sfpi*-1 but lack the corresponding normal allele (Paul et al., 1989; Spiro et al., 1989). Therefore, any RNA transcribed from this region must derive from the leukemogenically relevant *Sfpi*-1 allele that has an integrated provirus. Accordingly, cytogenetic analysis indicated that IP/IR cells contain only one copy of the *Sfpi*-1 locus in chromosome 2 (Paul et al., 1991). Recombinant lgt-10 phage plaques were screened with probes 1 and 2. Two positive phages were isolated, and inserts were sequenced by the dideoxynucleotide chain termination method by R. Paul (sequence is shown in figure 5). The cDNA sequence is similar to that obtained by Moreau-Gachelin and coworkers from the *Spi*-1 locus (Moreau-Gachelin et al., 1989) and is identical to the sequence encoding the PU.1 transcription activation factor (see below) that was previously isolated and described (Klemsz et al., 1990). However, the cDNA sequence obtained by Moreau-Gachelin and coworkers contained numerous errors that initially confused them (Moreau-Gachelin et al., 1989; Moreau-Gachelin et al., 1990b; Goebl, 1990). Interestingly, PU.1 mRNA and protein in IP/IR erythroleukemia cells appears to be identical to that in normal macrophages and implies that oncogenesis does not require any change in the mRNA or protein sequences (thesis results). A data search for proteins related to PU.1 revealed that PU.1 is significantly homologous to Ets proteins (Klemsz et al., 1990). Specifically, the DNA-binding domain of PU.1 is 38% identical to the DNA-binding domain of c-Ets-1, and PU.1 contains 2 of the 3 conserved tryptophan residues in the ETS-domain (Karim et al., 1990). The Ets family of proteins contains numerous members that bind to different DNA sequences that have a common purine-rich core sequence (5'-GGAA-3'); the Ets proteins function as sequence-specific

Figure 4. Map of the *Sfpi*-1 genomic region showing the locations of areas of active transcription in erythroleukemia cells. The sites of SFFV proviral integrations are also indicated. EcoRI-EcoRI fragments from throughout the genomic region were subcloned into pGEM-3 (Promega Biotec) and were used to detect unique transcripts. Single-copy probes 1 and 2, *Bam*HI-*Bam*HI fragments of approximately 800 and 500 bp, respectively were found to hybridize to a unique 1.5 kb mRNA that was transcribed in an orientation opposite to that of the integrated proviruses. Probe 3 is a 2.2 kbp *Sal*I-*Eco*RI fragment that hybridizes to a small (ca. 200-nucleotide) RNA. The cleavage sites for *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Sal*I (S) and *Xba*I (X) are indicated. Symbols: Δ, sites of proviral integration in erythroleukemias examined; →, orientation of proviruses 5' to 3'. Erythroleukemias C, D, E, F, G, I, J, K, L, M, O, P, T and U are labeled as previously described (Spiro et al., 1988a).

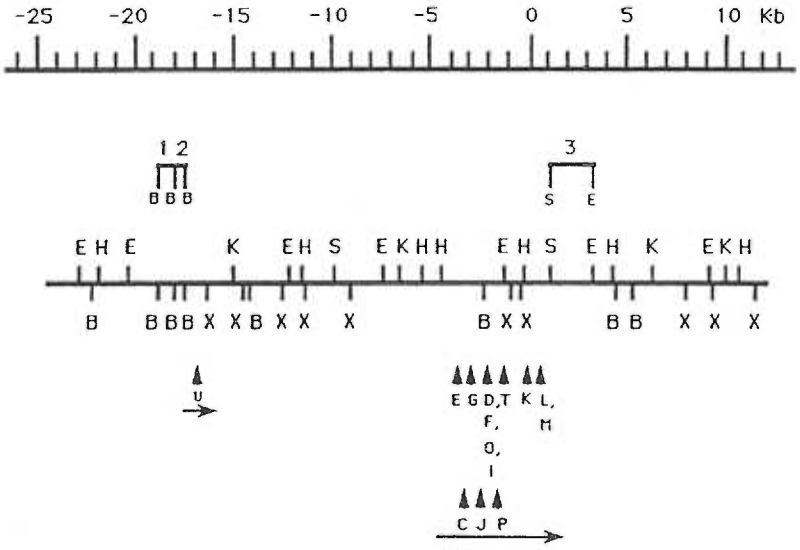
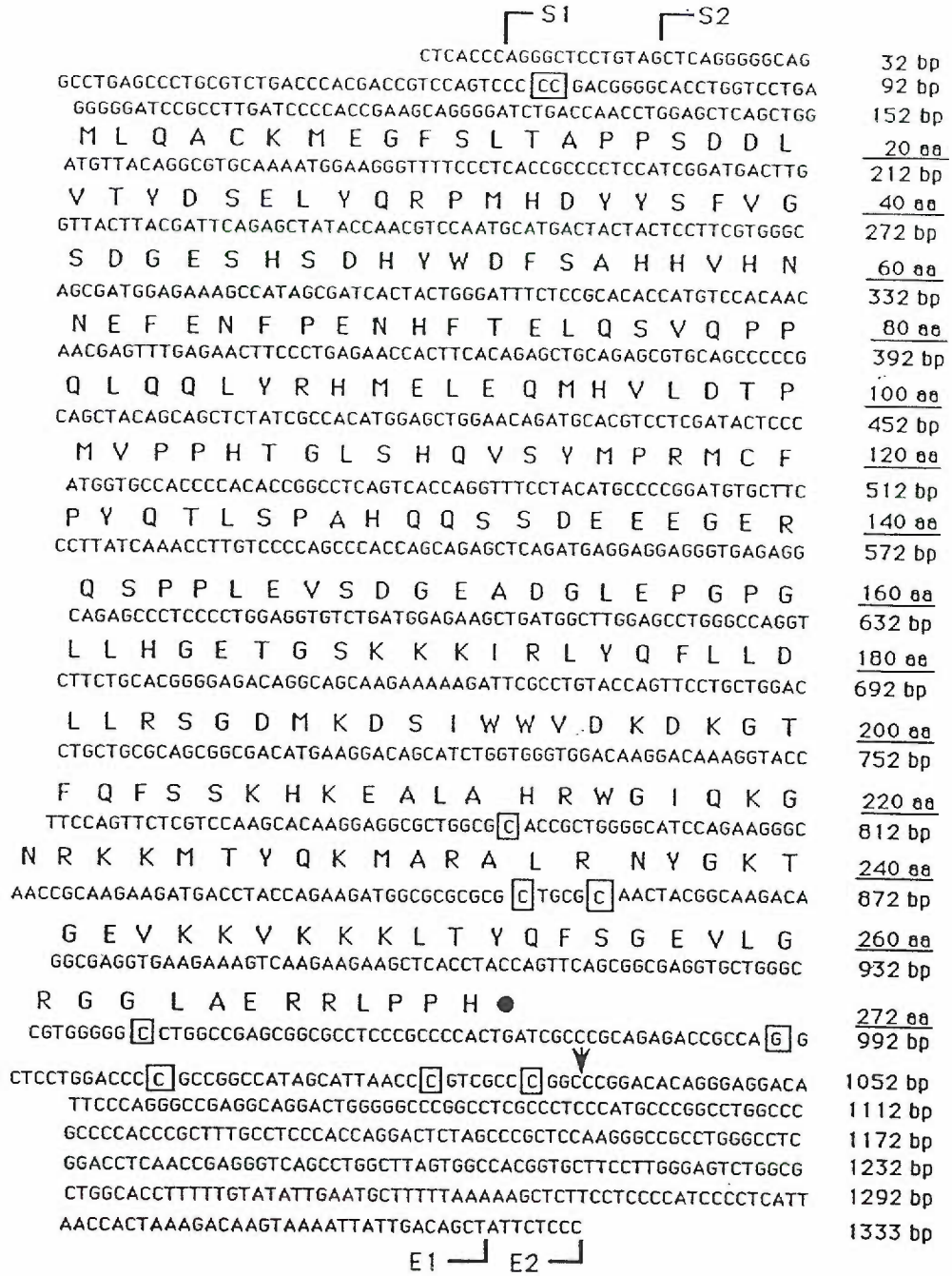

-25 -20 -15 -10 -5 0 5 10 Kb
1 2
B B B
3
S E
E H E K E H S E K H H E H S E H K E K H
B B B B X X B X X X B X X B B X X X
U
E G D, T K L,
F,
O,
I
C J P

Figure 5. Sequence of the *Sfpi*-1 cDNA. This sequence was determined from the IP/IR cDNA clone designated P15 and is numbered from the 5' nucleotide. A second IP/IR clone, P7, was also sequenced and extended from bp 28 to 1,333. A third clone, derived from an F-745 erythroleukemia cDNA library, had 100% homology with the P15 sequence throughout its length from bp 1 to 670. The predicted amino acid sequence is shown above the corresponding DNA sequence. The stop codon for the amino acid sequence shown is indicated (●). S1 and E1, start and end sites, respectively, of the *spi*-1 cDNA sequence reported by Moreau-Gachelin et al. (1989); S2 and E2, start and end sites, respectively, of the PU.1 sequence reported by Klemsz et al. (1990).; aa, amino acids. Nucleotides that were missing from the sequence reported by Moreau-Gachelin et al. (1989) are boxed. The arrowhead at bp 1,034 contained an extra T residue in the sequence reported by these same workers.


S1
S2
CTCACCCAGGGCTCCTGTAGCTCAGGGGGCAG
32 bp
GCCTGAGCCCTGCGTCTGACCCACGACCGTCCAGTCCCCCGACGGGGCACCTGGTCCTGA
92 bp
GGGGGATCCGCCTTGATCCCCACCGAAGCAGGGGATCTGACCAACCTGGAGCTCAGCTGG
152 bp
M L Q A C K M E G F S L T A P P S D D L
20 aa
ATGTTACAGGCGTGCAAAATGGAAGGGTTTTCCCTCACCGCCCCTCCATCGGATGACTTG
212 bp
V T Y D S E L Y Q R P M H D Y Y S F V G
40 aa
GTTACTTACGATTCAGAGCTATACCAACGTCCAATGCATGACTACTACTCCTTCGTGGGC
272 bp
S D G E S H S D H Y W D F S A H H V H N
60 aa
AGCGATGGAGAAAGCCATAGCGATCACTACTGGGATTTCTCCGCACACCATGTCCACAAC
332 bp
N E F E N F P E N H F T E L Q S V Q P P
80 aa
AACGAGTTTGAGAACTTCCCTGAGAACCACTTCACAGAGCTGCAGAGCGTGCAGCCCCCG
392 bp
Q L Q Q L Y R H M E L E Q M H V L D T P
100 aa
CAGCTACAGCAGCTCTATCGCCACATGGAGCTGGAACAGATGCACGTCCTCGATACTCCC
452 bp
M V P P H T G L S H Q V S Y M P R M C F
120 aa
ATGGTGCCACCCCACACCGGCCTCAGTCACCAGGTTTCCTACATGCCCCGGATGTGCTTC
512 bp
P Y Q T L S P A H Q Q S S D E E E G E R
140 aa
CCTTATCAAACCTTGTCCCCAGCCCACCAGCAGAGCTCAGATGAGGAGGAGGGTGAGAGG
572 bp
Q S P P L E V S D G E A D G L E P G P G
160 aa
CAGAGCCCTCCCCTGGAGGTGTCTGATGGAGAAGCTGATGGCTTGGAGCCTGGGCCAGGT
632 bp
L L H G E T G S K K K I R L Y Q F L L D
180 aa
CTTCTGCACGGGGAGACAGGCAGCAAGAAAAAGATTCGCCTGTACCAGTTCCTGCTGGAC
692 bp
L L R S G D M K D S I W W V D K D K G T
200 aa
CTGCTGCGCAGCGGCGACATGAAGGACAGCATCTGGTGGGTGGACAAGGACAAAGGTACC
752 bp
F Q F S S K H K E A L A H R W G I Q K G
220 aa
TTCCAGTTCTCGTCCAAGCACAAGGAGGCGCTGGCGCACCGCTGGGGCATCCAGAAGGGC
812 bp
N R K K M T Y Q K M A R A L R N Y G K T
240 aa
AACCGCAAGAAGATGACCTACCAGAAGATGGCGCGCGCGCTGCGCAACTACGGCAAGACA
872 bp
G E V K K V K K K L T Y Q F S G E V L G
260 aa
GGCGAGGTGAAGAAAGTCAAGAAGAAGCTCACCTACCAGTTCAGCGGCGAGGTGCTGGGC
932 bp
R G G L A E R R L P P H ●
272 aa
CGTGGGGGCCTGGCCGAGCGGCGCCTCCCGCCCCACTGATCGCCCGCAGAGACCGCCAGG
992 bp
CTCCTGGACCCCGCCGGCCATAGCATTAACCCGTCGCCCGGCCCGGACACAGGGAGGACA
1052 bp
TTCCCAGGGCCGAGGCAGGACTGGGGGCCCGGCCTCGCCCTCCCATGCCCGGCCTGGCCC
1112 bp
GCCCCACCCGCTTTGCCTCCCACCAGGACTCTAGCCCGCTCCAAGGGCCGCCTGGGCCTC
1172 bp
GGACCTCAACCGAGGGTCAGCCTGGCTTAGTGGCCACGGTGCTTCCTTGGGAGTCTGGCG
1232 bp
CTGGCACCTTTTTGTATATTGAATGCTTTTTAAAAAGCTCTTCCTCCCCATCCCCTCATT
1292 bp
AACCACTAAAGACAAGTAAAATTATTGACAGCTATTCTCCC
1333 bp
E1
E2

transcription activating factors (Karim et al., 1990; Klemsz et al., 1990; Gunther et al., 1990; Macleod et al., 1992).

5. SFFV proviral integration in *Sfpi*-1 activates expression of PU.1.

PU.1 mRNA levels in normal spleens, erythropoietically stimulated spleens and erythroleukemia cell lines were compared to determine if PU.1 expression is enhanced during erythroleukemogenesis (Paul et al., 1991). PU.1 mRNA occurs in a higher concentration in normal spleens than in erythropoietically active enlarged spleens from anemic mice and from preleukemic mice infected with Friend virus or with an erythropoietin-encoding retrovirus (see appendix 1, panel B). High levels of PU.1 mRNA in spleens is due, in a large part, to active expression of PU.1 in macrophages and B cells (Klemsz et al., 1990; Moreau-Gachelin et al., 1990a). The low levels of PU.1 mRNA in erythropoietically active spleens imply that normal differentiating erythroid cells either lack PU.1 mRNA or contain much smaller amounts than splenic macrophages and B cells. The high levels of PU.1 mRNA in immortalized erythroleukemia cells could be caused by enhancer-mediated constitutive expression as a result of proviral integration (e.g. in cell lines IP/IR, M1, and M4) or by alternative mechanisms (e.g. for cell lines M5 and M7). Also, an analysis of PU.1 protein levels in splenic tissue during Friend erythroleukemogenesis supports the above suggestion that PU.1 is aberrantly overexpressed in erythroleukemia cells (see thesis manuscript #1).

PU.1 mRNA is synthesized in bone marrow macrophages (Klemsz et al., 1990) and in macrophage and B lymphocyte cell lines (Klemsz et al., 1990; Moreau-Gachelin et al., 1990a). Furthermore, PU.1 mRNA is differentially expressed in murine tissues. Detectable levels of PU.1 mRNA occur in normal bone marrow, spleens, brains, hearts, lungs, large intestines, and in a B-cell lymphoma (see appendix 1, figure 2). PU.1 mRNA is absent in stomachs, livers, kidneys, and NIH 3T3 fibroblasts (see appendix 1, figure 2). PU.1 mRNA can be detected in thymic stromata but not in thymocytes (Paul et al., 1991). These findings indicate that PU.1 is differentially expressed in different cell types and

raise the possibility that SFFV proviral integration in *Sfpi*-1 may contribute to Friend leukemogenesis by activating expression of PU.1.

F. Ets superfamily of transcription factors; relevance to leukemogenesis.

The Ets superfamily of proteins are nuclear, sequence-specific DNA-binding proteins that share a homologous DNA binding domain but differ in putative transactivational domains (Karim et al., 1990; Macleod et al., 1992). Interestingly, members other than PU.1 have also been implicated in pathogenesis of cancer. Translocation of the 11q23 region of chromosome 11 containing the c-*ets*-1 locus to chromosome 4 is characteristic of certain human myelomonocytic leukemias, and a translocation of c-*ets*-2 from chromosome 21 to chromosome 8 is frequently found in certain human acute myelogenous leukemias (Sacchi et al., 1986). Molony MuLV proviral insertion near *tpl*-1 is associated with pathogenesis of rat thymic lymphomas (Bear et al., 1989). And, translocation involving *elk*-1 is associated with human synovial sarcomas (Rao et al., 1989). More important, other *ets*-related genes (*fli*-1 and v-*ets*) have been implicated in erythroleukemias induced in newborn mice by the Friend strain of MuLV in the absence of SFFV (Ben-David et al., 1990), and in chickens and mice by the E26 and ME26 viruses, respectively (Moscovici et al., 1981; Radke et al., 1982; Ruscetti et al., 1992). A chromosomal translocation at the *fli*-1 gene has recently been implicated in human Ewing's sarcoma (A. Bernstein, personal communication).

G. Thesis objectives.

Cancer is the second leading cause of death in the industrialized world, and its cure remains a formidable challenge for the medical and scientific communities. Considerable evidence indicates that carcinogenesis and leukemogenesis are progressive and multifaceted. While early investigations of the pathogenesis of cancer were principally descriptions of the evolution and course of disease, recent work has begun to

elucidate specific molecules involved in the processes of transformation and metastasis. Identification of proteins implicated in the induction and promotion of tumors has substantially aided our understanding of the mechanisms involved in the generation of malignancies and the particular molecular alterations associated with certain tumors. The discovery of common molecular and biochemical processes responsible for pathogenesis of certain cancers is of great importance if effective tumor-specific therapeutic agents are to be designed.

Study of virally induced malignancies has identified numerous oncogenes and their corresponding proto-oncogenes and has significantly furthered our understanding of the cellular processes resulting in tumor formation and growth. This approach has contributed substantially to cellular biology. However, the molecular alterations in tumors induced by viruses that lack oncogenes have remained elusive. The spleen focus-forming virus lacks a classical oncogene but causes a progressive erythroleukemia in susceptible mice. Extensive work in this laboratory and by others suggests that the glycoprotein gp55, encoded by the *env* gene of SFFV, induces proliferation and differentiation of erythroblasts. This effect of SFFV involves an interaction between gp55 and erythropoietin receptors on surfaces of infected erythroid cells. Work, in part by me, (see above) revealed that SFFV proviral integration in the *Sfpi*-1 locus results in transcription of the recently identified, putative transcription factor PU.1, and correlates with loss of the commitment of erythroblasts to differentiate and with their immortality. Based on these observed, potentially very important correlations, we proposed that constitutive expression of PU.1 was directly involved in development of the erythroleukemic stage of Friend disease. The potential role of PU.1 in erythroleukemogenesis had important implications and obviously deserved more direct analysis.

The following investigations were conducted to determine the relationship between PU.1 expression and the altered characteristics of Friend erythroleuekmia cells.

PU.1-specific antiserum was generated and used to analyze PU.1 protein levels throughout leukemic progression of Friend disease and during chemically induced differentiation of Friend erythroleukemia cell lines. Our results implied that expression of PU.1 was directly associated with the abrogation of differentiation of precommitted erythroblasts and with an immortal phenotype. Furthermore, the normal restriction of PU.1 expression to macrophages and B lymphocytes and the apparent, striking effect of PU.1 protein on erythropoiesis strongly suggested that this transcription activating protein serves a particular function in hematopoiesis and has unique effects in different types of cells. Therefore, we directly studied the effects of PU.1 expression, in the absence of gp55, in long-term bone marrow and fibroblast cultures to ascertain the role of PU.1 in pathogenesis of Friend disease and its affect on cell growth and differentiation. PU.1 protein expression was found to have remarkably distinct effects on the growth potential of different types of cells. Also, significant differences were identified in the metabolism of PU.1 in Friend erythroleukemia cells, immortalized erythroblasts and fibroblasts (where PU.1 is ectopically expressed) in contrast to bone marrow macrophages (where PU.1 is normally expressed). Results from these approaches have helped to define the role of PU.1 in Friend disease and have further clarified the multistage nature of leukemogenesis.

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Manuscript #1

Role of the PU.1 Transcription Factor in Controlling Differentiation of Friend Erythroleukemia Cells

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ABSTRACT

Both viral and cellular genes have been directly implicated in pathogenesis of Friend viral erythroleukemia. The virus-encoded gp55 glycoprotein binds to erythropoietin receptors to cause mitogenesis and differentiation of erythroblasts. However, if the provirus integrates adjacent to the gene for the PU.1 transcription factor, the cell loses its commitment to terminally differentiate and becomes immortal as indicated by its transplantability and by its potential for indefinite growth in culture (Spiro, C., B. Gliniak, and D. Kabat, J. Virol. 62:4129-4135, 1988; Paul, R., S. Schuetze, S.L. Kozak, and D. Kabat, J. Virol. 65:464-467, 1991). To test the implications of these results we produced polyclonal antiserum to bacterially-synthesized PU.1, and we used it to analyze PU.1 expression throughout leukemic progression and during chemically-induced differentiation of Friend erythroleukemia (F-MEL) cell lines. This antiserum identified three electrophoretically distinct PU.1 components in extracts of F-MEL cells and demonstrated their nuclear localization. Although PU.1 proteins are abundant in F-MEL cells, they are absent or present in only trace amounts in normal erythroblasts or in differentiating erythroblasts from the preleukemic stage of Friend disease. Furthermore, chemicals (dimethylsulfoxide or N,N'-hexamethylenebisacetamide) that overcome the blocked differentiation of F-MEL cells induce rapid declines of PU.1 mRNA and PU.1 proteins. The elimination of PU.1 proteins coincides with recommitment to the program of erythroid differentiation and with loss of immortality. These results support the hypothesis that PU.1 interferes with the commitment of erythroblasts to differentiate and that chemicals that reduce PU.1 expression reinstate the erythropoietic program.

INTRODUCTION

Friend viral erythroleukemia provides an excellent model for analyzing the multi-step process of leukemogenesis and the roles of host genes in controlling susceptibility to an oncogenic protein (see references 2 and 23 for reviews). The replication-defective viral component, the spleen focus-forming virus (SFFV), encodes a membrane glycoprotein (gp55) that causes proliferation of late burst-forming and colony-forming erythroblasts (23). Recent evidence indicates that gp55 acts by binding directly to erythropoietin receptors (5, 20, 32). Because erythroblasts are committed to differentiate and have limited self-renewal capacities and because SFFV does not generally alter these cellular properties, the initial stage of virus-induced disease is characterized by polyclonal proliferation of cells that continue to differentiate and that cannot be transplanted to secondary recipients (2, 23, 65, 70). In mice infected with mixtures of helper virus plus SFFV, this initial stage of disease is maintained by continuous infection of newly-forming erythroblasts. A second virus-induced change occurs if the SFFV provirus integrates by chance in the *Sfpi*-1 locus (37, 45, 65) adjacent to the gene for the *ets*-related transcription factor PU.1 (27, 46). This abrogates the erythroblast's commitment to differentiate and results in a transplantable, immortalized erythroleukemia (65, 66). Because *Sfpi*-1 proviral integration occurs at a very low frequency, the consequence is outgrowth of a rare immortalized clone that becomes a substantial proportion of the neoplastic population only after 4-8 weeks post-infection.

In support of the above conclusions, mice injected with large doses of helper-free SFFV (Lilly-Steeves strain) develop a polyclonal erythroblastosis that is followed by mild polycythemia and by complete recovery 10-14 days post-infection (65). A few mice, however, do not completely recover; these develop monoclonal transplantable erythroleukemias that weigh several grams by 30 days post-infection and that have proviral integrations in *Sfpi*-1. These leukemic cells are immortal and do not differentiate *in vivo*

(66). All immortal Friend erythroleukemias express large amounts of PU.1 mRNA as detected by RNA blotting (37, 38, 46).

Virus-induced changes have also been reported in the p53 anti-oncogene (reviewed in reference 2; 8, 41). However, these changes may not be obligatory and have not been correlated with specific phenotypic alterations in the leukemic cells.

The homology of PU.1 to *ets* is intriguing because v-*ets* contributes to erythroleukemia in chickens (40, 50). Indeed, v-*ets* was first identified as one element of the tripartite oncogene in the E26 avian leukemia virus that transforms hematopoietic cells of myeloid and erythroid lineages. The *ets* region of the E26 oncoprotein (the M_r 135,000 oncoprotein contains portions of chicken *ets*-1 and *myb* fused to a retroviral *gag* domain) is required for erythroblastosis but not for myeloid transformation (15, 16, 43). PU.1 and *ets*-1 are most conserved in their carboxyl terminal regions that bind to a common purine-rich core (5'-GGAA-3') DNA sequence (24). Studies of PU.1 (27) and other members of the *ets* family (4, 17) indicate that they are transcriptional regulators. Another *ets*-related protein, *fli*-1, contributes to erythroleukemias that form in newborn mice after infection with the Friend murine leukemia virus (MuLV) in the absence of SFFV (1, 2).

SFFV proviral integrations in *Sfpi*-1 occur several kilobases upstream of the PU.1 transcriptional initiation site (37, 45). PU.1 mRNA from erythroleukemias has the same size and sequence as PU.1 mRNA that occurs in normal macrophages (27, 46). These results suggest that the SFFV provirus activates PU.1 expression by an enhancer-mediated mechanism.

Although proviral integration near PU.1 has been associated with a block in differentiation (65, 66), the impediment is not absolute since it can be overcome by treatment of cultured Friend erythroleukemia (F-MEL) cells with dimethylsulfoxide (Me_2SO) and with certain other chemical inducers (12, 34, 52). Furthermore, although the studies mentioned above suggest that gp55 and PU.1 have critical roles in Friend viral erythroleukemia, little is known about the PU.1 protein or about its expression during

leukemogenesis or chemically-induced differentiation. To address these issues, we produced a polyclonal antiserum to PU.1. This antiserum identified three predominant PU.1 components in F-MEL cells. Rapid loss of PU.1 mRNA and proteins followed addition of chemical inducers to F-MEL cells. Loss of PU.1 proteins coincided with recommitment to the erythropoietic program.

MATERIALS AND METHODS

Cell lines and tissues. The F745-PC4-D2 (18) Friend leukemia cell line contains both SFFV and MuLV proviruses. The IP/IR F-MEL cell line was derived from a mouse infected with helper-free SFFV; it contains one SFFV provirus in *Sfpi*-1 on chromosome 2 and has lost the unmodified allele (45, 46). Ψ2 (33) and PA12 (36) are retroviral packaging derivatives of murine NIH3T3 fibroblasts. The pSFF retroviral expression vector (3) that contained PU.1 cDNA coding sequences cloned into the unique EcoRI site became amplified by ping-pong after transfection into a 1:1 coculture of Ψ2 and PA12 cells, as described elsewhere (3, 28). All cells were grown in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, 100 u/ml penicillin G and 100 μg streptomycin (DMEM/FBS).

Spleens were obtained from 4-6 week old female NIH/Swiss mice. A normal spleen (weight 0.15 g) was isolated from an untreated mouse that had a hematocrit of 47%. Erythropoietically active spleens were isolated from a mouse 48 h following two consecutive days of phenylhydrazine injections (60 mg/kg of body weight) (spleen weight 0.34 g; hematocrit 44%) and from a mouse 13 days after injection with 0.7 ml of passaged Friend virus (Lilly-Steeves polycythemia strain) via the tail vein (spleen weight 3.33 g; hematocrit 68%) as described (65).

Molecularly cloned probes used for hybridization. The β-actin probe, pBam-bA2, contained the coding region of mouse B-actin cDNA cloned into the BamHI site in pGEM (68). The B-globin probe consisted of the HindIII fragment (probe B) from the mouse cDNA encompassing the first two exons cloned into pBR322 (21). The c-*myc* plasmid, pM104BH, contained a 3.2 kilobase pair (kbp) BamHI-HindIII fragment from the mouse c-*myc* locus (61). A v-*myb* fragment was excised from pVM2 (26). The p53 probe pp53-208 was cloned from mouse cDNA (44). The *Sfpi*-1 probe was the BamHI-Apa1 fragment isolated from the PU.1 coding region of a mouse cDNA clone (46).

PU.1-specific antiserum. The PvuII-BamHI fragment containing the entire coding region of PU.1 was excised from our PU.1 cDNA construct (46). BamHI linkers (New England Biolabs, Beverly, MA) were ligated onto the PvuII digested blunt end and cleaved with BamHI restriction endonuclease. This modified fragment was ligated into the BamHI site of the expression plasmid pET-11d (Novagen, Madison, WI) which is based on the T7 polymerase system reported by Studier *et al* (67). The recombinant gene contained 15 codons fused to the first AUG codon at the 5' end of the PU.1 cDNA; the codons for the 13 N-terminal amino acids were from the T7 gene 10. The PU.1 cDNA codes for 272 amino acids (27, 46). For protein expression, the pET-11d-PU.1 vector was transformed into the BL21(DE3)pLysS lysogenic strain (Novagen) of *Escherichia coli* and a transformed clone was induced to express the PU.1 fusion product according to the manufacturer's recommendations. Bacterial cells were collected by centrifugation at 3,000 x g for 30 min, resuspended in 50 mM sodium chloride, 50 mM Tris hydrochloride (pH 8.0), 1 mM EDTA and 5 mM dithiothreitol, and lysed in a French press at 10,000 psi. Insoluble PU.1 protein was collected by centrifugation at 3,000 x g for 30 min and was solubilized by boiling in Laemmli modified buffer (0.0625 M Tris hydrochloride, [pH 6.8], 2.3% sodium dodecyl sulfate [SDS], 20% glycerol and 5% mercaptoethanol) for 10 min. PU.1 was separated from other proteins by electrophoresis through an 8% polyacrylamide-0.1% SDS gel (31), excised in a gel slice and either emulsified in phosphate-buffered saline solution, pH 7.4, (PBS) (JRH Biosciences, Lenexa, KS) at approximately 1 mg of protein per ml of PBS or electroeluted into 0.5x TAE (1x TAE is 40 mM Tris-acetate and 1 mM EDTA), dialyzed against water, lyophilized and resuspended at 1 mg/ml in PBS. 500 µg of PU.1 protein in polyacrylamide that was emulsified in PBS was diluted 4:1 in complete Freund's adjuvant and injected subcutaneously into a female New Zealand White rabbit. Booster immunizations of 500 µg of electroeluted PU.1 protein, diluted 1:1 in incomplete Freund's adjuvant, were given intramuscularly every 7 weeks. Rabbit serum was assayed for PU.1-specific antibodies by a two-step

immunoprecipitation-western blotting procedure (see below). Serum obtained after the second boost gave a strong positive response when diluted 10^3-fold and was designated PU.1-9794.

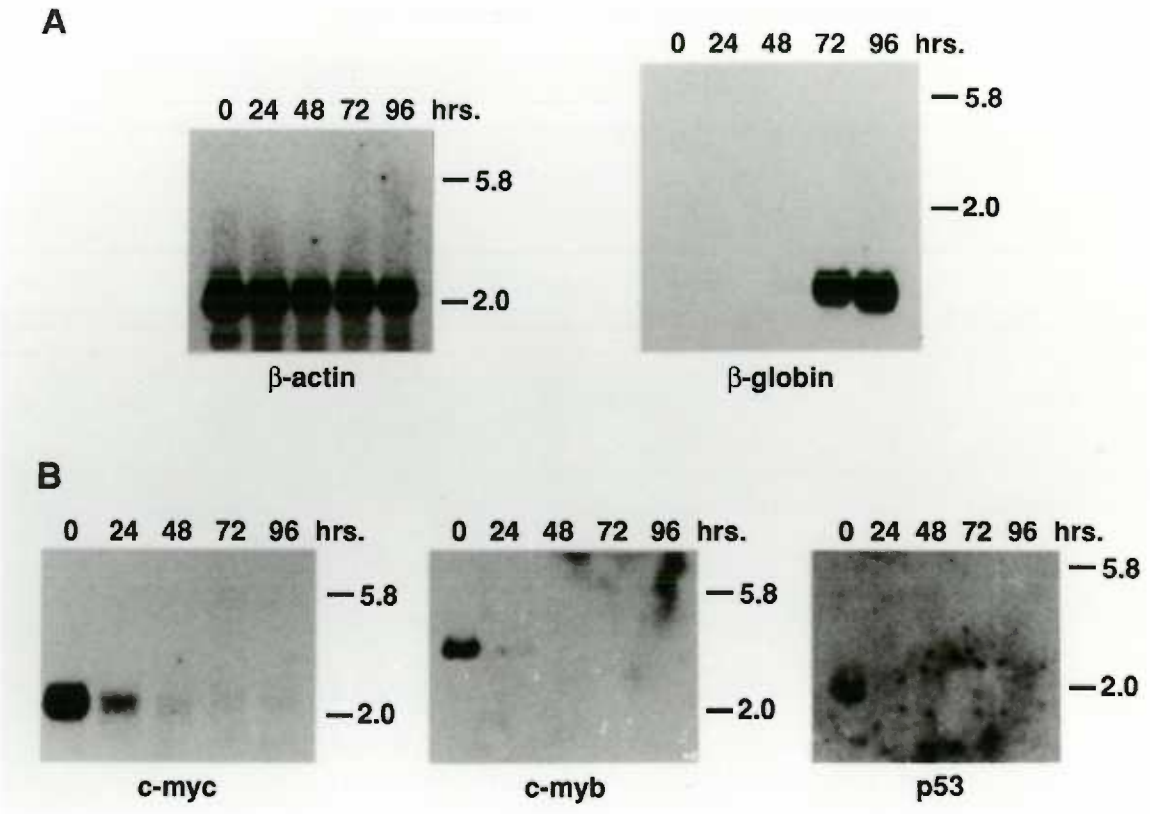
Analysis of proteins. F745 erythroleukemia cells were labeled by incorporation for 2 h with L-[^{35}S]methionine and L-[^{35}S]cysteine (Tran^{35}S-label, ICN Biochemicals Inc., Costa Mesa, CA) and lysed in immune precipitation buffer (IPB) as described (14). Lysates of tissue samples were also prepared as previously described (65). Lysates were precleared by adsorption with fixed *Staphylococcus aureus* (Pansorbin, Calbiochem Corp., La Jolla, CA) and by centrifugation prior to immunoprecipitation and to electrophoresis in polyacrylamide gels in the presence of 0.1% SDS (55, 56). For PU.1 detection, proteins were transferred onto nitrocellulose membranes (14). Nonradioactive samples were blocked, washed (57) and then immunoblotted with a 1:1000 dilution of PU.1-9794 antiserum in a solution of 5% nonfat milk, 0.01% antifoam A, 0.01% Tween 20 and 0.02% sodium azide in PBS. Blots with bound antibodies were then labeled by incubation with [^{125}I]protein A, and the labeled membranes were developed by autoradiography. Immunofluorescence was done using Ψ2/PA12 ping-pong cocultures (see above) in which approximately 10% of the cells were active in PU.1 expression. Cells growing on coverslips were rinsed with PBS, fixed with 3.7% paraformaldehyde in PBS for 20 min and then immersed in ice cold acetone for 1 min. Incubation for 1 h at 37°C with a 1:500 dilution of PU.1-9794 antiserum in DMEM/FBS was followed by rinsing with medium and then incubating for 1 h at 37°C with a 1:1000 dilution of fluorescein-labeled goat anti-rabbit immunoglobulin (TAGO, Inc., Burlingame, CA) prior to final rinsing.

Differentiation in F-MEL cultures. Erythroid differentiation was induced by addition of either 1.8% Me_2SO (Mallenckrodt, Paris, France) (12), or 5mM N,N'-hexamethylenebisacetamide (HMBA, Sigma, St. Louis, MO) (52) to exponentially growing cultures containing 0.7-1.0x10^6 cells/ml. In all studies, more than 70% of the

cells stained benzidine positive (42) by 120 h post-induction. To ensure that our F-MEL cell clones differentiated in a manner consistent with earlier studies that used other clones, RNA blots were analyzed for transcripts previously reported to be altered by inducers of differentiation. As shown in Figure 6, Me_2SO-induced differentiation in our clone of F745 cells resulted in accumulation of β-globin mRNA beginning at about 48 h while β-actin mRNA remained relatively constant. In agreement with previous reports, chemical induction resulted in depletion of transcripts for c-*myc* (30, 51), c-*myb* (25, 51) and p53 (51). Similar results were obtained using our clone of IP/IR cells (data not shown).

RNA analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate-CsCl method (7, 57). Poly(A)RNA was selected from total RNA by the oligo(dT) batch procedure (57). For Northern analysis, either total cellular RNA or poly(A) selected RNA samples were electrophoresed through a 1.2% agarose gel following denaturation with glyoxal and Me_2SO (57), transferred to nitrocellulose or nytran membranes (Schleicher and Schuell, Keene, NH), and fixed by baking for 2 h at 80°C or UV irradiating and baking, respectively. Nytran blots were stained with a solution of 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5) to visually inspect the RNAs for uniform transfer and integrity and then destained in 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were prehybridized at 42°C for 16 h in a solution consisting of 50% formamide, 2.5 x SSC, 2.5x Denhardts (50x Denhardts solution is 1.0% each of Ficoll, polyvinyl pyrrolidone and BSA-fraction V), 25 mM sodium phosphate (pH 6.6), 2.5% dextran sulfate (Pharmacia, Piscatawy, NJ), 0.5% SDS, and 100 μg/ml denatured herring sperm DNA. Hybridization was performed in the same buffer with the addition of 10^6 cpm/ml of [α-^{32}P]dCTP labeled nick-translated or randomly primed probe. The final wash was at 42-55°C in 0.1 x SSC, 0.1% SDS and 1 mM EDTA (pH 8.0).

Figure 6. Gene expression during Me_2SO-induced differentiation. F745 cells were cultured with 1.8% Me_2SO and RNA was isolated at 24 hour intervals through 96 h. 5 μg poly(A) selected RNA was loaded in each lane. Northern blot analysis was performed with radiolabeled probes (see Materials and Methods for a description of the probes used). (A) Expression of β-actin and β-globin. (B) Expression of cellular oncogenes *myc*, *myb*, and p53. Size markers are in kilobasepairs.


A
0 24 48 72 96 hrs.
5.8
2.0
β-actin
0 24 48 72 96 hrs.
5.8
2.0
β-globin
B
0 24 48 72 96 hrs.
5.8
2.0
c-myc
0 24 48 72 96 hrs.
5.8
2.0
c-myb
0 24 48 72 96 hrs.
5.8
2.0
p53

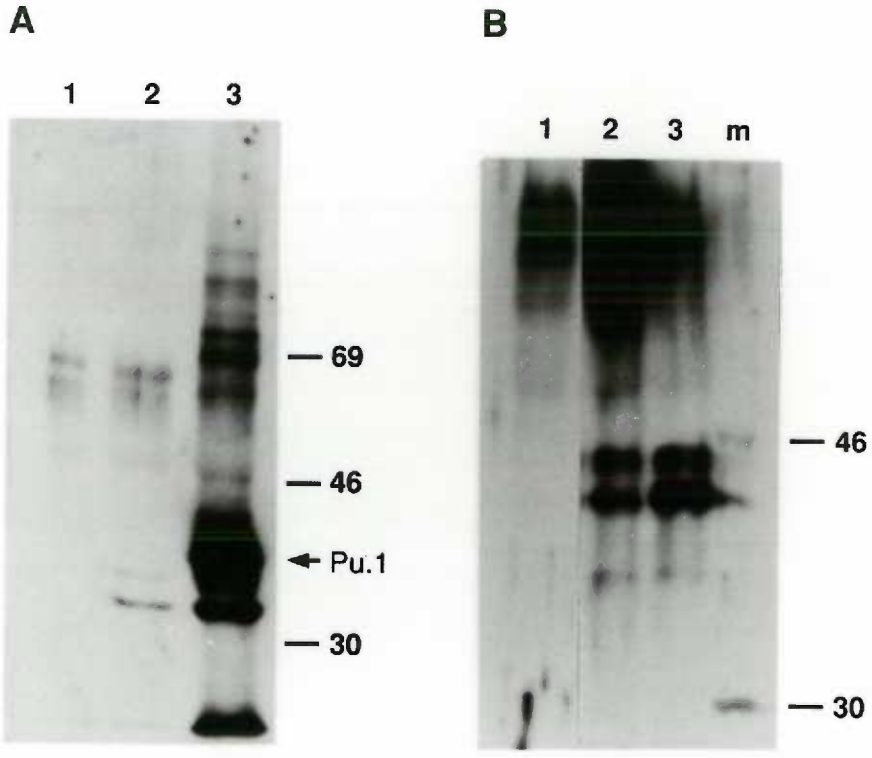
RESULTS

Immunological detection of PU.1 proteins. PU.1 was expressed in bacteria as the complete protein with an amino terminal extension of 15 amino acids and an apparent M_r 38,000. This size is in agreement with the expected PU.1 M_r of 36,500 based on its cDNA sequence (46). Maximum yields of the bacterial product (10-15% of the total cell protein as estimated from the Coomassie Blue staining pattern) were obtained 2-3 h after addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside inducer to the exponentially growing bacteria. As shown in Figure 7, our rabbit antiserum (named PU.1-9794) precipitated this M_r 38,000 protein from lysates of bacteria that express PU.1 (panel A, lane 3) but not from a lysate of control bacteria that lacks PU.1 (panel A, lane 2).

Both F-745 and IP/IR F-MEL cell lines contain substantial amounts of PU.1 mRNA (46). Figure 7 illustrates that PU.1-9794 antiserum also specifically precipitated proteins from lysates of IP/IR cells (panel B, lane 3). Three major PU.1-related proteins were reproducibly identified in F-MEL cell lysates (see below). The two predominant components seen in this protein blot migrate with apparent M_r of 42,000 and 44,000, substantially larger than expected for PU.1. A third PU.1-related protein (apparent M_r 37,000) is seen in lower concentrations in F-MEL lysates at the position expected for PU.1. The preimmune rabbit serum did not precipitate any of these components.

To determine which of these immunoprecipitated proteins in F-MEL lysates were encoded by the PU.1 gene, we expressed PU.1 cDNA in a coculture of Ψ2 and PA12 murine fibroblasts using a retroviral vector (see Materials and Methods) that becomes amplified in these cocultures (3, 28). Interestingly, all three of these PU.1-related protein components were detected in lysates from these fibroblasts (Fig. 7, panel B, lane 2) but not in lysates from control fibroblasts (e.g., panel B, lane 1).

Figure 7. Western blot analysis of proteins precipitated by the antiserum PU.1-9794. Cell lysates (1 mg of total protein except in panel A, lane 2 in which 100 μg of protein was used) were immunoprecipitated with a 1:500 dilution of antiserum and separated by electrophoresis through a 0.1% SDS-8% polyacrylamide gel. Transferred proteins were immunoblotted with a 1:1000 dilution of PU.1-9794 followed by ^{125}I-labeled protein A (see Materials and Methods). Panel A, lane 1, immune precipitation buffer treated with Pansorbin and antiserum but without cell lysate; lane 2, isopropyl-β-D-thiogalacto- pyranoside induced bacterial BL21(DE3)pLysS cells containing the control pET-11d plasmid; and lane 3, isopropyl-β-D- thiogalactopyranoside induced bacterial cells containing the recombinant pET-11d-PU.1 vector. Panel B, lane 1, a coculture of Ψ2 and PA12 fibroblasts that lack PU.1 expression; lane 2, a coculture of Ψ2 and PA12 fibroblasts containing PU.1 cDNA in a retroviral expression vector; lane 3, IP/IR erythroleukemia cells; and lane m, rainbow [^{14}C]methylated protein molecular weight standards (Amersham Corp., Arlington Heights, IL). Markers represent the positions of protein standards and indicate their sizes in kiloDaltons. In the analysis in Panel B, some [^{125}I]protein A bound to the upper portion of the blot; this occurs to a variable extent and is due to immunoglobulin and Pansorbin components that occur in the samples. The arrow indicates the position of the PU.1 fusion protein from induced bacterial cultures.


A
1
2
3
69
46
Pu.1
30
B
1
2
3
m
46
30

The three major forms of PU.1 were most clearly resolved when they were labeled directly by incorporation of L-[^{35}S]amino acids. Figure 8 shows these three labeled PU.1 proteins recovered from a lysate of IP/IR erythroleukemia cells that was immunoprecipitated with PU.1-9794 antiserum (lane 2) but not by the preimmune serum (lane 1). Additional minor PU.1 components were also observed in the M_r 42,000 - 44,000 region of the gel. Although we have not identified causes for the major size differences between PU.1 proteins, we have found that all three major components can be labeled by intracellular incorporation of [^{32}P]orthophosphate (data not shown). None of the PU.1 proteins appear to be glycosylated as determined by adsorption to wheat germ agglutinin (10, 22); nor could they be precipitated from cell lysates by antiserum to ubiquitin (data not shown).

Indirect immunofluorescence microscopy was used to determine subcellular localization of PU.1 protein. Figure 9 shows results using murine fibroblast cultures in which the retroviral vector amplification was allowed to proceed until approximately 10% of the cells expressed PU.1 (see Materials and Methods). Preimmune rabbit serum did not label any of the cells (panel A). Moreover, normal murine fibroblasts lack PU.1 and could not be labeled by the PU.1-9794 antiserum (panel B). In contrast, this PU.1-9794 antiserum strongly labeled the nuclei in the PU.1-expressing fibroblasts (panel C). The presence of nonexpressing cells in these same microscopic fields served as an internal control that confirms the specificity of the nuclear staining. Although PU.1 also appeared to be intranuclear in F-MEL cells, an ideal negative control for the latter immunofluoresence analysis was unavailable because all F-MEL cells contain PU.1 (39, 40, 47).

Expression of PU.1 during leukemogenesis. PU.1 mRNA quantities are very low in normal erythroblasts compared with erythroleukemia cell lines (46). Figure 10 shows the relative abundances of PU.1 proteins which were immunoprecipitated from extracts of mouse spleens and from erythroleukemia cell lines. A small amount of PU.1 protein (M_r 42,000 component) was present in a 0.15 g normal spleen (lane 2); presumably

Figure 8. Protein analysis of PU.1 products. Cell lysates from 5 x 10^6 F745 erythroleukemia cells that were labeled with [^{35}S]methionine and [^{35}S]cysteine (see Materials and Methods) were precipitated with a 1:500 dilution of either preimmune rabbit serum (lane 1) or PU.1-9794 immune serum (lane 2). Immune precipitates were separated by SDS-8% polycrylamide gel electrophoresis and transferred to nitrocellulose. Markers represent the positions of the rainbow [^{14}C]methylated ovalbumin and carbonic anhydrase protein standards and indicate their M_rs (46,000 and 30,000, respectively).

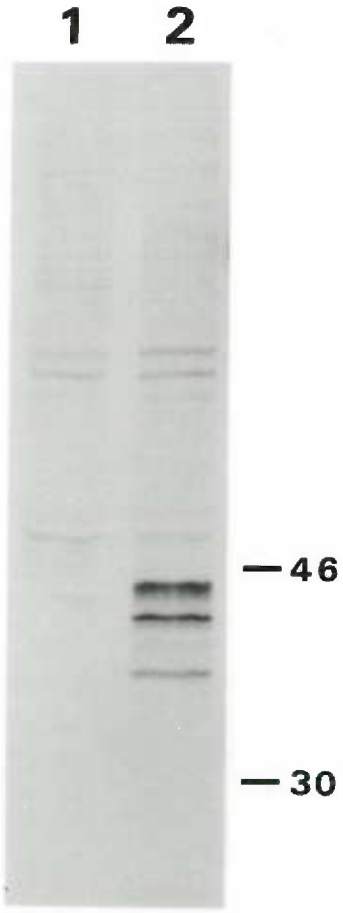
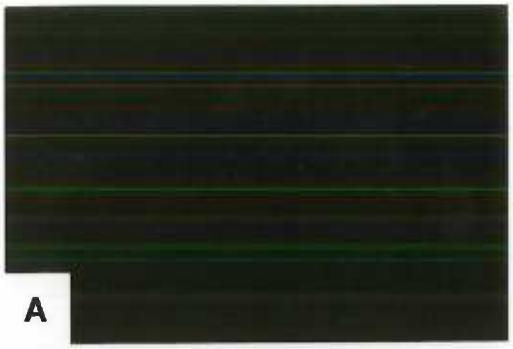
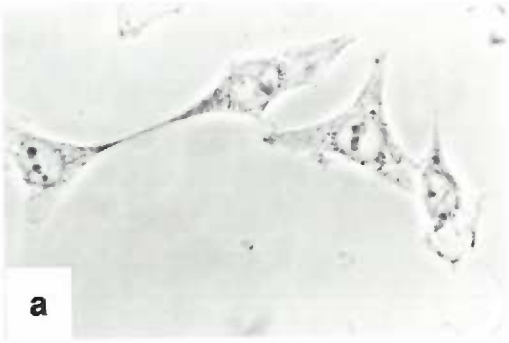
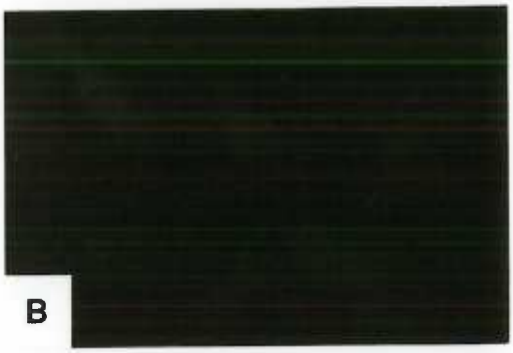
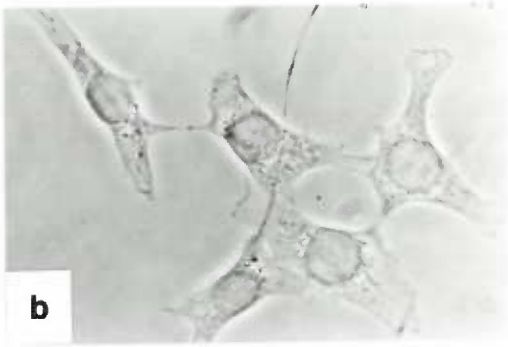

1
2
46
30

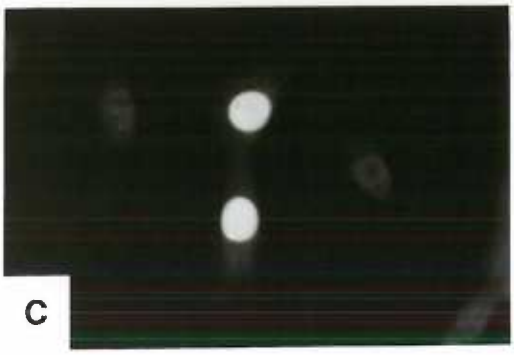
Figure 9. Nuclear localization of PU.1 by indirect immunofluorescent microscopy. Each microscopic field is shown in fluorescence (A,B,C) and in phase-contrast (a,b,c). Cells were incubated with preimmune rabbit serum (A,a) or PU.1-specific serum (B,b,C,c) followed by goat anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (see Materials and Methods). (A,a,C,c) cocultures of the Ψ2 and PA12 lines of mouse NIH3T3 fibroblasts expressing PU.1 from a retroviral vector. (B,b) a control coculture of Ψ2 and PA12 cells that lacks PU.1. Magnification is 400x.


A


a


B


b


C

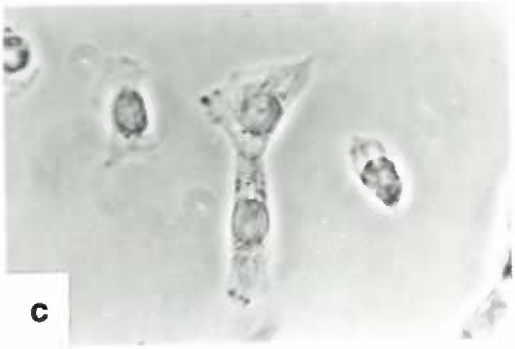
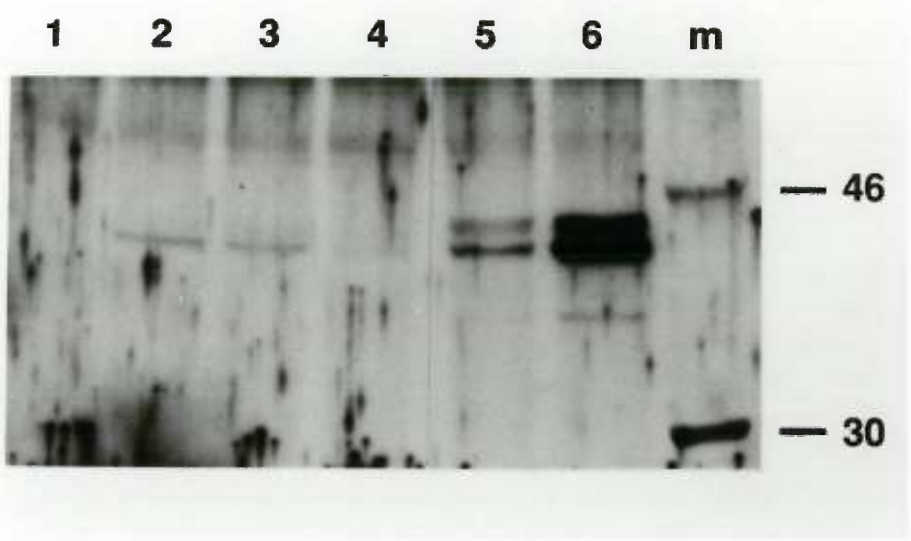

c

Figure 10. Western blot analysis of PU.1 protein levels during leukemic progression. Cell lysates (1 mg of total protein) from mouse spleens (lanes 2 through 4) and Friend erythroleukemia cell lines (lanes 5 and 6) were immunoprecipitated and immunoblotted with PU.1-specific antiserum as described in Materials and Methods. Lane 1, immune precipitation buffer treated with Pansorbin and antiserum but without cell lysate; lanes 2 through 4, lysates from a normal spleen, a spleen from a phenylhydrazine-treated mouse and a preleukemic spleen harvested 13 days after infection with Friend virus, respectively (see Materials and Methods for a more complete description); lanes 5 and 6, lysates from F745 and IP/IR erythroleukemia cells, respectively; lane m, rainbow [^{14}C]methylated protein molecular weight standards. Markers represent the positions of the ovalbumin and carbonic anhydrase protein standards and indicate their sizes (46 and 30 kDa, respectively).


1
2
3
4
5
6
m
46
30

this was in B lymphocytes and macrophages which are known to contain PU.1 mRNA (27). Anemia causes spleen enlargement due to immigration of proliferating and differentiating erythroblasts (19). A mouse that was recovering from phenylhydrazine-induced anemia had a mildly enlarged spleen (0.34 g) and this spleen (lane 3) contained slightly less PU.1 per mg of protein than the normal spleen. More significantly, much less PU.1 per mg of protein occurred in a greatly enlarged 3.3 g preleukemic spleen that was taken from a mouse 13 d after infection with Friend virus (lane 4). Since this enlargement is caused almost exclusively by proliferating and differentiating infected erythroblasts (70), this result implies that these cells lack or contain only trace amounts of PU.1 proteins. F-745 and IP/IR F-MEL cell lines (lanes 5 and 6, respectively) contain much more PU.1 than any of these spleens. The largest M_r 44,000 PU.1 component also occurs in a relatively higher proportion in the F-MEL cells.

PU.1 mRNA and protein levels decline during chemically-induced differentiation of F-MEL cells. Figure 11 (upper panels) shows changes in the abundance of PU.1 mRNA during the early phase of chemically-induced differentiation. PU.1 mRNA levels decline almost completely between 2 and 4 h and transiently reappear at about 5% of the initial level between 12 to 24 h post-induction. Similar changes in mRNAs for c-*myc* and c-*myb* were previously described (25, 30, 51). PU.1 mRNA levels remained very low after 48 h (data not shown). The decline in PU.1 mRNA levels occurred reproducibly whether differentiation was induced with Me_2SO or HMBA (panels A and B, respectively). Moreover, PU.1 mRNA levels declined and transiently reappeared with very similar kinetics for all of our F-MEL cell lines when either Me_2SO or HMBA was used to induce differentiation (data not shown).

Figure 12 shows the degradation of PU.1 mRNA following treatment of nondifferentiating IP/IR cells with actinomycin D in order to block new mRNA synthesis (64). Based on densitometric analysis, the half-life of PU.1 mRNA is approximately 2.0 h. This result was reproducible and similar to the half-life of the PU.1 mRNA measured in

Figure 11. Expression of PU.1 mRNA during chemically-induced differentiation. F745 erythroleukemia cells were cultured with 1.8% Me_2SO (panel A) or 5 mM HMBA (panel B) and RNA was isolated at the times indicated. 20 μg total cellular RNA was loaded in each lane. The upper panels represent RNA blot analysis performed with a radiolabeled fragment of *Sfpi*-1 cDNA (see Materials and Methods section). The middle panels represent the same blots hybridized with a radiolabeled mouse β-actin probe described in Materials and Methods. The lower panels show the filters stained with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5). Size markers are in kilobases and indicate the relative mobilities of the 28S and 18S ribosomal RNAs; hrs, hours.

F745 cells (data not shown). Thus, rapid degradation of PU.1 mRNA occurs normally and is not simply a consequence of adding chemicals that induce erythropoiesis.

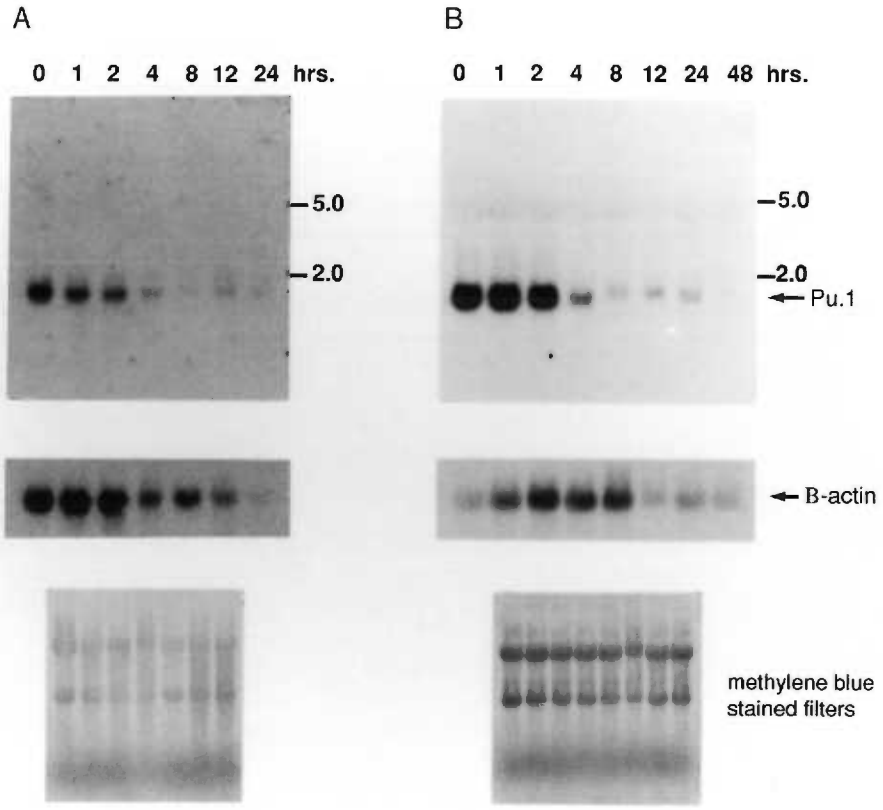
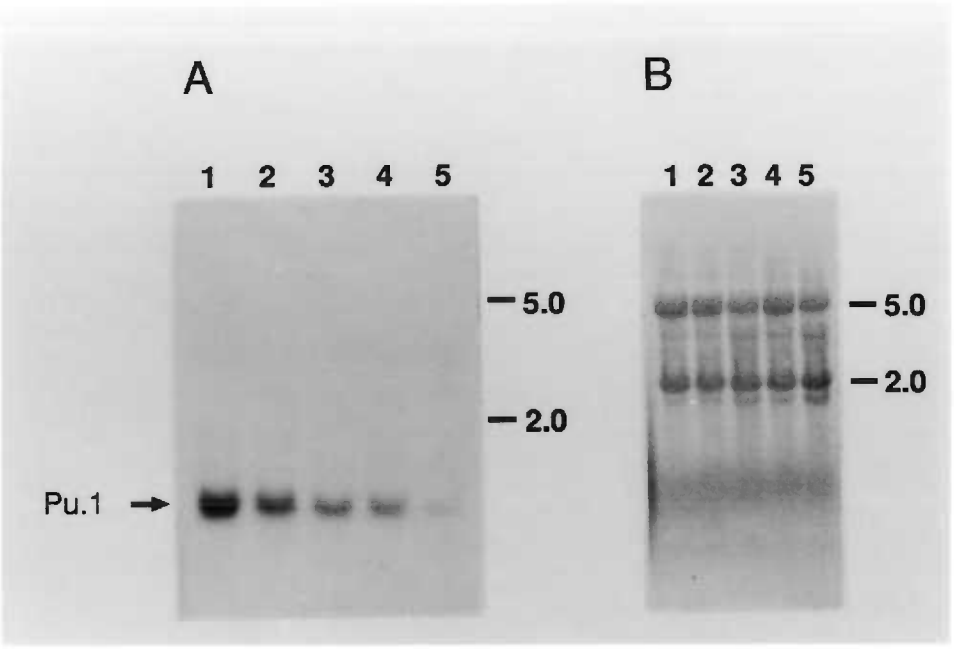
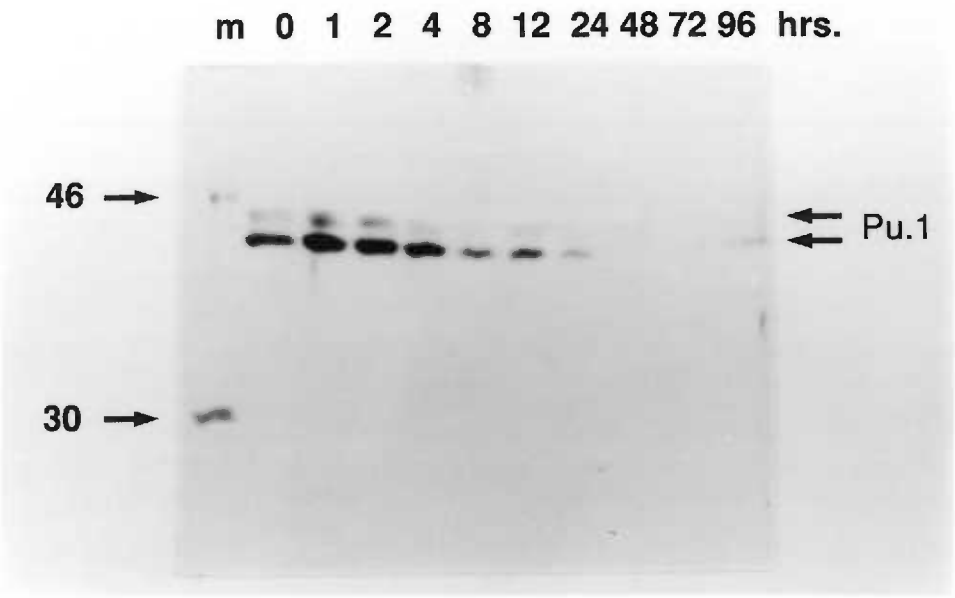

A
0 1 2 4 8 12 24 hrs.
5.0
2.0
B
0 1 2 4 8 12 24 48 hrs.
5.0
2.0
Pu.1
B-actin
methylene blue
stained filters

Figure 12. Northern blot analysis of PU.1 mRNA stability in IP/IR erythroleukemia cells. Actinomycin D was added to a final concentration of 5 μg/ml of media to cultures containing 1.5 x 10^6 cells/ml. RNAs were isolated at the following times after addition of the drug: lanes 1 through 5; 2, 3, 4, 5 and 7 h, respectively. 20 μg of total RNA was loaded in each lane. (A) The blot was hybridized with a radiolabeled *Sfpi*-1 probe. (B) The same blot was stained with methylene blue to visually inspect the RNAs for uniform transfer and integrity. Size markers are in kilobases and indicate the relative mobilities of the 28S and 18S ribosomal RNAs.


A
B
1 2 3 4 5
1 2 3 4 5
5.0
2.0
5.0
2.0
Pu.1

PU.1 proteins were also analyzed throughout the chemically-induced differentiation. Total protein synthesis in F-MEL cells treated with Me_2SO declines gradually to 75% of control levels by 48 h but remains constant thereafter (62). As shown in Figure 13, the amount of PU.1 in IP/IR cells cultured in the presence of Me_2SO remained unchanged for 1-2 h and then declined drastically to approximately 10% of the initial quantity by 8 h. PU.1 was undetectable after 24 h. On a much longer exposure of the protein blot in Figure 13, the form of PU.1 that migrated with an apparent M_r of 37,000 was also detected; it declined similarly to the other PU.1 components.

Figure 13. Immunoblot of PU.1 protein levels during Me_2SO-induced differentiation. IP/IR erythroleukemia cells were cultured in the presence of 1.8% Me_2SO and 5 x 10^6 cells were removed, washed in PBS and lysed in IPB at the times indicated. 900 μg of protein lysate from each sample was immunoprecipitated and immunoblotted with PU.1-specific antiserum as described in material and methods. Lane m, rainbow [^{14}C]methylated protein molecular weight standards. Markers show the positions of the ovalbumin and carbonic anhydrase protein standards. PU.1 forms that migrated with apparent M_rs of 44 and 42 kDaltons are indicated at the right by arrows; hrs., hours.


m 0 1 2 4 8 12 24 48 72 96 hrs.
46
30
Pu.1

DISCUSSION

This work provides an initial description of PU.1 protein and an analysis of its expression throughout leukemic progression and during the chemically-induced differentiation of cultured F-MEL leukemia cells. In all respects, our results are compatible with previous evidence (see Introduction; 39, 45, 65) that SFFV proviral integration adjacent to PU.1 may abrogate the commitment of erythroblasts to terminally differentiate and result in their immortal phenotype *in vivo*. Thus, our studies suggest that PU.1 proteins are absent or present in only trace quantities in normal erythroblasts or in SFFV-infected erythroblasts from preleukemic (nonimmortal) stages of Friend disease (e.g., see Fig. 10) but are abundantly expressed in permanent F-MEL cell lines. High expression occurs even in the few erythroleukemia cell lines that seem to lack *Sfpi*-1 proviral integrations (46); this suggests that PU.1 expression can be activated at a very low frequency by a mechanism that does not involve a provirus. Furthermore, chemically-induced differentiation of cultured F-MEL cells is associated with rapid degradation of PU.1 mRNA (Fig. 11) and protein (Fig. 13). Previous studies suggested that F-MEL cells recommit to the program of terminal differentiation between 12-24 h following treatment with chemical inducers (6, 18) and that this results in loss of immortality and inability of the cells to form tumors in mice (12). Commitment in this context is defined as the change(s) that occur while F-MEL cells are exposed to an inducer that renders differentiation irreversible even in its absence. Interestingly, this loss of leukemic properties of the cells appears to coincide with loss of PU.1. Thus, both induction of immortality during disease progression and its chemically-induced reversal in cell cultures appears to be associated with consistent changes in expression of PU.1 proteins.

Previous approaches to understanding Friend erythroleukemia have involved studies of oncogenes implicated in other cancers. These studies have shown that the proto-oncogenes c-*myc*, c-*myb*, K-*ras* and the nuclear protein p53 are expressed in large amounts

in Friend erythroleukemia cells compared to normal erythroblasts (25, 53), and that their expressions decline rapidly during chemically-induced differentiation (25, 51, 53, 60). Constitutive expression of c-*myc* (9, 11, 47) or c-*myb* (35) substantially inhibits cell differentiation. More detailed studies have shown, however, that constitutive expression of either c-*myc* (29) or c-*myb* (35) during the commitment phase of differentiation (up to 24 h post-induction) does not significantly impede differentiation, whereas constitutive expression during the post-commitment phase does. Therefore, commitment does not require loss of either c-*myc* or c-*myb*. Accordingly, suppression of either c-*myc* or c-*myb* expression does not cause spontaneous differentiation of F-MEL cells (48, 69).

Our results also demonstrate that PU.1 mRNA turns over relatively rapidly in F-MEL cells (Fig. 12). Rapid turnover rates have been reported for other *ets*-related transcription factors (13) and for other nuclear oncogenic mRNAs (49, 58) and proteins (54). However, PU.1 mRNA does not contain an AU-rich 3'-untranslated region nor the AU pentanucleotide consensus sequence that is partially responsible for rapid degradation of the c-*fos* transcript (47, 63).

We were surprised by the heterogeneity of PU.1 proteins and the existence of major components (e.g., M_r 42,000 and 44,000 forms) substantially larger than the full-length M_r 36,500 protein predicted from the cDNA sequence (see Figs. 7 and 8). This cannot be ascribed to an unusually slow electrophoretic mobility of the PU.1 protein because full-length PU.1 made in *E. coli* with a 15 amino acid extension on its amino terminus migrates as expected for a protein of M_r 38,000 (see Fig. 7). We have found that the PU.1 proteins are all phosphorylated and have obtained evidence that they are probably not glycosylated or ubiquitinylated. We are unaware of any studies that would indicate whether similar size modifications affect other *ets*-related proteins. Interestingly, the highest M_r 44,000 PU.1 component is relatively more abundant in F-MEL cells than in the RAW 264.7 macrophage cell line (unpublished results) or in normal spleen cells (e.g., see Fig. 10). Therefore, the structure of PU.1 is physiologically regulated.

Although our evidence is compatible with previous virological and pathogenic evidence that PU.1 may abrogate the commitment of erythroblasts to differentiate and lead to their immortality, additional studies will be required to thoroughly test this hypothesis. Recently, we have found that the PU.1-encoding retrovirus described here can immortalize erythroblasts in long-term murine bone marrow cultures (59).

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Manuscript #2

The Transcription Factor PU.1 is Hyperphosphorylated in Friend Erythroleukemia Cells in Contrast to Macrophages

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ABSTRACT

The PU.1 protein is an Ets-related transcription factor that is normally expressed in macrophages and B cells but is ectopically expressed in Friend virus-induced erythroleukemia. Four predominant species of PU.1, which migrate with apparent M_rs of 45,000, 44,000, 42,000 and 37,000 in sodium dodecyl sulfate-polyacrylamide gels, can be detected in metabolically labeled Friend erythroleukemia cells with PU.1-specific anti-serum. All of these forms of PU.1 are phosphorylated, exclusively on serine residues. Predicted casein kinase II serine target sites were mutated to alanines to determine the effect of inhibition of phosphorylation at specific sites on the mobility of PU.1 protein. Anaysis of the mutants demonstrated that the 45 kDa form results from phosphorylation of serines 41 and 45 and that PU.1 activity *in vitro* and *in vivo* requires the presence of both serine 41 and 45. Phosphorylation of PU.1 is differentially regulated in cells such that the 45 kDa form is much more abundant in Friend erythroleukemia cells than in macrophages. Futhermore, serum did not affect phosphorylation of PU.1 in Friend erythroleukemia cells but enhanced phosphorylation in macrophages. We propose that the oncogenic nature of PU.1 in Friend erythroleukemia cells may be partially due to the inability of these cells to negatively regulate PU.1 activity.

INTRODUCTION

A set of proto-oncogenes (*ets*, *myc*, *myb*, *fos* and *jun*) encoding nuclear DNA-binding proteins share specific properties including rapid turnover, phosphorylation and regulated expression in response to modulators of cell proliferation and differentiation (Bohman et al., 1987; Curran and Morgan, 1985; Fujiwara et al., 1988; Greenberg and Ziff, 1984; Kelly et al., 1983; Torelli et al., 1985). The transactivating function of certain transcription factors (e.g. p53, retinoblastoma gene product and Oct-1) is regulated by posttranslational phosphorylation of specific kinase target sites (Buchkovich et al., 1989; Ludlow et al., 1989; Meek et al., 1990; Roberts et al., 1991; Scheidtmann and Haber, 1990). In addition, phosphorylation can affect the affinity of transcription factors (e.g. E2F, CREB, Myb, Jun and Sp1) for DNA (Bagchi et al., 1989; Borellini et al., 1991; Boyle et al., 1991; Lüscher et al., 1990; Pulverer et al., 1991; Yamamoto et al., 1988) or the activity of bound factors (e.g. the yeast heat shock transcription factor and Oct-2) (Sorger and Pelham, 1988; Tanaka and Herr, 1990). The products of c-*ets*-1 and c-*ets*-2 are phosphorylated in chicken and in human cells (Leprince et al., 1988; Koizumi et al., 1990). Phosphorylation of Ets-1 and Ets-2 in T cells (probably via the mutifunctional Ca^{2+}-calmodulin-dependent protein kinase) occurs in response to mitogenic signals and results in loss of non-specific DNA-binding activity (Fujiwara et al., 1990; Pognonec et al., 1990). C-*ets*-1 is alternatively spliced in different tissues resulting in a p54 kDa or a p68 kDa protein, and the putative differential functions of the two distinct products is thought to be due to unique transactivation domains encoded by alternate 5' exons (Leprince et al., 1988; Schneikert et al., 1992).

The Ets superfamily of proteins contains over 12 family members of nuclear, sequence-specific DNA-binding proteins that share a homologous DNA binding domain but differ in putative transactivation domains (Karim et al., 1990; for a review see Macleod et al. 1992). The conserved DNA binding domain bears no structural homology

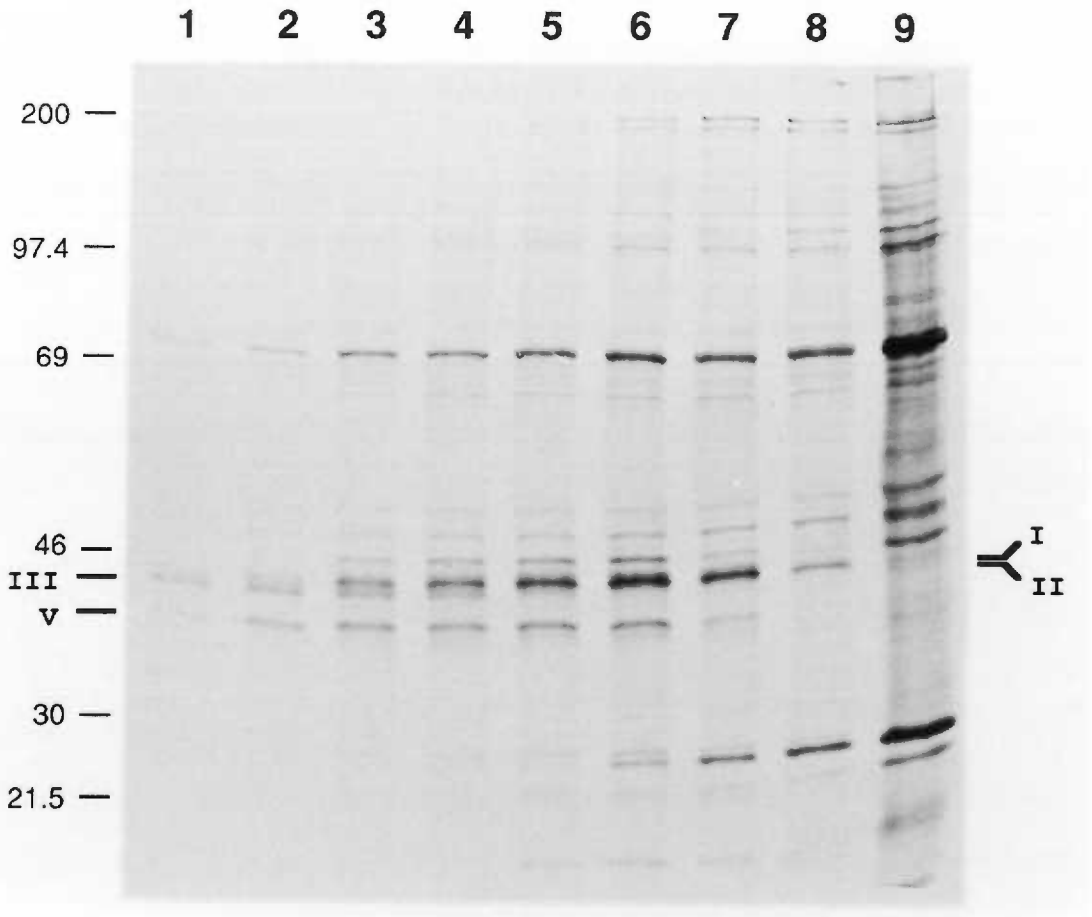
to other DNA-binding motifs of nuclear transcription factors such as the zinc-finger, helix-turn-helix, leucine zipper or homeodomain, but Ets-related proteins contain a series of tryptophan amino acids in conserved positions similar to the tryptophan cluster in the Myb protein (Karim et al., 1990).

PU.1 shows the least degree of sequence homology to c-*ets*-1 among the Ets-related proteins (Karim et al., 1990; Macleod et al., 1992). PU.1 is differentially expressed in mouse tissues and has been readily detected only in B cells, macrophages and the Friend erythroleukemias induced by the spleen focus-forming virus (Klemsz et al., 1991; Moreau-Gachelin et al., 1988; Paul et al., 1989). We previously identified multiple protein products of the PU.1 gene in erythroleukemia cells using a polyclonal antiserum specific for PU.1 (Schuetze et al., 1992). Although one product has a relative M_r of 37,000 which is consistent with the expected M_r based on the PU.1 cDNA sequence, the other predominant forms of PU.1 exhibit larger apparent M_rs. The nature of posttranslational modifications giving rise to these alternate PU.1 products have not been clearly defined, and the biologically important form(s) of PU.1 are unknown.

RESULTS AND DISCUSSION

We have extended our previously reported (Schuetze et al., 1992) characterization of PU.1 in an effort to determine the type(s) of modification of PU.1 precursor(s) and the relationship between protein modification and cellular regulation of PU.1 function in normal macrophages versus tumorigenic erythroleukemia cells. F-745 Friend erythroleukemia cells were pulse-labeled with L-[^{35}S]methionine and L-[^{35}S]cysteine and chased with cold medium to evaluate the precursor-product relationships between the various forms of PU.1. Cell lysates were immunoprecipitated with PU.1-specific polyclonal antibodies raised against bacterially expressed murine PU.1 protein. The results shown in fig. 14 indicated that two protein products with M_rs of 37, 000 (form V) and 42,000 (form III) are rapidly translated and appear simultaneously in erythroleukemia cells. Both forms of PU.1 can be detected within 10 minutes of labeling (fig. 14, lane 1). During chase of the radiolabeled proteins, the 42 kDa form quickly diminished in amount while levels of a larger 44 kDa protein (form II) increased. This result suggested that the 42 kDa protein is a precursor of the 44 kDa form of PU.1. A slightly larger 45 kDa protein (form I) was also detected after 60 minutes of labeling and continued to increase in amounts during the chase period; however, it is unclear whether this 45 kDa protein arose from the 44, 42 or 37 kDa precursor. A minor PU.1 protein of a 39.5 kDa mol. mass (form IV) is occasionally seen in anti-PU.1 immunoprecipitates of metabolically labeled cells (see below). 42 and 37 kDa proteins are initially synthesized in rabbit reticulocyte lysates from mRNA transcribed from PU.1 cDNA (C. Van Beveren, personal communication); thus the 42 kDa protein may be a cotranslationally modified form of PU.1. Previous comparison of PU.1 proteins in erythroleukemia cells, which were expressing endogenous PU.1, with PU.1 proteins expressed from cDNA in fibroblasts revealed that identical forms were produced in both types of cells (Schuetze et al., 1992). This implies that the 37 kDa and 42 kDa proteins are not the result of alternative splicing. Distinct Myc proteins, which differ in size, are generated in certain cells by initiation of

Figure 14. Pulse-chase protein analysis of PU.1. F-745 erythroleukemia cells were pulse-labeled with 100 μCi/ml of L-[^{35}S]methionine and L-[^{35}S]cysteine in methionine and cysteine-free Dulbecco's Modified Eagle Medium (DMEM) plus 10% dialyzed fetal bovine serum for 10, 20, 40, 60 min, and 2 hrs.. (lanes 1-4 and 9, respectively). After labeling, cells were washed free of medium containing radiolabeled amino acids and were chased in (DMEM) plus 10% fetal bovine serum for 15 min., 45 min., 2.5 hrs. and 7.5 hrs. (lanes 5-8, respectively). 5 x 10^6 cells per sample were lysed in ice-cold immune precipitation buffer and sheared through a 27 gauge syringe. Proteins were immunoprecipitated with a 1:500 dilution of anti-PU.1 serum (lanes 1-8) or preimmune serum (lane 9), extensively washed with immune precipitation buffer, electrophoresed in an 8.0% polyacrylamide-0.1% sodium dodecylsulfate gel and transferred to nitrocellulose as previously described (Schuetze et al., 1992). Size markers indicate the relative mobilities of rainbow ^{14}C-methylated protein standards (Amersham Corp., Arlington Heights, Ill.) and are expressed in kDa. Roman numerals mark the predominant forms of PU.1.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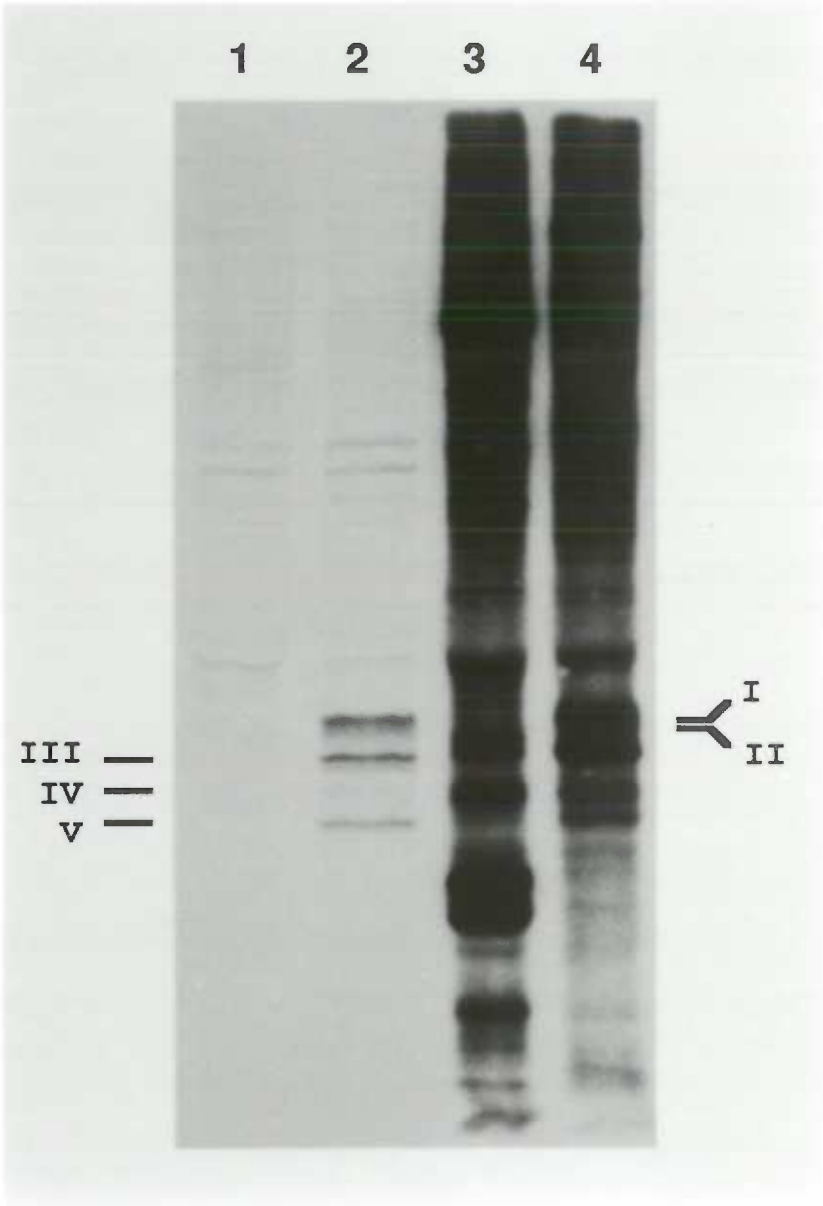
translation at alternative in-frame codons (Hann et al.,1988). We investigated the possibility that the higher M_r PU.1 proteins were due, in part, to translational initiation upstream of the putative ATG initiation codon at nucleotide 154 of the PU.1 cDNA (Paul et al., 1991). Expression of PU.1 cDNA lacking all of the sequence 5' of nucleotide 148 in fibroblasts resulted in PU.1 proteins identical in size to PU.1 in erythroleukemia cells (data not shown). Therefore, the higher M_r forms of PU.1 cannot be ascribed to initiation of translation at alternative upstream codons. PU.1 proteins were rapidly degraded over a few hours, and the kinetics of degradation were approximately the same for PU.1 forms II and V (see fig. 14).

Friend erythroleukemia cells were metabolically labeled with [^{32}P]ortho-phosphate or L-[^{35}S]methionine and L-[^{35}S]cysteine, and lysates were immuno-precipitated with PU.1-specific antiserum to determine if any of the PU.1 proteins are phosphorylated intracellularly. As shown in figure 15, forms V, III and II and/or I are all phosphoproteins; however, it is not clear whether phosphorylation alone accounts for the increase in M_r of forms I, II and III. Form IV may also be phosphorylated, but this is uncertain because a phosphoprotein that comigrates with form IV precipitated with preimmune serum (fig. 15, lanes 3 and 4). Phosphoamino acid analysis of $^{32}P_i$-labeled macrophages revealed that phosphate was exclusively bound to seryl residues in PU.1; radiolabeled phosphothreonine and phosphotyrosine were not detected (C. Van Beveren, personal communication).

The PU.1 protein sequence contains 9 serines within a consensus sequence [S*/T*-(D/E/S$^{\bullet}_{1-3}$)] (where the phosphoacceptor group* is followed by from 1 to 3 acidic residues in position(s) 1, 2, and/or 3; S$^{\bullet}$ is a phosphoserine) for potential phosphorylation by the ubiquitous nuclear protein kinase, casein kinase II (Kennelly and Krebs, 1991). Five of these serines are the mostly likely candidates for casein kinase II-mediated phosphorylation because an acidic residue is present in the third amino acid position carboxyl to the putative phosphoacceptor serine (Kuenzel et al., 1987). These serines lie

Figure 15. Immunoprecipitation of phosphorylated PU.1 proteins. 2.5 x 10^6 F-745 erythroleukemia cells were labeled for 2 hrs. with 100 μCi/ml of L-[^{35}S]methionine and L-[^{35}S]cysteine in methionine and cysteine-free DMEM plus 10% dialyzed fetal bovine serum (lanes 1 and 2) or were labeled for 4 hrs. with 500 μCi/ml of [^{32}P]orthophosphate in phosphate-free DMEM plus 10% dialyzed fetal bovine serum (lanes 3 and 4). [^{35}S]-labeled cells were then lysed in ice-cold immune precipitation buffer plus 1mM phenylmethylsulfonyl fluoride, and [^{32}P]-labeled cells were solubilized in modified RIPA buffer (50 mM Tris-HCL, pH 7.4, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 10 mM NaF, 4 mM EDTA, and 2 mM sodium vanadate)(Akiyama, 1988). Insoluble material was pelleted by ultracentrifugation for 35 min. at 40,000 x g. Supernatants were precleared with a 1:500 dilution of preimmune rabbit serum (lanes 1 and 3) and then immunoprecipitated with a 1:500 dilution of anti-PU.1 serum (lanes 2 and 4), electrophoresed in an 8.0% polyacrylamide-0.1% sodium dodecylsulfate gel and transferred to nitrocellulose. Roman numerals mark the predominant forms of PU.1.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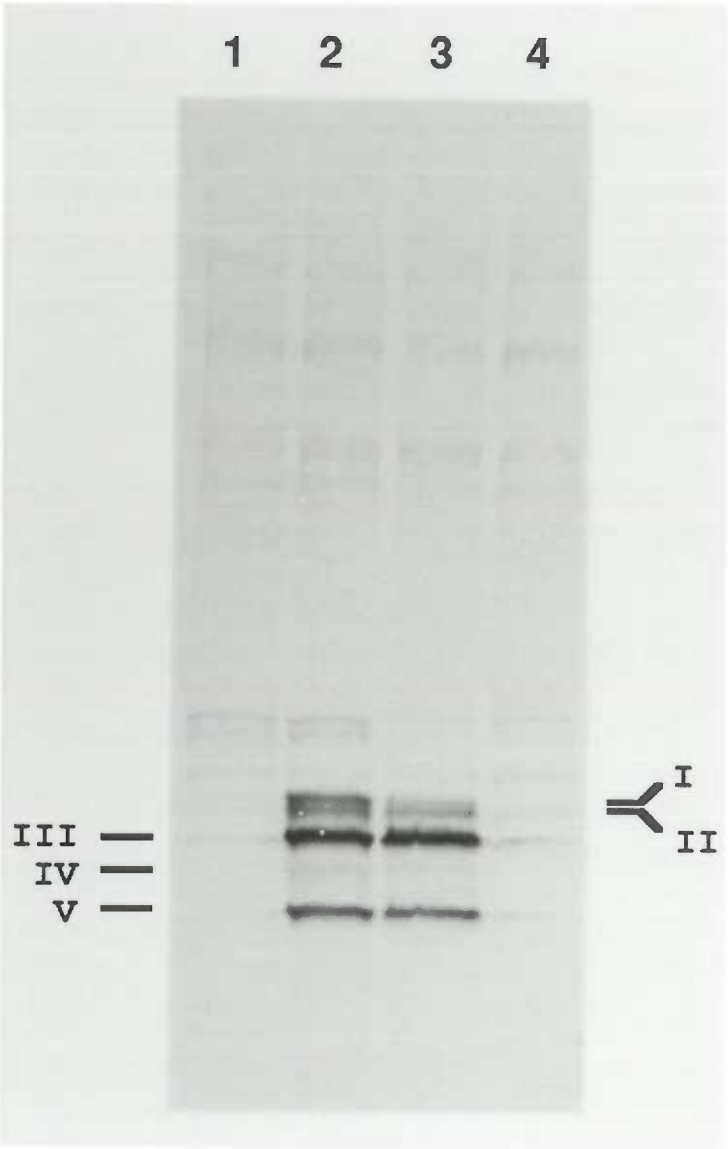
at amino acid positions 41, 45, 132, 133 and 148 in the PU.1 protein (Paul et al., 1991). Casein kinase II phosphorylates a broad range of nuclear transcription factors and nuclear oncogenic proteins including Myc (Lüscher et al., 1989), Myb (Krebs et al., 1988; Lüscher et al., 1990), Fos (Carroll et al., 1988), p53 (Meek et al., 1990), the adenovirus E1A protein (Carroll et al., 1988), the human papillomavirus E7 protein (Barbosa et al., 1990) and the SV40 large T antigen (Grasser et al., 1988; Krebs et al., 1988). Importantly, the activity of casein kinase II is significantly stimulated in response to several mitogens such as serum (Carroll and Marshak, 1989), the tumor promoting phorbol ester TPA (Carroll et al., 1988), insulin (Sommercorn et al., 1987; Klarlund and Czech, 1988) and epidermal growth factor (Sommercorn et al., 1987). Thus, casein kinase II mediated phosphorylation of transcription factors may play a crucial role in signal transduction to the nucleus. PU.1 protein expressed in bacteria can be labeled *in vitro* with casein kinase II and gamma-^{32}P-ATP and results in a phosphoprotein that comigrates with form V (Van Beveren, personal communication).

The following results stemmed from collaborative studies conducted by C. Van Beveren and R. Maki using anti-PU.1 serum generated by S. Schuetze (Van Beveren, in preparation). In order to study the role of phosphorylation in the regulation of PU.1 DNA binding and transcriptional activity, PU.1 cDNAs were selectively mutated at the 5 serine residues (ser to ala codon change) which are the most likely sites for casein kinase II-mediated phosphorylation. All of these serines lie outside of the DNA-binding domain (Klemsz et al., 1990). Expression of either the S41A or S45A mutant PU.1 constructs in COS cells failed to produce form I, and expression of a S41A and S45A double mutant in COS cells failed to produce both forms I and II. Furthermore, potato acid phosphatase treatment of [^{35}S]methionine labeled PU.1 proteins eliminated forms I, II and IV leaving forms III and V. Taken together, these results suggest that form I results from phosphorylation at both seryl residue 41 and 45, and form II results from phosphorylation at either serine 41 or 45. Alanine substitutions at serine positions 132 and 133 or 148 did

not significantly affect the M_rs of PU.1 proteins. Serine to alanine substitutions at positions 41 and/or 45 did not inhibit the ability of PU.1 to bind to its target DNA sequence nor its ability to translocate to the nucleus. However, mutations at serine 41 and/or 45 impaired the ability of PU.1 to transactivate expression of a reporter chloramphenicol acetyltransferase gene driven by a thymidine kinase promoter, and more important, suppressed proliferation of M-CSF stimulated bone marrow macrophages which express wild-type PU.1. Mutations at serine positions 132 and 133 or 148 had no effect on DNA binding, nuclear translocation, or growth of M-CSF stimulated macrophages. These results suggest that form I of PU.1 is biologically active and that cells may be able to regulate PU.1 activity via phosphorylation and dephosphorylation of PU.1. Different types of cells may differ in their abilities to regulate phosphorylation of form II or III (to form I), or dephosphorylation of form I.

To address this issue, the relative quantities of the various forms of PU.1 in macrophages were compared to the PU.1 proteins in Friend erythroleukemia cells. Cells were metabolically labeled with L-[^{35}S]methionine and L-[^{35}S]cysteine and equivalent amounts of radioactive trichloroacetic acid insoluble cell lysate were immunoprecipitated with PU.1 antiserum. A comparison of PU.1 proteins fractionated by SDS-PAGE shows that amounts of forms III, IV and V are approximately the same in IP/IR and RAW264.7 cells (fig. 16, lanes 2 and 3, respectively). Macrophage RAW264.7 cells, however, appear to express slightly lower levels of form II and substantially lower levels of form I than IP/IR erythroleukemia cells (fig. 16, lanes 3 and 2, respectively). This difference in the relative ratio of the different forms of PU.1 in RAW264.7 cells compared to IP/IR cells was seen in three independent analyses. Furthermore, the relative ratio of the different forms of PU.1 was similar in bone marrow macrophages compared to the RAW264.7 macrophage cell line (unpublished observation). The possibility that the quantity of form I in cells is partially regulated by serine phosphatase(s) was examined by C. Van Beveren. Treatment of murine bone marrow macrophages with calyculin A, an

Figure 16. Comparison of PU.1 in Friend erythroleukemia cells versus macrophages. IP/IR erythroleukemia cells (lanes 1 and 2) and macrophage RAW264.7 cells (lanes 3 and 4) were labeled for 2 hrs. with 100 μCi/ml of L-[^{35}S]methionine and L-[^{35}S]cysteine in methionine and cysteine-free DMEM plus 10% dialyzed fetal bovine serum, lysed in ice-cold immune precipitation buffer plus 1mM phenylmethylsulfonyl fluoride and ultra-centrifuged for 35 min. at 40,000 x g. Equivalent amounts of ice-cold 5% trichloroacetic acid insoluble counts of supernatant were immunoprecipitated with a 1:500 dilution of preimmune serum (lanes 1 and 4) or a 1:500 dilution of anti-PU.1 serum (lanes 2 and 3), electrophoresed in an 8.0% polyacrylamide-0.1% sodium dodecylsulfate gel and transferred to nitrocellulose. Roman numerals mark the predominant forms of PU.1.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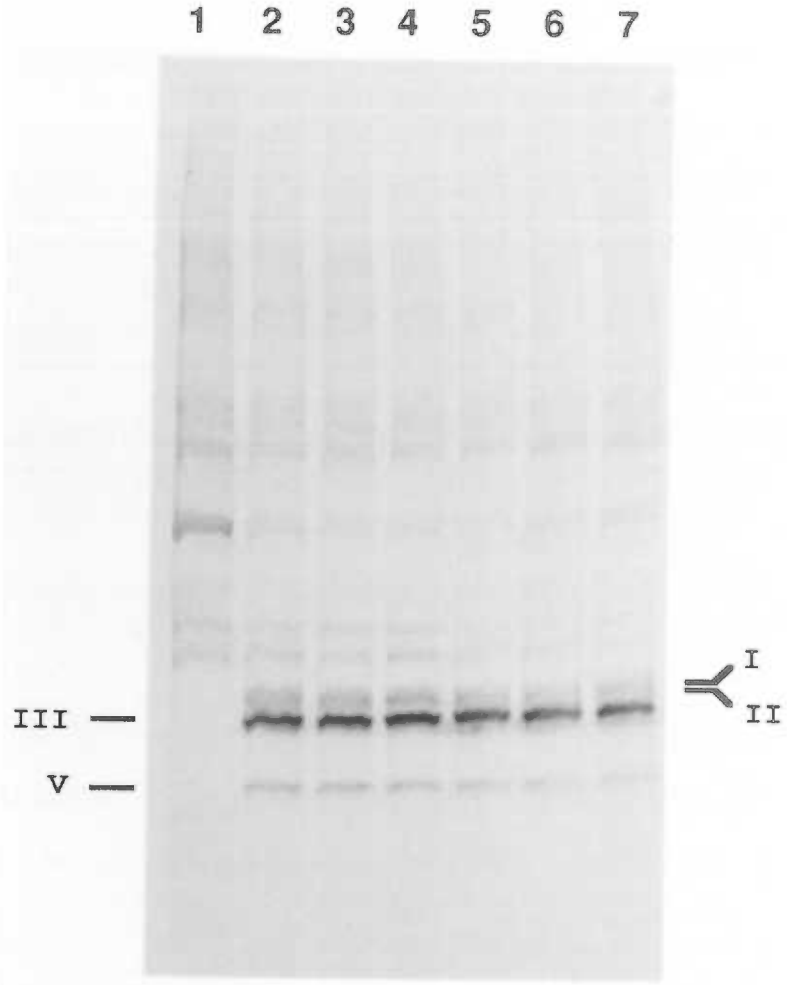
inhibitor of both protein phosphatase 1 and protein phosphatase 2A, produced a shift in PU.1 proteins to the hyperphosphorylated species (form I) in a dose-dependent manner. Okadaic acid, an inhibitor primarily of protein phosphatase 2A, has no discernible effect; therefore, levels of PU.1 form I may be directly or indirectly negatively regulated by a PP1-type activity. Further investigation of PU.1 processing in murine macrophages revealed that accumulation of form I is enhanced for up to 30 min. following the addition of fetal bovine serum to cells that were metabolically labeled with ^{32}P-orthophosphate in serum-free medium containing 0.4 mg/ml bovine serum albumin. The quantity of form I rapidly declined after 30 min. of serum stimulation. Interestingly, recombinant human CSF-1 poorly stimulated phosphorylation of PU.1 to form I in the absence of serum. The effects of serum on PU.1 processing in Friend erythroleukemia cells was also examined in a similar manner. IP/IR erythroleukemia cells were cultured for 4 hrs. in DMEM plus 0.4 mg/ml bovine serum albumin and then metabolically labeled with L-[^{35}S]methionine and L-[^{35}S]cysteine for 2 hours in the same medium. No discernible increase in the amount of form I was observed following stimulation with 10% fetal bovine serum in Friend erythroleukemia cells (figure 17, lanes 3-7 compared to lane 2). Rather, the radioactivity was gradually diminished in all PU.1 components as they were degraded during the cold chase. Likewise, serum stimulation did not alter the relative quantities of the PU.1 species in fibroblasts which were expressing PU.1 from a retroviral vector (data not shown). Thus, the ability of certain component(s) in serum to affect cellular regulation of PU.1 phosphorylation appears to be cell-type specific.

Studies by C. Van Beveren and R. Maki demonstrate that inhibition of phosphorylation at serines 41 or 45 prevents the appearance of the 45 kDa (form I) PU.1 protein and blocks the ability of PU.1 to activate transcription and to stimulate proliferation of macrophages. Furthermore, levels of the active, hyperphosphorylated form of PU.1 seem to be differentially regulated in specific cell types, and the relative amount of the active form I is substantially higher in Friend erythroleukemia cells than in

macrophages. Interestingly, phosphorylation of two serines in c-Jun occurs in response to serum via a c-Ha-Ras dependent pathway and is required for transcriptional activity (Smeal et al., 1991). Regulation of activity of transcription factors by phosphorylation/dephos-phorylation pathways appears to be a common mechanism of transcriptional control in response to extracellular signals. We propose that the oncogenic nature of PU.1 in Friend disease may be due, in part, to its expression in a cell type that lacks the phosphorylation/dephosphorylation controls that are operative in macrophages.

Figure 17. PU.1 protein modification is not affected by serum in Friend erythroleukemia cells. IP/IR erythroleukemia cells at a density of 10^6 cells/ml were cultured for 4 hours in DMEM plus 0.4 mg/ml bovine serum albumin fraction V (Sigma; St. Louis, Mo.) and then labeled for 2 hrs with 100 μCi/ml of L-[^{35}S]methionine and L-[^{35}S]cysteine in methionine and cysteine-free DMEM plus bovine serum albumin. After labeling, cells were washed free of radioactive amino acids, cultured in DMEM plus 10% fetal bovine serum for 0, 7.5, 15, 30, 60 and 90 min. (lanes 2-7, respectively) and then lysed in ice-cold immune precipitation buffer plus 1 mM phenylmethylsulfonyl fluoride. Samples were sheared through a 27 gauge syringe and equivalent amounts of 5% trichloroacetic acid insoluble counts of supernatant (approximately 1.5 x 10^6 cells per sample) were immunoprecipitated with preimmune serum (lane 1) or PU.1-specific antiserum (lanes 2-7). Precipitated proteins were fractionated on an 8.0% polyacrylamide-0.1% sodium dodecylsulfate gel and transferred to nitrocellulose. Roman numerals mark the predominant forms of PU.1.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Manuscript #3

The Ets-Related Transcription Factor PU.1 Immortalizes Erythroblasts but Kills Fibroblasts

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Running title: PU.1 Immortalizes Erythroblasts

SUMMARY

In vivo studies of Friend virus-induced erythroleukemia have suggested that proviral integrations adjacent to the gene for the Ets-related transcription factor PU.1 inhibit the commitment of erythroblasts to terminally differentiate and cause their immortality as indicated by their capability for indefinite transplantation [C. Spiro et al., J. Virol. 62, 4129-4135 (1988); R. Paul et al., J. Virol. 65, 464-467 (1991)]. To test the implications of these results, we ligated PU.1 cDNA into a retroviral vector and studied its effects on cultured cells. Infection of fibroblasts with PU.1-encoding retrovirus resulted in PU.1 synthesis followed by cell death. In contrast, infection of long-term bone marrow cultures caused specific immortalization of erythroblasts. The resultant cell lines contained PU.1, were morphologically blast-like, required erythropoietin and bone marrow stromal cells for survival and proliferation, and spontaneously differentiated at low frequency to synthesize hemoglobin. Frequency of differentiation was increased by treatment with 1% dimethylsulfoxide. Our results suggest that PU.1 perturbs pathway(s) that control potential for indefinite proliferation, with opposite effects on fibroblasts (killing) and erythroblasts (immortalization).

INTRODUCTION

Friend viral erythroleukemia provides an excellent model of multistage leukemogenesis (See Ben-David and Bernstein, 1991; and Kabat, 1989 for reviews). The replication-defective viral component, spleen focus-forming virus (SFFV), encodes a membrane glycoprotein gp55 that complexes with erythropoietin receptors (EpoR) to stimulate proliferation and differentiation of infected erythroblasts, thereby acting as an erythropoietin (Epo) agonist (Casadevall et al., 1991; Hoatlin et al., 1990; Li et al., 1990). However, erythroblasts normally have limited self-renewal capacities and are committed to differentiate. When treated with Epo or infected with SFFV, they proliferate until their replicative capacities are exhausted, and then they differentiate to form small hemoglobinized colonies (Hankins et al., 1978). Accordingly, virus infection initially causes polyclonal proliferation of SFFV-infected erythroblasts that terminally differentiate (Ben-David and Bernstein, 1991; Kabat, 1989; Spiro et al., 1988). During this initial polyclonal phase of disease, mice infected with a mixture of replication-competent helper murine leukemia virus (MuLV) plus SFFV contain neoplastic cells that are not transplantable, and the disease is maintained by continual infection of newly-formed erythroblasts (Hankins and Troxler, 1980; Spiro et al., 1988; Wendling et al., 1981).

Within several weeks of infection, in a second stage of disease, erythroleukemia cells of clonal origin begin to increase in proportion in the neoplastic population. These second-stage cells have unlimited self-renewal capacities, do not differentiate *in vivo*, can be transplanted indefinitely in syngeneic hosts, and in almost all cases contain SFFV proviral integrations in a single chromosomal site (*Sfpi*-1) adjacent to the gene for the Ets-related transcription factor PU.1 (Moreau-Gachelin et al., 1988; Paul et al., 1989; Spiro et al., 1988).

Evidence that proviral integrations in *Sfpi*-1 abrogate the commitment of erythroblasts to differentiate and cause their immortality was obtained using helper-free

SFFV (Spiro et al., 1988). Nearly all mice inoculated with such virus develop polyclonal erythroblastosis followed by mild polycythemia and complete recovery by 10-14 d post-infection. Such recovery is compatible with the limited replicative capacity of erythroblasts and with their commitment to differentiate. These first-stage cells contain proviruses integrated at essentially random positions in their genomes (Spiro et al., 1988). However, a few mice infected with large doses of helper-free SFFV do not completely recover. They develop monoclonal splenic leukemias that weigh several grams by 30 d post-inoculation (Spiro et al., 1988). These leukemic cells do not differentiate *in vivo*; they are transplantable; and they contain proviral integrations in *Sfpi*-1 (Spiro et al., 1988; Paul et al., 1989). Thus, the immortalization of single erythroblast clones correlates precisely with *Sfpi*-1 proviral integration events *in vivo*.

Unlike normal erythroblasts, immortal Friend erythroleukemia cells synthesize PU.1 mRNA and proteins (Moreau-Gachelin et al., 1990; Paul et al., 1991; Schuetze et al., 1992). When treated with chemical inducers that overcome their blocked differentiation, PU.1 mRNA and proteins are rapidly degraded before the cells lose their immortality and recommit to the program of erythropoiesis (Friend et al., 1971; Marks and Rifkind, 1978; Schuetze et al., 1992). Interestingly, other *ets*-related genes have been implicated in erythroleukemias induced in newborn mice by the Friend strain of MuLV in the absence of SFFV (Ben-David et al., 1991; Ben-David et al., 1990), and in chickens by the E26 virus (Moscovici et al., 1981; Radke et al., 1982).

Evidently, additional genetic changes can also contribute to malignant progression of Friend erythroleukemias. Mutations and proviral integrations often occur in the p53 gene of advanced Friend erythroleukemias (Chow et al., 1987; Mowat et al., 1985; reviewed in Ben-David and Bernstein, 1991). Mice with a dominant negative mutant p53 transgene develop advanced Friend erythroleukemias at an accelerated rate; yet most of these leukemias also have rearrangements in *Sfpi*-1 (Lavigueur and Bernstein, 1991). Also observed have been proviral integrations in the EpoR gene (Hino et al., 1991),

chromosomal rearrangements (Miller et al., 1979; Ostertag et al., 1972), and amplifications of the c-*myc* gene (Gliniak and Kabat, unpublished results). Conceivably, these and other genetic changes might facilitate rapid cell proliferation in different sites *in vivo* or erythroblast growth autonomous of hematopoietic stroma or of growth factors *in vivo* or in culture.

We have attempted to study these issues in long-term bone marrow cultures. In these cultures, adherent bone marrow stroma consisting of endothelial cells, adipocytes, fibroblasts and macrophages form an environment for proliferation and differentiation of hematopoietic stem cells (Dexter et al., 1977; Allen and Dexter, 1976). While granulopoiesis persists for many months, megakaryopoiesis progressively declines and erythropoiesis becomes blocked at the early burst-forming erythroblast (BFU-E) stage. Addition of Epo to long-term marrow cultures, however, stimulates BFU-E maturation and can restore complete erythropoiesis (Eliason et al., 1979). We found that infection of mouse bone marrow cultures with a retrovirus that encodes PU.1 results in sustained proliferation of Epo-dependent primitive erythroblasts. These immortal cells differentiate spontaneously at a low frequency and require bone marrow stroma for proliferation and viability. In contrast, PU.1 expression in fibroblasts causes the opposite effect, abrogation of immortality and cell death.

RESULTS

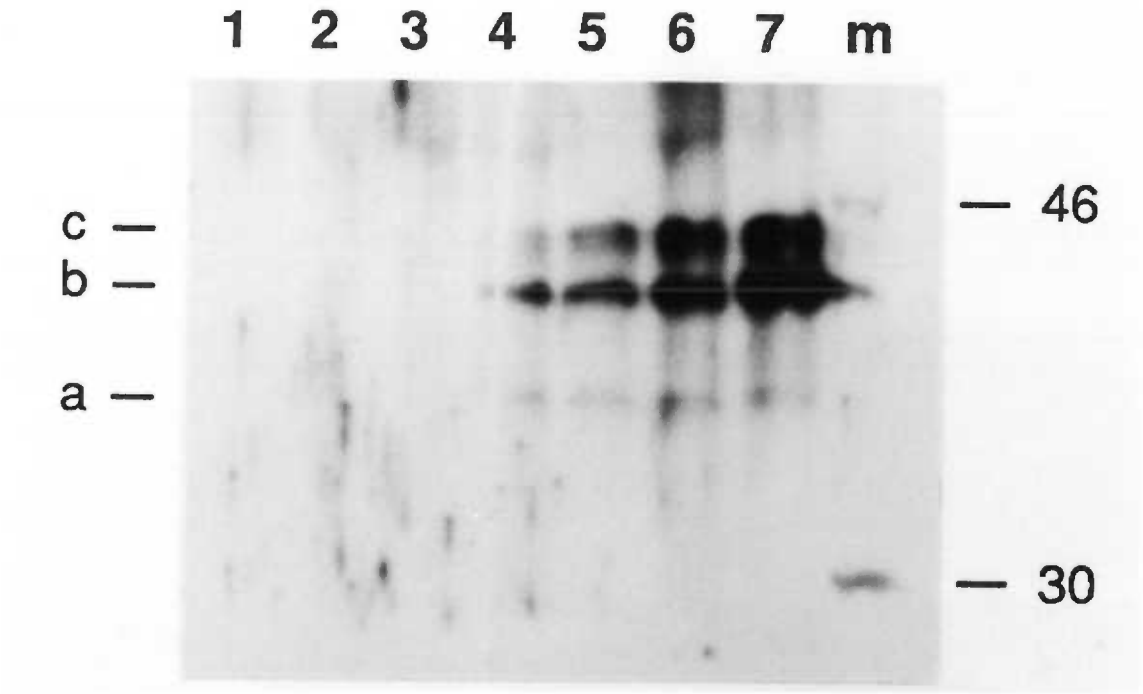
PU.1 Expression Kills Mouse Fibroblasts.

After certain retroviral vectors [e.g., pSFF (Bestwick et al., 1988)] are transfected into cocultures that contain cells for packaging retroviruses into distinct host-range envelopes [e.g., the Ψ2 and PA12 derivatives of mouse NIH/3T3 fibroblasts (Mann et al., 1983; Miller et al., 1985)], the encoded viruses become amplified by a potentially limitless back-and-forth (ping-pong) process of infection (Bestwick et al., 1988; Kozak and Kabat, 1990). For example, ecotropic host-range virions released from Ψ2 cells can infect PA12 amphotropic packaging cells without interference, and *vice-versa*, thus generating vector amplification in the absence of replication-competent helper virus (Bestwick et al., 1988). As illustrated in Fig. 18 (lane 1), PU.1 proteins are absent in control fibroblasts. However, following transfection of the pSFF-PU.1 expression vector into a Ψ2/PA12 coculture, PU.1 proteins of Mr 37,000, 42,000, 44,000 and 45,000 were evident by 6 days (lane 4). By 10 and 14 days (lanes 5 and 6, respectively), these PU.1 proteins were present in amounts similar to the highly expressive (Schuetze et al., 1992) IP/IR line of Friend erythroleukemia cells (lane 7). Immunofluorescence microscopy of the same cocultures used in Fig. 18 indicated PU.1 presence in 8.5%, 16%, and 28% of the cells at 6, 10, and 14 days post-transfection, respectively. The PU.1 components with different apparent Mr are all phosphorylated on serines, and their relative proportions are subject to rapid physiological control (Schuetze et al., 1992; Van Beveran et al., 1992). As expected, PU.1-encoding virions were present in the media of cocultures at 10-14 days post-transfection (see below).

Unexpectedly, cell morphology changed in cocultures that were synthesizing PU.1, and large quantities of cells and debris lifted into their media; these floating cells did not grow in liquid cultures or in soft agar. A substantial proportion of cells that remained in the PU.1-expressing fibroblast monolayers also had reduced adherence to the substratum and a

Figure 18. Amplification of PU.1 expression in murine fibroblast cocultures.

PU.1 protein levels were analyzed using PU.1-specific serum. 1 mg of total protein from each cell lysate was immunoprecipitated, electrophoresed in a 0.1% SDS-8.0% polyacrylamide gel and immunoblotted as described in Experimental Procedures. Lanes 1, 14 day control coculture of the Ψ2 and PA12 lines of mouse NIH/3T3 fibroblasts which lack a pSFF-PU.1 expression vector; 2-6, fibroblasts from a coculture harvested 3, 5, 6, 10 and 14 days posttransfection of a pSFF-PU.1 retroviral vector, respectively; 7, IP/IR erythroleukemia cells; and m, rainbow ^{14}C-methylated protein molecular weight standards (Amersham Corp., Arlington Heights, IL). Markers indicate the sizes of protein standards in kilodaltons. a, b and c mark the predominant forms of PU.1 which migrate with apparent M_rs of 37,000, 42,000, 44,000 and 45,000. c denotes the 44/45 kDa doublet. The percentage of fibroblasts positive for PU.1 expression as detected by indirect immunofluorescent staining at 6, 10 and 14 days posttransfection were 8.5, 16 and 28, respectively.

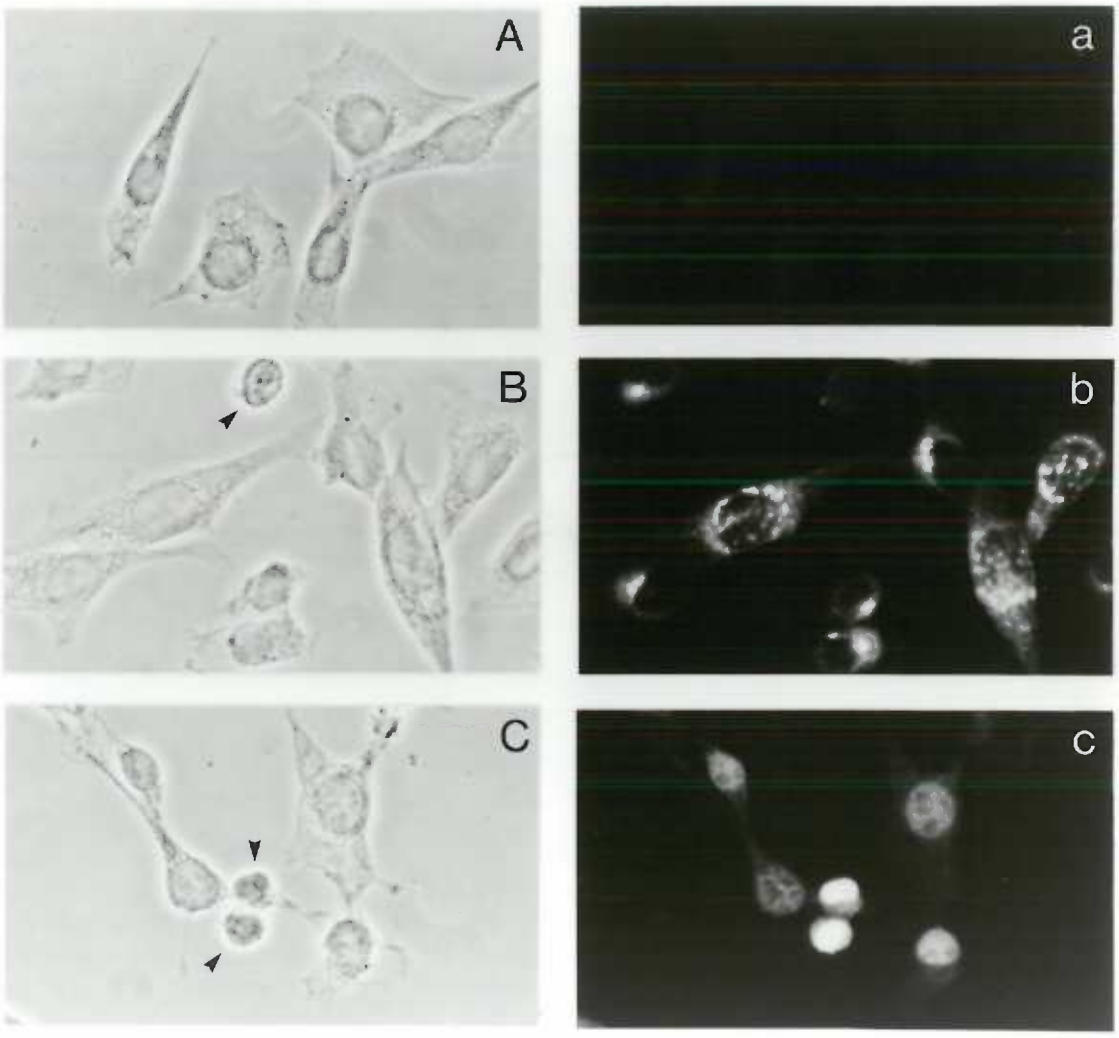

1
2
3
4
5
6
7
m
c
b
a
46
30

relatively rounded morphology, with small pyknotic nuclei (see cells marked with arrowheads in Fig. 19, panel C). These loosely adherent rounded cells, which were clearly selected against by standard methods for passaging fibroblasts, increased to percentages as high as 50% in some cocultures that were highly active in PU.1 synthesis. Such rounded cells were relatively rare (c.a., 5-10%) in control Ψ2/PA12 cocultures (Fig. 19, panel A) or in Y2/PA12 cocultures that had amplified pSFF-hGH and were actively synthesizing growth hormone (Fig. 19, panel B). In the latter case, there was no correlation between cell rounding (see arrowhead marker in panel B) and synthesis of growth hormone (see panel b). However, immunofluorescence analysis of the cocultures synthesizing PU.1 clearly showed a strong correlation between cell rounding with nuclear pyknosis and high levels of PU.1 (e.g., see Fig. 19, panels C, c). This correlation was not absolute, however, apparently because the process of infection and rounding is continuous, because it may take time for rounding to develop, and because cells with small amounts of PU.1 may also lose adherence slowly and lift into the medium.

Consistent with our interpretation that PU.1 expression causes cell rounding and release from the substratum, the percentages of cells that were round and that expressed PU.1 both declined substantially after approximately 14-20 days. By 60 days post-transfection, the cocultures appeared adherent and only 10% or fewer cells contained small amounts of PU.1 proteins. PU.1-encoding virus also appeared to be relatively scarce by this time. Attempts to isolate cell clones that stably express PU.1 were essentially unsuccessful, although a few Ψ2 or PA12 fibroblast clones expressed trace amounts that are detectable by RNA slot blotting. Similarly, we were unable to isolate NIH/3T3 or rat 208 F fibroblast transfectants that stably expressed PU.1. These studies suggest that PU.1 causes loss of adherence, nuclear pyknosis, and death of NIH/3T3 fibroblasts. The mechanism of cell death is difficult to study because it occurs asynchronously in the cultures; however, it has the morphological characteristics of apoptosis (Bursch et al., 1990; Wyllie, 1987).

Figure 19. Indirect immunofluorescence analysis of PU.1 expression in murine fibroblasts exhibiting altered cellular morphology.

Micrographs were taken on the 14th day of retroviral ping-pong amplification in 1:1 cocultures of the Ψ2 and PA12 lines of mouse NIH/3T3 fibroblasts. Each microscopic field is shown in phase-contrast (A, B and C) and in fluorescence (a, b and c). Cells were incubated with PU.1-specific serum (A, a, C and c) or human growth hormone-specific serum (B and b) followed by goat antirabbit immunoglobulin conjugated to fluorescein isothiocyanate (see Experimental Procedures). (A and a) control coculture which lacks a retroviral expression vector. Coculture expressing human growth hormone. (B and b) or PU.1 (C and c) from a pSFF-based retroviral vector. Cells with a rounded and condensed shape (arrowheads) are morphologically atypical compared to normal fibroblasts.

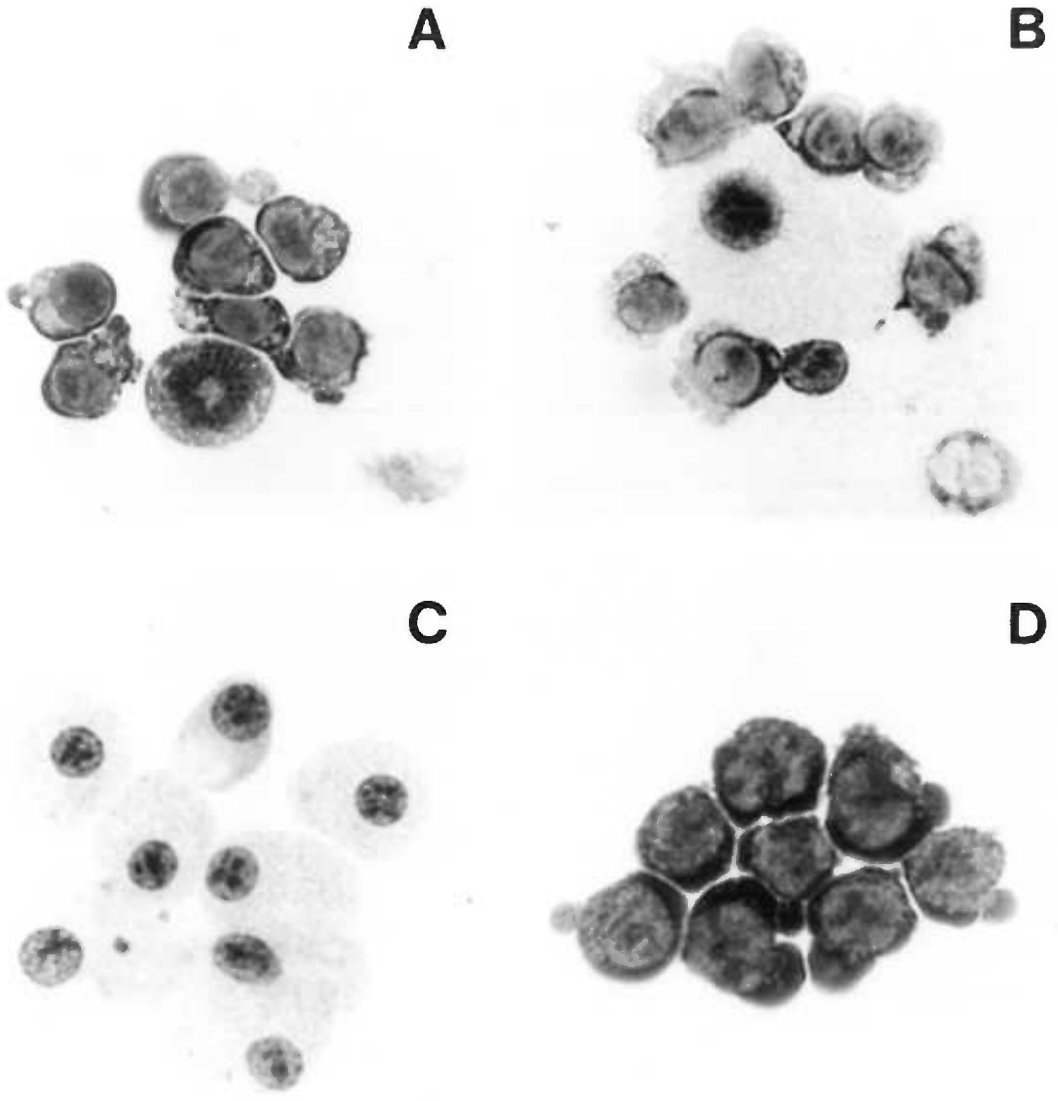

A
a
B
b
C
c

Effects of PU.1 Expression in Long-Term Mouse Bone Marrow Cultures.

To study effects of PU.1 in hematopoietic cells, long-term bone marrow cultures were infected with SFF-PU.1 virus, either in the presence or absence of a replication-competent helper MuLV. Within 2-3 weeks post-infection, those cultures that were supplemented with Epo developed an obvious preponderance of small blast-like cells (see Fig. 20, A) that were essentially absent from infected cultures lacking Epo or from control cultures in the presence or absence of Epo that had been mock-infected or infected only with MuLV. By 8 weeks, these small blast-like cells formed a thick confluent layer of weakly attached and unattached cells that overlay the adherent stromal cells, and they could be harvested every few days when the media were replaced on the stroma (see Experimental Procedures). Interestingly, they morphologically resembled Friend erythroleukemia cells; although, the mononuclear blasts are slightly smaller in general (Fig. 20, compare panel A to D). Approximately 5% of the cells in these harvests appeared to be macrophages in intimate association with the small blast-like cells (Fig. 20 B). On the contrary, in control cultures or in cultures lacking Epo, hematopoiesis was confined to sparse, small islands and the stromal layers were mostly exposed. In these cultures, larger cells resembling macrophages gradually became predominant (see Fig. 20 C).

The above results suggested that the small blast-like cells in the cultures infected with SFF-PU.1 virus might require Epo for viability and proliferation. Accordingly, when removed from their marrow stroma and plated on a fixed NIH/3T3 feeder layer (see below) in the absence of Epo, at least 97% of the blast-like cells died within 24-48 h as indicated by loss of ability to exclude trypan blue dye. In the same conditions supplemented with Epo, the cells continued to grow and remained substantially (c.a., 80%) viable. Erythropoietin dependence of the mononuclear blasts was also assessed by measuring ^{3}H-thymidine incorporation in cells grown in medium containing various concentrations of erythropoietin. Cells that were cultured in the presence of 0.1 U/ml or more of Epo

Figure 20. Photomicrographs of cells from murine long term bone marrow cultures. Epo-dependent cells derived from (A) helper-free pSFF-PU.1 virus or (B) pSFF-PU.1 virus plus F-MuLV infected bone marrow cultures. (C) cells from a F-MuLV infected bone marrow culture, and (D) IP/IR erythroleukemia cells. Cytocentrifuged preparations (500 x g for 5 minutes) of cells were fixed and stained with Wright-Giemsa. The larger cells with abundant cytoplasm resemble macrophages and were also observed in-uninfected bone marrow cultures.


A
B
C
D

incorporated approximately five times the amount of thymidine than cells grown without Epo (data not shown). Other growth factors [interleukin 3 (IL-3), stem cell factor (kit ligand), GM-CSF, WEHI-3 conditioned medium, spleen cell conditioned medium] could not supplant the Epo requirement of these cells.

We then tested these small blast-like cells for presence of PU.1 proteins. Fig. 21 shows an immunofluorescence analysis of the nonadherent cells from a bone marrow culture that had been infected with SFF-PU.1 virus and then incubated with Epo. Clearly, the nuclei of the small blast-like cells bound the PU.1-specific (Schuetze et al., 1992) antiserum (panels B, b) but not the preimmune control serum (panels A, a). The macrophage-like cells in these cultures bound some immunoglobulins nonspecifically (panels A, a), perhaps to their Fc receptors (Kurlander and Batker, 1982). However, as reported previously for normal macrophages (Klemsz et al., 1990), they also contained a detectable amount of intranuclear immunoreactive PU.1 (panels B, b).

Fig. 22A shows a Northern blot analysis of RNA extracted from cultured cells. RNA that hybridized to our PU.1 cDNA probe was absent from mouse fibroblasts or human K562 erythroleukemia cells (lanes 1 and 2, respectively), but was present in nonadherent cells harvested from a bone marrow culture that had been infected with SFF-PU.1 virus and maintained with Epo (lane 3). These PU.1-specific RNAs included components with sizes (Mr ~ 6.2 kb and 2.9 kb) expected for genomic and spliced subgenomic transcripts of the SFF-PU.1 provirus. In contrast, immortal Friend erythroleukemia cells contain a 1.5 kb mRNA encoded by the endogenous PU.1 gene (lanes 4 and 5). This endogenous 1.5 kb transcript is not detected in RNA extracted from the bone marrow cultures (lane 3), presumably because it occurs in relatively small amounts.

Electrophoretic mobilities and quantities of PU.1 proteins were indistinguishable in preparations derived from nonadherent populations of bone marrow cultures and from Friend erythroleukemia cells (Fig. 22, panel B, lanes 3 and 4, respectively). As shown

Figure 21. Indirect immunofluorescent staining of PU.1 in Epo-dependent cells derived from pSFF-PU.1 virus infected bone marrow culture.

Each microscopic field is shown in phase contrast (A and B) and in fluorescence (a and b). Cells in suspension were gently harvested from supporting fixed and adherent NIH/3T3 fibroblast monolayers, cytocentrifuged and incubated with either preimmune serum (A and a) or PU.1-specific serum (B and b). Bound antibodies were labeled with goat antirabbit immunoglobulin conguated to fluorescein isothiocyanate. The large cells with abundant cytoplasm resemble macrophages, are not dependent on erythropoietin and are also pervasive in F-MuLV infected and uninfected long term bone marrow cultures.

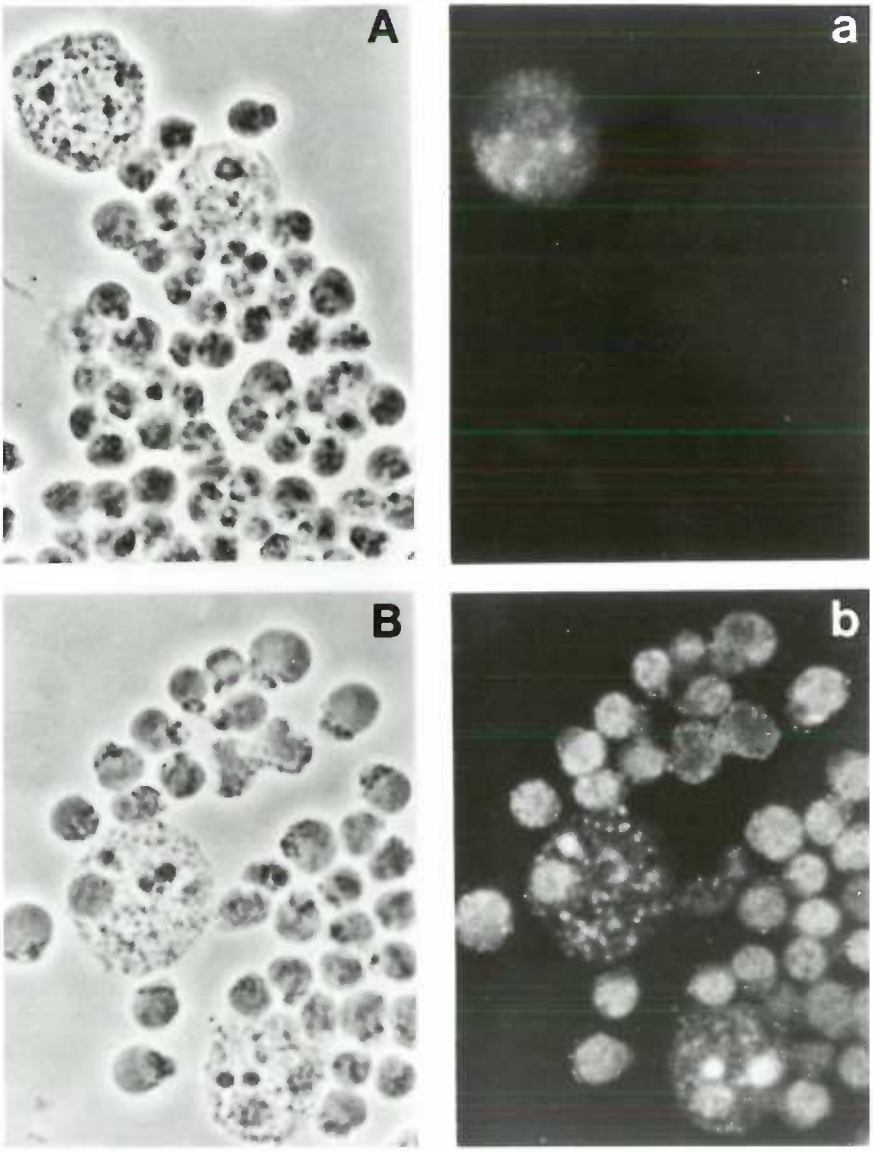
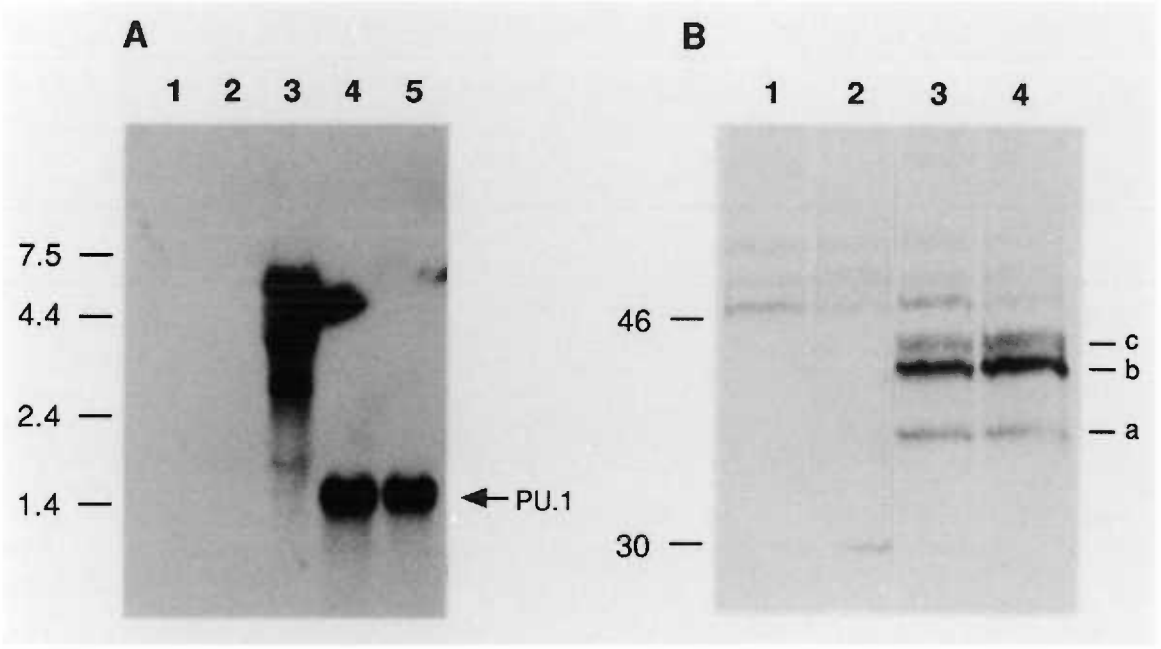

A
a
B
b

Figure 22. Analysis of PU.1 expression in Epo-dependent cells derived from a pSFF-PU.1 virus infected bone marrow culture.

(A) Northern blot analysis of PU.1 encoding transcripts. Five micrograms of poly(A)-selected RNA (lanes 1, 2, 4 and 5) and 20 micrograms of total RNA (lane 3) were electrophoresed through a 1.2% agarose gel, transferred to nylon membrane and hybridized to a radiolabeled *Sfpi*-1 probe. Lane 1, PA12 fibroblasts; Lane 2, human K562 erythroleukemia cells; Lane 3, Epo-dependent, pSFF-PU.1 virus infected cells; Lane 4, M-7 erythroleukemia cells; and Lane 5, IP/IR erythroleukemia cells. Size markers are in kilobases and represent the relative mobilities of a 9.5-0.24 kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD). The expected position of endogenous PU.1 transcripts is shown (arrow). (B) Protein analysis of PU.1 products in Epo-dependent, pSFF-PU.1 virus infected cells (lanes 1 and 3) versus IP/IR erythroleukemia cells (lanes 2 and 4). Equivalent amounts of acid insoluble radioactive counts in lysates of [^{35}S]methionine- and [^{35}S]cysteine-labeled cells were immunoprecipitated with 1:500 dilution of either preimmune rabbit serum (lanes 1 and 2) or PU.1 immune serum (lanes 3 and 4) and were electrophoresed through a 0.1% SDS-8.0% polyacrylamide gel. Left-sided markers represent the positions of the rainbow ^{14}C-methylated ovalbumin and carbonic anhydrase protein standards and indicate their respective M_r (46,000 and 30,000). a, b and c mark the predominant forms of PU.1 which migrate with apparent M_r of 37,000, 42,000, 44,000 and 45,000. c denotes the 44/45 kDa doublet.


A
1 2 3 4 5
7.5
4.4
2.4
1.4
PU.1
B
1 2 3 4
46
30
c
b
a

previously, component a has the same size (Mr ~ 37,000) as PU.1 protein made in bacteria, whereas components b and the doublet c (Mr ~ 42,000 and ~ 44,000/45,000) are substantially larger, in part due to their more extensive phosphorylation (Van Beveran et al., 1992; Schuetze et al., 1992). The Mr 45,000 component of doublet c occurs in barely detectable amounts in macrophages (our unpublished results).

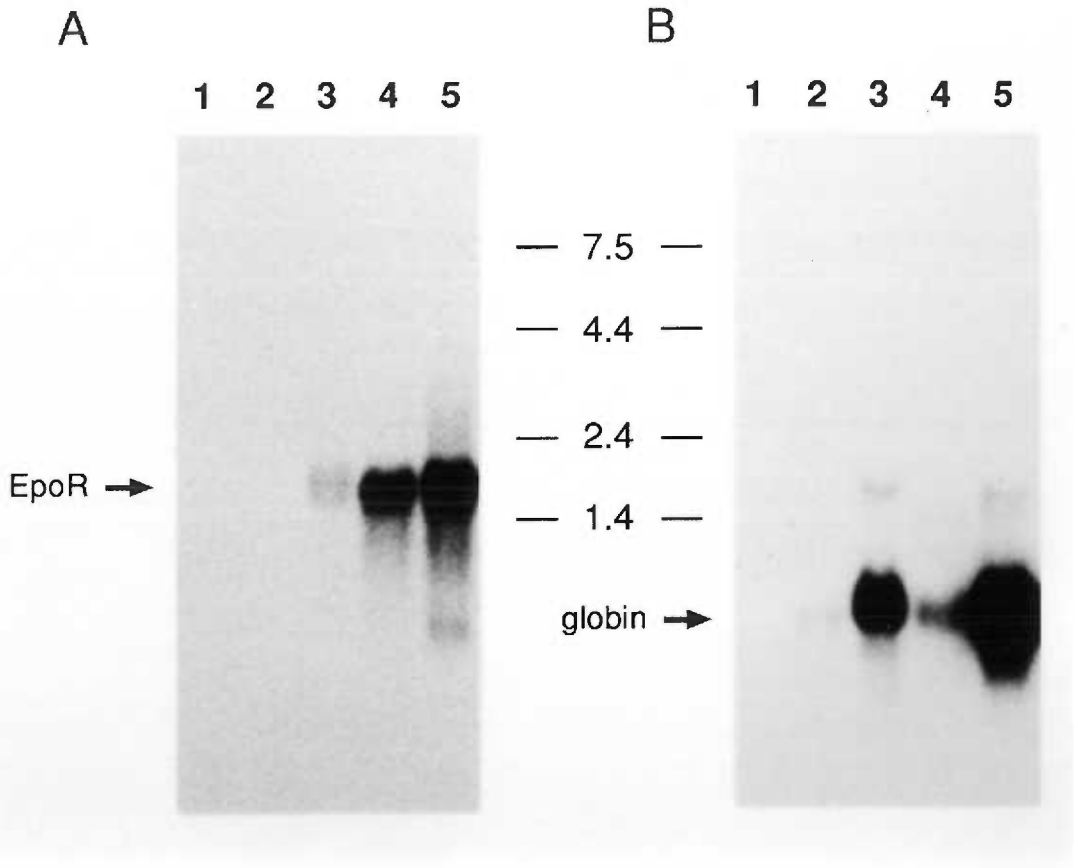
These results suggest that retroviral-encoded PU.1 protein may increase the proliferative potential of blast-like cells in long-term bone marrow cultures. Southern blot analysis of DNA from these nonadherent cell populations was compatible with this concept. When digested with Kpn I restriction endonuclease, which cuts in the retroviral long-terminal repeats, this DNA yielded the expected PU.1-containing fragment of 5.8 kilobase pairs that was absent from normal mouse DNA (results not shown). When digested with Hind III, which cuts only once in the SFF sequences but not within the PU.1 cDNA insert, a smear of different-sized DNAs was observed that hybridized to the PU.1 cDNA probe. This implies that the nonadherent cells are a polyclonal population, with SFF-PU.1 proviruses integrated at different positions in the chromosomes of different clones. As described below, we have not yet been able to isolate single cell clones in order to test this hypothesis.

The PU.1-Expressing Blasts Have Erythroid Characteristics and Resemble Friend Erythroleukemia Cells.

The RNA blot used in Fig. 22A was stripped of radioactive PU.1 probe and was then hybridized in sequential experiments with probes specific for EpoR and for mouse β-globin (Fig. 23, panels A and B, respectively). Erythropoietin receptors are apparently synthesized in small amounts in early hematopoietic progenitors and in much larger amounts in late burst-forming erythroblasts (BFU-E) (Broudy et al., 1991), whereas globin mRNA begins to accumulate much later during the proerythroblast-normoblast transition (Harrison et al., 1974; Ramirez et al., 1975). Although RNAs reactive with our

Figure 23. Analysis of erythroid-specific gene expression in pSFF-PU.1 and F-MEL cells.

RNA analysis of (A) EpoR and (B) β-globin expression. The Northern blot shown in Fig. 22 was stripped and was reprobed with radiolabeled EpoR or b-globin (see Experimental Procedures). Lanes: 1, PA12 derivative of mouse NIH 3T3 fibroblasts; 2, human K562 erythroleukemia cells; 3, pSFF-PU.1 cells; 4, M-7 erythroleukemia cells; and 5, IP/IR erythroleukemia cells.

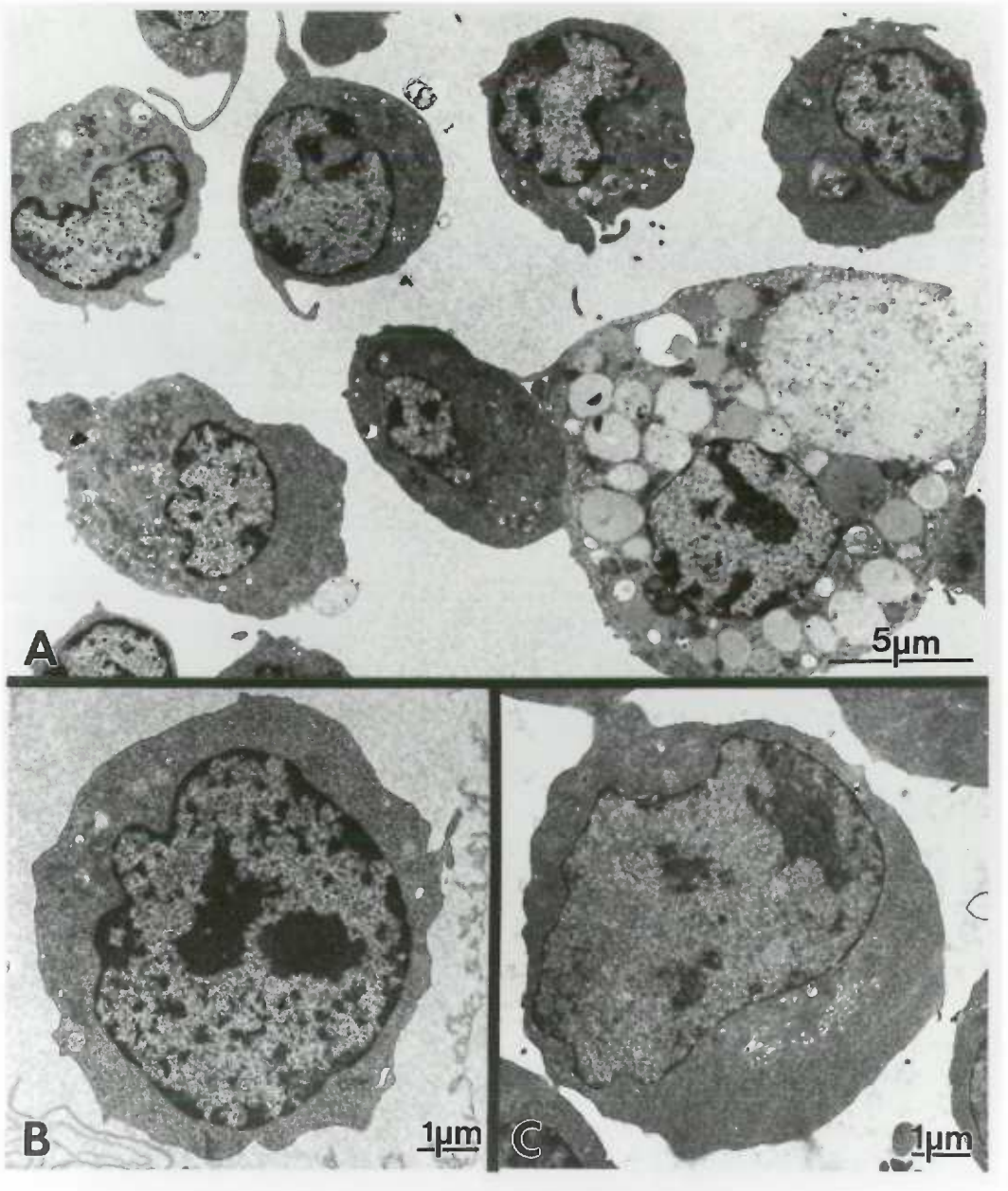

A
B
1 2 3 4 5
1 2 3 4 5
7.5
4.4
2.4
1.4
EpoR
globin

EpoR and β-globin probes were absent in fibroblasts (lane 1) and were absent or present in trace amounts in human K562 cells (lane 2), transcripts with the sizes expected for EpoR and β-globin mRNAs were present in RNAs of SFF-PU.1-infected nonadherent cells from bone marrow cultures that had been maintained in the presence of Epo (see lane 3). As expected (D'Andrea et al., 1989), identically-sized mRNAs were also present in Friend erythroleukemia cell lines (see lanes 4 and 5).

Presence of β-globin mRNA in nonadherent SFF-PU.1 cells from long-term marrow cultures was unexpected because globin synthesis occurs late in erythropoiesis in cells that can divide only a few times before exhausting their self-renewal capability (Gregory, 1976; Stephenson et al., 1971). Whereas, SFF-PU.1 blast-like cells maintain their proliferative potential indefinitely in marrow cultures. However, immortal Friend erythroleukemia cells synthesize small amounts of globin mRNA (Conkie et al., 1974) and differentiate spontaneously at a low frequency. Furthermore, they can be induced to differentiate in larger proportions by certain chemicals such as dimethylsulfoxide (Friend et al., 1971). Consequently, we inferred from Fig. 23B that a small percentage of the SFF-PU.1 cells might be spontaneously differentiating in the conditions of our long-term cultures. In agreement with this inference, only 1-2% of the SFF-PU.1 blast-like cells stained positive for hemoglobin with benzidine (not shown). To study effects of 1% dimethylsulfoxide without complications that might be caused by changes in the marrow stroma, a culture of nonadherent SFF-PU.1 cells was grown in the presence of Epo on a fixed fibroblast monolayer (see below). Initially, approximately 2% of the SFF-PU.1 cells stained positively with benzidine for hemoglobin. At 48 h and 96 h after addition of 1% dimethylsulfoxide, the percentage of benzidine-positive cells was 8% and 30%, respectively. Similar results were obtained with independently produced cultures of SFF-PU.1 blasts.

Ultrastructural analysis of SFF-PU.1 cells from the nonadherent population of a bone marrow culture is shown in Fig. 24. The SFF-PU.1 cells are, in general, slightly

Figure 24. Transmission electron micrographs of (A) loosely adherent cells from an Epo-supplemented, pSFF-PU.1 virus infected murine bone marrow culture. Magnification is x 4,500. Macrophages typically comprised 5-10% of the cell population. (B) An epo-dependent, pSFF-PU.1 blast. Magnification is x 8,500. (C) An IP/IR Friend erythroleukemia cell. Magnification is x 8,500.


A
5µm
B
1µm
C
1µm

smaller than IP/IR Friend erythroleukemia cells (Fig. 20, compare panels A and B *versus* panel D, respectively), but these cell types are otherwise very similar morphologically (Fig. 7). Both types of cells have irregularly shaped nuclei with patches of heterochromatin, fairly large nuclear to cytoplasm size ratios, cell surface blebbings, and a paucity of organelles. The cytoplasm of both cell types contain a few mitochondria, ribosomes and occasional Golgi complex profiles. These properties are compatible with a relatively undifferentiated state (Castoldi, 1981). The blast-like cells from marrow cultures are sometimes closely associated with larger macrophages (see panel A), as has been observed in normal erythropoiesis (Dexter et al., 1981b).

PU.1-Expressing Erythroblasts Require a Stromal Support.

Erythroblasts infected with SFF-PU.1 virus have continued to proliferate in media supplemented with Epo in their original infected long-term bone marrow cultures for more than 7 months. Transfer of these nonadherent cells onto uninfected marrow stroma has also resulted in their undiminished healthy proliferation. Apparently, the cells that constitute normal marrow stroma sequester growth factors and provide microenvironments for development of different hematopoietic lineages; however, for certain stem cells, exogenous growth factors can replace the stromal requirement (Dexter et al., 1990). In the case of these SFF-PU.1 blasts, however, no tested growth factor [i.e., IL-3, stem cell factor (kit factor), GM-CSF, WEH1-3 conditioned medium, spleen cell conditioned medium (IL-3 plus GM-CSF), or bone marrow stromal conditioned medium] was able to replace the stromal cell requirement.

After transferring nonadherent SFF-PU.1 blasts into plastic tissue culture dishes in medium with Epo, the majority of cells died within 3 days without differentiating, leaving only viable macrophages. Occasionally, stromal cells were spontaneously transferred along with the small SFF-PU.1 blasts, or stroma could be transferred by trypsinization and replating. In these cases, the SFF-PU.1 blasts grew actively in loose association with the

newly-seeded stromal islands. We have developed an uninfected line of stromal cells that also facilitates growth of SFF-PU.1. By these means, new cultures were prepared.

Fixed, metabolically inactive NIH/3T3 fibroblasts can support marrow-independent survival and proliferation of some hematopoietic stem cells (Roberts et al., 1987). Similarly, glutaraldehyde-fixed NIH/3T3 fibroblast monolayers supported SFF-PU.1 blast cells. The SFF-PU.1 blasts associated closely with the fibroblasts, and within 2-3 days islands of active proliferation were observed. However, it is uncertain whether stromal cells in addition to the SFF-PU.1 blasts had seeded these islands that formed on the NIH/3T3 fibroblast monolayers.

In previous analyses of Friend virus effects on long-term bone marrow cultures, the proliferating erythroblasts were also initially dependent on stroma; however, autonomous derivatives were isolated after 5-10 months (Dexter et al., 1981a). Autonomous derivatives of SFF-PU.1 blasts have not formed in our cultures.

DISCUSSION

PU.1 Protein Causes Fibroblast Killing.

Our results strongly suggest that PU.1 expression in mouse NIH/3T3 fibroblasts or in their Ψ2 and PA12 retroviral packaging derivatives causes weakening of their adherence to the substratum, pyknosis of their nuclei, and their eventual release into the culture medium as nondividing degenerating cells. Thus, as the SFF-PU.1 virus rapidly spreads into a substantial proportion of the Ψ2 and PA12 cells in a coculture, the degenerative changes ensued and there was a clear correlation between high-level PU.1 expression as seen by immunofluorescence and cell rounding with nuclear pyknosis (e.g., see Fig. 19). Control studies showed that these degenerative changes were not caused by the vector amplification method or by the SFF retroviral sequences. We were also unable to isolate clones of NIH/3T3 or rat 208F fibroblasts that stably express more than marginally detectable quantities of PU.1.

A few previous studies suggested that oncogenic proteins can cause cell death. For example, high-level expression of the *v-abl* oncogene kills fibroblasts (Ziegler et al., 1981; Watanabe and Witte, 1983) and *c-myc* causes apoptosis of growth-arrested fibroblasts (Evan et al., 1992). Protooncogene c-*fgr* inhibits fibroblast growth (Sartor et al., 1992). The normal p53 gene causes apoptosis in human colon tumor cells and in myeloid leukemia cells (Shaw et al., 1992; Yonish et al., 1991), and it has an antiproliferative effect in other cells (Ullrich et al., 1992).

PU.1 Protein Immortalizes Erythroblasts.

Infection of mouse bone marrow cultures with the SFF-PU.1 retrovirus results in outgrowth of Epo-dependent erythroblast-like cells that synthesize viral-encoded PU.1 protein and that remain dependent upon stromal cells for their long-term survival and proliferation. As expected of late BFU-E or CFU-E erythroblasts, these cells

morphologically resemble Friend erythroleukemia cells (Fig. 24); they contain EpoR (e.g., Fig. 23); they appear unresponsive to hemopoietins other than Epo; and they spontaneously differentiate at a low frequency to synthesize hemoglobin. Like Friend erythroleukemia cells, the frequency of their differentiation is increased by treatment with 1% dimethylsulfoxide.

In contrast to normal erythroblasts, these SFF-PU.1 virus-infected blasts clearly have a greatly increased self-renewal potential. Normal BFU-E or CFU-E erythroblasts incubated with Epo divide only for several days before exhausting their proliferative potentials and then differentiating (Gregory, 1976; Stephenson et al., 1971). When cultured in the presence of Epo or infected with SFFV, normal erythroblasts form only small hemoglobinized colonies (Hankins and Troxler, 1980) and these differentiated colonies lack any remaining stem cells. On the contrary, the SFF-PU.1 virus-infected blasts appear to be immortal as indicated by their indefinite potential for self-renewal.

Although the initial outgrowth of SFF-PU.1 virus-infected erythroblasts might have been interpreted as due to a relatively rapid and unregulated mitogenic stimulus, our results do not support this interpretation. Thus, the viability and proliferative rate of these cells remain completely dependent on Epo and on growth factors and microenvironmental supports provided by bone marrow stromal cells. Therefore, PU.1 does not abrogate the growth factor and microenvironmental dependencies of these cells. Rather, it seems to act principally or solely by increasing their self-renewal capacity and by concomitantly interfering with their commitment to terminally differentiate after a limited number of cell divisions.

We are uncertain whether the SFF-PU.1 virus acts exclusively on committed progenitors of the erythroid lineage or whether it might act more broadly. Recent studies of the Gag-Myb-Ets fusion protein encoded by E26 virus suggest that the virus infects uncommitted stem cells and then directs them into the erythroid lineage. In chickens, the resulting erythroblast-like cells are not irreversibly committed and they can be reverted into

other lineages by treatment with phorbol esters (Graf et al., 1992), whereas in mice the erythroid lineage commitment may be irreversible. Our preliminary studies suggest that the SFF-PU.1 virus differs from a Gag-Myb-Ets-encoding virus (Aurigemma et al., 1992; Ruscetti et al., 1992) in being unable to induce uncommitted mouse hematopoietic stem cells to synthesize EpoR (unpublished results). Nevertheless, we emphasize that our results do not exclude effects of PU.1 on nonerythroid hematopoietic lineages. Observation of such effects might require addition of hemopoietins other than Epo to the infected marrow cultures.

Mechanism of PU.1 Actions.

In considering the mechanisms of PU.1 effects, several facts seem pertinent. First, PU.1 and other members of the Ets family are transcription factors that bind to slightly different DNA sequences that contain a common ...GGAA... core (Klemsz et al., 1990; Karim et al., 1990). The Ets factors contain closely-related DNA binding domains at their carboxyl termini and divergent effector domains at their amino termini that are believed to interact with other transcription factors and adapter proteins in a combinatorial manner (Karim et al., 1990). Second, PU.1 is not normally expressed in either erythroblasts (Paul et al., 1991; Schuetze et al., 1992) or fibroblasts (see Fig. 18), suggesting that it is acting as an ectopic, abnormal protein. Third, it is striking that different members of the Ets family have powerful oncogenic effects in erythroblasts. These Ets proteins include the Gag-Myb-Ets fusion protein of the E26 virus (Moscovici et al., 1981; Radke et al., 1982), PU.1 (Klemsz et al., 1990), and *fli-1* in MuLV-induced erythroleukemias of newborn mice (Ben-David et al., 1990; Ben-David et al., 1991). These considerations suggest that PU.1 acts by altering gene expression. Conceivably, it might activate common or different genes that are otherwise quiescent in erythroblasts and fibroblasts. Alternatively, PU.1 might compete with an endogenously-expressed Ets-related protein for binding to a critical DNA site or to a limiting accessory factor. Thereby, it could reduce or block regulated

expression of an important gene. The strong oncogenic effects of different Ets-related factors in erythroblasts supports the latter model and raises the possibility that an endogenous Ets-related protein or binding factor plays a critical role in controlling the proliferative potential of normal erythroid progenitors.

EXPERIMENTAL PROCEDURES

Cells and Viruses.

IP/IR (Spiro et al., 1989) and M-7 (Wolff et al., 1986) (provided by S. Ruscetti, NCI, Frederick, MD) Friend erythroleukemia cell lines were derived from mice infected with helper-free SFFV. Ψ2 ecotropic (Mann et al., 1983) and PA12 amphotropic (Miller et al., 1985) retroviral packaging cell lines are derivatives of murine NIH/3T3 fibroblasts. Rat 208F cells (Miller et al., 1984) were a generous gift from A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA. These cells and NIH/3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin G and 100 μg/ml of streptomycin. Human erythroleukemia K562 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium (JRH Biosciences) supplemental with 10% fetal bovine serum. BaF3 IL-3 dependent cells (Mathey-Prevot et al., 1986) (provided by Alan D'Andrea, Dana-Farber Cancer Institute, Boston, MA) were grown in RPMI 1640 medium, 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol and 10% WEHI-3-conditioned medium to provide IL-3 (see below).

pSFF-PU.1 virus was generated by ping-pong amplification in cocultures of Ψ2 and PA12 cells as previously described (Schuetze et al., 1992). pSFF-ghGH (Bestwick et al., 1988) was maintained as a stably transfected clone in PA12 cells. Friend ecotropic MuLV (F-MuLV) was maintained in chronically infected NIH/3T3 fibroblasts. Culture medium was harvested to obtain viruses (Kozak and Kabat, 1990). Virus preparations were filtered (0.45 μm), and used immediately or stored at -80°C.

Growth Factors.

Recombinant mouse erythropoietin (m-Epo; Boehringer Mannheim, Indianapolis, IN) was used at a concentration of 0.5 U/ml. Recombinant mouse IL-3, stem cell factor

(m-SCF) and human GM-CSF (Genzyme, Cambridge, MA) were used at concentrations ranging form 1-500 U/ml, 1-100 ng/ml and 1-100 ng/ml, respectively. Murine spleen cell conditioned medium (IL-3/GM-CSF; Terry Fox Laboratory, Vancouver, B.C., Canada) was used at a dilution of 1:100. Mouse IL-3 was also provided as 10% WEHI-3 conditioned medium (Ihle et al., 1984).

Long-Term Bone Marrow Culture.

Cultures were prepared according to Mei and Burstein (1991) modification of the method of Dexter et al. (1977). Briefly, bone marrow cells from the femurs of 6-8 week old female NIH Swiss mice were flushed with Iscove's modified Dulbecco's medium (IMDM, JRH Biosciences), centrifuged and resuspended in IMDM supplemented with 25% horse serum (ICN Biochemicals, Inc., Costa Mesa, CA), 0.5 µg/ml hydrocortisone (Sigma, St. Louis, MO), 125 U/ml penicillin G and 125 µg/ml streptomycin (IMDM/HS). Bone marrow cells were cultured in 6-well tissue culture plates (no. 3046, Becton Dickinson & Co., Lincoln Park, NJ) in a 37°C, 5% CO_2, humidified incubator. After 1 week, cultures were recharged by removing the supernatant nonadherent cells and adding newly isolated bone marrow cells along with fresh medium. Bone marrow cultures were maintained by replacing one-half of the supernatant with fresh medium weekly.

Retroviral infection was performed, in the following manner, 3 weeks after bone marrow cultures were recharged. At this time, the stromal layer was confluent and islands of active hematopoiesis were observed. One-half of the medium (1.5 mls) from the cultures was removed and later replaced as conditioned cell-free supernatant. Duplicate cultures were infected with 1.5 mls of virus in the presence of 2.0 µg/ml Polybrene (Sigma) at 37°C for 4 hours. When F-MuLV was mixed with helper-free pSFF-PU.1, a ratio of 1:2 was used. The medium was then withdrawn, and cells were pelleted by centrifugation and resuspended in a 1:1 mixture of conditioned supernatant to fresh

medium. One set of infected cultures was grown in the presence of 0.5 U/ml erythropoietin.

Confluent monolayers of NIH/3T3 cells, which were fixed with 2% glutaraldehyde (Roberts et al., 1987), served as a supporting substratum for loosely adherent bone marrow culture cells in certain experiments.

Soft Agar Culture.

Cells were plated at densities of 10^4, 10^3, and 10^2 cells per ml in 60 mm tissue culture dishes containing IMDM/HS, 5 x 10^5 M 2-mercaptoethanol, 0.5 U/ml erythropoietin and 0.35% SeaPlaque agarose. Cells were grown in the absence or presence of 1% murine spleen cell conditioned medium and assayed for colony growth up to 6 weeks of incubation at 37°C.

Factor-independent growth assay.

Cells were mock infected or infected with pSFF-PU.1 virus in the presence of 8.0 μg/ml Polybrene for 4 hours and were then grown for 48 hours in medium containing IL-3. The cells were then washed extensively before replating in medium lacking added growth factors or medium containing IL-3 or Epo and observed for viability and growth as described previously (Kozak et al., submitted).

Electron Microscopy.

Cells from long-term bone marrow cultures were fixed in 1.5% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, plus 1% sucrose for 4 hours. They were then pelleted into 6% bovine serum albumin and fixed overnight in the same fixative. Subsequently, the cell pellets were briefly washed in 100 mM sodium cacodylate buffer and post-fixed in 2% osmium tetroxide. The pellets were dehydrated in a graded series of ethanols, infiltrated with toluene and then Epon resin, and finally embedded in Epon resin.

The polymerized blocks were sectioned, and the sections were stained with uranyl acetate and lead citrate and then viewed with a Philips 301 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.).

Analysis of Proteins.

Cells in suspension were labeled by incorporation for 2 hours with L-[^{35}]methionine and L-[^{35}S]cysteine (Tran^{35}S-label; ICN Biochemicals Inc., Costa Mesa, CA) and lysed in immune precipitation buffer as previously described (Gliniak and Kabat, 1989). Cell lysates were immunoprecipitated, electrophoresed in polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate (SDS) and transferred onto nitrocellulose membranes (Gliniak and Kabat, 1989). Nonradioactive samples were detected by immunoblotting with PU.1-9794 rabbit antiserum as previously described (Schuetze et al., 1992). Bound antibodies were labeled with [^{125}I]protein A and visualized by autoradiography. Indirect immunofluorescence was performed on Ψ2/PA12 cocultures that were grown on coverslips and on cytocentrifuged preparations (500 x g for 5 minutes) of suspension cells. PU.1 protein was detected with PU.1-9794 serum by a previously described technique (Schuetze et al., 1992). Human growth hormone was detected as previously described (Kozak and Kabat, 1990).

RNA and DNA Analysis.

Total cellular RNA was isolated by the guanidine thiocyanate-CsCl method (Chirgwin et al., 1979). Poly(A) RNA was selected from total RNA by the oligo(dT) batch procedure (Sambrook et al., 1989). For Northern (RNA) analysis, RNA samples were electrophoresed through a 1.2% agarose gel following denaturation with glyoxal and Me_2SO (Sambrook et al., 1989), transferred to Nytran membranes (Schleicher and Schuell, Keene, H.H.), and fixed by UV irradiating and baking. Blots were stained with a solution of 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5) to visually inspect the

RNAs for integrity and uniform transfer and then destained in 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were prehybridized overnight in a solution consisting of 50% formamide, 2.5x SSC, 2.5x Denhardt's solution (50x Denhardt's solution is 1.0% each Ficoll, polyvinyl pyrrolidone, and bovine serum albumin fraction V), 25 mM sodium phosphate (pH 6.6), 2.5% dextran sulfate (Pharmacia, Piscataway, NJ), 0.5% SDS, and 100 µg/ml of denatured herring sperm DNA. Hybridization was performed in the same buffer with the addition of 10^6 cpm of [a-^{32}P]dCTP-labeled nick-translated or randomly primed probe per ml. The final wash was at 50°C in 0.1x SSC-0.1% SDS. The *Sfpi-1* probe was the *Bam*HI-*Apa*I fragment isolated from the PU.1 coding region of a mouse cDNA clone (Paul et al., 1991). The β-globin probe consisted of the *Hind*III fragment (probe B) from mouse cDNA cloned into pBR322 (Hofer and Darnell, 1981). An EpoR cDNA fragment (D'Andrea et al., 1989) was used to detect EpoR transcripts, and *myb* transcripts were detected with pVM2 (Klempnauer, et al., 1982).

High molecular weight DNA was prepared by the technique of Laird et al. (1991) and was digested with restriction endonuclease (Promega, Madison, WI) according to the manufacturer's recommendations. For Southern (DNA) analysis, 15 µg of DNA per lane was electrophoresed in a 0.8% agarose gel, transferred to Nytran membrane and fixed by UV irradiating and baking. Filters were prehybridized, hybridized and washed as described above for RNA analysis. An 800 base pair region of genomic DNA from the 5' end of the PU.1 gene (probe 1, Paul et al., 1991) was used as a probe.

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III. SUMMARY AND CONCLUSIONS

Friend disease is a progressive erythroleukemia caused by infection with a retroviral complex in adult mice. To scientists, Friend disease is an excellent paradigm for the multistep nature of leukemogenesis, and study of pathogenesis of Friend erythroleukemia has recently revealed certain molecules important for generation of this malignancy. Work on Friend virus-induced cancer will likely provide significant insights into the molecular mechanisms of genesis, growth and progression of leukemia. The causative agent of Friend erythroleukmia is the replication-defective spleen focus-forming virus (SFFV). SFFV infection principally induces an erythroid lineage-specific pathology. SFFV encodes a functional membrane glycoprotein (gp55) that is inefficiently processed to the plasma membrane and complexes with erythropoietin receptors in infected erythroblasts. This complex at the cell surface mitogenically stimulates erythroblasts in the absence of physiologic levels of the normal regulatory hormone, erythropoietin, and induces their terminal differentiation. Thus, expression of the SFFV-specific gene causes an immediate erythroproliferative disease by circumventing hematopoietic regulatory mechanisms.

Subsequent to this early erythroblastosis, a preponderance of erythroleukemia cells emerge that do not differentiate and that exhibit indefinite self-renewal capabilities as indicated by their transplantability and potential for sustained growth in culture. This block to differentiation is not absolute, however, because treatment of erythroleukemia cells with certain chemicals reinstates the program to differentiate and results in programmed cell senescence and loss of immortality. Earlier studies in this laboratory demonstrated that the leukemic stage is due to outgrowth of a single infected cell when helper-free SFFV is used as the inoculum. These results supported the hypothesis that proviral integration in a particular region(s) of the genome caused a specific alteration(s) of gene structure or expression thereby contributing to the leukemic phenotype. We

showed that in the vast majority of independently induced erythroleukemias, a SFFV provirus had integrated in a common locus upstream of the newly described *ets*-related PU.1 gene. In Friend erythroleukemia cells, proviral integration does not disrupt the PU.1 coding sequence, rather it results in constitutive expression of high levels of PU.1, probably via enhancer mediated mechanisms. PU.1 is not expressed or is expressed in only trace amounts in differentiating erythroblasts and during the immediate "preleukemic" stage of Friend disease. Furthermore, loss of PU.1 mRNA and protein expression coincides with recommitment to differentiation when Friend erythroleukemia cells are cultured in the presence of certain differentiation-inducing chemicals. Thus PU.1 expression in erythroblasts correlates with the leukemic phenotype. Our results strongly suggested that aberrant expression of the cellular gene PU.1 causes the phenotypic changes (blocked differentiation and unlimited self-renewal capacity) characteristic of Friend erythroleukemia cells.

PU.1 is a member of the Ets superfamily of transcription activating factors. These factors bind to DNA at sequence-specific sites and can act alone or in cooperation with other factors to enhance transcription of adjacent genes. We identified multiple forms of PU.1 that are antigenically related and are derived from a common transcript. The higher molecular weight proteins appear to result from phosphorylation of seryl residues. PU.1 is normally expressed in macrophages and B lymphocytes and ectopically expressed in Friend erythroleukemia cells. In bone marrow macrophages, PU.1 enhances CSF-1-stimulated proliferation but does not cause immortality. This effect of PU.1 is dependent on phosphorylation of at least seryl residues 41 and 45. Interestingly, the majority of PU.1 in macrophages is not phosphorylated on serine 41 or 45. Phosphorylation of PU.1 in macrophages is stimulated by serum; whereas, dephosphorylation may be due to a protein phosphatase 1-type activity. A substantially greater percentage of PU.1 in Friend cells, as well as fibroblasts, occurs as the "hyperphosphorylated" form that contains phosphoserine 41 and 45. Phosphorylation of PU.1 in Friend cells or fibroblasts does not

appear to be affected by serum. Our preliminary results suggest that these two cell types negatively regulate PU.1 activity less effectively than macrophages. Yet, further studies are necessary to determine if the hyperphosphorylated form of PU.1 is required for its effects on growth of Friend cells and fibroblasts.

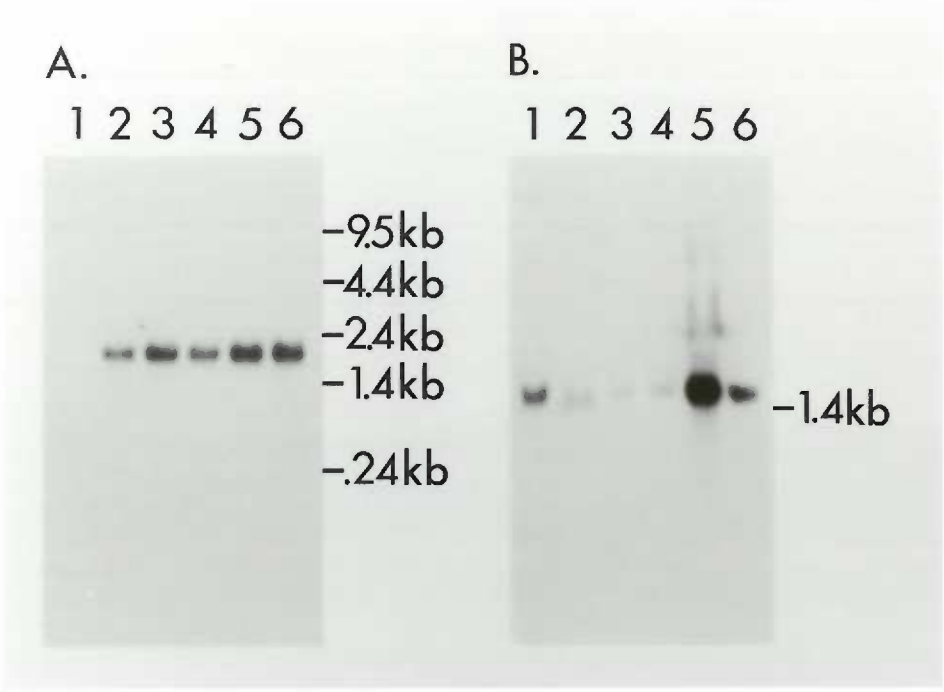
Studies of the effects of PU.1 expression in long-term murine bone marrow cultures have substantially clarified our understanding of the molecular basis of Friend viral erythroleukemogenesis. Our results imply that PU.1 is sufficient to abrogate the commitment of erythroblasts to terminally differentiate and that PU.1 is sufficient to sustain an indefinite self-renewal capability. Surprisingly, this effect is apparently unique to erythroblasts. Ectopic expression of PU.1 in fibroblasts leads to cell death that is morphologically characteristic of apoptosis. Importantly, PU.1 does not obviate the mitogenic requirement for erythropoietin, or presumably gp55, in erythroleukemia cells and does not overcome the apparent requirement for substratum-bound bone marrow element(s) for sustained cell viability. Additional molecular alterations are necessary for cell growth independent of marrow or splenic substrata. Our findings are consistent with earlier observations that Friend erythroleukemic cells initially develop in the spleen and bone marrow and are subsequently detected in peripheral blood. Thus, progression of Friend disease is not limited to two principal molecular events but involves further unidentified changes. p53 is often deleted or mutated in established Friend erythroleukemia cell lines. Certain mutant p53 proteins act as dominant negative suppressors of p53 function. It was recently proposed that p53 arrests the cell cycle in G1 to allow time for repair of DNA damage before replication commences. Tumor cells which have lost the function of p53 are unable to carry out this G1 arrest and may accumulate mutations at an accelerated rate. Loss of p53 in Friend erythroleukemia cells may, therefore, lead to rapid selection of malignant clones that are capable of growth autonomous of hematopoietic stroma.

In conclusion, we have identified and characterized biological effects of PU.1 in different cell types. Based on our results, we suggest that PU.1 perturbs pathway(s) that control potential for indefinite proliferation. Clearly, further studies are needed to identify the gene(s) or gene product(s) that is positively or negatively regulated by expression of PU.1. However, PU.1 expression appears to maintain the undifferentiated state of erythroblasts and contribute to their immortality thereby creating a perfect milieu for further genetic change in certain SFFV-infected erythroblasts and subsequent progression of Friend erythroleukemia.

IV. APPENDIX #1

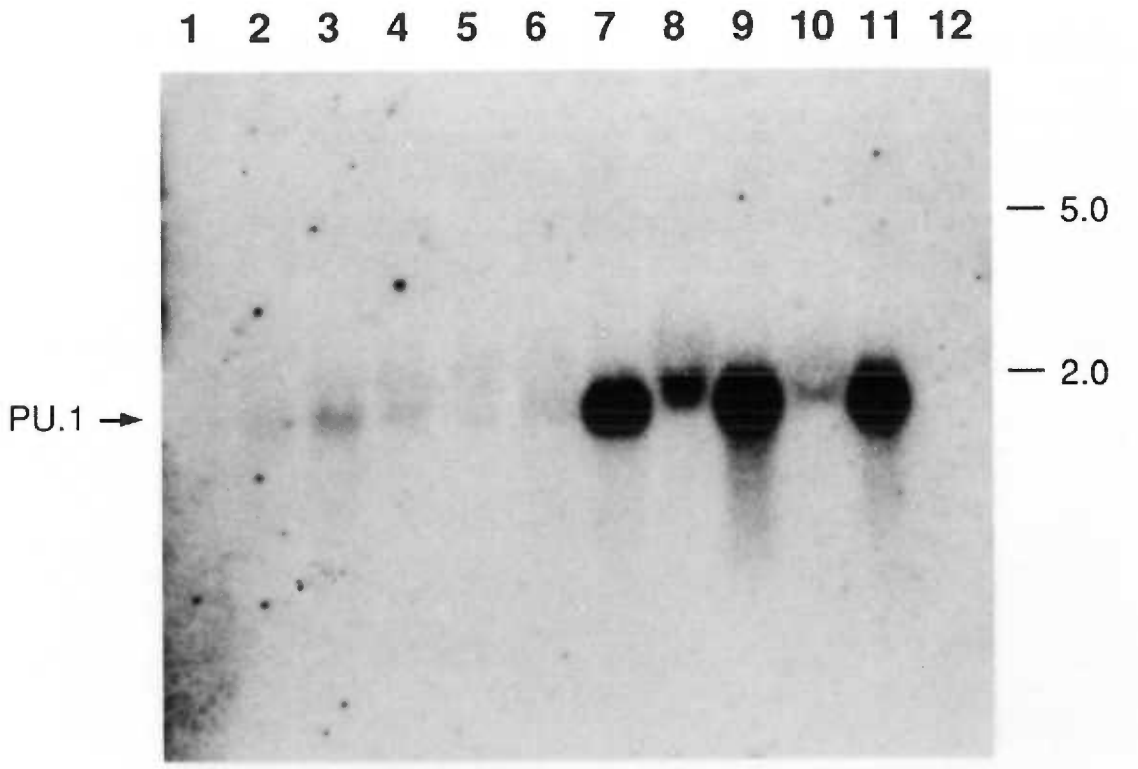
A. PU.1 mRNA Expression In Erythroleukemia Cell Lines and Spleens.

Figure 25. RNA analysis demonstrating active transcription in the *Sfpi*-1 locus. RNAs from mouse erythroleukemia cell lines (A) and from mouse tissues (B) were isolated by the guanidinium-isothiocyanate method (Chirgwin et al., 1979; Sambrook et al., 1989). Samples were electrophoresed through 1.2% agarose gels following denaturation with glyoxyl and dimethyl sulfoxide (Sambrook et al., 1989) and transferred to Nytran membrane (Schleicher and Schuell, Keene, N.H.). The uniform transfer and integrity of RNA were visually determined by soaking the membrane in 0.3 M sodium acetate (pH 5.5)-0.02% methylene blue. The blots were hybridized at 42°C with a radiolabeled *Sfpi*-1 cDNA probe. The final wash was at 53°C with 0.1x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate. Erythroleukemia cell lines previously described (Spiro et al., 1988; Paul et al., 1989) are identified here with the same nomenclature. (A) Five micrograms of poly(A)-enriched RNA. Lanes: 1, the PA12 line of mouse NIH 3T3 fibroblasts; 2 though 6, IP/IR, M1, M4, M5 and M7 erythroleukemia cells, respectively. Cell lines M5 and M7 lack any provirus in *Sfpi*-1 (Paul et al., 1989). (B) Twenty micrograms of total RNA. Lanes: 1, normal spleens; 2, spleens from phenylhydrazine-treated mice; 3, enlarged spleens harvested 10 days after infection with an erythropoietin-producing virus (Hoatlin et al., 1990); 4, preleukemic spleens harvested 14 days after infection with Friend virus; 5, IP/IR erythroleukemia cells; and 6, F-745 erythroleukemia cells. A 0.24- to 9.5-kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, Md.) was used as a size marker.


A.
1 2 3 4 5 6
~9.5kb
~4.4kb
~2.4kb
~1.4kb
~.24kb
B.
1 2 3 4 5 6
~1.4kb

B. PU.1 mRNA Expression In Murine Tissues.

Figure 26. RNA blot analysis of PU.1 expression in murine tissues and cell lines. RNAs from mouse livers, hearts, lungs, brains, kidneys, large intestines, bone marrow, spleens, IP/IR erythroleukemia cells, PS-C6 B cell lymphoma line, F-745 erythroleukemia cells and the PA12 line of NIH 3T3 fibroblasts (lanes 1-12, respectively) were isolated by the guanidinium-isothiocyanate method (Chirgwin et al., 1979; Sambrook et al., 1989). Twenty micrograms per sample were electrophoresed through 1.2% agarose gels following denaturation with glyoxyl and dimethyl sulfoxide (Sambrook et al., 1989) and transferred to Nytran membrane (Schleicher and Schuell, Keene, N.H.). The uniform transfer and integrity of RNA were visually determined by soaking the membrane in 0.3 M sodium acetate (pH 5.5)-0.02% methylene blue. The blots were hybridized at 42°C with a radiolabeled *Sfpi*-1 cDNA probe. The final wash was at 50°C with 0.1x SSC-0.1% sodium dodecyl sulfate. Size markers are in kilobases and indicate the positions of the 28S and 18S rRNAs.


1 2 3 4 5 6 7 8 9 10 11 12
PU.1
5.0
2.0

C. References.

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V. APPENDIX #2

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A Common Site for Immortalizing Proviral Integrations in Friend Erythroleukemia: Molecular Cloning and Characterization

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By using a tagged derivative of Friend spleen focus-forming virus, we previously obtained evidence that proviral integration(s) in the host genome can cause erythroblast immortality by abrogating the commitment of the cell to differentiate (C. Spiro, B. Gliniak, and D. Kabat, J. Virol. 62:4129–4135, 1988). Exploiting the fact that each leukemia was a single clone that contained one tagged provirus, we have now molecularly cloned and characterized one common genomic site for immortalizing proviral integrations.

Friend virus causes progressive erythroleukemia in susceptible mice (12, 14, 22, 25; D. Kabat, Curr. Top. Microbiol. Immunol., in press). Within 1 to 2 days, the spleens become engorged with numerous clones of proliferating erythroblasts that have only limited self-renewal capabilities (8, 21, 22, 24–26). After their proliferative potential is exhausted, these erythroblasts express their commitment to terminally differentiate (8, 24; Kabat, in press). Subsequently, between 3 to 8 weeks postinfection, immortal cells can be detected (12, 24–26). These leukemia cells have lost their commitment to differentiate, and they can be transplanted indefinitely in secondary recipients.

Several genetic factors have been implicated in leukemic progression. Although Friend virus is a mixture of a replication-defective spleen focus-forming virus (SFFV) and a replication-competent murine leukemia virus (MuLV), helper-free SFFV can occasionally induce transplantable erythroleukemias (24, 27, 28). In addition, advanced erythroleukemias caused by the viral complex usually have many and SFFV proviruses integrated into the cellular DNA (16–18). Often, these integrations occur in a common host genomic site, Spi-1 (18), and in the p53 proto-oncogene (4, 5, 9, 19). Additional important sites of integration might also occur in these leukemias. Advanced erythroleukemias also frequently have amplified copies of the c-*myc* proto-oncogene (B. Gliniak and D. Kabat, unpublished results) and various chromosomal rearrangements (15, 20). Because these changes in the genome of the cell can occur together, their individual roles in leukemogenesis have remained obscure. The clonal aspects of progression have also been difficult to analyze because SFFV contains only murine leukemia virus-related nucleic acid sequences that are present in many copies in the normal mouse genome (3, 16–18).

Recently, we described a method to address these issues (24). We modified a Lilly-Steeves polycythemia strain of SFFV by inserting a small tag of simian virus 40 DNA into its nonfunctional *pol* gene region. This SFFV clone was transfected into Psi-2 (13) retrovirus-packaging cells, which then released tagged helper-free SFFV virions. This tagged virus caused two types of disease in NIH Swiss mice. Most mice developed a transient mild polycythemia associated with polyclonal proliferation and subsequent differentiation of splenic erythroblasts. However, 20 to 30% of the mice developed severe anemias by 26 to 33 days postinfection and had large transplantable helper-free erythroleukemias in their spleens. Southern blot (hybridization) analyses indicated that these leukemias were all single clones with one tagged provirus per cell and that these proviruses were in a few tightly clustered sites in the host genome. Because these clonal erythroblast immortalization events were tightly associated with single proviral integrations at specific chromosomal sites, we proposed that these integrations were responsible for abrogating the commitment to differentiate and for the immortalization. We now describe one common site for SFFV proviral integrations in these immortalized erythroleukemias.

Genomic libraries were constructed in the EMBL-3 bacteriophage lambda vector by standard methods (7; Promega Biotec, Madison, Wis.) with DNAs prepared from leukemic spleen 33.1 (J) (24) and from erythroleukemia cell line IP/IR (24a). For the IP/IR library, two plaques that hybridized with simian virus 40 DNA were isolated from approximately 3×10^6 phage. For the J (33.1) library, one positive was isolated from approximately 4×10^6 phage. Restriction maps of the SFFV-host DNA junction fragment clones are presented in Fig. 1C.

Single-copy probes A, B, and C were prepared from these junction fragment clones (Fig. 1C). Probe B hybridized to both clones. These probes were then used to isolate additional host genomic clones from the integration site region. An extended map showing 30 kilobases of the region is shown in Fig. 1A; the maps of two overlapping genomic clones are shown in Fig. 1B.

By Southern blotting (23, 24), we then analyzed independently isolated Friend erythroleukemias to determine whether they also contained proviruses in this region. These leukemias included nine produced with our tagged helper-free SFFV, six (cell lines M-1, M-4, M-5, M-7, M-12, and M-13) that were produced with an untagged helper-free SFFV (28), and four other cell lines (FVT/A and FII [1], F745 [6], and F4N [20]) that contained both SFFV and MuLV proviruses. A typical Southern blot of *Kpn*I-digested genomic DNAs that was hybridized with probe B is shown in Fig. 2A; clearly, many of these samples contain rearranged loci, as indicated by extra DNA fragments (i.e., lanes 3 through 8, 12, and 13), in contrast to the single 12.5-kilobase fragment observed in the *Kpn*I digest of normal mouse DNA (lanes 2 and 14). By using DNAs digested with different restriction endonucleases (*Eco*RI, *Kpn*I, and *Bam*HI) and different single-copy probes, we were able to determine positions and orientations of proviruses in this gene region. Typical data are shown in Fig. 2. This information is

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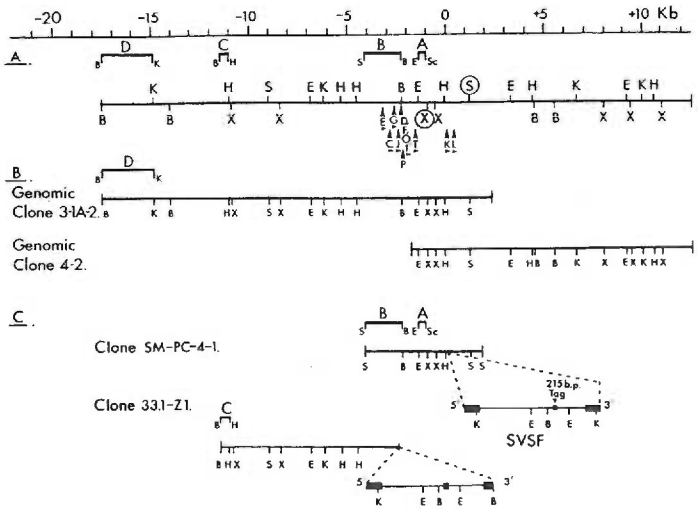

FIG. 1. (A) Map of the unrearranged imi-1 genomic site showing location and orientation of proviral integrations. Position zero is arbitrarily defined as the site of a tagged SFFV proviral integration in erythroleukemia cell line IP/IR (represented as integration K beneath the genomic map). The cleavage sites for *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Sal*I (S), *Xba*I (X), and *Sac*I (Sc) are indicated. Circled restriction sites identify some of the differences between the imi-1 and Spi-1 (21) maps. ▲, Sites of proviral integration in erythroleukemias examined; ►, orientation of provirus 5′ to 3′. Erythroleukemias C, D, E, F, G, I, and J, which have tagged SFFV proviral integrations, are labeled as previously described (27). Proviral integrations in additional erythroleukemias are labeled as follows: L, FVT/A cells and FII cells; O, MI cells; P, M4 cells; T, M13 cells. Orientation of provirus P is not assigned because this DNA did not cut in one *Eco*RI digestion. Fragments A through D (indicated by thick underline) were subcloned into the vector pGEM-4 (Promega Biotec) and used as probes for our DNA and RNA blots. Kb, Kilobases. (B) Maps of overlapping genomic clones 3-1A-2 and 4-2. Abbreviations are described in the legend for map A. (C) Maps of clones SM-PC-4-1 and 33.1-Z1, including tagged SFFV (called here SVSF) proviral sequences present in each. Clones SM-PC-4-1 and 33.1-Z1 were obtained by hybridizing the 215-base-pair simian virus 40 probe to genomic libraries made with DNA from leukemias K and J, respectively. Single-copy probes A, B, and C were derived from fragments of these clones as indicated. Abbreviations are described in the legend for map A.

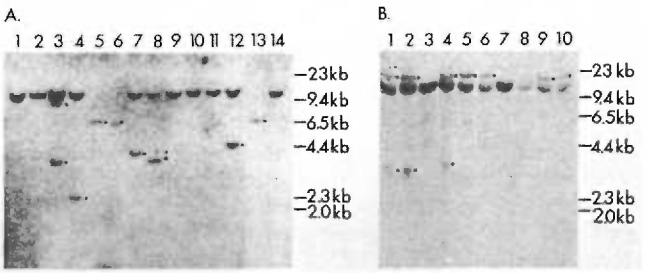

FIG. 2. Southern blot analyses of proviral integrations in the imi-1 genomic site. High-molecular-weight DNAs from SFFV-infected erythroleukemic spleens and from normal spleens were digested with *Kpn*I (A) or *Bam*HI (B). Blots were probed with the 1,900-base-pair probe B described in the legend to Fig. 1A. Leukemia DNAs described in the legend to Fig. 1A and by Spiro et al. (27) are identified here with the same nomenclature. (A) DNAs were as follows. Lanes: 1, F745 cells; 2, uninfected spleens; 3, tagged helper-free spleen C; 4, F-4N cells; 5, FVT/A cells; 6, FII cells; 7, MI cells; 8, M4 cells; 9, M5 cells; 10, M7 cells; 11, M12 cells; 12, M13 cells; 13, IP/IR cells; 14, uninfected spleens. Lanes 13 and 14 were from a reprobing of the same blot. This was done because these lanes were obscured by radioactive contamination in the original autoradiogram. (B) DNAs were as follows. Lanes: 1, tagged helper-free spleen C; 2, tagged helper-free spleen G; 3, tagged helper-free spleen H; 4, tagged helper-free spleen E; 5, tagged helper-free spleen F; 6, tagged helper-free spleen J; 7, uninfected spleen; 8, IP/IR cells; 9, tagged helper-free spleen I; 10, tagged helper-free spleen D. To ensure identification of faint bands that were clearly seen in the original autoradiograms, black dots were placed to the right of any such band.

summarized in Fig. 1A. Of the 18 leukemias studied, 12 contained assignable proviral integrations in this host genomic region. One additional leukemia that contained both MuLV and SFFV proviruses (F4N) also had a rearrangement (Fig. 2A, lane 4). However, this rearranged locus had an abnormal restriction map, and we were therefore unable to unambiguously locate and orient its provirus.

Eight of nine helper-free leukemias with tagged SFFVs contained tightly clustered integrations. Previously, we obtained evidence that the SFFV proviruses in leukemias C, G, and E and in D, F, and J were in tightly clustered groups (24). This confirms our earlier interpretations and also establishes close linkage of these groups. The C, G, and E group splits the B probe region, whereas the D, F, and J group occurs closer to its edge (compare lanes 1, 2, and 4 with lanes 5, 6, and 10, respectively, of Fig. 2B).

A significant proportion (ca. 5/18) of independently derived immortalized leukemias lack proviruses in this region. Southern blot analysis with a 900-base-pair *Bgl*II-*Pst*I fragment from the 27.1a molecular clone of p53 (11) suggested that the proviruses in these leukemias were not in the p53 oncogene (results not shown). Erythroleukemia cell lines with or without integrations in this common region differentiated similarly in response to dimethyl sulfoxide.

We infer that SFFV proviral integrations into one allele at this common site can immortalize erythroblasts. Thus, many immortalized leukemias contain both rearranged and normal alleles (Fig. 2). Nevertheless, loss of the normal allele had occurred in the erythroleukemia cell lines IP/IR, FVT/A, and FII (Fig. 2A, lanes 5, 6, and 13). Since FVT/A is a derivative of FII (1), the normal allele must have been lost before their divergence. A similar loss could have occurred in the splenic

leukemias, but it would have been masked by the presence of uninfected spleen cells (24a). The substantial frequency of this loss suggests that it might contribute to leukemic progression. According to this idea, the normal allele would act as a tumor suppressor gene whereas the allele with an integrated provirus would act as a dominant oncogene. Precedence for conversion of a tumor suppressor gene into an oncogene occurs with the p53 proto-oncogene (2, 10, 19).

This common integration site appears to be identical to the recently described Spi-1 site (18). First, the restriction map in Fig. 1 is similar but not identical to that of Spi-1. The circled *Xba*I site in Fig. 1A is absent in the Spi-1 map. Also, we find no other *Sal*I sites at the right of the circled *Sal*I site in Fig. 1A, whereas Spi-1 has two additional *Sal*I sites in this region. Our genomic clones also contain numerous *Sac*I sites, in contrast to the single *Sac*I site reported in Spi-1, and we also detected several additional *Pst*I and *Pvu*II sites that were absent from the Spi-1 map (data not shown). Because our clones and Spi-1 were derived from NIH Swiss and ICFW mice, respectively, these discrepancies could be due to strain differences. Our map also extends the published Spi-1 map. Second, S. Ruscetti had sent DNA from six helper-free erythroleukemias (M-1, M-4, M-5, M-7, M-12, and M-13) to F. Moreau-Gachelin and co-workers and had been informed which three of these contained Spi-1 integrations. In a blind study, we determined that the same three DNAs contained integrations in the region we had cloned. Finally, after all other aspects of this work were completed, we received single-copy Spi-1 probes and then confirmed the identity of these loci.

Although our data therefore generally support the conclusions of Moreau-Gachelin et al. (18), our results also differ significantly. Important differences include the restriction maps, the use of different leukemias, and our evidence for loss of the normal allele during progression. We have also found that probe D (corresponding to their probe C [18]) is not single copy. Consequently, the reported 4.0-kilobase RNA (18) appears not to be the true transcript of this gene. We are currently analyzing an abundant 1.5-kilobase poly(A)-containing RNA that is transcribed from the region adjacent to this probe. Most importantly, our approach with helper-free SFFV suggests that single integrations in Spi-1 contribute to immortalization of erythroblasts and that additional proviruses are unnecessary for leukemic progression.

We thank Sandra Ruscetti for kindly supplying DNAs from six erythroleukemia cell lines, Kris Krabill for technical assistance, and Craig Spiro for helpful discussions during the early stages of this work.

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The *Sfpi-1* Proviral Integration Site of Friend Erythroleukemia Encodes the *ets*-Related Transcription Factor Pu.1

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Previous studies identified a common site (*Sfpi-1*) for proviral integration in immortalized Friend erythroleukemias. cDNAs corresponding to a 1.5-kb *Sfpi-1* mRNA were isolated and sequenced. These were larger than an independently isolated *Sfpi-1* cDNA described by researchers from another laboratory, and they contained common differences from that sequence, including in the coding region four extra nucleotides that altered the reading frame. The properly translated protein is identical to Pu.1, a transcription activation factor that is related to the *ets* oncogene family. Genetic methods were used to map *Sfpi-1* with respect to other loci on mouse chromosome 2. Our results suggest that Pu.1 blocks erythroblast differentiation and thereby causes immortalization.

Previously, we (16, 21) and researchers from another laboratory (11, 12, 15) independently identified a common site for proviral integration (now designated *Sfpi-1* for SFFV proviral integration 1) in Friend erythroleukemias. Our evidence, which was based on studies using helper-free Friend spleen focus-forming virus (SFFV) that contained a small inserted tag of simian virus 40 DNA, suggested that proviral integration in *Sfpi-1* caused erythroblasts to lose their commitment to differentiate and to concomitantly become immortal (16, 21). We have now attempted to identify RNAs encoded by this region and to determine the role of the integrated provirus.

Figure 1 shows a map of the *Sfpi-1* genomic region, including the SFFV proviral integrations that we have identified in different erythroleukemias. Restriction endonuclease fragments from this entire region were used as hybridization probes for RNA blots. Only probes 1 through 3 hybridized under stringent conditions to discrete RNAs extracted from erythroleukemia cells. Probes 1 and 2 hybridized to 1.5-kb poly(A)-containing RNA that we previously described (16) and that was recently also analyzed by others (11, 12), whereas probe 3 identified an unusually small (ca. 200-nucleotide) RNA.

As described below and elsewhere (11, 12), these RNAs can be expressed in erythroleukemia cells by *Sfpi-1* alleles that lack an integrated provirus. Therefore, to ensure that we were analyzing RNAs transcribed from a leukemogenically relevant *Sfpi-1* allele, we used IP/IR erythroleukemia cells that contain an SFFV provirus in *Sfpi-1* but that lack the corresponding normal allele (16, 22). Accordingly, cytogenetic analysis indicated that IP/IR cells contain only one copy of mouse chromosome 2. Chromosome 2 contains *Sfpi-1* (12; also see below).

Poly(A)-containing RNA from IP/IR cells (2, 19) was used to construct a cDNA library of 2.5×10^6 phage in λgt-10 (5). Among 4.5×10^5 plaques screened with probes 1 and 2, two positive phages were isolated. A third positive phage was isolated from a cDNA library constructed from F-745 erythroleukemia cells (3). Inserts were subcloned into the *Eco*RI site of pGEM-3 (Promega Biotec, Madison, Wis.). Oligonucleotide primers (Midland Certified Reagent Co., Midland, Tex.) were used to sequence both strands of the cDNA clones by the dideoxynucleotide chain termination method (10, 20) with the Sequenase system (U.S. Biochemicals, Cleveland, Ohio). Regions of sequence compression were resolved by replacing dGTP with dITP in the reaction mixtures. The common sequence of these cDNAs and the sequence of the encoded protein are presented in Fig. 2. In general, the nucleotide sequence is similar to sequences recently reported by Moreau-Gachelin and coworkers (11, 12). However, our cDNAs are longer at both the 5′ and 3′ ends. Moreover, there are several differences between our sequences and theirs in the 5′ and 3′ untranslated regions. Most important, our cDNAs contain four additional nucleotides within the coding region at positions 789, 852, 857, and 941 that change the translational reading frame (Fig. 2).

Interestingly, the *Sfpi-1* protein encoded by our cDNAs is identical to the Pu.1 transcription activation factor that was recently described by Klemsz and coworkers (9). This identity also includes the 5′ and 3′ untranslated sequences of the cDNAs. However, our cDNA is 20 nucleotides longer at the 5′ end than the reported Pu.1 cDNA clone. Klemsz et al. (9) also presented evidence that Pu.1 binds to a purine-rich DNA sequence, 5′-GAGGAA-3′ (PU box), and that Pu.1 is related to the *ets* oncogene family of proteins. Structures and functions of other *ets*-related oncogenic proteins have also been described (1, 6, 9, 23). These proteins all contain PEST sequences (18) that cause them to turn over with short life spans, and they have been implicated in transcriptional control of mitosis and cellular differentiation (1, 6, 9, 23). Our results strongly suggest that Pu.1 is also an oncogenic protein. Moreover, since the Pu.1 in IP/IR erythroleukemia cells (Fig. 2) appears to be identical to that in normal cells (9), we suggest that oncogenesis does not require any change in the protein or mRNA sequences.

RNA blots that illustrate expression of the 1.5-kb *Sfpi-1*/Pu.1 RNA are shown in Fig. 3A and B. The RNA is synthesized in SFFV-infected erythroleukemia cells whether or not they contain a provirus in *Sfpi-1* (Fig. 3A). Moreover, this RNA occurs in a higher concentration in normal spleens than in erythropoietically active enlarged spleens from ane-

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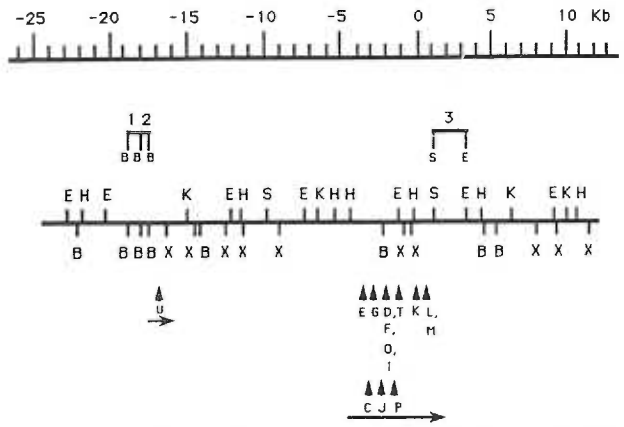

FIG. 1. Map of the *Sfpi-1* genomic region showing the location and orientation of proviral integrations. The cleavage sites for *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Sal*I (S), and *Xba*I (X) are indicated. Position zero is arbitrarily defined as the site of SFFV proviral integration in erythroleukemia cell line IP/IR (represented as integration K beneath the genomic map). Symbols: ▲, sites of proviral integration in erythroleukemias examined; →, orientation of proviruses 5′ to 3′. Erythroleukemias C, D, E, F, G, I, J, K, L, M, O, P, T, and U are labeled as previously described (21). Orientation of provirus P was not assignable because of an apparent partial deletion of the provirus. *Eco*RI-*Eco*RI fragments from throughout the genomic region were subcloned into pGEM-3 (Promega Biotec) and used as probes to detect unique transcripts. Single-copy probes 1 and 2, *Bam*HI-*Bam*HI fragments of approximately 800 and 500 bp, respectively, were found to hybridize to a unique 1.5-kb mRNA that was transcribed in an orientation opposite to that of the integrated proviruses. Probe 3 is a 2.2-kb *Sal*I-*Eco*RI fragment that hybridizes to a small (ca. 200-nucleotide) RNA.

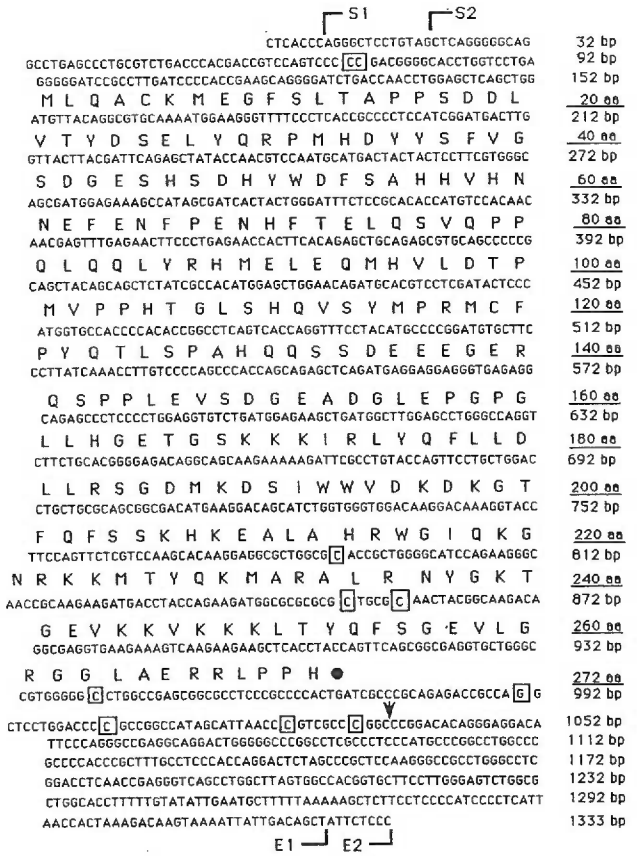

FIG. 2. Sequence of the *Sfpi-1* cDNA. This sequence was determined from the IP/IR cDNA clone designated P15 and numbered from the most 5′ nucleotide. A second IP/IR clone, P7, was also sequenced and extended from bp 28 to 1,333. A third clone, derived from an F-745 erythroleukemia cDNA library, had 100% homology with the P15 sequence throughout its length from bp 1 to 670. The predicted amino acid sequence is shown above the corresponding DNA sequence. The stop codon for the amino acid sequence shown is indicated (●). S1 and E1, Start and end sites, respectively, of the *Sfpi-1* cDNA sequence reported by Moreau-Gachelin et al. (12); S2 and E2, start and end sites of the Pu.1 sequence reported by Klemsz et al. (9); aa, amino acids. Nucleotides that were missing (boxed) from the *Sfpi-1* sequence reported by Moreau-Gachelin et al. (11, 12) and an additional T residue at bp 1,034 reported by Moreau-Gachelin et al. (11, 12) that was not observed in the clone P15, P7, or Pu.1 sequences (arrowhead) are shown.

mic mice and from preleukemic mice infected either with Friend virus or with an erythropoietin-encoding retrovirus (Fig. 3B, compare lane 1 with lanes 2 through 4). These results imply that normal differentiating erythroid cells either lack *Sfpi-1* mRNA or contain much smaller amounts than normal spleen cells. The increased amounts of *Sfpi-1* RNA in immortalized erythroleukemia cell lines could be caused by proviral integration (e.g., in cell lines IP/IR, M1, and M4) or by alternative mechanisms (e.g., for cell lines M5 and M7). We also detected *Sfpi-1* RNA in normal bone marrow, brains, hearts, lungs, large intestines, thymic stromata, and a B-cell lymphoma and in a Leydig cell testicular tumor. This RNA was absent in stomachs, livers, kidneys, and thymocytes and in NIH 3T3 fibroblasts. High levels of Pu.1 RNA were previously found in macrophages and in B lymphocytes (9, 17).

To chromosomally map the murine *Sfpi-1* gene, we analyzed DNAs from the progeny of an intersubspecies backcross (8). DNAs from the inbred strains NFS/N and from *Mus musculus musculus* produce 33- and 40-kbp *Hpa*I fragments, respectively, that hybridize on Southern blots with probe 2. Of 53 progenies of the backcross [(NFS/N × m) F_1 × m], 30 contained the 33-kbp fragment, consistent with the expected 1:1 ratio for a single gene. The inheritance pattern for *Sfpi-1* was compared with that of other markers on chromosome 2, including *Hc* (the fifth component of complement) by using probe pMC104 to identify a *Pvu*II polymorphism and *Hdc* (histidine decarboxylase) by using a rat cDNA probe to identify an *Xba*I polymorphism as described previously (8). Of the 53 mice, 8 recombinants were identified between *Hc* and *Sfpi-1* (percent recombination = 15.1 ± 4.9 [mean ± standard error]) and 7 other mice were recombinant between *Hdc* and *Sfpi-1* (percent recombination = 13.2 ± 4.6). These data are compatible with previous evidence (12) and suggest the gene order centromere–*Hc*–*Sfpi-1*–*Hdc*. The *Sfpi-1* map location confirms that *Sfpi-1* is distinct from other known oncogenes.

Our results suggest that SFFV proviral integration in *Sfpi-1* contributes to leukemogenesis without altering the structure of the *Sfpi-1* mRNA or Pu.1 protein. Presumably, the provirus causes enhancer-mediated constitutive expression of this gene. Our previous results imply that this expression may block the cellular commitment to terminally differentiate, thereby causing indefinite proliferation of immortal erythroleukemia cells (16, 21).

After this paper had been submitted, Goebl (4) described the similarity between the previously reported murine *Sfpi-1*

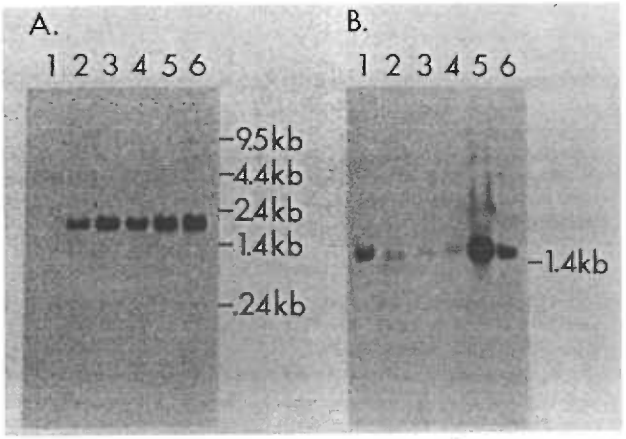

FIG. 3. RNA blot analysis demonstrating active transcription in the *Sfpi-1* locus. RNAs from mouse erythroleukemia cell lines (A) and from mouse tissues (B) were isolated (2, 19). Samples were electrophoresed through 1.2% agarose gels following denaturation with glyoxal and dimethyl sulfoxide (19) and transferred to a nytran membrane (Schleicher and Schuell, Keene, N.H.). The uniform transfer and integrity of RNA were visually determined by soaking the membrane in 0.3 M sodium acetate (pH 5.5)–0.02% methylene blue. The blots were hybridized at 42°C with a *Sfpi-1* cDNA probe. The final wash was at 53°C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate. Erythroleukemia cell lines previously described by Spiro et al. (22) and Paul et al. (16) are identified here with the same nomenclature. (A) Five micrograms of poly(A)-containing RNA. Lanes: 1, the PA12 line of mouse NIH 3T3 fibroblasts; 2 through 6, IP/IR, M1, M4, M5, and M7 erythroleukemia cells, respectively. Cell lines M5 and M7 lack any provirus in *Sfpi-1* (16). (B) Twenty micrograms of total RNA. Lanes: 1, normal spleens; 2, spleens from phenylhydrazine-treated mice; 3, enlarged spleens harvested 10 days after infection with an erythropoietin-producing virus (7); 4, preleukemic spleens harvested 14 days after infection with Friend virus; 5, IP/IR erythroleukemia cells; 6, F-745 erythroleukemia cells. A 0.24- to 9.5-kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, Md.) was used as a size marker.

(11, 12) and Pu.1 (9) cDNA sequences, and he inferred that the *Sfpi-1* sequences probably contained errors, including the four missing C residues in the coding region that we have observed (Fig. 2). In two letters (13, 14), Moreau-Gachelin et al. agreed that *Sfpi-1* and Pu.1 are identical, although they acknowledged only one missed C residue in their sequence at position 789 (our numbering system). Clearly, however, adding only this one C residue to their sequence would leave major differences from the Pu.1 cDNA and derived protein sequences, specifically, a string of 28 incorrect amino acids due to frameshifting and a protein shorter by 1 amino acid. The protein would be most altered in the region conserved in the *ets* oncogene family of proteins (23). A recently described human *Sfpi-1* cDNA sequence (17) also appears to have missing nucleotides at three positions identical to and at one site proximal to those missing in the two murine cDNA sequences (11, 12). Our results agree with the suggestions of Goebl (4) and provide strong evidence that the *Sfpi-1* and Pu.1 genes are the same.

Nucleotide sequence accession number. The nucleotide sequence accession number issued by GenBank for the sequence reported in this paper is M38252.

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Role of the PU.1 Transcription Factor in Controlling Differentiation of Friend Erythroleukemia Cells

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Both viral and cellular genes have been directly implicated in pathogenesis of Friend viral erythroleukemia. The virus-encoded gp55 glycoprotein binds to erythropoietin receptors to cause mitogenesis and differentiation of erythroblasts. However, if the provirus integrates adjacent to the gene for the PU.1 transcription factor, the cell loses its commitment to terminally differentiate and becomes immortal, as indicated by its transplantability and by its potential for indefinite growth in culture (C. Spiro, B. Gliniak, and D. Kabat, J. Virol. 63:4434–4437, 1989; R. Paul, S. Schuetze, S. L. Kozak, and D. Kabat, J. Virol. 65:464–467, 1991). To test the implications of these results, we produced polyclonal antiserum to bacterially synthesized PU.1, and we used it to analyze PU.1 expression throughout leukemic progression and during chemically induced differentiation of Friend erythroleukemia (F-MEL) cell lines. This antiserum identified three electrophoretically distinct PU.1 components in extracts of F-MEL cells and demonstrated their nuclear localization. Although PU.1 proteins are abundant in F-MEL cells, they are absent or present in only trace amounts in normal erythroblasts or in differentiating erythroblasts from the preleukemic stage of Friend disease. Furthermore, chemicals (dimethyl sulfoxide or *N,N'*-hexamethylenebisacetamide) that overcome the blocked differentiation of F-MEL cells induce rapid declines of PU.1 mRNA and PU.1 proteins. The elimination of PU.1 proteins coincides with recommitment to the program of erythroid differentiation and with loss of immortality. These results support the hypothesis that PU.1 interferes with the commitment of erythroblasts to differentiate and that chemicals that reduce PU.1 expression reinstate the erythropoietic program.

Friend viral erythroleukemia provides an excellent model for analyzing the multistep process of leukemogenesis and the roles of host genes in controlling susceptibility to an oncogenic protein (see references 1 and 23 for reviews). The replication-defective viral component, the spleen focus-forming virus (SFFV), encodes a membrane glycoprotein (gp55) that causes proliferation of late burst-forming and colony-forming erythroblasts (23). Recent evidence indicates that gp55 acts by binding directly to erythropoietin receptors (5, 20, 32). Because erythroblasts are committed to differentiate and have limited self-renewal capacities and because SFFV does not generally alter these cellular properties, the initial stage of virus-induced disease is characterized by polyclonal proliferation of cells that continue to differentiate and that cannot be transplanted to secondary recipients (1, 23, 65, 70). In mice infected with mixtures of helper virus plus SFFV, this initial stage of disease is maintained by continuous infection of newly forming erythroblasts. A second virus-induced change occurs if the SFFV provirus integrates by chance in the *Sfpi*-1 locus (39, 45, 65) adjacent to the gene for the *ets*-related transcription factor PU.1 (27, 46). This abrogates the erythroblast's commitment to differentiate and results in a transplantable, immortalized erythroleukemia (45, 65). Because *Sfpi*-1 proviral integration occurs at a very low frequency, the consequence is outgrowth of a rare immortalized clone that becomes a substantial proportion of the neoplastic population only after 4 to 8 weeks postinfection.

In support of the foregoing conclusions, mice injected with large doses of helper-free SFFV (Lilly-Steeves strain) develop a polyclonal erythroblastosis that is followed by mild polycythemia and by complete recovery 10 to 14 days postinfection (65). A few mice, however, do not completely recover; these animals develop monoclonal transplantable erythroleukemias that weigh several grams by 30 days postinfection and that have proviral integrations in *Sfpi*-1. These leukemic cells are immortal and do not differentiate in vivo (65). All immortal Friend erythroleukemias express large amounts of PU.1 mRNA, as detected by RNA blotting (38, 39, 46).

Virus-induced changes have also been reported in the p53 antioncogene (reviewed in reference 1; 8, 41). However, these changes may not be obligatory and have not been correlated with specific phenotypic alterations in the leukemic cells.

The homology of PU.1 to *ets* is intriguing because v-*ets* contributes to erythroleukemia in chickens (40, 50). Indeed, v-*ets* was first identified as one element of the tripartite oncogene in the E26 avian leukemia virus that transforms hematopoietic cells of myeloid and erythroid lineages. The *ets* region of the E26 oncoprotein (the 135,000-M_r oncoprotein contains portions of chicken *ets*-1 and *myb* fused to a retroviral *gag* domain) is required for erythroblastosis but not for myeloid transformation (15, 16, 43). PU.1 and *ets*-1 are most conserved in their carboxyl-terminal regions that bind to a common purine-rich core (5′-GGAA-3′) DNA sequence (24). Studies of PU.1 (27) and other members of the *ets* family (4, 17) indicate that they are transcriptional regulators. Another *ets*-related protein, *fli*-1, contributes to erythroleukemias that form in newborn mice after infection with the Friend murine leukemia virus in the absence of SFFV (1, 2).

SFFV proviral integrations in *Sfpi*-1 occur several kilobases upstream of the PU.1 transcriptional initiation site (39,

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45). PU.1 mRNA from erythroleukemias has the same size and sequence as does PU.1 mRNA that occurs in normal macrophages (27, 46). These results suggest that the SFFV provirus activates PU.1 expression by an enhancer-mediated mechanism.

Although proviral integration near PU.1 has been associated with a block in differentiation (65, 66), the impediment is not absolute since it can be overcome by treatment of cultured Friend erythroleukemia (F-MEL) cells with dimethyl sulfoxide (Me_2SO) and with certain other chemical inducers (12, 34, 52). Furthermore, although the studies mentioned above suggest that gp55 and PU.1 have critical roles in Friend viral erythroleukemia, little is known about the PU.1 protein or about its expression during leukemogenesis or chemically induced differentiation. To address these issues, we produced a polyclonal antiserum to PU.1. This antiserum identified three predominant PU.1 components in F-MEL cells. Rapid loss of PU.1 mRNA and proteins followed addition of chemical inducers to F-MEL cells. Loss of PU.1 proteins coincided with recommitment to the erythropoietic program.

MATERIALS AND METHODS

Cell lines and tissues. The F745-PC4-D2 (18) Friend leukemia cell line contains both SFFV and murine leukemia proviruses. The IP/IR F-MEL cell line was derived from a mouse infected with helper-free SFFV; it contains one SFFV provirus in *Sfpi*-1 on chromosome 2 and has lost the unmodified allele (45, 46, 66). ψ2 (33) and PA12 (36) are retroviral packaging derivatives of murine NIH 3T3 fibroblasts. The pSFF retroviral expression vector (3) that contained PU.1 cDNA coding sequences cloned into the unique *Eco*RI site became amplified by ping-pong after transfection into a 1:1 coculture of ψ2 and PA12 cells, as described elsewhere (3, 28). All cells were grown in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, Kans.) supplemented with 10% fetal bovine serum, 100 U of penicillin G per ml, and 100 μg of streptomycin (DMEM/FBS).

Spleens were obtained from 4- to 6-week-old female NIH/Swiss mice. A normal spleen (weight 0.15 g) was isolated from an untreated mouse that had a hematocrit of 47%. Erythropoietically active spleens were isolated from a mouse 48 h following 2 consecutive days of phenylhydrazine injections (60 mg/kg of body weight) (spleen weight, 0.34 g; hematocrit, 44%) and from a mouse 13 days after injection with 0.7 ml of passaged Friend virus (Lilly-Steeves polycythemia strain) via the tail vein (spleen weight, 3.33 g; hematocrit, 68%) as described previously (65).

Molecularly cloned probes used for hybridization. The β-actin probe, pBam-bA2, contained the coding region of mouse β-actin cDNA cloned into the *Bam*HI site in pGEM (68). The β-globin probe consisted of the *Hin*dIII fragment (probe B) from the mouse cDNA encompassing the first two exons cloned into pBR322 (21). The c-*myc* plasmid, pM104BH, contained a 3.2-kbp *Bam*HI-*Hin*dIII fragment from the mouse c-*myc* locus (61). A v-*myb* fragment was excised from pVM2 (26). The p53 probe pp53-208 was cloned from mouse cDNA (44). The *Sfpi*-1 probe was the *Bam*HI-*Apa*I fragment isolated from the PU.1 coding region of a mouse cDNA clone (46).

PU.1-specific antiserum. The *Pvu*II-*Bam*HI fragment containing the entire coding region of PU.1 was excised from our PU.1 cDNA construct (46). *Bam*HI linkers (New England Biolabs, Beverly, Mass.) were ligated onto the *Pvu*II-digested blunt end and cleaved with restriction endonuclease *Bam*HI. This modified fragment was ligated into the *Bam*HI site of the expression plasmid pET-11d (Novagen, Madison, Wis.), which is based on the T7 polymerase system reported by Studier et al. (67). The recombinant gene contained 15 codons fused to the first AUG codon at the 5′ end of the PU.1 cDNA; the codons for the 13 N-terminal amino acids were from the T7 gene 10. The PU.1 cDNA codes for 272 amino acids (27, 46). For protein expression, the pET-11d-PU.1 vector was transformed into the BL21(DE3)pLysS lysogenic strain (Novagen) of *Escherichia coli*, and a transformed clone was induced to express the PU.1 fusion product according to the manufacturer's recommendations. Bacterial cells were collected by centrifugation at 3,000 × *g* for 30 min, resuspended in 50 mM sodium chloride–50 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–5 mM dithiothreitol, and lysed in a French press at 10,000 lb/in^2. Insoluble PU.1 protein was collected by centrifugation at 3,000 × *g* for 30 min and was solubilized by boiling in Laemmli modified buffer (0.0625 M Tris hydrochloride [pH 6.8], 2.3% sodium dodecyl sulfate [SDS], 20% glycerol, 5% mercaptoethanol) for 10 min. PU.1 was separated from other proteins by electrophoresis through an 8% polyacrylamide–0.1% SDS gel (31), excised in a gel slice, and either emulsified in phosphate-buffered saline solution (pH 7.4) (PBS; JRH Biosciences, Lenexa, Kans.) at approximately 1 mg of protein per ml of PBS or electroeluted into 0.5× TAE (1× TAE is 40 mM Tris-acetate and 1 mM EDTA), dialyzed against water, lyophilized, and resuspended at 1 mg/ml in PBS. PU.1 protein (500 μg) in polyacrylamide that was emulsified in PBS was diluted 4:1 in complete Freund's adjuvant and injected subcutaneously into a female New Zealand White rabbit. Booster immunizations of 500 μg of electroeluted PU.1 protein, diluted 1:1 in incomplete Freund's adjuvant, were given intramuscularly every 7 weeks. Rabbit serum was assayed for PU.1-specific antibodies by a two-step immunoprecipitation-Western immunoblotting procedure (see below). Serum obtained after the second boost gave a strong positive response when diluted 10^3-fold and was designated PU.1-9794.

Analysis of proteins. F745 erythroleukemia cells were labeled by incorporation for 2 h with L-[^{35}S]methionine and L-[^{35}S]cysteine (Tran^{35}S-label; ICN Biochemicals Inc., Costa Mesa, Calif.) and lysed in immune precipitation buffer as previously described (14). Lysates of tissue samples were also prepared as previously described (65). Lysates were precleared by adsorption with fixed *Staphylococcus aureus* (Pansorbin; Calbiochem Corp., La Jolla, Calif.) and by centrifugation prior to immunoprecipitation and to electrophoresis in polyacrylamide gels in the presence of 0.1% SDS (55, 56). For PU.1 detection, proteins were transferred onto nitrocellulose membranes (14). Nonradioactive samples were blocked, washed (57), and then immunoblotted with a 1:1,000 dilution of PU.1-9794 antiserum in a solution of 5% nonfat milk, 0.01% antifoam A, 0.01% Tween 20, and 0.02% sodium azide in PBS. Blots with bound antibodies were then labeled by incubation with ^{125}I-protein A, and the labeled membranes were developed by autoradiography. The immunofluorescence assay was done by using ψ2/PA12 ping-pong cocultures (see above) in which approximately 10% of the cells were active in PU.1 expression. Cells growing on coverslips were rinsed with PBS, fixed with 3.7% paraformaldehyde in PBS for 20 min, and then immersed in ice-cold acetone for 1 min. Incubation for 1 h at 37°C with a 1:500 dilution of PU.1-9794 antiserum in DMEM/FBS was followed by rinsing with medium and then incubation for 1 h at 37°C with a 1:1,000 dilution of fluorescein-labeled goat

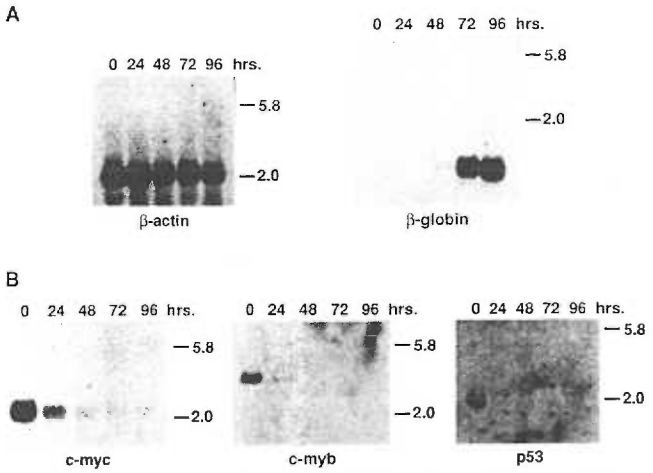

FIG. 1. Gene expression during Me_2SO-induced differentiation. F745 cells were cultured with 1.8% Me_2SO, and RNA was isolated at 24-h intervals through 96 h. Five micrograms of poly(A)-selected RNA was loaded in each lane. Northern blot analysis was performed with radiolabeled probes (see Materials and Methods for a description of the probes used). (A) Expression of β-actin and β-globin; (B) expression of cellular oncogenes *myc*, *myb*, and p53. Size markers are in kilobase pairs.

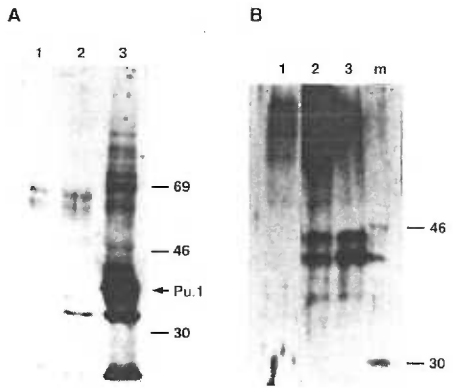

FIG. 2. Western blot analysis of proteins precipitated by the antiserum PU.1-9794. Cell lysates (1 mg of total protein except in panel A, lane 2, in which 100 μg of protein was used) were immunoprecipitated with a 1:500 dilution of antiserum and separated by electrophoresis through a 0.1% SDS–8% polyacrylamide gel. Transferred proteins were immunoblotted with a 1:1,000 dilution of PU.1-9794 followed by ^{125}I-labeled protein A (see Materials and Methods). (A) Lanes: 1, immune precipitation buffer treated with Pansorbin and antiserum but without cell lysate; 2, isopropyl-β-D-thiogalactopyranoside-induced bacterial BL21(DE)pLysS cells containing the control pET-11d plasmid; 3, isopropyl-β-D-thiogalactopyranoside-induced bacterial cells containing the recombinant pET-11d-PU.1 vector. (B) Lanes: 1, coculture of ψ2 and PA12 fibroblasts that lack PU.1 expression; 2, coculture of ψ2 and PA12 fibroblasts containing PU.1 cDNA in a retroviral expression vector; 3, IP/IR erythroleukemia cells; m, rainbow ^{14}C-methylated protein molecular weight standards (Amersham Corp., Arlington Heights, Ill.). Markers represent the positions of protein standards and indicate their sizes in kilodaltons. In the analysis in panel B, some ^{125}I-protein A bound to the upper portion of the blot; this occurs to a variable extent and is due to immunoglobulin and Pansorbin components that occur in the samples. The arrow indicates the position of the PU.1 fusion protein from induced bacterial cultures.

anti-rabbit immunoglobulin (TAGO, Inc., Burlingame, Calif.) prior to final rinsing.

Differentiation in F-MEL cultures. Erythroid differentiation was induced by addition of either 1.8% Me_2SO (Mallenckrodt, Paris, France) (12) or 5 mM *N,N'*-hexamethylenebisacetamide (HMBA; Sigma, St. Louis, Mo.) (52) to exponentially growing cultures containing 0.7×10^6 to 1.0×10^6 cells per ml. In all studies, more than 70% of the cells stained benzidine positive (42) by 120 h postinduction. To ensure that our F-MEL cell clones differentiated in a manner consistent with earlier studies that used other clones, RNA blots were analyzed for transcripts previously reported to be altered by inducers of differentiation. As shown in Fig. 1, Me_2SO-induced differentiation in our clone of F745 cells resulted in accumulation of β-globin mRNA beginning at about 48 h, while β-actin mRNA remained relatively constant. In agreement with previous reports, chemical induction resulted in depletion of transcripts for c-*myc* (30, 51), c-*myb* (25, 51), and p53 (51). Similar results were obtained by using our clone of IP/IR cells (data not shown).

RNA analysis. Total cellular RNA was isolated by the guanidine thiocyanate-CsCl method (7, 57). Poly(A) RNA was selected from total RNA by the oligo(dT) batch procedure (57). For Northern (RNA) analysis, either total cellular RNA or poly(A)-selected RNA samples were electrophoresed through a 1.2% agarose gel following denaturation with glyoxal and Me_2SO (57), transferred to nitrocellulose or Nytran membranes (Schleicher & Schuell, Keene, N.H.), and fixed by baking for 2 h at 80°C or UV irradiating and baking, respectively. Nytran blots were stained with a solution of 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5) to visually inspect the RNAs for uniform transfer and integrity and then destained in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were prehybridized at 42°C for 16 h in a solution consisting of 50% formamide, 2.5× SSC, 2.5× Denhardt's solution (50× Denhardt's solution is 1.0% each Ficoll, polyvinyl pyrrolidone, and bovine serum albumin fraction V), 25 mM sodium phosphate (pH 6.6), 2.5% dextran sulfate (Pharmacia, Piscataway, N.J.), 0.5% SDS, and 100 μg of denatured herring sperm DNA per ml. Hybridization was performed in the same buffer with the addition of 10^6 cpm of [α-^{32}P]dCTP-labeled nick-translated or randomly primed probe per ml. The final wash was at 42 to 55°C in 0.1× SSC–0.1% SDS–1 mM EDTA (pH 8.0).

RESULTS

Immunological detection of PU.1 proteins. PU.1 was expressed in bacteria as the complete protein with an amino-terminal extension of 15 amino acids and an apparent M_r of 38,000. This size is in agreement with the expected PU.1 M_r of 36,500, as judged from its cDNA sequence (46). Maximum yields of the bacterial product (10 to 15% of the total cell protein as estimated from the Coomassie blue staining pattern) were obtained 2 to 3 h after addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside inducer to the exponentially growing bacteria. As shown in Fig. 2, our rabbit antiserum (named PU.1-9794) precipitated this 38,000-M_r protein from lysates of bacteria that express PU.1 (Fig. 2A, lane 3) but not from a lysate of control bacteria that lacks PU.1 (lane 2).

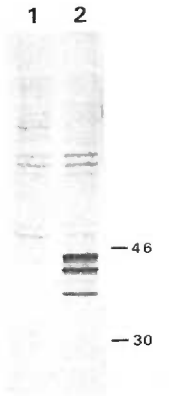

FIG. 3. Protein analysis of PU.1 products. Cell lysates from 5×10^6 F745 erythroleukemia cells that were labeled with [^{35}S]methionine and [^{35}S]cysteine (see Materials and Methods) were precipitated with a 1:500 dilution of either preimmune rabbit serum (lane 1) or PU.1-9794 immune serum (lane 2). Immune precipitates were separated by SDS–8% polyacrylamide gel electrophoresis and transferred to nitrocellulose. Markers represent the positions of the rainbow ^{14}C-methylated ovalbumin and carbonic anhydrase protein standards and indicate their M_rs (46,000 and 30,000, respectively).

Both F-745 and IP/IR F-MEL cell lines contain substantial amounts of PU.1 mRNA (46). Figure 2B (lane 3) illustrates that PU.1-9794 antiserum also specifically precipitated proteins from lysates of IP/IR cells. Three major PU.1-related proteins were reproducibly identified in F-MEL cell lysates (see below). The two predominant components seen in this protein blot migrate with apparent M_rs of 42,000 and 44,000, substantially larger than expected for PU.1. A third PU.1-related protein (apparent M_r of 37,000) is seen in lower concentrations in F-MEL lysates at the position expected for PU.1. The preimmune rabbit serum did not precipitate any of these components.

To determine which of these immunoprecipitated proteins in F-MEL lysates were encoded by the PU.1 gene, we expressed PU.1 cDNA in a coculture of ψ2 and PA12 murine fibroblasts, using a retroviral vector (see Materials and Methods) that becomes amplified in these cocultures (3, 28). Interestingly, all three of these PU.1-related protein components were detected in lysates from these fibroblasts (Fig. 2B, lane 2) but not in lysates from control fibroblasts (lane 1).

The three major forms of PU.1 were most clearly resolved when they were labeled directly by incorporation of L-^{35}S-labeled amino acids. Figure 3 shows these three labeled PU.1 proteins recovered from a lysate of IP/IR erythroleukemia cells that was immunoprecipitated with PU.1-9794 antiserum (lane 2) but not by the preimmune serum (lane 1). Additional minor PU.1 components were also observed in the 42,000- to 44,000-M_r region of the gel. Although we have not identified causes for the major size differences between PU.1 proteins, we have found that all three major components can be labeled by intracellular incorporation of $^{32}P_i$ (data not shown). None of the PU.1 proteins appear to be glycosylated, as determined by adsorption to wheat germ agglutinin (10, 22), nor could they be precipitated from cell lysates by antiserum to ubiquitin (data not shown).

Indirect immunofluorescence microscopy was used to determine subcellular localization of the PU.1 protein. Figure 4 shows results using murine fibroblast cultures in which the retroviral vector amplification was allowed to proceed until approximately 10% of the cells expressed PU.1 (see Materials and Methods). Preimmune rabbit serum did not label any of the cells (Fig. 4A). Moreover, normal murine fibroblasts lack PU.1 and could not be labeled by the PU.1-9794 antiserum (Fig. 4B). In contrast, this PU.1-9794 antiserum strongly labeled the nuclei in the PU.1-expressing fibroblasts (Fig. 4C). The presence of nonexpressing cells in these same microscopic fields served as an internal control that confirms the specificity of the nuclear staining. Although PU.1 also appeared to be intranuclear in F-MEL cells, an ideal negative control for the latter immunofluorescence analysis was unavailable because all F-MEL cells contain PU.1 (37, 40, 47).

Expression of PU.1 during leukemogenesis. PU.1 mRNA quantities are very small in normal erythroblasts compared with erythroleukemia cell lines (46). Figure 5 shows the relative abundances of PU.1 proteins which were immunoprecipitated from extracts of mouse spleens and from erythroleukemia cell lines. A small amount of PU.1 protein (42,000-M_r component) was present in a 0.15-g normal spleen (lane 2); presumably this was in B lymphocytes and macrophages, which are known to contain PU.1 mRNA (27). Anemia causes spleen enlargement due to immigration of proliferating and differentiating erythroblasts (19). A mouse that was recovering from phenylhydrazine-induced anemia had a mildly enlarged spleen (0.34 g), and this spleen (lane 3) contained slightly less PU.1 per milligram of protein than did the normal spleen. More significantly, much less PU.1 per milligram of protein occurred in a greatly enlarged 3.3-g preleukemic spleen that was taken from a mouse 13 days after infection with Friend virus (lane 4). Since this enlargement is caused almost exclusively by proliferating and differentiating infected erythroblasts (70), this result implies that these cells lack or contain only trace amounts of PU.1 proteins. F-745 and IP/IR F-MEL cell lines (lanes 5 and 6, respectively) contain much more PU.1 than do any of these spleens. The largest 44,000-M_r PU.1 component also occurs in a relatively higher proportion in the F-MEL cells.

PU.1 mRNA and protein levels decline during chemically induced differentiation of F-MEL cells. Figure 6 (upper panels) shows changes in the abundance of PU.1 mRNA during the early phase of chemically induced differentiation. PU.1 mRNA levels decline almost completely between 2 and 4 h and transiently reappear at about 5% of the initial level between 12 to 24 h postinduction. Similar changes in mRNAs for c-*myc* and c-*myb* were previously described (25, 30, 51). PU.1 mRNA levels remained very low after 48 h (data not shown). The decline in PU.1 mRNA levels occurred reproducibly whether differentiation was induced with Me_2SO (Fig. 6A) or HMBA (Fig. 6B). Moreover, PU.1 mRNA levels declined and transiently reappeared with very similar kinetics for all of our F-MEL cell lines when either Me_2SO or HMBA was used to induce differentiation (data not shown).

Figure 7 shows the degradation of PU.1 mRNA following treatment of nondifferentiating IP/IR cells with actinomycin D in order to block new mRNA synthesis (64). Densitometric analysis indicates that the half-life of PU.1 mRNA is approximately 2.0 h. This result was reproducible and similar to the half-life of the PU.1 mRNA measured in F745 cells (data not shown). Thus, rapid degradation of PU.1 mRNA occurs normally and is not simply a consequence of adding chemicals that induce erythropoiesis.

PU.1 proteins were also analyzed throughout the chemically induced differentiation. Total protein synthesis in

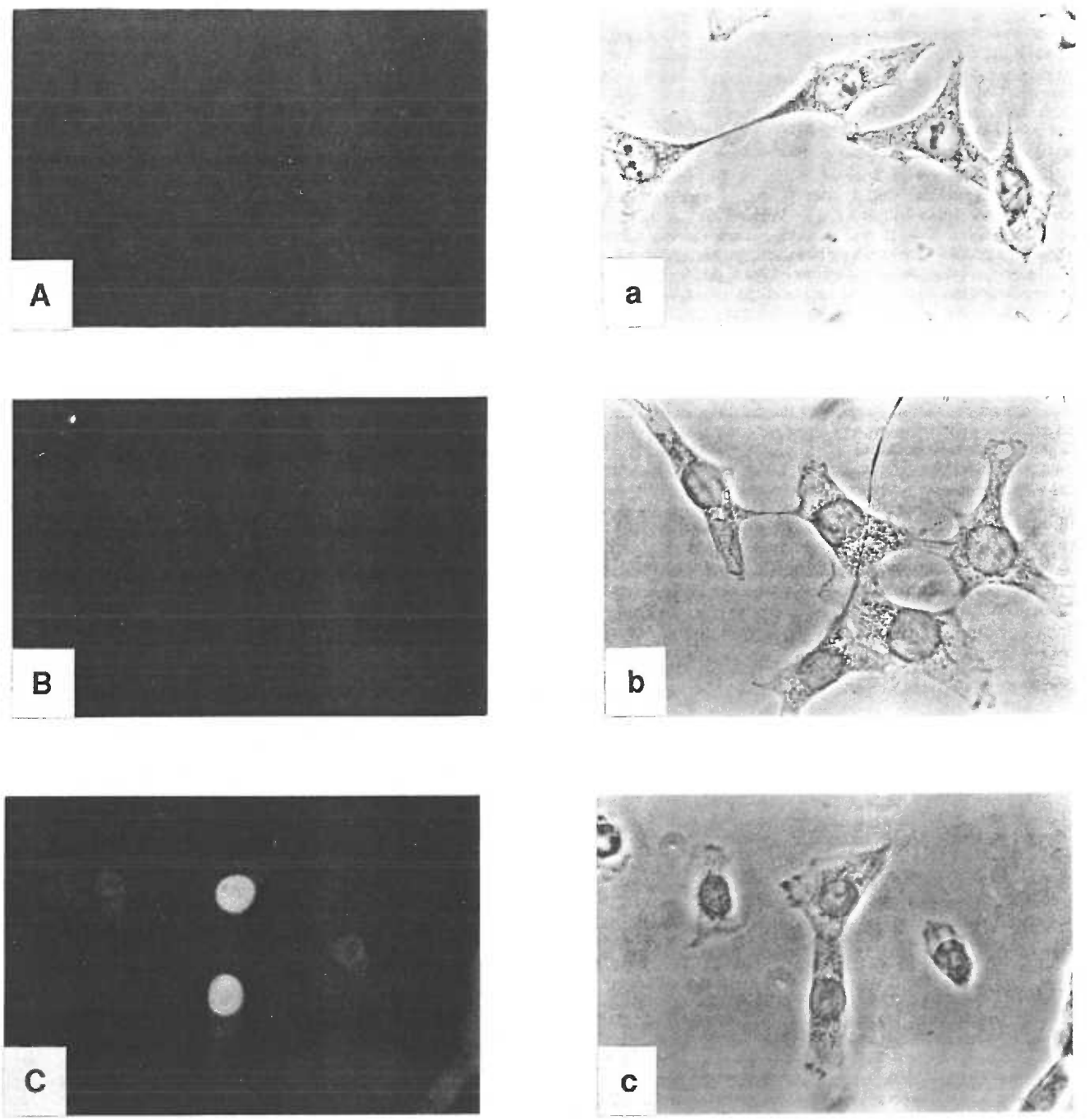

FIG. 4. Nuclear localization of PU.1 by indirect immunofluorescence microscopy. Each microscopic field is shown in fluorescence (A, B, and C) and in phase-contrast (a, b, and c). Cells were incubated with preimmune rabbit serum (A and a) or PU.1-specific serum (B, b, C, and c) followed by goat anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (see Materials and Methods). (A, a, C, and c) Cocultures of the ψ2 and PA12 lines of mouse NIH 3T3 fibroblasts expressing PU.1 from a retroviral vector; (B and b) control coculture of ψ2 and PA12 cells that lacks PU.1. Magnification is ×400.

F-MEL cells treated with Me_2SO declines gradually to 75% of control levels by 48 h but remains constant thereafter (62). As shown in Fig. 8, the amount of PU.1 in IP/IR cells cultured in the presence of Me_2SO remained unchanged for 1 to 2 h and then declined drastically to approximately 10% of the initial quantity by 8 h. PU.1 was undetectable after 24 h. On a much longer exposure of the protein blot in Fig. 8, the form of PU.1 that migrated with an apparent M_r of 37,000 was also detected; it declined similarly to the other PU.1 components.

DISCUSSION

This work provides an initial description of PU.1 protein and an analysis of its expression throughout leukemic progression and during the chemically induced differentiation of

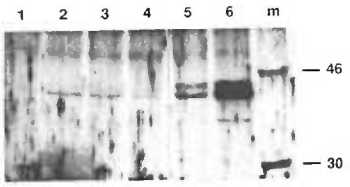

FIG. 5. Western blot analysis of PU.1 protein levels during leukemic progression. Cell lysates (1 mg of total protein) from mouse spleens (lanes 2 through 4) and Friend erythroleukemia cell lines (lanes 5 and 6) were immunoprecipitated and immunoblotted with PU.1-specific antiserum as described in Materials and Methods. Lanes: 1, immune precipitation buffer treated with Pansorbin and antiserum but without cell lysate; 2 through 4, lysates from a normal spleen, a spleen from a phenylhydrazine-treated mouse, and a preleukemic spleen harvested 13 days after infection with Friend virus, respectively (see Materials and Methods for a more complete description); 5 and 6, lysates from F745 and IP/IR erythroleukemia cells, respectively; m, rainbow ^{14}C-methylated protein molecular weight standards. Markers represent the positions of the ovalbumin and carbonic anhydrase protein standards and indicate their sizes (46 and 30 kDa, respectively).

cultured F-MEL leukemia cells. In all respects, our results are compatible with previous evidence (see introduction) (37, 45, 65) that SFFV proviral integration adjacent to PU.1 may abrogate the commitment of erythroblasts to terminally differentiate and result in their immortal phenotype in vivo. Thus, our studies suggest that PU.1 proteins are absent or present in only trace quantities in normal erythroblasts or in

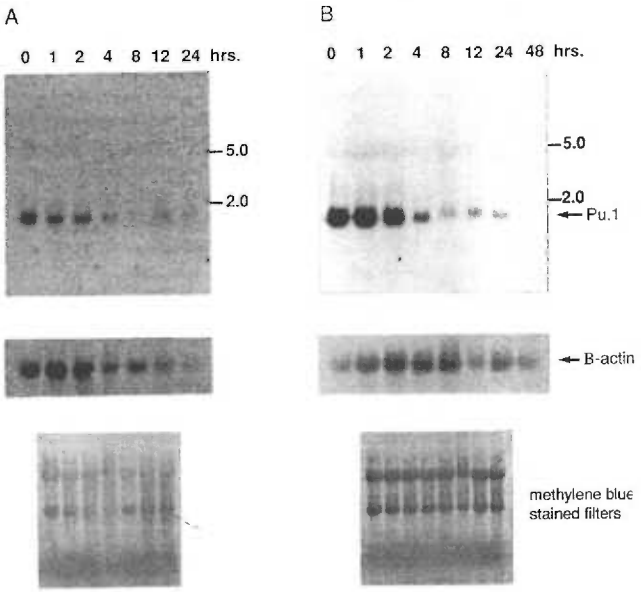

FIG. 6. Expression of PU.1 mRNA during chemically induced differentiation. F745 erythroleukemia cells were cultured with 1.8% Me_2SO (A) or 5 mM HMBA (B), and RNA was isolated at the times indicated. Twenty micrograms of total cellular RNA was loaded in each lane. The upper panels represent RNA blot analysis performed with a radiolabeled fragment of *Sfpi*-1 cDNA (see Materials and Methods); the middle panels represent the same blots hybridized with a radiolabeled mouse β-actin probe described in Materials and Methods; the lower panels show the filters stained with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5). Size markers are in kilobases and indicate the relative mobilities of the 28S and 18S rRNAs.

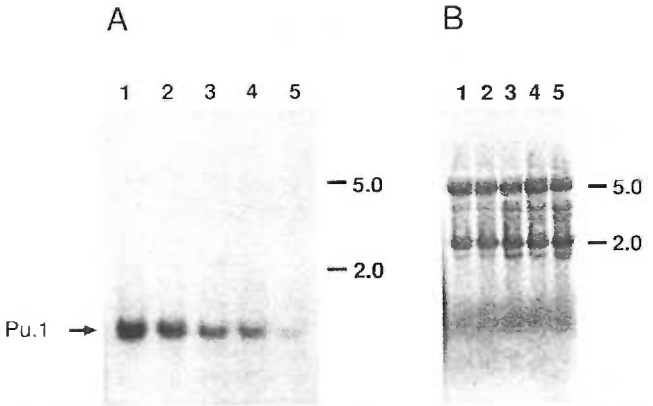

FIG. 7. Northern blot analysis of PU.1 mRNA stability in IP/IR erythroleukemia cells. Actinomycin D was added to a final concentration of 5 μg/ml of medium to cultures containing 1.5×10^6 cells per ml. RNAs were isolated at the following times after addition of the drug: lanes 1 through 5, 2, 3, 4, 5 and 7 h, respectively. Twenty micrograms of total RNA was loaded in each lane. (A) The blot was hybridized with a radiolabeled *Sfpi*-1 probe. (B) The same blot was stained with methylene blue to visually inspect the RNAs for uniform transfer and integrity. Size markers are in kilobases and indicate the relative mobilities of the 28S and 18S rRNAs.

SFFV-infected erythroblasts from preleukemic (nonimmortal) stages of Friend disease (e.g., Fig. 5) but are abundantly expressed in permanent F-MEL cell lines. High expression occurs even in the few erythroleukemia cell lines that seem to lack *Sfpi*-1 proviral integrations (46); this finding suggests that PU.1 expression can be activated at a very low frequency by a mechanism that does not involve a provirus. Furthermore, chemically induced differentiation of cultured F-MEL cells is associated with rapid degradation of PU.1 mRNA (Fig. 6) and protein (Fig. 8). Previous studies suggested that F-MEL cells recommit to the program of terminal differentiation between 12 and 24 h following treatment

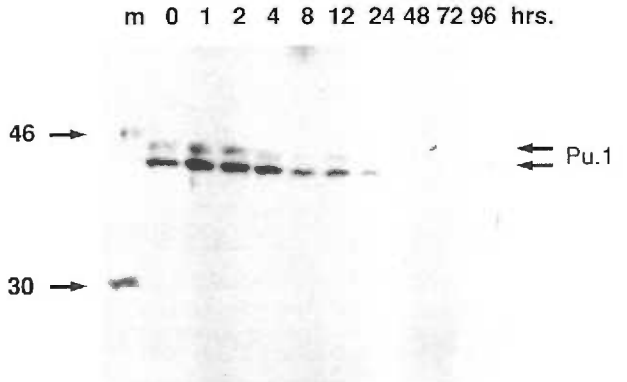

FIG. 8. Immunoblot of PU.1 protein levels during Me_2SO-induced differentiation. IP/IR erythroleukemia cells were cultured in the presence of 1.8% Me_2SO, and 5×10^6 cells were removed, washed in PBS, and lysed in immune precipitation buffer at the times indicated; 900 μg of protein lysate from each sample was immunoprecipitated and immunoblotted with PU.1-specific antiserum as described in Materials and Methods. Lane m, rainbow ^{14}C-methylated protein molecular weight standards. Markers show the positions of the ovalbumin and carbonic anhydrase protein standards. PU.1 forms that migrated with apparent M_rs of 44,000 and 42,000 are indicated at the right.

with chemical inducers (6, 18) and that this results in loss of immortality and inability of the cells to form tumors in mice (12). Commitment in this context is defined as the change(s) that occurs while F-MEL cells are exposed to an inducer that renders differentiation irreversible even in its absence. Interestingly, this loss of leukemic properties of the cells appears to coincide with loss of PU.1. Thus, both induction of immortality during disease progression and its chemically induced reversal in cell cultures appear to be associated with consistent changes in expression of PU.1 proteins.

Previous approaches to understanding Friend erythroleukemia have involved studies of oncogenes implicated in other cancers. These studies have shown that the proto-oncogenes c-*myc*, c-*myb*, and K-*ras* and the nuclear protein p53 are expressed in large amounts in Friend erythroleukemia cells compared with normal erythroblasts (25, 53) and that their expressions decline rapidly during chemically induced differentiation (25, 51, 53, 60). Constitutive expression of c-*myc* (9, 11, 47) or c-*myb* (35) substantially inhibits cell differentiation. More detailed studies have shown, however, that constitutive expression of either c-*myc* (29) or c-*myb* (35) during the commitment phase of differentiation (up to 24 h postinduction) does not significantly impede differentiation, whereas constitutive expression during the postcommitment phase does. Therefore, commitment does not require loss of either c-*myc* or c-*myb*. Accordingly, suppression of either c-*myc* or c-*myb* expression does not cause spontaneous differentiation of F-MEL cells (48, 69).

Our results also demonstrate that PU.1 mRNA turns over relatively rapidly in F-MEL cells (Fig. 7). Rapid turnover rates have been reported for other *ets*-related transcription factors (13) and for other nuclear oncogenic mRNAs (49, 58) and proteins (54). However, PU.1 mRNA does not contain an AU-rich 3′-untranslated region or the AU pentanucleotide consensus sequence that is partially responsible for rapid degradation of the c-*fos* transcript (47, 63).

We were surprised by the heterogeneity of PU.1 proteins and the existence of major components (e.g., 42,000- and 44,000-M_r forms) substantially larger than the full-length 36,500-M_r protein predicted from the cDNA sequence (Fig. 2 and 3). This cannot be ascribed to an unusually slow electrophoretic mobility of the PU.1 protein because full-length PU.1 made in *Escherichia coli* with a 15-amino-acid extension on its amino terminus migrates as expected for a protein with an M_r of 38,000 (Fig. 2). We have found that the PU.1 proteins are all phosphorylated and have obtained evidence that they are probably not glycosylated or ubiquitinylated. We are unaware of any studies that would indicate whether similar size modifications affect other *ets*-related proteins. Interestingly, the highest 44,000-M_r PU.1 component is more abundant in F-MEL cells than in the RAW 264.7 macrophage cell line (unpublished results) or in normal spleen cells (e.g., Fig. 5). Therefore, the structure of PU.1 is physiologically regulated.

Although our evidence is compatible with previous virological and pathogenic evidence that PU.1 may abrogate the commitment of erythroblasts to differentiate and lead to their immortality, additional studies will be required to thoroughly test this hypothesis. Recently, we have found that the PU.1-encoding retrovirus described here can immortalize erythroblasts in long-term murine bone marrow cultures (59).

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