The Hematopoietic Transcription Factor PU.1 Is Downregulated in Human Multiple Myeloma Cell Lines

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PU.1 is a hematopoietic transcription factor belonging to the Ets-family. It is identical to the Spi-1 oncogene, which is implicated in spleen focus-forming virus-induced murine erythroleukemias. PU.1 seems to be required for early development of multiple hematopoietic lineages, but its expression in mature cells is preferentially observed in cells of the B-cell- and monocyte/macrophage-differentiation lineage. It binds the so-called Pu box, an important tissue-specific regulatory DNA element present in a number of genes expressed in these cell lineages. We have analyzed the expression and activity of PU.1 during human B-cell development using a panel of B-cell lines representing different stages of maturation, from early precursors to differentiated plasma cells. PU.1 mRNA expression and PU.1 DNA binding activity, as measured by Northern blot analysis and electrophoretic mobility shift assay, respectively, were evident in cell lines representing pro-B, pre-B, and mature B cells. We could also show Pu box-dependent transactivation of a reporter gene in transient transfections in these cell lines. In contrast, in a number of multiple myeloma cell lines, representing differentiated, plasma cell-like B cells, PU.1 mRNA expression, Pu box-dependent transactivation were absent or detectable at a very low level. In lymphoblastoid cell lines, which exemplify an intermediate stage of B-cell differentiation, a reduced expression and activity were observed. The findings in the human multiple myeloma cell lines represent the first examples of B cells with downregulated PU.1 expression and apparently contradict observations in the murine system in which PU.1 is expressed and active in plasmacytoma cell lines. At present, it is unclear whether the lack of PU.1 expression and activity in human multiple myeloma cell lines represents a malignancy-associated defect in these cells or exemplifies a normal developmental regulation in terminally differentiated B cells. © 1995 by The American Society of Hematology.

AN IMPORTANT AREA of research on cellular differentiation is the identification of transcription factors regulating cell lineage- and differentiation stage-specific gene expression. Examples of such factors in the hematopoietic system are the Oct-2 and GATA family proteins. Another transcription factor specific for hematopoietic cells is PU.1, originally described as a lymphoid- and macrophage-specific factor. However, by gene targeting, PU.1 was recently shown to be required for the development of multiple hematopoietic lineages. PU.1 belongs to the ets proto-oncogene family (including Ets1, Ets2, Erg, Elf1, GABPα, PEA3, Fli-1, E74A, Elk1, and SAP1) characterized by the well-conserved ETS domain that mediates specific DNA binding to the core sequence GGA. The N-terminal part of the protein, which is only weakly or not at all homologous to other Ets-proteins, contains a glutamine-rich transactivation domain and is able to bind to the TATA-box-binding protein and the retinoblastoma protein Rb in vitro. PU.1 also contains a motif that mediates protein-protein interactions with the B-cell-specific transcription factor NF-EM5.

PU.1 is identical to the oncogene Spi-1 implicated in the development of spleen focus-forming virus-induced murine Friend erythroleukemia. Spleen focus-forming virus was found to integrate in the Spi-1 locus in 95% of the tumors, resulting in an elevated expression of Spi-1 mRNA. The observations that overexpression of Spi-1/PU.1 immortalizes primary erythroblasts and that antisense oligonucleotides inhibit the proliferation of Friend murine erythroleukemia cell lines further indicated a role for Spi-1/PU.1 in the growth control of immature erythroid cells. The Ets family members v-Ets and Fli-1 have been implicated in the development of erythroid tumors in chickens and mice, respectively.

The expression of PU.1 in mature hematopoietic cells was reported to be confined to B cells, monocytes/macrophages, and mast cells, whereas it seems to be more broadly expressed at early stages of lymphoid and myeloid development. This would also agree with the gene-targeting data which suggested that it is required for the early development of several lineages. The PU.1 binding site, the so-called Pu box, has been identified as an important tissue-specific regulatory element in the promoters or enhancers of a number of differentiation-associated genes in different hematopoietic cell types. Thus, PU.1 has been implicated as an important tissue-specific factor regulating these genes in intimate cooperation with other transcription factors (such as NF-EM5, or Spi1) or other Ets-family members (such as Ets1, Erg-3, or GABPα). We are interested in the role of PU.1 during development of human B-lineage cells and its possible involvement in tumor formation in these cells. A panel of human B-cell lines representing different stages of development, from early precursors to differentiated plasma cells, was used for studies of PU.1 expression and activity. We found differences in PU.1 mRNA expression, PU.1 DNA binding, and transcriptional activity in cell lines at different maturation stages. In particular, we can show that PU.1 activity and expression was shut off in a panel of multiple myeloma cell lines, representing differentiated B cells. The implications of these findings will be discussed.

MATERIALS AND METHODS

Cell lines. All cell lines were maintained in RPMI 1640 (Flow Laboratories, Ayrshire, UK) supplemented with 7.5% fetal calf se-

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rum (GIBCO, Grand Island, NY), glutamine, 100 IU/mL penicillin, and 50 μg/mL streptomycin. A panel of cell lines was selected representing different stages of B-cell development. KM3 is derived from a non-B, non-T acute lymphocytic leukemia and represents a lymphoid precursor cell line.26 BJA-B, Raji, Daudi, and Ramos are B-lymphoma cell lines representing pre-B to mature B cells.27 Karpas 422 and MN60 are B-lymphoma and leukemia cell lines, respectively,27,28 representing mature B cells. U-255 and Corinna II are Epstein-Barr virus-immortalized Ig-secreting lymphoblastoid cell lines,29,30 representing B cells at the B-blast/immunoblast stage. Karpas 707, L363, U-1996, and U-266 are multiple myeloma cell lines19,31 and represent plasmablasts/plasma cells. U-266-1970 and U-266-1984 are early and late passages, respectively, of an IgE myeloma cell line, the phenotype of which has been described.32

**Transfection and chloramphenicol acetyltransferase (CAT) assay.** Transfections were performed using the diethyl aminoethyl (DEAE)-dextran method.33 The cells were fed 24 hours before transfection, and 20 × 10⁶ cells were mixed with a solution containing 300 μg DEAE-dextran (Pharmacia, Sollentuna, Sweden) and 5 μg DNA. The mixture was incubated for 30 minutes at room temperature, followed by glycerol treatment for 3 minutes. The cells were washed once in phosphate-buffered saline before fresh medium was added. Seventy-two hours postinfection, the cells were harvested, and a CAT assay was performed.35

**Nuclear extracts and electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared from 40 × 10⁶ cells using the lysis/cellithin procedure described by Zervitz and Akusjärvi.36 Ten micrograms of extract was used for each binding reaction, and 1 μg poly(dI-dC)poly(dI-dC) (Pharmacia) and 10 pmol single-stranded oligonucleotide were included as nonspecific competitors. EMSA, with the Pu box probe, was performed in a buffer containing 10 mM HEPES (pH 7.9), 10% glycerol, 20 mM KCl, 4 mM MgCl₂, 10 mM EDTA, 0.25 mM dithiothreitol, 4 mM spermidine, and 100 μg/mL bovine serum albumin. The Sp1 EMSA was performed in 10 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 5 mM MgCl₂, 0.6 mM dithiothreitol, and 200 μg/mL ZnSO₄. The extract was added to the buffer including the nonspecific competitor and was incubated for 20 minutes at room temperature. Pu box or the Sp1 oligonucleotide (1 to 2 fmol), end-labeled with Klenow DNA polymerase or T4 DNA polynucleotid kinase, respectively, was added, and the mixture was further incubated for 20 minutes at room temperature. Samples were analyzed on a 4% polyacrylamide gel in 0.25x TBE (0.022 mol/L Tris-borate and 0.5 mM EDTA). The Pu box oligonucleotide sequence (5'TCGACT- CTTAGAAGAGGAACTCTCGAGCT) is derived from the SV40 control region.37 The mutated Pu box sequence is 5'TCGACT-CGTCGCCAGAGGAACTCTCGAGCT. The Sp1 oligonucleotide (5'CCTGGGCCCCGCGCATCTCGGGCCCGCATCC) contains a dimer of a binding site found in the immediate early gene 3 of herpes simplex virus. Antibody 1297 (kindly provided by Dr van Beveren, La Jolla Cancer Research Foundation, La Jolla, CA) is specific for the N-terminal region of PU.1, and antibody T-21 (Biotechnology, Santa Cruz, CA) recognizes the C-terminal part of the protein.

**Plasmid constructions.** pCAT-Control plasmid (Promega, Madison, WI) with the SV40 early promoter and enhancer sequences served as the control plasmid in the transfection experiments. An Xho I linker was cloned into the Bgl II site of pCAT-Promoter plasmid (Promega), and the new plasmid was designated pPromX-CAT. Four copies of the Pu box oligonucleotides (wild-type and mutated) were cloned into the oligonucleotide vector (OVEC) plasmid.37 The resulting Xho I-Sal I fragment was then inserted into the Xho I site of pPromXCAT.

**Northern analysis.** Total RNA was extracted from exponentially growing cells by the LiCl/urea method.38 RNA (15 μg) was denatured in formamide and fractionated in an 1% agarose gel containing formaldehyde (4 mL/100 mL gel). After electrophoresis, the RNA was transferred to a nitrocellulose filter. The probes were ³²P-labeled by the random priming method (Amersham, Buckinghamshire, UK). Hybridization was performed at 42°C in a solution containing 50% formamide, 1X Denhardt’s solution, 2X SSC (2X SSC: 0.3 mol/L sodium chloride and 30 mM sodium citrate), 5 mM NaPO₄, 0.1% sodium dodecyl sulfate, and 200 μg/mL of salmon sperm DNA. Filters were washed in 0.5% sodium dodecyl sulfate and 2X SSC at 50°C and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). A murine PU.1 cDNA clone (unpublished, Dr L. Hellman, Department of Medical Immunology and Microbiology, University of Uppsala, Sweden) and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA clone37 were used as probes.

**RESULTS**

**Absence of PU.1 DNA binding activity in multiple myeloma cell lines.** To establish the conditions for studies of PU.1 DNA binding, we used the human BJA-B cell line known to express PU.1.3 A ³²P-labeled oligonucleotide containing the Pu box sequence from the SV40 control region was mixed with nuclear extracts from BJA-B cells and analyzed by EMSA. The SV40 Pu box binds PU.1 with high affinity but is a poor binding site for many other Ets-family proteins.40-42 Figure 1A shows that two major retarded complexes, a and b, were formed. These complexes were competed by an access unlabeled Pu box oligonucleotide but not by a mutated oligonucleotide or an oligonucleotide containing an Sp1 binding site, thus showing the specificity of the binding. The mutated oligonucleotide, previously shown to eliminate binding of PU.1, contains 4 substitutions 4-7
PU.1 DNA binding activity in B-cell nuclear extracts. EMSA using the Pu box oligonucleotide was performed with nuclear extracts prepared from the following B-cell lines: lane 1, no extract; lane 2, KM3; lane 3, BJA-B; lane 4, Daudi; lane 5, Raji; lane 6, Ramos; lane 7, Karpas 422; lane 8, MN60; lane 9, U-255; lane 10, Corinna II; lane 11, Karpas 707; lane 12, L363; lane 13, U-1996; lane 14, U-266-1970; and lane 15, U-266-1984.

nucleotides 5' of the the GGA core binding site for Ets-family proteins. Addition to the binding reaction of antibodies directed to the N-terminus of PU.1 resulted in a supershift of the upper but not the lower Pu box binding complex, thus confirming the presence of PU.1 or an antigenically closely related protein in the former (Fig 1B). Addition to the binding reaction of antibodies specific for the C-terminus of PU.1 prevented formation of both the upper and lower complexes (data not shown), indicating that the lower complex represents a degradation product of PU.1 containing the DNA binding domain but lacking the N-terminal antibody-binding epitope. PU.1 contains a so-called PEST sequence suggested to play a role in protein degradation.

To investigate the DNA binding activity of PU.1 in relation to B-cell differentiation, nuclear extracts were prepared from various human B-cell lines representing different stages of B-cell differentiation. These included the pro-B-cell line KM3; the B-lymphoma cell lines BJA-B, Raji, Daudi, and Ramos representing pre-B to mature B cells; the Karpas 422 follicular lymphoma cell line and B-leukemia cell line MN60 representing mature B cells; the U-255 and Corinna II Ig-secreting lymphoblastoid cell lines and, finally, the multiple myeloma cell lines Karpas 707, L363, U-1996, U-266-1970, and U-266-1984 representing plasmablasts-plasma cells. The extracts were analyzed by EMSA using the Pu box sequence as above. Figure 2 shows that a Pu box-binding complex comigrating with the upper PU.1 complex in BJA-B cells was clearly shown in the cell lines representing early and mature B cells (Fig 2, lanes 2-8). In contrast, three of the myeloma cell lines, U-1996, U-266-1970, and U-266-1984, were completely negative; in the other two, L363 and Karpas 707, a very faint band could be detected (Fig 2, lanes 9 and 10). Compared with early and mature B-cell lines, a reduced PU.1 binding activity was observed in the lymphoblastoid cell lines, in particular in Corinna II (Fig 2, lanes 9 and 10).

Figure 3 shows that an oligonucleotide containing an Sp1 binding site showed specific Sp1 binding activity in the myeloma cell extracts as well as in BJA-B extracts, thus ruling out a general degradation of the myeloma extracts.

Absence of Pu box-dependent transcriptional activity in multiple myeloma cell lines. We next investigated whether the observed binding of PU.1 to the Pu box in the various extracts correlated with transcriptional activity. Four copies of the Pu box oligonucleotide and a mutated version thereof were cloned upstream of the SV40 early promoter in a CAT gene construct. The plasmids were transfected into selected B-cell lines, and, 72 hours after transfection, the cells were harvested and assayed for CAT activity. A construct with the SV40 early promoter and enhancer sequences was
transfected in parallel and served as a positive control. In the BJA-B, Raji, U-255, and Karpas 422 cell lines, the presence of the Pu boxes led to an enhanced transcription from the CAT gene to various degrees, whereas mutations introduced in the Pu box reduced the activity to basal level (Fig 4). Densitometrical scanning of the autoradiogram and normalizing to the activity of pPromXCAT vector lacking Pu boxes showed that four copies of the Pu box stimulated transcription 28 times in BJA-B cells, 8 times in Raji cells, and 3 times in U-255 and Karpas 422 cells. Transfection of the myeloma cell lines L363 and U-266-1984 with the Pu box-containing plasmids did not lead to activation of the CAT gene, thus correlating with the lack of Pu box binding in these cells. A control CAT reporter plasmid containing SV40 promoter and enhancer sequences strongly stimulated transcription in the myeloma cell lines. We were also unable to detect any Pu box-dependent transcriptional activity in the Corinna I lymphoblastoid cell line, which has a reduced PU.1 binding activity.

The PU.1 gene is downregulated in multiple myeloma cell lines. The lack of PU.1 activity in the myeloma cells could be because of a downregulation of the PU.1 gene expression or, alternatively, because the protein is present in an inactive form. To investigate the expression of PU.1 in the panel of B-cell lines used above, RNA was prepared, and a Northern blot analysis was performed using a mouse PU.1 cDNA as probe. Figure 5A shows that the 1.4-kb PU.1 transcript was expressed in all B-cell lines with the exception of the myeloma cell lines, in which it was undetectable even after prolonged exposure. The lymphoblastoid cell line U-255 showed a reduced expression, and the level of PU.1 mRNA was very low, but detectable, in Corinna II. As a control, the same filter was rehybridized with a GAPDH probe, showing that this gene was expressed in all cell lines (Fig 5B). We conclude that the PU.1 gene is shut off in the myeloma cell lines, thus explaining the lack of PU.1 transcriptional activity and DNA binding in these cells.

DISCUSSION

PU.1 is a transcription factor specific for hematopoietic cells that seems to be required for development of early
precursor cells. It has also been suggested to play a role in mature B cells and macrophages. We have undertaken a study to investigate the presence and activity of PU.1 during human B-cell development using a panel of human B-cell lines representing the B-cell lineage from early precursors to differentiated plasma cells.

Our results show major differences in the expression and activity of PU.1 in cell lines representing different levels of B-cell development. PU.1 mRNA expression and PU.1 DNA binding activity was shown in the pro-B-cell line KM3 as well as in pre-B and mature B-cell lines. Pu box-dependent transcrional activity of a CAT reporter gene was also shown in these cell lines in transient transfections. In contrast, in a panel of multiple myeloma cell lines representing differentiated B cells, no or only very low mRNA expression, DNA binding activity, and Pu box-dependent transactivation were detectable. A reduced PU.1 activity and expression were observed in the lymphoblastoid cell lines U-255 and Corinna II, representing an intermediate stage of differenntiation. This reduction was most pronounced in Corinna II, which seems further differentiated than many other Epstein-Barr virus-transformed lymphoblastoid cell lines in that it lacks CD37 expression (our unpublished observations). However, Corinna II is clearly less differentiated than the myeloma cell lines.

Our observations in human pro-B, pre-B and mature B cells are in good agreement with previous studies in murine B-cell lines. However, the lack of PU.1 expression and activity in multiple myeloma cell lines is a unique finding and represents the first example of B cells with down-regulated PU.1 expression. Our results are apparently contradictory to results obtained in the murine system, in which PU.1 is expressed and active in most plasmacytoma cell lines.

Our finding in human multiple myeloma cell lines may be interpreted in at least two different ways. (1) Because PU.1 seems generally expressed in other B cells, one interpretation is that the lack of PU.1 is a malignancy-associated defect common to human multiple myeloma. If PU.1 is an important transcription factor involved in commitment along the B lineage, it is conceivable that loss of PU.1 may result in problems for the cell to recognize itself as a B cell, and, thus, it may be unable to complete the differentiation program and develop into terminally differentiated, resting plasma cells. However, from Southern analysis (data not shown) and cytogenetic analysis of chromosome 11 we have found no evidence for chromosomal aberrations of the PU.1 loci in the myeloma cell lines. This does not exclude minor alterations such as small deletions or point mutations.

(2) An alternative interpretation is that the shut off of the PU.1 gene is a normal event occurring at a late stage of B-cell development corresponding to that of the myeloma cell lines. This interpretation is supported by the reduced expression and activity in the U-255 and Corinna II cell lines representing an intermediate stage of differentiation. Data from homozygous gene-targeted Pu.1 mouse embryos suggest that Pu.1 is a very early hematopoietic transcription factor required for generation of B- and T-lymphocyte, monocyte, and granulocyte progenitors, thus acting already at a stage of a multipotential stem cell. This interpretation would be in agreement with previous expression studies suggesting that PU.1 is expressed in early myeloid/lymphoid cells committed to the erythroid, granulocytic, monocyctic, mast cell, B- and T-lymphoid lineages. It is then shut off early in T-cell, erythroid, and granulocytic differentiation but is expressed at later stages of monocyctic, mast cell, and B-cell differentiation. The present data may suggest that it is shut off late during B-cell differentiation, thus performing its function at preterminal stages of B-cell development. From this perspective, the differences in expression in human myelomas and murine plasmacytomomas may be caused by slight differences in maturation stage or a malignancy-associated inability to shut off PU.1 in the plasmacytomomas or might represent a species difference. Studies of PU.1 expression in normal human and mouse plasma cells are required to clarify these points.

It may seem remarkable that PU.1 is shut off in human myeloma cell lines that produce Ig at a high level, because PU.1 has been suggested to be one important transcriptional activator of both the heavy- and light-chain Ig genes. However, there are alternative pathways of Ig gene regulation mediated by, for instance, Oct-2 and NF-kB, acting on the heavy chain enhancer and on the k-chain intron enhancer, respectively, that seem to increase in importance at late stages of B-cell differentiation. Therefore, such alternative pathways may be in operation in the human myeloma cell lines. Other genes suggested to be regulated by PU.1 in B cells are the J-chain, the mb-1 and B29 antigen receptor-associated signaling proteins, and the major histocompatibility complex class II antigens. The expression of these genes seems to be connected to B-cell activation, proliferation, and IgM secretion at preterminal stages of differentiation rather than to terminally differentiated plasma cells or myelomas.

Therefore, one interesting possibility is that PU.1 might be a transcription factor which integrates differentiation and cell growth at proliferative stages along the B-cell differentiation lineage. The oncogenic potential of PU.1/Spi-1 in erythroleukemia and its ability to interact with Rb in vitro would point in this direction. Immunohistochemical studies in normal murine bone marrow also suggest the highest expression of PU.1 in dividing cells. An attractive hypothesis is that PU.1 is replaced by an alternative Ets-family or a non-Ets transcription factor such as Oct-2 or NF-kB, lacking proliferative or having an antiproliferative capacity, at terminal stages of B-cell differentiation in which high expression of the differentiation-associated genes is connected to irreversible growth-arrest. Examples of other Ets-family members expressed in hematopoietic cells are Ets1, Ets2, Erg, Flt-1, Elf1, GABPz, and/or Sp1. The EMISA and transactivation assays in the myelomas suggest that PU.1 is not replaced by an Ets-family protein with capacity to bind the SV40 Pu box. However, other PU.1 binding sites in relevant cellular target genes may have a broader Ets-family binding specificity that could result in a competition between different Ets-family proteins or a sequential role of these during B-cell development. Further studies of PU.1 and other Ets-family proteins during normal B-cell development is re-
required to establish whether our findings in multiple myeloma cell lines represent a malignancy-associated deregulation of PU.1 in these cells or exemplify a normal phase of B-cell differentiation.

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