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(54) Title: NOVEL TRANSCRIPTION FACTOR, BP1

(57) Abstract: An isolated DNA of SEQ ID NO: 1 is provided that encodes the transcription factor BP1, which is believed to be a repressor of the  $\beta$ -globin gene. A host cell that is transformed with a vector that contains the DNA may be used to produce BP1. Vectors having a controllable promoter operably connected to the BP1 open reading frame may be used to transform  $\beta$ -globin producing cells of patients with sickle cell anemia, thereby providing a treatment. Because BP1 is overexpressed in leukemia and breast cancer cells, acute myeloid leukemia, acute lymphocytic leukemia, and breast cancer can be screened for and diagnosed by determining whether BP1 is overexpressed in cell samples of patients who may have these conditions. An antisense DNA or RNA to the DNA encoding BP1 may be used as a treatment for acute myeloid leukemia, acute lymphocytic leukemia, and breast cancer.

## NOVEL TRANSCRIPTION FACTOR, BP1

5 The present application claims the benefit of the filing date of United States Provisional Application No. 60/148,940, filed August 13, 1999. The provisional application is incorporated by reference herein.

**Statement As To Federally Sponsored Research**

Work described herein was supported by NIH grant R01DK53533. The U.S. Government has certain rights in the invention.

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**Technical Field**

15 The present invention relates to a DNA that encodes the transcription factor BP1, a vector containing the DNA and a host cell containing the DNA. The invention also relates to an antisense DNA or RNA to the DNA encoding BP1, methods for treating sickle cell anemia by administering an effective amount of BP1, and methods for screening for acute myeloid leukemia, acute lymphocytic leukemia, and breast cancer.

20 (In the provisional application, the transcription factor BP1 was sometimes called "BP1/D1x9" or "BP1/D1x9". Herein, for greater clarity, the transcription factor is simply referred to as "BP1".)

25 Expression of globin genes in the  $\beta$ -globin cluster is restricted to erythropoietic cells, with five different genes expressed during embryonic ( $\epsilon$ ), fetal ( $G\gamma$  and  $A\gamma$ ) and adult ( $\delta$  and  $\beta$ ) development. Transcriptional activation of globin genes occurs not only by binding of transcriptional activator proteins to the promoter of the gene being activated, but also by a regulatory element located 6 - 18 kb upstream of the  $\beta$ -globin cluster, the Locus Control Region (LCR) (See, for example, **Berg,**  
30 **P. E. and A. N. Schechter.** 1992. Molecular genetics of disorders of hemoglobin. In T. Friedmann (ed), Molecular Genetic

Medicine. Academic Press, San Diego.; **Forrester, W. C., C. Thompson, J. T. Elder, and Groudine, M.** 1986. A developmentally stable chromatin structure in the human  $\beta$ -globin gene cluster. Proc. Natl. Acad. Sci. USA **83**: 1359-1363.; and **Tuan, D., W. Soloman, Q. Li, and I. M. London.** 1985. The " $\beta$ -like-globin" gene domain in human erythroid cells. Proc. Natl. Acad. Sci. USA **82**: 6384-6388.). Sequential activation of the  $\beta$ -globin cluster genes during ontogeny must be countered by repression of the globin genes inactive during a given developmental stage. Repression is caused by binding of repressor proteins to promoter/upstream DNA and, in the case of the adult  $\beta$ -globin gene, is probably also due to lack of activation by the LCR (see, for example, **Crossley, M. and S. H. Orkin.** 1993. Regulation of the  $\beta$ -globin locus. Curr. Opin. Gen. Dev. **3**: 232-237.). While much is known about transcriptional activators that bind to DNA sequences near the  $\beta$ -globin gene, little is known about the proteins that repress its transcription.

As discussed below, BP1 is shown to bind to two silencer DNA sequences upstream of the  $\beta$ -globin gene and therefore, there is strong evidence suggesting that BP1 protein is a repressor of the  $\beta$ -globin gene. The present invention provides for a DNA sequence that encodes BP1, and methods of using information derived from knowledge of the DNA sequence to screen for conditions such as breast cancer, acute myeloid leukemia and acute lymphocytic leukemia. The DNA sequence was found to be closely related to two other human genes, DLX4 and DLX7, described in **Quinn, L. M., B. V. Johnson, J. Nicholl, G. R. Sutherland, and B. Kalionis.** 1997. Isolation and identification of homeobox genes from human placenta including a novel member of the Distal-less family, DLX4. Gene **187**: 55-61 and **Nakamura S, Stock DW, Wydner KL, Bollekens JA, Takeshita K, Nagai BM, Chiba, Kitamura T, Freeland TM, Zhao Z, Minowada J, Lawrence JB, Weiss KB, and Ruddle FH.** Genomic analysis of a new mammalian Distal-less gene: Dlx-7. *Genomics* 1996; **38**: 314-324.

Survival rates for many types of cancers correlate with early detection and treatment. Further, it is helpful to monitor ongoing cancer treatments to determine effectiveness. Accordingly, there is a continuing need for reliable cancer cell screening methods. One method of screening is to detect and monitor the expression of genes that are overexpressed or underexpressed in particular types of cancer cells, in comparison to normal cells. Detection techniques and particular markers have been disclosed, for example, in the following U.S. patents, incorporated herein by reference: U.S. Patent No. 5,776,683 to Smith et al; U.S. Patent No. 5,965,409 to Pardee et al; U.S. Patent No. 6,037,129 to Cole, et al; U.S. Patent No. 5,677,125 to Holt, et al; U.S. Patent No. 6,004,756 to Watson, et al; U.S. Patent No. 5,994,062 to Mulshine, et al; U.S. Patent no. 5,700,927 to Zon, et al; and U.S. Patent No. 5,981,218 to Rio et al.

Because no marker or method of screening is completely reliable, there is a continuing need in the art for additional genetic markers. In particular, there is a continuing need for genetic markers for identifying breast cancer, acute myeloid leukemia and acute lymphocytic leukemia. The present invention overcomes this problem in the art by providing DNA BP1 compositions and methods for screening for breast cancer, acute myeloid leukemia and acute lymphocytic leukemia.

The problem also exists in the art of lack of information regarding repression of the  $\beta$ -globin gene. As discussed below, there are medical conditions such as sickle cell anemia that can be treated by repressing the expression of  $\beta$ -globin and the present invention, provides for the production of BP1, a putative  $\beta$ -globin repressor.

#### Disclosure of Invention

It is an object of the present invention to provide a DNA sequence encoding BP1.

It a further object of the present invention to provide a

marker and a method for detecting acute myeloid leukemia and acute lymphocytic leukemia.

It is a further object of the present invention to provide a marker and a method for detecting breast cancer.

5 It is a further object of the present invention to provide a treatment for conditions that can be alleviated by repressing the expression of the  $\beta$ -globin gene.

It is a further object of the present invention to provide a treatment for sickle cell anemia.

10 It is a further object of the present invention to provide a treatment for conditions that can be alleviated by blocking the expression of BP1.

These and other objects are achieved in present invention by providing an isolated DNA having SEQ ID NO: 1, encoding BP1. BP1 exhibits a repressor function: it binds to Silencers I and II upstream of the  $\beta$ -globin gene and binds to a sequence of -530 bp upstream of the  $\delta$ -globin gene.

The invention also includes a vector comprising the DNA encoding BP1 and a host cell transformed with the vector.

20 In an alternative embodiment, the invention is directed to an antisense DNA or an antisense RNA of a DNA having SEQ ID NO: 1, encoding BP1.

The invention further provides a method of treating sickle cell anemia comprising administering an effective amount of BP1.

25 The invention provides a method of screening for and/or diagnosing acute myeloid leukemia or acute lymphocytic leukemia, the method comprising the steps of (a) obtaining a cell sample from a patient, and (b) determining whether BP1 is overexpressed by said cell sample as compared to normal cells.

30 The invention also provides a method of screening for and/or diagnosing breast cancer, the method comprising the steps of (a) obtaining a cell sample from a patient, and (b) determining whether BP1 is overexpressed by said cell sample as compared to normal cells.

35 The invention further provides for a polyclonal antibody to BP1.

The invention further provides a set of PCR primers for amplifying the DNA of SEQ ID NO. 1.

The above and other objects of the invention will become readily apparent to those of skill in the relevant art from the following detailed description and figures, wherein only the preferred embodiments of the invention are shown and described, simply by way of illustration of the best mode of carrying out the invention. As is readily recognized the invention is capable of modifications within the skill of the relevant art without departing from the spirit and scope of the invention.

### Brief Description of the Drawings

Figure 1 is a schematic representation of the region -100 to -600 bp upstream of the  $\beta$ -globin, showing that BP1 binds within Silencer I at -530 bp relative to the cap site (+1) and within Silencer II at -300 bp, and that BP2, another DNA-binding protein, binds within Silencer II at -270 bp.

Figure 2 is an autoradiogram showing the results of an EMSA competition assay to verify that the cDNA encoded a protein with the expected binding specificities of BP1, namely, the ability to bind to Silencers I and II upstream of the  $\beta$ -globin gene and to a sequence -530 bp upstream of the  $\delta$ -globin gene. The putative BP1 protein, fused to  $\beta$ -galactosidase, was expressed from a  $\lambda$  lysogen. This fusion protein was partially purified and incubated with the Silencer II probe used for screening the library, resulting in a single shifted band, indicated by the arrow, lane 1.

Figure 3A is an autoradiogram showing the results of a Northern blot of K562 RNA using part of the 630 bp BP1 cDNA (obtained as described herein) as a probe. An RNA band of 2.1 kb was recognized.

Figure 3B is an autoradiogram showing the results of Northern blot of fetal tissue, using the same probe as described in Figure 3A

Figure 4 is the sequence of BP1. This sequence is also set

forth in the attached sequence listing as SEQ ID NO:1. The open reading frame of the sequence is indicated as well as the predicted protein sequence, which is also set forth in the sequence listing as SEQ ID NO:2 and SEQ ID NO: 12.

5 Figure 5A and Figure 5B are graphs showing the relative CAT activity of K562 cells in transient transfection assays in relation to increasing amounts of pRSV/BP1-ORF (sub-cloned as described herein) or to an empty vector. In Figure 5A, pCAT/SI was the target DNA; in Figure 5B, pCAT/SII was the target DNA.  
10 K562 cells were transiently co-transfected with increasing amounts of either an empty vector or pRSV/BP1-ORF, along with a target plasmid.

Figure 6 is an autoradiogram showing the expression of BP1 in erythroid and myeloid lineage cell lines, as determined by RT-PCR.  
15

Figures 7A and 7B are autoradiograms showing the expression of BP1, DLX7 and DLX4 in T-cell ALL cell lines and erythromyloid cell lines, as determined by RT-PCR.

Figure 8 is an autoradiogram showing the expression of BP1, DLX7 and DLX4 in normal bone marrow, B-cells and T cells. Semi-quantitative RT-PCR was used to measure expression.  
20

Figure 9 is an autoradiogram showing the expression of BP1, DLX7 and DLX4 in acute myeloid leukemia. Samples from six patients are shown. (-) indicates expression equal to or less than normal controls; (+) indicates expression greater than controls; **(+)** indicates an expression ratio at least three times greater than the ratio for (+).  
25

Figure 10 is a graph comparing the expression of BP1, DLX7 and DLX4 in AML and T-cell ALL. Black bars represent adult AML, and white bars represent pediatric T-cell ALL.  
30

Figure 11 is an autoradiogram showing BP1 expression in CD34<sup>+</sup> and CD34<sup>-</sup> cells.

Figure 12 is a graph showing the percent viability over the course of four days of cell lines transformed with an BP1 antisense-producing plasmid (10B and 10D) in comparison with  
35

control cells receiving an empty vector(9A and 9B).

Figure 13 is an autoradiogram showing BP1 expression of the cell lines 9A - 9B receiving an empty vector (lanes 1 - 2) and cell lines transformed with an BP1 antisense-producing plasmid 10B-10D(lanes 3 - 4).

Figure 14 is an autoradiogram showing the expression of BP1 by several breast cancer cell lines, including breast cancer lines MCF7 ADR, MDA468 and T47D, and no expression in novel breast tissue.

Figure 15 is an autoradiogram showing the expression of BP1 in normal and malignant breast tissues.

#### **Best Mode For Carrying Out The Invention**

The present invention relates to an isolated DNA, having or consisting essentially of SEQ ID NO:1 and encoding the transcription factor BP1. The transcription factor BP1 exhibits  $\beta$ -globin repressor function and binds to Silencers I and II upstream of the  $\beta$ -globin gene, to a sequence of -530 bp upstream of the  $\delta$ -globin gene and to an Indian haplotype D sequence. BP1 is typically expressed in placenta and kidney. The predicted sequence of BP1, deduced from the open reading frame of the isolated DNA of SEQ ID NO: 1, is given as SEQ ID NOs: 2 and 12. Both the DNA sequence and the protein sequence are shown together in Fig. 4. As used herein, the term "isolated DNA" refers to DNA that is not in its native environment.

In a preferred embodiment of the invention, the isolated DNA encoding BP1 is a cDNA of 1251 bp. The isolated DNA also comprises an open reading frame (ORF).

The present invention further relates to a vector containing the DNA and to a host cell transformed with the vector containing the DNA. In a preferred embodiment, the transformed host cell is a eukaryotic host cell.

The invention further includes an antisense DNA or an antisense RNA of a DNA having SEQ ID NO:1.

The invention further includes a method of treating sickle cell anemia, the method comprising the step of administering an

effective amount of BP1 to a patient in need thereof. In the method of the invention, BP1 decreases  $\beta$ -globin expression in the patient and decreases intracellular concentrations of HbS.

5 The invention further includes a method of screening for and diagnosing acute myeloid leukemia or acute lymphocytic leukemia, the method comprising the steps of

(a) obtaining a cell sample from a patient, and

(b) determining if BP1 is overexpressed by said cell sample as compared to normal cells.

10 The invention also includes a method of screening for and diagnosing breast cancer, the method comprising the steps of

(a) obtaining a cell sample from a patient, and

(b) determining if BP1 is overexpressed by said cell sample as compared to normal cells.

15 In the methods for detecting leukemia and breast cancer, the overexpression of BP1 may be determined by detecting BP1-encoding mRNA by reverse transcriptase polymerase chain reaction (RT-PCR) or by detecting BP1 itself by immunohistochemical detection.

20 The invention further includes PCR primers that amplify a unique DNA molecule from a substrate of cDNA or mRNA that encodes for BP1. Such PCR primers can be readily derived from the DNA sequence of SEQ ID NO.: 1. As used herein, the term "unique DNA molecules" means that the DNA is of sufficient specificity to determine that the substrate being amplified originated from the  
25 sequence of SEQ ID NO:1.

The invention further includes polyclonal and monoclonal antibodies to BP1. A polyclonal antibody can be obtained by immunizing a mammal with BP1 or an antigenic fragment thereof in order to induce the production of sera containing polyclonal  
30 antibodies, as described below. A monoclonal antibody to BP1 may be obtained by hybridoma technology, which typically includes the further steps of isolating splenocytes from immunized mammals, fusing them with myeloma cells, then identifying fused cells that secrete antibodies that bind to BP1.

35 The invention further includes a method of repressing the  $\beta$ -globin gene comprising administering an effective amount of

BP1.

These aspects of the invention are further characterized below. First, it is shown that the cloning of BP1 cDNA and that its overexpression in a transient co-transfection assay causes repression of a reporter gene linked to either Silencer I or Silencer II DNA of the  $\beta$ -globin gene. This provides evidence that BP1 is a repressor of the  $\beta$ -globin gene. Sequencing reveals that the DNA encoding BP1 contains a homeobox, placing the DNA in a family of conserved genes that regulate each other and that regulate unrelated genes as well (see, for example, **Jaynes, J. B. P. H. and O'Farrell.** 1988. Activation and repression of transcription by homeodomain-containing proteins that bind a common site. *Nature* **336**: 744-749. and references therein; **Levine, M. and T. Hoey.** 1988. Homeobox proteins as sequence-specific transcription factors. *Cell* **55**: 537-540.). Sequence comparison indicates that the DNA encoding BP1 belongs to the Distal-less family of homeobox genes, which are expressed during early development (see, for example, **Cohen, S. M. and G. Jurgens.** 1989. Proximal-distal pattern formation in *Drosophila*: cell autonomous requirement for Distal-less gene activity in limb development. *EMBO J* **8**: 2045-2055; **Cohen, S. M., G. Bronner, F. Kuttner, G. Jurgens, and H. Jackle.** 1989. *Distal-less* encodes a homeodomain protein required for limb development in *Drosophila*. *Nature* **338**: 432-434; **Dolle, P., M. Price and D. Duboule.** 1992. Expression of the murine *Dlx-1* homeobox gene during facial, ocular, and limb development. *Differentiation* **49**: 93-99; **Robinson, G. W. and K. Mahon.** 1994. Differential and overlapping expression domains of *Dlx-2* and *Dlx-3* suggest distinct roles for Distal-less homeobox genes in craniofacial development. *Mech. Dev.* **48**: 199-215; **Simeone, A., D. Acampora, M. Pannese, M. D'Esposito, A. Stornaiuolo, M. Gulisano, A. Mallamaci, K. Kastury, T. Druck, and K. Huebner.** 1994. Cloning and characterization of two members of the vertebrate *Dlx* gene family. *Proc. Natl. Acad. Sci. USA* **91**: 2250-54; and **Stock, D. W., D. L. Ellies, Z. Zhao, M. Ekker, F. H. Ruddle, and K. M. Weiss.** 1996. The evolution of the

vertebrate Dlx family. Proc. Natl. Acad. Sci. USA **93**: 10858-10863.). The expression of the DNA encoding BP1 is highly restricted, which is a characteristic of developmentally important genes.

## 5 Cloning and Characterizing BP1

### Materials and Methods:

Cloning BP1: A  $\lambda$ gt11 K562 cell cDNA expression library (Clontech, Inc.) was screened with an oligonucleotide probe containing the BP1 binding site located in Silencer II DNA, which included sequences between -336 and -278 bp upstream of the  $\beta$ -globin gene. Screening was as described (see, for example, **Vinson, C. R., K. L. LaMarco, P. F. Johnson, W. H. Landschulz, and S. L. McKnight**. 1988. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. Genes & Devel. **2**: 801-806), with several modifications. Filters were prehybridized in binding buffer (see, for example, **Berg, P. E., D. M. Williams, R.-L. Qian, R. B. Cohen, S. X. Cao, M. Mittelman, and A. N. Schechter**. 1989. A common protein binds to two silencers 5' to the human  $\beta$ -globin gene. Nucl. Acids Res. **17**: 8833-8852) containing 5% Carnation instant dry milk at 4°C for 30 minutes, then hybridized in the same buffer for 2 to 12 hours at 4°C with shaking. Positive plaques were purified and subjected to three additional rounds of screening.

Electrophoretic mobility shift assay (EMSA): The binding reaction included 500 pg of fusion protein or 250 ng of nuclear extract added to binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 5% glycerol, 1mM DTT, 1 $\mu$ l of 1mg/ml BSA, 1  $\mu$ g poly(dI-dC)) and 5,000-10,000 cpm of <sup>32</sup>P-dCTP labeled probe. Incubation was for 30 minutes at room temperature. Nuclear extracts were prepared according to Dignam et al (**Dignam, J. D., R. M. Lebovitz, and R. G. Roeder**. 1983. Accurate transcription initiation by RNA

polymerase II in a soluble extract from isolated mammalian nuclei. Nucl. Acids Res. **11**: 1475-1489). Protein extracts from lysogens were prepared as described (see, for example, **Cowell, I. G. and H. C. Hurst.** 199. Cloning transcription factors from a cDNA expression library, p.120-122. In D. S. Latchman (ed.), Transcription factors: a practical approach. IRL Press, New York). Competitors were pre-incubated with nuclear extract for 20 minutes prior to addition of the probe. Oligonucleotide sequences of probes and competitors for EMSA analyses are shown below.

<u>Name</u>	<u>Sequence</u>
-530 Reference	TGTATATATACACATATATATATATATATATTTTTTTTT CTTTTCTTACCAGAAGGTTT (SEQ ID NO:3)
-530 Indian	TGTACATATACACATATATATATATATATA TTTTTTCTTTTCTTACCAGAAGGTTT (SEQ ID NO: 4)
-300 beta	TTCTTATTTGTGTAATAAGAAAATTGGGAAAACG ATCTTCAATATGCTTACCAAGCTG (SEQ ID NO: 5)
-530 delta	TTCTTTTAA TGGATATTTATTTCAATATAAT AAAAAATTAGAGTTTTA (SEQ ID NO: 6)
non-specific	TGCATATATATGTATATGTATGTGTGTATA (SEQ ID NO: 7)

Plasmid construction: To construct a plasmid expressing the BP1 cDNA open reading frame, BP1 cDNA was amplified by RT-PCR from K562 cell mRNA. The resulting 1000 bp product was cloned into pGEM7 vector (Promega, Inc.) and its identity confirmed by sequencing. This plasmid was cleaved with *Hind* III and *Xba* I to release the BP1 ORF, which was directionally cloned into pRc/RSV (Invitrogen, Inc.).

Transfections and CAT assays: Transient transfection assays were performed in K562 cells using DMR1E-C reagent (Gibco-BRL, Inc.). Cells were grown to a density of  $5 \times 10^5$  cells/ml and a total of

2 X 10<sup>6</sup> cells were used per transfection reaction. Transfections were performed in six well plates using OPTI-MEM serum-free medium (Gibco-BRL, Inc.). A total of 8 µg of plasmid DNA and salmon sperm DNA were added to complex with 16 ul of DMRIE-C reagent in 1 ml of OPTI-MEM I Reduced Serum Medium (Gibco-BRL, Inc.) for 15-45 min. at room temperature. 2 X 10<sup>6</sup> cells in 0.2 ml serum-free medium were added to each well and plates were incubated for 4-5 hrs at 37°C. Two ml of growth medium were added and cells were harvested after 48 hr. CAT assays were performed as described (see, for example, **Berg, P. E., D. M. Williams, R.-L. Qian, R. B. Cohen, S. X. Cao, M. Mittelman, and A. N. Schechter**. 1989. A common protein binds to two silencers 5' to the human β-globin gene. *Nucl. Acids Res.* **17**: 8833-8852).

Northern blot analysis: RNA was isolated using RNeasy and Oligotex kits (Qiagen, Inc.). Two micrograms of mRNA was electrophoresed per lane, and then transferred to Hybond N membrane (Amersham) by capillary action. RNA was crosslinked to the membrane using a Stratolinker (Stratagene), and the filter was hybridized using either standard methods or a sandwich method, which eliminates the background (see, for example, **Wu, S., Q. Lu, and A. L. Kriz**. 1995. Multiple-sandwich, one-step hybridization of Northern and Southern blots. *BioTechniques* **18**: 585-586.). An RNA dot blot was purchased (Clontech, Inc.); the amounts of RNA loaded on the blot had been normalized by the manufacturer to eight different housekeeping genes, obviating the need to normalize the samples. The probe was a 630 bp DNA fragment obtained by PCR amplification of BP1 cDNA cloned in pBluescript with subsequent *Eco* RI digestion to release a partial 3' BP1 cDNA lacking homeobox sequences. It was labeled by random priming and hybridized overnight at 65°C. Blots were washed and exposed to X-ray film.

RT-PCR: Total RNA was isolated using TRIzol Reagent (GIBCO BRL) according to the manufacturer's specifications. One microgram of RNA was reverse transcribed using SuperScript II RT (GIBCO BRL)

in a total reaction volume of 20  $\mu$ l. PCR was performed with 1  $\mu$ l of reverse-transcription product in 25  $\mu$ l total reaction volume consisting of 16.8  $\mu$ l of distilled H<sub>2</sub>O, 2.5  $\mu$ l of 10 X PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 1.5  $\mu$ l 25 mM MgCl<sub>2</sub>, 0.5  
5  $\mu$ l 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP), 0.2  $\mu$ l (1 unit) Ampli-Taq DNA polymerase (Perkin Elmer) and 1  $\mu$ l each of 10  $\mu$ M  $\beta$ -actin or BP1 forward and reverse primers, respectively. Based on linearity assay results, the following PCR conditions for BP1 were used. Each PCR cycle consisted of a denaturation  
10 step (94°C, 1 min), an annealing step (58°C, 1 minute) and an elongation step (72°C, 1.5 minute) for 27 cycles, followed by an additional extension (72°C, 5 minutes). Primers, designed to amplify a product of 581 bp, were: forward: 5'-CACCTCCTGTCTTACCCCTACACC-3' SEQ ID NO:8; reverse: 5'-GCCCTTCCCCAGATTACATCATC-3' SEQ ID NO:9. PCR products were  
15 electrophoresed on a 2% agarose gel and visualized with ethidium bromide. The product was verified by cleavage with restriction enzymes and hybridization with an internal probe.

Alternatively, a product of 225 bp may be obtained with the  
20 following primers: forward 5'-GTATGGCCACCTCCTGTCTT-3' (SEQ ID NO: 10) and reverse: 5'-GAGTAGATGGTCCTCGGCTT-3' (SEQ ID NO: 11) under the following conditions: 94° C for 2 minutes; then 94° C for 1 minute, 62° C for 1 minute and 72° C for 1.5 minutes, 30 cycles; 72° C for 10 minutes.

25

#### Cloning of BP1 cDNA:

A  $\lambda$ gt11 cDNA expression library made from human K562 cells was probed using a multimerized oligonucleotide containing the -300 bp BP1 binding site. Two million plaques were screened and  
30 one positive plaque was isolated that expressed a protein that recognized the -300 bp BP1 binding site but not a negative control sequence.

To verify that the cDNA encoded a protein with the expected binding specificities of BP1, namely, the ability to bind to  
35 Silencers I and II upstream of the  $\beta$ -globin gene and to a sequence -530 bp upstream of the  $\delta$ -globin gene (see, for example,

**Berg, P.E., S. Abhyankar, and M. Chase.** 1994. The high mobility group protein HMG-I(Y) binds to a silencer DNA sequence upstream of the human  $\beta$ -globin gene. *Blood* **84** Suppl 1: 262a), an EMSA competition assay was performed. The putative BP1 protein, fused to  $\beta$ -galactosidase, was expressed from a  $\lambda$  lysogen, as described above. This fusion protein was partially purified and incubated with the Silencer II probe used for screening the library, resulting in a single shifted band, indicated by the arrow (Fig. 2, lane 1). The band at the top marks the position of the wells, and the band at the bottom contains the unshifted probe. Cold competitors were added at a 100 X and 200 X molar excess to determine specificity. The shifted band was competed by self DNA (unlabeled -300 binding site, lanes 2 -3), the BP1 binding site at -530 bp in Silencer I containing either the reference (wild type) or Indian haplotype sequences (lanes 6-9), and the BP1 binding site found upstream of the  $\delta$ -globin gene (lanes 10-11), but not to a non-specific DNA (lanes 4-5). Note that the Indian sequence is a better competitor than the reference sequence, indicating tighter binding of BP1 to the Indian sequence, as was previously observed using K562 nuclear extracts (see, for example, **Elion, J., P. E. Berg, C. Lapoumeroulie, G. Trabuchet, M. Mittelman, R. Krishnamoorthy, A. N. Schechter, and D. Labie.** 1992. DNA sequence variation in a negative control region 5' to the  $\beta$ -globin gene correlates with the phenotypic expression of the  $\beta^s$  mutation. *Blood* **79**: 787-792.). A negative control, pure  $\beta$ -galactosidase protein, did not exhibit binding activity towards the probe (data not shown). The EMSA analysis thus demonstrated that the binding properties of the fusion protein conform to those expected for BP1.

The BP1 cDNA obtained was 630 bp in length. When part of this sequence was used as a probe in a Northern blot containing K562 RNA, an RNA band of 2.1 kb was recognized (Fig. 3A). This result indicated an incomplete cDNA had been cloned. To extend the cDNA sequence, rapid amplification of cDNA ends (RACE) was used. Additional sequences in both the 5' and 3' directions were

obtained, giving a cDNA of 1366 bp. Subsequently fine-tuning led to the 1251 bp sequence of SEQ ID:1 (and Figure 4). Further, the predicted open reading frame (ORF) was obtained.

5 Computer analysis using BLAST indicated that BP1 contains a homeobox (HB), placing it in a family of genes known to be important in development. Several sub-families of homeobox genes have been defined. BP1 is a member of the Distal-less (Dlx) family, based on sequence homology to the homeobox of these genes. In Figure 4, the homeobox is underlined and the amino  
10 acids comprising the three predicted  $\alpha$  helices found in homeodomains (see, for example, **Pabo, C. O. and R. T. Sauer. 1992. Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. 61: 1053-1095.**) are also indicated. The second and third helices comprise the helix-turn-helix (HTH) motif which is characteristic of homeodomains. A  
15 putative translational start site was determined by homology with the Kozak consensus sequence (see, for example, **Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucl. Acids Res. 15: 8125-8148.**) The open reading  
20 frame (ORF) and its predicted amino sequence are shown.

#### Transient transfection assays:

To examine the function of the cloned BP1 ORF, transient transfection assays were performed. The open reading frame (ORF) described above was sub-cloned to create pRSV/BP1-ORF. K562 cells  
25 were transiently co-transfected with increasing amounts of either an empty vector or pRSV/BP1-ORF, along with a target plasmid (Figs. 5A and 5B). The target DNA consisted of Silencer I (p $\epsilon$ CAT/SI) or Silencer II (p $\epsilon$ CAT/SII) cloned into an expression  
30 vector containing the epsilon globin promoter fused to the CAT reporter gene. (This plasmid was originally used in experiments which defined Silencers I and II (see, for example, **Berg, P. E., D. M. Williams, R.-L. Qian, R. B. Cohen, S. X. Cao, M. Mittelman, and A. N. Schechter. 1989. A common protein binds to two  
35 silencers 5' to the human  $\beta$ -globin gene. Nucl. Acids Res. 17:**

8833-8852). CAT activity was normalized against a co-transfected internal standard, pCMV/ $\beta$ gal. Relative CAT activity was calculated as the ratio of CAT activity in cells receiving pRSV/BP1-ORF to the CAT activity of cells receiving only the empty vector. When increasing amounts of pRSV/BP1-ORF DNA were added to p $\epsilon$ CAT/SI (Fig. 5A), CAT activity was repressed in a dose dependent manner, with a plateau beginning at 3  $\mu$ g of pRSV/BP1-ORF. Likewise, increasing addition of pRSV/BP1-ORF DNA to p $\epsilon$ CAT/SII caused increasing repression of the CAT reporter gene (Fig. 5B), but the lowest amount added, 1  $\mu$ g, caused a sharp decrease in CAT expression. A control, an  $\epsilon$ CAT plasmid lacking silencer DNA, was unresponsive to the addition of pRSV/BP1-ORF (data not shown). Thus, it was shown that BP1 exhibits repressor activity towards both silencers of the  $\beta$ -globin gene.

#### Expression Analysis of BP1:

Northern analysis was performed to determine the pattern of BP1 expression in human tissues and cell lines. In placenta, the BP1 probe recognized three bands, of 2.1, 2.6 and 3.2 kb (Fig. 3A). A Northern blot of fetal tissue was also probed, with the results shown in Fig. 3B. There was expression in both fetal kidney and lung (lanes 1 and 3), with bands of 2.1 and 6.3 kb, while no expression was detected in fetal liver or brain (lanes 2 and 4). The 2.1 kb band likely corresponds to BP1, since it is the sole band in K562 cells from which BP1 was cloned. The other bands may represent additional isoforms, commonly associated with homeobox genes (see, for example, **Cohen, S. M. and G. Jurgens. 1989. Proximal-distal pattern formation in *Drosophila*: cell autonomous requirement for Distal-less gene activity in limb development. EMBO J 8: 2045-2055; Lowney, P., J. Corral, M. M. LeBean, L. Deaven, H. J. Lawrence, and C. Largman. 1991. A human Hox1 homeobox gene exhibits myeloid-specific expression of alternative transcripts in human hematopoietic cells. Nucl. Acids Res. 19: 3443-3449. and O'Connor, M. B., R. Binari, L. A. Perkins, and W. Bender. 1988. Alternative RNA products from the**

Ultrabithorax domain of the bithorax complex. EMBO J 7: 435-445.)

To analyze expression in a broad spectrum of tissues, a dot blot containing normal human RNA from 50 tissues was probed (Table 1, below). Strong expression was seen in placenta and kidney, and weak expression was observed in cerebral cortex, spleen, mammary gland, small intestine, lung, fetal lung and fetal kidney (the latter two being present on both the Northern blot of Fig. 3B and the RNA dot blot). RNA from 41 additional tissues showed no expression.

TABLE 1 Tissue specific expression of BP1

<u>Strong</u>	<u>Weak</u>	<u>Negative</u>	<u>Negative</u>
placenta	cerebral cortex	whole brain	thyroid gland
kidney	spleen	amygdala	salivary gland
	mammary gland	caudate nucleus	pituitary gland
	small intestine		trachea
	lung	cerebellum	peripheral
	fetal lung	hippocampus	leukocyte
	fetal kidney	frontal lobe	lymph node
		medulla oblongata	bone marrow
			appendix
		occipital lobe	thalamus
		putamen	liver
		substantia nigra	spinal cord
			fetal spleen
		temporal lobe	fetal thymus
		subthalamic	fetal brain
		nucleus	fetal heart
		thymus	fetal liver
		heart	
		aorta	
		skeletal muscle	
		colon	
		bladder	
		uterus	
		prostate	
		stomach	
		testis	
		ovary	
		pancreas	
		adrenal gland	

Thus far, twenty-five human homeobox genes belonging to the HOX family have been described that are expressed during normal

hematopoietic development and in leukemic cell lines, often in a lineage-specific manner (see, for example, **Lawrence, H. J. and C. Largman**. 1992. Homeobox genes in normal hematopoiesis and leukemia. *Blood* **80**: 2445-2453). Therefore, the expression of BP1 in erythroid and myeloid lineage cell lines were examined by RT-PCR (Fig. 6). The strongest expression was seen in erythroid K562 and HEL cells and monocytic THP-1 and U937 cells, with less expression in megakaryocytic MEG-01 cells and monocytic/granulocytic HL60 cells. Although the cell lines used in this experiment are often examined for expression of genes in hematopoietic lineages, it should be noted that they are derived from leukemias.

As described above, BP1 exhibits repressor function. Using increasing amounts of a plasmid containing the ORF causes increasing repression of a target  $\epsilon$ CAT reporter gene containing Silencer I or II DNA. Expression of the reporter genes was not completely extinguished, which is believed to be due to a possible requirement for binding of additional, low abundance repressor proteins. One such protein may be BP2, which also binds to Silencer II DNA and appears to have repressor function (**Berg, P. E., D. M. Williams, R.-L. Qian, R. B. Cohen, S. X. Cao, M. Mittelman, and A. N. Schechter**. 1989. A common protein binds to two silencers 5' to the human  $\beta$ -globin gene. *Nucl. Acids Res.* **17**: 8833-8852 and **Ebb, D., D. C. Tang, L. Drew, K. Chin, P. E. Berg, and G. P. Rodgers**. 1998. Identification of regulatory elements that repress adult beta-like globin genes. *Blood Cells, Mol., Dis.* **24**: 356-369.). The data and the fact that an intact BP1 binding site is required for Silencer II function (see, for example, **Ebb, D., D. C. Tang, L. Drew, K. Chin, P. E. Berg, and G. P. Rodgers**. 1998. Identification of regulatory elements that repress adult beta-like globin genes. *Blood Cells, Mol., Dis.* **24**: 356-369.) shows that BP1 is most likely a repressor of the  $\beta$ -globin gene. As such, it is the first  $\beta$ -globin repressor to be cloned.

BP1 is a homeobox gene and a member of the Distal-less family (see, for example, **Cohen, S. M., G. Bronner, F. Kuttner, G.**

**Jurgens, and H. Jackle.** 1989. *Distal-less* encodes a homeodomain protein required for limb development in *Drosophila*. *Nature* **338**: 432-434.). Homeobox genes are known as master regulator genes during development because, upon mutation, vast morphologic abnormalities occur. Distal-less genes are found in organisms as diverse as mice, *Xenopus*, zebrafish and humans (see, for example, **Stock, D. W., D. L. Ellies, Z. Zhao, M. Ekker, F. H. Ruddle, and K. M. Weiss.** 1996. The evolution of the vertebrate Dlx family. *Proc. Natl. Acad. Sci. USA* **93**: 10858-10863). In *Drosophila*, Distal-less (called D11) is required for the normal development of larval sensory organs of the head and thorax. In larval and adult flies, D11 plays an important role in the development of the distal regions of the limbs (see, for example, **Cohen, S. M. and G. Jurgens.** 1989. Proximal-distal pattern formation in *Drosophila*: cell autonomous requirement for Distal-less gene activity in limb development. *EMBO J* **8**: 2045-2055 and **Cohen, S. M., G. Bronner, F. Kuttner, G. Jurgens, and H. Jackle.** 1989. *Distal-less* encodes a homeodomain protein required for limb development in *Drosophila*. *Nature* **338**: 432-434.). There is a single D11 gene in *Drosophila*, but multiple Distal-less genes in other organisms. In mammals, seven Distal-less (called Dlx in mammals) genes have been identified so far. During murine development, Dlx genes are expressed in branchial arches, forebrain, limbs, eyes, teeth, bones and facial mesenchyme (see, for example, **Dolle, P., M. Price and D. Duboule.** 1992. Expression of the murine Dlx-1 homeobox gene during facial, ocular, and limb development. *Differentiation* **49**: 93-99, **Robinson, G. W. and K. Mahon.** 1994. Differential and overlapping expression domains of Dlx-2 and Dlx-3 suggest distinct roles for Distal-less homeobox genes in craniofacial development. *Mech. Dev.* **48**: 199-215 and **Simeone, A., D. Acampora, M. Pannese, M. D'Esposito, A. Stornaiuolo, M. Gulisano, A. Mallamaci, K. Kastury, T. Druck, and K. Huebner.** 1994. Cloning and characterization of two members of the vertebrate Dlx gene family. *Proc. Natl. Acad. Sci. USA* **91**: 2250-54).

Bp1 and two recently discovered human genes, DLX7 (see, for

example, Nakamura, S., D. W. Stock, K. L. Wydner, J. A. Bollekens, K. Takeshita, B. M. Nagai, S. Chiba, T. Kitamura, T. M. Freeland, Z. Zhao, J. Minowada, J. B. Lawrence, K. B. Weiss, and F. H. Ruddle. 1996. Genomic analysis of a new mammalian Distal-less gene: Dlx-7. *Genomics* **38**: 314-324) and DLX4 (formerly called Dlx8; see, for example, Quinn, L. M., B. V. Johnson, J. Nicholl, G. R. Sutherland, and B. Kalionis. 1997. Isolation and identification of homeobox genes from human placenta including a novel member of the Distal-less family, Dlx4. *Gene* **187**: 55-61) appear to be isoforms, since their homeoboxes are identical and they exhibit extensive areas of homology. Using fluorescence in situ hybridization, BP1 was mapped to chromosome 17q21-23, also the locus of DLX7 and DLX4. Further, BP1 and DLX7 (see, for example, Nakamura, S., D. W. Stock, K. L. Wydner, J. A. Bollekens, K. Takeshita, B. M. Nagai, S. Chiba, T. Kitamura, T. M. Freeland, Z. Zhao, J. Minowada, J. B. Lawrence, K. B. Weiss, and F. H. Ruddle. 1996. Genomic analysis of a new mammalian Distal-less gene: Dlx-7. *Genomics* **38**: 314-324 and Price, J. A., D. W. Bowden, J. T. Wright, M. J. Pettenati, and T. C. Hart. 1998. Identification of a mutation in Dlx3 associated with tricho-dento-osseous (TDO) syndrome. *Hum. Mol. Gen.* **7**: 563-569) share complete sequence identity from nucleotides 565 to 1250 of BP1 while, further upstream of nt 565, there is no homology. Sequence homology between BP1 and DLX4 also begins at nt 565 of BP1 and includes the homeobox. The functions of DLX7 and DLX4 are not known. However, abrogation of DLX7 expression in K562 cells causes apoptosis (see, for example, Shimamoto, T., S. Nakamura, J. Bollekens, F. H. Ruddle and K. Takeshita. 1997. Inhibition of Dlx-7 homeobox gene causes decreased expression of GATA-1 and c-myc genes and apoptosis. *Proc. Natl. Acad. Sci. USA* **94**: 3245-3249). Preliminary data suggests lack of BP1 causes apoptosis in K562 cells.

A striking feature of BP1 is its restricted expression. BP1 mRNA is most strongly expressed in placenta and kidney (Table 1 and Fig. 3). Although no homeobox sequences were present in the

probe, there was partial sequence homology to DLX7. Thus, expression of BP1 in placenta and bone marrow by RT-PCT was assessed using BP1-specific primers; a band of the correct size was observed. The seven tissues exhibiting weak hybridization may be expressing BP1 and/or DLX7.

Forty one tissues lacked BP1 expression. It seemed surprising that BP1 mRNA was absent in fetal liver and fetal spleen, if BP1 represses adult globin gene expression. However, both of these tissues were obtained from 24 week fetuses, by which time  $\beta$ -globin expression has already begun (see, for example, **Ley, T. J., K. A. Maloney, J. I. Gordon and A. L. Schwartz**. 1989. Globin gene expression in erythroid human fetal liver cells. *J. Clin. Invest.* **83**: 1032-1038, **Mavilio, F., A. Giampaolo, A. Care, G. Migliaccio, M. Calandrini, G. Russo, G. L. Pagliardi, G. Mastroberardino, M. Marinucci, and C. Peschle**. 1983. Molecular mechanisms of human hemoglobin switching: selective undermethylation and expression of globin genes in embryonic, fetal, and adult erythroblasts. *Proc. Nat. Acad. Sci. USA* **80**: 6907-6911, and **Papayannopoulou, T., T.H. Shepard, and G. Stamatoyannopoulos**. 1983. Studies of hemoglobin expression in erythroid cells of early human fetuses using anti- $\gamma$ - and anti- $\beta$ - globin chain fluorescent antibodies, p.421-430. *In* G. Stamatoyannopoulos and A.W. Nienhuis (ed.), *Globin Gene Expression and Hematopoietic Differentiation*. Alan R. Liss, New York). To examine this point further, RT-PCR was performed on four 10 week fetal liver samples. Even by 10 weeks,  $\beta$ -globin transcription has begun (see, for example, **Ley, T. J., K. A. Maloney, J. I. Gordon and A. L. Schwartz**. 1989. Globin gene expression in erythroid human fetal liver cells. *J. Clin. Invest.* **83**: 1032-1038) and BP1 expression using 30 cycles of amplification samples were not detected; at 35 cycles a detectable band of the correct size in each of the four was observed. Thus, there are very low amounts of BP1 in fetal liver; yolk sac tissue is not available to better trace its pattern of expression.

At least 25 HOX genes, the largest family of human homeobox genes, are expressed during hematopoietic development, often in a

lineage-specific manner (reviewed in **Lawrence, H. J. and , C. Largman.** 1992. Homeobox genes in normal hematopoiesis and leukemia. *Blood* **80**: 2445-2453. ). However, the functions of these genes are unknown. The identification of BP1 as a regulator of the  $\beta$ -globin gene makes this the first human homeobox gene with a known function in hematopoiesis.

In addition to the Silencer I and Silencer II binding sites discussed above, additional potential gene targets that may be regulated by BP1 have been identified. Sequence analysis was performed to identify DNA sequences with at least 75% homology to known BP1 binding sites. Three sites were identified upstream of the gamma globin genes. These sites are located at -1427 bp and -1091 bp upstream of the Gy-globin gene and at -1091 bp upstream of the Ay-globin gene. These sites were shown to be binding sites for BP1 protein using the electrophoretic mobility shift assay.

#### Creation of a Polyclonal Antibody, Immunoprecipitation and Western Blotting

A polyclonal antibody was raised in rabbits against the unique N-terminal BP1 peptide SYPYTEPANPGDSYLSCQQ (SEQ ID NO:13) (Research Genetics). Freshly prepared K562 cell protein lysate was immunoprecipitated using Protein A Sepharose beads (Biorad). Equal amounts of K562 protein lysate, immunoprecipitated lysate and cold *in vitro* transcription/translation protein product were used for analysis by 12.5% SDS-PAGE gel. The proteins were electrophoretically transferred to nitrocellulose membranes. BP1 antibody was diluted in PBS (1:2500) and added for 2 hours at room temperature, then the anti-rabbit secondary antibody was added. Detection of the signal was accomplished using an ECL kit according to the recommended protocol (Amersham Pharmacia Biotech)

#### Creation of a Plasmid containing Antisense BP1 DNA

A BP1 DNA fragment of 239 bp (nt 219 to 558) was cloned into the vector pMT in an antisense orientation, under control of the sheep metallothionein promoter, as follows. RT-PCR was performed

using primers to amplify DNA between nt 219 and nt 1231 of the BP1 cDNA. This fragment was blunt ended and cloned into pGEM7. A 5' BamHI site in the polylinker of pGEM7 and an Xho I site at nt 558 (internal to BP1), were used to release BP1 DNA from the vector.

5 This BP1 fragment of 239 bp was directionally cloned in an antisense orientation into pMT. (see, for example, Canelles, M., Delgado, M.D., Hyland, K.M. Lerga, A., Richard, C., Dang, C.V. and Leon, J. (1997) Max and inhibitory cMyc mutants induce erythroid differentiation and resistance to apoptosis in human myeloid

10 cells. Oncogene 14, 1315 - 1327.)

#### Treatment of Sickle Cell Anemia

A mutation in the sixth codon of the beta globin gene causes sickle cell anemia. Current treatments of sickle cell anemia are focused on reactivation of the fetal hemoglobin genes. Such

15 treatments may include gene therapy, which would be used either to replace the beta globin gene or to add a fetal globin gene engineered to overexpress fetal globin protein. However, these treatments are hampered by the presence of the defective sickle cell beta protein, which interferes with the formation of normal

20 hemoglobin.

As discussed above, there are two silencer regions upstream of the  $\beta$ -globin gene and two proteins which bind to them, BP1 and BP2 (see, for example, **Berg, P. E., D. M. Williams, R.-L. Qian, R. B. Cohen, S. X. Cao, M. Mittelman, and A. N. Schechter.** 1989. A

25 common protein binds to two silencers 5' to the human  $\beta$ -globin gene. Nucl. Acids Res. **17**: 8833-8852). BP1 binds within Silencer I at -530 bp relative to the cap site (+1) and within Silencer II at -300 bp, while BP2 binds within Silencer II at -270 bp, shown in Fig. 1. Interestingly, it was found that High Mobility Group Protein I (HMG-I(Y)), an architectural protein, binds to and bends the DNA at or near the BP1 binding site in Silencer I, but not in

30 Silencer II (see, for example, **Chase, M. B., S. Haga, W. D. Hankins, D. M. Williams, Z. Bi, J. W. Strovel, C. Obriecht, and P. E. Berg.** 1999. Binding of HMG-I(Y) elicits structural changes in

a silencer of the human  $\beta$ -globin gene. Am. J. Hem. **60**: 27-35). HMG-I(Y) may facilitate binding of BP1 and possibly other repressor proteins in this region. Mutation of the BP1 site in Silencer II partially relieves repression (see, for example, **Ebb, D., D. C. Tang, L. Drew, K. Chin, P. E. Berg, and G. P. Rodgers.** 1998. Identification of regulatory elements that repress adult beta-like globin genes. Blood Cells, Mol., Dis. **24**: 356-369), suggesting that BP1 may act as a repressor of the  $\beta$ -globin gene. Furthermore, BP1 binds in a negative regulatory region upstream of the other adult globin gene, delta (see, for example, **Berg, P.E., S. Abhyankar, and M. Chase.** 1994. The high mobility group protein HMG-I(Y) binds to a silencer DNA sequence upstream of the human  $\beta$ -globin gene. Blood **84** Suppl 1: 262a). The inventor believes that BP1 coordinately regulates adult globin gene expression.

BP1 protein may be directly involved in the switch from fetal to adult hemoglobin during development since it binds to DNA near the fetal gamma globin genes, as well as near the adult delta and beta globin genes. Experiments have demonstrated that BP1 binding activity is increased upon pharmacologic reactivation of fetal globin genes.

Since BP1 is believed to activate fetal genes while repressing adult genes, it can potentially be used to treat sickle cell anemia, as an alternative to treatments involving adding normal beta gene or gamma gene.

An inverse correlation between the binding affinity of BP1 and the severity of sickle cell anemia (SCA) has been observed (see, for example, **Elion, J., P. E. Berg, C. Lapoumeroulie, G. Trabuchet, M. Mittelman, R. Krishnamoorthy, A. N. Schechter, and D. Labie.** 1992. DNA sequence variation in a negative control region 5' to the  $\beta$ -globin gene correlates with the phenotypic expression of the  $\beta^s$  mutation. Blood **79**: 787-792.). In sickle cell anemia, the mutated  $\beta$ -globin gene ( $\beta^s$ ) is in strict linkage disequilibrium with five restriction haplotypes (see, for example, **Kulozik, A. E., J. S. Wainscoat, G. R. Serjeant, B. D. Kar, B. Al-Awamy, G. J. F. Essan, A. G. Falusi, S. K. Haque, A. M. Hilali,**

**S. Kate, W. A. E. P. Ranasinghe, and D. J. Weatherall.** 1986. Geographical survey of  $\beta^s$ -globin gene haplotypes: Evidence for an independent Asian origin of the sickle-cell mutation. *Am. J. Hum. Genet.* **39**: 239-244; **Lapoumeroulie, C., O. Dunda, G. Trabuchet, M. Mony-Lobe, D. Labie, J. Elion, and R. Krishnamoorthy.** 1989. A novel sickle gene of yet another origin in Africa: The Cameroon type. *Blood* **74**: 225a and **Pagnier, J., J. G. Mears, O. Dunda-Belkhodja, K. E. Schaefer-Rego, C. Beldjord, R. L. Nagel, and D. Labie.** 1984. Evidence of the multicentric origin of the hemoglobin S gene in Africa. *Proc. Natl. Acad. Sci. USA* **81**: 1771-1773.). There are differences in the clinical severity of sickle cell anemia among the haplotypes, with the Indian-Arabo (hereafter referred to as the Indian haplotype) being the mildest and the Bantu the most severely affected (see, for example, **Ali, S. A.** 1970. Milder variant of sickle cell disease in Arabs in Kuwait associated with unusually high level of foetal haemoglobin. *Br. J. Haematol.* **19**: 613-619 and **Perrine, R. P., M. E. Pembrey, S. Perrine, and F. Shoup.** 1978. Natural history of sickle cell anemia in Saudi Arabs. A study of 270 subjects. *Ann. Internal. Med.* **88**: 1-6). A sequence polymorphism within the -530 bp binding site for BP1 is also in linkage disequilibrium with these five SCA haplotypes (see, for example, **Chebloune, Y., J. Pagnier, G. Trabuchet, C. Faure, G. Verdier, D. Labie, and V.M. Nigon.** 1988. Structural analysis of the 5' flanking region of the  $\beta$ -globin gene in African sickle cell anemia patients: further evidence for three origins of the sickle cell mutation in Africa. *Proc. Natl. Acad. Sci. USA* **85**: 4431-4435).

Using electrophoretic mobility shift competition assays, it was found that BP1 binds five to six times more tightly to the Indian haplotype sequence than to the normal or reference sequence (see, for example, **Berg, P. E., M. Mittelman, J. Elion, D. Labie, and A. N. Schechter.** 1991. Increased protein binding to a -530 mutation of the human  $\beta$ -globin gene associated with decreased  $\beta$ -globin synthesis. *Am. J. Hematol.* **36**: 42-47; **Elion, J., P. E. Berg, C. Lapoumeroulie, G. Trabuchet, M. Mittelman, R.**

**Krishnamoorthy, A. N. Schechter, and D. Labie.** 1992. DNA sequence variation in a negative control region 5' to the  $\beta$ -globin gene correlates with the phenotypic expression of the  $\beta^s$  mutation. *Blood* **79**: 787-792 and **Zeng, F.-y., G. P. Rodgers, S.-z. Huang, A. N. Schechter, M. Salamah, S. Perrine, and P. E. Berg.** 1994. Sequence of the -530 region of the beta globin gene of sickle cell anemia patients with the Arabian haplotype. *Human Mutation* **3**: 163-165). On the other hand, BP1 binds two to three times more weakly to the Bantu haplotype sequence than the reference sequence (see, for example, **Elion, J., P. E. Berg, C. Lapoumeroulie, G. Trabuchet, M. Mittelman, R. Krishnamoorthy, A. N. Schechter, and D. Labie.** 1992. DNA sequence variation in a negative control region 5' to the  $\beta$ -globin gene correlates with the phenotypic expression of the  $\beta^s$  mutation. *Blood* **79**: 787-792), which may allow more transcription of  $\beta^s$  resulting in an increased concentration of Hemoglobin S. This correlates with the increased severity of sickle cell disease observed in Bantus.

As stated above, BP1 is a strong candidate for use in therapy of sickle cell anemia (SCA). Decreasing  $\beta^s$ -globin expression is expected to decrease the intracellular concentration of HbS, in turn decreasing the intracellular HbS concentration and polymer formation, believed to be the primary cause of the clinical effects of SCA (see, for example, **Schechter, A. N., C. T. Noguchi and G. P. Rodgers.** 1987. Sickle cell disease, p. 179-218. In G. Stamatoyannopoulos, A. W. Nienhuis, P. Leder, Majerus, P. W. (ed.), *The Molecular Basis of Blood Diseases.* Saunders, Philadelphia). In addition, treatment with a repressor such as BP1 may also indirectly reactivate fetal globin genes. This idea is based on well established observations, both *in vivo* and *in vitro*, which demonstrate reciprocal regulation of fetal and adult globin genes. Dover and Boyer (see, for example, **Dover, G. J. and S. H. Boyer.** 1987. Fetal hemoglobin-containing cells have the same mean corpuscular hemoglobin as cells without fetal hemoglobin: a reciprocal relationship between gamma- and beta-globin gene expression in normal subjects and in those with high fetal

hemoglobin production. Blood **69**: 1109-1113) demonstrated that reciprocal regulation exists in individuals who express high levels of HbF and low levels of HbA due to SCA, heterocellular hereditary persistence of fetal hemoglobin (HPFH), and  $\beta$ -thalassemia. Perrine et al (see, for example, **Perrine, S. P., B. A. Miller, M. F. Greene, R. A. Cohen, N. Cook, C. Shackleton, and D. V. Faller**. 1987. Butyric acid analogues augment  $\gamma$  globin gene expression in neonatal erythroid progenitors. Biochem. Biophys. Res. Comm. **148**: 694-700.) showed that two factors,  $\alpha$ -amino-n-butyrac acid and insulin, present in high levels in the plasma of infants born to diabetic mothers, increase  $\gamma$ -globin expression accompanied by decreased  $\beta$ -globin expression. Furthermore, cultured erythroid progenitors from  $\beta$ -thalassemia and SCA patients, when treated with sodium butyrate, 4-phenyl butyrate or phenylacetate exhibited an increase in  $\gamma$ -globin expression and a reciprocal decrease in  $\beta$ -globin expression (see, for example, **Fibach, E., P. Prasanna, G.P. Rodgers, and D. Samid**. 1993. Enhanced fetal hemoglobin production by phenylacetate and 4-phenylbutyrate in erythroid precursors derived from normal donors and patients with sickle cell anemia and  $\beta$ -thalassemia. Blood **82**: 2203-2209 and **Perrine, S.P., B. A. Miller, D. V. Faller, R. A. Cohen, E. P. Vichinsky, D. Hurst, B. H. Lubin, and T. Papayannopoulou**. 1989. Sodium butyrate enhances fetal globin gene expression in erythroid progenitors of patients with HbSS and  $\beta$  thalassemia. Blood **74**: 454-459). Since current treatments of SCA focus on chemical reactivation of fetal globin genes, repression of HbS may reduce or eliminate the need for such chemicals.

As a treatment of sickle cell anemia, the preferred method is to incorporate DNA encoding BP1 into a vector that can be administered to a patient by gene therapy, preferably targeted to tissues that express globin genes. To avoid potential problems caused by overexpression of BP1, the DNA could be linked to a controllable promoter.

Screening of Leukemia

Acute myeloid leukemia (AML) is the second most frequent pediatric leukemia and the most frequent adult leukemia (see, for example, **Pui C-H**. Childhood leukemias. *New Eng J Med* 1995; **332**: 1618-1630 and **Karp JE**. Acute leukemia: mechanisms of cell survival as targets for therapy. *Int J Oncol* 1997; **11**: 657-674.). Survival is poor in AML, with only 30-40% survival in children and 10-35% survival in adults. On the other hand, acute lymphocytic leukemia (ALL) is the-most frequent leukemia in children, but is rare in adults. Although several molecular markers have been associated with AML and ALL (see, for example, **Look AT**. Oncogenic transcription factors in the human acute leukemias. *Science* 1997; **278**: 1059-1064; **Tenen DG**, Hromas R, Licht JD, Zhang D-E. Transcription factors, normal myeloid development and leukemia. *Blood* 1997; **90**: 489-491; and **Lawrence HJ, Sauvageau G, Humphries RK, Largman C**. The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells* 1996; **14**: 281-291), there is clearly a need to identify additional markers which could aid in diagnosis and in determining appropriate therapy, and/or potentially serve as therapeutic targets.

Altered expression of transcription factors, including homeobox genes (HB), has been implicated in acute leukemias, and aberrant expression of homeobox genes has been described in leukemia cell lines and in primary leukemia blasts. Homeobox genes are characterized by a conserved 180 bp DNA sequence encoding the DNA binding domain (see, for example, **Levine M, Hoey T**. Homeobox proteins as sequence-specific transcription factors. *Cell* 1988; **55**: 537-540). They are "master regulator genes" which are extremely important to the physical development of a wide range of organisms. Recent studies have demonstrated that HB genes are also expressed during hematopoiesis (see, for example, **Lawrence HJ, Sauvageau G, Humphries RK, Largman C**. The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells* 1996; **14**: 281-291). There are several families of HB genes, defined by the extraordinary sequence conservation among their homeoboxes. The largest family, consisting of 39 members in humans, is called HOX.

Some leukemias aberrantly express homeobox genes as fusion proteins (reviewed in **Look AT**. Oncogenic transcription factors in the human acute leukemias. *Science* 1997; **278**: 1059-1064). In these cases, DNA encoding the activation domain from one gene is fused to DNA encoding the DNA binding region of another, bringing a different function to the new protein product. The downstream targets of fusion genes associated with AML or ALL have yet to be determined. In pre-B-cell lymphoblastic leukemia, for example, the HB gene Pbx1 is fused to E2A (see, for example, **Lu Q, Wright DD, Kamps MP**. Fusion with E2A converts the Pbx1 homeodomain protein into a constitutive transcriptional activator in human leukemias carrying the t(1;19) translocation. *Mol Cell Biol* 1994; **14**: 3938-3948) and, in acute myeloid leukemia (AML), HOXA9 is fused to NUP98-HOXA9 (see, for example, **Borrow J, Shearman A.M., Stanton Jr. V.P., Becher R, Collins T, Williams AJ, Dube I, Katz F, Kwong YL, Morris C, Ohyashiki K, Toyama K, Rowley J, Housman DE**. The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. *Nature Genetics* 1996; **12**: 159-167 and **Nakamura T, Yamazaki Y, Hatano Y, Miura I**. NUP98 is fused to PMX1 homeobox gene in human acute myelogenous leukemia with chromosome translocation t(1;11)(q23;p15). *Blood* 1999; **94**: 741-747). In other cases of leukemia not involving obvious cytogenetic changes, a gene which is normally silent is either activated or perhaps not downregulated in an early hematopoietic progenitor. This was observed for four of nine HOXB genes which were expressed in T- or B-ALL, but not in normal T or B lymphocytes (see, for example, **Petrini M, Quaranta MT, Testa U, Samoggia P, Tritarelli E, Care A, Cianetti L, Valtieri M, Barletta C, Peschle C**. Expression of selected human HOX genes in B/T acute lymphoid leukemia and interleukin-2/interleukin-1  $\beta$ -stimulated natural killer lymphocytes. *Blood* 1992; **80**: 185-193).

BP1 expression was investigated in erythromyeloid and lymphoid leukemia cell lines and in bone marrow samples from adult and pediatric acute myeloid leukemia (AML), pediatric acute T-cell

lymphocytic leukemia (ALL) and pediatric pre-B-cell ALL. Because there are two apparent isoforms of BP1 called DLX7 and Dlx4, their expression in the same samples was measured. All three are co-expressed in erythroid and monocytic cell lines and in at least half of the lymphoid cell lines. They are barely if at all detectable in normal bone marrow, PHA-stimulated T cells or B cells. BP1 RNA was found in 63% of AML cases, including 81% of the pediatric and 47% of the adult cases, and in 32% of T-ALL cases, but was not expressed in any of the pre-B ALL cases. Similar results were obtained with DLX7 and Dlx4, with co-expression of all three in a significant number of leukemias. The data shows that BP1 expression occurs in early progenitors. The presence of BP1 RNA in leukemic blasts may therefore be a molecular marker for primitive cells and/or may indicate BP1 is an important upstream factor in an oncogenic pathway.

The hypothesis that BP1 is leukemogenic is supported by the present studies showing that overexpression of BP1 causes increases clonogenicity in the cell line K562, as well as causing decreased ability of these cells to undergo erythroid differentiation. Overexpression of BP1 in the erythroleukemia cell line K562 results in increased growth in soft agar, which is an indicator of leukemogenic potential.

BP1 expression is believed to cause increased proliferation and survival and decreased differentiation in K562 cells. Similar results were found in K562 cells when either of the two well known oncogenes were overexpressed, c-myc and c-myb. Therefore, the data suggests that BP1 functions as an oncogene in AML and T-cell ALL conditions.

The studies carried out to determine BP1 expression are described in greater detail below:

#### Materials and Methods:

Culture of leukemic cell lines: T and B-ALL cell lines were grown in RPMI 1640 (Gibco-BRL) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM non-essential

amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. Erythromyeloid cell lines were cultured in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, 10 mM HEPES and 10% fetal bovine serum.

5 Clinical samples: Normal bone marrows (BM) were obtained from the Brain Tissue Bank for Developmental Disorders at the University of Maryland. Adult AML bone marrow samples were obtained from the University of Maryland Greenebaum Cancer Center and The Johns Hopkins Cancer Center Cytogenetics Core Laboratory.  
10 Bone marrow samples from pediatric T and B- ALL patients were obtained from the Pediatric Oncology Group (POG). Informed consent was obtained from each patient or guardian at their local institutions. Leukemia samples contained at least 85% blasts. T and B lymphocytes from peripheral blood of normal donors were  
15 obtained by countercurrent elutriation followed by lineage specific separation using magnetic beads (Miltenyi Biotec, Auburn, CA).

RT-PCR: Mononuclear cells were isolated for RNA analysis by Ficoll-Paque. Total RNA was extracted from all samples using  
20 TRIZOL Reagent (Gibco-BRL, Gaithersburg, MD). For the reverse transcription (RT) reaction, one microgram of DNase I-treated RNA was added to 2.5  $\mu$ M oligo-d(T) primers (PE Biosystems), 10 U RNase inhibitor (Gibco-BRL), 0.5 mM dNTPs, and 100 U MMLV-reverse transcriptase (Promega). The RT reaction was performed in a  
25 thermal cycler at 25° C for 10 minutes, 4 minutes ramp, 42° C for 50 minutes, and 99° C for 5 minutes. One to two microliters of this reaction was used in the PCR reaction. Primers specific for BP1, DLX4 and DLX7 were designed, and the PCR products were verified by restriction enzyme analysis. Linearity assays for each  
30 primer set have been performed and cycling conditions adjusted accordingly (data not shown). Primer sequences and PCR cycling conditions are in the following Table 2.

TABLE 2: PCR Primers

<u>Primers</u>	<u>Sequence</u>	<u>PCR conditions</u>	<u>Product</u>	<u>Reference</u>
BP1	Upper: 5'CACCTCCTGTCTTACC CCTACACC3' (SEQ ID NO: 8) Lower: 5'GCCCTTCCCCAGATTC ACATCATC3' (SEQ ID NO: 9)	94° C/1 min.; 62° C/1 min.; 72° C/1.5 min. for 30 cycles	581 bp	
DLX7	Upper: 5'CCTACACCGTGTGTG CTGC3' (SEQ ID NO: 14) Lower: 5'CTGTTGCCATAGCCAC TG3' (SEQ ID NO: 15)	94° C/1 min.; 60° C/1 min.; 72° C/1.5 min. for 30 cycles	406 bp	
DLX4	Upper: 5'CACGGTGTGGCGGGG AGACAT3' (SEQ ID NO: 16) Lower: 5'CTGCGGTGGGAGGTCG GAGTTC3' (SEQ ID NO: 17)	94° C/1 min.; 60° C/1 min.; 72° C/1.5 min. for 30 cycles	350 bp	
5	$\beta$ -actin	Upper: 5'GGATCTTCATGAGGTA GTCAGTC3' (SEQ ID NO: 18) Lower: 5'CCTCGCCTTTGCCGAT CC3' (SEQ ID NO: 19)	94° C/1 min.; 60° C/1 min.; 72° C/1.5 min for 20 cycles	626 bp Raff et al, 1996
	c-myb	Upper: 5'ATTAGGTAATGAATTG TAGCCAG3' (SEQ ID NO: 20) Lower: 5'ACTTAGAGTAATGCTT TTACTGA3' (SEQ ID NO: 21)	94° C/30 sec; 55° C/30 sec.; 72° C/1 min. for 28 cycles	228 bp Majello et al, 1986  Shimamoto et al, 1997
	GATA-1	Upper: 5'CCATTGCTCAACTGTA TGGAGGG3' (SEQ ID NO: 22) Lower: 5'ACTATTGGGGACAGGG AGTGATG3' (SEQ ID NO: 23)	94° C/30 sec; 58° C/30 sec; 72° C/1 min. for 28 cycles	249 bp Tsai et al, 1989  Shimamoto et al, 1997
	SCL	Upper: 5'CAATCGAGTGA AGAGGAGACCTCC3' (SEQ ID NO: 24) Lower: 5'TTGCGGAGCTCGGCAA AGGC3' (SEQ ID NO: 25)	94° C/45 sec; 55° C/45 sec.; 72° C/1.5 min for 30 cycles	144 bp Chen et al, 1990

Semi-quantitative RT-PCR: An oligonucleotide specific for each product was end-labelled with  $\gamma$ - $^{32}\text{P}$ -dATP and added to 10 percent of the PCR product (2.5  $\mu\text{l}$ ). Hybridization was in the thermal cycler (94° C for 15 seconds; 42° C for 1 minute). The hybridized product was electrophoresed on a 5% polyacrylamide gel, the gel was dried, and then exposed to film. The autoradiograph was aligned with the gel and bands were excised and quantitated by scintillation counting.

Expression was scored as negative (-), positive (+) or ambiguous (+/-) by normalizing against  $\beta$ -actin: a ratio was calculated by dividing the cpm in the band representing BP1, DLX7 or DLX4 by the cpm in the  $\beta$ -actin band from the same sample. One normal BM and one remission BM were included in each AML experiment; their ratios (6 repeats of each) averaged 0.01 for BP1, DLX7 and DLX4, and were the guide against which a sample was scored as -(0.0-0.10), +/- (0.11-0.15), +(>0.15) or ++(>0.45). Two phytohemagglutinin (PHA)-stimulated normal T cell cultures were the control for T-cell ALL; their average ratios (10 repeats) were 0.05; the same criteria were used for scoring BP1, DLX7 and DLX4 as above. *c-myb* and GATA-1 are expressed in normal BM (our observations and also noted in **Gewirtz AM, Calabretta B. A *c-myb* antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro. *Science* 1988; 242: 1303-1306. Guerrasio A, Saglio G, Rosso C, Alfarano A, Camaschella C, Lo Coco F, Biondi A, Ranbaldi A, Nicolis S, Ottolenghi S. Expression of GATA-1 mRNA in human myeloid leukemic cells. *Leukemia* 1994; 6: 1034-1038).** The average ratios in BM for *c-myb* and GATA-1 were 0.57 and 2.1, respectively. Samples with a ratio between 0.10 and 1.14 (*c-myb*) or 0.10 and 4.2 (GATA-1) were scored as (+), i.e., within the range of normal BM, and ratios greater than 1.14 (*c-myb*) or 4.2 (GATA-1) were scored as (++). Each RT-PCR and hybridization was performed twice, independently.

Isolation of CD34 cells: Bone marrow cells were aspirated from the posterior iliac crest of consenting healthy adult donors

following guidelines approved by the Institutional Review Board for NIH. Cells were first prepared with Histopaque-1077 density gradient centrifugation. Mononuclear cells were then incubated with CD34 (QBEnd10)-conjugated magnetic microbeads (AmCellCorp, Sunnyvale, CA) and processed through a MACS magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain purified CD34+ cells. For higher purity of CD34+ cells, a second column run was used. Purity of isolated CD34+ cells was generally greater than 90%, and cell viability as evaluated by propidium iodide exclusion was always higher than 95%. Purity of the remaining CD34- cells was greater than 95%.

Construction of cell lines overexpressing BP1: A BP1 cDNA fragment of 1013 bp containing the complete open reading frame was amplified by RT-PCR from K562 cell RNA using primers designed from the original cDNA. This fragment was cloned into pGEM7, sequenced, then sub-cloned into pRC/RSV (Invitrogen) using *Hind* III and *Xba* I.

Statistical Analysis: The significance of correlation between expression of BP1, DLX4, and DLX7 was assessed by Fisher's exact test. All p-values reported were two-sided.

#### Detailed Results:

Expression of BP1 in normal cells: RNA levels for BP1 were determined in four normal bone marrow samples and one bone marrow from an AML patient in remission, and in five PHA-stimulated T-cell and five B-cell preparations from normal bone marrow. Results obtained after semi-quantitative RT-PCR analysis of six representative samples are shown in Fig. 8. BP1 RNA was barely visible in any of these control samples; results were similar for DLX4 and DLX7. After a longer exposure of the autoradiograph, a faint band was seen in all six samples for several of the isoforms (data not shown).

Expression of BP1 in cell lines: Expression of BP1 was examined by RT-PCR in a number of leukemia cell lines. Fourteen cell lines of lymphoid origin were analyzed for BP1 expression. BP1 RNA was present in four of eight T-cell ALL cell lines and in three of four lymphoma cell lines (Table 3).

TABLE 3. Expression of BP1 in Hemopoietic Cell Lines

<u>Lymphoid</u>	<u>BP1 Expression</u>	<u>Myeloid</u>	<u>BP1 Expression</u>
B Cell Lineage		Erythroid	
Normal	+/-	K562	+
ALL		HEL	+
REH	+	Monocytic	
RS4;11	+	THP-1	+
Burkett's lymphoma		U937	+
Raji	-	Megakaryocytic	
Daudi	+	MEG-01	+/-
		Monocytic/granulocytic	
		HL60	+/-
T Cell Lineage			
Normal	+/-		
ALL			
Jurkat	+		
MOLT-3	+		
MOLT-4	+		
MOLT-13	+		
CCRF-CEM	-		
HSB2	-		
MOLT-16	-		
RPMI 8402	-		
Lymphoma			
HUT78	+		
Sup-T1	+		

DLX7 and DLX4 RNA levels were also determined in the T-cell ALL cell lines (Fig. 7A), with co-expression of the three isoforms in the same cell lines. BP1 mRNA was also readily detectable in four of six erythromyeloid cell lines (K562, HEL, THP-1 and U937), with less expression in MEG-01 and HL60 cells.<sup>1</sup> Analysis of DLX7 and DLX4 in those cell lines showed that BP1, DLX7 and DLX4 were frequently co-expressed, with greatest expression of all three in K562 and U937 cells (Fig 7B). The observation that there was little or no expression of any of the isoforms in normal bone marrow (described above), compared with the expression found in diverse myeloid and lymphoid leukemia cell lines, led us to examine the relative expression of BP1, DLX7 and DLX4 in the bone marrow of acute leukemia patients using semi-quantitative RT-PCR.

Expression of BP1 in acute myeloid leukemias: Expression of BP1 was examined in both pediatric and adult AML patients. A total of 39 AML patients were studied, of whom 18 were under the age of 18 (pediatric; cases 8-27) and 21 were 18 years of age or older (adult; cases 29-49). Table 4, below, summarizes the analysis of the AML samples and the clinical data available for each patient, including cytogenetics, expression of surface markers and initial response to treatment; outcome data was not available for all patients and thus was excluded. Data are grouped according to the French-American-British (FAB) criteria. (see, for example, **Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C.** Proposed revised criteria for the classification of the French-American-British Cooperative Group. *Ann Intern Med* 1985; **103**: 620-625. Assignment of expression levels was made as described in Materials and Methods. For this analysis, normal bone marrow was used as a negative baseline against which the clinical samples were compared. Samples classified as +/- were excluded from statistical analysis. BP1 was overexpressed in a definitive manner in 81% (13/16) of the pediatric bone marrow samples and in 47% (9/19) of the adult cases. DLX7 was overexpressed in 59% (10/17) of pediatric and 38% (6/16) of adult cases, while DLX4 was

overexpressed in 79% (11/14) of pediatric and 79% (15/19) of adult cases. An example of RT-PCR analysis showing expression in selected samples is seen in Fig. 9. Here, patient 15 showed the greatest BP1 and DLX7 levels, with less DLX4 expression. The correlation between BP1 and DLX7 ( $p=0.0002$ ), BP1 and DLX4 ( $p=0.0016$ ), and DLX7 and DLX4 ( $p=0.023$ ) were statistically significant by Fisher's exact test.

Table 4. Expression of BP1, DLX& and DLX4 and clinico-biological features in patients with AML

FAB	Patient	BP1	DLX7	DLX4	c-myb	GATA-1	CD34	CD33	CD13	Karyotype	Response**	
Normal B.M.		-	-	-	+	+						
M0	12*	++	+	+/-	++	+	-	+	+	ND	PR	
	37	+/-	-	+	++	+	+	+	+	46.XY,t(1;15),trisomy 1q	NR	
	47	-	-	-	ND	ND	+	+	+	47,XY,+13	NR	
5	M1	15*	++	++	+	++	+	+	+	46.XY,t(11;9)(q23;p13.1)	CR	
		17*	+/-	-	+/-	++	+	+	+	46,Y,t(X;7)(q13;p15),dup(12)(p11p13)	CR	
		19*	-	-	-	++	+	+	+	46,XX	CR	
		22*	++	++	++	+++	+	+	+	47,XX,+21	ND	
		26*	+	+	+/-	+	+	+	+	+	46,XX	CR
		27*	-	-	+	+	+	+	+	+	46,XY	CR
		29	-	-	+	++	+	+	+	+	46,XY,del(5)(q31q34)	NR
		40	++	++	++	++	+/-	-	+	+	46,XY,t(1;12)	CTC
		46	+	+	+	ND	ND	-	+	+	46.XY,del(1)(q32),del(13)(q12-22)	CR
		M2	18*	-	-	-	+/-	+	+	+	+	47,XX,+8
20*	+		-	+	+	-	-	+	+	46.XY	CR	
44	-		ND	+/-	ND	ND	+	+	+	46,XY	CR	
45	-		-	-	ND	ND	+	+	-	45,X,-Y	CR (<6 mo.)	
48	+		+/-	ND	ND	ND	+	+	+	47,XY,+8	NR	
M3	41	+	+	++	+	+	-	+	+	46,XX,t(15;17)(q21;q12-21)	CR	
M4	23*	+/-	-	-	+	-	+	+	+	46,XX,inv(16)(p13q22)	CR	
	31	-	-	-	+	+	-	+	+	45,XY,-7	NR	
	32	-	-	-	+	+	-	+	+	45,XX,-16	NR	
	35	+	+/-	+	+	+	-	+	+	46,XX	NR	
	49	-	-	+	ND	ND	+	+	+	46,XY,inv(16)(p13q22)	CR	
M5	8*	+	+	+	++	-	-	+	-	46,XX,t(11;17)(q23;q25)	CR	
	9*	+	+/-	+/-	+	-	-	+	-	ND	CR	
	11*	+	+	+	++	-	-	+	+	46,XY	NR	
	13*	+	+	+	++	+	-	+	-	46,XY,der(1)t(1;6)(q32;p21.1), add(11)(q23),der(22)t(1;22)(q23;q11.2)	CR	
	14*	+	+	+	+	+	-	+	+	46,XX	CR	
	16*	+	-	+	+	+	-	+	+	46,XX	NA	
	30	-	+/-	+	+	-	-	+	+	47,XY,+3	NA	
	33	-	-	+	+	+	+	+	+	46,XY	NR	
	34	+	+	+	+	+	+	+	+	48,XX,add(4)(p15.1),del(5)(q21q33), t(7;12)(p10,q10).add(11)(q23),+i(12)(p10), add(16)(q11.2),-17,+18,-20, +der(21)t(17;21)(q10;q10),+mar	NR	
	36	+	-	+	+	+	+	+	-	46,XY,del(7)(q22),+(8)(q24.3)	NR	
	38	+/-	-	+	+	+	+	+	+	46,XX	CR	
	39	-	+/-	++	+	+	-	+	+	48,XY,t(1;16),+7,+19	CTC	
	42	+	+	+	+	-	13%	+	62%	47,XY,t(6;9)(q23;q34),+8	RD	
43	+	+	+	ND	ND	-	+	+	46,XY,t(3;5)(q25;q34)	NR		
10	M7	21*	+	++	++	+++	+++	+	+	-	46,XY	CR
		24*	+	+	+	++	+	+	+	+	45,XY,t(3;3)(q21;q26),-7	NR

\* AGE LESS THAN 18

\*\*COMPLETE RESPONSE(CR), NO RESPONSE (NR), PARTIAL RESPONSE (PR),

COMPLETE TUMOR CLEARANCE (CTC)

RESIDUAL DISEASE(RD), NOT DETERMINED (ND), DATA NOT AVAILABLE(NA)

Analysis of GATA-1 and c-myb, markers of primitive cells, was performed on the AML samples to indicate the differentiation state of cells expressing BP1. GATA-1 is

believed to be involved in positive regulation of myeloid development and thought to be expressed in early progenitors, then downregulated in myeloid (but not erythroid) differentiation, making its expression in the myeloid pathway a sign of immaturity. (see, for example, **Crotta S, Nicolis S, Ronchi A, Ottolenghi S, Ruzzi L, Shimada Y, Migliaccio AR.** Progressive inactivation of the expression of an erythroid transcriptional factor in GM- and G-CSF-dependent myeloid cell lines. *Nucl Acids Res* 1990; **18**: 6863-6869) It is detected in acute leukemias characterized by expansion of early progenitors. (see, for example, **Guerrasio A, Saglio G, Rosso C, Alfarano A, Camaschella C, Lo Coco F, Biondi A, Ranbaldi A, Nicolis S, Ottolenghi S.** Expression of GATA-1 mRNA in human myeloid leukemic cells. *Leukemia* 1994; **6**: 1034-1038) *c-myb* is expressed in immature hematopoietic cells, and its expression decreases as cells differentiate. (see, for example, **Gewirtz AM, Calabretta B.** A *c-myb* antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro. *Science* 1988; **242**: 1303-1306; **Gonda T, Metcalf D.** Expression of *myb*, *myc* and *fos* proto-oncogenes during the differentiation of a murine myeloid leukemia. *Nature* 1984; **310**: 249-251 and **Luscher B, Eisenman RN.** New light on Myc and Myb. Part II. Myb. *Genes & Devel* 1990; **4**: 2235-2241.) Since *c-myb* and GATA-1 are expressed in normal bone marrow (data not shown), (see, for example, **Gewirtz AM, Calabretta B.** A *c-myb* antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro. *Science* 1988; **242**: 1303-1306 and **Guerrasio A, Saglio G, Rosso C, Alfarano A, Camaschella C, Lo Coco F, Biondi A, Ranbaldi A, Nicolis S, Ottolenghi S.** Expression of GATA-1 mRNA in human myeloid leukemic cells. *Leukemia* 1994; **6**: 1034-1038.) A classification of positive was given to samples expressing *c-myb* at the level of normal cells (see above for details). It was found that every AML sample exhibited *c-myb* expression at least equal to that in normal bone marrow, and 42% (13/31) exhibited greater expression. A higher proportion of pediatric than adult cases showed high level *c-myb* expression, 59% in children compared

with 21% in adults. GATA-1 was present in 77% (24/31) of AML cases, with 72% expression in children and 85% in adults.

Co-expression of DLX7 and DLX4 with BP1 was striking: 84% of BP1+ samples were DLX7+ and 100% were DLX4+. GATA-1 was co-expressed in 74% of the samples, and all were *c-myb* positive, with 45% exhibiting high levels of *c-myb*. Another parameter in this study was analysis of surface markers. Interestingly, 64% of the BP1 positive samples were CD34 negative, while 73% were CD13 positive and 100% were CD33 positive.

Among the 39 AML patients, two had an abnormal chromosome 17q arm, the locus of BP1. (see, for example **Fu S, Strovel JW, Haga SB, Stamberg J, Berg PE**. Mapping of a new homeobox gene, BP1, near its isoform DLX7 and characterization of their roles in repression of the beta globin gene. *Am. J. Hum. Gen.* 1998; **63**: A181.) Patient 41, with acute promyelocytic leukemia, exhibited a t(15;17) translocation presumably involving the retinoic acid receptor on chromosome 17. (see, for example, **Look AT**. Oncogenic transcription factors in the human acute leukemias. *Science* 1997; **278**: 1059-1064.) Patient 8 exhibited a t(11;17)(q23;q25) translocation. This translocation has been identified as fusing the MLL gene on chromosome 11q23 to either the AF17q25 gene or to MSF, both located on 17q25 and differing by a single base. (see, for example, **Baer MR, Stewart CC, Lawrence D, Arthur DC, Mrozek K, Strout MP, Davey FR, Schiffer CA, Bloomfield CD**. Acute myeloid leukemia with 11q23 translocations: myelomonocytic immunophenotype by multiparameter flow cytometry. *Leukemia* 1998; **12**:317-325; **Taki T, Ohnishi H, Shinohara K, Sako M, Bessho F, Yanagisawa M, Hayashi Y**. AF17q25, a putative septin family gene, fuses the MLL gene in acute myeloid leukemia with t(11;17)(q23;q25). *Cancer Res* 1999; **59**: 4261-4265 and **Osaka M, Rowley JD, Zeleznik-Le NJ**. MSF (MLL septin-like fusion), a fusion partner gene of MLL, in a therapy-related acute myeloid leukemia with a t(11;17)(q23;q25). *Proc Natl Acad Sci USA* 1999; **96**: 6428-6433.) Since the MLL gene is known to activate several HOX

genes, (see, for example, **Yu BD, Hess JL, Horning SE, Brown GAJ, Korsmeyer S.** Altered Hox expression and segmental identity in Mll-mutant mice. *Nature* 1995; **378**: 505-508.) it is tempting to speculate that the fusion protein may activate BP1 in this case.

Expression of BP1 in acute lymphoid leukemias: For comparison with the myeloid lineage leukemias, nineteen cases of pediatric T-cell ALL were examined (Table 5).

TABLE 5. Expression of BP1, DLX7 and DLX4 in Children with T-Cell ALL

	<u>Patent</u>	<u>BP1</u>	<u>DLX7</u>	<u>DLX4</u>
10	1	-	-	-
	2	-	+	-
	3	-	+	-
	4	+	-	-
15	5	+	+	+
	6	-	-	-
	7	-	+	+
	8	-	-	-
	9	-	-	-
20	10	+	+	+
	11	-	nd	-
	12	+	-	-
	13	-	-	+
	14	+	+	+
25	15	-	nd	nd
	16	+	+	+
	17	-	nd	nd
	18	-	nd	nd
30	19	-	nd	nd

Here, 32% (6/19) were BP1 positive. Outcome data were not available for these patients. Analysis of DLX7 and DLX4 showed that 40% (6/15) of the cases were DLX7 positive, and 40% (6/15)

were DLX4 positive. A comparison of expression of the three isoforms in AML and T-cell ALL is seen in Fig. 10.

Next, 19 pediatric patients with pre B-ALL were analyzed. No detectable BP1 was observed in any of these cases, although  
5 the  $\beta$ -actin controls were normally expressed (data not shown).

BP1 expression in CD34+ and CD34- cells: To examine more precisely the reason for very low BP1 expression in normal bone marrow and to determine whether BP1 is expressed in early progenitors, BP1 expression was measured in CD34+ and CD34-  
10 cells. There was clear expression in two independent isolates of CD34- cells (Fig. 11, lanes 2 and 3). In contrast, there was barely detectable BP1 mRNA in three independent samples of CD34+ bone marrow (lanes 4, 5 and 6). Expression in K562 cells is shown for comparison in lane 1, and b-actin was measured as  
15 a loading control.

Clonogenicity of K562 cells overexpressing BP1: It has been found that enforced expression of BP1 in K562 cells leads to decreased differentiation and increased clonogenicity. The human erythroleukemia cell line K562 was used to examine the  
20 effects of ectopic BP1 expression on erythroid differentiation. Four independent K562 clones overexpressing BP1 were isolated and compared with two clones containing an empty vector. All of the overexpressing cell lines were found to exhibit a diminished ability to undergo erythroid differentiation (data  
25 not shown).

Clonogenicity, considered to be a measure of oncogenicity, can be measured by the ability of leukemia cells to grow in 0.5% soft agar. Three of the cell lines overexpressing BP1 exhibited significantly increased numbers of colonies able to  
30 grow in soft agar per 15,000 cells plated compared with controls (Table 6, below). These results suggest BP1 overexpression may be associated with increased oncogenicity in K562 cells and may increase proliferative capacity or cell survival. The average number of cells per colony (an indicator  
35 of proliferation) was determined by pooling 30 colonies to

obtain an average number of cells per colony (Table 6). This number did not increase in the overexpressing cell lines, and the proliferation curves were the same for the overexpressing cell lines and the controls (data not shown), making it likely that the overexpressors show increased survival relative to 7a and 7b rather than increased proliferation. Taken together, these data imply that BP1 expression may direct K562 cells towards survival at the expense of differentiation, a characteristic exhibited by some oncogenes.

TABLE 6. Clonogenicity of K562 cells overexpressing BP1

	<u>Cell Line plated</u>	<u>Relative BP1 Expression</u>	<u>Col./15,000</u>	<u>Avg. no. cells/colony</u>
15	7a (control)	1	23±15	4400
	7b (control)	1	21±7	1300
	8a	21	517±108	2900
	8c	12	924±199	5300
	8d	5	223±34	5800
20	8e	7	57±18	2300

K562 cells are BCL-2 negative, p53 negative and contain the BCR-ABL translocation; the effects of overexpressing BP1 in K562 cells occur against this transformed background. For this reason, NIH 3T3 cells, which are immortalized but not transformed (they do not cause malignancy if injected into mice), were used. Enforced expression of BP1 in those cells led to increased growth in soft agar, but at a reduced level of about 4-fold. NIH 3T3 cells overexpressing BP1 also exhibited about a 4-fold increase in focus formation. The difference in the effect of high BP1 expression on K562 cells and NIH 3T3 cells may mean that additional genetic changes are required for the approximately 10-fold larger increase in clonogenicity seen in K562 cells.

Discussion:

To summarize, significant RNA expression of BP1 was detected in the bone marrow of 81% of pediatric AML patients, compared with 47% of adult patients. In contrast, expression of BP1 was not reproducibly found in normal bone marrow. The highest percentage of BP1 positives occurred in the FAB classification M5 (monocytic), in which 77% of AML cases were BP1 positive; bone marrow cells from 100% of the children in this category were BP1 positive. Two splice variants, DLX7 and DLX4, were co-expressed in 48% and 79% of AML patients, respectively. BP1, DLX7 and DLX4 levels were also assessed in 19 cases of pediatric T-cell ALL. Although the frequency of expression was less in comparison with AML, BP1 was overexpressed in 32%, DLX7 in 50% and DLX4 in 40% of T-cell ALLs, compared with weak or no expression in normal PHA-stimulated T lymphocytes. In sharp contrast, no BP1 expression was detected in pre-B ALL. Although the reason for expression of BP1 in T-cell ALL but not in pre B-cell ALL is not obvious, this difference may provide a useful diagnostic distinction.

*c-myb* was expressed in all AML samples, either at a level comparable to normal bone marrow or at a higher level. Expression of *c-myb* is associated with immaturity, (see, for example, **Gewirtz AM, Calabretta B.** A *c-myb* antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro. *Science* 1988; **242**: 1303-1306; **Gonda T, Metcalf D.** Expression of *myb*, *myc* and *fos* proto-oncogenes during the differentiation of a murine myeloid leukemia. *Nature* 1984; **310**: 249-251 and **Luscher B, Eisenman RN.** New light on Myc and Myb. Part II. Myb. *Genes & Devel* 1990; **4**: 2235-2241.) so those cases within the normal range may be arrested at an early progenitor stage. Higher *c-myb* expression may be part of the leukemogenic process since activation of *c-myb* causes leukemia in mice. (see, for example, **Wolff L, Koller R, Bies J, Nazarov V, Hoffman B, Amanullah A, Krall M, Mock B.** Retroviral insertional mutagenesis in murine promonocytic leukemias: *c-myb* and *Mml1*. *Curr Topics Micro Immuno* 1996; **211**: 191-199.) In this regard, of 11 samples which highly expressed *c-myb* and could

be evaluated for BP1 expression, 9 were BP1 positive. There is a substantial body of data on expression of HOX genes in malignant hematopoietic cell lines. The HOX genes are clustered on four chromosomes, and the DLX genes are located in pairs on the same chromosomes. (see, for example **Lawrence HJ, Sauvageau G, Humphries RK, Largman C**. The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells* 1996; **14**: 281-291 and **van Oostveen JW, Biji JJ, Raaphorst FM, Walbooners JJM, Meijer CJLM**. The role of homeobox genes in normal hematopoiesis and hematological malignancies. *Leukemia* 1999; **13**: 1675-1690; **Nakamura S, Stock DW, Wydner KL, Bollekens JA, Takeshita K, Nagai BM, Chiba , Kitamura T, Freeland TM, Zhao Z, Minowada J, Lawrence JB, Weiss KB, and Ruddle FH**. Genomic analysis of a new mammalian Distal-less gene: Dlx-7. *Genomics* 1996; **38**: 314-324.; **Simeone A, Acampora D, Pannese M, D'Esposito M, Stornaiuolo A, Gulisano M, Mallamaci A, Kastury K, Druck T, Huebner K**. Cloning and characterization of two members of the vertebrate Dlx gene family. *Proc Natl Acad Sci USA* 1994; **91**: 2250-2254.) BP1 is situated at the 3' end of the HOXB cluster on chromosome 17. (see, for example **Fu S, Strovel JW, Haga SB, Stamberg J, Berg PE**. Mapping of a new homeobox gene, BP1, near its isoform DLX7 and characterization of their roles in repression of the beta globin gene. *Am. J. Hum. Gen.* 1998; **63**: A181.) HOXB genes are preferentially expressed in erythroid cells, including K562 and HEL cell lines. (see, for example, **Lawrence HJ, Sauvageau G, Humphries RK, Largman C**. The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells* 1996; **14**: 281-291; **Shen W-F, Largman C, Lowney P, Corral JC, Detmer K, Hauser CA, Simonitch TA, Hack FM, Lawrence HJ**. Lineage-restricted expression of homeobox-containing genes in human hematopoietic cell lines. *Proc Natl Acad Sci USA* 1989; **86**: 8536-8540; **Magli CM, Barba, P, Celetti A, De Vita G, Cillo, C, Boncinelli E**. Coordinate regulation of HOX genes in human hematopoietic cells. *Proc Natl Acad Sci USA* 1991; **88**: 6348-6352; and **Mathews CHE, Detmer K, Boncinelli E, Lawrence HJ**,

**Largman C.** Erythroid-restricted expression of homeobox genes of the human HOX2 locus. *Blood* 1991; **78**: 2248-2252) It is believed that genes in the HOX clusters are switched off or on in blocks in myeloid cells. (see, for example, 38,40 The data presented herein suggest that BP1 may be part of this coordinate regulation since its pattern of expression in erythromyeloid cell lines is similar to that of the adjacent HOXB genes. Transcripts of HOX genes have also been found in AML, T-ALL and pre B-ALL but, unlike BP1, they are readily detectable in normal bone marrow. (see, for example, **Petrini M, Quaranta MT, Testa U, Samoggia P, Tritarelli E, Care A, Cianetti L, Valtieri M, Barletta C, Peschle C.** Expression of selected human HOX genes in B/T acute lymphoid leukemia and interleukin-2/interleukin-1  $\beta$ -stimulated natural killer lymphocytes. *Blood* 1992; **80**: 185-193; **Lawrence HJ, Sauvageau G, Ahmadi N, Lopez AR, LeBeau MM, Link M, Humphries K, Largman C.** Stage- and lineage-specific expression of the HOXA10 homeobox gene in normal and leukemic hematopoietic cells. *Exp Hem* 1995; **23**: 1160-1166; **Biji JJ, van Oostveen JW, Walboomers JMM, Brink ATP, Vos W, Ossenkuppele GJ, Meijer CJLM.** Differentiation and cell-type-restricted expression of HOXC4, HOXC5 and HOXC6 in myeloid leukemias and normal myeloid cells. *Leukemia* 1998; **12**: 1724-1732; **Kawagoe H, Humphries RK, Blair A, Sutherland HJ, Hogge DE.** Expression of HOX genes, HOX cofactors, and MLL in phenotypically and functionally defined subpopulations of leukemic and normal human hematopoietic cells. *Leukemia* 1999; **13**: 687-698; **Salvati PD, Ranford PR, Ford J, Kees UR.** HOX11 expression in pediatric acute lymphoblastic leukemia is associated with T-cell phenotype. *Oncogene* 1995; **11**:1333-1338) Notably, the expression of HOX genes is downregulated during normal hematopoietic differentiation but not in AML. (see, for example, **Kawagoe H, Humphries RK, Blair A, Sutherland HJ, Hogge DE.** Expression of HOX genes, HOX cofactors, and MLL in phenotypically and functionally defined subpopulations of leukemic and normal human hematopoietic cells. *Leukemia* 1999; **13**: 687-698). BP1 RNA expression in acute

leukemias may represent a marker for the differentiation stage of the leukemic blasts and/or may be directly involved in leukemogenesis. The data presented herein point to the possibility that BP1 expression in AML occurs in early progenitors: (i) As described above, all of the BP1 positive cells are also *c-myb* positive and 74% are GATA-1 positive, two indicators of early progenitors. (see, for example, 20,22-24 (ii) The barely detectable expression of BP1 seen in normal bone marrow is compatible with expression in primitive cells, which comprise a very small sub-population of normal bone marrow. (iii) Overexpression of BP1 in both myeloid and lymphoid leukemia argues that leukemogenesis may occur in a stem cell or multipotent hematopoietic progenitor. (iv) Further supporting this idea is the observation that 59% of BP1 positive blasts are found in FAB classes considered to be primitive and associated with stem cell leukemias, i.e., M0 (minimally differentiated), M5 (monocytic) or M7 (megakaryocytic). (see, for example, **Cuneo A, Mecucci C, Kerim S, Vandenberghe E, Dal Cin P, Van Orshoven A, Rodhain J, Bosly A, Michaux JL, Martiat P., Boogaerts M, Carli MG, Castoldi G, Van Den Berghe H.** Multipotent stem cell involvement in megakaryoblastic leukemia: cytologic and cytogenetic evidence in 15 patients. *Blood* 1989; **74**: 1781-1790; **Bonnet D, Dick JE.** Human acute leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* 1997; **3**: 730-737; **Venditti A, Del Poeta G, Buccisano F, Tamburini A, Cox MC, Stasi R, Bruno A, Aronica G, Maffei L, Suppo G, Simone MD, Forte L, Cordero V, Postorino M, Tuffilli V, Isacchi G, Masi M, Papa G, Amadori S.** Minimally differentiated acute myeloid leukemia (AML-M0): Comparison of 25 cases with other French-American-British subtypes. *Blood* 1997; **89**: 621-629.) (v) 64% of BP1 positive cases are CD34 negative. CD34+ stem cells express several HOX genes, and this expression is down-regulated in CD34- cells. (see, for example, **van Oostveen JW, Biji JJ, Raaphorst FM, Walbooners JJM, Meijer CJLM.** The role of homeobox genes in normal hematopoiesis and

hematological malignancies. *Leukemia* 1999; **13**: 1675-1690 and **Sauvageau G, Lansdorp PM, Eaves CJ, Hogge DE, Dragowska WH, Reid DS, Largman C, Lawrence J, Humphries RK**. Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proc Natl Acad Sci USA* 1994; **91**: 12223-12227.) In contrast, BP1 is expressed in CD34- cells and down-regulated in CD34+ cells. These results are in agreement with the data in AML samples (point v), in which BP1 was primarily found in CD34- cells. The very low expression in CD34+ cells could represent either expression in a few CD34+ cells or contamination of the CD34+ cells with CD34- cells. Recent papers support the existence of a subpopulation of primitive CD34- lin- stem cells with repopulating ability in both mice and humans. (see, for example, 49-51 In mice, CD34- lin- stem cells can convert to CD34+ stem cells upon activation; this has not been investigated in humans. (see, for example, **Goodell MA**. CD34+ or CD34-: does it really matter? *Blood* 1999; **94**: 2545-2547 and **Sato T, Laver JH, Ogawa M**. Reversible expression of CD34 by murine hematopoietic stem cells. *Blood* 1999; **94**: 2548-2554) Since our CD34- cells contain both lin+ and lin- subpopulations, it is not known whether BP1 is expressed in stem cells. However, it is clear that BP1 is activated early in hematopoiesis. It is therefore hypothesized that that BP1 expression is then repressed during differentiation. This idea is strengthened by the observation that BP1 is down-regulated during erythroid differentiation of the cell line MB-02. In support of a possible oncogenic role for BP1, it was observed that stable cell lines overexpressing BP1 exhibited up to a 45-fold increase in clonogenicity. Moreover, its high frequency of expression in AML may indicate BP1 is an upstream factor in an oncogenic pathway. Further experiments are needed to delineate the roles of BP1 in normal hematopoiesis, to directly determine if it plays a role in neoplastic transformation, and to examine the clinical significance of its expression in acute leukemias.

In view of the above findings of increased expression of BP1 in leukemias, a method of screening for acute myeloid

leukemia or acute lymphocytic leukemia includes the steps of obtaining a cell sample from a patient and determining whether BP1 is overexpressed by cells in the cell sample, as compared to normal cells. Typically, to test for leukemias, the cell sample is taken from appropriate sources such as bone marrow or peripheral blood. The determination of whether BP1 is overexpressed is preferably carried out by measuring BP1 RNA levels or BP1 protein levels in a cell sample. RNA levels can be determined by RNA assays known in the art such as Northern blot analysis, slot and dot blot analysis, RT-PCR and in situ hybridization. For example, the technique of reverse transcription polymerase chain reaction (RT-PCR), such as in the typical examples described above, can be used to determine whether cells are producing BP1 RNA. Specifically, RNA is isolated from the sample cells and transcribed to obtain a reverse-transcription product (cDNA). A polymerase chain reaction is then carried out using forward and reverse PCR primers derived from SEQ ID NO: 1. Suitable PCR primer pairs for BP1 include, for example, SEQ ID NOs 8 and 9, which amplify a product of 581 bp and SEQ ID NOs 10 and 11, which amplify a product of 225 bp. The polymerase chain reaction is carried out under any suitable reaction conditions for amplifying the BP1 product. For example, for the primers of SEQ ID NOs 8 and 9, typical reaction conditions comprise a denaturation step (94°C, 1 min), an annealing step (58°, 1 min) and an elongation step (72°C, 1.5 min) for 27 cycles, followed by an additional extension (72°C, 5 min). For the primers of SEQ ID NOs 10 and 11, typical reaction conditions comprise holding the sample at 94° C for 2 minutes; then carrying out 30 cycles of 94° C for 1 minute, 62° C for 1 minute and 72° C for 1.5 minutes, 30 cycles; and then holding the sample at 72° C for 10 minutes. The PCR products are then separated from the sample and visualized or quantified by typical methods such as electrophoresis. BP1 protein levels may be measured directly by protein assays known in the art such as immunohistochemical assays. To provide polyclonal or monoclonal antibodies for immunoassays, the BP1 DNA disclosed herein can be used, by

techniques known in the art, to produce sufficient quantities of substantially purified BP1 to use to inoculate a mammal to produce polyclonal or monoclonal antibodies that bind specifically to BP1.

#### 5 Treatment of Leukemia

Decreasing the BP1 expression in K562 leukemia cells causes apoptosis. To demonstrate this, a vector containing an inducible metallothionein promoter regulating antisense BP1 expression was constructed and stably introduced it into K562  
10 cells. Four cell lines were studied, two controls containing an empty vector (9A and 9B) and two containing the antisense plasmid (10B and 10D). CdSO<sub>4</sub>, an inducer of the metallothionein promoter, was added at 50μM for up to four days to induce antisense BP1 expression. Induction of antisense BP1  
15 led to loss of viability, measured by trypan blue (Fig. 12). There was some loss of viability of the controls, but significantly greater cell death for 10B and 10D. This correlated with a great reduction of BP1 mRNA at this time. Fig. 13 shows the expression of BP1 mRNA assessed by RT-PCR for  
20 the controls, 9A and 9B (lanes 1 and 2, respectively), compared with 10B and 10D (antisense, lanes 3 and 4, respectively) in the presence of 50μM CdSO<sub>4</sub>. BP1 RNA expression is almost extinguished in 10B and 10D. To determine whether loss of viability was due to increased apoptosis, cells were assessed  
25 using Annexin V and scored for Annexin V positive, propidium iodide negative cells; apoptosis of the antisense-containing cells significantly increased (data not shown). These experiments indicate that decreasing BP1 expression is associated with apoptosis and, in conjunction with the fact that  
30 cells overexpressing BP1 appear to exhibit increased survival, confirm that modulation of BP1 expression has strong consequences for cell survival in K562 cells.

TABLE 7. Percent apoptosis of K562 cells after induction of BP1 antisense RNA

<u>Cell Line</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>
9A	9±1	7±3	13±3
9B	5±3	9±1	13±1
10B	6±1	15±2	53±4
10D	9±1	20±1	68±1

These results suggest that a possible treatment for leukemia may be to introduce antisense oligonucleotides of the DNA encoding BP1 into a patient whereby the antisense oligonucleotides block the expression of BP1 in leukemia cells, thereby causing apoptosis of these cells.

It has also been found that enforced expression of BP1 causes increased sensitivity of leukemia cells to cytosine arabinoside (Ara C), a drug used to treat AML patients. K562 cell lines overexpressing BP1 were challenged with different concentrations of ara C. After challenge with 50  $\mu\text{m}$  ara C for 4 days, the viability of control cells was 82-86% while the viability of cells overexpressing BP1 ranged 27-29% to 66%, i.e., there was up to a three-fold decrease in viability (data not shown). No significant differences in the data were seen at 100  $\mu\text{m}$  Ara C. In an Annexin V assay at 30 minutes and 60 minutes, the highest frequency of apoptotic cells was observed for the cells with the lowest viability. Thus, it is clear that the cell lines with enforced BP1 expression exhibit increased sensitivity to Ara C, evidenced by an increase in apoptosis.

The above findings can be used therapeutically to optimize the dosage of Ara C given to a patient in accordance with the BP1 expression level of that patient.

#### Screening and Treatment of Breast Cancer

In order to determine whether BP1 is overexpressed in breast cancer cell lines, the inventor conducted studies to detect overexpression of BP1, as compared to normal breast tissue. Figure 14 shows that expression of BP1 by several

breast cancer cell lines, including breast cancer lines MCF7 ADR, MDA468 and T47D. In further studies, BP1 expression was examined by RT-PCR in breast cancer cell lines (Table 8).

TABLE 8. BP1 expression in breast cancer cell lines

	<u>Cell line</u>	<u>ER</u>	<u>PR</u>	<u>Malignancy</u>	<u>Tumorigenic<sup>+</sup></u>	<u>BP1</u>
5	Hs578T	-	-	ductal ca.	no	+/-
	MCF7	+	+	ductal ca.	**	+
	MCF7ADR	*		ADR resistant	yes	+++
	MDA-MB-231	-	-	ductal ca.	yes	++
10	MDA-MB-435s	-	-	ductal ca.	no	+
	MDA-MB-468	-	-	ductal ca.	yes	++
	T47D	+	+	ductal ca.	NA	+++
	MCF10A	+	+	normal	no	+/-

\* non-responsive to estradiol

15 + Data from the American Type Culture Collection. NA, data not available.

\*\* Not tumorigenic without the addition of estradiol

20 The most striking correlation was between BP1 expression and the ability of a cell line to cause mammary tumors in mice. Interestingly, high BP1 expression was observed in the adriamycin (ADR) resistant MCF7 cell line, MCF7ADR. Whether there is any relationship between BP1 expression and ADR resistance is unknown. Cell line MCF10A, which is derived from  
25 normal breast epithelium, showed barely detectable BP1 mRNA.

To further demonstrate that BP1 is expressed in breast cancer, BP1 expression was examined in frozen breast tumor tissues and in surrounding normal tissue. A total of 15 tumor tissues were analyzed (Table 9). BP1 was expressed in all 12  
30 of the ER negative cases, but in only one of three ER positive cases. These data indicate a trend of BP1 expression in high grade, ER-, PR- tumor tissues.

TABLE 9. Comparison of BP1 expression with ER and PR status in breast cancer

	<u>BP1+</u>	<u>BP1-</u>
	<u>ER+PR+</u> 1	2
5	<u>ER-PR-</u> 12	0

RT-PCR analysis of representative samples from normal and malignant breast tissues are shown in Fig. 15. Normal tissue is indicated by N and tumor by T. All of the tumor tissues shown are ER- PR-. In lanes 1-4, tumor and corresponding normal tissues are shown. No expression was seen for the normal samples, while BP1 was expressed in all of the tumors. A total of six normal breast tissues have been analyzed. Five of the six were BP1 negative and one showed low BP1 expression (data not shown). Three additional tumor tissues are shown in lanes 5-7.  $\beta$ -actin expression demonstrates the integrity of the RNA and serves as a loading control for each sample.

In view of the above, the detection of overexpression of BP1 is a screening tool for breast cancer. The method of screening is carried out in the same manner as the method for screening for leukemia, except that, in the present method, the cell samples are taken from breast tissue.

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The purpose of the above description and examples is to illustrate some embodiments of the present invention without implying any limitation. It will be apparent to those of skill in the art that various modifications and variations may be made to the composition and method of the present invention without departing from the spirit or scope of the invention. All patents and publications cited herein are incorporated by reference in their entireties.

## I Claim:

1. An isolated DNA having SEQ ID NO:1, encoding BP1.
2. The isolated DNA according to claim 1, wherein said BP1 exhibits repressor function.
3. The isolated DNA according to claim 1, wherein said BP1 binds to Silencer I at -530 bp upstream of the  $\beta$ -globin gene and binds to Silencer II at -300 bp upstream of the  $\beta$ -globin gene.
4. The isolated DNA according to claim 1, wherein said BP1 binds to a DNA sequence at -530 bp upstream of the  $\delta$ -globin gene.
5. The isolated DNA according to claim 1, wherein said BP1 binds to sequences at -1400 bp and -1100 upstream of the  $\epsilon$ -globin gene and to a sequence at -1100 of the  $\lambda$ -globin gene.
6. The isolated DNA according to claim 1, wherein said DNA is a cDNA of 1251 bp.
7. The isolated DNA according to claim 1, wherein said DNA contains an open reading frame (ORF).
8. A vector comprising the isolated DNA of claim 1.
9. A host cell transformed with the vector of claim 8.
10. A host cell according to claim 9, wherein said host cell is a eukaryotic host cell.
11. An antisense DNA or an antisense RNA complementary to the DNA having SEQ ID NO:1.
12. A method of treating sickle cell anemia, the method

comprising the step of administering an effective amount of BP1 to a patient.

13. The method of claim 12 wherein the BP1 is administered by transforming  $\beta$ -globin-producing cells of a patient with a vector containing an open reading frame of SEQ ID NO. 1 and a controllable expression promoter, so that BP1 is controllably produced in the  $\beta$ -globin-producing cells of the patient.

14. The method of claim 12, wherein said BP1 decreases  $\beta$ -globin expression and decreases intracellular concentrations of HbS.

15. A method of screening or diagnosing acute myeloid leukemia or acute lymphocytic leukemia, the method comprising the steps of

- (a) obtaining a cell sample from a patient, and
- (b) determining whether BP1 is overexpressed by said cell sample as compared to normal cells, wherein overexpression, if any, of BP1 indicates a positive diagnosis of acute myeloid leukemia or acute lymphocytic leukemia.

16. The method of claim 15 wherein the cell sample is obtained from bone marrow or peripheral blood.

17. The method according to claim 15, wherein step (b) of determining whether BP1 is overexpressed is accomplished by carrying out RT-PCR (reverse transcriptase polymerase chain reaction) using PCR primers derived from SEQ ID NO:1.

18. The method of claim 17 wherein the PCR primers are the oligonucleotides of SEQ ID NOs 8 and 9 and wherein the expression of BP1 is indicated by a PCR product of 581 base pairs (bp).

19. The method of claim 17 wherein the PCR primers are the oligonucleotides of SEQ ID NOs 10 and 11 and wherein the

expression of BP1 is indicated by a PCR product of 225 base pairs (bp).

20. The method of claim 15 wherein the method further includes the step of providing an antibody to BP1 and wherein step (b) of determining whether BP1 is overexpressed by said cell sample as compared to normal cells is accomplished by immunohistochemical detection of BP1 using the antibody to BP1.

21. A method of screening/diagnosis of breast cancer, the method comprising the steps of

(a) obtaining a cell sample from breast tissue of a patient, and

(b) determining whether BP1 is overexpressed by said cell sample as compared to normal cells, wherein the overexpression, if any, of BP1 indicates a positive diagnosis of breast cancer.

22. The method according to claim 21, wherein step (b) of determining whether BP1 is overexpressed is accomplished by carrying out RT-PCR (reverse transcriptase polymerase chain reaction) using PCR primers derived from SEQ ID NO:1.

23. The method of claim 22 wherein the PCR primers are the oligonucleotides of SEQ ID NOs 8 and 9 and wherein the expression of BP1 is indicated by a PCR product of 581 base pairs (bp).

24. The method of claim 22 wherein the RT-PCR primers are the oligonucleotides of SEQ ID NOs 10 and 11 and wherein the expression of BP1 is indicated by a PCR product of 225 base pairs (bp).

25. The method of claim 21 wherein the method further includes the step of providing an antibody to BP1 and wherein step (b) of determining whether BP1 is overexpressed by said cell sample as compared to normal cells is accomplished by immunohistochemical detection of BP1 using the antibody to BP1.

26. An mRNA fragment encoding BP1 protein.

27. The isolated DNA according to claim 1, wherein said BP1, wherein said BP1 is a homeobox gene and a member of the distal-less family.

28. The isolated DNA according to claim 1, wherein said BP1, wherein said BP1 maps to chromosome 17q21-22.

29. A polyclonal antibody to BP1.

30. A monoclonal antibody to BP1

31. A composition comprising a set of PCR primers that amplifies a unique DNA molecule from a substrate of cDNA of mRNA that encodes for BP1.

32. The composition of claim 31 wherein the set of PCR primers comprises the oligonucleotides of SEQ ID NO: 8 and SEQ ID NO: 9.

33. The composition of claim 31 wherein the set of PCR primers comprises the oligonucleotides of SEQ ID NOs: 10 and SEQ ID NO: 11.

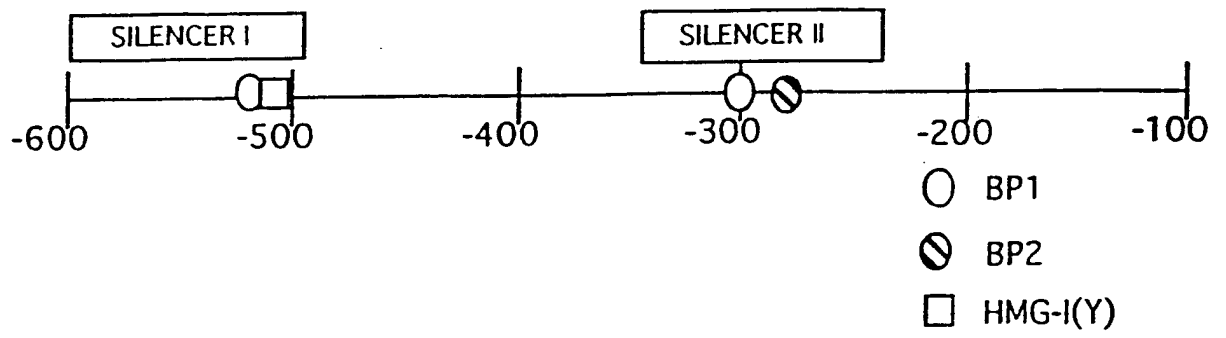


Fig. 1

2/15

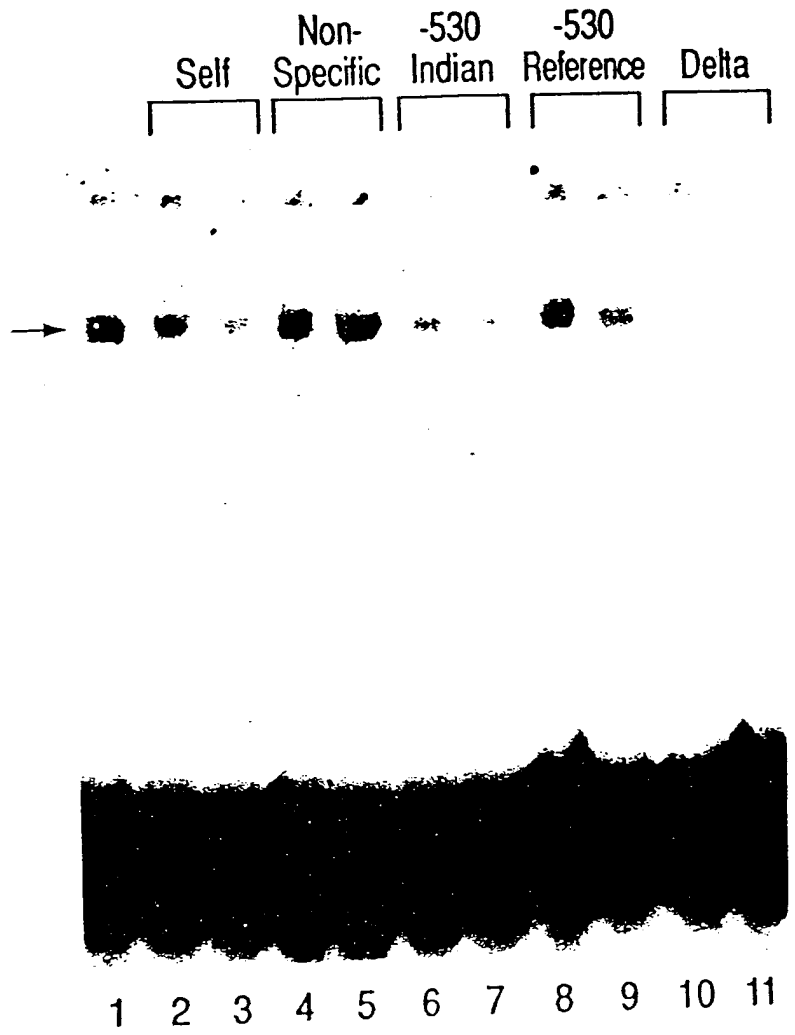
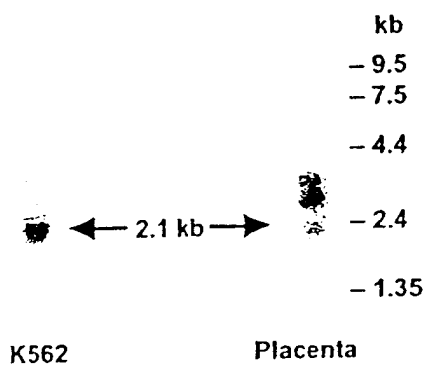


Fig. 2

A.



B.

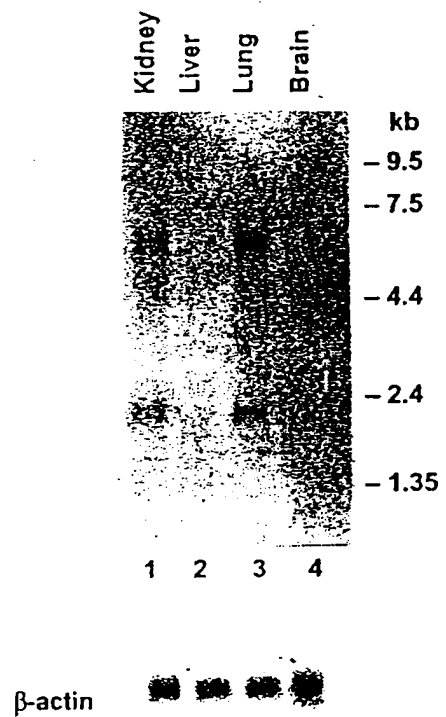


Fig. 3

CCGCCCGGGC AGGTGGGAAC CGAACCCGAT GGAGAGGAGG GGGCCCCCAT GGATTTAGGG 60  
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 CCGCTGAAAG AGGCTCAGAG AGACACTTTC TCCGGGATCT TAAGTGTGGG GGCTGCTGGC 180  
 TGGGGGGCCC GTCCGGCCA ACGCCGGAGG CTTGAAAAG AGAGTTAGCA GCGGGAGCGG 240  
 ACTACGTGCC GGGCCATGGC CTTTCTGCCC GGGCCCTGGC CACA 284

ATGACCTCTTTGCCCTGCCCCCTCCCCGGCCGGGACGCCTCCAAAGCTGTCTTCCCAGACCTC 347  
 M T S L P C P L P G R D A S K A V F P D L (21)

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FIGURE 4

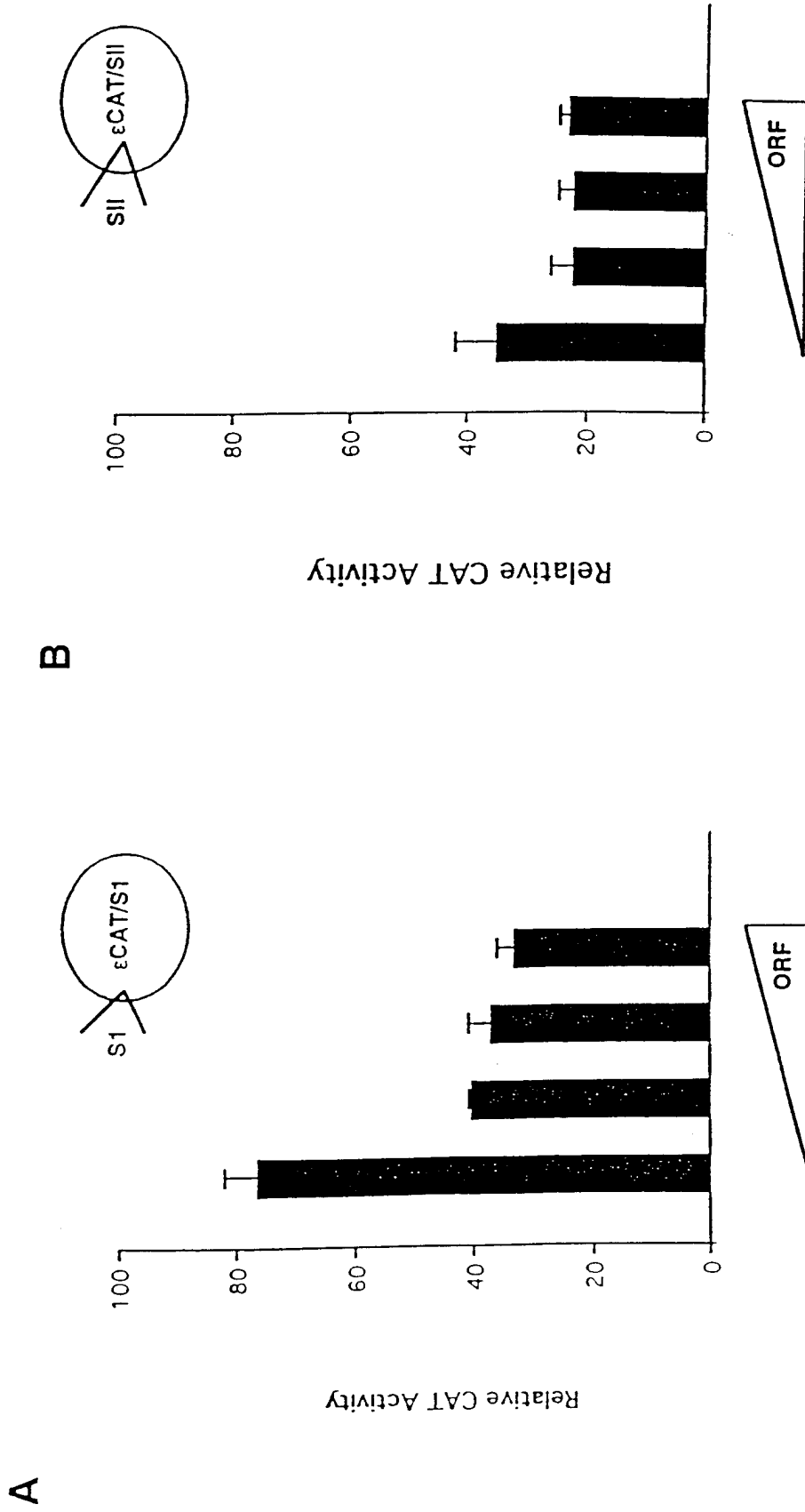


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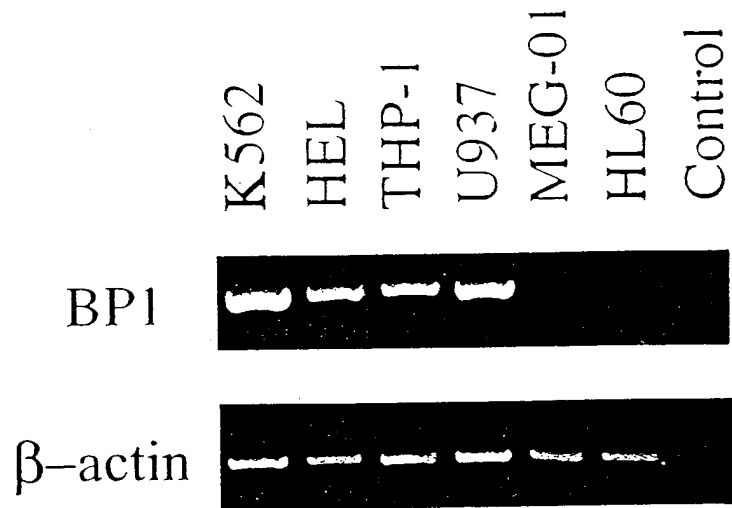


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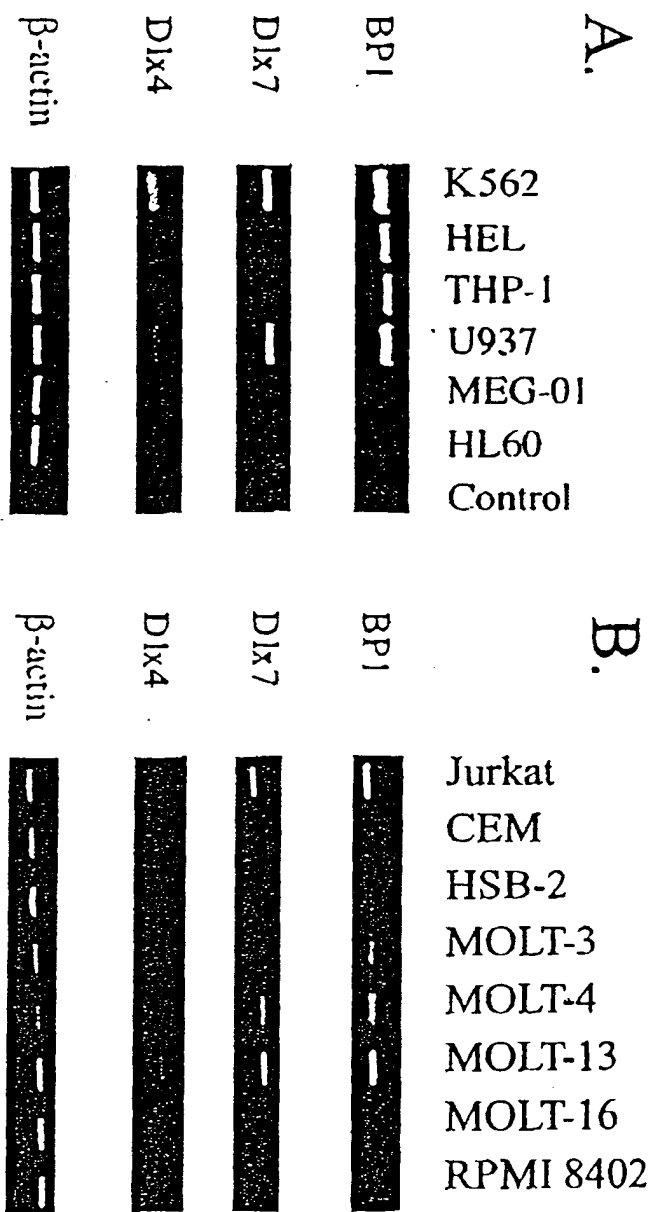


Fig. 7

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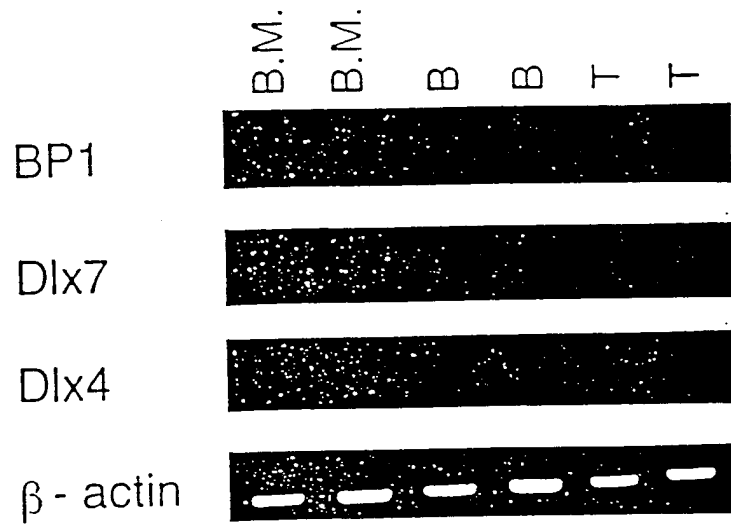


Fig. 8

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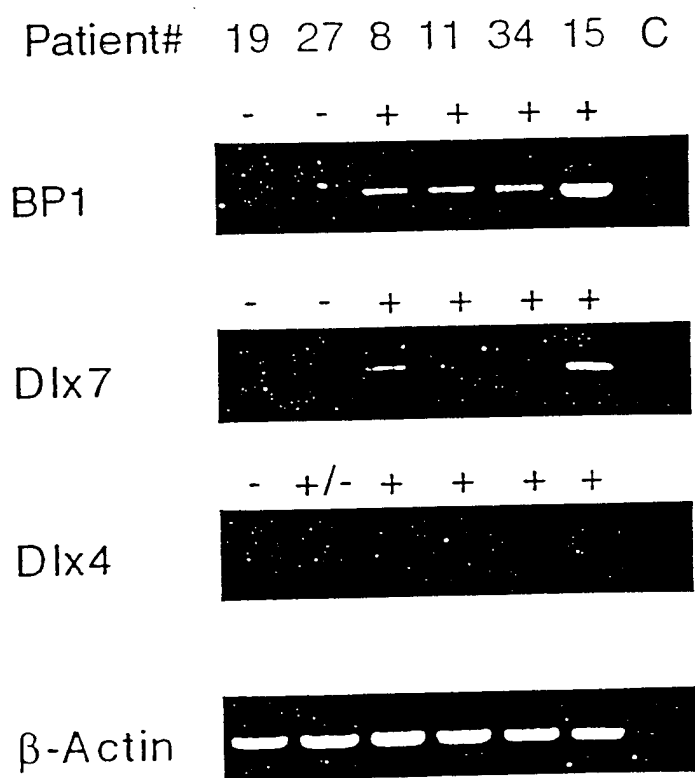


Fig. 9

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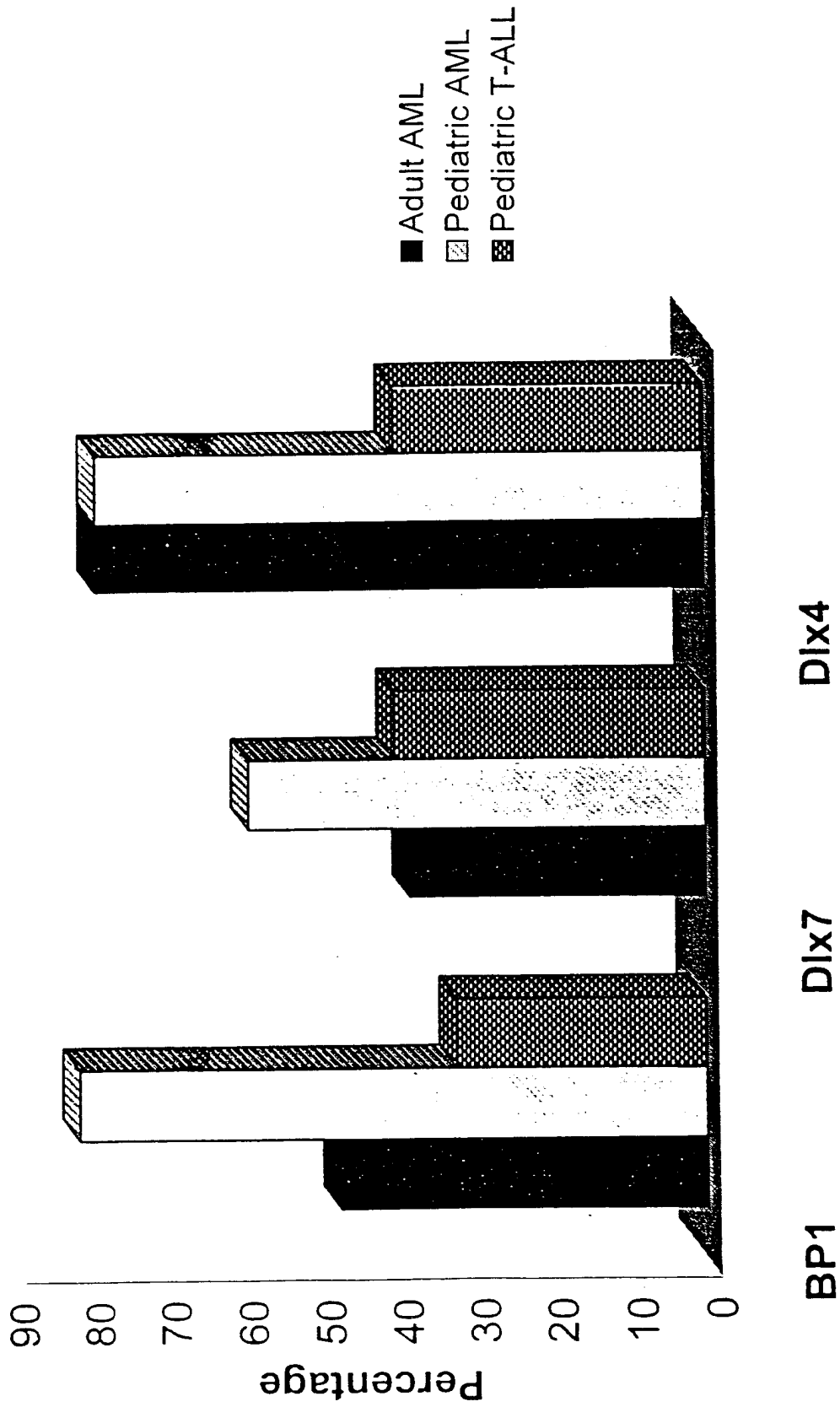


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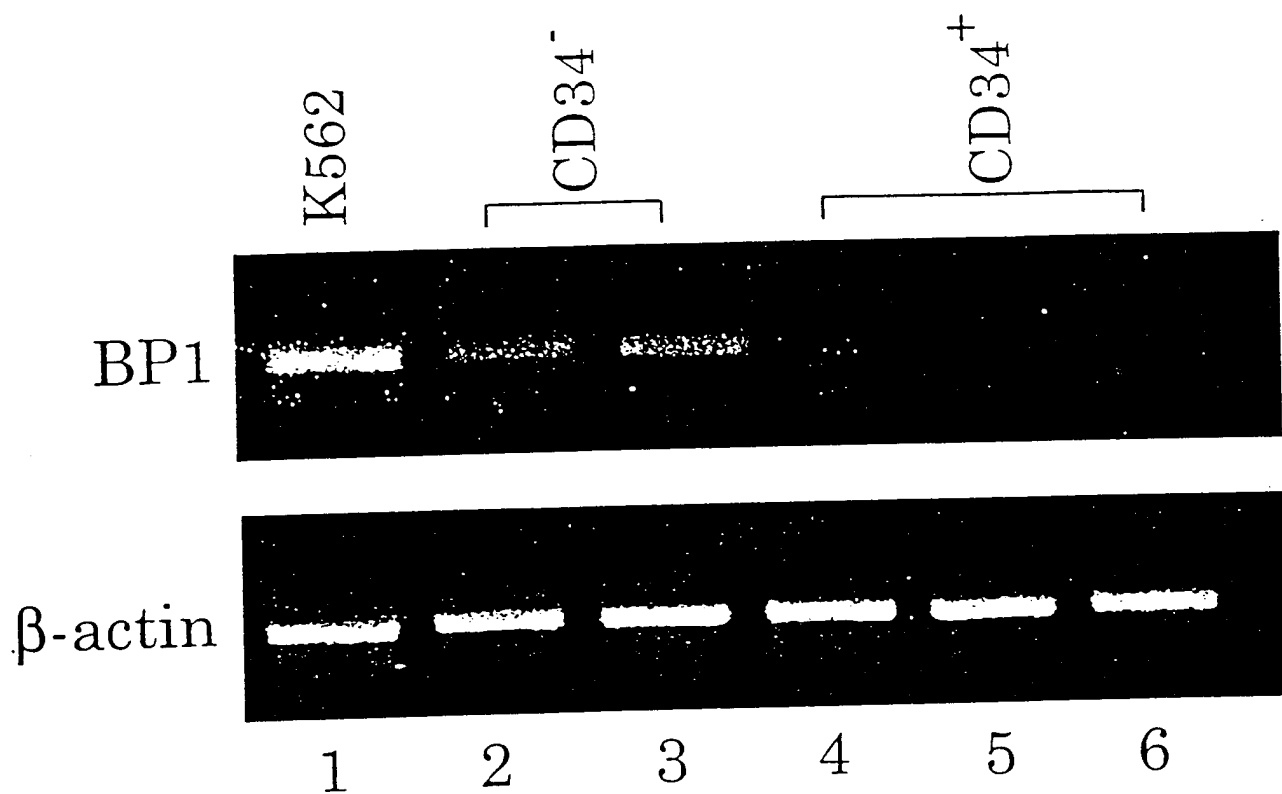


Fig. 11

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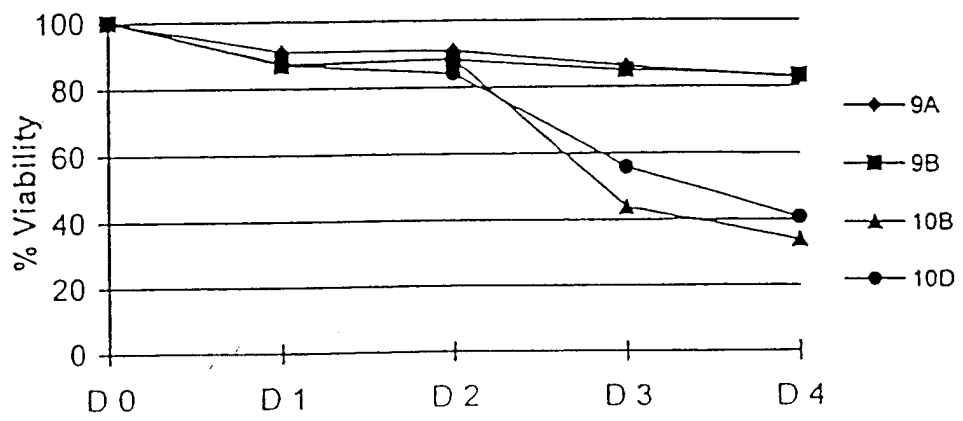


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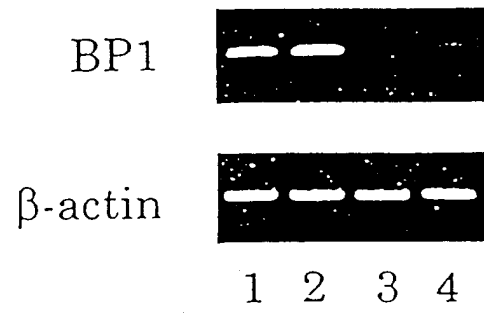


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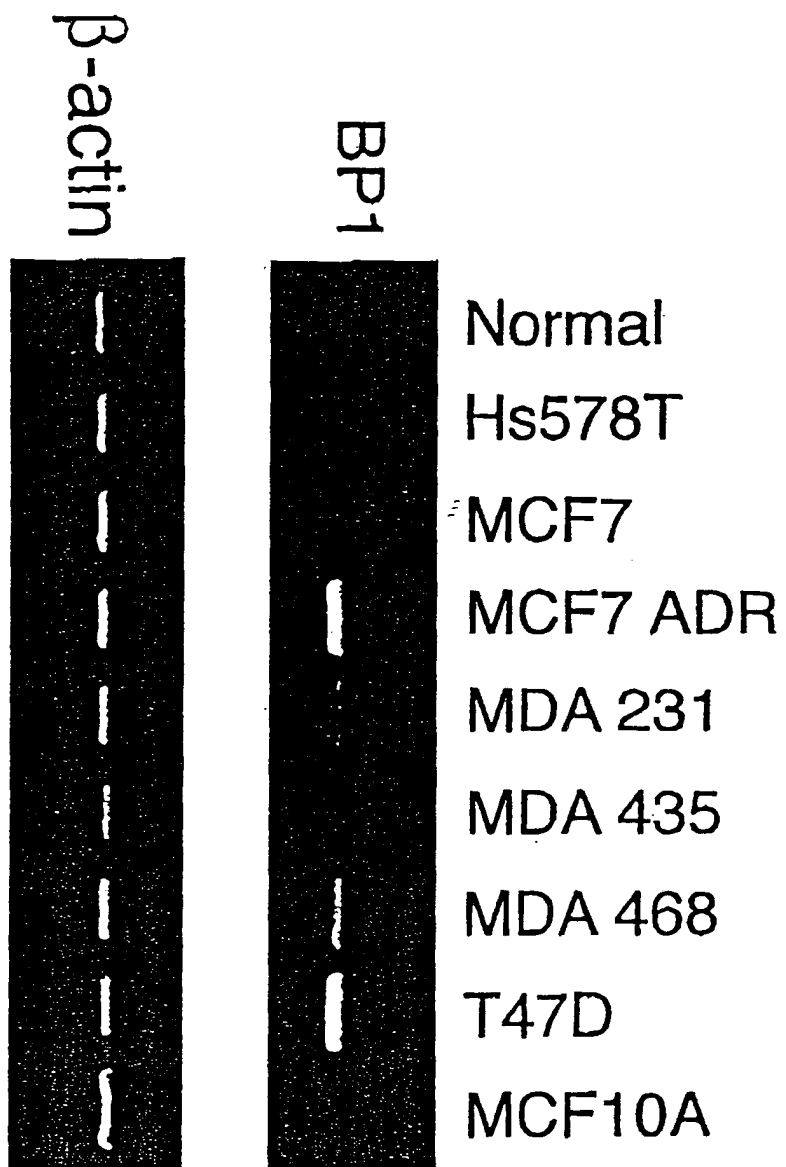


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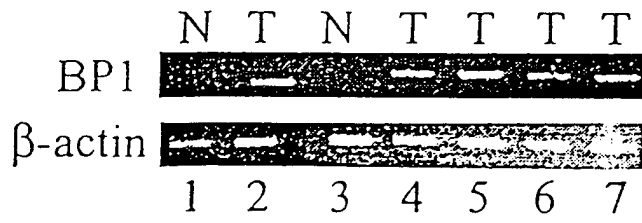


Fig. 15

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/22093

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC(7) : C07H 21/04; C12Q 1/68; G01N 33/53; C07K 16/00 US CL : 536/23.5; 435/6, 7.1; 530/387.1 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) U.S. : 536/23.5; 435/6, 7.1; 530/387.1		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) MPSRCH, DIALOG, WEST search terms: transcription factor, homeobox gene, distal-less		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Database Genbank, Accession No. U73328, NAKAMURA et al. 'Genomic analysis of a new mammalian distal-less gene: Dlx7. Gene sequence'. Genomics. 1996, Vol. 38, No. 3, pages 314-324. See entire document.	11, 26 ----- 15, 17, 20, 29, 30
Y	SHIMAMOTO et al. Inhibition of DLX-7 homeobox gene causes decreased expression of GATA-1 and c-myc genes and apoptosis. Proc. Natl. Acad. Sci. USA. April 1997, Vol. 94, pages 3245-3249. See entire document.	15, 17
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* "A" "B" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
Date of the actual completion of the international search 02 NOVEMBER 2000		Date of mailing of the international search report 05 DEC 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer MINH-TAM DAVIS Telephone No. (703) 308-0916

专利名称(译)	新型转录因子 , bp1		
公开(公告)号	<a href="#">EP1633769A1</a>	公开(公告)日	2006-03-15
申请号	EP2000957403	申请日	2000-08-14
申请(专利权)人(译)	乔治华盛顿大学		
当前申请(专利权)人(译)	乔治华盛顿大学		
[标]发明人	BERG PATRICIA E		
发明人	BERG, PATRICIA, E.		
IPC分类号	C07H21/04 C12Q1/68 G01N33/53 C07K16/00 G01N33/48 A61K38/00 A61K48/00 A61P7/06 A61P35/02 C07K14/47 C07K16/18 C12N1/15 C12N1/19 C12N5/08 C12N5/10 C12N15/09 C12P21/08 C12Q1/02 C12Q1/6886 G01N33/574 G01N33/577		
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其他公开文献	EP1633769A4		
外部链接	<a href="#">Espacenet</a>		

#### 摘要(译)

提供了SEQ ID NO : 1的分离的DNA，其编码转录因子BP1，其被认为是β-珠蛋白基因的阻遏物。用含有DNA的载体转化的宿主细胞可用于产生BP1。具有与BP1开放阅读框可操作连接的可控启动子的载体可用于转化患有镰状细胞性贫血的患者的β-珠蛋白产生细胞，从而提供治疗。因为BP1在白血病和乳腺癌细胞中过表达，可以通过确定PB1是否在可能患有这些病症的患者的细胞样品中过表达来筛选和诊断急性髓性白血病，急性淋巴细胞白血病和乳腺癌。编码BP1的DNA的反义DNA或RNA可用于治疗急性髓性白血病，急性淋巴细胞白血病和乳腺癌。