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p53 IN CHRONIC MYELOGENOUS LEUKEMIA

Study of Mechanisms of Differential Expression

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The p53 is a 53-kD nuclear phosphoprotein whose expression is associated with cellular proliferation (1–3). While the physiologic functions of p53 and its role in malignancy are as yet unclear, evidence suggests that the gene product can participate in the transforming event; cotransfection experiments showed that p53 can complement the Ha-*ras* protooncogene in transformation of primary rodent cells (4–6). The p53 is expressed in a variety of leukemic cell lines and the abnormal cells from patients with various hematologic malignancies (7–9). The normal counterparts of many of these transformed hematopoietic cells express negligible levels of p53 protein (7). We have found that cells from several lymphoid but not myeloid lines synthesize the p53 protein (7). Lack of expression of p53 can occur in cell lines with structural alterations of the p53 gene region, as was shown for the human promyelocytic cell line HL-60 (10), murine lymphoma (11), and erythroleukemia (12) cell lines and human osteosarcoma cell lines (13).

Chronic myelogenous leukemia (CML)[1] displays a clonal population of cells evolved from a transformed pluripotent hematopoietic stem cell (14). This clone can be identified by the presence of a specific chromosomal translocation t(9;22) (Philadelphia chromosome [Ph^1]). After a clinically benign phase, characterized by an increased number of immature myeloid precursors in the bone marrow and peripheral blood, a terminal "blast crisis" phase develops that resembles acute leukemia. In this phase, the blast cells appearing in bone marrow and peripheral blood are predominantly myeloid (70% of cases) or lymphoid (30% of cases). In this study, we used CML cells as a model to examine expression of p53 in two different cell lineages. Cell lines established from patients in either myeloid or lymphoid blast crisis of CML, and fresh cells from CML patients were screened for presence of p53 mRNA and protein, and experiments were performed to study the differential expression of p53 in these hematopoietic cells.

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[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; CML, chronic myelogenous leukemia; HTF, Hpa II tiny fragments; HTLV-I, human T lymphotropic virus I; Ph^1, Philadelphia chromosome; TdT, terminal deoxynucleotidyltransferase.

Materials and Methods

Cell Lines. The following Ph^{1+} cell lines were examined for p53 expression: K562, EM-2, EM-3, KCl-22 (myeloid lines, [15–17]) and BV173, NALM-1, MBL, SK-CML8-Bt(9;22) (lymphoid lines, [18–21]). Furthermore, we studied somatic hybrids formed from fusions of myeloid (p53 nonexpressor) and lymphoid (p53 expressor) parental lines; Dutko and Putko are hybrids of K562 (myeloid, p53 mRNA nonexpressor) and PUT or Daudi (lymphoid expressors), respectively (22, 23). HP-1 is a hybrid of HL-60 (promyelocyte with a deletion and non-expression of p53) and PUT (24). Also, we examined p53 expression in EBV-immortalized, Ph^{1-} SK-CML8-BN lymphocytes (21), which are from the same individual as Ph^{1+} SK-CML8-Bt(9;22) lymphocytes (generous gifts of B. Clarkson, Memorial Sloan Kettering, NY). In addition, the promyelocytic cell line HL-60 (25), myeloblastic KG-1 (27), Burkitt's lymphoma lines Daudi and PUT (22, 26), and human T lymphotropic virus (HTLV-I) immortalized T-lymphocytic S-LB1 (28) were tested. Cells were grown in α medium (Flow Laboratories, Inc., McLean, VA) enriched with 10% FCS in a 7.5% CO_2 humidified atmosphere.

Patients. After receiving informed consent from the patients with CML, heparinized venous blood was drawn and subjected to Ficoll density gradient separation (29). The differential count of the nonadherent, mononuclear fraction was determined by morphological inspection of Giemsa-stained cytospin preparations: myeloid cells comprised between 94 and 96% of the leukocytes. Diagnosis and staging of the patients was performed by using clinical, morphological, cytogenetic, and immunohistochemical criteria including examination for terminal deoxynucleotidyl transferase (TdT) and expression of the common ALL antigen (14).

Antibody. p53 mAb (PAb421) was affinity purified and concentrated from the supernatant of an established hybridoma cell line (generous gift of E. Harlow, Cold Spring Harbor, NY) (30) by $(NH_4)_2SO_4$ precipitation and binding to Sepharose–protein A columns (Sigma Chemical Co., St. Louis, MO) (31).

Recombinant DNA Constructs. The following p53 clones were used as probes: the complete insert of the cDNA clone pR4-2 (32) (complementary to exons II–XI); the 350-bp Bam HI–Xba I fragment of the clone pBT53 (p53 promoter and part of exon I) (33). For detection of p53 mRNA, the 480-bp Nco I–Nco I fragment of pR4-2 (complementary to exons II–V) was used. For the transfection experiments we used the plasmid constructs pBXB53 and pRXB53, containing 350 bp and 2.4 kb, respectively, of the 5′ p53 sequences with promoter activity (33). The pSV2-CAT containing the SV40 early promoter was used to determine transfection efficiency and chloramphenicol acetyltransferase (CAT) enzyme stability (34); pSVO-CAT (lacking promoter sequences) was used as a negative control (34). The 414-bp Pst I/Pst I fragment detecting exon II of the *c-myc* gene (35) was generated from the plasmid pHSRI and used to probe for *c-myc* mRNA.

p53 Protein Synthesis. Cells (5×10^6) were washed several times with PBS, resuspended (1 ml) in methionine-free α medium with 10% FCS and 200 μCi [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) or [$^{32}P_i$]orthophosphate (500 μCi). After a 5-h incubation, the cells were washed several times and lysed as described previously (7). The cell lysate was precleared by absorption on *Staphylococcus aureus* and nonimmune serum. Equal amounts of labeled protein were then precipitated with mAb PAb421. SDS-PAGE was performed according to Laemmli (36). Precipitated proteins were visualized by autoradiography.

Inhibition of New Protein Synthesis. K562 cells were seeded at 4×10^5 cells/ml in α medium with 10% FCS 4 h before treatment with the protein synthesis inhibitor cycloheximide (CHX) (Sigma Chemical Co.). At 0 h, CHX was added to the culture medium to a final concentration of 20 μg/ml. After 3 h the cells were washed two times in PBS and RNA was extracted.

DNA and RNA Isolations. Extraction of genomic DNA from cell lines was performed by lysing the cells with 3% NP-40 and digesting the nuclei for 16 h at 50°C with proteinase K (Bethesda Research Laboratories, Bethesda, MD) (37). The DNA was precipitated by adjustment of the solution to a final concentration of 2 M ammonium acetate and

subsequent addition of 1 vol isopropanol, followed by several rinses in 2 M ammonium acetate/80% ethanol, and 80% ethanol, as described by Hardy et al. (38).

Total cellular RNA was extracted from cell lines and fresh human hematopoietic cells by the hot phenol method (37). Briefly, cells were lysed at 60°C in guanidine isothiocyanate (Eastman Kodak Co., Rochester, NY) and extracted into phenol–chloroform. After several extractions the solution was precipitated with 2 vol ethanol. The precipitate was redissolved and digested with proteinase K for 2 h at 37°C. After several subsequent extractions in phenol–chloroform and chloroform, the aqueous phase was again precipitated with 2 vol ethanol.

Southern and Northern Blot Analyses. Restriction endonuclease digestions of genomic DNA were performed following manufacturer specifications. For methylation studies, 5–10× excess of Hpa II and Hha I (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used for digestion, and plasmid DNA was added to aliquots of the reaction mix to monitor completeness of digestion. DNA was size fractionated on Tris–acetate or tris–borate agarose gels. Total RNA was size fractionated on denaturing formaldehyde gels as described (37). Nucleic acids were transferred to nylon-based filters (NCI; Biodyne, Irvine, CA). Hybridization with ^{32}P-oligolabeled (39) p53-specific probes was carried out as described (37); filters were rinsed to a final stringency of 0.25× SSC (DNA filters) or 0.1× SSC (RNA filters) at 65°C for 15 min (1× SSC: 0.15 mol/liter NaCl; 0.015 mol/liter trisodium citrate).

DNA Transfection and CAT Assay. The BV173 lymphoid and K562 myeloid cell lines were transfected by a modification of the DEAE–dextran method (40). Briefly, 10^7 cells were washed four times with serum-free medium and incubated for 4.5 h in 10 ml serum-free medium with 0.25 mg/ml DEAE–Dextran (Sigma Chemical Co.), 0.1 M Tris (pH 7.3), 10^{-4} M chloroquin (Sigma Chemical Co.), and 10 μg of plasmid DNA. The cells were washed and incubated in α medium with 10% FCS for 42 h, then lysed by freeze-thawing, and cell extract was used to acetylate [^{14}C]chloramphenicol (Amersham Corp.). Labeled chloramphenicol and its acetylated forms were separated by ascending silica gel thin layer chromatography (TLC) (with solvent chloroform/methanol 95:5) and visualized by autoradiography.

Results

Expression of p53 mRNA and Protein in CML Cell Lines and Cells from Patients with CML. A number of leukemic cell lines established from patients with CML were screened by Northern blot analysis for expression of p53 mRNA (Fig. 1*A*, Table I). T-lymphocytic S-LB1, immortalized by infection with HTLV-I, was used as a positive control and the HL-60 myeloid cell line (with a deletion of the p53 gene) served as negative control. The p53 mRNA was detected in all the lymphoid lines, which includes SK-CML8-Bt(9;22), MBL, BV173, SK-CML8-BN and S-LB1 (Fig. 1*A*, lanes *1–3*, *8*, and *10*), and EM-2 and EM-3 myeloid lines (lanes *5* and *6*). Negligible p53 mRNA was detectable in the myeloid CML cell lines K562 and KCl-22 (lanes *4* and *7*). The p53 mRNA was not detected in K562 cells even after treatment of the cells with an inhibitor of protein synthesis (cycloheximide) (data not shown). The filter was rehybridized with c-*myc* (Fig. 1*B*). HL-60 cells expressed the largest amounts of c-*myc* mRNA, followed by EM-2, EM-3 (Fig. 1*B*, lanes *7–9*), KCl-22, and K562. Small to undetectable amounts of c-*myc* mRNA were found in the lymphoid lines.

We studied the ability of the CML cell lines to synthesize p53 protein by metabolic labeling and immunoprecipitation, using the mAb PAb421 (Fig. 2). The p53 protein was detected in the lymphoid cell lines BV173, NALM-1, MBL (lanes *4–6*). No p53 protein was detected in the myeloid lines including K562, EM-2, EM-3 (lanes *7–9*), and KCl-22 (data not shown). Of note, in lane *6* (MBL),

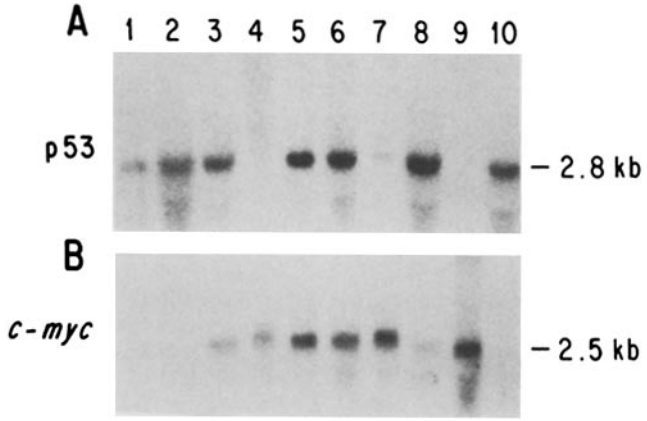

FIGURE 1. (*A*) p53 mRNA accumulation in myeloid and lymphoid CML cell lines. Total RNA (20 μg per lane) from SK-CML8-Bt(9;22) (lymphoid, CML, lane *1*), MBL (lymphoid, CML, lane *2*), BV173 (lymphoid, CML, lane *3*), K562 (myeloid, CML, lane *4*), EM-2 (myeloid, CML, lane *5*), EM-3 (myeloid, CML, lane *6*), KCl-22 (myeloid, CML, lane *7*), SK-CML8-BN (lane *8*), HL-60 (lane *9*), S-LB1 (lane *10*) was electrophoresed, blotted, and hybridized with the 480-bp Nco I–Nco I fragment of the oligolabeled (39) p53 cDNA clone pR4-2 (32). The filter was then washed and autoradiographed for 4 d. The 28S and 18S bands of rRNA served as molecular weight markers. (*B*) The same filter was rehybridized with the 414-bp Pst I–Pst I fragment (exon 2) of the human c-*myc* clone pHSRI (35).

TABLE I

Expression of p53 and c-myc in Cells of Leukemic Lines, Summarizing Figs. 1–3, 5, and 6

Cell line	Reference	Characteristics	Ph^1	p53 mRNA	p53 protein	c-*myc* mRNA
HL-60	25	Myeloid	–	–	–	+
K562	15	Myeloid	+	–	–	+
KCL:22	17	Myeloid	+	±	–	+
EM-2	16	Myeloid	+	+	–	+
EM-3	16	Myeloid	+	+	–	+
Putko	22	Hybrid	+	+	+	NT
Dutko	23	Hybrid	+	+	+	NT
BV173	18	Lymphoid	+	+	+	±
MBL	20	Lymphoid	+	+	+	–
SK-CML8-Bt(9;22)	21	Lymphoid	+	+	+	–
SK-CML8-BN	21	Lymphoid	–	+	+	±
S-LB1	28	Lymphoid	–	+	+	–

The characteristics or the cell lines are provided in their corresponding references. Abbreviations: NT, not tested; ±, only trace amounts detectable.

a 70-kD band is quite prominent. We noted this band to coprecipitate with p53 in five separate experiments; it is the correct molecular weight to be the major heat shock protein Hsp70. Prior studies have shown that the p53 protein can complex with this protein (41).

p53 expression was also studied in two EBV-immortalized B lymphocyte lines that differ in the presence [SK-CML8-Bt(9;22)] or absence (SK-CML8-BN) of the Ph^1 chromosome. Both were established from a 63 yr old male patient with CML in the chronic phase (21). The SK-CML8-Bt(9;22) line represents the

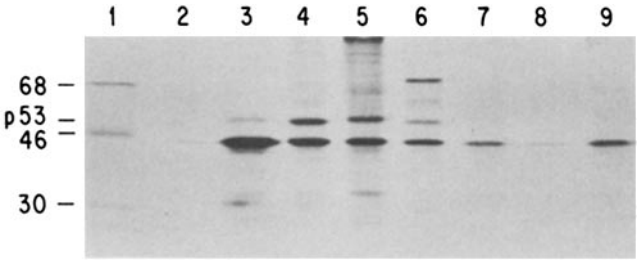

FIGURE 2. p53 protein synthesis in myeloid and lymphoid CML cell lines. 5×10^6 cells from HL-60 (lane *2*), S-LB1 (lane *3*), BV173 (lane *4*), NALM-1 (lane *5*), MBL (lane *6*), K562 (lane *7*), EM-2 (lane *8*), EM-3 (lane *9*), were labeled metabolically with [^{35}S]methionine for 5 h, then immunoprecipitated with PAb421 (30), electrophoresed on a SDS/PAGE and autoradiographed. (Lane *1*) Molecular weight markers ($M_r \times 10^{-3}$). The ~43 kD band in lanes *3–10* probably represents actin, which can polymerize during immunoprecipitation. The ~70-kD band visible in lane *6* may represent the major heat shock protein Hsp 70.

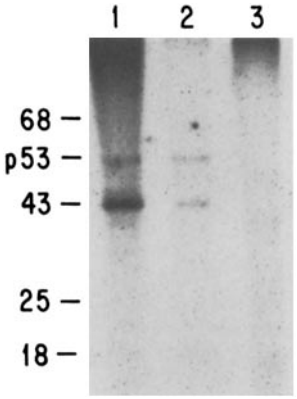

FIGURE 3. Synthesis of phosphorylated p53 protein in Ph^{1+} and Ph^{1-} B lymphocyte lines from the same patient. 5×10^6 cells were labeled metabolically with [$^{32}P_i$]orthophosphate for 5 h. Ph^{1+} SK-CML8-Bt(9;22) (lane *1*) and Ph^{1-} SK-CML8-BN (lane *2*) cells were immunoprecipitated with PAb 421; SK-CML8-Bt(9;22) was also precipitated with calf serum (lane *3*); each immunoprecipitate was size fractionated by SDS/PAGE and autoradiographed. The ~43-kD band visible in lanes *1* and *2* probably represents polymerized actin.

malignant clone of the disease, while SK-CML8-BN was immortalized from the untransformed, normal B lymphocyte population. Northern blot analysis showed presence of p53 mRNA in both cell lines (Fig. 1, lanes *1* and *8*). Using metabolic labeling with [$^{32}P_i$]orthophosphate, we also detected synthesis of a phosphorylated p53 protein in both of these lines (Fig. 3, lanes *1*, *2*).

We also performed Northern blot analysis of p53 expression in fresh CML cells from three patients in myeloid blast crisis and one patient in the chronic phase of Ph^{1+} CML (Table II). We found that p53 mRNA was expressed in myeloid cells from two patients in myeloid blast crisis and one patient in chronic phase, while no p53 mRNA could be detected in cells from patient 4 (Fig. 4, lanes *1–4*). The amounts of mRNA expressed were comparable to that detected in the T-lymphocyte line S-LB1 (lane *S*). HL-60 cells expressed no p53 mRNA (lane *H*).

Analysis of Expression of p53 mRNA in Ph^{1+} Somatic Hybrids and Their Parental Lines. Somatic hybrids (Dutko, Putko) formed between K562 (myeloid, p53 mRNA nonexpressor) and one of two lymphoid lines (Daudi, PUT; p53 expres-

TABLE II

Expression of p53 mRNA in Leukemic Cells from Patients with CML

Patient		Sex	Age	Phase of CML	Ph[1]	p53 mRNA detected
Number	Name					
1	RH	M	76	Myeloid blast crisis	+	+
2	BH	F	15	Chronic	+	+
3	EL	M	35	Myeloid blast crisis	+	+
4	MR	M	49	Myeloid blast crisis	+	−

See Fig. 4 as well.

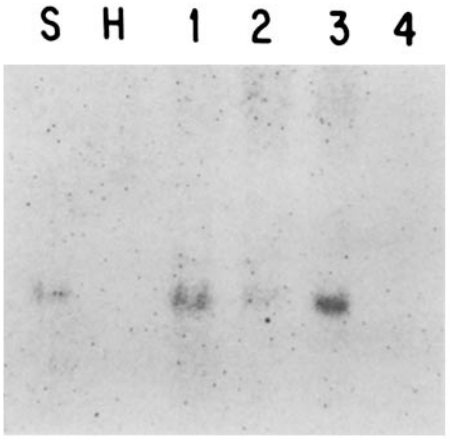

FIGURE 4. p53 mRNA accumulation in leukemic cells from patients (Pt.) with CML. Total RNA (20 μg per lane) from Pt. 1, CML myeloid blast crisis (lane *1*), Pt. 2, CML chronic phase (lane *2*), Pt. 3, CML myeloid blast crisis (lane *3*), Pt. 4, CML myeloid blast crisis (lane *4*), HL-60 (lane *H*), S-LB1 (lane *S*) was electrophoresed, transferred, and hybridized with the Nco I–Nco I fragment of the p53 cDNA clone pR4-2.

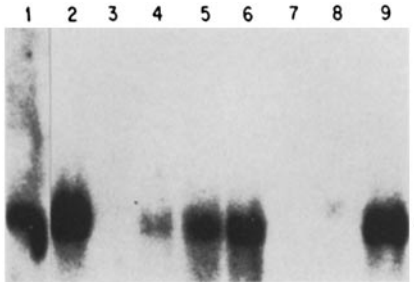

FIGURE 5. p53 mRNA accumulation in somatic hybrids between myeloid and lymphoid cells. Total RNA (20 μg per lane) from Putko (lane *1*), PUT (lane *2*), K562 (lane *3*), Dutko (lane *4*), Daudi (lane *5*), HP-1 (lane *6*), KG-1 (lane *7*), HL-60 (lane *8*), S-LB1 (lane *9*) was electrophoresed, blotted and hybridized with the Nco I–Nco I fragment of the p53 cDNA clone pR4-2.

sors) were used to study the mechanisms governing expression of p53 mRNA. In addition, we studied expression in a somatic hybrid (HP-1) between HL-60 (myeloid nonexpressor with loss of one and grossly rearranged remaining p53 allele) and PUT. The p53 mRNA was present in the lymphoid parental cells, PUT and Daudi (Fig. 5, lanes *2* and *5*; respectively), but not in the myeloid parental cells K562 and HL-60 (lanes *3* and *8*). The p53 transcripts were present in the three hybrid lines (Putko, lane *1*; Dutko, lane *4*; HP-1, lane *6*).

Analysis of p53 protein synthesis in these lines and somatic hybrids by means of metabolic labeling and immunoprecipitation showed that all lines that expressed p53 mRNA also expressed p53 protein (Fig. 6). Southern blot analysis of these cell lines using Eco RI digestion and probes detecting the 3′ and 5′ regions of the gene showed that the 3′ region of p53 was grossly intact, except

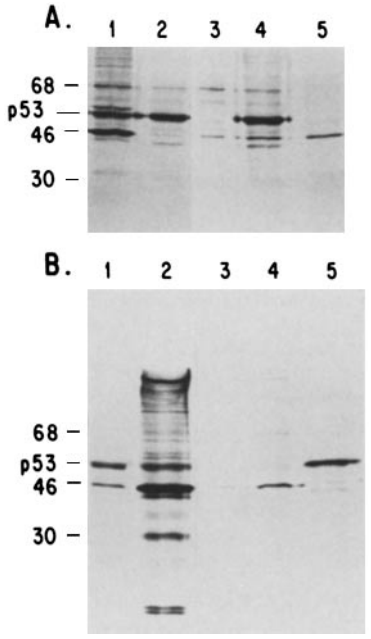

FIGURE 6. p53 protein synthesis in somatic hybrids between myeloid and lymphoid cells. (*A*) [^{35}S]methionine-labeled cells from Putko (lane *1*), PUT (lane *2*), K562 (lane *3*), HP-1 (lane *4*), HL-60 (lane *5*) were analyzed by SDS/PAGE. (*B*) Same analysis with Daudi (lane *1*), Dutko (lane *2*), HL-60 (lane *3*), K562 (lane *4*), HP-1 (lane *5*). The band at ~43 kD probably represents actin, which can polymerize during immunoprecipitation.

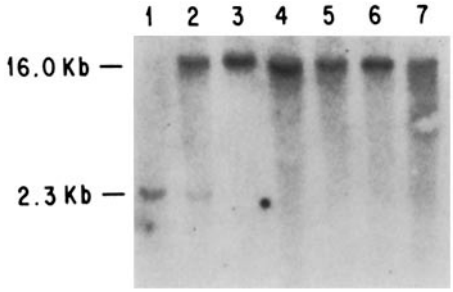

FIGURE 7. Structure of the p53 gene in p53 expressing and nonexpressing myeloid and lymphoid cells. Eco RI–digested DNA (10 μg per lane) from HL60 (lane *1*), HP-1 (lane *2*), Daudi (lane *3*), Dutko (lane *4*), K562 (lane *5*), PUT (lane *6*), Putko (lane *7*) was electrophoresed, blotted, and hybridized with the oligo-labeled p53 cDNA pR4-2. The filter was then washed, and autoradiography was performed.

for the p53 deletion found in both HL-60, as described previously (10), and HP-1 (somatic hybrid of HL-60 and PUT). Both showed a truncated 2.3-kb Eco RI fragment (Fig. 7). The somatic hybrid HP-1 also retained the normal p53 alleles from the PUT parental line as demonstrated by the the presence of the normal 16-kb Eco RI band. The 5′ region of all lines appeared grossly intact (data not shown).

Activation of the p53 Promoter Linked to an Indicator Gene (CAT) in Both a p53 mRNA Expressor and Nonexpressor Line. Gene expression can be either positively or negatively regulated by the interaction of nuclear proteins with promoter sequences (42, 43). We sought to study whether a difference could be detected in the ability of K562 (myeloid p53 nonexpressor) and BV173 (lymphoid p53 expressor) to activate the p53 promoter. To this end, two plasmid constructs (p53BXB and p53RXB) carrying p53 promoter sequences cloned in front of the CAT gene (33) were transfected into each cell type. Activation of the promoter was determined by assaying for CAT activity. The results are shown in Fig. 8. Both p53/CAT constructs showed equal activity in each of the two cell lines

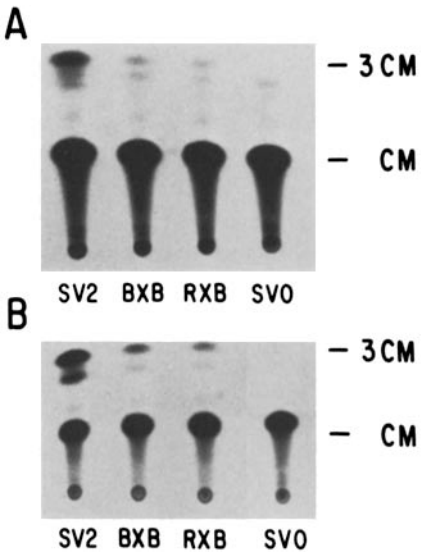

FIGURE 8. Activity of the p53 promoter in p53 expressing and nonexpressing cells. (*A*) K562 cells were transfected with pSV2-CAT (lane *1*), p53BXB (lane *2*), p53RXB (lane *3*), pSVO-CAT (lane *4*) and analyzed for CAT activity. The cells were lysed 48 h after transfection, and the cell extract was used to acetylate [^{14}C]chloramphenicol. Silica gel thin-layer chromatography was performed and the silica gel plate was autoradiographed. (*B*) BV173 cells were transfected with pSV2-CAT (lane *1*), p53BXB (lane *2*), p53RXB (lane *3*), pSVO-CAT (lane *4*) and assayed for CAT activity. *CM*, chloramphenicol; 3CM, 3-acetyl-chloramphenicol.

(p53BXB and p53RXB in BV173: panel *A*, lanes *2* and *3*, respectively; p53BXB and p53RXB in K562; panel *B*, lanes *2* and *3*, respectively). No significant difference between the strength of the two p53/CAT constructs was observed. An overall stronger CAT enzyme activity in BV173 than K562 was observed in repeated experiments and is most clearly reflected by the signal strength with pSV2-CAT (lane *1* of panels *A* and *B*).

DNA Methylation Pattern in a p53 mRNA Expressor and Nonexpressor Cell Line. The pattern of demethylated CpG dinucleotides was studied in the p53 gene of BV173 (p53 expressor, CML lymphoid line) and in K562 (p53 mRNA nonexpressor, CML myeloid line). The DNA from both lines was digested with the methylcytosine-sensitive restriction enzymes Hpa II (recognition site CCGG) and Hha I (recognition site GCGC) and with the methylcytosine-insensitive enzyme Msp I (isoschizomer of Hpa II, recognition sites CmCGG and CCGG). Hybridization of a Southern blot containing these DNAs with the 3′-specific probe pR4-2 yielded a band >23 kb that was identical in size for both lines in Hpa II and Hha I digests (Fig. 9, lanes *1–4*). Msp I digests yielded a number of small bands of identical size in both cell lines (Fig. 9, lanes *5* and *6*). When studying the 5′ portion of the gene using the clone pBT53 as probe, we detected a band of ~700 bp in K562, BV173, and S-LB1 cells after digestion with either Hpa II or Msp I (Fig. 10, lanes *1–6*) (inspection of the ethidium bromide–stained gel showed that in lane *6*, less material was run, resulting in the faint 700-bp band in that lane). Digesting the DNA of BV173, K562, and S-LB1 with Hha 1 and probing the resulting Southern blot with pBT53, we found one band of ~670 bp in each of the digests (Fig. 10, lanes *7–9*). This finding indicates the presence of four demethylated CpG sites. The orientation of the fragments and the position of the restriction sites flanking them were deduced from the published sequence (33) and from double restriction enzyme digestions. The 700-bp Hpa II/Msp I band was diminished in size with Bam HI but not Xba I, and double digestion with Hha I and Xba I yielded a smaller fragment of 325

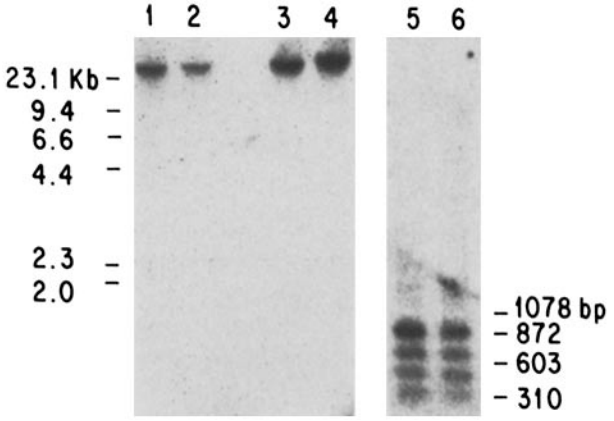

FIGURE 9. DNA methylation of the p53 gene (exons 2–11) in p53-expressing (BV173) and nonexpressing (K562) cells. DNA was digested with methylcytosine-sensitive and -insensitive restriction enzymes: K562 with Hpa II (lane *1*), BV173 with Hpa II (lane *2*), K562 with Hha I (lane *3*), BV173 with Hha I (lane *4*), K562 with Msp I (lane *5*), BV173 with Msp I (lane *6*), then electrophoresed (10 μg per lane), transferred, and hybridized to the p53 cDNA pR4-2. Molecular weight markers: Hind III–digested λ phage (*left*), Hae III–digested ØX 174 RF DNA (*right*).

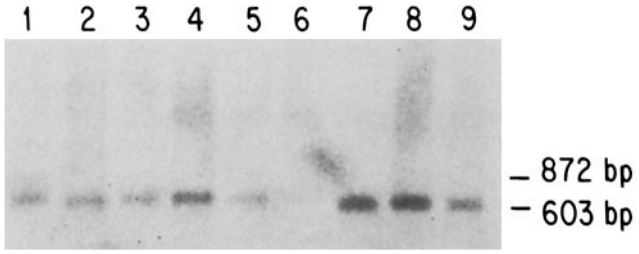

FIGURE 10. DNA methylation of the p53 gene (promoter region and exon 1) in p53 expressing (BV173, S-LB1) and nonexpressing (K562) cells. DNA was digested: S-LB1 with Hpa II (lane *1*), K562 with Hpa II (lane *2*), BV173 with Hpa II (lane *3*), S-LB1 with Msp I (lane *4*), K562 with Msp I (lane *5*), BV173 with Msp I (lane *6*), S-LB1 with Hha I (lane *7*), K562 with Hha I (lane *8*), BV173 with Hha I (lane *9*), then electrophoresed (10 μg per lane), transferred, and hybridized to the Bam HI–Xba I fragment of the p53 clone pBT53. The size marks represent two fragments of ØX 174 RF digested with Hae III.

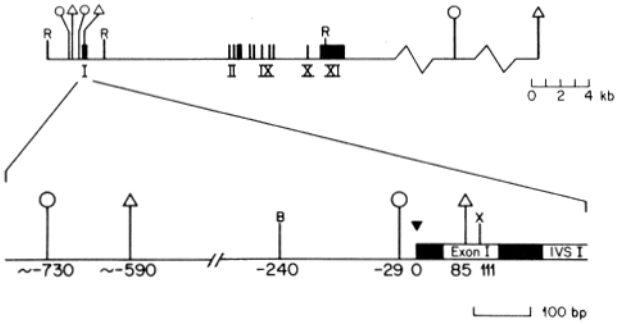

FIGURE 11. Distribution of demethylation sites in the p53 gene. (*Open circles*) demethylated CCGG sites; (*open triangles*) demethylated GCGC sites; (*R*) Eco RI restriction sites; (*B*) Bam HI restriction site; (*X*) Xba I restriction site; (*inverted solid triangle*) cap site [position 312 in (33)]. Positions of sites are given relative to the cap site.

bp (data not shown). Three of the demethylated sites map to a sequence in the promoter region starting ~730 bp upstream of the hypothetical cap site; the fourth site of demethylation is located 85 bp downstream of the cap site (Fig. 11). The methylation status of the CCGG site 155 bp downstream of the cap site

could not be determined because the fragment size of 180 bp was below our detection limit on Southern blot. Also, we did not evaluate other sites of demethylation upstream of the CCGG sequence at approximately −730 of the cap site.

Discussion

Prior studies by others (8, 9) as well as ourselves (7) have found that cells of lymphoid origin can express high levels of p53 protein, whereas cells of myeloid origin express negligible amounts of p53. In the present study we investigated the p53 expression using CML cells as a model. This malignancy originates from a single pluripotent hematopoietic stem cell that can differentiate into either lymphoid or myeloid cells. We found that all lymphoid CML lines expressed p53 mRNA and protein. None of the myeloid lines expressed detectable p53 protein; and negligible levels of p53 mRNA were found in two myeloid CML lines K562 and KCl-22, even after treatment of K562 cells with CHX, an inhibitor of protein synthesis. Accumulation of mRNA coding for other transiently expressed oncogenes and growth factors is increased when the cells are exposed to an inhibitor of protein synthesis (44, 45). Surprisingly, however, two myeloid CML lines (EM-2, EM-3) and primary leukemic cells from three of four patients with CML expressed p53 mRNA. Our present results, taken together with our prior findings, suggest that two groups of myeloid leukemic lines can be found with respect to expression of p53: those that express negligible p53 mRNA and protein (e.g., K562, KCl-22, KG-1, ML-3, U937, A7) and those that accumulate p53 transcripts but no protein (EM-2, EM-3). Possibly, cells belonging to the latter group synthesize an antigenically altered p53 protein that is not recognized by the PAb421 mAb. This phenomenon has been described in HeLa cells (46). The appearance of p53 in myeloid cells might mean that expression of the gene is not regulated normally in CML.

To study the regulatory mechanisms responsible for p53 expression, we examined two somatic hybrid lines that were the fusion products of K562 (myeloid, p53 RNA nonexpressor) and either of two lymphoid lines (p53 expressors). Our results indicate that p53 expression is a dominant feature, with the somatic hybrids expressing both p53 mRNA and protein. This suggests that lack of p53 expression in K562 is probably not the result of an inhibitory *trans*-acting protein.

We also studied the ability of the p53 mRNA expressor and nonexpressor CML lines to activate the p53 promoter attached to the CAT gene. Two plasmid constructs containing 350 bp and 2.4 kb of the 5′ p53 region (part of exon 1 and 5′ flanking sequences, both shown to contain the p53 promoter [33]) were transfected into K562 (myeloid, p53 nonexpressor) and BV173 (lymphoid, p53 expressor). We found that both of these constructs were about equally active in BV173 and K562, indicating that the lack of p53 expression by K562 was not because of either the lack of a positive or presence of a negative *trans*-activator of p53.

The distribution pattern of demethylated CpG dinucleotides is a feature of genes that is correlated with their expression (47, 48). In DNA of vertebrates, cytosine is methylated up to 90% of the time, and this modification is almost

always found in the sequence mCpG. Demethylation of this sequence at certain sites in the gene may be necessary for gene expression; and for a number of tissues, this demethylation occurs in genes actively expressed, whereas the same sites are methylated in nonexpressing tissues (47). For many genes, however, no differences in the methylation pattern between expressing and nonexpressing tissues have been detected. Of these, so-called "housekeeping genes" (genes expressed in a variety of tissues often at a variety of stages of differentiation) have a specific distribution pattern of methylated and demethylated cytosine/guanine dinucleotides (CpGs) (48). Sites of demethylation are clustered in a CpG-rich 5′ region of an otherwise hypermethylated and CpG-poor gene (48, 49). These clusters have been termed Hpa II Tiny Fragments (HTF) islands (49). Our analysis showed no difference in the methylation pattern of BV173 (lymphoid, p53 expressor) and K562 (myeloid, p53 nonexpressor). Furthermore, we found that the p53 gene is highly methylated over its entire coding region but demethylated at least in four sites in and 5′ of the noncoding first exon. This pattern of hypomethylation is reminiscent of a HTF island in a housekeeping gene. We have found the same pattern of methylation in another system of differential p53 expression, quiescent (p53 mRNA nonexpressor) and actively proliferating peripheral blood T lymphocytes (p53 expressor) (manuscript in perparation).

Based on the notion that p53 and c-*myc* may have similar functions, and the observation that HL-60 cells lack p53 expression but exhibit c-*myc* amplification, Wolf and Rotter (10) speculated that lack of p53 expression may be compensated by overexpression of c-*myc*. Our results are consistent with this notion. All myeloid lines lacking p53 protein synthesis (as detected by PAb421) strongly express c-*myc* mRNA, while all lymphoid lines express p53 protein but negligible amounts of c-*myc* mRNA. Cells of none of the lines lack expression of both p53 and c-*myc* (see Table I).

Summary

The p53 is a nuclear protein that is associated with normal cellular proliferation and can cooperate with Ha-*ras* in causing cellular transformation in vitro. Lineage association is known to exist between p53 expression and normal lymphopoiesis, but not myelopoiesis. We studied the expression of p53 using chronic myelogenous leukemia (CML) cell lines, somatic hybrids of these cells, and leukemic cells from CML patients. Lymphoid CML lines expressed both p53 mRNA and protein. We also analyzed p53 synthesis by two B-lymphoid lines from the same CML patient; cells of one line were derived from the neoplastic clone, cells of the other were derived from the normal clone. Both synthesized equal amounts of a phosphorylated p53 protein. None of the myeloid CML lines expressed detectable p53 protein and two of four expressed negligible p53 mRNA. Two other myeloid CML lines and myeloid cells from three of four patients expressed p53 mRNA. These findings suggest that expression of the gene is not regulated normally in CML. Several approaches were pursued to explore the differential expression of p53. Southern blot analyses showed no gross alterations in the p53 gene from cells of either the expressing or the nonexpressing lines. No difference in the pattern of demethylated CpG sites was noted in the region of the p53 gene in cells from K562 (myeloid p53 nonexpressor) and in BV173 (lymphoid

p53 expressor). The sites of demethylation clustered in and around the p53 promoter in both cell lines. Somatic hybrids formed between a p53 mRNA nonexpressor myeloid line (K562) and the parental p53 expressor lymphoid lines (Daudi, PUT) produced p53 mRNA and protein, suggesting that p53 is a dominantly expressed protein and that lack of expression in myeloid cells is not mediated by a *trans*-acting negative regulatory protein. DNA transfection experiments performed using the indicator gene chloramphenicol acetyltransferase attached to promoter sequences of p53 showed that these constructs were equally activated in BV173 (p53 expressor) and K562 (p53 mRNA nonexpressor). The mechanism of p53 regulation in CML remains unclear.

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