

Alteration and Abnormal Expression of the *c-myc* Oncogene in Human Multiple Myeloma

By Peter Selvanayagam, Mark Blick, Franco Narni, Peter van Tuinen, David H. Ledbetter, Raymond Alexanian, Grady F. Saunders, and Bart Barlogie

Structural alterations of the *c-myc* oncogene in human Burkitt's lymphoma and mouse plasmacytoma suggest that this oncogene is involved in several B cell neoplasms. The possibility of *c-myc* alterations in human myeloma has not been explored, probably because the low proliferative activity characteristic of this tumor impairs the propagation of representative cell lines for the performance of adequate cytogenetic studies. This report describes alterations in the *c-myc* locus with concomitant elevated expression of mRNA in the tumor cells of two of 37 patients with multiple myeloma. In one case, somatic cell hybrid studies

revealed that the cloned rearranged DNA was entirely derived from chromosome 8, thus indicating a novel mechanism of *c-myc* activation different from that in Burkitt's lymphoma. Seven other patients exhibited five- to 12-fold overexpression of *c-myc* RNA when compared with normal marrow cells. Elevated mRNA expression in about one fourth of our patients suggests that the *c-myc* oncogene has a pathogenetic role in the evolution of multiple myeloma.

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MULTIPLE MYELOMA is a neoplasm of differentiated B (plasma) cells that is characterized by monoclonal immunoglobulin (Ig) production.¹ There is growing evidence that cellular oncogenes are altered or overexpressed in several human malignancies, although their role in neoplastic transformation remains to be determined.² Oncogenes are activated by several mechanisms, most notably by chromosomal translocation, as has been observed frequently in human lymphoma and leukemia.³⁻⁵ Similar data are not available for myeloma, probably because no consistent chromosomal anomalies have been found.⁶ Myeloma plasma cells typically have a low proliferative activity that renders karyotypic studies difficult, and only a few representative cell lines have been established.⁷⁻⁹ Yet, an unequivocal increase in the nuclear DNA content of plasma cells has been observed in 80% of patients.¹⁰ We analyzed the structure and expression of the *c-myc* oncogene in myeloma plasma cells because *c-myc* is involved frequently in other human and animal B cell neoplasms.^{11,12} We report here alterations in the *c-myc* gene locus with a concomitant elevated expression of mRNA in the tumor cells of two of 37 patients. Seven other myeloma samples also showed an abnormally high expression of *c-myc* mRNA without alteration in gene structure. These data indicate that the *c-myc* oncogene is involved in human myeloma.

From the Departments of Hematology, Biochemistry and Molecular Biology, and Clinical Immunology and Biological Therapy, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute; and the Institute for Molecular Genetics, Baylor College of Medicine, Houston.

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Address reprint requests to Bart Barlogie, MD, Department of Hematology, University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, 1515 Holcombe Blvd, Houston, TX 77030.

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MATERIALS AND METHODS

Bone marrow aspirates were studied in 37 patients, in eight at the time of diagnosis and in 29 during later advanced stages of disease. Informed consent was obtained before the bone marrow aspiration procedures. The degree of marrow plasmacytosis was usually marked (median, 55%). Myeloma protein was phenotyped by immunoelectrophoresis, and cytogenetic studies were conducted on several samples (Table 1). Bone marrow cells and placental tissue from normal healthy individuals served as controls. HL-60 cells with abundant *c-myc* mRNA were used as a positive control for RNA expression studies. Informed consent was obtained before bone marrow aspiration procedures.

Nucleic acid analysis. High-molecular weight DNA and total RNA were coextracted from bone marrow cells by the method of Erisman et al.¹³ DNA was digested with restriction enzymes as recommended by the manufacturer. Size fractionation in agarose gel and transfer to nitrocellulose filter were carried out by the method of Southern.¹⁴ For Northern blot analysis, 20 µg of total RNA was denatured in 1 mol/L glyoxal, 50% dimethylsulfoxide, and 10 mmol/L phosphate buffer, pH 7.0, at 50°C for one hour; size-fractionated in 1.2% agarose gel; and transferred to nitrocellulose filter by the method of Thomas.¹⁵ Hybridization of the DNA and RNA filters with ³²P-labeled gene probes and subsequent washing were carried out according to the method of Maniatis et al.¹⁶ Autoradiography on x-ray films was performed at -70°C for various durations. Gene expression was quantitated by densitometric scanning of autoradiograms.

Isolation of the rearranged gene. Tumor DNA digested with *Hind* III enzyme was fractionated in a sucrose density gradient, and the fraction enriched for the rearranged *myc* fragment was cloned into λ2001 phage vector.¹⁷ Screening of the library and purification of the recombinant phage were performed by published protocols.¹⁸

Somatic cell hybrid analysis. Construction and analysis of Chinese hamster-human and mouse-human somatic cell hybrids have been reported previously.^{19,20} G-banding analysis was performed on at least 20 cells in each hybrid and was followed by sequential G-11 staining of selected G-banded cells. Chromosomes were scored as absent if they were found in less than 20% of the cells scored.

Gene probes. The *c-myc* probe used in our experiments is a 1.6 kilobase (kb) *Cl*a I-*E*co RI fragment (MC 41'3rC) consisting of the third exon and the 3' flanking sequences of the human *c-myc* gene.²¹ To detect structural abnormalities at the 5' portion of *myc*, we used a 0.8-kb *Pvu* II fragment consisting of the first exon and the 5' flank of the *c-myc* gene.²² Immunoglobulin gene rearrangements were examined with human J_H,²³ S_μ,²⁴ C_μ,²⁴ C_{α1},²⁵ C_{γ4},²⁶ C_κ,²⁷ and C_λ²⁸ probes. For determining the proliferative activity, an S-phase-specific gene encoding histone H3 was used.²⁹

Table 1. Clinical, Cytogenetic, and Molecular Features in 37 Patients With Multiple Myeloma

No.	% PC	c-myc		Ig			M Protein	Chromosome 8 Anomalies
		RNA Expression ($\pm 15\%$)	DNA Rearrangement	J _H Rearrangement	L Rearrangement			
1	76	10	+	+	κ		A ₂	+8
2	10	10	+	+	κ		G ₂	ND
3	65	12	—	+	λ		A ₂	t(8;14)(q24;q32)
4	90	10	—	+	κ		A ₂	—8
5	21	8	—	+	λ		A ₂	Normal
6	26	8	—	+	κ		A ₂	Normal
7	21	8	—	+	κ		A ₂	Normal
8	86	6	—	+	κ		G ₂	ND
9	43	5	—	+	κ		G ₂	Normal
10	48	1	—	+	κ		A ₂	t(8;14)(q24;q32)
11	73	1	—	+	κ		G ₂	+8; +8
12	48	1	—	+	κ		A ₂	der(8);t(8;7)(q24;?)
13	80	1	—	—	nd		$\phi\lambda$	der(8);t(8;7)(q22;?)
14	77	1	—	+	κ		A ₂	der(8); —8;
								t(1;8)(p11;p23)
15	44	1	—	+	λ		A ₂	—8
16-25	>10	1	—	+, 8; —, 2	k,6; λ ,1; nd, 3		A ₂ , 1; A ₂ ,2; G ₂ ,5; BJP,2	Normal
26-37	>10	1	—	+	k,8; nd, 2; ND, 2		A ₂ ,4; G ₂ ,4; G ₂ ,4	ND

Abbreviations: X, -fold increase over normal marrow; nd, not detected; ND, not done; BJP, Bence Jones protein; % PC, plasma cells.

RESULTS

Analysis of Ig (and more recently of T cell receptor) gene rearrangement has helped determine the clonality and differentiation stage in lymphoproliferative disorders.^{23,30} When applied to bone marrow samples from 37 myeloma patients, discrete rearrangements of Ig heavy- and light-chain genes were observed in most cases and corresponded to immunoelectrophoretic results (Table 1 and Fig 1). The autoradiographic signal intensity of rearranged Ig gene bands corresponded with the degree of bone marrow plasmacytosis.

For *c-myc* gene analysis, DNA was digested with *Bam* HI, *Eco* RI and *Hind* III restriction enzymes and hybridized to the probe MC 41'3rC. One of the 37 samples (myeloma 1), digested with *Hind* III, showed a rearranged 14-kb *c-myc* fragment as well as the germline 11.6-kb band. This finding was corroborated by additional analysis with *Kpn* I and *Bgl* II, which revealed rearranged *myc* fragments of 15 and 9 kb in size, respectively (Fig 2A). Further analysis of the gene locus by using additional restriction enzymes localized the aberration to a site between the *Xba* I and *Bgl* II at the 3' flanking region of the gene (Fig 2B). In comparison with their germline counterparts, the rearranged *myc* fragments always exhibited a reduced signal intensity (Fig 2A). The germline bands had the same size as those from normal human placental DNA analyzed in parallel (data not shown).

To examine whether the rearranged *myc* DNA in myeloma was associated with Ig genes frequently observed in other B cell malignancies,¹¹ filters were rehybridized to several Ig heavy- and light-chain gene probes. As seen in Fig 3, the rearranged *myc* fragment did not react with C_H and J_H as well as S_H, C_{H1}, C_{H2}, C_{H3}, and C_{H4} probes (data not shown), thereby implying a novel rearrangement of *c-myc* in myeloma 1. To confirm this, the novel DNA situated at the 3' flank of the altered *myc* was isolated by means of cloning into a phage vector and probed with a panel of hamster-human

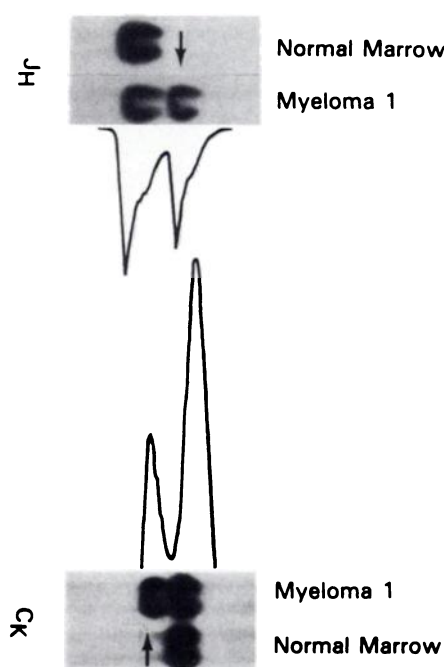


Fig 1. Detection of monoclonal plasma cells in the bone marrow of a patient with IgA κ myeloma. *Bgl* II- and *Bam* HI-digested DNA from myeloma 1 was hybridized to ³²P-labeled J_H and C_κ probes which detected 5.5-kb and 10.5-kb rearranged Ig heavy- and κ light-chain gene bands, respectively (arrows). The probes also revealed 4.2-kb heavy-chain and 12-kb κ light-chain germline gene bands, probably from the unrearranged alleles of the 75% plasma cells and normal hemopoietic cells. Hybridization to the C_λ probe revealed a germline configuration (not shown). DNA from bone marrow cells of a healthy donor was used as control.

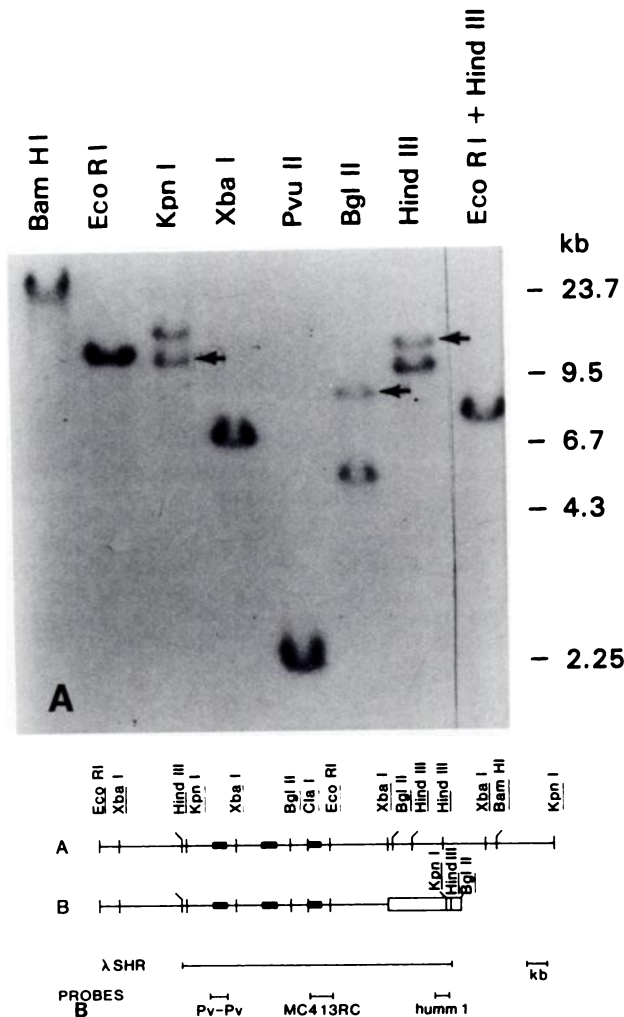


Fig 2. (Top) Southern blot analysis of myeloma 1 DNA with *c-myc* probe MC 41'3rC. *Kpn* I, *Bgl* II, and *Hind* III restriction enzyme digestions revealed a rearranged *c-myc* fragment (arrows) with reduced signal intensity when compared with germline bands. No rearranged *c-myc* band was detectable after *Bam* HI digestion, probably reflecting a similar or large DNA fragment size. The germline fragments resulting from digestion with different enzymes matched the sizes of control human placental DNA analyzed in parallel (not shown). (Bottom) Partial restriction map of the human germline *c-myc* gene (A) and the rearranged *myc* DNA from myeloma 1 showing the alteration in the 3' flanking region of the gene (B; open bar). The location of the probes used is indicated. λ SHR is the cloned *Hind* III fragment.

and mouse-human somatic hybrid cell DNA. As seen in Table 2, the probe *humm* 1 isolated from the novel sequences in myeloma 1 (Fig 2B) was localized to chromosome 8 with 100% concordance. The probe hybridized to an 18-kb human DNA fragment generated by *Bam* HI. The probe also hybridized to a 23-kb mouse band, whereas hamster DNA failed to react with this probe (Fig 4).

In a second more recently studied case (myeloma 2), the tumor DNA revealed additional *myc* fragments 21, 5, and 10 kb in size after digestion with *Bam* HI, *Eco* RI and *Kpn* I, respectively (Fig 5). Due to a shortage of material, detailed characterization of this rearrangement by using primary tumor DNA was not possible.

When DNA was digested with *Pvu* II enzyme and hybridized to the first exon-specific *myc* probe,²² all 37 samples exhibited a germline configuration indicating the absence of structural changes characteristic of endemic Burkitt's lymphoma. There was no gene amplification evident in any of these other samples investigated.

The two cases with *myc* DNA rearrangement showed increased transcriptional activity on Northern analysis (Fig 6A). Seven other patients, or a total of 24%, also exhibited higher *myc* RNA expression in comparison with that of normal bone marrow (Fig 6B). RNA overexpression ranged from five- to 12-fold using slot blot analysis (Table 1). Bone marrow cells from a healthy individual were chosen as reference tissue in the absence of significant quantities of normal plasma cells. Elevated *c-myc* expression in our myeloma samples was not proliferation related because the expression of the S-phase-specific histone H3 gene was consistently lower in myeloma samples compared with normal bone marrow (Fig 6C). Although (8;14) translocations have recently been observed in human myeloma,³¹ including our two cases (see Table 1), high *myc* expression was also noted with other chromosome 8 abnormalities; conversely, only one of the two cases with t(8;14) translocations was associated with elevated *myc* transcriptional activity. Although there was no apparent clinical association with increased *c-myc* expression, the IgA myeloma protein type was more frequent among such patients in comparison with the remaining 28 individuals with low *myc* gene activity (67% v 39%, $P = .29$).

DISCUSSION

There is growing evidence that cellular oncogenes are altered or amplified in several human malignancies. The *c-myc* oncogene, which is closely associated with Burkitt's lymphoma,³ is also altered in other human B cell tumors.³² In human plasma cell myeloma, however, gross alterations of the *myc* gene were infrequent, with only two of 37 patients showing rearrangement within the 30-kb region analyzed. Sumegi et al³³ found no alteration of the *c-myc* locus among 21 patients with myeloma.³³ Recently, Gazdar et al observed *c-myc* gene rearrangement in a myeloma cell line that they considered responsible for maintaining continuous growth in culture.⁹ Interestingly, this cell line as well as six of our nine patients with elevated *myc* expression secreted IgA myeloma protein (Table 1). Similarly, recent cytogenetic studies from this laboratory revealed that all of four t(8;14) anomalies were associated with an IgA phenotype.³¹

The *c-myc* gene alteration in myeloma 1 with 75% marrow plasmacytosis appeared to be tumor specific because the hybridization signal intensities of the rearranged *myc* fragments (and of the rearranged Ig gene bands) were consistently less than those of their germline counterparts (Figs 1 and 2). This observation ruled out the possibility of a constitutive *c-myc* aberration and indicated involvement of only one allele. The 5' portion of the *myc* gene, which is frequently altered in endemic Burkitt's lymphoma, was intact in myeloma. In contrast to similar alterations in a few variant Burkitt's lymphomas¹¹ and mouse plasmacytomas³⁴ with chromosomal translocations involving Ig light-chain genes, alteration at the 3' flank of the *myc* gene from

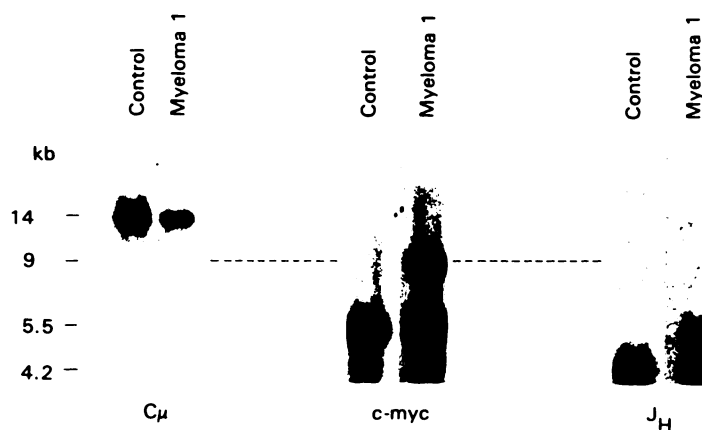


Fig 3. Cohybridization analysis demonstrating the lack of Ig gene elements within the rearranged *c-myc* fragment in myeloma 1. The filter containing *Bgl* II-digested DNA was hybridized to the *c-myc* probe MC41'3rC and revealed a 9-kb altered *myc* fragment (center). After the *c-myc* probe was stripped from the filter, the filter was rehybridized to *C_μ* (left) and *J_H* (right) probes. Similar negative results were obtained after hybridization to the *S_μ*, *C_{α1}*, and *C_{α2}* subunits of the Ig heavy-chain gene as well as the *C_κ* and *C_λ* light-chain gene probes (not shown). The less intense germline *C_μ* signal is probably due to deletion of the *C_μ* region during B cell maturation of myeloma plasma cells. Equal amounts of placental DNA from a normal individual were used as control.

Table 2. Somatic Cell Hybrid Mapping of the *hum* 1 Probe

Hybrid	Probe	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
C ₁ 1A	—	—	—	+	+	+	+	—	—	—	—	+	+	—	+	+	+	+	+	—	—	+	+	+	—
1.4	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—	+	—	—	+	—	+	—	+	—
8.2	+	—	+	+	+	—	—	+	+	—	+	+	—	—	+	+	+	—	—	—	+	—	—	+	—
16.1	—	—	+	+	+	+	+	—	—	—	—	+	—	—	—	—	+	—	+	+	—	—	+	—	+
MR2.2	—	—	—	+	—	—	—	+	—	—	+	+	—	+	—	—	—	—	—	—	+	—	+	+	—
MR7.11	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—	—	—	+	—	—	—	+	+	—
MR1.21	—	—	—	—	—	—	+	+	—	—	—	—	+	—	+	—	—	—	—	—	—	—	—	+	—
SA-5	—	—	—	—	—	—	—	+	—	—	—	—	+	—	—	+	—	+	—	—	+	—	—	—	—
MH-18	+	—	—	+	—	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	—	+	+	—	—

*This hybrid contains the translocation chromosome 15qter → 15q15:17p13 → 17qter.

†This hybrid contains the translocation chromosome 9qter → 9q12:17p11 → 17qter.

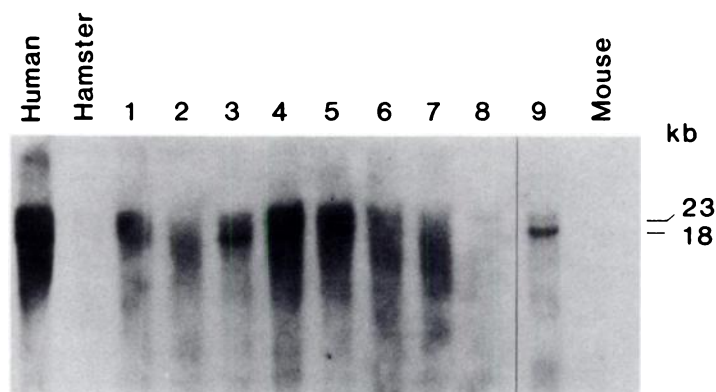


Fig 4. High-molecular weight DNA from hamster-human and mouse-human somatic cell hybrids was digested with *Bam* HI and transferred to nitrocellulose filter. Hybridization conditions with the *hum* 1 probe was as described in Materials and Methods. The size of the human and mouse fragments hybridizing to the probe was determined by comparison with standard size markers resolved in parallel. Lanes 1 to 9 contain DNA from somatic cell hybrids described in Table 2. Discrete bands reacting with the *hum* 1 probe can be detected in lanes 3 and 9.

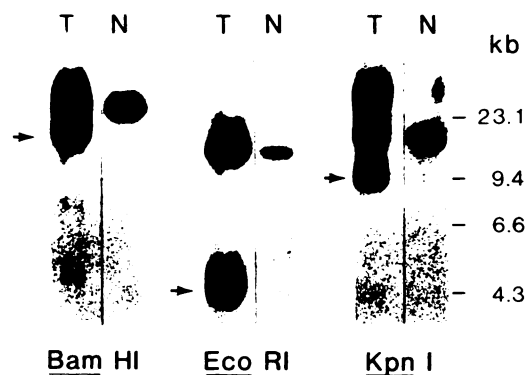


Fig 5. Detection of *c-myc* gene alteration in myeloma 2. DNA from the bone marrow cells of the patient (T) and of a healthy donor (N) was analyzed with the probe MC41'3rC by methods described earlier. Rearranged *myc* fragments in the patient's DNA are indicated by arrows.

myeloma 1 did not show cohybridization with either light- or heavy-chain gene probes. Instead, somatic cell hybridization experiments revealed that the cloned rearranged DNA originated entirely from chromosome 8, thus suggesting inversion or deletion as a possible novel mechanism of *myc* gene deregulation. Similar alterations at the 3' portion of the *myc* gene, associated with enhanced transcriptional activity, have been reported in T cell leukemia lines.^{35,36} Enhancer elements are capable of controlling gene transcription in an orientation-independent manner over long distances,³⁷ and the possibility that the novel DNA isolated from the 3' part of the rearranged *myc* gene in myeloma 1 has enhancer properties is being investigated. A second case of *myc* gene alteration with associated RNA overexpression likewise revealed a lack of Ig gene involvement. The high *myc* RNA expression in the absence of DNA rearrangement could be due to alterations at large distances from the analyzed area.³⁶ The occurrence of elevated *c-myc* expression in about one fourth of our patients with or without demonstrable gene alterations indicates a possible pathogenetic role of the *c-myc* oncogene in multiple myeloma.

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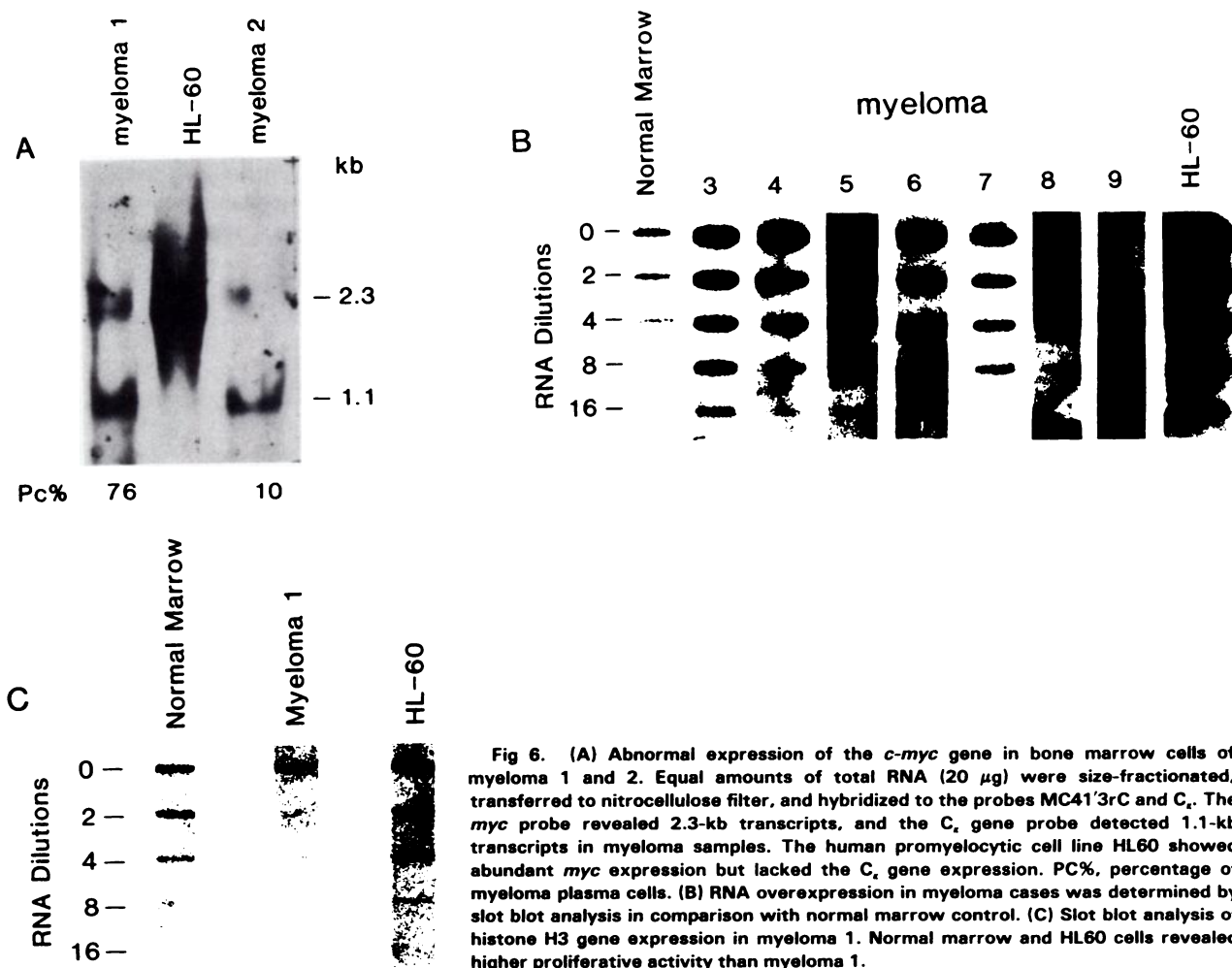


Fig 6. (A) Abnormal expression of the *c-myc* gene in bone marrow cells of myeloma 1 and 2. Equal amounts of total RNA (20 μ g) were size-fractionated, transferred to nitrocellulose filter, and hybridized to the probes MC41'3rC and C_α . The *myc* probe revealed 2.3-kb transcripts, and the C_α gene probe detected 1.1-kb transcripts in myeloma samples. The human promyelocytic cell line HL60 showed abundant *myc* expression but lacked the C_α gene expression. PC%, percentage of myeloma plasma cells. (B) RNA overexpression in myeloma cases was determined by slot blot analysis in comparison with normal marrow control. (C) Slot blot analysis of histone H3 gene expression in myeloma 1. Normal marrow and HL60 cells revealed higher proliferative activity than myeloma 1.

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