stimulated to proliferate (*30*). Expression of the gene appears to be coupled with DNA replication since inhibition of DNA synthesis results in a rapid loss of hybridizable cellular H1 histone mRNA's. However, because of the heterogeneity of H1 histone proteins and the degeneracy of the genetic code, not all H1 histone mRNA's may have been detected by this H1 histone gene hybridization probe. Therefore, it would be premature to conclude that the pattern of expression observed for this gene is a general feature of all human H1 sequences. A more complete understanding of the structural and functional properties of human H1 histone genes and their relation to the organization and expression of core histone sequences must await isolation and characterization of additional H1 genes.

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Amplification of the c-*myb* Oncogene in a Case of Human Acute Myelogenous Leukemia

Abstract. *Amplification is one of the mechanisms by which cellular oncogenes may be altered in their function, possibly leading to neoplastic transformation. The oncogenes* c-myc, c-abl, *and* c-*Ki*-ras *are amplified in several different human neoplasias. The oncogene* c-myb, *which is specifically expressed and regulated in hematopoietic cells, was found to be amplified in cell lines ML-1, ML-2, and ML-3, which were separately cultured from cells of a patient with acute myelogenous leukemia (AML). A five- to tenfold amplification was correlated with high levels of expression of normal size* c-myb *messenger RNA and with chromosomal abnormalities in the region 6q22–24, where the* c-myb *locus is normally located. Amplification and cytogenetic abnormalities were detected in DNA's from primary and secondary cultures of ML cells, suggesting that they may have contributed to leukemogenesis. The similar AML cell lines HL-60 and ML's contain different amplified oncogenes:* c-myc *and* c-myb, *respectively. Alternative activation of structurally and possibly functionally similar oncogenes may distinguish—at the pathogenetic level—phenotypically similar tumors.*

Structural or regulatory alterations of cellular oncogenes appear to be associated with several human tumors (*1, 2*). Qualitative changes—that is, point mutations—have been found by isolating oncogene alleles that act in transforming NIH/3T3 fibroblasts in transfection assays in vitro (*2*). Alternatively, chromosomal translocations involving cellular oncogenes have been found in a number of different tumors and are likely to cause changes in normal oncogene regulation (*3*). Gene amplification represents an additional event that could influence oncogene expression. This mechanism has been shown for various genetic loci (*4*) and more recently has been reported to involve different oncogenes in several human or mouse tumors (*5–11*). Examples of oncogene amplification include c-*myc* amplification in an acute promyelocytic leukemia cell line (*5, 6*), in neuroendocrine tumors (*7*), and in small cell lung cancer cell lines (*8*); c-Ki-*ras* amplification in a neuroendocrine tumor (*9*); c-*abl* amplification in an erythroleukemia cell line (*10*); and c-N-*myc* amplification in neuroblastoma cell lines (*11*).

Although a causative link between oncogene amplification and the pathogenesis of these neoplasias has not been conclusively shown, the same genes that are found amplified are clearly associated with transformation when activated by mechanisms other than amplification. For example, the c-*myc* oncogene is activated by promoter-enhancer insertions in chicken B-cell lymphomas (*12*) and by chromosomal translocations in murine and human B-cell lymphomas (*13*). Different mechanisms, including amplification, may result in analogous regulatory alterations in development or maintenance (or both) of the malignant phenotype. Determining which oncogenes can regularly or occasionally be amplified in certain tumors may aid in the development of a functional classification of oncogenes and of molecular pathways in different tumors.

In view of the finding of c-*myc* oncogene amplification in a few cases of human myelogenous leukemia (*6*), we investigated another oncogene, c-*myb*, for amplification in human hematopoietic neoplasias. This gene has structural and possibly functional similarities to the c-*myc* gene (*14*), is specifically expressed in hematopoietic cells (*15*), and appears to be tightly regulated during myeloid cell differentiation and proliferation (*15*). We now report that the c-*myb* oncogene is amplified in a case of human acute myelogenous leukemia, both in the independently derived cell lines ML-1, ML-2, and ML-3 and in the primary cells

from which these cell lines were derived.

The c-*myb* oncogene is homologous to the transforming gene (v-*myb*) of avian myeloblastosis virus (AMV) (*16*). The human locus has been mapped to the region q22–24 of chromosome 6 (*17*) and the gene has been cloned and preliminarily characterized (*18*). A 4.5-kilobase (kb) c-*myb* messenger RNA (mRNA) appears to be specifically expressed in hematopoietic cells (*15*) and regulated during myeloid differentiation, since its transcription is turned off when the human promyelocytic cell line HL-60 is induced to terminal granulocytic differentiation in vitro (*15*).

We analyzed the c-*myb* locus in a number of fresh and cultured acute myelogenous leukemia (AML) cells. The cultured cells include cell lines HL-60 (*19*), ML-1, ML-2, and ML-3 (*20*). The ML cells represent three different lines independently originated from peripheral blood leukemia cells of the same AML patient (*20*). Although the HL-60 cell line is usually described as a promyelocytic leukemia (APL) line (*19*) and the ML lines are described as APL/AML lines (*20*), these lines are quite indistinguishable by cytochemical and immunophenotypical analysis (*20*). Thus, in this context, we describe them with the more general term of AML lines. DNA from these cell lines, from four other fresh AML samples, and from normal peripheral blood lymphocytes was digested with Hind III restriction endonuclease and analyzed by the Southern blot hybridization technique (*21*) with c-*myb*–specific probes (Fig. 1A).

Representative results (Fig. 1B) indicate that the intensity of the 7.1-kb band, corresponding to the central portion of the gene, is markedly increased in all the DNA's from ML cells. The increased intensity can be demonstrated by comparison with the same band in other DNA's and with an internal single-copy-gene control represented by the 11.6-kb band generated by hybridization to the normal c-*myc* locus (*22*). Amplification of this gene is detectable in the HL-60 genome (*5, 6*). Analogous results were consistently obtained with early and late passages of ML-1, ML-2, and ML-3 lines and also with primary and secondary cell culture passages. The latter were frozen before the establishment of the ML-1 cell line (see Fig. 1B for representative results). Although uncultured leukemic cells taken directly from the peripheral blood or bone marrow of this AML patient were not available, our results suggest that the c-*myb* gene was amplified in the original malignant clone (see below for further discussion on this point).

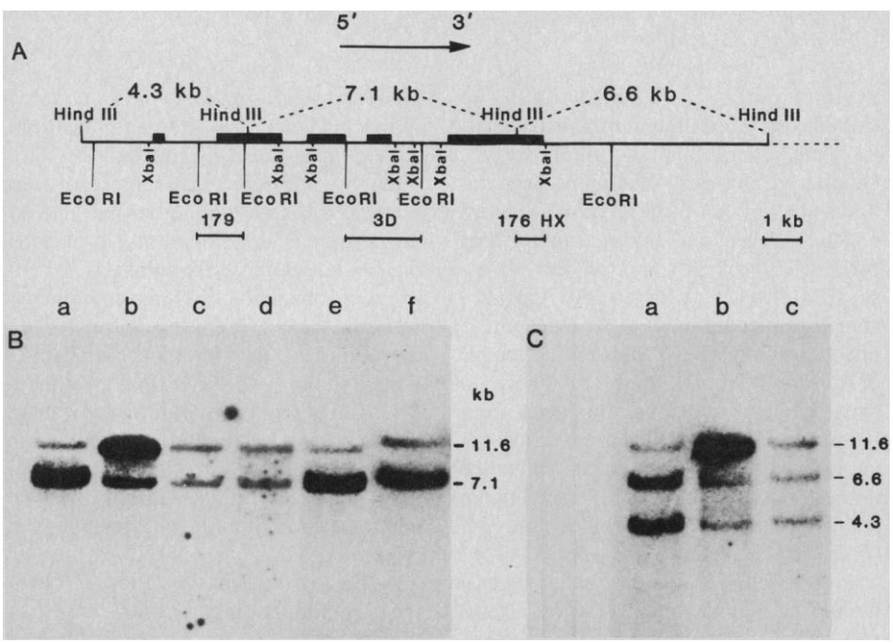

Fig. 1. Genomic organization of the human c-*myb* locus and amplification in ML cells. (A) Diagrammatic representation of the human c-*myb* locus with restriction enzyme sites, as derived from (*18*). Black boxes indicate the approximate position of putative exons identified by their homology with the v-*myb* oncogene of AMV (*18*). The c-*myb* probes used for these studies are shown below the map. Their construction has been described (*18*), except for that of probe 176 HX, which is derived from the recombinant plasmid p176 (*18*) by isolation of the 0.8-kb Hind III–Xba I fragment. The respective size and position of the three Hind III fragments is indicated on top. (B) Amplification of the c-*myb* locus in ML DNA's. DNA (10 μg) was extracted from (lane a) ML-3 cells, (lane b) HL-60 cells, (lane c) AML primary cells (case 34), (lane d) normal human peripheral blood lymphocytes, (lane e) ML-2 cells, and (lane f) primary and secondary cells from ML-1. Each DNA sample was digested with Hind III restriction endonuclease and analyzed by the Southern blot hybridization technique (*21*). The recombinant plasmids p3D (7.1-kb c-*myb* band) and pMC413RC (11.6-kb c-*myc* band) (*6*), labeled by nick translation (*31*), were hybridized in the same experiment. (C) DNA's as indicated in (B) were analyzed with probes p179 (4.3-kb band) and p176 (6.6-kb band); pMC413RC was again used as a probe for the c-*myc* gene.

The degree of c-*myb* amplification by determining the size of the amplification unit with respect to the c-*myb* gene and by quantifying the degree of amplification. The first determination was performed by using 5′- and 3′-specific c-*myb* probes, which, in combination with Hind III restriction endonuclease digestion, allowed us to explore the entire c-*myb* gene (as defined by v-*myb* homologous regions) plus 3′ and 5′ flanking sequences (*18*). With these probes, amplification was detectable in both the 5′ (4.3 kb) and 3′ (6.6 kb) Hind III fragments in ML-3 DNA (Fig. 1C) and in all the ML DNA's tested (not shown). The human c-*myc* locus was again used as an internal control. Although the exact borders of the human c-*myb* locus have not yet been defined (*18*), these results indicate that the entire c-*myb* gene, as defined by v-*myb* homologous regions, is amplified. The size of the amplification unit is larger than the 18-kb DNA segment explored by Hind III digestion, since no rearrangement was detectable within these borders.

The degree of c-*myb* amplification in ML cells was quantified by dilution experiments. Hind III–digested DNA (10 μg) was sequentially diluted, and the intensity of the hybridization band was compared with that obtained with an equivalent amount of normal human DNA. Hybridization to a probe derived from the human c-*myc* locus was again used as a control in the same experiment (Fig. 2). A five- to tenfold dilution is necessary to bring the c-*myb* band to the same apparent level as the control DNA, which indicates a five- to tenfold amplification of the c-*myb* gene in ML cells. Similar estimates were obtained by densitometric scanning of x-ray films of the same experiments (Fig. 2B). The degree of amplification was consistent among different ML lines.

The effects of amplification on *myb* mRNA expression were also studied in ML cells by Northern blot hybridization (*23*). Since a suitable normal control (normal myeloblast at the same stage of differentiation) is not available, *myb* mRNA blots of ML-3 cells were compared with those of HL-60 cells which are phenotypically similar (AML) but do not contain an amplified c-*myb* oncogene. As additional controls we included malignant cells of different hematopoiet-

ic lineage (Daudi B cell lymphoma) (*24*) and a nonhematopoietic cell line (*25*) (see Fig. 3). The intensity of the 4.5-kb band corresponding to c-*myb* polyadenylated [poly(A)$^+$] mRNA (*15*) is significantly higher in ML-3 than in HL-60 mRNA. A faint hybridization signal is detectable in the Daudi Burkitt lymphoma cell line but not in the glioblastoma cell line A172. As previously described (*15*), c-*myb* expression is suppressed in terminally differentiated HL-60 cells treated with dimethyl sulfoxide (DMSO). Hybridization of the same filter to a human β-actin probe (*26*) (Fig. 3B), yielding a similarly intense 1.9-kilodalton band in all samples, indicates that an equivalent amount of RNA is present on the filter for all of the samples tested. Densitometric scanning of x-ray films from this experiment suggests a fivefold increase in *myb* mRNA levels in ML-3 as compared to mRNA levels in HL-60 cells. In conclusion, our results indicate that c-*myb* gene amplification correlates with high levels of c-*myb* transcription in ML cells.

Finally, we examined the karyotype of different ML lines. This analysis was suggested by two considerations. First, we wanted to exclude the possibility that the observed amplification was due to a simple hyperploidy of a single chromosome, chromosome 6. Second, c-*myb* amplification could have occurred as part of a chromosomal rearrangement detectable by cytogenetic analysis. In fact, in several instances, oncogene amplification was located within an abnormally banded chromosomal region correlated with the presence of double minute chromosomes (*7, 9, 11, 27*).

Karyotype analysis of primary and secondary cultures of ML-1, ML-2, and ML-3 cell lines at different passages excluded the presence of double minute chromosomes and revealed an unstable modal number varying from a slight hyperploidy [present in primary cells at early and late passages (*20*)] to near tetraploidy (present in ML-3 late passages). Despite this instability, no variation was observed in the degree of c-*myb* amplification and in no case was a relative hyperploidy of chromosome 6 observed. Moreover, a human HLA probe that hybridizes to several genomic regions on chromosome 6 (*26*), did not detect any amplified band in ML DNA as opposed to control DNA's (data not shown). These data confirm that the amplification specifically involves a region containing the c-*myb* locus and is not due to hyperploidy of chromosome 6. The karyotypes of ML primary cultures and of an ML-3 late passage are shown in Fig. 4A and Fig. 4B, respectively. The first karyotype (Fig. 4A) is representative of the most commonly observed pattern in ML-1, ML-2, and ML-3 lines and in primary and secondary cell cultures. It is characterized (among other features described in detail in Fig. 4) by an abnormal chromosome 6 carrying a deletion of band q24 (arrow in Fig. 4A; see also Fig. 4C), the normal site of the c-*myb* locus (*17*). The same chromosomal region appears to be involved in a different rearrangement in ML-3 late-passage cells, which are also characterized by a nearly tetraploid modal number (Fig. 4B). These cells carry an additional marker, M2, represented by chromosome 6 from 6pter to 6q22–24, beyond which there is considerable material whose origin was not clearly identified. Again, the site of rearrangement correlates with the region to which the c-*myb* locus has been mapped (*17*). Although the link between these cytogenetic abnormalities involving region 6q24 and the amplification event awaits further confirmation by hybridization experiments in situ, these findings are provocative in suggesting that this site may be recombinationally active in these cells. Analogous findings have been recently obtained in HL-60 cells in which the amplified c-*myc* locus is associated with the appearance of new marker chromosomes during cell cul-

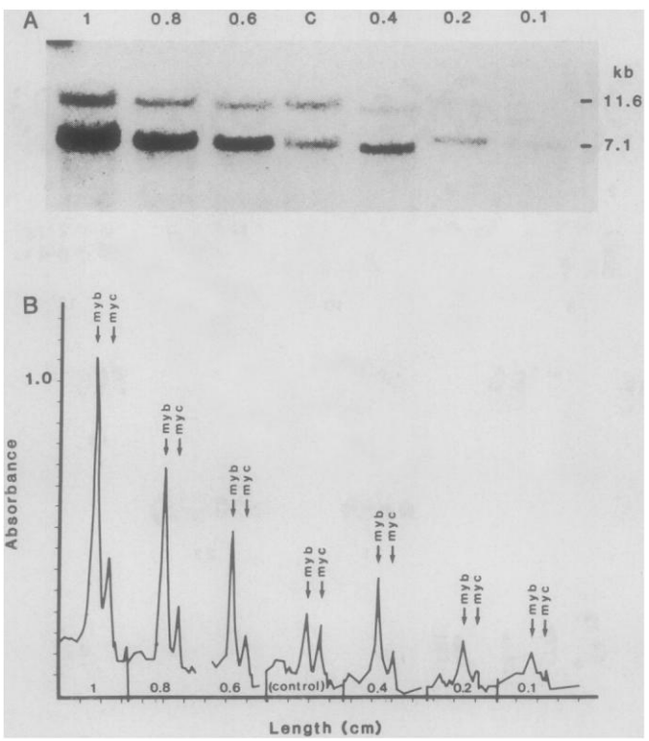

Fig. 2. (A) Estimate of c-*myb* copy number in ML-3 DNA. For this Southern blot dilution experiment, all the DNA's described below were digested with Hind III and simultaneously hybridized to p3D (c-*myb*) and pMC413RC (c-*myc*) probes (see Fig. 1). The intensity of c-*myb* and c-*myc* hybridization bands in 10 μg of DNA from normal human lymphocytes was used as standard to be compared with that obtained with the same amount of DNA from ML-3 cells (lane 1) and dilutions of the latter. Each number shown above the lanes indicates the dilution factor with respect to the 10 μg in lane 1 (for example, lane 0.4 contains 4 μg of ML-3–digested DNA). (B) Densitometric scanning of x-ray film from the experiment shown in (A). The absorbance of *myc* and *myb* bands is plotted for the different lanes described in (A).

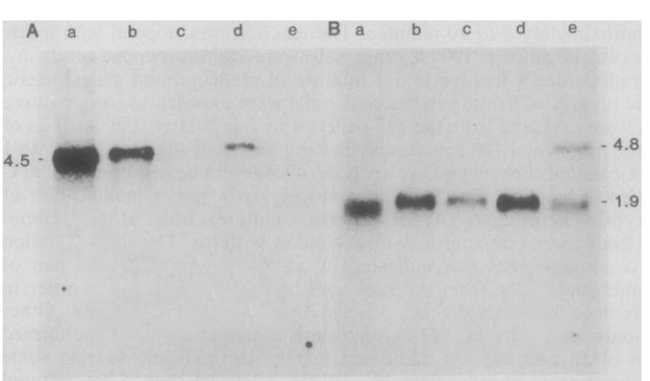

Fig. 3. (A) High levels of expression of c-*myb* mRNA in ML-3 cells. Total cellular RNA from (lane a) ML-3 cells, (lane b) HL-60 cells, (lane c) DMSO-treated HL-60 cells, (lane d) Daudi cells, and (lane e) A172 glioblastoma cells was extracted by the guanidine HCl method, poly(A)$^+$-selected, subjected to electrophoresis on a formaldehyde-containing gel, transferred to nitrocellulose filter, and hybridized to p3D (*myb*) probe according to established procedures (*23*). (B) Control hybridization of filter used in (A) with human β-actin probe. The filter used in (A) was boiled for 10 minutes in distilled water to remove the c-*myb* probe and subsequently hybridized to the labeled β-actin probe. The significance of the additional 4.8-kb band present in A172 cells is not known and has not been further investigated in this study.

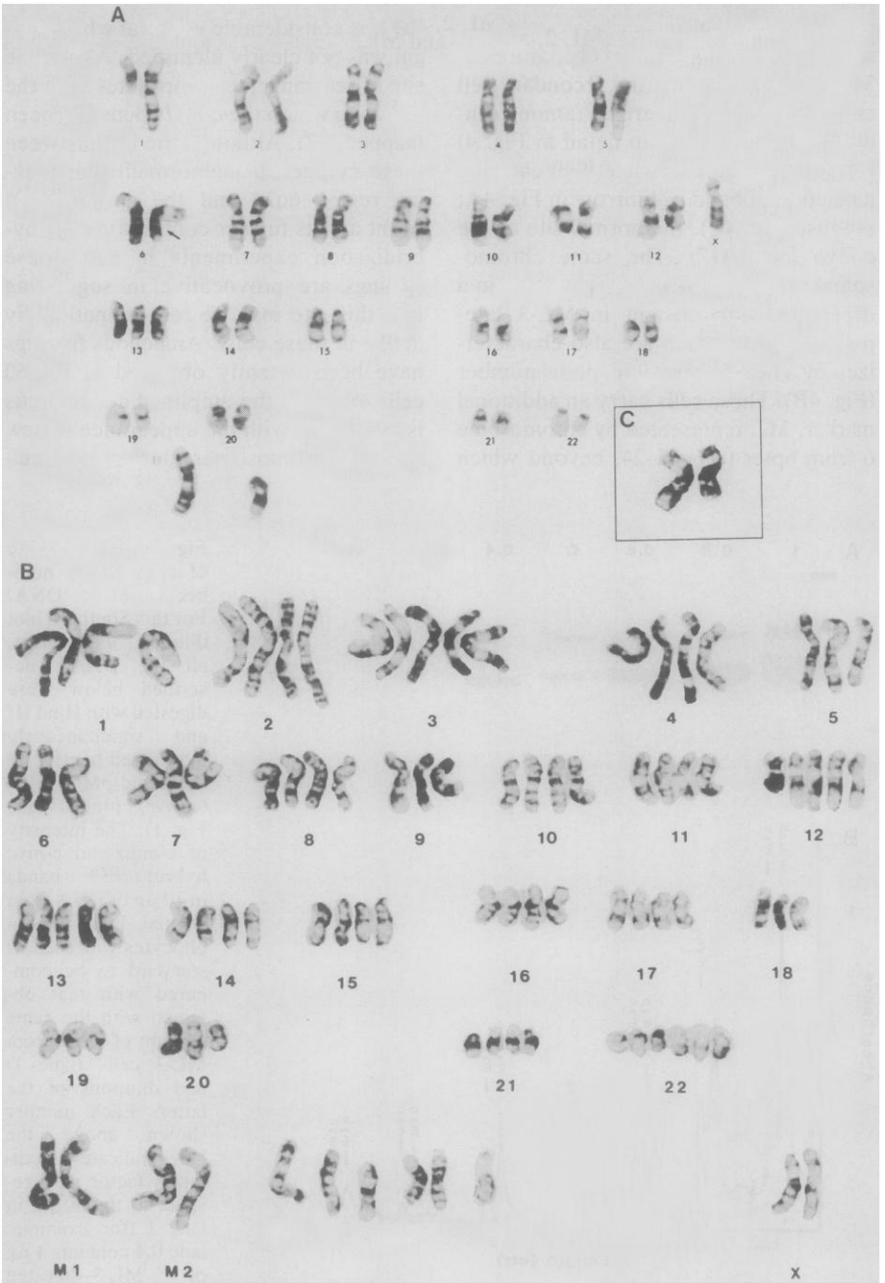

Fig. 4. Karyotypic analysis of (A) ML-1 primary cultures and of (B) ML-3 late passage. Cultures were split and fed 24 to 48 hours before chromosome harvest. Colcemid at a concentration of 0.1 μg/ml was added to the cultures 15 minutes before harvest. The cultures were centrifuged at 100 rev/min and cells were then suspended gently in 0.56 percent KCl and exposed to the hypotonic solution for approximately 8 to 10 minutes. The reaction was stopped with a few drops of fixative and, after centrifugation at 1000 rev/min, cells were again suspended gently in a solution of freshly prepared Carnoy's fixative (a 3:1 mixture of methanol and glacial acetic acid). After two 15-minute periods of fixation in the cold, cells were exposed to fresh fixative and left overnight. Slides were prepared from the cell pellets 4 to 7 days later. For analysis of double minute chromosome fragments, 100 consecutive metaphases from slides stained with 1 percent acetic orcein were counted. For metaphase analysis, slides were heated briefly at 90°C for 10 minutes before Giemsa banding. The staining protocol was a minor modification of Seabright's method for trypsin-Giemsa (*32*). (A) ML-1 primary cultures. Most of the chromosomes show no deviation from normal intrachromosomal banding patterns. The site of deletion (6q24) on one of the two chromosomes 6 is indicated by an arrow. An additional pair of chromosome 6 from another metaphase from the same cell line is shown in (C) in order to further illustrate the difference between the normal and the deleted chromosome. Other abnormalities include trisomy for the number 13 chromosome, a marker chromosome formed by centric recombination of 1q and 14q, an additional marker chromosome bearing some resemblance in size and banding to chromosome 12. (B) ML-3 late passage. Like cultured primary cells, most chromosomes of ML-3 late passage cells show no structural alterations from their normal counterparts. Trisomy, tetrasomy, or pentasomy for most of the normal chromosomal pairs is present. A consistent pair of marker chromosome (M1) was formed by centric recombination of 1q and 14q. The second marker pair (M2) is described in the text. Several other unidentified markers were seen.

ture, as seen by hybridization experiments in situ (*27, 28*). Moreover, consistent with these observations is the report that gene amplification is sometimes accompanied by chromosomal translocations at or near the site of the amplified gene (*29*). We suggest that the amplification of ML cells was originally associated with the deletion site (Fig. 4A), which has then further recombined, generating the M2 marker.

In summary, we have shown moderate levels of c-*myb* oncogene amplification in the human ML cell lines. As is the case with other tumor-related oncogene amplifications, we were unable to establish whether c-*myb* amplification had already occurred at the time of leukemogenic transformation; that is, whether amplification is present in nonleukemic cells from the same individual. The consistency in both the detection and the levels of amplification in primary cells and early and late passages of independently derived cell lines indicates that oncogene amplification did not occur during maintenance of this cell line in vitro but rather occurred early in the transformation event. However, alternative hypotheses such as a germ-line amplification in this individual or a differentiation-related event cannot be ruled out at present. In general, these findings indicate amplification as a mode of activation of the c-*myb* oncogene. The only other reported example of tumor-associated c-*myb* activation involves c-*myb* rearrangements in mouse plasmocytoid lymphosarcomas (ABPL), an infrequently occurring subtype of mouse lymphosarcomas (*30*). In analogy with ABPL's, c-*myb* amplification in ML cells may identify a particular subset of AML's that we cannot now characterize by morphological or biochemical analysis. This possibility can be evaluated by analyzing a larger number of AML cases for c-*myb* amplification, since the limited survey reported here is not informative in this respect. Alternatively, the fact that c-*myb* amplification was not detectable in other AML DNA's may indicate that this event is only occasionally associated with this tumor, as it is the case of c-*myc* amplification in AML and other tumors (*30*).

Since c-*myb* and c-*myc* code for protein products that have some degree of structural homology (*14*), and since their expression appears to be analogously regulated during myeloid differentiation (*15*), the finding of unlike amplification in similar tumors (that is, in AML cell line HL-60, ML-1, ML-2, and ML-3) suggests that these two oncogenes may be alternatively activated in the same

pathogenetic step. In particular, amplification may prevent the physiologic c-*myc* or c-*myb* suppression that in myeloid cells appears to correlate with the switch from a proliferative to a differentiative program (*15*).

Finally, the finding of alternative c-*myb* or c-*myc* oncogene amplification in two phenotypically similar tumors suggests that tumor subtypes that are indistinguishable by current morphological or biochemical techniques may be characterized by different genetic markers and pathogenetic pathways. This form of genetic heterogeneity could be further explored in relation to variability in other parameters; for example, differences in drug sensitivities or in the properties of malignant cells in vitro, which sometimes occur in phenotypically similar tumors.

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Amplification of N-*myc* in Untreated Human Neuroblastomas Correlates with Advanced Disease Stage

Abstract. *A domain of DNA designated N-*myc *is amplified 20- to 140-fold in human neuroblastoma cell lines but not in cell lines from other tumor types. N-*myc *has now been found to be amplified in neuroblastoma tissue from 24 of 63 untreated patients (38 percent). The extent of amplification appears to be bimodal, with amplification of 100- to 300-fold in 12 cases and 3- to 10-fold in 10 others. Amplification was found in 0 of 15 patients with stage 1 or 2 disease, whereas 24 of 48 cases (50 percent) with stage 3 or 4 had evidence of N-*myc *amplification. These data indicate that N-*myc *amplification is a common event in untreated human neuroblastomas. Furthermore, N-*myc *amplification is highly correlated with advanced stages of disease (*$P < 0.001$*) and with the ability to grow in vitro as an established cell line, both of which are associated with a poor prognosis.*

Aberrant expression of cellular oncogenes has been implicated in the causation of various types of malignant disease. Oncogenes appear to be activated from their normal quiescent or regulated state by (i) increased expression of a single gene, (ii) gene amplification, and (iii) gene alteration resulting in a product with increased oncogenic potential. Some of these events have cytogenetic correlates. For example, the increased expression of c-*myc* in Burkitt lymphoma is frequently associated with a specific 8;14 translocation (*1*). Instances of oncogene activation by gene amplification may be manifested cytogenetically by the presence of homogeneously staining regions (HSR's) on specific chromosomes, or by extrachromosomal double minutes (DM's) (*2*). As expected, a cytogenetic correlate of gene alteration has not been observed, but this appears to be the most common mechanism for activation of oncogenes of the *ras* family (*3*).

Amplification of the oncogene c-*myc* has been demonstrated in several human tumor cell lines, including the colon tumor COLO-320, the lung tumor NCI-417, and the promyelocytic leukemia cell line HL-60 (*2*, *4*). Recently we have identified amplification of a c-*myc*-related sequence called N-*myc* in eight of nine human neuroblastoma cell lines, but not in HSR-bearing cell lines derived from other tumor types (*5*). In addition, we have mapped the normal single-copy locus of N-*myc* to the short arm of chromosome 2, bands 2p23 or 24 (*6*). These findings are in agreement with other recent reports (*7*). Although we demonstrated one case of amplification of N-*myc* in neuroblastoma tissue from an untreated patient (*5*), our previous study was confined almost exclusively to the analysis of established cell lines.

We have now examined 63 primary untreated neuroblastomas for N-*myc* amplification. Our result shows that amplification of this sequence is a common event in vivo, and that it is associated with advanced stages of disease.

Neuroblastoma tissue was obtained from the primary tumors of 63 untreated patients undergoing diagnostic surgery; it was frozen until the time of analysis. Most of the samples were obtained from member institutions of the Children's Cancer Study Group (CCSG), and addi-