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Germ Line c-*myc* Is Not Down-Regulated by Loss or Exclusion of Activating Factors in *myc*-Induced Macrophage Tumors

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As in tumors with c-*myc* chromosomal translocations, c-*myc* retrovirus-induced monocyte tumors constitutively express an activated form of c-*myc* (the proviral gene), whereas the normal endogenous c-*myc* genes are transcriptionally silent. Treatment of these retrovirus-induced tumor cells with a number of bioactive chemicals and growth factors that are known to induce c-*myc* expression in cells of the monocyte lineage failed to induce the endogenous c-*myc* gene. In contrast, the same treatments induced the c-*fos* gene in both tumors and a control macrophage line. To investigate c-*myc* suppression further, a normal copy of the human c-*myc* gene was introduced into tumor and control cell lines by using a retrovirus with self-inactivating long terminal repeats. This transduced normal gene was expressed at equivalent levels in all cells, regardless of the state of endogenous c-*myc* gene expression, and was strongly induced by agents that induce the normal gene in the control cells. These results indicate that the signal transduction pathways that normally activate the c-*myc* gene are functional in *myc*-induced tumor cells and suggest that endogenous c-*myc* is actively suppressed. An examination of the c-*myc* locus itself showed that the lack of transcriptional activity correlated with the absence of several prominant DNase I-hypersensitive sites in the 5′-flanking region of the gene but without loss of general DNase sensitivity. Furthermore, analysis of 22 methylation-sensitive restriction enzyme sites in the 5′-flanking region, first exon, and first intron indicated that the silent c-*myc* genes remained in the same unmethylated state as did actively expressed genes. Thus, c-*myc* suppression does not appear to result from the most frequently described mechanisms of gene inactivation.

The c-*myc* proto-oncogene has been implicated in the genesis of a diverse spectrum of tumors, particularly those of hemopoietic origin (reviewed in reference 13). In nontransformed cells, c-*myc* expression is elevated in dividing cells and stimulated in response to treatment with growth factors, indicating that c-*myc* expression is coupled to cellular proliferation (18, 32, 33). Conversely, inappropriate expression of c-*myc* in nondividing cells increases the probability that these cells will undergo DNA replication (2). This observation, and the finding that *myc*-associated tumors generally have alterations that induce constitutive *myc* expression rather than change the protein structure, argue that the c-*myc* gene is activated by deregulation of expression. Thus, elucidation of the normal regulatory mechanisms that govern c-*myc* expression is critical to understanding the role of the c-*myc* oncogene in tumorigenesis.

A consistent observation in *myc*-induced tumors is that although the activated c-*myc* allele is expressed constitutively, the normal proto-oncogene is virtually silent (1, 3, 49, 50). One model proposed to explain this observation is that c-*myc* down-regulation is linked to the developmental stage of the cells from which *myc*-induced tumors commonly arise (13). Supporting this hypothesis are studies that show that c-*myc* does indeed turn off during the differentiation of a number of cell lines in culture (35, 53). A second model argues that c-*myc* protein produced by the activated oncogene, which is not responsive to normal regulatory signals, is involved in a feedback regulatory loop that leads to repression of the proto-oncogene (36). However, viral promoter-linked c-*myc* genes introduced into lines by transfection usually have no effect on either absolute c-*myc* mRNA levels or general patterns of regulation (14, 31), although there have been some reports to the contrary (12). Independent of whether differentiation or autoregulation is responsible for the loss of c-*myc* transcripts, it is not yet clear whether down-regulation is due to an absence of the stimulatory signals or factors that turn c-*myc* on or to a new, dominant suppressing activity that can turn c-*myc* off. The work presented here indicates that signal transduction pathways capable of activating c-*myc* remain functional in *myc*-induced tumor cells and that *cis*-acting sequences beyond 600 base pairs (bp) from the c-*myc* promoter may be responsible for suppression.

Expression of the c-*myc* proto-oncogene is regulated at both the transcriptional and posttranscriptional levels. Run-on transcription assays have demonstrated that c-*myc* is induced transcriptionally in response to serum stimulation of mouse fibroblasts (18, 25), and a block to transcriptional elongation is largely responsible for a reduction in c-*myc* RNA levels at early stages of HL60 differentiation (6). In contrast, c-*myc* transcriptional activity remains constant during induced differentiation of the embryonal carcinoma line F9 even though c-*myc* mRNA completely disappears, which implicates posttranscriptional control mechanisms (19). Similarly, the decrease in *myc* mRNA levels after treatment of Daudi cells with interferon results from a reduction of the already short half-life of c-*myc* message (17, 34). In B-cell tumors that carry a highly expressed, translocated c-*myc* gene, it is generally difficult to determine the transcriptional activity of the untranslocated allele, for which there is no specific monitor of activity. Although it is generally believed that the normal gene is transcriptionally off, it has been shown that altered transcripts of the activated oncogene are more stable than normal, allowing them to accumulate to high levels (43, 44). To unambiguously address whether transcriptional or posttranscriptional mecha-

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nisms are responsible for suppression of endogenous c-*myc* expression, we have taken advantage of a set of clonal monocyte-macrophage tumors produced by intraperitoneal infection with a c-*myc* retrovirus (4, 5). As with plasmacytomas and Burkitt lymphomas, there are no detectable transcripts of the normal c-*myc* proto-oncogene in these tumors (5). However, unlike other tumor systems, the activated c-*myc* allele (the integrated provirus) does not include sequences of the untranslated first exon and thus is easily distinguished from the endogenous allele. These cells have allowed us to study the transcription, methylation status, and chromatin structure of the endogenous c-*myc* gene.

MATERIALS AND METHODS

Cell lines and culture conditions. BM-M8 cells and monocyte-macrophage tumors induced by intraperitoneal infection with a mouse c-*myc* retrovirus have been previously described (4). The macrophage cell line P388D1 and the myelomonocytic line WEHI-3B were obtained from the American Type Culture Collection, Rockville, Md. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.J.).

Cells were made quiescent in 0.5% serum for 48 h and then treated as follows. Medium that had been conditioned for 24 h by mouse L cells (LCM) was used as a source of colony-stimulating factor 1. The synthetic polyribonucleotide poly(I-C) (Pharmacia, Inc., Piscataway, N.J.) was added to 50 μg/ml. The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 25 to 75 ng/ml from a 25-μg/ml stock solution (in dimethyl sulfoxide, stored at −20°C). The calcium ionophore A23187 (Sigma) was kept in a 2 mM stock solution (in dimethyl sulfoxide, stored at −20°C) and added to a final concentration of 2 μM. Dibutyryl cyclic AMP (Bt_2-cAMP; Sigma) was added to 0.5 to 1.0 mM from a 0.5 M aqueous stock solution.

Transcription in isolated nuclei and analysis of RNA. Transcription in isolated nuclei was performed as recently described (46), using linearized, double-stranded target plasmids. RNA was prepared and analyzed as described elsewhere (46). Filters were hybridized to probes generated by random hexamer priming from gel-purified DNA fragments according to the directions of the manufacturer (Pharmacia). RNase protection assays were performed as described previously (55). The following probes were used. For c-*myc*, a 419-bp *Pst* insert from the second exon of human c-*myc* into pT71 (United States Biochemical Corp., Cleveland, Ohio) generates a 470-base probe and 419-base (human) and approximately 155-base (mouse) protected fragments (28). For *neo*, two probes were used. One (*Hin*dIII to *Bgl*II) protects a 320-base fragment from the 5′ end; the other protects 270 bases from an internal region of the gene (*Bgl*II to *Pvu*II). For *fos* and retroviral sequences, a 609-base *Bgl*II insert from FBJ murine sarcoma virus containing retroviral and 5′ v-*fos* sequences cloned into pT71 gives rise to a 480-base probe and 303-base (*fos*) and 144-base (retroviral) protected fragments. Bands were quantitated by densitometry.

Retroviral transduction of the human c-*myc* gene. A 6.6-kbp *Acc*I-*Eco*RI fragment from the human c-*myc* gene (H-*myc*) covering sequences from 600 bp 5′ of P1 to 500 bp 3′ of the polyadenylation site was cloned into a vector containing an intact Moloney murine leukemia virus 5′ long terminal repeat, a herpes simplex virus thymidine kinase promoter-driven *neo* gene, and a 3′ long terminal repeat from which the enhancer and G+C-rich promoter region had been deleted so that the retroviral control sequences would be inactivated during infection and reverse transcription (a generous gift of I. Lemischka). The construct was transfected into Ψ2 cells (38), and a single G418-resistant colony was used to produce viral stocks. The indicated cells were infected with the H-*myc*-*neo* virus, and single G418-resistant clones were expanded for DNA and RNA. No differences in retroviral titer were found for the different cells. Southern blot analysis using several restriction enzymes confirmed that each line harbored a single provirus with the structure shown in Fig. 4A.

Analysis of DNA methylation. Cells were lysed in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate, digested overnight with 100 μg of proteinase K per ml, extracted repeatedly with phenol, and precipitated with ethanol. Samples that had been treated with RNase A and the restriction enzyme of interest were run on 6% denaturing polyacrylamide gels, electrophoretically transferred to Nytran, and immobilized as described by Church and Gilbert (11). Probes were labeled by random hexamer priming and hybridized as described above. Regions analyzed included the c-*myc* 5′ end (*Xba* −1491 to *Bam*HI +116, where 1 is the position of the first mRNA start site) and the first exon-intron (*Bam*HI +116 to *Bam*HI +1270).

Mapping of DNase I-hypersensitive sites. Nuclei were prepared as described above and washed once in digestion buffer (15 mM Tris hydrochloride [pH 7.4], 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM dithiothreitol) before being resuspended at a density of 2×10^8 nuclei per ml in the same buffer. DNase I (Sigma) was added to equal portions of the nuclear suspension at concentrations ranging from 1 to 10 U/ml. After incubation at 25°C for 3 min, the reactions were terminated by the addition of EDTA to 10 mM and sodium dodecyl sulfate to 0.5%. Further purification of the partially digested DNA was carried out by treatment with proteinase K, repeated phenol extraction, and ethanol precipitation. Sites of preferential DNase I cleavage were localized by the technique of indirect end labeling (54).

RESULTS

Transcriptional inactivity of the c-*myc* proto-oncogene. By using the nuclear run-on assay, it was possible to unambiguously assess the transcriptional activity of the normal c-*myc* allele in monocyte tumors induced by a c-*myc* retrovirus. This was possible because the integrated provirus contains only the second and third (protein-coding) c-*myc* exons, whereas the untranslated first exon is unique to the normal c-*myc* transcription unit (5). A set of four representative tumors (1.1, 2.3, 7.1.3, and 9.1.1) was analyzed, along with the macrophage cell line P388D1 and the myelomonocytic line WEHI-3B. Hybridization to the first exon, which was derived solely from the endogenous gene, was very low to virtually nonexistent (at least 20-fold reduced) in the *myc*-induced tumors compared with either P388D1 or WEHI-3B (Fig. 1). Thus, suppression of the endogenous c-*myc* genes in these *myc* retrovirus-induced tumors resulted from the lack of transcriptional initiation, including all initiation from both P1 and P2. As controls for transcription, DNAs homologous to *fos*, KC, and histone H3 were included on the filters, and pBR322 was used to monitor nonspecific binding. Hybridization to the c-*myc* second-exon probe represented the sum of endogenous and exogenous transcription and showed that the retrovirus-transduced

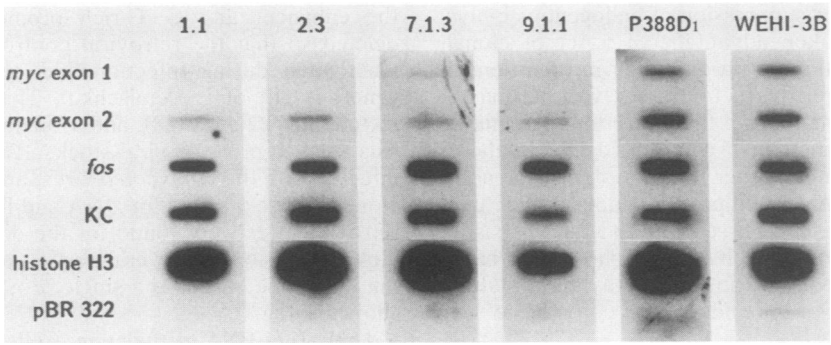

FIG. 1. Run-on transcription analysis of macrophage cell lines. Mouse c-*myc* retrovirus-induced macrophage tumors (1.1, 2.3, 7.1.3, and 9.1.1), the macrophage line P388D1, and the myelomonocytic line WEHI-3B were subjected to a nuclear run-on assay. Radiolabeled transcription products were hybridized to filters containing c-*myc* first exon (subcloned 0.46-kbp *Bam*HI-*Bgl*II fragment), c-*myc* second exon (subcloned 0.58-kbp *Pst*I fragment), *fos* (1.3-kbp region of FBJ murine leukemia virus homologous to c-*fos*), KC (full-length cDNA; 0.5 kbp), histone H3 (a complete mouse gene; 0.54 kbp), and plasmid pBR322. The c-*myc* second exon probe can hybridize to transcripts from the endogenous gene in P388D1 and from both the endogenous and viral genes in the tumors.

gene was transcribed, although the signal was consistently less than that for the endogenous gene. Despite the low levels of transcription, the virus-derived c-*myc* RNA levels were as high as or higher than the endogenous levels in P388D1 because the viral transcripts are more stable than the endogenous mRNA due to the loss of signals for rapid mRNA turnover (28).

Lack of inducibility of c-*myc* by bioactive compounds. One possible explanation for the lack of transcription of the endogenous c-*myc* genes is an absence of the stimuli that normally activate the gene. We attempted to reactivate the silent c-*myc* genes by using a number of factors and chemicals that induce c-*myc* in monocytic or other cell lineages; many of these compounds induce c-*fos* expression as well. The inducers tested included serum, specific growth factors such as colony stimulating factor 1, TPA, Bt_2-cAMP, the calcium ionophore A23187, and the double-stranded synthetic RNA poly(I-C) (8, 18, 25, 33, 39, 40, 41, 51). Also, labile mRNAs such as c-*myc* and c-*fos* mRNAs can accumulate in the presence of the translational inhibitor cycloheximide, which stabilizes otherwise short-lived messages (16). Cycloheximide could also conceivably induce gene expression by blocking the synthesis of a labile repressor.

Each of the compounds was tested for the ability to increase c-*myc* and c-*fos* mRNA levels in serum-suppressed cells. Most of the treatments caused a detectable increase in c-*myc* mRNA in the P388D1 cell line (Fig. 2; see Fig. 4); particularly effective were TPA and Bt_2-cAMP, which gave 50- and 20-fold increases, respectively. None of the treatments, however, were able to induce detectable endogenous c-*myc* mRNA in the tumor lines. In contrast, c-*fos* expression was modulated similarly in both control and tumor cells. In some cases two treatments, such as TPA plus cycloheximide or TPA plus Bt_2-cAMP, were given simultaneously on the basis of the idea that multiple intracellular signaling pathways might cooperate to lead to c-*myc* transcription. None of these combinations, however, was able to activate c-*myc* (data not shown). Finally, to ensure that we had not missed a transcriptional induction resulting from an additional posttranscriptional down-regulation, tumor line 7.1.3 was analyzed by the nuclear run-on assay after treatment with either TPA or Bt_2-cAMP for various lengths of time. Neither of these treatments, however, led to an increase in transcription of the endogenous c-*myc* gene, whereas the c-*fos* gene was stimulated by both (data not shown). Although we concede that some as yet untried treatment may allow c-*myc* to be expressed, it appears that the endogenous c-*myc* gene is in a noninducible state in these tumors.

Expression and inducibility of a normal c-*myc* gene in monocyte tumors. The observation that c-*fos* is inducible in the macrophage tumor lines suggests that the growth factor transduction pathways in these cells are at least partially

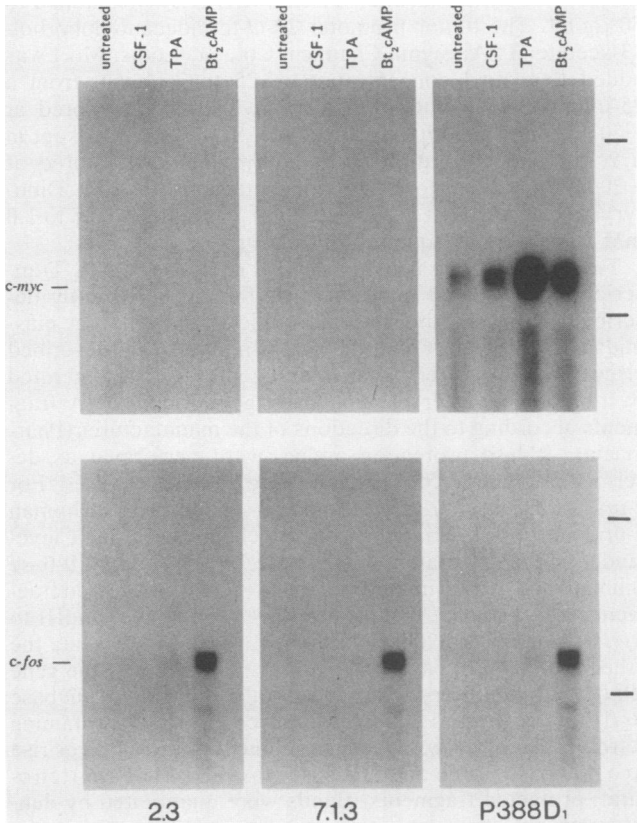

FIG. 2. Inducibility of c-*fos* but not c-*myc* mRNA. Quiescent cultures of 2.3, 7.1.3, and P388D1 cells were stimulated with colony-stimulating factor-1 (CSF-1) (10% LCM), TPA (25 ng/ml), or Bt_2-cAMP (0.5 mM). After 2.5 h, cytoplasmic RNA was isolated and subjected to Northern (RNA) blot analysis. The filter was first probed with a mouse c-*myc* first-exon probe and then stripped and reprobed with a *fos* probe. Bars on the right mark positions of the 18S and 28S rRNA species.

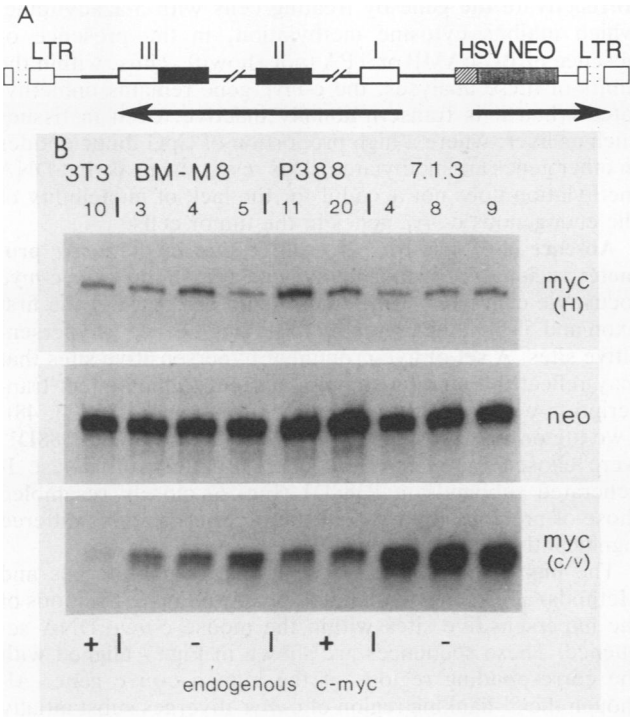

FIG. 3. Steady-state levels of exogenous human c-*myc* mRNA. (A) Map of the retrovirus-transduced human c-*myc* gene. LTR, Long terminal repeat. (B) Expression of human c-*myc*, *neo*, and mouse c-*myc* RNAs in isolated clones harboring the provirus shown in panel A. Total cytoplasmic RNA (30 μg) from subconfluent cultures of A31 (3T3), BM-M8, P388D1 (P388), and 7.1.3 cells was harvested and analyzed by RNase protection assays. The c-*myc* probe is specific for the second exon of human c-*myc* [myc (H)] but cross-reacts with the murine transcript [myc (c/v)] to generate fragments of 419 and approximately 155 bases, respectively. The 155-base band is derived from the endogenous gene in P388D1 and NIH 3T3 cells and from the transforming mouse *myc* virus in the tumors. The status of the endogenous gene is indicated at the bottom. All panels are from the same gel. The number at the top of each lane refers to individual G418-resistant clones of the indicated cell type.

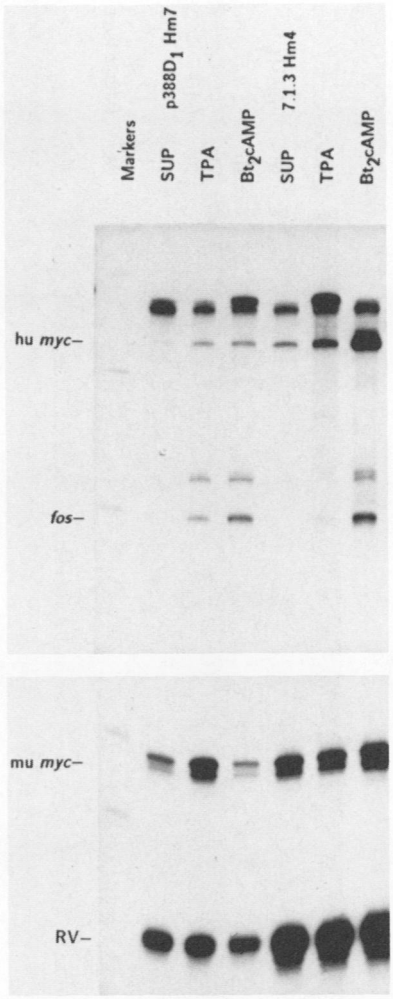

FIG. 4. Inducibility of exogenous c-*myc* mRNA. Two human *myc*-containing subclones, 7.1.3Hm4 and P388D1Hm7, were made quiescent in 0.5% serum for 48 h (SUP) and then stimulated with TPA (75 ng/ml) or Bt_2-cAMP (1 mM) for 1.5 h. Total cytoplasmic RNA was analyzed by RNase protection. The *myc* probe is as in Fig. 3B. The *fos*-retrovirus probe protects fragments of 303 bases (*fos* RNA) and 144 bases (endogenous retroviral transcripts as a normalizing control [RV]).

active. To test this idea further, we introduced a normal human c-*myc* gene into cells that suppress endogenous c-*myc* expression (Materials and Methods; Fig. 3A) and examined whether it was sensitive to the compounds tested above. Equivalent levels of both human c-*myc* and *neo* RNAs were produced in clones of cells that did (P388D1 and BALB/c 3T3) and did not (BM-M8 and 7.1.3) express their endogenous c-*myc* genes (Fig. 3B). Thus, retroviral transfer of the c-*myc* gene did not allow recreation of the endogenous gene regulation. This result is consistent with the findings from a number of laboratories that have attempted to analyze suppression of the c-*myc* promoter by DNA transfection into tumor and normal cells (10, 27, 37, 45).

We next asked whether the transduced human gene was responsive to agents that normally induce the c-*myc* gene but failed to do so in *myc*-transformed cells. Expression of the human c-*myc* gene in both cell types was induced sixfold by Bt_2-cAMP and twofold by TPA, as was expression of c-*fos* (Fig. 4). The same treatments induced the endogenous c-*myc* gene in P388D1 (Fig. 4, lanes 1 to 3), whereas *neo* mRNA levels remained constant (data not shown). Nuclear run-on assays indicated that a significant portion of this induction was at the transcriptional level. Thus, since both the exogenous human gene and c-*fos* can be induced, the signal transduction pathways that can activate the c-*myc* gene must still be functional in *myc*-transformed cells.

Absence of methylated sequences in both active and inactive c-*myc* genes. Since the endogenous c-*myc* alleles in the tumor cells could not be activated by agents that normally induce the gene, we considered potential *cis*-acting modifications that might preclude the binding of transcription factors. The most widely studied modification is methylation of CpG dinucleotides, which frequently correlates with gene inactivity (9). To investigate the methylation status of active versus silent c-*myc* genes, we analyzed DNAs from P388D1 (active), monocyte tumors 7.1.3 and 2.3 (silent), and liver (down-regulated but inducible). DNAs were digested to completion with either *Hpa*II (sensitive to CpG methylation) or *Msp*I (insensitive to methylation) and hybridized to probes from the c-*myc* gene which span 1.6 kbp of the 5′-flanking sequences, the first exon, and the first intron, all of which are unique to the endogenous gene. If the c-*myc* gene is unmethylated, all of the restriction enzyme fragments

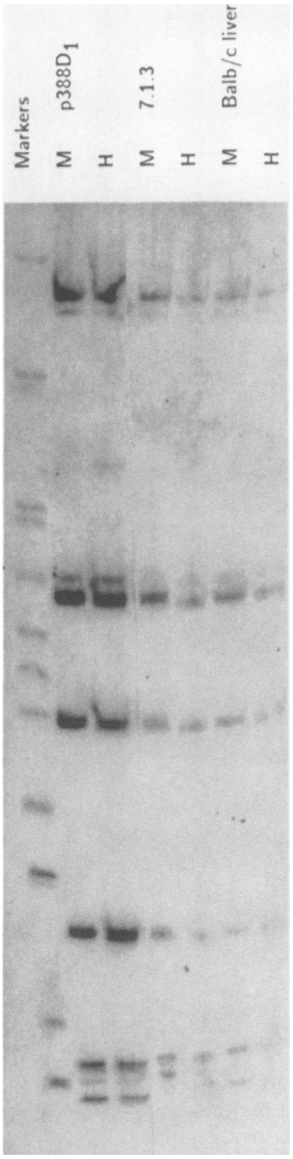

FIG. 5. Methylation status of the c-*myc* 5′ region. Genomic DNAs from line 7.1.3 or P388D1 or from BALB/c liver tissue were digested with *Hpa*II (H) or *Msp*I (M), and the fragments were visualized by Southern blotting. The probe used was a 1.6-kbp *Xba*I-*Bam*HI fragment (nucleotides −1491 to +116 with respect to the c-*myc* promoter).

predicted from the sequence should be detected in both the *Hpa*II and *Msp*I digests; if the gene is methylated, some fragments should be missing or submolar in hybridization intensity in the *Hpa*II digest. Somewhat surprisingly, all *Hpa*II-*Msp*I sites within the c-*myc* gene regions analyzed were completely unmethylated in each of the DNAs, even in liver tissue, which is highly methylated. When the filter was hybridized with a 5′-flanking region probe, all fragments predicted from the sequence were identifiable in both the *Hpa*II and *Msp*I digests (Fig. 5). A cluster of fragments of approximately 50 bases was not well resolved, but there was no difference in hybridization intensity among lanes. Other methylation-sensitive restriction sites, such as *Sma*I and *Xho*I, were also unmethylated in the each of the DNAs described above (not shown). We also tried without success to reactivate the gene by treating cells with 5-azacytidine, which inhibits cytosine methylation, in the presence or absence of Bt_2-cAMP or TPA (not shown). Thus, within the limits of these analyses, the c-*myc* gene remains unmethylated when it is transcriptionally inactive, even in tissues such as liver, where a high proportion of CpG dinucleotides in other genes are methylated. This result suggests that DNA methylation does not account for the lack of inducibility of the endogenous c-*myc* genes in the tumor cells.

Absence of DNase I-hypersensitive sites in the c-*myc* promoter region. To further investigate the suppressed c-*myc* locus, we characterized the chromatin structure of the first exon and 5′-flanking region by analyzing DNase I-hypersensitive sites. A set of five prominent hypersensitive sites that may reflect the binding sites for proteins which affect transcription was previously mapped in this region (22, 23, 48). Two tumor lines, 7.1.3 and 9.1.1, and a control line, P388D1, were chosen for study. The overall pattern of DNase I-generated subbands in P388D1 (Fig. 6) closely resembled those of previous reports, but the precise positions differed significantly.

The high resolution of the mapping (see Materials and Methods) allowed us to identify the approximate positions of the hypersensitive sites within the mouse c-*myc* DNA sequence. These sequences are shown in Fig. 7 aligned with the corresponding regions of the human c-*myc* gene. Although the 5′-flanking region of c-*myc* diverges substantially between mouse and human genomes, the DNase I-hypersensitive sites are within or adjacent to small regions that are evolutionarily conserved. The region just upstream of site I is 91% conserved over 45 bp, and there is a region of 41 bp adjacent to site II_1 that is 88% identical. Sites III_1 and III_2 map to the two promoters, which are also quite well conserved. Moreover, site II_2 corresponds to the binding site of a plasmacytoma-specific factor, termed PCF, which has been suggested to play a role in c-*myc* down-regulation in mature plasma cells (29).

The results of DNase treatment for the two tumor lines, in which c-*myc* is transcriptionally silent, revealed a strikingly different picture. Only hypersensitive site I was present in tumor line 9.1.1, and all sites were absent in tumor 7.1.3. The relevance of hypersensitive site I for c-*myc* expression is somewhat controversial, with some reports claiming that its presence correlates with suppression (48) and others finding that it does not (21, 23). Our results are consistent with the latter view, and we can conclude that the lack of transcriptional activity correlates with the absence of all of the hypersensitive sites.

It is important to note that the c-*myc* region was sensitive to DNase I digestion in all cells, as indicated by the rate of disappearance of the intact *Nhe*I fragment, even though few sites of preferential cleavage were present. This result contrasts with findings for plasmacytomas, in which the region surrounding the unrearranged c-*myc* gene is insensitive to DNase I (30) and is therefore considered to be in a condensed configuration. To ensure that the general degree of DNase digestion was the same between the monocyte tumors and P388D1, the blots in Fig. 6 were stripped and rehybridized with a probe from the c-*fos* gene, which gave similar patterns for all three (data not shown).

DISCUSSION

The goal of this study was to understand the mechanism by which the normal c-*myc* gene is suppressed in tumors caused by activation of a c-*myc* oncogene through chromo-

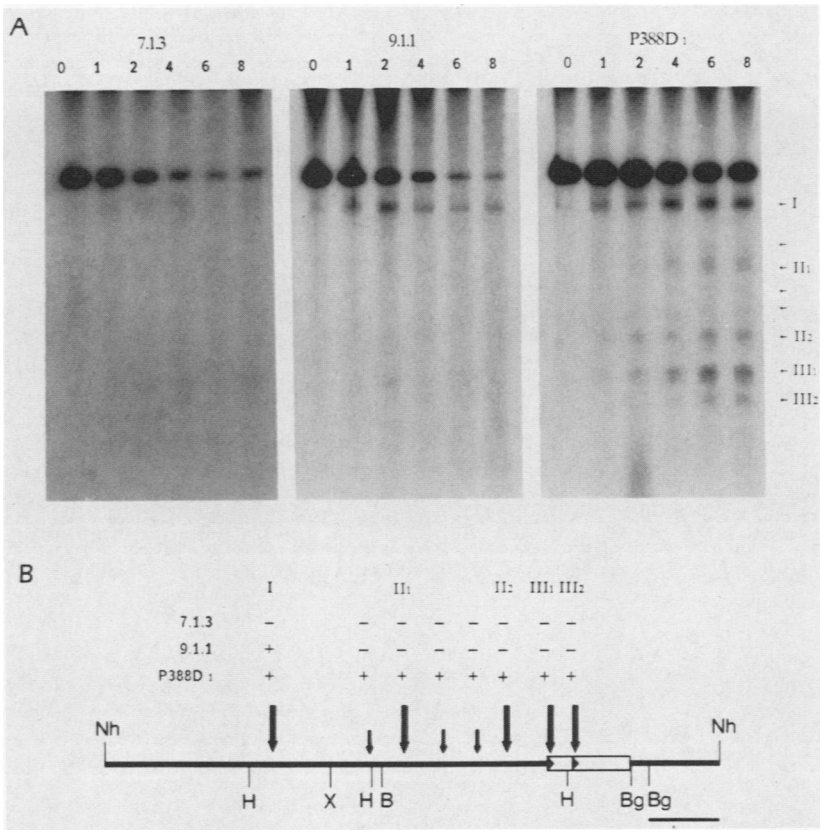

FIG. 6. Localization of DNase I-hypersensitive sites in the c-*myc* 5′ region. (A) Nuclei isolated from *myc*-induced macrophage tumor lines 7.1.3 and 9.1.1 and from the control macrophage line P388D1 were treated with increasing amounts of DNase I (shown above each lane in units per milliliter), and sites of preferential cleavage were localized by Southern blot analysis of *Nhe*I-digested DNAs, using the technique of indirect end labeling. The major bands are indicated by the established nomenclature, and several fainter additional bands are marked with arrows. (B) The presence or absence of hypersensitive sites in each cell line is indicated by a plus or minus sign over each site (▼). The restriction map spans the 3.7-kbp *Nhe*I fragment that was monitored in the analysis. The precise distances for the hypersensitive sites were based on adjacent lanes in which the same DNA samples were digested with *Bam*HI and *Hin*dIII, which generated hybridizing fragments of known length (see B above). Symbols: □, first c-*myc* exon with its two RNA initiation sites (▶); ▬, the 479-bp *Bgl*II-*Nhe*I fragment used as a hybridization probe. Restriction enzyme abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Nh, *Nhe*I; X, *Xba*I.

somal translocation or retroviral integration. Our analysis of the c-*myc* retrovirus-induced monocyte tumors demonstrated unambiguously that suppression of the germ line c-*myc* gene occurs at the transcriptional level. There is virtually no transcription of either the sense or antisense strand and no transcription of only the first exon such as is found in early differentiating HL60 cells (6, 42). In addition, we cannot reinduce the gene with any of the substances known to induce c-*myc* in other contexts, an observation similar to that of Siebenlist and co-workers with late-differentiating HL60 cells (47). Such down-regulation could theoretically be due either to a loss of the signaling pathway(s) or transcription factors that normally stimulate c-*myc* expression or to the addition of a new suppressing activity that renders the transactivators ineffectual. Our finding that c-*fos* and the retrovirus-transduced human c-*myc* gene are still inducible in the monocyte tumors favors the latter hypothesis.

We recognize, however, that we cannot rule out the possibility that the expression of the human gene has not been affected by sequences surrounding it. Factors binding to the herpes simplex virus thymidine kinase promoter, for example, may also interact with sites or proteins at the human c-*myc* promoter to induce expression. As an argument against such cooperative effects, however, we note that *neo* mRNA levels remain constant under conditions in which human c-*myc* is induced. More globally, the chromosomal locus into which the retrovirus has integrated may influence expression (52). For example, a large domain structure is required for proper regulation of globin expression in addition to the sequences located near the gene itself (26). Our construct probably would not contain the sequences required to reconstruct a hypothetical c-*myc* domain such as this, and the human gene may instead be influenced by the new domain into which it has inserted. Alternatively, though present in the nucleus, the factors required for c-*myc* transcription may be sequestered from the gene itself. Inconsistent with this view, however, is the observation that all of the clones we tested behaved identically regardless of the site of integration. Finally, suppression may be actively established at a particular stage of cell growth or differentiation, after which this state is maintained in the absence of the factors that were responsible for the initial down-regulation. However, somatic cell hybrid experiments between c-*myc*-expressing and -nonexpressing lines suggest that cells carrying a silent c-*myc* gene are still capable of turning off a newly introduced active allele (15, 24).

If we accept that the endogenous c-*myc* gene is suppressed, we are faced with the question of how. In contrast

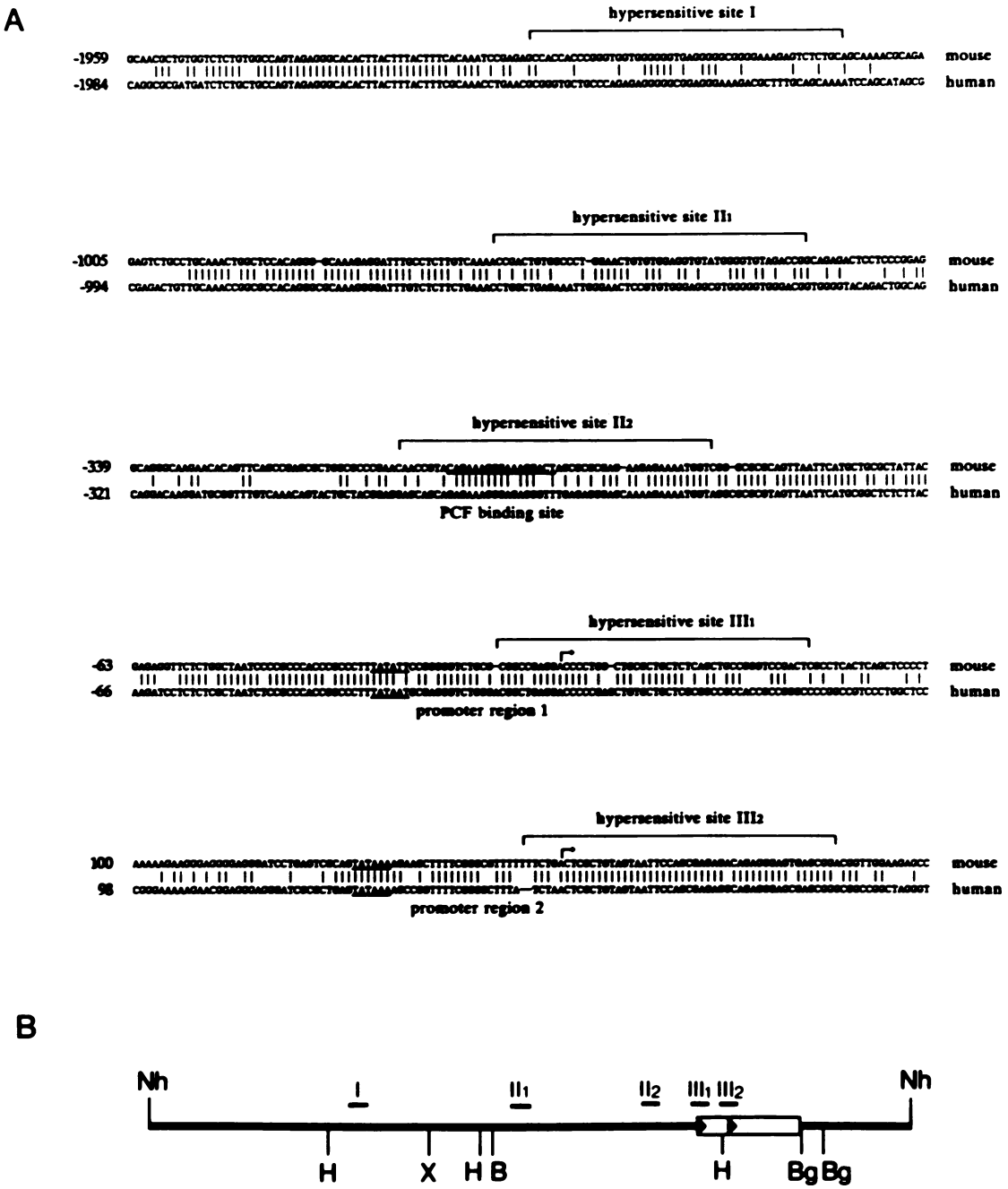

FIG. 7. Sequences associated with the DNase I-hypersensitive sites. (A) Sequences of the mouse c-*myc* gene preferentially cleaved by DNase I in isolated nuclei of P388D1 cells (see Fig. 6) are shown aligned with the corresponding sequences of the human c-*myc* gene. Vertical bars between the sequences mark regions of identity. Numbering is relative to the RNA start site of promoter P1. The binding site for plasmacytoma-specific factor PCF (29) maps to hypersensitive site II_2 (underlined region). Within each of the promoter elements, the TATA box is underlined and an arrow marks the initiation site for transcription. (B) The position of each sequence segment in panel A is indicated by the bar over the restriction map of the mouse c-*myc* 5′-flanking region. Restriction enzyme abbreviations are as given in the legend to Fig. 6.

to previous studies with mouse plasmacytomas (20, 30), we find that the gene is not methylated and is not insensitive to general DNase digestion. This implies that the gene is not condensed into inactive chromatin or chemically modified so as to exclude transcription factors. The absence of all hypersensitive sites in the tumor cells, however, suggests that transcription factors, though apparently present and functional in the cells, are not bound to the upstream region of the c-*myc* gene. If the down-regulation of the germ line c-*myc* gene is being actively suppressed by a repressor molecule, then the complete absence of hypersensitive sites is surprising, since one might have expected to find a suppression-specific hypersensitive site that represented the binding site of the repressor. Of course, suppression may well be due to a repressor, but the binding site(s) are not marked by a hypersensitive site. Alternatively, some global

mechanism other than methylation or chromatin condensation may be responsible. Models concerning such mechanisms, however, must be able to accommodate the observation that the weak upstream promoter P0 remains active in Burkitt lymphoma cells, in which P1 and P2 are silent (7).

The positions, and hence sequences, of the hypersensitive sites that we have mapped in the active alleles differ significantly from those described previously, although the pattern itself is the same. This result may be due to a species-specific difference or to the location of the restriction sites and indirect end-labeled fragment used in our analysis, which created small subbands that could be mapped with high resolution. The sequences at or adjacent to the hypersensitive sites are among the most highly conserved sequences (between mouse and human genomes) of the 5′-flanking region of the c-*myc* gene.

When the data presented here and from other laboratories are considered in toto, it remains unclear how the c-*myc* gene is suppressed in tumor cells. None of the mechanisms that are usually associated with suppression (such as methylation or condensed chromatin) appear to contribute to maintaining the inactive state. Furthermore, since reintroduced c-*myc* genes are invariably expressed and, as shown here, inducible, factors capable of activating the c-*myc* promoter are still present. How are these factors prevented from activating the endogenous gene? It appears that c-*myc* suppression may involve some novel mechanism that requires sequences outside of the immediate promoter or perhaps some currently unrecognized feature of the c-*myc* chromosomal domain.

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LITERATURE CITED

1. **Adams, J., S. Gerondakis, E. Webb, L. M. Corcoran, and S. Cory.** 1983. Cellular *myc* oncogene is altered by chromosome translocation to the immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc. Natl. Acad. Sci. USA **80**:1982–1986.
2. **Armelin, H. A., M. C. S. Armelin, K. Kelly, T. Stewart, P. Leder, B. Cochran, and C. D. Stiles.** 1984. Functional role for c-*myc* in mitogenic response to platelet-derived growth factor. Nature (London) **310**:655–660.
3. **ar-Rushdi, A., K. Nishikura, J. Erikson, R. Watt, G. Rovera, and C. Croce.** 1983. Differential expression of the translocated and untranslocated c-*myc* oncogene in Burkitt lymphoma. Science **222**:390–393.
4. **Baumbach, W. R., E. J. Keath, and M. D. Cole.** 1986. A mouse c-*myc* retrovirus transforms established fibroblast cell lines in vitro and induces monocyte-macrophage tumors in vivo. J. Virol. **59**:276–283.
5. **Baumbach, W. R., E. R. Stanley, and M. D. Cole.** 1987. Induction of clonal monocyte-macrophage tumors in vivo by a mouse c-*myc* retrovirus: rearrangement of the CSF-1 gene as a secondary transforming event. Mol. Cell. Biol. **7**:664–671.
6. **Bentley, D. L., and M. Groudine.** 1985. A block to elongation is largely responsible for decreased transcription of c-*myc* in differentiated HL60 cells. Nature (London) **321**:702–706.
7. **Bentley, D. L., and M. Groudine.** 1986. Novel promoter upstream of the human c-*myc* gene and regulation of c-*myc* expression in B-cell lymphomas. Mol. Cell. Biol. **6**:3481–3489.
8. **Bravo, R., M. Neuberg, J. Buckhardt, J. Almendral, R. Wallich, and R. Müller.** 1987. Involvement of common and cell type-specific pathways in c-*fos* gene control: stable induction by cAMP in macrophages. Cell **48**:251–260.
9. **Cedar, H.** 1988. DNA methylation and gene activity. Cell **53**:3–4.
10. **Chung, J., E. Sinn, R. R. Reed, and P. Leder.** 1986. Trans-acting elements modulate expression of the c-*myc* gene in Burkitt lymphoma cells. Proc. Natl. Acad. Sci. USA **83**:7918–7922.
11. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
12. **Cleveland, J. L., M. Huleihel, P. Bressler, U. Siebenlist, L. Akiyama, R. N. Eisenman, and U. R. Rapp.** 1988. Negative regulation of c-*myc* transcription involves *myc* family proteins. Oncogene Res. **3**:357–375.
13. **Cole, M. D.** 1986. The *myc* oncogene: its role in transformation and differentiation. Annu. Rev. Genet. **13**:361–384.
14. **Coppola, J. A., and M. D. Cole.** 1986. Constitutive c-*myc* oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. Nature (London) **320**:760–763.
15. **Croce, C. M., J. Erikson, A. ar-Rushdi, D. Aden, and K. Nishikura.** 1984. Translocated c-*myc* oncogene of Burkitts lymphoma is transcribed in plasma cells and repressed in lymphoblastoid cells. Proc. Natl. Acad. Sci. USA **81**:3170–3174.
16. **Dani, C., J. M. Blanchard, M. Piechaczyk, S. E. Sabouty, L. Marty, and P. Jeanteur.** 1984. Extreme instability of *myc* mRNA in normal and transformed human cells. Proc. Natl. Acad. Sci. USA **81**:7046–7050.
17. **Dani, C., N. Mechti, M. Piechaczyk, B. Lebleu, P. Jeanteur, and J. M. Blanchard.** 1985. Increased rate of degradation of c-*myc* mRNA in interferon-treated Daudi cells. Proc. Natl. Acad. Sci. USA **82**:4896–4899.
18. **Dean, M., R. A. Levine, W. Ran, M. S. Kindy, G. E. Sonenshein, and J. Campisi.** 1986. Regulation of c-*myc* transcription and mRNA abundance by serum growth factors and cell contact. J. Biol. Chem. **261**:9161–9166.
19. **Dony, C., M. Kessel, and P. Gruss.** 1985. Post-transcriptional control of *myc* and p53 expression during differentiation of the embryonal carcinoma line F9. Nature (London) **317**:636–639.
20. **Dunnick, W., J. Baumgartner, L. Fradkin, C. Schultz, and P. Szurek.** 1985. Methylation of plasmacytoma c-*myc* genes. Gene **39**:287–292.
21. **Dyson, P. J., T. D. Littlewood, A. Forster, and T. H. Rabbitts.** 1985. Chromatin structure of transcriptionally active and inactive human c-*myc* alleles. EMBO J. **4**:2885–2891.
22. **Dyson, P. J., and T. H. Rabbitts.** 1985. Chromatin structure around the c-*myc* gene in Burkitt lymphomas. Proc. Natl. Acad. Sci. USA **82**:1984–1988.
23. **Fahrlander, P. D., M. Piechaczyk, and K. B. Marcu.** 1985. Chromatin structure of the c-*myc* locus: implications for the regulation of normal and chromosomally translocated genes. EMBO J. **4**:3195–3202.
24. **Feo, S., A. ar-Rushdi, K. Huebner, J. Finan, P. C. Nowell, B. Clarkson, and C. M. Croce.** 1985. Suppression of the normal mouse c-*myc* oncogene in human lymphoma cells. Nature (London) **313**:493–495.
25. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of mouse 3T3 cells induces transcription of the c-*fos* oncogene. Nature (London) **311**:433–438.
26. **Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51**:975–985.
27. **Hay, N., J. M. Bishop, and D. Levens.** 1987. Regulatory elements that modulate expression of human c-*myc*. Genes Dev. **1**:659–671.
28. **Jones, T. J., and M. D. Cole.** 1987. Rapid cytoplasmic turnover of c-*myc* mRNA: requirement of the 3′ untranslated sequences. Mol. Cell. Biol. **7**:4513–4521.
29. **Kakkis, E., and K. Calame.** 1987. A plasmacytoma-specific factor binds the c-*myc* promoter region. Proc. Natl. Acad. Sci. USA **84**:7031–7035.
30. **Kakkis, E., J. Prehn, and K. Calame.** 1986. An active chromatin structure acquired by translocated c-*myc* genes. Mol. Cell. Biol. **6**:1357–1361.

31. **Keath, E. J., P. G. Caimi, and M. D. Cole.** 1984. Fibroblast lines expressing activated c-*myc* oncogenes are tumorigenic in nude mice and syngeneic animals. Cell **39**:339–348.
32. **Keath, E., A. Kelekar, and M. D. Cole.** 1984. Transcriptional activation of the translocated c-*myc* oncogene in mouse plasmacytomas: similar RNA levels in tumor and proliferating normal cells. Cell **37**:521–528.
33. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
34. **Knight, E., Jr., E. D. Anton, D. Fahey, B. K. Friedland, and G. J. Jonak.** 1985. Interferon regulates c-*myc* gene expression in Daudi cells at the post-transcriptional level. Proc. Natl. Acad. Sci. USA **82**:1151–1154.
35. **Lachman, H. M., and A. I. Skoultchi.** 1984. Expression of c-*myc* changes during differentiation of mouse erythroleukemia cells. Nature (London) **310**:592–594.
36. **Leder, P., J. Battey, G. Lenoir, C. Moulding, W. Murphy, H. Potter, T. Stewart, and R. Taub.** 1983. Translocations among the antibody genes in human cancer. Science **222**:765–771.
37. **Lipp, M., R. Schilling, S. Wiest, G. Laux, and G. W. Bornkamm.** 1987. Target sequences for the cis-acting regulation within the dual promoter of the human c-*myc* gene. Mol. Cell. Biol. **7**:1393–1400.
38. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper free defective retrovirus. Cell **33**:153–159.
39. **Mitchell, R. L., L. Zokas, R. D. Schreiber, and I. M. Verma.** 1985. Rapid induction of the expression of proto-oncogene *fos* during human monocytic differentiation. Cell **40**:209–217.
40. **Moore, J. P., J. A. Tood, T. R. Hesketh, and J. C. Metcalfe.** 1986. c-*fos* and c-*myc* gene activation, ionic signals, and DNA synthesis in thymocytes. J. Biol. Chem. **261**:8158–8162.
41. **Müller, R., T. Curran, D. Müller, and L. Guilbert.** 1985. Induction of c-*fos* during myelomonocytic differentiation and macrophage proliferation. Nature (London) **314**:546–548.
42. **Nepveu, A., and K. B. Marcu.** 1986. Intragenic pausing and anti-sense transcription within the murine c-*myc* locus. EMBO J. **5**:2859–2865.
43. **Piechaczyk, M., Y.-Q. Yang, J.-M. Blanchard, P. Jeanteur, and K. B. Marcu.** 1985. Posttranscriptional mechanisms are responsible for accumulation of truncated c-*myc* RNAs in murine plasmacytoma cells. Cell **42**:589–597.
44. **Rabbits, P. H., A. Forester, M. A. Stinson, and T. H. Rabbits.** 1985. Truncation of exon 1 from the c-*myc* gene results in prolonged c-*myc* mRNA stability. EMBO J. **4**:3727–3733.
45. **Remmers, E. F., J.-Q. Yang, and K. B. Marcu.** 1986. A negative transcriptional control element located upstream of the murine c-*myc* gene. EMBO J. **5**:899–904.
46. **Schuler, G. D., and M. D. Cole.** 1988. GM-CSF and oncogene mRNA stabilities are independently regulated in trans in a mouse monocytic tumor. Cell **55**:1115–1122.
47. **Siebenlist, U., P. Bressler, and K. Kelly.** 1988. Two distinct mechanisms of transcriptional control operate on c-*myc* during differentiation of HL60 cells. Mol. Cell. Biol. **8**:867–874.
48. **Siebenlist, U., L. Hennighausen, J. Battey, and P. Leder.** 1984. Chromatin structure and protein binding in the putative regulatory region of the c-*myc* gene in Burkitt lymphoma. Cell **37**:381–391.
49. **Stanton, L. W., R. Watt, K. B. Marcu.** 1983. Translocation breakage and truncated transcripts of c-*myc* oncogene in murine plasmacytomas. Nature (London) **303**:401–406.
50. **Taub, R., C. Moulding, J. Battey, W. Murphy, T. Vasicek, G. M. Lenoir, and P. Leder.** 1984. Activation and somatic mutation of the translocated c-*myc* gene in Burkitt lymphoma cells. Cell **36**:339–348.
51. **Tramontano, D., W. W. Chin, A. C. Moses, and H. I. Sidney.** 1986. Thyrotropin and dibutyryl cyclic AMP increase levels of c-*myc* and c-*fos* mRNAs in cultured rat thyroid cells. J. Biol. Chem. **261**:3919–3922.
52. **Weintraub, H.** 1985. Assembly and propagation of repressed and derepressed chromosomal states. Cell **42**:705–711.
53. **Westin, E. H., F. Wong-Staal, E. P. Gelman, R. Dalla-Favera, T. S. Papas, J. A. Lautenberger, A. Eva, E. P. Reddy, S. R. Tronick, S. A. Aaronson, and R. C. Gallo.** 1982. Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79**:2490–2494.
54. **Wu, C.** 1980. The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. Nature (London) **286**:854–860.
55. **Zinn, K. D., D. DiMaio, and T. Maniatis.** 1983. Identification of two distinct regulatory regions adjacent to the human -interferon gene. Cell **34**:865–879.