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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

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A mouse retrovirus containing the c-*myc* oncogene was found to induce tumors of mononuclear phagocytic cells in vivo. All tumors expressed the c-*myc* retroviral gene but not the endogenous c-*myc* gene (with one exception), and virtually all tumors were clonal with a unique proviral integration. This observation, coupled with a lag time in tumor formation, suggests that a second event, in addition to c-*myc* proviral integration, is necessary for the generation of neoplastic cells in vivo. All of the tumor cells expressed high levels of mRNA for both the putative colony-stimulating factor 1 (CSF-1) receptor (c-*fms* proto-oncogene product), as well as the c-*fos* proto-oncogene. Although all of the tumor cells proliferated in culture without the addition of exogenous CSF-1, which is required for the proliferation of primary macrophages partially transformed by the same c-*myc* retrovirus, several phenotypes were observed with respect to the expression of CSF-1 and granulocyte-macrophage CSF and to their growth factor responsiveness. The proliferation of one tumor, which secreted high levels of CSF-1, was blocked by specific anti-CSF-1 serum. This tumor was found to express altered CSF-1 mRNA and to have a DNA rearrangement at the CSF-1 locus. In this particular case, the data indicate that a CSF-1 gene rearrangement was the secondary event in development of the tumor. The pleiotropy of phenotypes among the other tumors indicated that there are a variety of other mechanisms for such secondary events which can be investigated with this system.

Activation of the c-*myc* proto-oncogene has been linked to neoplastic transformation in many different systems, although the primary targets are cells of the hemopoietic lineage. The c-*myc* gene can be activated by proviral insertion in lymphomas (12, 23, 51), by chromosomal translocation in plasmacytomas (1, 35, 44) and Burkitt lymphomas (16, 52), and, less frequently, by gene amplification (10, 14, 34). Several avian *myc* retroviruses have been isolated which induce primarily myelocytomatosis (reviewed in reference 55). In addition, high levels of normal c-*myc* RNA are induced in lymphocytes and fibroblasts by mitogens (28). A central issue of neoplastic transformation is the relative contributions of c-*myc* activation and other genetic changes in the development of malignant tumors in vivo.

Different biological activities have been ascribed to activated c-*myc* oncogenes. Studies of avian *myc* retrovirus-infected cells have shown morphological transformation of fibroblasts and macrophages in vitro, but these cells are neither immortalized nor tumorigenic (39). Conversely, when cotransfected with activated *ras* oncogenes, c-*myc* is capable of inducing the establishment of secondary fibroblasts morphologically transformed by *ras* (31) and hence has been linked to immortalization. In support of this, transfection of activated *myc* genes into secondary fibroblasts has been shown to provide a long-term growth potential (38, 41). More recently, we have shown that established fibroblast lines expressing activated c-*myc* genes were induced to become tumorigenic but were not morphologically transformed (26). Thus, c-*myc* genes appear to have both transforming and immortalizing activities, and the expression of either of these activities may depend on the presence of other genetic changes or on the lineage and stage of differentiation of the target cell.

Although activated c-*myc* genes have been found in several types of hemopoietic tumors, transformation has frequently been found to involve late developmental stages within each lineage. Both plasmacytomas and Burkitt lymphomas are derived from mature B cells, in contrast to pre-B cells, which are transformed by Abelson murine leukemia virus (MuLV) (46). Mature cells of the monocytic lineage also serve as target cells for *myc*-containing virus transformation in avian bone marrow; again, this constrasts with the more immature cells of the myeloid lineage transformed by v-*myb* (reviewed in reference 19). Thus, a second important question that emerges is why oncogenic transformation in vivo and in vitro is dependent on a particular developmental stage for different oncogenes.

In this study, we characterized monocyte-macrophage tumors induced by a murine retrovirus which contains the c-*myc* oncogene (6). The tumors were found to be clonal, they expressed the c-*myc* provirus, and they could be directly established in culture. Molecular characterization of oncogene expression in the tumor cells provided insight into the stage specificity of transformation, and evidence is presented for specific secondary events in transformation. In one case, the secondary event was shown to be a rearrangement of the CSF-1 gene, resulting in inappropriate expression of colony-stimulating factor 1 (CSF-1) and autocrine growth.

MATERIALS AND METHODS

Tumor lines and virus. The monocyte-macrophage tumors in this study were induced by intraperitoneal infection of BALB/c mice with a mouse c-*myc* retrovirus (6). Tumor

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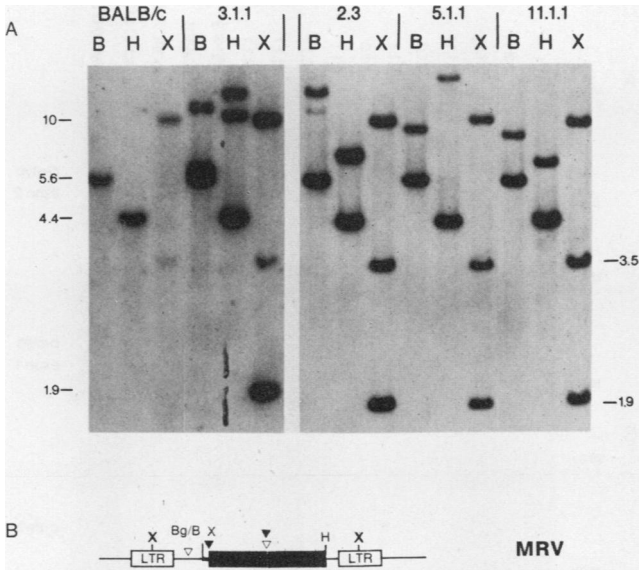

FIG. 1. (A) Southern blots of DNA from monocyte-macrophage tumors showing a single c-*myc* provirus in each line. The DNAs were digested with *Bam*HI (B), *Hin*dIII (H), or *Xba*I (X), electrophoresed on 0.7% agarose gels, and hybridized to a probe from c-*myc* exon 2 (1.4-kb *Sac*I-*Sac*I). The endogenous c-*myc* bands at 10.0 kb (*Xba*I), 5.6 kb (*Bam*HI), and 4.4 kb (*Hin*dIII) are indicated. The 1.9-kb *Xba*I band in each tumor line derives from the identical proviruses. The 3.5-kb *Xba*I band arises from cross-hybridization between a (CA)20 sequence in the probe and a repeated element in mouse DNA. Only two bands of hybridization are found with *Hin*dIII and *Bam*HI. (B) Restriction enzyme map of the c-*myc* provirus (6).

cells were derived from the ascites fluid and plated in RPMI 1640 medium supplemented with 10% fetal calf serum at 2×10^6 cells per 10-cm plate. The cells immediately adhered loosely to the plates and were passaged by being scraped with a rubber policeman or being triturated with medium. The tumor cells had a doubling time in culture of 18 to 20 h. Tumor lines 7.1.3 and 9.1.1 were derived from helper-free virus infection of spleen cells, which were subsequently injected intraperitoneally. BM-M8 is a line of bone-marrow-derived macrophages partially transformed by a helper-free stock of the c-*myc* retrovirus and propagated for 2 months with L-cell-conditioned medium. The morphology and growth rate of these cells are indistinguishable from those of the tumor lines. RNA and DNA were extracted and analyzed as described previously (26). Tumor RNA and DNA were extracted from ascites fluid after the tumor cells were pelleted.

Cell lines were obtained from the following sources: $\psi 2$, R. Mulligan (Whitehead Institute); P388D1 and WEHI-3, American Type Culture Collection, Rockville, Md.; 32D and FDCP1, J. Pierce (National Cancer Institute). The 32D and FDCP1 cells were maintained in RPMI 1640 medium supplemented with 15% fetal calf serum (GIBCO Laboratories) and 20 or 5% WEHI-3 conditioned medium, respectively.

Growth factor analysis. Murine L cell CSF-1 was purified as described previously (49). Its purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions and by complexing with a goat anti-CSF-1 antibody. The CSF-1 concentration was determined by radioimmunoassay (48). Antiserum to purified murine L cell CSF-1 was raised in a goat as described previously (49). The production of granulocyte macrophage (GM)-CSF and interleukin-3 (IL-3) was assayed by plating 32D or FDCP1 cells at 10^5/ml in 24-well trays in the presence of 20 or 5% conditional medium from various cell lines, respectively. After 3 days, 1.0 μCi of [^{3}H]thymidine was added overnight, and trichloroacetic acid-precipitable counts were determined. 32D cells respond to IL-3 (24), and FDCP1 cells respond to IL-3 or GM-CSF (15). Conditioned medium (CM) from WEHI-3 cells was used as a positive control for each line. The only CM that induced a positive growth response was from tumor 2.3 (see Table 2), which stimulated FDCP1 cells but not 32D cells approximately as well as WEHI-3 CM did.

Tumor cells were analyzed for anchorage-independent growth by being suspended at 6.7×10^3/ml in 0.26% agar (Difco Laboratories) over a 0.65% agar lower layer. Soft-agar cultures were plated in RPMI 1640 medium supplemented with 20% fetal calf serum and CM, growth factors, or antiserum, where indicated. Colonies were counted 1 to 3 weeks after being seeded.

Cloning of the mouse CSF-1 locus. The mouse CSF-1 locus was cloned from a bacteriophage lambda library of plasmacytoma DNA by using a synthetic 49-nucleotide probe derived from the sequence of a human CSF-1 cDNA (25). The sequence used was AAGGAGGTGTCGG AGCACTGTAGCCACATGATTGGGAATGGACACCTAG, which includes some changes from the human sequence to account for differences in the mouse protein (25). The phage containing the CSF-1 locus was plaque purified, and the 1.0-kilobase (kb) *Bam*HI fragment containing the region of homology to the oligonucleotide probe was subcloned into pUC18. This fragment was then used as a probe for both DNA and RNA. The same CSF-1-specific bands that were detected with this genomic probe were also detected with the oligonucleotide probe (data not shown). A cDNA clone of the granulocyte-macrophage CSF (18) was used to assay GM-CSF mRNA levels.

RESULTS

Induction of clonal monocytic tumors. We have shown recently that a murine retrovirus containing the c-*myc* oncogene induces ascites tumors with a latency period of 8 to 10 weeks (6). Morphological (data not shown) and biochemical (6) characterization demonstrated that the tumor cells are mature members of the monocyte-macrophage hemopoietic lineage. Cells derived from the ascites fluid of c-*myc* retrovirus (MRV)-infected mice could be passaged intraperitoneally through non-pristane-treated mice or grown directly in culture with no media supplements beyond 10% fetal calf serum.

Analysis of c-*myc* proviral sequences in DNA from the ascites cells confirmed the involvement of the virus in the neoplastic transformation and indicated that nearly all of the tumors were clonal. DNA was extracted directly from ascites cells and subjected to Southern analysis to determine the frequency of proviral integration within the tumor cell populations. Using a c-*myc* exon 2 probe, we found one novel *myc*-containing restriction fragment in 12 of 14 tumors, with a unique junction fragment in each tumor (Fig. 1). Conversely, digestion with *Xba*I gave an identical *myc* fragment of 1.9 kb for each tumor (Fig. 1), which was consistent with the provirus structure (Fig. 1B) (6). Only one of the tumors gave submolar proviral bands with single-cleavage enzymes, but it gave the expected proviral fragment with *Xba*I (data

not shown), indicating a possible multiclonal origin. In addition, one cell line derived from a single soft-agar colony (3.1.1) displayed two exogenous c-*myc* proviruses (Fig. 1).

DNA from the tumor cells was analyzed further for the presence of an activated transforming gene by using the NIH 3T3 cell focus formation assay. None of the tumor DNAs tested induced foci (not shown), which indicated primarily that no *ras* oncogenes were activated in these tumors, given the preferential detection of mutated *ras* genes in this assay.

RNA expression in the mononuclear phagocytic tumor cells. RNAs from the MRV-induced tumor lines were analyzed by using several oncogene probes to examine the expression of cellular genes that are associated with the control of cell proliferation. For comparison, control cell lines were included which represent known differentiative stages of murine hemopoiesis. The myelomonocytic leukemia WEHI-3 cell line represents a stage prior to commitment to either granulocytic or mononuclear phagocytic pathways (37), and P388D1 is a murine macrophage cell line (30). To distinguish between mRNA levels of the endogenous and exogenous c-*myc* genes, we probed identical filters with nick-translated fragments corresponding to c-*myc* exon 1 or 2. Both the viral and cellular *myc* RNAs contain exon 2, while only the endogenous transcripts contain the noncoding exon 1. All cell lines expressed high levels of c-*myc*, but the levels were lower than or, at most, equal to those found in plasmacytomas or *myc*-transformed fibroblasts (Fig. 2A). The MRV-induced tumor lines expressed the 2.5- and 3.0-kb RNAs that are consistently found wth the c-*myc* retrovirus construct (6). WEHI-3 and P388D1 expressed the normal 2.4-kb *myc* transcript. In contrast, none of the MRV-induced tumor lines expressed detectable endogenous c-*myc* transcripts, with the exception of 9.1.1, which had a low but significant level (Fig. 2B). The latter line consistently exhibited lower levels of mature macrophage-specific markers, indicating an earlier stage of differentiation (4).

We also examined the expression of three other cellular proto-oncogenes, c-*myb*, c-*fos*, and c-*fms*, which have been linked to different stages of myeloid cell differentiation. For c-*myb*, only WEHI-3, which is at the earliest stage of differentiation of the lines tested here, displayed evidence of transcriptional activity (Fig. 2C). On the other hand, high constitutive levels of c-*fos* mRNA were uniformly observed in all of the MRV-induced tumors (Fig. 2D) and were observed in some lines at many times the level found in the macrophage line P388D1 or in WEHI-3, in which virtually no c-*fos* RNA was found. It is necessary to examine c-*fos* protein synthesis to determine whether the gene is subject to translational control. The c-*fms* gene, which is likely to encode the receptor for the growth factor CSF-1 (45), is also transcriptionally active in all of the c-*myc* retrovirus-induced tumor lines (Fig. 2E). The expression of the c-*fms* gene by the tumor cells is consistent with their characterization as mononuclear phagocytes, since the CSF-1 receptor or c-*fms* mRNA or both have been shown to be specifically expressed at high levels in mononuclear phagocytes and at substantially lower levels in more primitive cells (4, 5, 8, 20, 42). Correspondingly, a lower level of c-*fms* expression was found in WEHI-3 cells, but P388D1 displayed mRNA levels which are comparable to the levels seen in the c-*myc* transformed macrophages. All of the lines were also found to express high levels of p53 mRNA (not shown).

CSF-1 expression in the monocyte tumors. Certain growth characteristics of the c-*myc* retrovirus-induced tumors suggested that an altered growth factor response might play a role in tumorigenesis. In particular, the tumor cells could be established in culture directly from the animal, while, in contrast, mouse macrophages transformed by *myc* viruses in vitro require supplementation of the culture medium with CSF-1 for growth (6, 54). This result prompted an examination of the monocyte-macrophage tumors for the production of CSF-1 or IL-3 (or multi-CSF) or both and GM-CSF (36). Growth factor production was analyzed by testing tumor cell CM or by using Northern blots. Extracellular levels of CSF-1 were determined by radioimmunoassay (48), and GM-CSF and IL-3 were assayed by using cell lines dependent upon these growth factors (15, 24). Four of the tumor lines were found to secrete moderate to high levels of CSF-1, with two of the lines (9.1.1 and 7.1.3) producing as much of the factor as L cells (Table 1). Other tumors produced low but detectable levels. One tumor line (2.3) was found to secrete both CSF-1 and GM-CSF, while none of the lines produced IL-3. Since mononuclear phagocytes do not produce CSF-1 in vitro (53), production of high levels of this factor by tumor cells could lead to autocrine growth.

Northern blot analysis was used to further investigate

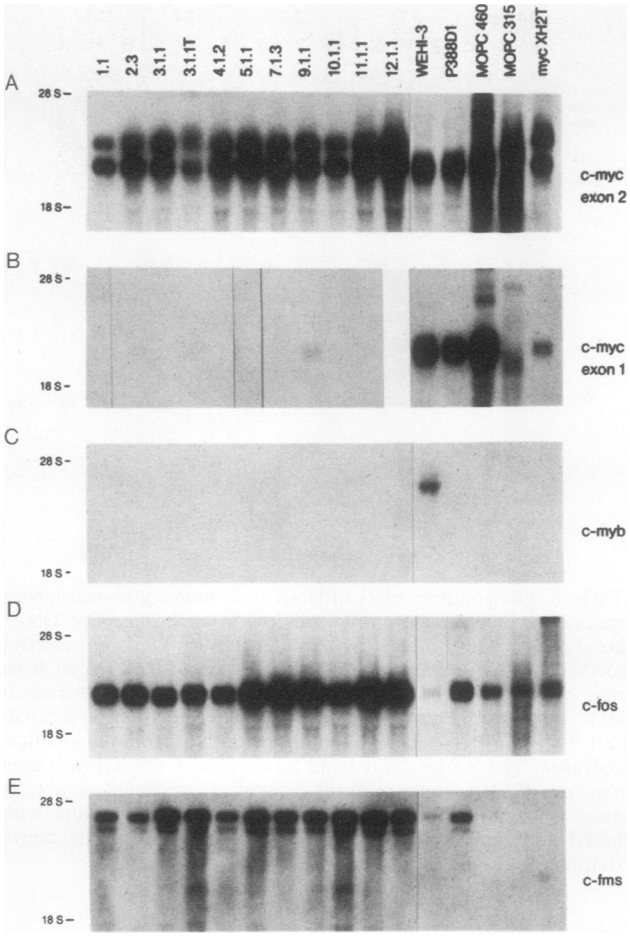

FIG. 2. Northern blots of monocyte-macrophage tumor RNAs with cellular oncogene probes. (A) c-*myc* exon 2 (0.98-kb *Xba*I-*Sac*I); (B) c-*myc* exon 1 (0.45-kb *Bam*HI-*Bgl*II); (C) v-*myb* (1.3-kb *Kpn*I-*Sac*I) (29); (D) v-*fos* (1.0-kb *Pst*I) (13); (E) v-*fms* (1.6-kb *Pst*I from pSM3) (21). For comparison, RNA is also included from WEHI-3, a monomyelocytic cell line; P388D1, a macrophage tumor line; MOPC 315, a plasmacytoma with a c-*myc* translocation (44); and *myc*XH2-T, a nude-mouse tumor induced by transfection of the pEVX-XH *myc* construct into NIH 3T3 cells (26).

TABLE 1. Hemopoietic growth factor production by c-*myc* retrovirus-induced monocyte-macrophage tumors

Tumor line	Amt of CSF-1 produced (U/ml)[a]	Production of[b]:	
		GM-CSF	IL-3
1.1	72	–	–
2.3	257	+	–
3.1.1	221	–	–
4.1.2	70	–	–
5.1.1	137	–	–
7.1.3	1,496	–	–
9.1.1	2,391	–	–
10.1.1	18	–	–

[a] CSF-1 production was assayed from medium conditioned by confluent tumor cells in RPMI 1640 medium for 2 days. CSF-1 concentrations were determined on triplicate sets of CM by radioimmunoassay (48); 1 U = 0.44 fmol of CSF-1 protein.

[b] Production of the hemopoietic growth factors GM-CSF and IL-3 was assayed as described in Materials and Methods.

CSF-1 production in the tumors. The mouse genomic CSF-1 locus was cloned by using an oligonucleotide probe derived from the sequence of a human CSF-1 cDNA (25; see Materials and Methods). A coding region probe was then used to examine the production of CSF-1 mRNA in the tumor cells. L cells were found to transcribe two CSF-1 mRNAs (4.3 and 2.3 kb), with the 4.3-kb species being more abundant (Fig. 3A). The level of CSF-1 mRNA in the tumors was found to correspond well to the amount of factor secreted into the medium, with the exception of the level in tumor line 9.1.1. This tumor line produced the highest levels of the factor, yet had only a moderate level of CSF-1 mRNA (discussed in more detail below). Notably, tumor line 7.1.3 had an altered pattern of CSF-1 RNA, with extremely abundant 2.3-kb RNA and a lower level of 4.3-kb RNA. The production of high levels of CSF-1 RNA and protein is not a direct consequence of c-*myc* retrovirus infection or of culturing the tumor cells, because in vitro-transformed macrophages grown for a similar length of time in culture (in the presence of CSF-1) did not express CSF-1 RNA (Fig. 3, lane BM-M8).

DNA rearrangement at the CSF-1 locus in tumor 7.1.3. The abundant and altered CSF-1 expression in 7.1.3 suggested the possibility that transcription from the gene was enhanced as a result of a DNA rearrangement in the tumor cells. A fragment from the CSF-1 gene was used to probe a Southern blot of tumor and normal cellular DNAs (Fig. 3B). DNA from 7.1.3 was found to contain an altered *Eco*RI restriction fragment when compared with DNAs from normal cells and tumors which had no altered CSF-1 RNAs. Southern analysis of the primary tumor DNA also exhibited the same *Eco*RI rearrangement (data not shown). A preliminary restriction map of the mouse CSF-1 locus shows that the rearrangement occurs approximately 8 kb from the segment used as the probe (Fig. 3C). Consistent with this map, a probe from the rearranged region of the CSF-1 locus detected altered fragment sizes with other restriction enzymes (data not shown). The relationship between the observed DNA rearrangement, the exon structure of the mouse CSF-1 locus, and the altered CSF-1 expression in 7.1.3 is currently under investigation.

Growth factor responsiveness of tumor cells. Growth factor independence could arise through different mechanisms such as autocrine growth factor production, mutations in the growth factor receptor yielding a constitutive signal, or mutations that activate the growth signal pathway. Although all of the monocyte-macrophage tumors exhibited autologous growth in culture, we observed four different phenotypes when individual lines were tested for anchorage-independent growth in response to their own CM, purified CSF-1, and anti-CSF-1 serum (Table 2). Tumor line 2.3, which produces GM-CSF and a low level of CSF-1, was stimulated both by CM and, even more dramatically, by purified CSF-1, but the response to CM was not blocked by anti-CSF-1 serum. This raises the possibility that 2.3 is autocrine for GM-CSF and that the low level of CSF-1 produced is not important for tumor cell growth. In contrast to 2.3, the majority of the tumor lines, such as 3.1.1, which produced very low levels of CSF-1 (Table 1; Fig. 3A), grew to a significant degree in the absence of CM, did not respond to purified CSF-1, and were not inhibited by anti-CSF-1

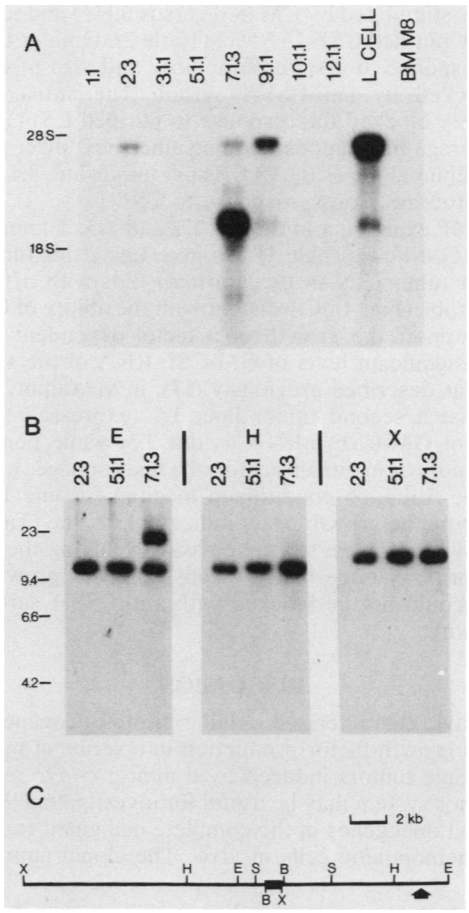

FIG. 3. (A) CSF-1 RNA synthesis in c-*myc* retrovirus-induced monocyte-macrophage tumors. Poly(A)$^+$ RNA was analyzed as for Fig. 2, by using a fragment from the CSF-1 coding region (*Bam*HI-*Bam*HI in panel C) as a hybridization probe. BM-M8 is a line of bone-marrow-derived macrophages partially transformed in vitro by the c-*myc* retrovirus and propagated for 2 months in the presence of CSF-1-supplemented medium. (B) DNA rearrangement at the CSF-1 locus in tumor line 7.1.3. The CSF-1 coding-region probe shown in panel C was hybridized to Southern blots of monocyte tumor DNAs cleaved with the indicated restriction enzymes. DNA rearrangement was found with this probe only in tumor line 7.1.3. (C) Restriction enzyme map of the mouse CSF-1 locus derived from an isolated clone and from digestion of genomic DNA. The black bar indicates the fragment which hybridizes to the oligonucleotide probe from the protein coding region. The arrow indicates the approximate position of the DNA rearrangement in tumor line 7.1.3.

serum (Table 2). Thus, a secondary event other than autocrine growth factor production may be responsible for these tumors, and the low level of CSF-1 produced is apparently not required for growth. Tumor 9.1.1 exhibited a distinctly different phenotype in that it failed to respond to purified CSF-1 and growth was not blocked by anti-CSF-1 serum, even though this line produced the highest level of CSF-1 in the medium. However, the growth of 9.1.1 was stimulated by CM (Table 2). One interpretation of this response is that 9.1.1 produces a growth factor in addition to the three tested or produces an altered form of CSF-1. In this respect, it is important to note that 9.1.1 is a more primitive cell of the monocyte lineage with lower levels of nonspecific esterase, lysozyme synthesis, and phagocytosis (6).

The data obtained for tumor 7.1.3, which produces high levels of CSF-1 as a consequence of the DNA rearrangement described above, are particularly interesting. The growth of 7.1.3 was stimulated by CM (8.0 versus 0.8%) and to a lesser extent by purified CSF-1 (2.5%) (Table 2). Greater than 80% of the response to CM (both number and size of colonies) was blocked by anti-CSF-1 serum. The antiserum also completely blocked the response to purified CSF-1 to yield an anchorage-independent cloning efficiency lower than that with medium alone (<0.01%). Thus, tumor line 7.1.3 exhibits an autocrine growth response to CSF-1.

GM-CSF expression in tumors 1.1 and 2.3. Tumor line 2.3 secretes GM-CSF (Table 1). To investigate this further, we analyzed tumor RNAs by Northern blot with a GM-CSF cDNA probe (Fig. 4). Consistent with the ability of CM from 2.3 to support the growth of a factor-dependent line, we found a significant level of GM-CSF RNA of the same size (1.2 kb) as described previously (17), in the tumor. We also found that a second tumor line, 1.1, expressed the same amount of GM-CSF mRNA as did 2.3, while none of the other tumors or in vitro-transformed macrophages expressed the gene. Although conditioned medium for line 1.1 could not support the growth of an indicator cell line (Table 1), it was active in a more sensitive assay involving the stimulation of primary bone marrow cells, and this growth factor activity could not be blocked with anti-CSF-1 serum (data not shown).

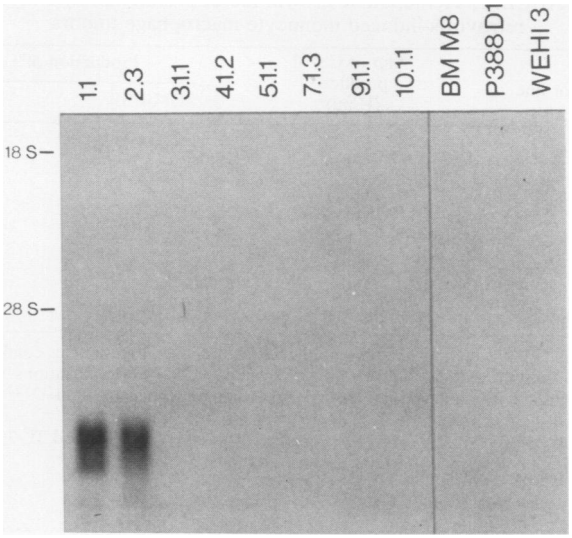

FIG. 4. Expression of granulocyte-macrophage CSF mRNA in the monocyte tumors. Tumor RNAs were analyzed by Northern blot and hybridized to a GM-CSF probe. The 1.2-kb GM-CSF mRNA is evident in tumor lines 1.1 and 2.3 but not in other tumors or in in vitro c-*myc* transformed macrophages.

DISCUSSION

We have characterized cellular proto-oncogene expression and growth factor production in a series of monocyte-macrophage tumors induced by a mouse c-*myc* retrovirus. This tumor system may be useful for investigating the role of individual oncogenes in the complete malignant transformation of hemopoietic cells in vivo. The clonal nature of the tumors and the characteristic lag time in tumor formation are consistent with the requirement for a second event for complete transformation. In particular, we have identified a novel rearrangment of the mononuclear phagocyte growth factor CSF-1 gene which is likely to represent the principal secondary event for complete transformation in one tumor.

TABLE 2. Anchorage-independent growth response of monocyte tumor lines to CM and blocking by antiserum to CSF-1

Addition[a]	Growth response of tumor line[b]:			
	7.1.3	2.3	9.1.1	3.1.1
None	0.8	0.3	1.1	7.5
CM (20% vol/vol)	8.0	2.5	16.0	12.6
CM + preimmune serum	5.9	2.0	13.5	11.7
CM + anti-CSF-1 antiserum	1.6[c]	2.5	9.1	9.7
CSF-1 (2,000 U/ml)	2.5	13.0	0.6	3.8
CSF-1 + anti-CSF-1 antiserum	<0.01[c]	1.4	0.4	4.6

[a] Tumor cells were plated in 0.26% agar with Dulbecco modified Eagle medium and 10% fetal calf serum plus the indicated additional reagents.

[b] Values represent the percentage of the cells plated which formed colonies of >30 cells.

[c] Colony size was reduced dramatically.

An intriguing question is that of what determines the stage specificity of the c-*myc* retrovirus-induced tumors. One explanation, which we consider unlikely, is the relative susceptibility of different cell stages to virus infection. Alternatively, the characterization of cellular oncogene expression offers a possible model for the *myc*-induced tumors as being mature monocytic phagocytes. We suggest that the deregulation of c-*myc* expression (in the form of the *myc* provirus) may influence only the control of proliferation when the cells reach a stage in which the endogenous c-*myc* gene is inactive, e.g., in terminally differentiated or quiescent cells. Several lines of evidence support this proposal. First, the exogenous c-*myc* provirus is expressed at a level not significantly different from that of the endogenous gene in cells with no apparent c-*myc* activation, such as WEHI-3 and P388D1. Thus, transformation does not seem to be the result of exceptionally high levels of c-*myc* protein, although actual protein levels have not yet been analyzed. Second, the endogenous c-*myc* gene is not expressed in the tumor cells, similar to the findings for plasmacytomas and Burkitt lymphomas (1, 3, 50). It has been shown that in several studies of myeloid and other cell types, induction of terminal differentiation and cessation of proliferation are accompanied by dramatic decreases in c-*myc* expression (9, 40, 56). Thus, a deregulated c-*myc* gene may influence growth preferentially in cells approaching terminal differentiation, which become quiescent and inactivate the endogenous gene (or vice versa).

It should be pointed out that there is another possible explanation for the lack of endogenous c-*myc* expression, namely that the normal c-*myc* gene may be shut off owing to repression by an activated c-*myc* gene (33). However, we recently showed that the constitutive expression of an

exogenous c-*myc* gene does not alter the regulation or expression of the endogenous gene in fibroblast or mouse erythroleukemia lines expressing similar c-*myc* constructs (11, 26, 27). Furthermore, the 9.1.1 tumor cell line expressed the endogenous c-*myc* gene at a low level, even though the exogenous, activated c-*myc* gene was expressed at the same level as in the other lines (Fig. 2A and B). Thus, the activity of the endogenous gene in this line correlates with its less differentiated phenotype as measured by several stage-specific parameters (6), and it is likely that the control of normal c-*myc* expression is linked primarily to the developmental stage.

One of the most striking findings of the present study was the difference in growth factor dependence between the *myc*-induced myeloid tumors and the partially transformed macrophages derived from in vitro infection. Evidence from several systems indicates that deregulation of c-*myc* expression is not sufficient to relieve the requirement for exogenous growth factors in monocytes. For example, macrophages partially transformed by the c-*myc* retrovirus must be cultured in L cell CM, which provides CSF-1 (6), and macrophages transformed in vitro by a v-*myc* retrovirus exhibit a similar phenotype (54). Moreover, avian tumor cells transformed in vivo by *myc*-containing retroviruses still require a growth factor for in vitro propagation (2), although this requirement can be eliminated by infection with a second oncogenic virus.

Different phenotypes were observed among the mononuclear phagocyte tumor lines with respect to response to, and production of, monocyte-specific growth factors, and these different phenotypes are consistent with alternative secondary events in tumor induction. For one tumor line, 7.1.3, we have identified a distinct secondary genetic change involving the CSF-1 gene. The DNA rearrangement at the CSF-1 locus, the high level of growth factor production (1,500 U/ml), and the inhibition of proliferation by anti-CSF-1 serum suggest that the secondary event in this tumor was the activation of CSF-1 expression in a monocyte that had been partially transformed by the c-*myc* virus. Other tumor lines were found to produce low levels (20 to 250 U/ml) of CSF-1, and the failure to inhibit the growth of these lines with anti-CSF-1 serum and the inability of CM from these lines to induce proliferation of primary bone marrow cultures (data not shown) suggest that this level is not essential for tumor cell growth. Lines 1.1 and 2.3, but not control cells or other tumors, expressed GM-CSF, making it possible that these tumors are autocrine for this second myeloid lineage-specific growth factor.

The discovery of autocrine stimulation in the development of tumors in vivo appears to be unique. Autocrine mechanisms have been suggested for tumor growth in several systems (reviewed in reference 47). Furthermore, several studies of nontumorigenic cell lines that are dependent on specific factors for in vitro growth have shown that acquisition of the ability to produce the required growth factor leads to a malignant phenotype (7, 22, 32, 43). However, it seems likely that many kinds of secondary changes can promote malignant transformation of partially transformed macrophages. For example, if autocrine production of CSF-1 or GM-CSF are possible events, alterations that lead to constitutive activation of the receptors would be expected to yield a similar growth stimulation. In some cases, a macrophage growth factor other than the three tested (CSF-1, GM-CSF, and IL-3) might be produced. Alternatively, the receptor to a growth factor naturally produced by macrophages (e.g., platelet-derived growth factor) might be activated. Furthermore, if autocrine stimulation of a growth factor receptor can promote tumor cell growth, other lesions could occur in the pathway mediating the transmission of the receptor signal through the cytoplasm to the nucleus. Because of the variety of tumor phenotypes, further investigation into this tumor system should provide additional insight into the multiple steps involved in malignant transformation in vivo.

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