Transfection of mouse erythroleukemia cells with \textit{myc} sequences changes the rate of induced commitment to differentiate

\textbf{(antisense RNA/metallothionein promoter)}

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\textbf{ABSTRACT} We have examined the role of the c-\textit{myc} protooncogene in chemically induced differentiation of mouse erythroleukemia (MEL) cells by transfecting the cells with recombinant plasmids in which \textit{c-myc} coding sequences were cloned downstream from the mouse metallothionein I promoter in sense and antisense orientations. We previously showed that treatment of MEL cells with inducer of differentiation leads to a rapid (<2 hr) decrease in the level of c-myc mRNA. c-myc mRNA is then transiently restored to pretreatment levels ~12–18 hr later. These events occur prior to the detection of cells that are irreversibly committed to erythroid differentiation. MEL cell transfectants containing the plasmid with \textit{myc} in the sense orientation express a chimeric MT-\textit{myc} mRNA, which also decreases shortly after addition of inducer. However, these clones reexpress \textit{myc} mRNA more rapidly than the parental line and they also differentiate more rapidly. On the other hand, transfectants containing the plasmid with \textit{myc} in the antisense orientation exhibited a delay in the reexpression of c-myc mRNA and were found to differentiate more slowly than parental cells. Thus, we find a correlation between the time at which \textit{myc} RNA is reexpressed following inducer treatment and the rate of entry of cells into the terminal differentiation program.

We have been studying the expression of the c-\textit{myc} protooncogene in mouse erythroleukemia (MEL) cells. These cells are capable of unlimited proliferative potential \textit{in vitro} and are tumorigenic in mice (1). Upon treatment with a variety of chemical inducing agents such as hexamethylenobisacetamide (HMBA) or dimethyl sulfoxide (Me₂SO), the cells reinitiate a program of erythroid differentiation culminating in terminal cell divisions and extensive hemoglobin accumulation (2). The continuous presence of an inducer is not required for terminal differentiation of MEL cells. Following a latent period of inducer treatment lasting ~18 hr, an increasing number of cells becomes irreversibly committed to differentiate (3, 4). The commitment process results in irreversible changes in the pattern of erythroid-specific gene expression. For example, accumulation of globin mRNA, which is due to increased transcription of the globin genes (5), proceeds in the absence of inducer once commitment occurs. The molecular mechanisms that lead to the irreversible commitment of MEL cells are not well understood.

We have previously reported that the level of c-myc mRNA undergoes rapid changes in the period before MEL cells become committed (6, 7). Within 1–2 hr following the addition of HMBA or Me₂SO, c-myc mRNA levels decrease by a factor of ~10. At about 12–18 hr of treatment, c-myc mRNA is transiently reexpressed to a level similar to that found in uninduced cells. After 24–30 hr, c-myc mRNA levels decrease again and remain low during terminal differentiation. These changes in c-myc mRNA levels, occurring during the first 18 hr of inducer treatment, are seen before cells can be detected in the culture that are irreversibly committed to a program of terminal erythroid differentiation. The timing of these changes led us to consider the possibility that the early decline in c-myc RNA and its subsequent reexpression may be important events in the decision of a MEL cell to enter the differentiation program. To test this possibility we have introduced additional copies of c-myc coding sequences driven by heterologous transcription signals into MEL cells, and we have examined the ability of the transfected cells to undergo the commitment to terminal differentiation.

\textbf{METHODS}

Cell Culture and Transfection Protocol. MEL cells (clone DS19) were grown as described (6). Cells (10⁶) were transfected with 50 μg of \textit{myc} recombinant plasmids and 5 μg of pSV2neo (8) linearized with \textit{EcoRI}, by electroporation as described by Potter et al. (9). Transfectants were selected in medium containing 2 mg of geneticin (G418) per ml.

RNA Analysis. Total cellular RNA was isolated by the hot phenol extraction method of Soeiro and Darnell (10). RNA transfer blot analysis was performed as described (6). Endogenous and transfected mRNAs were analyzed simultaneously using an RNase mapping procedure. We cloned a 700-base-pair \textit{Pat I} restriction fragment from pMC-myc54 (11) into pGEM-1. Following digestion of pGEM-myc with \textit{Hind III}, a 606-base ³²P-labeled RNA probe was generated with \textit{T7} RNA polymerase. Labeled probe (2–5 × 10⁵ cpm) was hybridized with 20 μg of total cellular RNA in 30 μl of 80% formamide/40 mM Pipes, pH 6.4/1 mM EDTA/0.4 M NaCl at 45°C for 12–14 hr. The mixture was treated with 50 μg of RNase A per ml and 100 units of RNase T1 per ml at 30°C for 30 min. The digestions were terminated by treatment with 100 μg of proteinase K and 0.5% NaDodSO₄ for 15 min at 37°C. RNA-RNA hybrids recovered by ethanol precipitation were denatured in 90% formamide at 95°C and fractionated through 5% acrylamide/7 M urea gels at 100–125 V for 12 hr.

\textbf{Commitment Assay}. Cells maintained in medium with 400 μg of G418 per ml were passaged twice in the absence of G418 and treated with 5 mM HMBA. Cells in logarithmic growth were treated with 5 mM HMBA and incubated at 37°C in 10% CO₂ in air. At various time intervals, 15 μl of culture (1000–2000 cells) was added to plates containing 1.5 ml of a mixture of Dulbecco's modified Eagle's medium, 15% fetal bovine serum, and 1.5% methylcellulose without HMBA. After incubation for 5 days at 37°C, cell differentiation was scored by benzidine staining (4).

Abbreviations: MEL, mouse erythroleukemia; HMBA, hexamethylenobisacetamide; MT, metallothionein; Me₂SO, dimethyl sulfoxide.
RESULTS

Introduction of MT-myc Recombinants into MEL Cells. To express the mouse c-myc gene from heterologous transcription signals, we constructed an expression vector, pMT-myc (Fig. 1), in which c-myc coding sequences were cloned 65 bases from the transcription start site of the mouse metallothionein (MT)-1 gene (12). To minimize the inclusion of potential c-myc regulatory sequences, we omitted almost all of the 5' and 3' untranslated regions of the c-myc cDNA (13). Though some mammalian cell clones transfected with MT recombinants exhibit varying degrees of heavy metal inducibility of the chimeric mRNA, many clones express the mRNA constitutively in the absence of heavy metals (12). MEL cells were cotransfected by electroporation with pSV2neo (8) and pMT-myc. Twenty independent G418-resistant clones were isolated. We identified seven cotransfectants that incorporated pMT-myc by Southern blot analysis (data not shown). Most clones contained 1–2 copies of pMT-myc but clone 6 contained about 10 copies.

We also transfected MEL cells with a plasmid in which myc coding sequences were placed in the antisense orientation with respect to the MT-1 promoter. Six independent clones containing ≈2–20 copies of the pMT-myc (antisense) DNA were identified by Southern blot analysis (data not shown).

Expression of myc mRNAs in Transfected Cells. The relative level of expression of the transfected MT-myc gene was evaluated by hybridization with a uniformly labeled RNA probe that detects the MT-myc chimeric mRNA and the endogenous c-myc mRNA. The RNA probe was generated by T7 RNA polymerase transcription from a recombinant pGEM-1 vector containing a segment of myc coding sequences (see Methods). A 600-base 32P-labeled RNA that spans the myc coding sequences from the HindIII site in exon 1 to the PstI site in exon 2 (Fig. 2, Lower) was synthesized. After hybridization and RNAse digestion the expected 32P-labeled protected fragments are 538 bases long, derived from endogenous c-myc mRNA, and 185 bases long, derived from the MT-myc chimeric mRNA. The 5' end of the 538-base protected fragment maps 24 bases 3' of the HindIII site. Fig. 2 shows the results obtained by hybridization with total cellular RNA from parental MEL cells and seven pMT-myc transfecteds. All of the samples contain the expected 538-base protected fragment derived from the endogenous gene. There is also a band of relatively low intensity, 562 bases long extending to the HindIII, that is derived from endogenous c-myc RNA initiated at an upstream promoter previously described (13–15). In addition, there is an intense band at 410 bases, which is probably not due to excessive RNAse digestion, since it was present in samples digested with smaller amounts of RNAse. We have not investigated further the origin of this band; it may be due to a polymorphism between MEL cell c-myc RNA and the probe derived from mouse plasmacytoma myc sequences.

In addition to the common bands seen in all samples, hybridization with RNA from the seven transfected lines containing pMT-myc DNA produced the 185-base protected fragment expected from reaction with MT-myc mRNA. The relative levels of MT-myc mRNA in the transfected clones were estimated by densitometric analysis of autoradiograms in the linear exposure range. Since the RNA probe used was uniformly labeled, the intensities of the bands must be normalized for the different fragment lengths. By comparing the intensities of the 185-base bands to that of the 538- and 410-base protected fragments we estimate that the levels of MT-myc mRNA ranged from 15% (Fig. 2, lane 6) to 150% (Fig. 2, lane 2) of endogenous c-myc mRNA.

To examine the expression of the MT-myc gene in inducer-treated transfected clones, we analyzed the levels of MT-myc and c-myc mRNAs with the RNAse protection assay at various time intervals after HMBA treatment. Fig. 3A shows the results of this analysis in the parental MEL cell line. As we reported previously, the level of c-myc mRNA decreases by a factor of ≈10 shortly after HMBA treatment and remains low until 12–18 hr, when the mRNA begins to accumulate again. Fig. 3 B–D show the results obtained with three pMT-myc transfected clones. These lines also exhibited a rapid decline in c-myc mRNA. In addition, the level of MT-myc mRNA expressed from the transfected gene is also markedly reduced following the addition of HMBA. However, in contrast to the parental cells, which begin to reexpress c-myc mRNA at ≈12 hr, the transfected clones reexpressed either MT-myc mRNA or c-myc and MT-myc
mRNAs at earlier times. In clone 6, Fig. 3B, MT-myc mRNA begins to be reexpressed after only 4 hr of HMBA treatment and is restored to preinduction levels after only 8 hr. Other clones (Fig. 3 C and D) showed a coordinate reexpression of c-myc and MT-myc mRNAs beginning at 8 hr of HMBA treatment. Thus, in contrast to the relatively low level of c-myc mRNA present in parental MEL cells at 8 hr of HMBA treatment, the transfectant clones contain significant amounts of c-myc encoding mRNA at this time. The increased levels are due to a more rapid reexpression of either MT-myc mRNA or a combination of MT-myc and c-myc mRNAs. These data also indicate that the separation of c-myc coding sequences from putative 5' regulatory sequences is not sufficient to confer completely autonomous regulation of c-myc gene expression.

We also analyzed expression of myc RNAs in cells that had been transfected with pMT-myc (antisense), in which the myc coding sequences were inserted in the antisense orientation with respect to the MT-1 promoter. To analyze for expression of an antisense myc RNA we synthesized a 32P-labeled RNA complementary to antisense myc RNA by transcribing the pGEM-myc recombinant plasmid described above in the opposite direction with SP6 RNA polymerase. Following hybridization and RNase digestion we were unable to detect any antisense RNA in uninduced cells that had incorporated pMT-myc (antisense) DNA. However, when RNA isolated from such cells after treatment with HMBA for 8–15 hr was analyzed, we did detect very low levels of the antisense RNA (data not shown). Note that at these times of HMBA treatment the level of c-myc mRNA is normally quite low. Since the MT-1 promoter is capable of producing high levels of sense myc transcripts, the failure to detect antisense myc transcripts from the MT-1 promoter in most samples suggests that such transcripts are extremely unstable in MEL cells. Perhaps the instability is due to the binding of antisense myc RNA to excess c-myc RNA. This explanation is consistent with our finding that small amounts of antisense transcripts could be detected in inducer-treated cells when c-myc RNA levels were very low.

Even though it was difficult to detect transcripts from the pMT-myc (antisense) plasmid, we investigated potential effects of its expression on c-myc mRNA levels. Fig. 4 shows an RNA transfer blot analysis of c-myc mRNA in two pMT-myc (antisense) transfectants treated for various lengths of time with HMBA. These clones also showed a rapid decline in c-myc mRNA following HMBA treatment. However, in contrast to parental MEL cells and pMT-myc transfectants, c-myc mRNA reexpression did not occur until 23–31 hr. These data suggest that transfection of MEL cells with pMT-myc-antisense DNA results in a significant delay in the reexpression of c-myc mRNA. We cannot adequately explain why transfection with pMT-myc (antisense) DNA affects the expression of c-myc mRNA in inducer-treated cells, whereas there is no apparent effect on c-myc mRNA levels in uninduced cells. Perhaps feedback mechanisms control the level of c-myc expression more effectively in uninduced cells than in inducer-treated cells.

**Effect of myc Transfection on the Commitment to Differentiate.** Treatment of sense and antisense myc transfectants with HMBA for 5 days resulted in 90% differentiated cells in most lines. To examine more carefully the differentiation program of these cells we analyzed the kinetics of accumulation of committed cells following HMBA treatment by plating the cells in semisolid medium without the inducer. In this way the fate of individual cells treated for various times with inducer can be followed, allowing one to determine the proportion of cells that have undergone an irreversible commitment event leading to terminal erythroid differentiation (3). Cells that are irreversibly committed give rise to small colonies with 4–32 cells, all of which stain positively for hemoglobin with the benzidine reagent. Uncommitted cells produce large colonies of several hundred cells, most of which are benzidine negative.

In cultures of the parental MEL cells, an increase in the percentage of committed cells is first detected between 18 and 24 hr of HMBA treatment (Fig. 5A). With increasing times of exposure to HMBA there is a gradual increase in the percentage of committed cells, such that by 36 hr nearly all of the cells have become committed. The duration of the latent period, defined as the time before an increase in

**Fig. 3.** Effect of HMBA treatment on c-myc and MT-myc mRNA levels. Total cellular RNA was extracted from parental cells (A) and pMT-myc transfectant clones 6, 3, and 2 (B, C, and D, respectively) that had been treated with 5 mM HMBA for the indicated period of time. The RNAs were analyzed by the RNase protection assay. The 538- and 410-nucleotide protected fragments are derived from c-myc mRNA and the 185-nucleotide fragment is produced from MT-myc mRNA.

![Fig. 3](image)

**Fig. 4.** RNA transfer blot analysis of c-myc mRNA levels in pMT-myc (antisense) transfectants. Total cellular RNA was extracted from pMT-myc antisense clones 3 (A) and 4 (B) that had been treated with 5 mM HMBA for the indicated period of time. Twenty micrograms of total cellular RNA was fractionated in 0.9% agarose gels containing 3% formaldehyde. The RNA was transferred to nitrocellulose filters and hybridized with a 32P-labeled Xho I fragment from pMC-myc 34 (11). The positions of the 28S and 18S rRNA bands in the gel were visualized by staining with ethidium staining.

![Fig. 4](image)
committed cells is detected, and the kinetics of accumulation of committed MEL cells shown here are similar to those found by other investigators (3, 4).

When we subjected the pMT-myc transfectants to this analysis, a striking pattern emerged. Six of seven of these clones exhibited a decrease in the latent period, resulting in more rapid commitment kinetics compared with the parental line. Relatively high percentages of committed cells were detected in most transfectant cultures after only 12–18 hr of HMBA treatment. Cultures of parental MEL cells required about 29 hr of HMBA treatment to accumulate 50% committed cells, whereas with one exception the pMT-myc transfectants produced 50% committed cells after 17–27 hr of treatment. We obtained similar results in three independent commitment experiments. The more rapid commitment kinetics exhibited by these clones was not due simply to the transfection and G418 selection procedures. We have isolated and analyzed 21 pSV2neo transfectants that did not receive exogenous myc DNA. Among these clones only four lines were found with commitment kinetics more rapid than the parental MEL cells. However, as shown below, these clones reexpress c-myc mRNA earlier than parental cells. In contrast to the rapid kinetics of commitment observed with pMT-myc transformants, four of six pMT-myc (antisense) clones exhibited delayed commitment kinetics compared with parental MEL cells (Fig. 5B).

Thus, we find a correlation between the timing of reexpression of myc RNA following inducer treatment and the rate of entry of cells into the terminal differentiation program. pMT-myc transfectants, which reexpress MT-myc mRNA or MT-myc and c-myc mRNAs between 4 and 8 hr of inducer treatment when c-myc mRNA is normally low in the parental MEL cells, exhibited significantly more rapid commitment kinetics. In contrast, pMT-myc antisense transfectants, which have a delay in reexpression of c-myc mRNA, exhibited delayed accumulation of committed cells.

Further support for the correlation between early reexpression of myc RNA and rapid commitment was obtained by studying the kinetics of c-myc mRNA reexpression in two G418-resistant subclones that spontaneously exhibited relatively rapid commitment but did not contain pMT-myc DNA. These clones had a latent period of only 12 hr in HMBA before committed cells were detected (data not shown). RNA transfer blot analysis showed that these clones exhibited the early decline in c-myc mRNA but, like the pMT-myc transfectants, they reexpressed c-myc mRNA after only 8 hr of HMBA treatment (Fig. 6). Thus, clones that spontaneously exhibit rapid commitment also reexpress c-myc mRNA earlier than the parental line.

**DISCUSSION**

Clonal analysis of MEL cell populations undergoing differentiation has shown that in vitro differentiation includes a latent period of about 18 hr, during which irreversible changes in cell fate do not occur, followed by a second phase in which cells become irreversibly committed and then can complete differentiation in the absence of the inducer (3, 4). We have previously shown that the c-myc mRNA level drops by a factor of ~10 shortly after addition of an inducer and then rises again to pretreatment levels at 18 hr, just before committed cells can first be detected in the culture (6). This suggests a possible dual role for c-myc in which it exerts positive and negative effects at different stages of the commitment program. According to this model, if myc expression was made to be constitutive at early times, so that it did not decline immediately after inducer treatment, MEL cell differentiation might be inhibited. On the other hand, if restoration of myc levels at about 18 hr is an important event in the preparation of cells for terminal differentiation, then altering the timing of myc reexpression might be expected to change the kinetics of differentiation in the same direction. Recently, Coppola and Cole (16) reported that constitutive expression of a simian virus 40-promoted c-myc gene results in the inhibition of differentiation, supporting the view that myc can have a negative effect on differentiation.

To investigate the potential effects of myc expression on MEL cell differentiation we introduced additional copies of myc coding sequences driven by a MT-1 transcription unit. However, analysis of chimeric mRNA levels following inducer treatment showed that, like c-myc mRNA, it also rapidly declined following HMBA treatment. Thus, with this particular construct, we were not able to study the effect of constitutive myc expression on differentiation. Nevertheless, we did find that, in contrast to the parental MEL cells, which reexpress c-myc mRNA 12–18 hr after the addition of HMBA, these transfectants reexpressed the chimeric mRNA

![Fig. 5. Commitment assay of pMT-myc and pMT-myc antisense transfectants. Cells in logarithmic growth were treated with 5 mM HMBA. At the indicated times, 1000–2000 cells were plated in growth medium containing 1.5% methylcellulose without HMBA. After 5 days of incubation the cultures were stained with the benzidine reagent and 100 colonies were scored by inverted phase microscopy. The percentage of colonies derived from committed cells was determined by comparing the number of benzidine-positive colonies to the total number of colonies scored. The commitment curve for the parental line DS19 is depicted by the solid lines in A and B. (A) pMT-myc clones 1 (c), 2 (c), 3 (e), 4 (o), 5 (o), 6 (a), 7 (•). (B) pMT-myc (antisense) clones 1 (o), 2 (c), 3 (c), 4 (e), 5 (c), and 6 (a).](image)

![Fig. 6. RNA transfer blot analysis of c-myc mRNA levels in rapidly committing MEL subclones. Total cellular RNA was extracted from cells treated with 5 mM HMBA for the indicated time. Electrophoresis and hybridization with a 32P-labeled myc probe were performed as described in the legend to Fig. 4.](image)
and, in some cases, c-myc mRNA as well, after only 4–8 hr of HMBA treatment. When these transfected lines were analyzed for the kinetics of differentiation, six of seven lines exhibited a significant reduction in the length of the latent period. The one exceptional clone in this series had the lowest level of MT-myc RNA among the transfected lines isolated. Thus, altering the timing of myc reexpression to earlier times appears to correlate with a shortening of the latent period, supporting the view that myc also can have a positive effect on the rate of differentiation. Two other lines of evidence support the correlation between the time of myc reexpression and the length of the latent period.

First, MEL cell lines were transfected with a plasmid in which myc coding sequences were placed in an antisense orientation with respect to the MT-1 promoter. Although it was difficult to detect the antisense transcripts, we did find that the reexpression of the endogenous c-myc mRNA was significantly delayed. With one exception these clones showed a significant shortening of the latent period. In fact, the exceptional clone in this series, which appears to differentiate more rapidly than the parental cells, did not exhibit delayed reexpression of c-myc mRNA (data not shown). Second, we isolated several pSV2neo transflectants that spontaneously exhibited a shortened latent period. Although these lines had not incorporated pMT-myc DNA, we found that c-myc mRNA was reexpressed in these lines after only 8 hr of HMBA treatment. We assume that these clones are spontaneously arising variants that are commonly found in cultured somatic cells. The behavior of these variants further supports the correlation between the timing of c-myc reexpression and the length of the latent period.

It is difficult to speculate on the mechanism by which the c-myc protooncogene might influence MEL cell differentiation because the function of the protein product is very poorly understood. c-myc is thought to belong to a class of nuclear proteins that may participate in determining a cell’s potential for proliferation (14, 17). Some of these proteins have been found to have positive and negative effects on the expression of other genes (18). Based upon our results and the results of Coppola and Cole (16), we suggest that c-myc may have positive and negative regulatory roles in the expression of subsets of genes that are necessary for commitment to occur in MEL cells. Recent experiments with other oncogenes have also suggested that they can, under certain circunstances, promote differentiation (19, 20).

It is also worth noting that the MT-myc chimeric mRNA is subject to rapid decline and reexpression during differentiation even though 5′ and 3′ untranslated regions of c-myc mRNA were omitted from the fusion gene. Recent experiments indicate that the transcription of c-myc measured in nuclear run-on experiments changes only by a factor of 2 during HMBA treatment, suggesting that posttranscriptional mechanisms determine changes in the level of c-myc mRNA in MEL cells (unpublished observations). Other studies have also suggested posttranscriptional regulation of c-myc mRNA levels in other cells (15, 21). The results with the MT-myc fusion gene suggest that part of the recognition signal for such posttranscriptional changes may reside in the coding sequences of c-myc mRNA.

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