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c-*myc* Protooncogene Expression in Mouse Erythroleukemia Cells

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Murine erythroleukemia (MEL) cells are erythroid progenitors whose program of erythroid differentiation has been interrupted by transformation with the Friend virus complex. As a result of the ability of certain chemicals such as dimethylsulfoxide (DMSO) to induce terminal erythroid differentiation, the cells have been used as a model for understanding the molecular basis of cellular differentiation. Recent work on MEL cells as well as other differentiating systems indicates that expression of cellular protooncogenes is implicated in chemically mediated differentiation. In MEL cells the expression of the c-*myc* protooncogene undergoes unusual biphasic changes following inducer treatment. Levels of c-*myc* mRNA decrease 10- to 20-fold between 1 and 2 hr and are then reexpressed between 12 and 24 hr. These changes occur as a result of complex transcriptional and posttranscriptional regulatory events. Recent DNA transfection experiments, in which MEL cells were transfected with *myc* expression vectors, indicate that both the early decrease in c-*myc* expression and its subsequent reexpression are important events in the differentiation pathway. The work on MEL cells, as well as on other models of differentiation, is directed at understanding the molecular basis of leukemogenic transformation and cellular differentiation. The ability of c-*myc*, as well as other protooncogenes, to influence both of these events indicates that cellular protooncogenes play a central role in their regulation.

Introduction

We have been interested in the role of protooncogenes, in particular the c-*myc* gene, in the differentiation of mouse erythroleukemia (MEL) cells. MEL cells are erythroid precursors that are arrested at an early stage of differentiation as a result of viral transformation. They were isolated by the late Charlotte Friend from mice infected with a virus derived from a cell-free tumor extract (*1*). Initially, morphological analysis suggested that the cells were either a myeloid or lymphoid leukemia line. However, the discovery that MEL cells contain hemoglobin suggested that they were erythroid in origin (*2*). In 1971, Dr. Friend and her colleagues at the Mt. Sinai School of Medicine made an important observation. During attempts at superinfecting the cells using dimethylsulfoxide (DMSO), they discovered that more than 95% of the cells stained positively for hemoglobin with benzidine reagent. By contrast, less than 1% of untreated MEL cells contain enough hemoglobin to become benzidine positive (*3*). Perhaps a more important observation was that DMSO-treated cells had reduced leukemogenic potential. Thus DMSO treatment results in the conversion of erythroleukemia cells, that are easily passaged between suitable mouse strains, to cells that exhibit a terminally differentiated phenotype. Since these observations were made, the MEL cell system has been used as a model for understanding the molecular basis of leukemic transformation and cellular differentiation.

Although it is not the purpose of this review to explore in much depth the hundreds of papers that have been published on this system, some important features that should serve as a background for the major concern of this paper, the role of the c-*myc* protooncogene in MEL cell differentiation, will be mentioned. For more specific details concerning biochemical events during differentiation and the nature of the various chemical agents used to induce differentiation, the reader is referred to the excellent Rifkind and Marks review (*4*).

Before discussing the role of cellular protooncogenes in differentiation, it is important to understand the basis of viral transformation of MEL cells. The Friend viral complex is composed of two retroviruses: a replication defective, spleen-focus-forming virus (SFFV) and a replication-competent murine leukemia virus (MuLV) (*5,6*). Although the Friend virus complex is capable of rapidly inducing leukemic transformation, it differs from other acutely transforming retroviruses in that neither SFFV nor MuLV contains transduced, cellular-derived oncogenes.

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The transforming ability of SFFV appears to reside in the *env* gene, which encodes a glycoprotein, gp55, found on the surface of infected cells. Mutations in gp55 are capable of abolishing the ability of SFFV to induce erythroleukemia (*7,8*). It is thought that the glycoprotein may be involved in sending a constitutive growth signal in infected cells (*9*), perhaps similar to retroviruses that contain transduced cellular derived oncogenes encoding growth factors or growth factor receptors, such as simian sarcoma virus (*10,11*) or avian erythroblastosis virus (*12,13*). The MuLV functions as a replication-competent helper virus but may also be involved in pathogenicity since it is capable, on its own, of inducing erythroid leukemias after a relatively prolonged latent period (*14*). Presumably, MuLV induces malignant transformation by proviral insertional mutagenesis of a cellular oncogene, similar to avian leukosis virus (*15*). Since SFFV also integrates as a provirus (*16*), it could function in this way as well. The cellular genomic targets of these integration events have not yet been identified.

Concept of Commitment

Typically when MEL cells are induced to differentiate, cells are treated with hexamethylene bisacetamide (HMBA) or DMSO for 5 days during which an arrest in cell growth and the accumulation of large amounts of hemoglobin are observed (*4,17*). However, 5 days of continuous inducer treatment is not needed for the cells to differentiate. If a culture is treated with inducer for a certain period of time, then plated in semisolid medium in the absence of inducer, individual cells will multiply into a colony whose phenotype reflects the capacity of the parent cell to differentiate. When MEL cells are treated with inducer for less than 10 hr, then plated without inducer, 100% of the colonies will be composed of benzidine negative, undifferentiated cells. However, after 10 to 12 hr of inducer treatment, a small percentage of cells gives rise, after 5 days of growth, to colonies of cells that are fully hemoglobinized and differentiated. The parent of the hemoglobinized colony of cells is said to be committed to erythroid differentiation (*18,19*). After 12 hr, an increasing percentage of cells becomes committed until approximately 48 hr when virtually 100% of the cells develop into differentiated colonies. Two important features of a committed cell include its restriction to about four or five cell divisions, whereas uncommitted cells will continue to proliferate indefinitely, and the irreversibility of the commitment program (*18*).

There are many intriguing questions one can ask regarding commitment of MEL cells to differentiate. For example, what determines the length of the latent period, which lasts for approximately 10 hr after inducer is added, before committed cells can be detected? What are the changes in gene expression that occur, during the latent and commitment periods, and how are these genes regulated? And finally, why do MEL cells become committed asynchronously such that some cells require 12 hr and others 48 hr of inducer treatment? It has been suggested that the commitment program develops stochastically with the probability that a cell will become committed increasing with prolonged inducer treatment (*18*). Data will be presented later in this review that demonstrate that the expression of the *c-myc* protooncogene could be involved in the decision of cells to become committed.

Early and Late Changes during the Differentiation Program

Rifkind and Marks have suggested that the changes that occur in MEL cells during chemically induced differentiation could be separated into early and late events (*4*), a distinction that is useful in presenting the differentiation process. A brief summary of some of the early changes that occur in inducer treated MEL cells include: increased Ca^{2+} uptake (*20*) and cAMP levels (*21*), a decrease in phosphatidylinositol turnover (*22*), and increased expression of some erythroid-associated proteins such as spectrin, glycophorin and histone H1° (*23-25*).

The importance of the changes in Ca^{2+} are supported by the findings that treatment of MEL cells with a Ca^{2+} ionophore increases the rate of inducer-mediated entry into a commitment program, whereas EGTA inhibits entry (*26*). The decrease in phosphotidylinositol turnover also appears to be an early critical event. The products of phosphotidylinositol breakdown, diacylglycerol (DAG) and inositol triphosphate (IP_3) are important intracellular mediators of the growth response. Protein kinase C is the target of DAG, whereas IP_3 mobilizes intracellular Ca^{2+} (*27-29*). The importance of the early decrease in DAG in the differentiation scheme is supported by the finding that treatment of MEL cells with DAG analogues inhibits differentiation (*22*). These early changes occur during the latent period of inducer treatment, before committed cells can be detected.

Late events in the differentiation process include the accumulation of globin mRNA and protein (*30-32*), enzymes involved in heme synthesis (*33*), and band 3, the transmembrane anion exchanger (*34*). Also occurring during this time are the chromatin and cytoskeletal changes that are associated with terminal erythroid differentiation (*35*). The increase in globin mRNA is due largely to an increase in globin gene transcription (*36,37*) which appears to be correlated with a change in globin chromatin structure manifested by the appearance of DNAse 1 hypersensitive sites in the β major gene locus (*38*). The increase in band 3 expression also results from an increase in band 3 gene transcription (*33*).

Arbitrarily separating the early and late events in time is a G1 prolongation in which cells accumulate in the G_1 phase of the cell cycle between 12 and 24 hr of inducer treatment (*39*). Although the timing of this event is correlated with the ability to detect the earliest committed cells, the importance of G_1 prolongation in the commitment process is not known.

c-*myc* Expression during MEL Cell Differentiation

The earliest direct evidence that oncogenes could be involved in leukemic transformation and cellular differentiation is from work with acutely transforming retroviruses containing viral oncogenes. A temperature-sensitive mutant of E26 leukemia virus, which contains the v-*myb* oncogene, induces leukemia at the permissive temperature. Following a switch to the nonpermissive temperature, infected cells spontaneously differentiate (*40*). This suggests that the expression of v-*myb* at the permissive temperature could inhibit the differentiation process and its inactivation at the nonpermissive temperature could switch the cells to a differentiated phenotype. Another finding that suggests that oncogenes have a role in differentiation is the observation that DMSO induction of the human promyelocytic leukemia cell line HL-60 to mature granulocytes is accompanied by a large decrease in c-*myc* expression (*41,42*). Similarly retinoic acid induction of F9 teratocarcinoma cells leads to a large decrease in c-*myc* expression (*43*). These findings suggest an association between the differentiation of cells with a decrease in the expression of certain protooncogenes. In other cases, the expression of oncogenes appears to promote differentiation. For example, PC-12 cells infected with *ras*-containing retroviruses or microinjected with a *ras* oncogenic protein spontaneously undergo neuronal differentiation, independent of nerve growth factor (*44,45*). Similarly, F9 teratocarcinoma cells transfected with the adenovirus E1A gene also undergo spontaneous differentiation (*46*).

Our work on the role of oncogenes in MEL cell differentiation began by screening MEL cell RNA with a variety of oncogene probes, which revealed that c-*myc* mRNA was easily detected in uninduced MEL cells and decreased about 10-fold in terminally differentiated cells. Although this result was similar to the findings in HL-60 cells, it was not terribly revealing since the level of many mRNAs decrease in terminally differentiated MEL cells. As we were interested in the relationship between commitment and the expression of oncogenes, we examined more carefully the expression of c-*myc* during the latent and commitment periods of inducer treatment. We found a curious pattern of expression. Within 2 hr of DMSO treatment there is a 10- to 20-fold decrease in c-*myc* mRNA. The level remains low until 12 to 24 hr, when the mRNA is transiently reexpressed to the level found in uninduced cells. Thereafter, c-*myc* mRNA levels decrease again as the cells differentiate (*47*) (Fig. 1). A particularly striking aspect of this observation was the temporal relationship between the early changes in c-*myc* mRNA levels and the detection of the earliest committed cells. It led us to consider the possibility that the changes in c-*myc* expression could be important in the commitment process. The changes in c-*myc* mRNA we observed initially appeared to be specific for this RNA in MEL cells and not due to a general effect of DMSO on RNA expression in MEL and other cells. For example, DMSO does not induce similar c-*myc* mRNA changes in a lymphoid line that does not differentiate in response to DMSO. Also, the level of β globin, actin, and histone H3 mRNAs did not undergo similar changes in MEL cells (*47*). Recently, however, we have detected some RNAs whose expression is similar to c-*myc*, so the effect is not as specific as we once thought. Biphasic c-*myc* expression occurs with other inducers of differentiation such as HMBA and hypoxanthine, although the kinetics of the changes may differ among different agents (*48*).

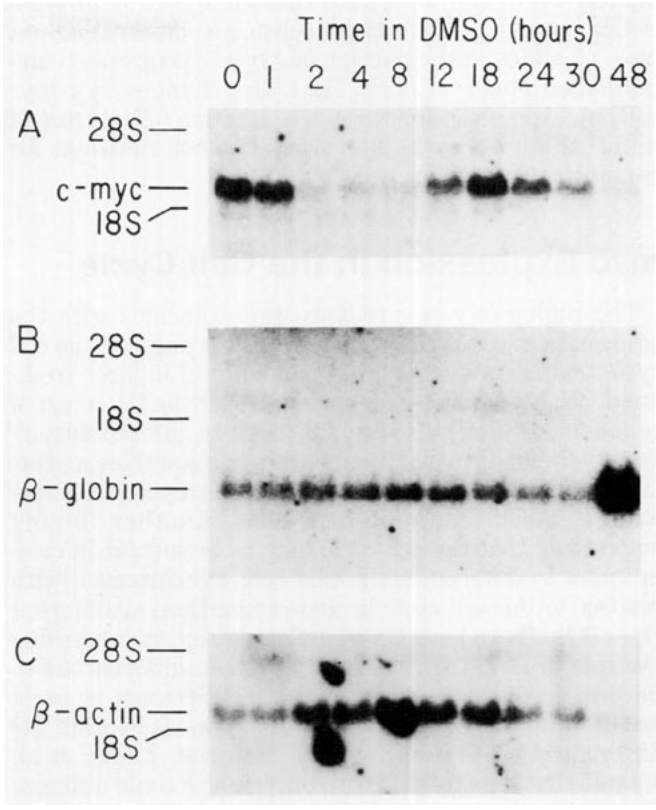

FIGURE 1. Changes in c-*myc* mRNA during inducer treatment. Total cellular RNA extracted from DMSO-treated cells was separated by gel electrophoresis, transferred to nitrocellulose filters, and hybridized with radiolabeled c-*myc*, β-globin, and β-actin-specific probes (Northern filter hybridization).

In order to determine whether the changes in c-*myc* expression are important for commitment and differentiation, we studied the effects of various inhibitors of MEL cell differentiation. We found that one potent inhibitor, cycloheximide, which inhibits *de novo* protein synthesis, completely blocks the reexpression of c-*myc* mRNA that occurs between 12 and 24 hr of inducer treatment, suggesting a correlation between inhibition of differentiation and the reexpression of c-*myc*. Baseline levels of c-*myc* mRNA in uninduced cells are unaffected by cycloheximide treatment. Two other inhibitors of differentiation, EGTA and phorbol esters, also affect the reexpression of c-*myc* mRNA, but not to the extent of cycloheximide. These agents delay *myc* reexpression such that the usual peak, which occurs at 16 hr, is not detected until approx-

imately 24 hr. However, two other inhibitors of differentiation, growth in 1% fetal calf serum and dexamethasone, have no effect on the pattern of c-*myc* reexpression (unpublished observations). Thus, the changes in c-*myc* mRNA expression following the addition of inducers of differentiation may be necessary but not sufficient for commitment to occur.

myc Expression in the Cell Cycle

The timing of c-*myc* reexpression coincides with the transient accumulation of cells in the G1 phase of the cell cycle that occurs between 12 and 24 hr of inducer treatment (*39,49*). It has been suggested that the G1 arrest of cells could be involved in commitment to differentiate, although the finding that the inducers hypoxanthine and actinomycin D do not prolong the G1 phase argues somewhat against this hypothesis (*50*). Another finding suggesting that the cell cycle may be important in commitment to differentiate is that cells synchronized with respect to the cell cycle become committed at different rates (*51*). There have been conflicting reports regarding the role of DNA synthesis in MEL cell differentiation. Several groups have reported that DMSO must be present for at least one round of DNA synthesis in order for differentiation to occur (*52,53*). However, Leder et al. showed that cells treated with butyric acid could differentiate in the absence of cell division (*54*), and Levenson et al. found that DNA synthesis is not required for commitment (*55*). In order to study the relationships between the cell cycle, *myc* expression, and its relationship to commitment, we measured c-*myc* mRNA levels in cells synchronized by centrifugal elutriation. In this technique, cells are separated into different cell cycle fractions based on their size, which is correlated with DNA content. We separated a logarithmic culture of uninduced MEL cells into cell-cycle-specific fractions, isolated total cellular RNA, and measured the relative level of c-*myc* mRNA in each fraction by Northern filter hybridization. As a control for the quality of the elutriation, we also measured histone H3 mRNA levels, because previous studies had shown that H3 expression in MEL cells primarily occurs in S phase cells (*56*). The analysis revealed that c-*myc* mRNA levels varied less than 2-fold across the cell cycle, in contrast to H3 mRNA in which there was 8- to 10-fold more H3 mRNA in S compared to G1 cells (*48*) (Fig. 2). These data agreed with the findings of several groups that expression of c-*myc* mRNA and protein in replicating cells is constitutive across the cell cycle (*57,58*).

A different pattern of c-*myc* expression is found in inducer-treated cells. MEL cells were separated into cell-cycle-specific fractions following treatment with hypoxanthine for 7 hr, a period of time sufficient for c-*myc* mRNA levels to decrease then reaccumulate in response to this particular inducer. In contrast to the absence of significant variation in c-*myc* mRNA levels across the cell cycle in uninduced cells, we detected 8-fold more c-*myc* mRNA in cells in G1 compared with cells in S (*48*) (Fig. 2).

The change in the cell cycle distribution of c-*myc* mRNA occurs without any change in H3 mRNA expression. The reexpression of c-*myc* mRNA in G1 cells following inducer treatment was confirmed using a different experimental approach with another inducer. Uninduced cells were separated into G1, S, and G2 fractions. The synchronized populations of cells were then treated with DMSO and aliquots were removed every 4 hr for total cellular RNA extraction. We also followed the cells' positions in the cell cycle by flow microfluorometry analysis of propidium iodide-stained cells. We found that the timing of c-*myc* mRNA reexpression was dependent on the cells' positions in the cell cycle when first exposed to inducer (*48*) (Fig. 3). For example, cells that were in S when first exposed to DMSO reexpress c-*myc* mRNA after only 8 hr, coinciding with the time of maximum accumulation of these cells in G_1. Cells that were in G_1 when DMSO was added reexpress c-*myc* mRNA between 12 and 16 hr, a time that corresponds to an accumulation of the cells in the next G_1 phase. Finally, cells that started in G_2 reexpress c-*myc* mRNA between 16 and 24 hr, a period of time corresponding to the passage of the cells through a com-

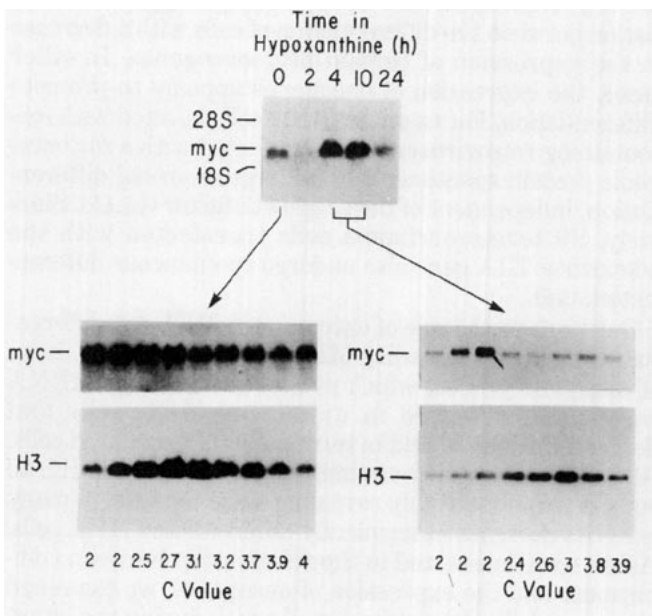

FIGURE 2. Change in cell cycle distribution of c-*myc* mRNA during hypoxanthine induction. (*A*) Uninduced MEL cells were separated into cell cycle fractions by centrifugal elutriation. The C-values at the bottom reflect the average DNA content of cells in each fraction determined by flow microfluorometry analysis of propidium iodide stained cells. A C-value of 2 reflects the diploid content of DNA (cells in G0 or G1); a C-value of 4 is found in cells in G_2/M. Total cellular RNA was extracted from each fraction and anlyzed by Northern filter hybridization. The 10-fold increase in H3 histone mRNA in mid-S (C = 2.5–3.1), determined by densitometric analysis of the hybridizing bands, is consistent with previous findings concerning H3 expression in the cell cycle. In contrast, c-*myc* mRNA levels vary less than 2-fold across the cycle. (*B*) Cells were also separated into cell cycle specific fractions following 7 hr of treatment with the inducer hypoxanthine. During this time, c-*myc* mRNA levels have decreased and then are reexpressed (upper panel). Northern filter hybridization revealed that while H3 mRNA is predominantly found in mid-S cells (C = 2.4–3.0) as in uninduced cells, 8-fold more c-*myc* mRNA is now found in G_1 cells compared with cells in S.

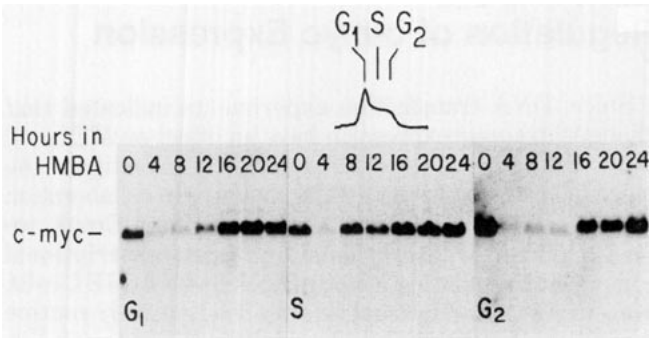

FIGURE 3. Timing of c-*myc* mRNA reexpression depends on the cell's position in the cell cycle before inducer treatment. Uninduced cells were synchronized into G_1, S, and G_2 cells by centrifugal elutriation. The cells were then treated with the inducer HMBA and total cellular RNA was extracted every 4 hr. The cell's progression through the cell cycle was followed by flow microfluorometry analysis (data not shown). C-*myc* reexpression occurs at 8, 12–16, and 16 hr for cells starting in S, G_1, and G_2, respectively, corresponding to the time that the cells accumulate in the G_1 phase of the cell cycle.

plete cell cycle, then back to G_1. The inability of G_2 cells to reexpress c-*myc* in the first G_1, after 4 hr of DMSO treatment, may reflect a latent period before reexpression can occur. These data support the hypothesis that the reexpression of *myc* mRNA occurs primarily in G_1 cells and demonstrates that the gradual reaccumulation of c-*myc* mRNA between 12 and 24 hr of DMSO treatment is apparently due to the different times nonsynchronous populations of MEL cells arrest in G_1 and reexpress c-*myc* mRNA.

The consequence of the change in expression of c-*myc* with respect to the cell cycle is not known. The coincidental timing of this change with the detection of the earliest committed cells could indicate that the decrease in c-*myc* expression in S cells or the increase in G_1 cells that occur during inducer treatment, may be important signals in commitment to differentiate.

Effect of Transfected *myc* Expression Vectors on Commitment

The most direct experimental approach to determine the effect of *myc* on commitment to differentiate is to transfect MEL cells with a *myc* recombinant vector whose expression is not influenced by the factors that presumably regulate endogenous *myc* expression in differentiating MEL cells. We constructed a *myc* expression vector which we thought satisfied all the requirements for autonomous regulation. A 1.36 kb XhoI fragment from a c-*myc*, c-DNA clone (*59*), which contains the *myc* coding sequences and minimal 5′ and 3′ noncoding information, was cloned near a promoter from the mouse metallothionein (MT)-1 gene. The 5′ noncoding exon which contains the c-*myc* promoters, and an AU rich 3′ end, which may be involved in c-*myc* mRNA instability, are almost entirely omitted from the construct (*60,61*). The MT-1 promoter is capable of driving the expression of heterologous genes, following treatment with heavy metals (*62*). Prior to the transfection we found that endogenous MT-1 is induced following treatment with cadmium sulfate, at concentrations that did not inhibit MEL cell differentiation. The stage seemed set for a well thought out transfection experiment. However, the biology of the system did not follow the plans we made for it. Although we obtained 9 transfectant clones that expressed a chimeric MT-*myc* mRNA, only two clones were inducible with cadmium sulfate. Unfortunately, the concentration necessary to induce MT-*myc* expression inhibited differentiation in parental cells and therefore could not be used to study the effect of *myc* expression on commitment. The other 7 clones constitutively express MT-*myc* mRNA in uninduced cells.

We next studied the effect of HMBA treatment on MT-*myc* expression and found that expression of MT-*myc* mimicked the biphasic changes that occur in endogenous c-*myc* mRNA (Fig. 4). A chimeric *myc* gene was constructed in which c-*myc* coding exons were cloned near the metallothionein I (MT-1) promoter. The resultant recombinant plasmid, pMT-*myc* was transfected into MEL with pSV2neo and selected in G 418. Total cellular RNA was extracted at various intervals of HMBA treatment and assayed using an RNAse protection assay. There is a decrease in the level of MT-*myc* mRNA within 2 hr of inducer treatment, followed by its reexpression (*63*). Fortunately there was a critical difference between MT-*myc* and c-*myc* reexpression that we were able to exploit. Whereas c-*myc* mRNA is reexpressed between 12 and 24 hr of HMBA treatment, with a peak at 16 hr, MT-*myc* mRNA is reexpressed between 4 and 8 hr. We were therefore able to determine the effect of early *myc* reexpression on commitment. Indeed, we found that 6/7 MT-*myc* transfectant clones that reexpress MT-*myc* mRNA between 4 and 8 hr have a more rapid rate of entry into the commitment program compared with parental cells and control transfectant lines (Fig. 4). This suggests that the rate of entry into a commitment program is correlated with the timing of *myc* reexpression. This hypothesis was supported when c-*myc* mRNA reexpression was measured in spontaneously derived, rapidly committing subclones of the parent MEL cell line, DS19, which commit as rapidly as MT-*myc* transfectant lines and reexpress c-*myc* mRNA early, between 8 and 12 hr. In addition, we isolated lines that exhibited a delay in c-*myc* mRNA reexpression and were found to commit more slowly than parental cells (*63*).

Because MT-*myc* expression is not constitutive during inducer treatment, we were not able to determine the effect of the early decline on commitment. However, almost simultaneously with the publication of our transfection results, three groups reported the successful transfection of MEL cells with recombinant *myc* expression vectors whose expression is constitutive during inducer treatment (*64–66*). These groups found that constitutive expression of chimeric *myc* mRNAs inhibits MEL cell differentiation. These data, in combination with the MT-*myc* transfectant results therefore suggest that both phases of the changes in c-*myc* expression, transient

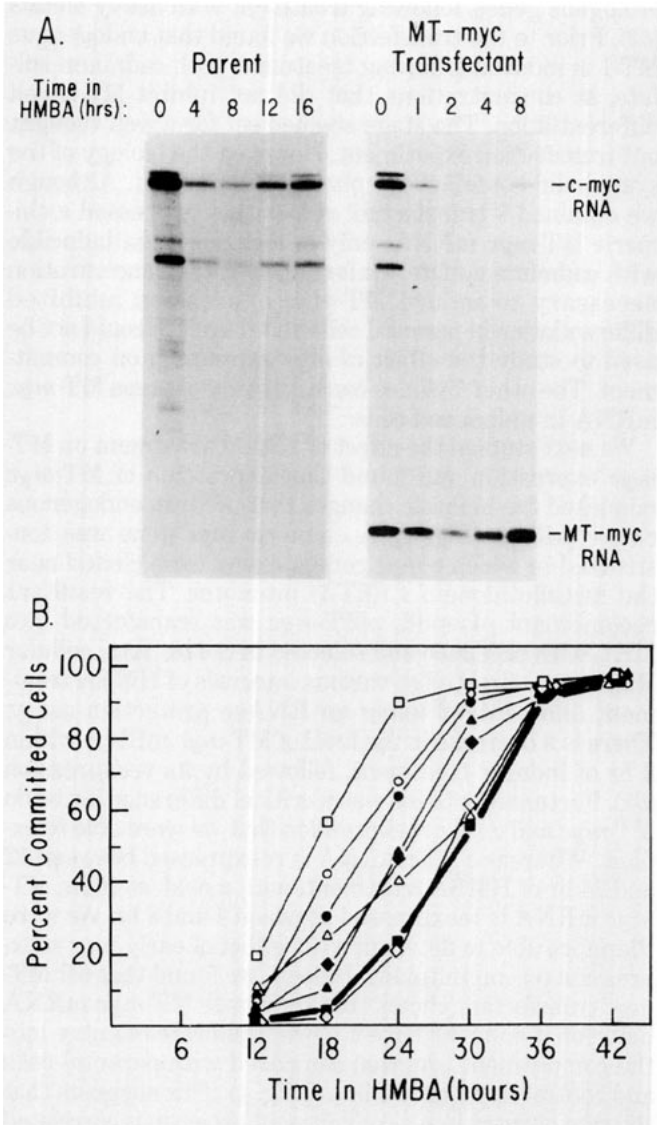

FIGURE 4. Effect of transfected *myc* sequences on MEL cell commitment. (*A*) C-*myc* mRNA has two protected fragments seen at the top of the gel. There are also two subbands generated by RNAse treatment that have not been fully characterized. In addition to the c-*myc* protected fragments, the MT-*myc* transfectant line shown on the right also contains bands corresponding to MT-*myc* mRNA. Note that the decline and reexpression of MT-myc mRNA occur more rapidly than c-*myc* mRNA. (*B*) Commitment assays were performed by treating cells with HMBA for 12–42 hr with subsequent plating in methycellulose without inducer. Colonies were scored for the presence (committed) or absence (uncommitted) of hemoglobin after 5 days in methylcellulose. The thick line is a typical commitment curve for the parent cell line, DS19. The thin lines represent analysis of 7 MT-myc transfectant clones. Control transfectant lines commit with similar kinetics as the parent in over 80% of examples analyzed (data not shown).

downregulation, and *myc* reexpression are important events in determining the differentiated phenotype of MEL cells. Presumably, these effects are due to the influence of *myc* protein on the expression of commitment and differentiation specific genes.

Regulation of c-*myc* Expression

Since, DNA transfection experiments indicated that changes in c-*myc* expression have an effect on MEL cell differentiation, we decided to carefully investigate the mechanisms responsible for the changes. In collaboration with Ken Marcu and Alain Nepveu at Stony Brook, we measured the transcriptional and posttranscriptional components regulating c-*myc* mRNA levels in MEL cells. Measuring transcriptional activity is a relatively routine procedure in which isolated nuclei are labeled with ^{32}P-uridine triphosphate (UTP) *in vitro*. The labeled RNAs reflect transcriptional elongation of previously initiated transcription units (*67*). Labeled RNA is recovered and annealed to membrane-bound DNA probes containing genes of interest. The assay has been complicated following the discovery by Bentley and Groudine that c-*myc* gene transcription could be regulated at the level of transcription elongation (*68*). The c-*myc* gene is made up of three exons in which exons 2 and 3 encode *myc* protein (*59*). The first exon is a long, 5′ untranslated sequence that contains two transcriptional start sites.

Bentley and Groudine found a molar excess of exon 1 transcription compared with transcription in exons 2 and 3, resulting from an intragenic block in RNA elongation near the exon 1 - intron 1 boundary, an effect also called transcriptional pausing. The mechanism of this phenomenon is not clear. One possibility is that a transcriptional pausing signal in exon 1 could block RNA polymerase II progression through the gene. In fact, poly T sequences, which block RNA polymerase III in Xenopus 5S RNA genes, are found in human c-*myc* exon 1 (*68*). The importance of the block in elongation in regulating c-*myc* mRNA levels was demonstrated by the finding that the decrease in c-*myc* mRNA in differentiating HL-60 cells is due to an increase in the degree of block (*68*). Marcu has extended these findings and demonstrated that transcriptional pausing of c-*myc* transcription occurs in many cell lines (*69*). Transcriptional pausing is not unique to c-*myc*, as Bender et al. has described a similar effect on c-*myb* gene transcription (*70*). It is conceivable that regulation by transcriptional pausing may provide a rapid mechanism of controlling the expression of these important regulatory genes (*71*).

In addition to transcriptional controls *myc* mRNA levels also appear to be regulated by posttranscriptional events. For example, the large decrease in c-*myc* mRNA that occurs when Daudi cells are treated with β interferon is unaccompanied by changes in c-*myc* gene transcription (*72*). Also, the decrease in c-*myc* mRNA that occurs in differentiating F9 cells, appears to be posttranscriptional (*73*). However, the discovery of transcriptional pausing casts some doubt on previous attempts to measure c-*myc* gene transcription using DNA probes that contain the entire c-*myc* gene.

In order to determine whether the changes in c-*myc* expression in differentiated MEL cells are due to transcriptional or posttranscriptional events, we measured c-*myc* gene transcription using an *in vitro* nuclear run on assay.

Multiple single-stranded segments of the c-*myc* gene were used as probes for *in vitro* labeled RNA, to determine whether transcriptional pausing occurs in MEL cells. In addition, single-stranded probes of the antisense strand were also used in order to detect antisense RNA transcription. We included this in the analysis because Marcu had detected significant *myc* antisense transcription in many cell lines (*74*). Our transcriptional analysis revealed that similar to other cells, MEL cells transcribe the first exon more efficiently than exons 2 and 3. Further, when the cells are treated with HMBA, there is an increase in transcriptional pausing, resulting in a dramatic decrease in effective c-*myc* transcription; a phenomenon that can be detected as early as 20 min following the addition of inducers (*75*). Thus, the early decrease in c-*myc* mRNA that occurs following exposure to inducers is initiated by a block in c-*myc* transcript elongation, similar to HL-60 cells. However, after 2 hr of HMBA treatment, effective transcription returns to the level found in uninduced MEL cells, yet c-*myc* mRNA levels remain quite low. This suggests that although the early decrease in c-*myc* mRNA is initiated by an increase in transcriptional pausing, the maintenance of the decrease is posttranscriptional. Furthermore, the 10-fold increase in c-*myc* mRNA levels between 12 and 24 hr of inducer treatment and the second decline which occurs as the cells terminally differentiate, are also not associated with significant changes in effective transcription, indicating that these changes in c-*myc* mRNA are due to posttranscriptional events (Fig. 5). In our experiments we also detected significant *myc* antisense transcription. However, the changes in c-*myc* mRNA could not be correlated with fluctuations in antisense transcription (*75*).

Posttranscriptional regulation is a general term implying regulation at the level of nuclear RNA processing, transport or mRNA stability. Experimental approaches to distinguish between these possibilities are limited by our lack of understanding of mRNA transport and the instability of precursor RNAs. Recent work concerning the regulation of tubulin mRNA demonstrates that barriers to the understanding of posttranscriptional regulation are being lifted by improved experimental strategies (*76*).

In the case of c-*myc* mRNA, we focused on the possibility that mRNA stability could be a factor in regulating c-*myc* expression because several authors have suggested that the c-*myc* mRNA levels in other cell lines could be regulated at this level (*77,78*). The half life ($T_{1/2}$) of c-*myc* mRNA in uninduced MEL cells was measured using an actinomycin D chase protocol in which the disappearance of an mRNA from the cell, which occurs at a rate proportional to its intrinsic $T_{1/2}$, is measured following actinomycin D-induced transcription inhibition. Using this approach, we obtained a value of 13 min, indicating that the mRNA is quite unstable in these cells. Since treatment of MEL cells with HMBA results in the virtual disappearance of c-*myc* mRNA we could not directly measure the $T_{1/2}$ of c-*myc* mRNA at those times.

However, several experiments indicated to us that the mRNA is not more unstable during HMBA treatment. First, the decrease in c-*myc* mRNA in the first 2 hr of HMBA treatment occurs with a rate of decay of 10 min, similar to the actual $T_{1/2}$ of c-*myc* mRNA in uninduced cells. Since effective c-*myc* transcription during this time is only 5% the level found in uninduced cells, a value similar to the degree of inhibition of effective c-*myc* transcription resulting from actinomycin D treatment, the rate of the decrease in c-*myc* mRNA during this time approaches the actual $T_{1/2}$.

Second, we measured the $T_{1/2}$ of c-*myc* mRNA during its reexpression between 12 and 24 hr of HMBA treatment and obtained the identical value of 13 min we found in uninduced cells. Third, we measured the $T_{1/2}$ of a chimeric MT-*myc* mRNA that is reexpressed during the time that endogenous c-*myc* mRNA is quite low. The $T_{1/2}$ of MT-*myc* mRNA was not significantly different from that found in uninduced cells, demonstrating that during the time when c-*myc* mRNA levels are decreased, a *myc*

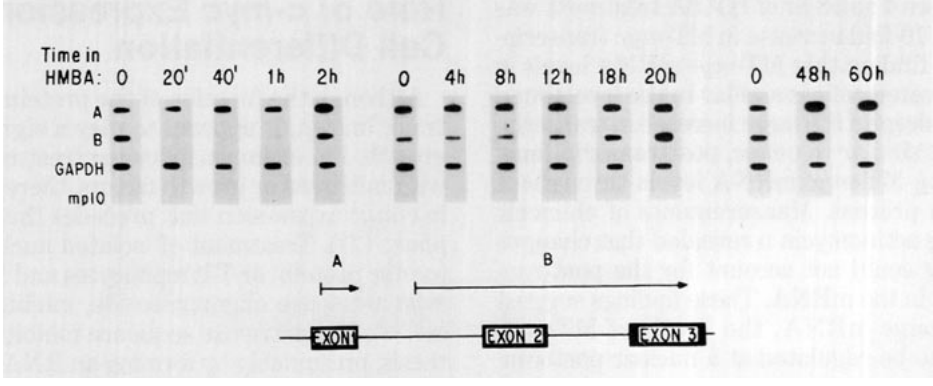

FIGURE 5. Demonstration of c-*myc* transcriptional pausing in MEL cells. Single-stranded probes for the noncoding first exon (*A*) and the coding exons (*B*) were prepared from M13 subclones and slotted onto nitrocellulose filters. GAPDH is a control gene whose expression varies little during inducer treatment while mp10 is a negative control. After 20 min, transcription into the coding exons markedly decreases, whereas transcription in exon 1 is constitutive, indicating a block in transcriptional elongation. By 2 hr, the rate of transcription into the coding exons has returned to the level found in uninduced cells. Note that in uninduced cells there is also a degree of block. This is seen more easily in the 0 time points corresponding to the experiments in which transcription was measured between 2–20 hr and at 48 and 60 hr. The intensity of the probe A signal underestimates the actual transcription rate compared with probe B because probe B contains 5 times more labeling sites than probe A.

containing mRNA can be detected whose stability is unaffected by inducer treatment (unpublished observations). These data indicate that the stability of c-*myc* is unchanged during inducer treatment and suggests that posttranscriptional regulation of c-*myc* mRNA in inducer-treated cells occurs at a nuclear level, such as RNA processing or transport (*75*).

There have been two other reports that suggested that the changes in c-*myc* mRNA levels in MEL cells are due to transcriptional events and changes in mRNA stability (*79,80*). In both reports, gene transcription was only measured early in inducer treatment, thus the return in effective transcription we detected after 2 hr of HMBA treatment was not observed. By not measuring c-*myc* gene transcription later in the differentiation pathway, the transcriptional component of its regulation was overemphasized. The role of c-*myc* mRNA stability in one of the reports was based on only one time point on a Northern filter hybridization of RNA from actinomycin D-treated cells, which makes these data difficult to interpret (*79*). Nevertheless, it is conceivable that different isolates of MEL cells could regulate *myc* mRNA differently.

Mechanism of Nuclear Posttranscriptional Regulation

In order to understand the mechanism of nuclear posttranscriptional regulation, we have begun to study the expression of chimeric *myc* genes in differentiating MEL cells. As mentioned earlier, a chimeric *myc* mRNA containing the *myc* coding exons and metallothionein-1 sequences (MT-*myc*) undergoes biphasic changes in inducer-treated cells similar to endogenous c-*myc* mRNA. Using *in vitro* nuclear run analysis we determined that the early decrease in MT-*myc* mRNA was posttranscriptional (unpublished observations). Transcriptional pausing is not observed with MT-*myc* because the chimeric gene does not contain the exon I sequences where pausing has been mapped. We also found that the early reexpression of MT-*myc* mRNA between 4 and 8 hr of HMBA treatment was due to a large 5- to 10-fold increase in MT-*myc* transcription. However, the finding that MT-*myc* mRNA levels in 4 to 8 hr HMBA-treated cells is similar to the level found in uninduced cells, despite the large increase in transcription, suggests that similar to c-*myc*, posttranscriptional events are affecting MT-*myc* mRNA levels throughout the differentiation process. Measurements of chimeric MT-*myc* $T_{1/2}$ using actinomycin D revealed that changes in mRNA stability could not account for the posttranscriptional changes in the mRNA. These findings suggest that similar to c-*myc* mRNA, the levels of MT-*myc* mRNA appeared to be regulated at a nuclear posttranscriptional level. The data also imply that the sequences responsible for nuclear posttranscriptional regulation could be contained within the 1.36 kb Xho fragment, which includes the c-*myc* coding exons, the only sequences common to c-*myc* and MT-*myc*.

An alternative explanation is that a regulatory event common to both mRNAs is being affected. For example, c-*myc* mRNAs and MT-*myc* mRNAs are both spliced, c-*myc* at its normal splice donor and acceptor sites, and MT-*myc* within residual MT sequences in the 3′ end of MT-*myc* RNA. MT-*myc* does not contain *myc* splice sites, since the *myc* containing sequences were derived from a cDNA clone.

In order to determine whether a splicing defect induced by HMBA treatment could be involved in nuclear posttranscriptional regulation, we transfected MEL cells with an intronless chimeric *myc* gene we constructed called CLAHX3-*myc*, which also contains the 1.36 myc Xho fragment. We found that expression of CLAHX3-*myc* mRNA undergoes the same biphasic changes in differentiating MEL cells as c-*myc* and MT-*myc* mRNAs demonstrating that the absence of introns does not prevent the biphasic change in *myc*-containing mRNAs from occurring. The data also lends further support to the hypothesis that the 1.36 kb Xhol fragment is involved in regulating *myc* mRNA levels, possibly at the level of RNA transport. Our results with a variety of *myc* expression vectors are in contrast to the findings of the groups who have been able to obtain clones of MEL cells that constitutively express chimeric *myc* genes during inducer treatment (*64–66*). There is not an adequate explanation for these differences, especially without data concerning the transcription rates of the other chimeric *myc* genes. It is conceivable that an increase in transcription sufficient to overcome nuclear posttranscriptional regulation could account for the ability of some of these chimeric *myc* genes to be expressed during inducer treatment. Alternatively differences in DNA copy number or genomic integration sites could be a factor.

In summary, the regulation of c-*myc* mRNA is a rather complex mixture of transcriptional and posttranscriptional events. A new concept to the understanding of *myc* regulation is the idea that the *myc* coding sequences may be a determinant in regulating *myc* mRNA levels through a poorly defined nuclear posttranscriptional process.

Role of c-*myc* Expression in MEL Cell Differentiation

Although the function of the protein encoded by c-*myc* is not known, it appears to play a significant role in cell growth. For example, following treatment of resting cells with mitogens or growth factors, there is a large increase in c-*myc* expression that precedes the cells' entry into S phase (*77*). Treatment of isolated nuclei with antibodies to *myc* protein, or T-lymphocytes and HL-60 cells with c-*myc* antisense oligonucleotide, inhibits DNA synthesis (*82,83*). The antisense sequence inhibits *myc* protein synthesis, presumably by forming an RNA:RNA hybrid with complementary nucleotides in *myc* mRNA, preventing its translation. Also, transfection of 3T3 cells with an inducible *myc* chimeric gene results in a partial reduction in the requirement for platelet-derived growth factor (PDGF) to stimulate cell division (*84*). Finally, an inappropriate growth signal by abnormally regulated *myc* sequences translocated into immunoglobulin loci appears to

be a major factor in the pathogenesis of human Burkitt's lymphoma and murine plasmacytoma (*85,86*).

There is also some evidence, although less convincing, that c-*myc* protein affects gene expression. The *myc* gene has been found to increase transcription from a heat shock gene promoter (hsp 70) and to decrease metallothionein (MT) 1 gene expression in a transient expression system (*87*). Of interest is that the inhibitory effect of *myc* proteins on MT-1 expression is abolished by mutations in exon 2, whereas hsp 70 activation is not affected, suggesting that the inhibiting and activating domains are encoded by different sequences. In some cells expression of *myc* or *myc*-related transfected genes appears to downregulate endogenous *myc* (*88*). It is believed that *myc* protein could interfere with *myc* gene regulatory elements by negative feedback inhibition (*89*). However, so far, there is no direct proof that this occurs. Certainly, in MEL cells we and others have not detected any evidence that expression of transfected *myc* genes suppresses endogenous c-*myc*.

Despite the relative dearth of evidence that *myc* regulates gene expression, the effect of transfected *myc* sequences on MEL cell differentiation is most easily explained by a role in gene expression. The DNA transfection experiments point to two different effects of *myc* expression. First, the finding that constitutive *myc* expression during inducer treatment inhibits differentiation (*64*–66) suggests that *myc* inhibits a subset of genes, which are designated group 1 and are activated during the early inducer-mediated decline in c-*myc* expression. These genes are inhibited in uninduced cells as a result of constitutive *myc* expression. Perhaps during normal erythroid differentiation there is a signal that results in rapid c-*myc* downregulation which triggers group 1 gene activation. However, viral transformation may prevent MEL cells from differentiating by delivering an inappropriate growth signal, the consequence of which is constitutive *myc* expression and group 1 gene suppression. Inducing agents of differentiation may be capable of bypassing the viral activation of c-*myc* to induce an early decline in its expression by initiating a block in transcriptional elongation and activating nuclear posttranscriptional controls on c-*myc* gene expression. As a consequence of this series of events, c-*myc* is downregulated and group 1 genes are activated (Fig. 6).

The activation of putative group 1 genes appears to be insufficient for commitment to occur. If MEL cells are plated in the absence of inducer after 12 hr of treatment, a time which includes the entire period of the c-*myc* mRNA decline, few, if any cells are committed to differentiate. Clearly, other events must occur in order for MEL cells to become committed.

The data from our MT-*myc* transfection experiment suggest that *myc* reexpression also plays a role in regulating commitment. The timing of *myc* reexpression in parental cells coincides with the ability to detect committed cells, and rapid reexpression of MT-*myc* or c-*myc* is associated with an increase in the rate cells become committed. The model most consistent with this picture is that there is a subset of genes (group 2) that are activated

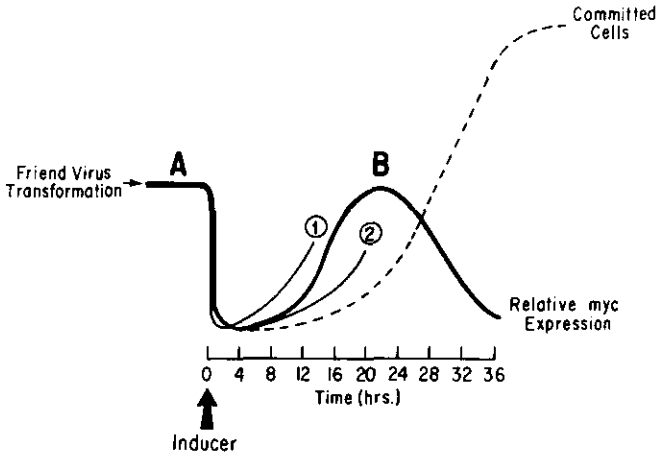

FIGURE 6. Hypothesis for the role of c-*myc* in generating committed cells. As a result of Friend virus transformation, MEL cells receive a constitutive growth signal, the consequence of which is the constitutive expression of c-*myc* (*A*). Following inducer treatment, c-*myc* expression decreases, activating group 1 genes which had been previously inhibited by *myc*. During the reexpression of *myc*, which occurs primarily in G_1 cells, group 2 genes, dependent on inducer plus *myc* reexpression, are activated. The combination of group 1 and group 2 gene expression drives the cell into a commitment program.

because of *myc* reexpression. The activation of group 2 genes may be a cell-cycle-mediated event, since *myc* reexpression occurs primarily in G_1 cells. Since the timing of *myc* reexpression depends on a cell's position in the cell cycle when first exposed to an inducer, one may expect group 2 genes to become activated at different times in an asynchronous population of cells, an assumption that could explain why MEL cells become committed at different rates. The combination of group 1 and group 2 gene expression is needed for cells to enter a commitment program (Fig. 6).

The ability of *myc* to influence both replication and cellular differentiation is consistent with the model of the gene as a multipurpose regulatory molecule. In this way it is analogous to the adenovirus EIA gene that influences viral replication and has both inhibitory and activating effects on cellular gene expression (*90,91*). The functional analogy between *myc* and EIA is also demonstrated by the finding that EIA can substitute for *myc* in the cotransformation of primary fibroblasts with an activated *ras* gene (*92,93*).

Although this paper has focused on the c-*myc* gene, it is not the only protooncogene involved in MEL cell differentiation. It is only one of at least four nuclear protooncogenes that are expressed in MEL cells. Indeed, the pattern of expression of other nuclear protooncogenes such as c-*fos*, c-*myb* and p53 suggests that these genes could also be involved in regulating the differentiation process. For example c-*fos* mRNA increases 5- to 10-fold within 2 to 4 hr of inducer treatment, p53 expression decreases approximately 20-fold (unpublished observations), and c-*myb* undergoes biphasic changes, similar to c-*myc* mRNA (*80*). The importance of these changes in

the commitment program is being investigated by several groups.

Because of the unusual nature of c-*myc* expression in differentiating MEL cells, the system provides a unique opportunity to understand the physiological role and regulation of c-*myc* in cells. By applying recombinant DNA strategies in studying the c-*myc* gene, we hope to understand the basis of malignant transformation of leukemia cells and the ability of these cells to terminally differentiate *in vitro*.

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