Jpn. J. Cancer Res. 83, 61–65, January 1992

c-Myc Interferes with the Commitment to Differentiation of Murine Erythroleukemia Cells at a Reversible Point

Shinji Takada, Tohru Yamamoto, Yasufumi Ohmori, Yasuhisa Matsui and Masuo Obinata[1]

Department of Cell Biology, The Research Institute for Tuberculosis and Cancer, Tohoku University, Seiryo-machi 4-1, Sendai 980

When murine erythroleukemia (MEL) cells, containing the transferred rat c-*myc* gene under the control of human metallothionein II gene promoter, are induced to differentiate with dimethyl sulfoxide, the level of differentiation is dependent on the c-Myc level, which is modulated by the addition of Zn ions. In this work, we examined the point of inhibition of differentiation by elevated levels of c-Myc in one (clone 38-2) of the typical transformants. Commitment assay indicated that elevated levels of c-Myc interfere with entry of the transformant into the commitment event, but when c-*myc* expression was reduced by removing Zn ions from the medium, the cells could reenter the commitment program. However, once the cells were committed, such cells could not return to the uncommitted state. In addition, time-dependent expression of two erythroid specific genes was inhibited by elevated levels of c-Myc in time-dependent manner. These results suggest that c-Myc modulates MEL cell differentiation at a reversible point of commitment.

Key words: c-*myc* — Murine erythroleukemia — Differentiation — Commitment

c-Myc is a nuclear oncoprotein present in nearly all cell types. The functions of c-Myc appear to be linked to the control of proliferation and differentiation. However, the molecular function of c-Myc in cell differentiation is not known. In the early stage of the induction of mouse erythroleukemia (MEL) cell differentiation,[1] c-*myc* mRNA levels show a drastic change. Within 1–2 h following the addition of dimethyl sulfoxide (DMSO), c-*myc* mRNA levels decrease by a factor of 10. After about 12–18 h of treatment, c-*myc* mRNA is transiently re-expressed at a level similar to that found in uninduced cells; then, its level again declines.[2,3] Previous studies showed that the forced expression of exogenously transfected c-*myc* gene inhibited the differentiation of MEL cells.[3–6] We have introduced the c-*myc* gene into MEL cells under the control of human metallothionein II promoter. In these MEL cell transformants, the exogenous c-*myc* gene was inducible by the addition of Zn ions and we have demonstrated that the elevated levels of c-Myc inhibit commitment and differentiation.[3] In this work, we examined the point of action of c-Myc on the MEL cell differentiation using a typical MEL cell transformant.

MATERIALS AND METHODS

Culture and induction of the MEL cell transformant The MEL cell transformant (clone 38-2) was cultivated as described previously.[3] For the examination of the time-dependent effect of the transferred c-*myc* gene on the MEL cell differentiation, MEL cells were induced with the addition of 1.5% DMSO and 180 μM $ZnCl_2$. To remove $ZnCl_2$, the cells were washed twice with the medium by centrifugation, and the pelleted cells were resuspended in the medium containing 1.5% DMSO. The percentage of differentiated cells was determined by scoring the hemoglobinized cells after staining with benzidine dye.

Commitment assay The cells were induced with 1.5% DMSO in the presence or absence of 180 μM $ZnCl_2$ in the liquid culture, and the cells were collected by centrifugation and resuspended in the semisolid medium containing methylcellulose (0.8%) without DMSO and $ZnCl_2$ at a concentration of 1×10^4 cells/ml. After 2 days of culture, colonies with 4 to 16 cells were formed. The colonies were stained with benzidine dye and the positive and negative colonies were scored. The percentage of committed cells was calculated as benzidine-positive colonies/positive+negative colonies $\times$ 100 (%).

Northern blot hybridization The levels of endogenous and exogenous c-*myc* mRNA were estimated by Northern blot hybridization as described.[3] Total cellular RNA was extracted and separated in an agarose gel under denaturing conditions. The c-*myc* DNA fragment was labeled with ^{32}P-dCTP by nick-translation and used as the hybridization probe.

RESULTS

Inhibition of commitment of MEL cells to differentiation by elevated levels of c-*myc* mRNA In this work, clone 38-2 was used throughout. This is a typical transformant

[1] To whom correspondence should be addressed.

showing inhibition of MEL cell differentiation when Zn ions were added together with DMSO. No effect on differentiation was apparent in non-transformed MEL cells.[3] In this clone, the addition of Zn ions to the culture can induce expression of the transferred rat c-*myc* gene, resulting in elevated c-*myc* mRNA levels. In the continuous presence of Zn ions, the level of c-Myc measured by Western blotting was shown to be elevated in parallel with the mRNA level (data not shown). Elevated levels of c-Myc were shown to inhibit the differentiation of 38-2 cells. At first, we examined the effect of elevated levels of c-Myc on the commitment to differentiation of this transformant. To determine the commitment,[7] the cells were induced with DMSO in the presence or absence of Zn ions in the liquid medium and after an interval, the cells were transferred to semi-solid medium without DMSO and Zn ions. After 2 days of culture in the semi-solid medium, the colonies formed were stained with benzidine and the benzidine-positive or negative colonies were scored. The result (Fig. 1) clearly indicates that elevated levels of c-Myc interfere with the commitment step in the differentiation of 38-2 transformant.

Reentry of transformant to the differentiation program after removal of $ZnCl_2$ in the culture with DMSO Then we examined whether the interference by c-Myc is reversible. Zn ions were added together with DMSO to the culture of clone 38-2 and at appropriate time intervals, Zn ions were removed by washing. The cells were then cultivated in the presence of DMSO. The time dependence of reentry of the cells into the differentiation program after removal of Zn ions in the continuous presence of DMSO was monitored in terms of the appearance of hemoglobin-positive cells (Fig. 2). In the continuous presence of both Zn ions and DMSO, the cells only slightly differentiated. Upon removal of Zn ions after 2 days of exposure, the accumulation of differentiated cells showed a delay of 2 days. Similarly, upon removal of the Zn ions after 3 days or 5 days of exposure, the cells showed a delay of 3 or 5 days, respectively. Thus, even after a prolonged exposure of the cells to Zn ions during induction with DMSO, the commitment of MEL cells to differentiate was reversible and occurred with kinetics identical to that of normal induction if Zn ions were removed.

Inability of committed cells to return to the uncommitted state The result shown in Fig. 2 indicated that if the cells were prevented from commitment by elevated levels of c-Myc, such cells could reenter the commitment program if the levels of c-Myc were reduced. This suggests that c-Myc interferes at a point prior to or close to the commitment. Then, we asked whether the commitment of cells could be reversed if levels of c-Myc were elevated. The cells were induced with DMSO and then Zn ions were added 1, 2 and 4 days after induction. In each case, Zn ions were removed after 1 day of exposure by washing. The effect of the pulse-addition of Zn ions on the differentiation is shown in Fig. 3. The pulse-addition in the period from 1 day to 2 days, when most of the cells

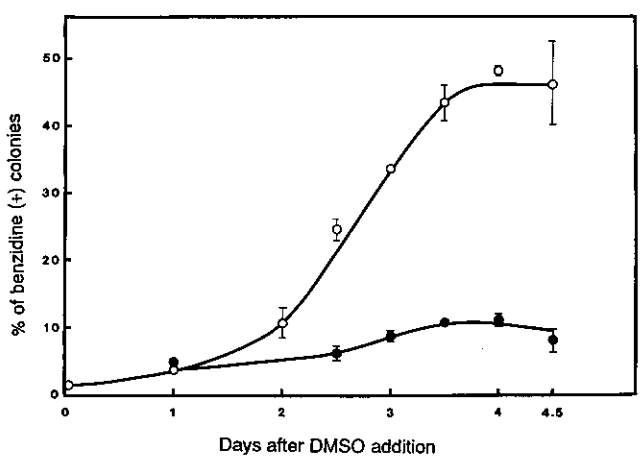

Fig. 1. Commitment of MEL cell transformant (38-2). Clone 38-2 was induced with DMSO (1.4%) in the presence (●) or absence of 180 μM $ZnCl_2$ (○). At various time intervals, the cells were taken from the liquid culture, transferred to semi-solid medium without DMSO and incubated for 2 days to form colonies. The percentage of benzidine-positive colonies amongst the total colonies was estimated.

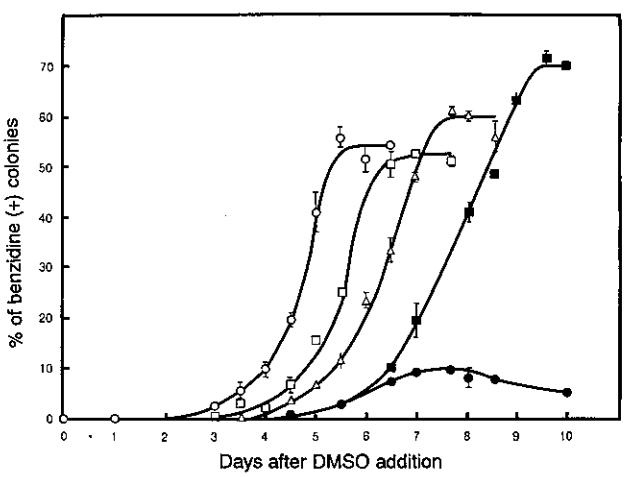

Fig. 2. Reentry of clone 38-2 into the differentiation program after removal of Zn ions. The cells were induced with DMSO (1.4%) in the presence of $ZnCl_2$ (180 μM). At various time intervals, the cells were collected, washed and transferred to a new medium containing DMSO, but not $ZnCl_2$. Then, the cells were incubated and the time-dependent accumulation of benzidine-positive cells was estimated. Zn ions were removed at 0 (○), 2 days (□), 3 days (△) and 5 days (■) or were present throughout the incubation (●).

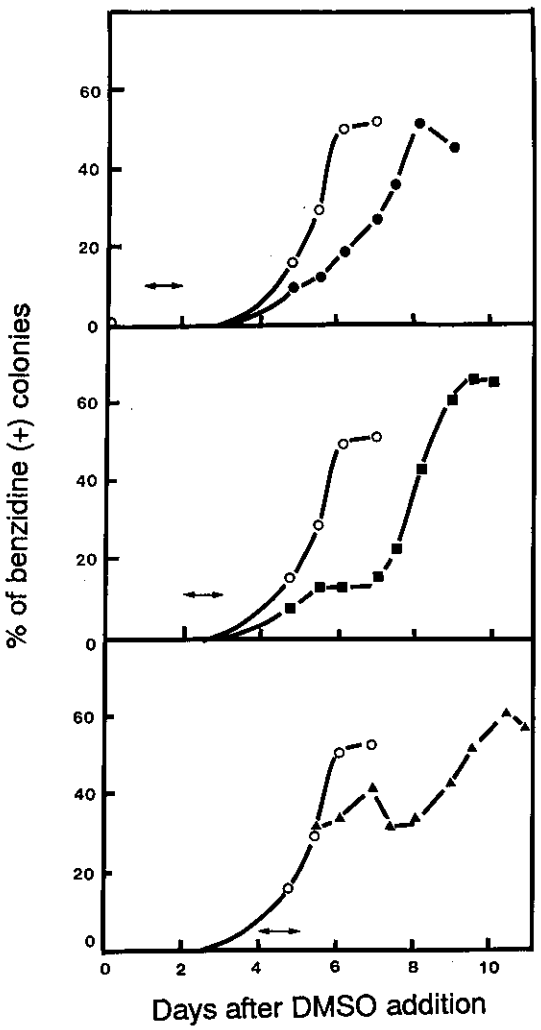

Fig. 3. Pulse-addition of Zn ions during induction of clone 38-2. The cells were induced with DMSO in the absence of $ZnCl_2$ and at day 1 (top figure), at day 2 (middle figure) and at day 4 (bottom figure), $ZnCl_2$ (180 μM) was added to the culture. After incubation for 1 day, the cells were washed and transferred to a new medium. Then the accumulation of benzidine-positive cells was estimated. Open circles show the percentage of benzidine-positive cells in the cells without pulse-addition of Zn ions and closed symbols show the results in the cells exposed to the pulse-addition of Zn ions. The time of the exposure of the cells to Zn ions is indicated by ⟷.

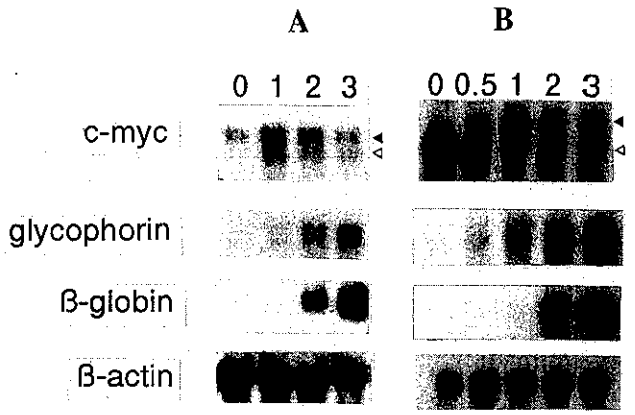

Fig. 4. Expression of two erythroid-specific genes during induction of clone 38-2. (A) The cells were induced with DMSO in the absence of Zn ions and total RNAs were isolated at 0, 1, 2, 3 days after induction. (B) The cells were induced with DMSO in the presence of Zn ions. Zn ions were removed at day 2 and transferred to a new medium. Then, total RNAs were extracted from the cells at 0, 0.5, 1, 2, and 3 days after the transfer. Total RNA was separated in agarose gel and blotted onto a nitrocellulose filter. Hybridization was performed[16] with ^{32}P-labeled rat c-*myc* gene, mouse β-globin gene, mouse glycophorin gene and chicken β-actin gene probes. ◁ indicates the band for exogenous c-*myc* mRNA and ◀ indicates the band for endogenous c-*myc* mRNA.

did not commit (see Fig. 1), caused a delay in the appearance of hemoglobin-postive cells. Upon pulse-addition between 2 and 3 days, a part of the cells (approximately 15%) showed no delay of differentiation and the rest showed a delay. Upon pulse-addition between 4 and 5 days, when most of the cells had been committed, approximately 50% of cells showed differentiation without delay and the rest showed a delay. These results indicated that once the cells are committed, such cells can not revert to the uncommitted state. This in turn indicates that c-Myc may act on the commitment point or at a point just prior to the commitment, but not after the commitment.

Changes in the c-*myc* mRNA level and the timing of expression of two erythroid-specific genes We measured the levels of exogenous and endogenous c-*myc* mRNAs by Northern blot hybridization to examine whether the reentry of the cells into the differentiation program after removal of Zn ions is caused by the decrease in the expression of the transferred c-*myc* gene (Fig. 4). Whereas the endogenous c-*myc* mRNA showed a 2.5 kb band, the exogenous c-*myc* mRNA showed a 2.0 kb band because it lacks the 1st exon.[3] In the course of induction with DMSO, c-*myc* mRNA levels show a biphasic change; soon after the addition of DMSO, c-*myc* mRNA decreases, but after about 1 day of treatment, c-*myc* mRNA is transiently re-expressed, and then its level again declines.[2,3] Without the addition of Zn ions, 38-2 cells showed essentially the same change in c-*myc* mRNA; it increased on day 1 after DMSO addition and decreased thereafter (Fig. 4A). Zn ions were removed after 2 days of induction with DMSO in the presence of

Zn ions, and the cells were cultured in the presence of DMSO. Under these conditions, accumulation of differentiated cells showed 2 days of delay, as shown in Fig. 2. Just after removal of Zn ions (0 in the figure), expression of the exogenous c-*myc* mRNA was high, but 0.5 days after removal, the level of the exogenous mRNA rapidly dropped. On day 1, expression of the endogenous and exogenous genes was induced, but decreased thereafter (Fig. 4B). The change in c-*myc* mRNA after removal of Zn ions is thus essentially similar to the usual induction shown in Fig. 4A. This result indicates that elevated levels of c-*myc* mRNA inhibit the commitment. If the cells that were blocked by elevated levels of c-*myc* were released by removing Zn ions, the initial reduction in the c-*myc* mRNA itself did not directly induce the differentiation program, but the commitment and differentiation were induced after transient expression of the c-*myc* gene followed by a decline. This is consistent with our result that the second decline in c-*myc* mRNA after 1 day is required for the commitment and differentiation of MEL cells.[3]

Then we examined the relation of c-*myc* gene expression to the timing of expression of two erythroid-specific genes (Fig. 4). Expression of glycophorin gene started on day 1, while that of β-globin gene started on day 2 after induction with DMSO. We thus examined whether the timing of expression of the two erythroid-specific genes could be altered by the overexpression of c-*myc* mRNA. After 2 days of induction with DMSO in the presence of Zn ions, Zn ions were removed. The appearance of the two erythroid-specific mRNAs showed a delay of 2 days (Fig. 2). Timing of expression of the two erythroid-specific genes seemed to be strictly regulated by the c-*myc* level during the induced differentiation of MEL cells.

DISCUSSION

During the induced differentiation of MEL cells, c-*myc* mRNA levels change in a biphasic fashion. This drastic change is presumed to be important for the commitment of MEL cells to differentiate. The possibility of the direct involvement of c-Myc was examined by transferring c-*myc* gene into MEL cells.[3-6] We have shown that commitment and differentiation were inhibited in MEL cell transformants having the exogenous c-*myc* gene under the control of human metallothionein gene promoter. Our previous work showed that the late decline, but not the early decline, in c-*myc* mRNA was required for differentiation.[3] Recently it has been reported that only a slight reduction was detected in the c-Myc protein level during the early phase as compared with the mRNA reduction.[8] On the contrary, the late decline in mRNA coincided well with the change of protein level, and thus the late decline in c-*myc* mRNA seems to be essential for the differentiation. Coppola and Cole[4] have shown that constitutively elevated expression of c-*myc* gene inhibited the differentiation of MEL cells, but not the commitment of MEL cells. Thus, it is likely that commitment may require regulatable expression of c-*myc* gene, whereas induction of differentiation may require only the late decline in c-*myc* mRNA.

In this work, we have demonstrated that the commitment of the transformants induced by DMSO is blocked by the presence of Zn ions, but the uncommitted cells can reenter the differentiation program if Zn ions are removed. c-*myc* mRNA levels were high in the presence of Zn ions, but were reduced after removal of Zn ions. Thus, the commitment is regulated by the level of c-*myc* mRNA in MEL cells. Interestingly, there is a time lag to the start of differentiation after removal of Zn ions from the culture. The kinetics of induction of differentiation with DMSO after removal of Zn ions is the same as that of the normal induction process. The high levels of the transferred c-*myc* mRNA transcripts rapidly dropped after removal of Zn ions and instead, the endogenous c-*myc* mRNA was transiently induced. The late decline in both c-*myc* transcripts seems to be required for the induction of the transcription of the two erythroid-specific genes. Our results indicated that there is some coordinated regulation of the transferred and the endogenous c-*myc* genes during MEL cell induction. Since transcription of the transferred c-*myc* gene is regulated by the human metallothionein gene promoter, post-transcriptional and/or some specific autoregulatory control must be involved in the coodinated regulation of both c-*myc* genes.[9-11] From these results, we speculate that the early decline may be a clue to the transient expression of the c-*myc* gene, and the transient increase may be a prerequisite for the late decline of c-*myc* mRNA. Such a regulatory mechanism may be required for the commitment, since a high level of constitutive expression of the transferred c-*myc* gene inhibited the differentiation, but not the commitment.[4]

Gene expression may be controlled sequentially during the differentiation process of cells, including erythroid cells,[12-14] but it is not known how the differential timing of gene expression is determined. During MEL cell differentiation, glycophorin and β-globin genes showed differential timing in their expression; expression of the glycophorin gene started on day 1, while that of the β-globin gene started on day 2 after induction with DMSO.[15, 16] After removal of Zn ions, differential timing of the expression of the two erythroid-specific genes is the same as that in the normal process. Thus, the differential timing of the two erythroid-specific genes is well correlated with the change in c-*myc* mRNA level. Expression of the glycophorin gene seems to occur prior to the commitment event, while that of the β-globin gene occurs

at or after the commitment event. When the transformants were induced with DMSO in the presence of Zn ions, expression of both erythroid-specific genes was repressed (data not shown). It is possible that expression of the glycophorin gene may not be repressed through the inhibition of commitment of cells by elevated levels of c-*myc* mRNA, whereas that of β-globin gene may be repressed through the commitment event. From the timing of expression of both erythroid-specific genes, they can be induced only when c-Myc levels are reduced. Thus, c-Myc may repress both erythroid-specific genes. Previous studies suggest that c-Myc may have a function in the transcriptional regulation of several genes,[17] such as heat shock gene,[18] metallothionein gene[19] and histone gene,[20] although the precise role of c-Myc is not known. A recent report suggested that c-Myc may be a sequence-specific DNA binding transcription factor.[21] It would be interesting to know how c-Myc regulates these erythroid-specific genes.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

(Received August 26, 1991/Accepted October 17, 1991)

REFERENCES

1) Marks, P. and Rifkind, R. Erythroleukemic cell differentiation. *Ann. Rev. Biochem.*, **47**, 419–448 (1978).
2) Lachman, H. M. and Skoultchi, A. I. Expression of c-*myc* changes during differentiation of mouse erythroleukemia cells. *Nature*, **310**, 592–594 (1984).
3) Kume, U. T., Takada, S. and Obinata, M. The probability that the commitment of murine erythroleukemia cell differentiation is determined by the c-*myc* level. *J. Mol. Biol.*, **22**, 779–786 (1988).
4) Coppola, J. A. and Cole, M. D. Constitutive c-*myc* oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. *Nature*, **320**, 761–763 (1986).
5) Dmitrovsky, E., Kuehl, W. M., Hollis, G. F., Kirsch, I. R., Bendoer, T. P. and Segal, S. Expression of a transfected human c-*myc* oncogene inhibits differentiation of a mouse erythroleukemia cell line. *Nature*, **322**, 748–750 (1986).
6) Prochownik, E. V. and Kurowska, J. Deregulated expression of c-*myc* by murine erythroleukemia cells. *Nature*, **322**, 848–850 (1986).
7) Gusella, J., Geller, R., Clarke, B., Weeks, V. and Housman, D. Commitment to erythroid differentiation by Friend erythroleukemia cells: a stochastic analysis. *Cell*, **9**, 221–229 (1976).
8) Spotts, G. and Hann, S. R. Enhanced translation and increased turnover of c-*myc* proteins occurs during differentiation of murine erythroleukemia cells. *Mol. Cell. Biol.*, **10**, 3952–3964 (1990).
9) Wingrove, T. G., Watt, R., Keng, P. and Macara, I. G. Stabilization of myc proto-oncogene proteins during Friend murine erythroleukemia cell differentiation. *J. Biol. Chem.*, **263**, 8918–8924 (1988).
10) Mechti, N., Piechczyk, M., Blanchard, J. M., Mart, L., Bonnieu, A., Jeanteur, P. and Lebleu, B. Transcriptional and post-transcriptional regulation of c-*myc* expression during the differentiation of murine erythroleukemia Friend cells. *Nucleic Acids Res.*, **14**, 9653–9666 (1986).
11) Darling, D., Tavassoli, M., Linskens, M. H. K. and Farzaneh, F. DMSO-induced modulation of c-*myc* steady-state RNA levels in a variety of different cell lines. *Oncogene*, **4**, 175–179 (1989).
12) Mishina, Y., Matsui, Y. and Obinata, M. Stage-specific gene expression in erythroid progenitor cells (CFU-E). *Cell. Differ.*, **22**, 259–266 (1988).
13) Mishina, Y., Natori, S. and Obinata, M. Modulation of non-globin mRNA levels in erythropoiesis. *Dev. Biol.*, **106**, 343–350 (1984).
14) Obinata, M. and Ikawa, Y. Change in message sequences during erythrodifferentiation. *Nucleic Acids Res.*, **8**, 4271–4282 (1980).
15) Matsui, Y., Natori, S. and Obinata, M. Isolation of the cDNA clone for mouse glycophorin, erythroid-specific membrane protein. *Gene*, **77**, 325–332 (1989).
16) Yamamoto, T., Matsui, Y., Natori, S. and Obinata, M. Cloning of a house-keeping gene (*MER5*) preferentially expressed in murine erythroleukemia cells. *Gene*, **80**, 337–343 (1989).
17) Luster, B. and Eisenman, R. N. New light on Myc and Myb. Part I. Myc. *Genes Dev.*, **4**, 2025–2035 (1990).
18) Kingston, R. E., Baldwin, A. S., Jr. and Sharp, P. A. Regulation of heat shock protein 70 gene expression by c-*myc*. *Nature*, **312**, 280–282 (1984).
19) Kaddurah-Daouk, R., Greene, J. M., Baldwin, A. S., Jr., Kingston, R. E. Activation and repression of mammalian gene expression by the c-*myc* protein. *Genes Dev.*, **1**, 347–357 (1987).
20) Cheng, G. and Skoultchi, A. I., Rapid induction of polyadenylated H1 histone mRNAs in mouse erythroleukemia cells is regulated by c-*myc*. *Mol. Cell. Biol.*, **9**, 2332–2340 (1989).
21) Blackwood, E. M. and Eisenman, R. N. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science*, **251**, 1211–1217 (1991).