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Effect of c-*myc* Gene Expression on Early Inducible Reactions Required for Erythroid Differentiation In Vitro

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By employing cell fusion between two genetically marked mouse erythroleukemia (MEL) cells in which an artificially introduced c-*myc* gene had been placed under the control of human metallothionein promoter, we investigated the mechanism of the suppressive action of c-*myc* gene expression in erythroid differentiation. The results indicated that the expression of the c-*myc* gene blocked the induction of dimethyl sulfoxide-inducible activity, one of the two early activities required for triggering the differentiation.

There have been a number of reports concerning the change in oncogene expression during cellular differentiation. In mouse erythroleukemia (MEL) cells (3), the cellular oncogene expression changes dramatically following exposure of the cells to erythroid-inducing agents such as dimethyl sulfoxide (DMSO) (4) or hexamethylenebis(acetamide) (13). For example, the level of c-*myc* gene expression fluctuates in a complex manner during the in vitro differentiation process triggered by these agents (7, 8, 12). The mechanism and biological significance of the change, however, still remain unknown. On the other hand, constitutive expression of the c-*myc* gene when it is artificially introduced into MEL cells suppresses the differentiation, strongly suggesting that the c-*myc* gene product prevents the cells from differentiating into erythroid cells (1, 2, 5a, 6, 11). These results are consistent with the widely accepted view that differentiation processes are, directly or indirectly, linked to cellular proliferation.

Terada et al. (15) and Scher and Friend (14) first showed that in vitro erythroid differentiation of MEL cells induced by DMSO is greatly stimulated by UV irradiation. By using cell and cytoplast fusion, we have confirmed their finding and further shown that the in vitro differentiation is triggered by a synergistic reaction of two basically different early inducible reactions (5, 9, 16). One reaction is induced when DNA replication (or cell division as a consequence) is blocked by UV irradiation or by mitomycin C (MMC) treatment, whereas the other is induced by a still unidentified signal generated by erythroid-inducing agents such as DMSO or hexamethylenebis(acrylamide). More recently, two different proteinaceous factors (DIF-I and DIF-II) which apparently correspond to these reactions were detected in cell extracts (10, 17). To obtain a clue about the mechanism of the suppressive action of the c-*myc* gene product in MEL cell differentiation, we constructed an MEL cell line in which c-*myc* gene expression was under the control of outside inducers. Then, by using cell fusion, we attempted to determine which of the two inducible reactions is suppressed by the c-*myc* gene product, thereby eventually leading to the inhibition of erythroid differentiation.

The two inducible activities required for the erythroid differentiation were demonstrated by cell fusion utilizing genetically marked MEL cells. In a typical experiment (Fig. 1A), MEL cells (DS19, TK^- $HPRT^+$) were treated with MMC, and then at different time intervals after treatment (as indicated in the figure) they were fused with other, differently marked MEL cells (TK^+ $HPRT^-$) which had been briefly exposed to DMSO. The cells were then incubated in hypoxanthine-aminopterin-thymidine (HAT) medium to select for fused cells. Among the surviving (fused) cells, those reactive with benzidine (B^+ cells), an indication of hemoglobin accumulation, were scored 5 days later. An activity was induced following the MMC treatment which was detected only when the partner cells (TK^+ $HPRT^-$) had been pulsed with DMSO (Fig. 1A). In the control experiment, the DMSO pulse alone did not induce erythroid differentiation (data not shown; see also reference 5). The induced activity reached a maximum at approximately 24 h of incubation after the treatment and was induced in a wide variety of mammalian cells, suggesting that the activity was not specific to MEL cells (9).

Another early erythroid-inducing activity was detected by an experiment similar to that described above, but with an opposite combination of cells. MEL cells (TK^- $HPRT^+$) were pulsed with DMSO and then, at different time intervals after the pulse, fused with the partner MEL cells (TK^+ $HPRT^-$), which had been treated with MMC 24 h before. The cells were incubated in HAT medium for 5 days, and B^+ cells were counted. A short-lived activity with appearance kinetics quite different from those of the MMC-inducible activity was induced (Fig. 1B). Furthermore, in contrast to that induced by MMC treatment, the induction was specific to MEL cells (9) and sensitive to biologically active phorbol esters (5), suggesting the involvement of protein kinase C.

To study the effect of c-*myc* gene expression on erythroid differentiation, we examined whether either one or both of the two inducible reactions required for MEL cell differentiation were inhibited by increased expression of the c-*myc* gene. For this, an MEL cell line was constructed in which expression of the c-*myc* gene was controlled by outside inducers, thus enabling us to study the effect of c-*myc* gene expression against an identical cellular background. We constructed a composite DNA in which the c-*myc* structural gene was placed downstream of the human metallothionein promoter, so that the gene was inducibly expressed when heavy-metal ions such as Zn^{2+} were supplied. The structure of the composite DNA (pHMT-*myc*S) is shown in Fig. 2. The DNA (pHMT-*myc*S) was introduced into MEL cells (B8,

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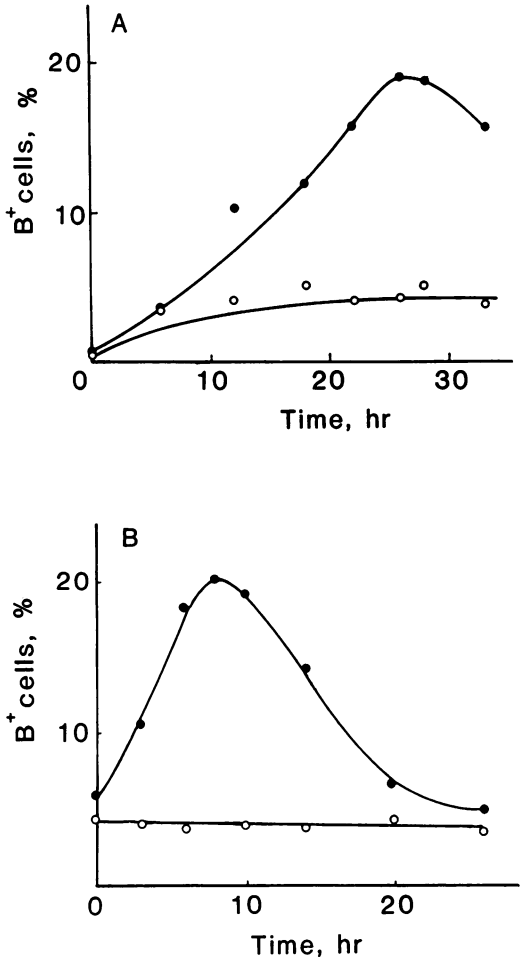

FIG. 1. Induction of the activities leading to erythroid differentiation revealed by cell fusion. (A) MMC (1 μg/ml) was added to a culture of MEL cells (TK^- $HPRT^+$), and at different time intervals, as indicated in the figure, a portion of the cells were fused with MEL cells (TK^+ $HPRT^-$) that had been incubated with DMSO for 8 h. The cells were then incubated in HAT medium for 5 days, and benzidine-positive (B^+) cells were counted. Cell fusion was performed as described previously (5). Symbols: ●, fusion with the cells that had been incubated with DMSO; ○, fusion with the control (without DMSO) cells. (B) MEL cells (TK^- $HPRT^+$) were incubated in the presence of DMSO (1.8%) for the period indicated in the figure and then fused with MEL cells (TK^+ $HPRT^-$) that had been incubated in the presence of MMC (1 μg/ml) for 24 h. The cells were then incubated in HAT medium for 5 days, and benzidine-positive (B^+) cells were counted (5). Symbols: ●, fusion with MMC-treated cells; ○, fusion with control (without MMC) cells.

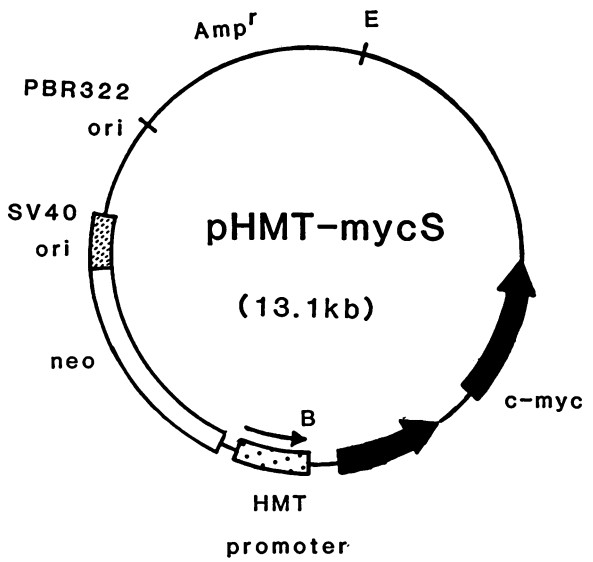

FIG. 2. Construction and structure of a c-*myc* gene under the control of the human metallothionein (HMT) promoter. A composite DNA (pHMT-*myc*S) with a c-*myc* gene under the control of the human metallothionein promoter was constructed as follows and as detailed in a recent report (5a). A 0.8-kilobase (kb) *Eco*RI-*Hin*dIII fragment containing the human metallothionein-IIA gene promoter was inserted into pSVneo. A 0.7-kilobase *Bam*HI-*Eco*RI fragment containing exons 2 and 3 of the rat c-*myc* gene was then inserted between the *Bam*HI and *Eco*RI sites. The direction of the human metallothionein promoter is indicated by an arrow. E, *Eco*RI site; B, *Bam*HI site; *neo*, the selective marker gene (neomycin resistance); ori, the origin of replication; Amp^r, ampicillin resistance gene. The positions of exons 2 and 3 of the c-*myc* gene (and the direction of transcription) are indicated as large solid arrows. The simian virus 40 (SV40) promoter is located immediately upstream of the *neo* gene.

TK^- $HPRT^+$) by protoplast fusion, and among the G418-resistant transformants, a clone (38-2) in which c-*myc* gene transcripts were induced in the presence of Zn^{2+} was isolated. The c-*myc* transcripts in clone 38-2 were greatly increased in the presence of Zn^{2+} (Fig. 3). The effect of Zn^{2+} on erythroid induction in clone 38-2 is shown in Table 1. Whereas Zn^{2+} had no effect on the erythroid induction of the parental strain (B8), it suppressed the differentiation substantially in clone 38-2. More detailed studies on the induction of the c-*myc* gene in clone 38-2 with respect to the mechanism of erythroid differentiation are reported elsewhere (5a).

For the first experiment examining the effect of c-*myc* expression on the induction of the early activities required for differentiation, MEL cells (38-2, TK^- $HPRT^+$) were exposed to MMC and incubated in the presence or absence of $ZnCl_2$. At different time intervals, the cells were fused with the partner MEL cells (TK^+ $HPRT^-$), which had been

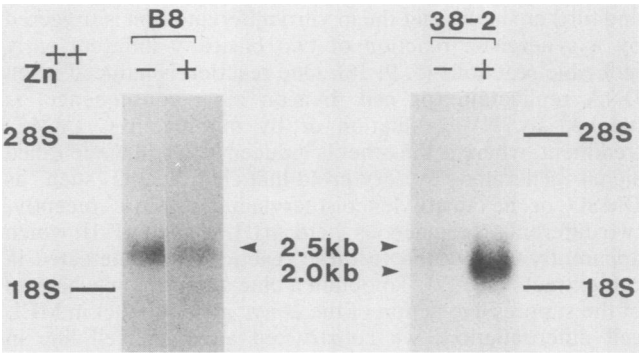

FIG. 3. Induction of the exogenous c-*myc* gene transcripts with heavy metals. The parental MEL cell (B8) and transformant (38-2) were cultured in the presence (+) or absence (−) of $ZnCl_2$ (200 μM) for 16 h. From the total RNA, poly(A)$^+$ RNA was purified with an oligo(dT)-cellulose column, and a portion was subjected to gel electrophoresis. The c-*myc*-specific transcripts were then identified by using a c-*myc*-specific ^{32}P-labeled riboprobe. The transcripts of the endogenous kilobases (2.5 [kb]) and exogenous (2.0 kilobases) c-*myc* genes differ in size because of the absence of exon 1 in the exogenous gene. The positions of molecular weight markers (18S and 28S) are also indicated.

TABLE 1. Erythroid differentiation of B8 (the parental cells) and 38-2 (bearing inducible c-*myc* gene)

Cell line	Conditions	B^+ cells (%)[a]
B8	No addition	<1.0
	DMSO (1.4%)	78.6
	DMSO (1.4%) + $ZnCl_2$ (200 μM)	74.8
38-2	No addition	<1.0
	DMSO (1.4%)	75.0
	DMSO (1.4%) + $ZnCl_2$ (200 μM)	21.9

[a] Percentage of benzidine-positive (B^+) cells after 5 days of incubation.

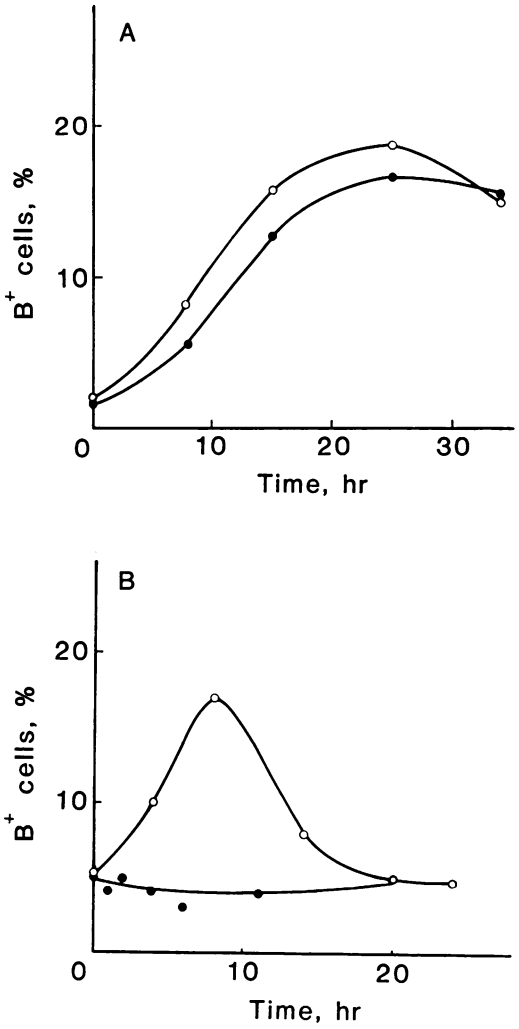

FIG. 4. Effect of c-*myc* gene expression on the induction of erythroid-inducing activities. (A) MEL cells (38-2, TK^- $HPRT^+$) were incubated with MMC (1 μg/ml) in the presence (200 μM) or absence of $ZnCl_2$ for different periods as indicated in the figure, and each sample was fused with MEL cells (TK^+ $HPRT^-$) that had been incubated with DMSO for 8 h. The cells were then incubated in HAT medium for 5 days, and B^+ cells were counted. Symbols: ●, the cells incubated with MMC and $ZnCl_2$ were fused; ○, the cells incubated with MMC alone were fused. (B) MEL cells (38-2, TK^- $HPRT^+$) were incubated with DMSO (1.4%) in the presence (200 μM) or absence of $ZnCl_2$ for different periods as indicated in the figure, and each sample was fused with MEL cells (TK^+ $HPRT^-$) that had been incubated with MMC (1 μg/ml) for 24 h. The cells were then incubated in HAT medium for 5 days, and B^+ cells were counted. Symbols: ●, the cells incubated with DMSO and $ZnCl_2$ were fused; ○, the cells incubated with DMSO alone were fused.

pulsed with DMSO 8 h before. The fused cells were incubated in HAT medium, and B^+ cells among the surviving cells were then counted. The induction of the MMC-induced activity was not significantly affected by the presence of $ZnCl_2$ (Fig. 4).

In the experiments with the opposite combination, in which 38-2 cells were pulsed with DMSO, incubated in the presence of $ZnCl_2$, fused with MMC-pretreated partner cells (TK^+ $HPRT^-$) at different time intervals after the pulse, and then incubated in HAT medium, we found that induction of the DMSO-inducible activity was completely blocked (Fig. 4B). Induction of the activity was normal in the control (without $ZnCl_2$) cells (Fig. 4B). We also performed experiments with another MEL cell line (28-1) with the same inducible c-*myc* gene as clone 38-2 and obtained essentially the same results as described above (data not shown). These results indicate that c-*myc* gene expression suppresses one of the two reactions (the DMSO-inducible activity) required at the early stage of MEL cell differentiation and that this eventually leads to the inhibition of differentiation.

Previous reports have suggested that the c-*myc* gene product somehow interferes with or antagonizes one or more of the intracellular reactions required for differentiation (1, 2, 5a, 6, 11). These reports, however, failed to provide information regarding the nature of the reaction inhibited by the c-*myc* gene product or the stage of differentiation at which the gene product functions. The cell fusion experiments described above allowed us to elucidate these aspects to some degree. First, induction of the c-*myc* gene product prevented induction of the intracellular reaction induced by DMSO, whereas it had little effect on the induction of the other early reaction triggered by inhibition of DNA replication. Second, the effect seemed to be quite rapid and effective, since the addition of Zn^{2+} together with the inducer (DMSO) immediately (less than 1 h; Fig. 4B) and completely blocked the DMSO-induced activity. This also suggests that the effect of c-*myc* expression is exerted at the very early stage of MEL cell differentiation, although it requires at least 3 to 4 days to complete.

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