Overexpression of c-Myc Inhibits the Appearance of a Specific DNase I Hypersensitive Site in the β-Globin Chromatin in Murine Erythroleukemia Cells

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To investigate the molecular mechanism of regulation of differentiation by c-Myc, we examined the acquisition of DNase I hypersensitivity by β-globin chromatin in an MEL cell transformant (38-2) in which the c-*myc* gene was placed under the control of a metallothionein gene promoter. Of the three DNase I hypersensitive sites around the β-globin gene which were detected in MEL cells after DMSO treatment, the appearance of a specific DNase I hypersensitive site near the promoter was greatly reduced by the overexpression of c-Myc. This result suggests that c-Myc regulates β-globin expression by interfering with the establishment of an active chromatin conformation around the β-globin gene.

Key words: Murine erythroleukemia cell — c-Myc — β-Globin gene — DNase I hypersensitive site — Chromatin

Murine erythroleukemia (MEL) cells can be induced to undergo a program of differentiation that resembles the final stages of erythropoiesis by incubation with dimethyl sulfoxide (DMSO) or a variety of other chemicals.[1] This includes a large increase in the production of hemoglobin. It has been reported that activation of β-globin chromatin structure precedes the large increase in the production of β-globin mRNA.[2-6] The active conformation measured in terms of DNase I hypersensitive sites located 5' to the β-blobin gene is detected only after the addition of DMSO, and the appearance of DNase I hypersensitive sites is well correlated with the commitment event.[6] In the early event of MEL cell differentiation induced by DMSO, the level of c-*myc* mRNA changes dramatically[7] and the commitment and the expression of globin genes are inhibited by elevated levels of c-Myc in MEL cell transformants in which the c-*myc* gene was placed under the control of metallothionein gene promoter.[8,9] Thus, it is likely that c-Myc may inhibit the commitment by interfering with the establishment of an active chromatin conformation of the β-globin gene. In this work, we examined whether the overexpression of c-Myc inhibits the appearance of DNase I hypersensitivity of the β-globin gene chromatin during the differentiation of MEL cells.

The MEL cell transformants were obtained by transfer of a rat c-*myc* gene fused to the promoter of the human metallothionein II gene.[8] In these transformants, the transferred c-*myc* expression could be manipulated artificially by the addition of Zn ions in a time-dependent and dose-dependent manner. One clone, 38-2, was a typical transformant showing a c-*myc*-dependent inhibi-

tion of differentiation and was used in this work. When the exogenous c-*myc* mRNA was induced in the continuous presence of Zn ions, the level of β-globin mRNA was approximately 1/5 of that in the absence of Zn ions, as shown in Fig. 1. The continuous presence of Zn ions did not affect the induction of differentiation of non-transfected cells (B8/3) as previously reported.[8] Thus, elevated levels of c-Myc inhibit expression of the β-globin gene. For the commitment assay, cells induced with DMSO in the presence or absence of Zn ions for various times were transferred to semi-solid medium without DMSO and Zn ions and incubated for 2 days to form colonies. In this assay, if the cells are committed at the time of transfer, such cells could form benzidine-positive colonies on the semi-solid medium, but if the cells are not committed, they might form benzidine-negative colonies. After induction with DMSO in the absence of Zn ions, the content of committed cells increased from day 2 and reached the maximum level on day 4 (Fig. 2). In the presence of Zn ions, however, the content of committed cells was 1/5 of that in the absence of Zn ions. Thus, elevated levels of c-Myc inhibit the commitment and therefore may reduce the expression of globin gene.

We next examined whether elevated levels of c-Myc inhibit conversion of the β-globin gene chromatin from an inactive to an active conformation. We analyzed the conformation of chromatin by a DNase I hypersensitivity assay.[4-6] 38-2 cells were induced with DMSO in the presence or absence of Zn ions. Nuclei were isolated from samples taken at various times after DMSO treatment until 4 days after DMSO addition and digested with increasing concentrations of DNase I. The DNA was
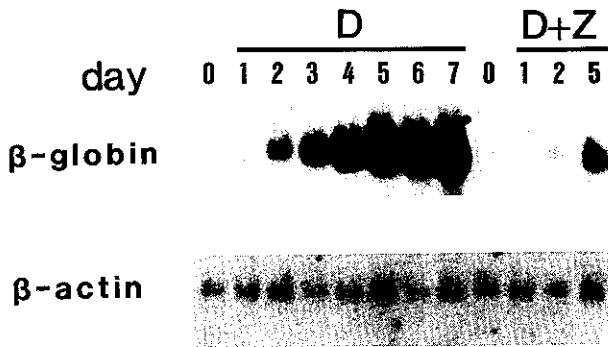
Fig. 1. Titration of β-globin and actin mRNAs after induction with DMSO in the presence or absence of ZnCl₂. Clone 38-2 cells were induced with DMSO (1.4%) in the presence of 180 μM ZnCl₂ (D+Z) or in the absence of ZnCl₂ (D). At one-day intervals (0, 1, 2, 3, 4, 5, 6, and 7 days for DMSO alone, and 1, 2, and 5 days for DMSO with ZnCl₂), the cells were collected and the RNA was isolated from the MEL cells by guanidine hydrochloride extraction, followed by CsCl density centrifugation. The RNA was separated in a 1.5% agarose gel containing MOPS and formaldehyde, then transferred to a nitrocellulose filter. The filter was hybridized with ^{32}P-labeled β-globin gene probe and chicken β-actin gene grobe as described previously.[20]
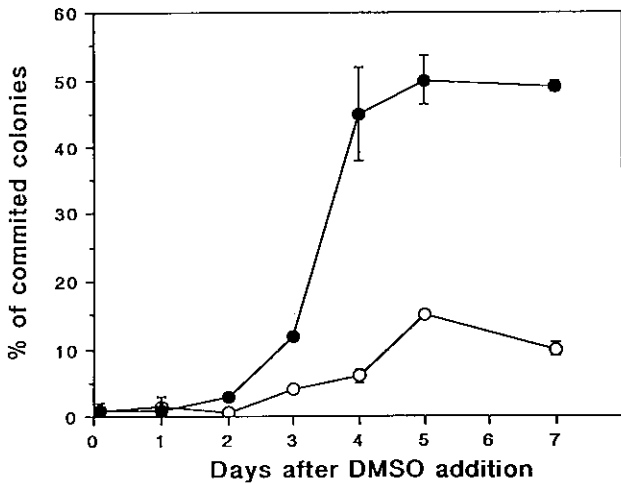

Fig. 2. Commitment of MEL cell transformant (clone 38-2) in the presence or absence of ZnCl₂. MEL cell transformant (clone 38-2) with the transferred c-myc gene under the human metallothionein promoter was grown in ES medium supplemented with 10% fetal bovine serum (Gibco).[8] Clone 38-2 cells were induced with DMSO (1.4%) in the presence of 180 μM ZnCl₂(○) or in the absence of ZnCl₂ (●). At various time intervals, the cells were taken from the liquid culture and transferred to the semisolid medium without DMSO as previously described.[21] After 2 days of incubation, the percentage of benzidine-positive colonies amongst the total colonies was estimated.
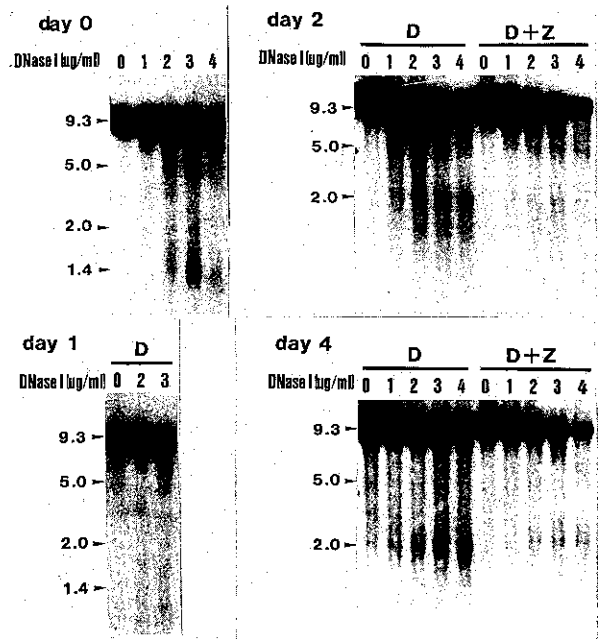

Fig. 3. DNase I hypersensitive sites of β-globin gene during MEL cell differentiation in the presence or absence of ZnCl₂. Clone 38-2 cells were induced with DMSO (1.4%) in the presence or absence of ZnCl₂ (180 μM) and the cells were collected at daily intervals. Isolation of nuclei from MEL cells was performed by the method of Smith and Yu.[10] Briefly, the cells were washed three times in buffer C (1 mM Tris-HCl, pH 7.6, 25 mM KCl, 0.9 mM MgCl₂, 0.9 mM CaCl₂, 0.14 mM spermidine), and once in buffer C plus phenylmethylsulfonyl fluoride. The cells were lysed in a Dounce homogenizer, and the nuclei were pelleted and washed twice in buffer C. DNase I digestion of the isolated nuclei was performed at 20°C for 7 min in 10 mM Tris-HCl, pH 7.6, 10 mM NaCl, and 5 mM MgCl₂ with various concentrations of DNase I. The reaction was terminated by the addition of 20 mM EDTA and 0.1% SDS. DNA extracted from the DNase I-treated nuclei was cleaved with XbaI, size fractionated in a 0.7% agarose gel and transferred to a nitrocellulose filter. For the detection of the β-globin gene, the DNA fragment of mouse β major globin gene was labeled with ^{32}P-dCTP by nick-translation and hybridization was performed as described.[20] The hypersensitivity assay was done in the cells just before the addition of DMSO (day 0), or 1-4 days after the addition of DMSO. Clone 38-2 cells were treated with DMSO alone (D) or with DMSO and ZnCl₂ (D+Z). The concentration of DNase I is given at the top of each lane. The uncleaved 9.3 kbp, cleaved 5, 2.0 and 1.4 kbp fragments are indicated by arrowheads.

purified, digested with XbaI, and subjected to Southern blot analysis (Fig. 3). The β-major globin gene lies on a 9.3 kb XbaI restriction fragment. Before and after induction with DMSO, three sub-bands, 5.0 kb, 2.0 kb and 1.4 kb in length, generated by cutting at the DNase I hypersensitive sites within the 9.3 kb fragment, could be

visualized as shown in Fig. 3. We tentatively call the site generating the 5.0 kb band, site I; that generating the 2.0 kb band, site II and that generating the 1.4 kb band, site III. Before induction (day 0), sites I and III were detected, but the 2.0 kb band (site II) was not. In the case of induction with DMSO in the absence of Zn ions, the 2.0 kb band (site II) became detectable after 2 days and increased in intensity with increasing time of induction. The kinetics of appearance of the 2.0 kb band correlated well with the commitment to differentiate, as shown in Fig. 2, in agreement with previous reports.[6, 10] The 1.4 kb band (site III), which was detected at day 0, disappeared soon after induction with DMSO (Fig. 3) as reported previously.[4] The 5.0 kb band (site I) was detectable before induction and its intensity did not change during induction. This band, which was weaker than the other two bands, was not mentioned in the reports of Sheffery *et al.*[5, 6] and Salditt-Georgieff *et al.*,[4] whereas Smith and Yu reported that this band was enhanced after induction with hexamethylenebisacetoamide.[10] The discrepancy concerning this band in the previous reports may be due to the difference in the cell lines and inducers.

Elevated levels of c-Myc had significantly different effects on the three DNase I hypersensitive sites. In the presence of Zn ions (D+Z in Fig. 3), the appearance of the 2.0 kb band (site II) was strongly inhibited by elevated levels of c-Myc. From densitometric measurements, the level of the band in the Zn-treated cells was approximately 1/5 of that in untreated cells and this value is well correlated with the extent of the inhibition of commitment (Fig. 2) as well as expression of β-globin gene (Fig. 1). We have shown that the transformant (38-2) can grow continuously in the presence of both DMSO and Zn ions and the proportion of differentiated cells in a population in continuous culture is strongly affected by the concentration of Zn ions.[9] Thus, the reduction to 1/5 in the globin gene expression and the intensity of the 2.0 kb band may be due to the presence of 20% fully-expressing cells with 80% non-expressing cells.

On the contrary, the disappearance of the 1.4 kb band (site III) was also observed in the presence of Zn ions. The effect of elevated levels of c-Myc on the 5.0 kb band (site I) was not clear because the band was only weakly detected, but it seems that it was not strongly affected by elevated levels of c-Myc. Thus, the overexpressed c-Myc

inhibited a specific DNase I hypersensitive site surrounding the β-globin gene. It is interesting that only site II was inhibited by elevated levels of c-Myc in MEL cells, since site II is located near the promoter region of the β-globin gene and this region is expected to be required for the erythroid-specific expression of the β-globin gene, as previously reported.[2, 11] The other two sites that were not affected by elevated levels of c-Myc do not contain the elementary sequences required for the erythroid-specific expression of this gene. Thus, c-Myc may have an important role in the formation of an active chromatin structure at the specific sites of erythroid-specific genes and may link the activation of these genes to the commitment of MEL cell differentiation.

Little is known about the mechanisms that establish, maintain and propagate hypersensitive sites, but once formed, sites can be propagated for at least 20 generations, even in the absence of an associated function.[12, 13] Many *trans*-acting factors may be associated with the resident DNA sequences of active genes and may form hypersensitive sites. Whereas c-Myc has DNA binding ability, it has not been shown to bind specific DNA sequences required for transcription.[14] In this respect, it might be important to examine whether several *trans*-acting factors which bind to the 5'-flanking region of the β-globin gene near site II could be affected by the elevated level of c-Myc in direct or indirect ways. Such an investigation is now under way. Active genes are located near the nuclear periphery[15] and thus may be advantageously situated to interact optimally with regulatory proteins upon entry to the nucleus from their cytoplasmic site of synthesis.[6] In addition, active genes replicate in early S phase, in contrast to inactive sequences.[16] A possible mechanism for propagation of hypersensitive sites has been proposed in which crucial transcription factors, which are believed to exist in limiting amounts, are titrated by the early replicating sequences.[17, 18] This provides a mechanism for inheritance of specific chromatin structures. c-Myc may regulate the propagation of hypersensitive sites on chromatin since c-Myc is believed to be located within the nucleus[14] and may have functions in cell cycle control.[19]

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