A Direct Role of Transcription Factor E2F in c-myc Gene Expression during Granulocytic and Macrophage-like Differentiation of HL60 Cells

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Abstract

The transcription factor E2F is known to play an important role in cell cycle progression through interaction with retinoblastoma protein. HL60 cells are able to differentiate into a granulocytic lineage by prolonged exposure to retinooids and into a macrophage-like lineage by exposure to tumor promoter 12-O-tetradecanoylphorbol 13-acetate, with a rapid decrease of c-myc gene expression. In this study, we assessed the changes of the E2F-binding pattern to the P2 promoter region of the c-myc gene during differentiation into both lineages. The observed changes of the E2F-binding pattern were a decrease of free E2F and an appearance of retinoblastoma protein-containing E2F complexes in both lineages. The effects of the anti-c-myc antibody and the recombinant c-Myc protein on the E2F-binding patterns suggest that the c-Myc protein is not involved directly in these changes. These changes also led the suppression of transcriptional initiation from the P2 promoter. The results indicate that, in the course of HL60 cell differentiation, E2F plays a direct role in the transcriptional control of the c-myc gene through interaction with the retinoblastoma protein. A potential role for the c-Myc protein is discussed in relation to an existing state of E2F and E2F-RB complexes in the HL60 cells.

Introduction

Retinooids, RA3 and its synthetic analogues, and tumor promoter TPA affect a wide array of biological processes in relation to cell growth and differentiation. When human promyelocytic leukemia cell line HL60 is cultured with a physiological concentration of RA, a rapid decrease of c-myc mRNA is observed within 24 h (1). We reported previously that this rapid decrease was partly attributed to the control at the level of transcriptional initiation (2), i.e., a treatment of HL60 cells with RA suppressed the transcription initiation from the P2 promoter of c-myc gene. The responsive element was identified in the P2 promoter region and tentatively designated RARE-myc, which corresponded to the E2F-binding site existing in the c-myc P2 promoter region (Fig. 1). E2F is one of the cellular transcription activators which play an important role in cell cycle control, and the complex formation with RB abolishes its transcriptional activation function (3-5). RA treatment reduced free E2F and induced the formation of E2F-RB complex on the E2F-binding site in RARE-myc; thus, the changes induced by RA led to the suppression of transcription initiation from the P2 promoter. Since the prolonged cultivation of HL60 cells with RA brings about granulocytic differentiation, one interesting question is whether the E2F-binding activity changes according to the progression of the granulocytic differentiation induced by retinooids. A relationship between the state of E2F and cell cycle progression has been studied extensively (6, 7), but the connection between E2F and cell differentiation is poorly understood. Thus far, Reichel (8) has reported on the RA-induced differentiation of mouse P19 teratocarcinoma cells. HL60 cells are also differentiated into macrophage-like cells, with a concomitant decrease of c-myc gene expression when treated with tumor promoter TPA (9). Another interesting question is whether a similar change of the E2F-binding activity would be observed during macrophage-like differentiation as compared to granulocytic differentiation.

In this report, we studied the changes of the E2F-binding activity during the differentiation of HL60 cells into a granulocytic lineage, induced by RA and Am80, or differentiation into a macrophage-like lineage by TPA. We show that the E2F-binding activity was changed in a similar manner during the progression of differentiation into either lineage, i.e., an increase of E2F-RB complexes and a decrease of free E2F. c-Myc protein, which was reported to be complexed with RB in vitro (10), was not involved in these changes. We show that these changes also led to the suppression of transcription initiation from the c-myc P2 promoter. These results indicate that the expression of the c-myc gene fluctuates with the changes in E2F-binding activity. Here we discuss the role of the c-Myc protein during terminal differentiation and in the progression of the cell cycle in relation to the state of E2F.

Results

Time Course of the Terminal Differentiation of HL60 Cells.

In order to examine the time course of differentiation into a granulocytic lineage, HL60 cells were cultured with two retinooids, RA and Am80, at a concentration of 0.1 μM for 4 days. After treatment, a percentage of differentiated cells was measured by means of the NBT reduction assay (Fig. 2B), which showed a good correlation with granulocytic differentiation. The number of total cells was also counted at the same time (Fig. 2A). Both retinooids induced differentiation, but the time course of differentiation or growth was found to be quite different in RA- and Am80-treated cells. After 2 days, about 90% of the cells were differentiated by

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3 The abbreviations used are: RA, retinoic acid; TPA, 12-O-tetradecanoylphorbol 13-acetate; RB, retinoblastoma protein; NBT, nitroblue tetrazolium; DOC, sodium deoxycholate; PACE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase.

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RA, while the differentiated cells were less than 20% by Am80. More than 70% of HL60 cells differentiated after 4 days by both retinoids. The time course of the growth suppression was also different in both cases. Data indicate that RA effectively suppressed the growth of HL60 cells after 4 days, but on the other hand, HL60 cells treated with Am80 for 4 days were still able to grow, although most of the cells became NBT positive. When those treated cells were harvested and cultured again in each corresponding fresh medium containing RA or Am80 for another 3 days, almost all of the cells died by further treatment with RA, but the number of cells treated with Am80 was significantly increased, despite the slow growth rate.\(^4\) The time course of the differentiation into a macrophage-like lineage was also examined by treating HL60 cells with 20 nM TPA. Most of the HL60 cells ceased to proliferate and attached to the plastic plate after a 24-h treatment of TPA (Fig. 2A).\(^4\) Results indicate that the macrophage-like differentiation of HL60 cells was completed within 24 h.

**Change of the E2F-binding Activity during Terminal Differentiation of HL60 Cells.** To examine a change of the E2F-binding activity during the granulocytic differentiation induced by RA or Am80, nuclear extracts were prepared from HL60 cells after treatment with 0.1 μM RA or Am80 for 1, 2, or 4 days. Each nuclear extract was incubated with a \(^{32}\)P-labeled X-3 probe, which contained the E2F-binding site in the P2 promoter region of the c-myc gene (Fig. 1), and then subjected to the mobility shift assay. As shown in the left panel of Fig. 3, a significant change of the E2F-binding activity was observed, according to the progression of the differentiation. Based on the previous assignment of retarded bands in the acrylamide gel (2), bands A, B, and C corresponded to the E2F-containing complexes, while band D was nonspecific and unchanged throughout the differentiation process. The change of E2F-binding activity observed after treatment with 0.1 μM RA for 1 day was the same as that in the previous data (2). Band B was increased, while band C decreased. After 1 day, less than one-fourth of HL60 cells was estimated to be differentiated by RA (Fig. 2A), but after 2 days, most of the HL60 cells were differentiated. In relation to this morphological change, a drastic change of the E2F-binding activity was observed. Prominent changes are a disappearance of band C and an appearance of the new band (band B'; Fig. 3, left panel). From the competition experiment, this new band also corresponded to the E2F-containing complex (Fig. 4).\(^4\) Besides these changes, band B increased, but band A did not change. HL60 cells treated with RA for 4 days exhibited complete differentiation and ceased to proliferate. The pattern of the E2F-binding activity of HL60 cells treated with RA for 4 days was about the same as that of the cells treated for 2 days. However, the intensity of each band slightly decreased, although the same amount of the extracted protein was used in each case.

The decreased intensity of each band by the fourth day is due to a certain change of the amount of E2F bound to DNA, because among the E2F-containing complexes, the...
component that directly binds to DNA is known to be E2F (11). Treatment of the reaction mixture with ionic detergent DOC following anionic detergent NP40 or with adenovirus E1A protein was able to dissociate protein-protein interactions of E2F-containing complexes but not DNA-protein interaction, and subsequently gave a single band in the mobility shift assay (11). Free E2F seems to be a complex consisting of an E2F-related protein (E2F-1, -2, or -3) and DP-1 (12). Although the effect of DOC or E1A protein on the formation of the E2F/DP-1 heterodimer was not yet clarified, the residual band might reflect the amount of E2F/DP-1 heterodimer. In the present experiments, DOC treatment gave only one band in all cases throughout the process of granulocytic differentiation, where the amount of E2F was almost unchanged for the 2 days after RA or Am80 treatment but was slightly decreased by the fourth day. One possible explanation for such decrease is that the reduced expression of E2F-1, -2, -3, or DP-1 gene at this period may cause the decrease, or another explanation is that it is due to a change of the phosphorylation state of these proteins (13–15).

The rate of granulocytic differentiation of HL60 cells induced by Am80 was slower than that of RA (Fig. 2A). After 2 days, less than 20% of HL60 cells were differentiated by Am80, and an increase of band B and a new appearance of band B' was observed. By 4 days, the cells were still able to grow, and band C could be observed, but the intensity of the band was reduced. In spite of the fact that differentiation reached up to 70%, bands B and B' showed no further increase. In contrast, when the changes of E2F-binding activity induced by RA were compared with those by Am80 in relation to the changes of HL60 cell growth and differentiation, the increase of band B and the appearance of band B' correlated well with the progression of differentiation and the decrease of band C with the cessation of proliferation, as was described above.

In the case of macrophage-like differentiation, HL60 cells were treated with 20 nM TPA for 24 h; then the nuclear extracts were prepared and subjected to the mobility shift assay. To compare this case with the granulocytic differentiation, the nuclear extract from HL60 cells treated with RA for 4 days was assayed at the same time. As shown in Fig. 4, each band was assigned on the basis of the mobility compared to the results of RA-treated cells. Prominent changes observed during the macrophage-like differentiation were a disappearance of bands A and C and an increase of band B, with a new appearance of band B'. When the amount of E2F bound to DNA was measured by DOC treatment, it showed a slight increase after TPA treatment in some experiments (Fig. 4) but almost no change or a slight decrease in other experiments. From these results, no significant change occurred in the amount of E2F through-

![Fig. 3](image1.png) Changes of the binding pattern of the X-3 probe during the granulocytic differentiation of HL60 cells. Left panel, nuclear extracts were prepared from HL60 cells cultured with 0.1 μM RA or 0.1 μM Am80 for 1, 2, and 4 days or with the same volume of ethanol for 4 days. Each nuclear extract was incubated with the 32P-labeled X-3 probe and then subjected to electrophoresis. Bands A, B, and C were assigned according to our previous report (2) and are indicated at the left side of the panel. Differentiation-specific DNA-protein complexes were identified and are indicated as B and B'. These bands correspond to E2F-containing complexes, while band D represents a nongenomic complex. Free probe was run off the bottom of the gel to fully resolve the retarded band. Right panel, change of the total amount of E2F protein, according to the differentiation, was assayed by treating the binding reaction mixture with 0.4% DOC.

![Fig. 4](image2.png) Changes of the binding pattern of the X-3 probe during the macrophage-like differentiation of HL60 cells and the characteristics of the DNA-protein complexes. Nuclear extract was prepared from HL60 cells cultured with or without 20 nM TPA for 24 h and subjected to the mobility shift assay. Various treatments of the binding reaction mixtures are indicated above each set of lanes. From the competition assay using E2-1, DOC treatment, or anti-RB supershift experiment, shifted bands were characterized; band C corresponds to free E2F, and bands B and B' are E2F-RB complexes. In the competition assay, 100-fold molar excess of E2-1 competitor was used.
out the process of the macrophage-like differentiation under the conditions used.

To confirm the assignment of each band, the competition mobility shift assay and the supershift assay were performed, as described previously (2). As shown in Fig. 4, the E2-1 competitor, which is a synthetic oligonucleotide containing an E2F-binding site derived from an adenovirus E2 promoter region (Fig. 1), competed with bands A, B, B', and C but not band D. The addition of the anti-RB antibody to the reaction mixture caused the disappearance of bands B and B' and an appearance of a new band in the higher molecular weight region. From these results, bands B and B' were assigned to RB-containing E2F complexes, in contrast to band C of free E2F. Therefore, common changes in the differentiation processes into both lineages were the disappearance of free E2F (band C) and the appearance of RB-containing E2F complexes (bands B and B'). We unexpectedly observed that band B' also contained RB besides band B. The difference in mobility between the two bands indicated that the component of each band was different each other. Although the exact difference must be clarified, one possible explanation is that the component of E2F (E2F-1, -2, -3, and DP-1) in bands B and B' is different. Another possibility is that band B contains a different factor from band B'. A candidate for such a factor would be RBP60, which was reported to promote the binding of E2F-RB complex to DNA (16). Existence of such a factor in band B may also explain why the intensity of band B is stronger than that of band B'. Results indicate that the change of E2F-binding activity is not specific for a certain differentiation lineage but rather a more general phenomenon in the terminal differentiation of HL6O cells. It should be noted, however, that disappearance of band A was not observed in the granulocytic differentiation by RA treatment but was found in the macrophage-like differentiation. Based on the supershift assay using anti-p107 antibody, band A probably contains p107, as will be described later (Fig. 6).

Relationship between the Formation of RB-containing E2F Complex and the Amount of c-Myc Protein and Phosphorylation of RB Protein. The next question is by what mechanism the change in the binding pattern of the E2F-containing complex is brought about. It was reported that RB could form a complex with c-Myc protein in vitro (10) as well as with E2F. Thus, it is plausible that the change in the E2F-binding activity is controlled by the change of interaction between E2F, RB, and c-Myc proteins. For example, one possible case is that the c-Myc protein forms a ternary complex with RB and E2F. Another possibility is that the c-Myc protein tends to dissociate the E2F-RB complexes. If the E2F-binding activity is controlled in such a way, it is expected that E2F, RB, and c-Myc proteins exist at the same time in the cell. First, we compared the change in the E2F-binding activity with the state of E2F, c-Myc, or RB, according to the progression of terminal differentiation.

HL6O cells were cultured with 20 nm TPA for 1, 4, 8, and 24 h, and the nuclear extract was prepared from each culture. The E2F-binding activity was then assayed using each nuclear extract (Fig. 5A, left panel) by mobility shift assay. A significant change of the E2F-binding activity became apparent between the 8- and 24-h treatments. To see a change of E2F protein bound to DNA, the reaction mixture was treated with DOC. The amount of binding was found to be unchanged throughout the process of macrophage-like differentiation (Fig. 5A, right panel).

Each nuclear extract was also subjected to SDS-PAGE, and c-Myc protein or RB was examined by Western blot analysis using appropriate antibodies [anti-c-Myc antibody, Rb(Ab-2); Oncogene Science]. Each assay system varies in the sensitivity of detection level; therefore, the amount of each protein could not be directly compared to each other. As shown in Fig. 5B, a significant decrease of the c-Myc protein became apparent after the 24-h treatment with TPA. As will be seen in Fig. 5C, the amount of total RB was little changed throughout the differentiation process, although...
the phosphorylated state was significantly changed, according to the progression of the macrophage-like differentiation. When the cells were cultured without TPA, the majority of RB existed in a hyperphosphorylated form. However, when the cells were cultured with TPA, dephosphorylation of RB took place gradually, and RB entirely changed into the hypophosphorylated form at the end of the 24-h culture with TPA (Fig. 5C). Within 8 h of treatment with TPA, the amount of E2F-RB complexes stayed at the low level (Fig. 5A), even if RB was gradually dephosphorylated (Fig. 5C). On the other hand, the c-Myc protein existed in abundance at this period (Fig. 5B). This implies that the c-Myc protein interferes with the formation of E2F-RB complexes.

**No Association of c-Myc Protein with RB-containing E2F Complexes.** If the c-Myc protein forms a ternary complex with E2F and RB, the anti-c-Myc antibody may bring about the supershift of bands B or B' in the mobility shift assay. In order to test this possibility, the anti-c-Myc antibodies were added to the reaction mixture. At the same time, the anti-RB or the anti-p107 antibody was used as a positive and negative control, respectively. As shown in Fig. 6A, bands B and B' were supershifted by the addition of the anti-RB antibody, consistent with previous results (Fig. 4). On the other hand, the anti-p107 antibody gave no change to bands B, B', and C, which were thought to be involved in HL60 cell differentiation. The addition of the anti-c-Myc antibody [c-myc(Ab-2); Oncogene Science] to the reaction mixture did not shift the mobility of the bands (Fig. 6A). Another anti-c-Myc antibody [c-myc(AB-1)] also gave a negative result (Fig. 6B). These results indicated that anti-c-Myc antibodies might not have any significant effect on E2F-RB complex formation. Data implies that there is little possibility that the c-Myc protein forms a ternary complex with E2F and RB. The Max protein hetero-dimerizes with the c-Myc protein through its helix-loop-helix leucine zipper domain but lacks the region homologous to the amino terminal domain of the c-Myc protein (17), which was reported previously to interact with RB in vitro (10). The addition of the anti-Max antibody did not cause any change in bands B, B', and C. Data indicate that the Max protein is not a constituent of the E2F-RB complex. With respect to the anti-p107 antibody, band A was partly supershifted, indicating that a part of band A contains the p107 protein. Band A changed only during the macrophage-like differentiation by TPA. The meaning of this change remains to be clarified.

Because the Max protein is known to be an in vivo partner of the c-Myc protein and the resulting heterodimer strongly binds to the E-box DNA sequence (CACGTG; Ref. 17), the state of the Max protein was also examined in the present experiments. When the nuclear extract from normally growing HL60 cells was incubated with the E-box probe (18) and subjected to the mobility shift assay, three main bands were observed (Fig. 7A). Among these bands, the fast-migrating one correlated to the c-Myc-Max heterodimer, because only this band was supershifted by both the anti-c-Myc and the anti-Max antibodies.4 When the nuclear extract from HL60 cells treated with TPA was used in this mobility shift assay, this fast-migrating band diminished dramatically (control in Fig. 7A). In other words, an excess amount of Max protein is probably present without forming the complex with the c-Myc protein in the nuclear extracts from TPA-treated cells.

To examine whether the heterodimer formation of the exogenously added c-Myc protein with the endogenous

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**Fig. 6.** Supershift of E2F-containing complexes by antibody in the mobility shift assay. A, anti-c-Myc antibody (Ab-2), anti-Max antibody, anti-RB antibody, or anti-p107 was used. B, anti-c-Myc antibody (Ab-1) was used instead of anti-c-Myc antibody (Ab-2).

Max protein is a good indicator to assess the ability of the Escherichia coli-expressed c-Myc protein to form a complex with other proteins, various amounts of the E. coli-expressed c-Myc protein were added into the TPA-un-treated or -treated nuclear extract, and the mobility shift assays were performed using the E-box as a probe. As shown in Fig. 7A, as little as a nanogram order of the recombinant c-Myc protein could restore the band corresponding to the c-Myc-Max heterodimer, because the restored band could be supershifted by the addition of either anti-c-Myc antibody or anti-Max antibody.4 Results clearly indicate that the recombinant c-Myc protein is able to form
a heterodimer with the Max protein under the conditions used in the mobility shift assay, suggesting that this protein is probably able to form complexes with other proteins in vitro as well. Then, if the c-Myc protein and RB could form a heterodimer and if this heterodimer formation could perturb the existing state of the E2F-RB complex, it was expected that the addition of the recombinant c-Myc protein into the reaction mixture of the gel shift assay would change the E2F-binding activity. However, as shown in Fig. 7B, the addition of the recombinant c-Myc protein up to 1 µg did not alter the band pattern of E2F-containing complexes under the conditions used. Present data suggests that recombinant c-Myc protein was unable to dissociate the E2F-RB complex in vitro. Is there any other factor that controls the E2F-binding activity? The nature of such a factor might be either inhibitory to the formation of the E2F-RB complex in the control nuclear extract or stimulatory in the TPA-treated nuclear extract. If an adequate amount of such a factor existed in the control extract, mixing the nuclear extract from control cells with that from TPA-treated cells would lead to further change of the band intensity in the mobility shift assay than that expected from an additive effect of the binding activity of each nuclear extract. When two different nuclear extracts, one from TPA-treated HL60 cells and the other from untreated cells, were mixed in the ratio of 1:3 to 1:0.25, no further change of the E2F-binding activity was observed than that expected from the mixing ratio.4 This result also suggests that the E2F-binding pattern observed during the differentiation of HL60 cells may not be controlled by the c-Myc protein, which is present in excess in the TPA-untreated cells.

Suppression of Transcription Initiation from the P2 Promoter of the c-myc Gene during Terminal Differentiation. E2F is known to be a transcriptional activator through the E2F-binding site upstream the P2 promoter of the c-myc gene (19, 20), and the complex formation of E2F with RB causes a loss of its transcriptional activation function (3–5). When HL60 cells were treated with 1 µM RA for 1 day, the E2F-RB complex formation was induced, resulting in the suppression of transcription initiation from the P2 promoter of the c-myc gene (2). Treatment of HL60 cells with a reduced concentration of RA (0.1 µM) for a longer period of time or with TPA for 24 h brought about a marked change in the E2F-binding activity. This change probably led to the strong suppression of transcription initiation from the P2 promoter of the c-myc gene. To test this point, CAT assays were performed using the pmycXCAT plasmid (Fig. 8A) as a reporter, which contains 95 base pairs upstream and 350 base pairs downstream of the P2 promoter (but not the P1 promoter) of the c-myc gene (2). HL60 cells were transfected with pmycXCAT DNA and then equally split into

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Fig. 8. Suppression of transcription from the P2 promoter of pmycXCAT plasmid by treatment with 20 µg TPA. A, map of the human c-myc gene and structure of pmycXCAT. B, the effect of TPA or RA on the transcription from the P2 promoter of pmycXCAT plasmid transected into HL60 cells. HL60 cells were transfected with pmycXCAT or pSV2CAT, then split into three parts and cultured with 1 µM RA, with 20 µg TPA, or with an equal volume of ethanol (--) for 24 h. Cell extracts were prepared, and CAT assays were performed.
three parts in order to culture with 1 μM RA, 20 nM TPA, or an equal volume of ethanol for 24 h. Cell extracts were prepared, and CAT assays were performed using the same amount of each cell extract. As shown in Fig. 6B, the CAT activity obtained from the TPA-treated cells was suppressed several fold from that of the control cells. pmycCAT, which contains both P1 and P2 promoters and the 5' flanking region up to the HindIII site (nucleotide –2328), gave the same results. This decrease by TPA treatment was not due to the suppression of general protein synthesis, because the CAT activity of the extract from HL60 cells transfected with pSV2CAT, which contains a SV40 early promoter, was increased by TPA treatment. As expected, the suppression of CAT activity by TPA was greater than that by RA. When the E2F-binding activities in the HL60 cells treated with 1 μM RA or 20 nM TPA for 24 h were compared, the amount of free E2F was less, and more E2F-RB complexes were present in TPA-treated cells than in RA-treated cells (2). It appears that there exists a correlation between the transcriptional initiation from the c-myc P2 promoter and the state of E2F binding. Data are consistent with an idea that the free E2F is a transcriptional activator, while the E2F-RB complex functions as a suppressor.

Discussion
In this study, we demonstrated that E2F-binding activity in HL60 cells was changed in a similar manner during the progression of differentiation into either a granulocytic lineage or macrophage-like lineage. Changes observed in the mobility shift assay were an increase of band B, an appearance of band B', and a decrease of band C (Figs. 3 and 4). From the results of the competition assay, the DOC treatment, and the supershift assay with the anti-RB antibody, bands B and B' corresponded to the RB-containing E2F complexes, and band C corresponded to free E2F (Fig. 4). Retinooids, both RA and Am80, are thought to exert their biological effects via specific nuclear receptors, RARs and RXRs, and cause HL60 cells to differentiate into the granulocytic lineage. The first step of TPA action is known to be the activation of protein kinase C, and subsequently, HL60 cells differentiate into the macrophage-like lineage. Although the initial step and the differentiation lineage are different, retinooids and TPA caused an increase of E2F-RB complexes during the progression of the differentiation and a concomitant disappearance of free E2F with the cessation of the proliferation (Figs. 2–4). These results indicate that the change of E2F-binding activity is not a specific phenomenon for a certain differentiation lineage but is a more general and important one during the terminal differentiation of HL60 cells.

E2F was originally identified as the promoter-binding factor of the adenovirus E2 gene and is now thought to play a central role in the control of the cell cycle (6, 7). According to the numerous data reported previously, E2F exists in many forms, e.g., free E2F, E2F-RB complex, E2F-p107 complex, or E2F-p130 complex (21–23), during the cell cycle progression. Among these complexes, the E2F-RB complex was thought to be restricted to the G1 phase of the cell cycle. But a previous report by Schwarz et al. (24) indicated that the E2F complex contained p107 restricted to the G1 phase of the cell cycle; on the other hand, the E2F complex contained RB protein formed in the G1 phase and which persisted through S phase. Together with the fact that E2F forms a large complex with cyclins, CDK2, and p107 protein in the S phase, Schwarz et al. (24) claimed that the association and dissociation of E2F and p107 protein showed a good correlation to the progression of the cell cycle. Then, what is the role of the RB-containing E2F complexes? According to the progression of HL60 cell differentiation, RB-containing E2F complexes appeared, and these changes were observed no matter which lineage of HL60 cells was differentiated (Figs. 3 and 4; bands B and B'). This indicates that the RB-containing E2F complexes may play an important role in the terminal differentiation of HL60 cells. Band B was observed at a reduced level in the undifferentiated cells, while band B' was not; therefore, their function might be different. A precise study of their composition and expression pattern will help to clarify this difference.

The formation of E2F-RB complexes is controlled by the phosphorylation state of the RB protein, i.e., a hypophosphorylated form of RB protein is able to complex with E2F, while the hyperphosphorylated form is not. In fact, changes of the phosphorylation state of the RB protein during terminal differentiation were observed in HL60 cells (Fig. 5C), and E2F existed throughout this period (Figs. 3 and 5A). A similar change of the phosphorylation state of RB protein was observed during the terminal differentiation of the muscle cell, where hypophosphorylated RB protein formed during the terminal differentiation could complex with MyoD; this change was thought to be a critical step for terminal differentiation (25). MyoD is a member of the basic helix-loop-helix protein family, as is E2F and the c-Myc protein. These facts imply that c-Myc protein itself is possibly involved in the E2F-RB complex formation. But, the results of the supershift assay with anti-c-Myc antibodies (Fig. 6) and the addition of the recombinant c-Myc protein into the reaction mixture of the mobility shift assay (Fig. 7B) disprove this possibility that c-Myc protein is directly involved in complex formation, although the interaction between the c-Myc protein and RB was reported in vitro (10). The present results indicate that the RB-containing E2F complexes are formed because of the increase of the hypophosphorylated form of RB, although it is not clear whether RA or TPA activates RB phosphatase or inhibits the RB kinase. However, the dephosphorylation of RB seems to be critical for the terminal differentiation of HL60 cells. In HL60 cells, the partner of the hypophosphorylated RB is E2F, like MyoD in the muscle cells.

During HL60 cell differentiation induced by either retinoids or TPA, disappearance of free E2F became evident after differentiation progressed considerably (Figs. 3 and 4). Complete disappearance was finally observed in the cells treated with RA or TPA for a longer period of time, but free E2F was still observed at the reduced level in the cells treated with Am80. At this stage, RA- or TPA-treated cells ceased proliferation, but the cells treated with Am80 were still able to proliferate, although Am80 had a strong retinoidal activity. From these results, the existence of free E2F is necessary for HL60 cell proliferation.

Free E2F is a transcriptional activator (19, 20), while the formation of the complex with RB causes the loss of its activation ability (3–5). Genes whose expression are probably controlled by E2F were listed by Mudryj et al. (26), and they are important for cells to progress from G1 into S phase, like dihydrofolate reductase (DHFR) or the proto-oncogene, c-myb. The c-myc gene is also included among them. In fact, the results of the transient transfection assay using pmycCAT (Fig. 8) indicated that the transcriptional initia-
tion from the c-myc P2 promoter is activated by free E2F existing in the normally growing HL60 cells, while it is suppressed by the RB-containing complexes formed during differentiation. In the cell cycle, E2F-RB complexes are formed at the early stage of the G1 phase, and free E2F exists at the G1→S transition (21, 22, 24, 27). These facts, together with the results of the transient transfection assay, suggest the possibility that the expression of the c-myc gene is suppressed during the G1 phase but up-regulated at the G1→S transition. Regulation of gene expression in such a manner is reported in the case of the DHFR gene (28). Control of the G1→S transition is thought to be an important step in the progression of the cell cycle (29). The c-myc gene may also be one of such genes, the expression of which is controlled by E2F in the G1→S transition. Present results predict that the c-Myc protein is one of the key proteins with which to elucidate the mechanism of the G1→S transition. The genes controlled by the c-Myc protein are under investigation.

Materials and Methods

Chemicals. RA and Am80 were prepared as described previously (30). TPA was obtained from Sigma Chemical Co.

Antibodies. Rb(AB-2) polyclonal antibody, c-myc(AB-1) monoclonal antibody and c-myc(AB-2) monoclonal antibody were obtained from Oncogene Science. Max(C-17) polyclonal antibody and p107(C-18) polyclonal antibody were obtained from Santa Cruz Biochemistry.

Plasmid. The construction and preparation of pmycX-CAT was described previously (2).

Cells. HL60 cells were maintained in RPMI 1640 supplemented with 5% FCS in 5% carbon dioxide at 37°C. Induction of cell differentiation was carried out by adding retinoids or TPA dissolved in ethanol to the medium. Control culture was added ethanol only. NBT reduction was assayed as described (31) in the presence of TPA. Transfection of the plasmid into HL60 cells was carried out by an electroporation method, and a CAT assay was performed as described previously (2).

Synthesis and Purification of the c-Myc Protein. The c-Myc protein expression plasmid pGD-myc1 for E. coli was constructed by inserting the full-length c-myc coding sequence downstream of the T7 promoter in the vector plasmid pGD1. The construction of pGD-myc1 was verified by nucleotide sequencing. The expression plasmid pGD-myc1 carrying bacteria [JM109(DE3)] was grown in LB with 100 µg/ml ampicillin. Wild-type c-Myc protein was expressed at a high level without isopropylthio-β-D-galactoside induction, but the reason for the high level of expression is not yet clarified.

For the purification of the c-Myc protein, bacterial cells were lysed by sonication in TEN (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, and 1 mM DTT), c-Myc protein found in the insoluble fraction was spun down, washed with 2% Triton X-100 and 10 mM EDTA, and then solubilized with MEN (50 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 0.5 mM EDTA, 50 mM NaCl, and 1 mM DTT) containing 8 mM urea. The solubilized c-Myc protein was applied onto a HiTrap SP column (Pharmacia) and eluted with a 50 mM to 0.5 mM NaCl gradient in MEN containing 8 mM urea. The peak fractions (assayed by SDS-PAGE) were pooled, dialyzed against MEN containing 8 mM urea, and then applied onto the HiTrap SP column again. The peak fractions were pooled and dialyzed against 1/2TEN containing 4 mM urea, then applied to a HiTrap Q column (Pharmacia) and eluted with a 50 mM to 0.5 mM NaCl gradient in 0.5 x TEN containing 4 mM urea. The peak fractions were pooled and used for the mobility shift assay. The purity of the c-Myc protein thus purified was more than 90% as judged by SDS-PAGE.

Mobility Shift Assay. Nuclear extracts were prepared according to Schreiber et al. (32). The mobility shift assay was carried out as described previously (2). The sequence of the E-box probe was GTAACGAACCACGTCAGGCTAGC (18).

The introduction of recombinant c-Myc protein to the reaction mixture was done as follows. An appropriate amount of c-Myc protein dissolved in 1/2TEN with 4 mM urea directly added to the reaction buffer of the mobility shift assay. The nuclear extract was then added to the mixture, followed by incubation for 5 min at room temperature. After the addition of the probe, the reaction mixture was incubated for 30 min and then applied to PAGE. The presence of urea up to 400 mM gave no effect on the E2F-binding pattern.

Western Blot Analysis. Nuclear extracts from HL60 cells were separated by 7.5% SDS-PAGE. Proteins were transferred to an Immobilon (Millipore) membrane. The membrane was probed with the anti-RB antibody or the anti-c-myc antibody and visualized by using a Vectastain ABC kit (Vector Laboratories) according to the manufacturer’s instructions.

References


