c-Myc-mediated expression of nucleophosmin/B23 decreases during retinoic acid-induced differentiation of human leukemia HL-60 cells

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Abstract The retinoic acid-induced differentiation of human leukemia HL-60 cells towards mature granulocytic cells was accompanied by the decline in the protein levels of c-myc, nucleophosmin/B23 and its promoter activity. These RA-induced effects were further enhanced by the concurrent treatment of HL-60 cells with p38 map kinase inhibitor SB203580 (SB). It seems that there is a strong correlation of nucleophosmin/B23 and c-Myc expressions in cells under RA treatment. Furthermore, nucleophosmin/B23 promoter activity decreased upon c-Myc antisense-mediated reduction of intracellular amount of c-Myc. CHIP assays showed that binding of c-Myc to the nucleophosmin/B23 promoter decreased in RA-treated cells. Thus, nucleophosmin/B23 expression is targeted by c-Myc during RA-induced differentiation. These results provide evidence for a novel mechanism of transcriptional downregulation of nucleophosmin/B23 and the functional role of c-Myc in RA-induced differentiation.

Keywords: c-Myc; Nucleophosmin/B23; Transcriptional regulation; Retinoic acid; Differentiation

1. Introduction

One important difference between cancer and normal cells is hyperactivity and pleomorphism of the nucleoli [1]. The nucleolus in cancer cells undergoes extreme variations in size, shape, fine structure, and cytochemical composition [2]. Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired damage to genes that directly regulate their cell cycles and cell growth. Although rRNA transcription, processing and ribosome assembly have been established as major functions of nucleolus, previous studies suggest that nucleolus participates in many other aspects of gene expression as well [3]. New results indicate that biosyntheses of signal recognition particle RNA and telomerase RNA involve a nucleolar stage and that nucleolus is a site critical to cellular aging [4]. A number of studies including ours indicate that nucleophosmin/B23, one of the major nucleolar phosphoproteins, plays a role in increased nucleolar activity that is necessary for cell proliferation [5]. The steady-state level of nucleophosmin/B23 mRNA is significantly higher in abnormal growth than in normal growth [6]. In the analysis of clinical cancer tissues, cancers of later stages or recurrent cancers seem to have higher nucleophosmin/B23 mRNA levels [7]. The functional association of nucleophosmin/B23 with cancer suggests that excess of nucleophosmin/B23 could be an important cause of cancer and not just a consequence.

Several signaling pathways are activated by retinoic acid (RA) to mediate induction of differentiation. ERK/MAPK activation is needed to elicit RA-induced cellular differentiation [8]. P38 MAP kinase pathway is activated and exhibits negative regulatory effects on RA-induced differentiation [9]. Previously, we have found that nucleophosmin/B23 is transcriptionally downregulated during RA-induced differentiation [10]. Nucleophosmin/B23 antisense oligomer treatment significantly potentiates RA-induced differentiation. Thus, nucleophosmin/B23 plays a role in control of cellular susceptibility to RA. The biochemical and molecular events involved in cellular response to RA include a modulation of nucleophosmin/B23 gene expression. In this study, attempts were therefore made to elucidate how nucleophosmin/B23 was affected by RA. Since the human nucleophosmin/B23 locus contains c-Myc binding site [11]. Antisense-mediated reduction and chromatin immunoprecipitation (CHIP) were carried out to determine whether c-Myc indeed binds and regulates nucleophosmin/B23 promoter during RA-induced differentiation. Our results show that the cellular protein levels of c-Myc and nucleophosmin/B23 decrease during RA-induced differentiation. Nucleophosmin/B23 promoter activity decreases upon RA treatment and upon antisense-mediated reduction of the intracellular amount of c-Myc. Moreover, the binding of c-Myc to nucleophosmin/B23 promoter decreases upon RA treatment. Our results functionally implicate c-Myc in transcriptional regulation of nucleophosmin/B23 during RA-induced differentiation.

2. Materials and methods

2.1. Cell culture and antibody

HL-60 leukemic cells were grown in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 0.5% antibiotics and 2.0 g/l sodium bicarbonate in a 5% CO_2 humidified incubator at 37 °C. The monoclonal antibody (MAb) to nucleophosmin/B23 (37/5.1) was kindly provided by Dr. P.K. Chan, Department of Pharmacology, Baylor College of Medicine, Houston, Texas. Characterization of nucleophosmin/B23 MAb has been reported previously [12].

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2.2. Oligonucleotides

The phosphorothioate analogs of deoxyoligonucleotides corresponding to nucleotides −2 to 18 of the c-myc cDNA were synthesized in both of the reverse (5′-GCT ACG GGG AGT TGC AAT CG-3′) and antisense (5′-GCT AAC GTT GAG GGG CAT CG-3′) orientations (ASIA Company Ltd., Oregon-Wilsonville).

2.3. Induction of differentiation

For the induction of differentiation, HL-60 cells (2×10^5 cells/ml) were incubated with 1 or 5 μM RA (all-*trans*). The percentages of CD-11b-positive cells and fluorescence intensity were evaluated by FACScan (FACScan, Beckton-Dickinson) using specific anti-CD11b FITC-conjugated MAb (Sigma).

2.4. Western blotting

Separated proteins on SDS–PAGE were electrotransferred to Hybond-PVDF membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). The PVDF membrane was then soaked in a blocking solution [5% (w/v) non-fat milk in TBST buffer (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, and 0.1% (v/v) Tween 20)] for 1 h at room temperature. The soaked PVDF membrane was then incubated with MAb against nucleophosmin/B23 (diluted 1:2000 in 3% (w/v) non-fat milk in TBST) for 1 h at room temperature, washed with TBST buffer three times for 15 min each and incubated at room temperature for 1 h in horseradish peroxidase conjugated goat anti-mouse IgG antibody (diluted 1:2000 in TBST buffer). The membrane was washed three times with TBST for 15 min each. Immunobands were identified by enhanced chemiluminescence reaction (ECL, Amersham Pharmacia Biotech).

2.5. Transfection with antisense c-Myc oligonucleotides

HL-60 cells were seeded at a density of 3×10^5 per well in 1.0 ml OPTI-MEM I reduced serum medium (Gibco). One to 5 micromolar oligonucleotide (c-Myc reverse or antisense) and 6 μg lipofectin reagents in OPTI-MEM I reduced serum medium were mixed gently. The mixture was incubated for 45 min at room temperature, added to the HL-60 cells and was incubated for 24 h at 37 °C in the CO_2 incubator.

2.6. Plasmid

The 5′ region of nucleophosmin/B23 gene (−552/+2217) containing c-Myc binding site was cloned into luciferase reporter gene vector pGL3 vector. Genome nucleophosmin/B23 was amplified by PCR using 5′-CGA GGT ACC TGA ACT TTG GGG TAA-3′ and 5′-TAG TCC ATG GGC CTT TAG TTC ACA ACC-3′ as primers. Amplified PCR products were then separated and isolated from 1% agarose gel. The 2.7 kb nucleophosmin/B23 genome region was then subcloned into the cloning site of the vector pGL3 supplied in the Eukaryotic TA cloning kit. The orientation of the cDNA in pGL3 was determined by nucleotide sequencing using the sequence kit (Amersham Pharmacia Biotech).

2.7. Transient transfection by electroporation

5×10^7cells were washed twice with ice-cold PBS, resuspended in 0.3 ml PBS and then transferred to electrochamber containing 40 μg of reporter plasmid. Electroporation was performed using the GIBCO-BRL gene pulser with a capacitance setting of 1180 μF and a voltage setting of 260 V. After electroporation, cells were incubated on ice for 10 min and transferred to 35 ml fresh RPMI 1640 medium containing 10% FBS. After 24 h, cells were then treated with RA for various times.

2.8. Luciferase and β-galactosidase activity assays

The cells were washed with PBS twice and lysed in Reporter Lysis Buffer (Promega, Madison, WI) after transfection. 40 μl of cell lysate was automatically mixed with the luciferase assay substrate (Promega, Madison, WI). Luciferase activity was quantified in a luminomer AutoLunmat LB953 (Berthold, Norwalk, CT). For β-galactosidase activity assay, 40 μl of cell lysate was mixed with the reaction buffer [200 mM sodium phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml ONPG (*o*-nitrophenyl-β-D-galactopyranoside)] and was incubated at 37 °C for 30 min. 500 μl of Na_2CO_3 was added to the reaction mixture and the optical density at a wavelength of 420 nm was recorded. Luciferase activity was normalized to the β-galactosidase activity of the same sample.

2.9. Chromatin immunoprecipitation

Cells were cross-linked by adding formaldehyde (final concentration, 1%) directly to the cells in a culture flask for 20 min at 37 °C. Cells were collected by centrifugation and washed with ice cold PBS. Cells were lysed in SDS lysis buffer (Upstate, Lake Placid, NY) [1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1), and 1 mM phenylmethylsulfonyl fluoride] and incubated on ice for 20 min. Samples were then sonicated on ice and then centrifuged at 14 000 rpm. The chromatin solution was precleared with Salmon Sperm DNA/Protein A Agarose – 50% Slurry for 30 min at 4 °C with agitation. Precleared supernatant from 2.5×10^7 cells was incubated with 4 μg of rabbit polyclonal antibody (Santa Cruz: anti-Myc sc-764), or with no antibody and rotated at 4 °C for 12 h. Immunoprecipitation, washing, and elution of immune complexes with 1% SDS and 0.1 M $NaHCO_3$ were carried out. Crosslinks were reversed by addition of NaCl to a final concentration of 200 mM and RNA was removed by addition of 10 μg of RNase A per sample followed by incubation at 65 °C for 5 h. 10 μl of 0.5 mM EDTA, 20 μl of 1 M Tris–HCl, pH 6.5, and 2 μl of 10 mg/ml Proteinase K to the eluates (500 μl) and the samples were incubated at 45 °C for 1 h. Samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and resuspended in 20 μl of H_2O, and analyzed by using PCR. Total input samples were resuspended in 100 μl of H_2O before PCR. PCRs contained 1 μl of immunoprecipitate, 50 ng of each primer, 0.88 mM $MgCl_2$, 2 mM each of dATP, dCTP, dGTP, dTTP, and 1X Thermophilic buffer (Promega), and 1.25 units *Taq* DNA polymerase (Promega) in a total volume of 25 μl. After 32–35 cycles of amplification, PCR products were run on a 2.5% agarose gel and analyzed by ethidium bromide staining.

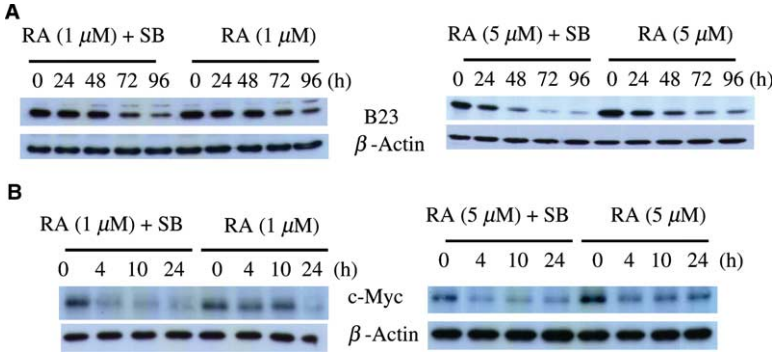

Fig. 1. Effect of RA on the levels of nucleophosmin/B23 and c-Myc proteins. HL-60 cells were pretreated without or with 20 μM SB203580 (SB) for 1 h. The cells were then treated with 1 or 5 μM RA for 24–96 h (A) or with 1 or 5 μM for 4–24 h (B). Cells were then harvested at indicated times, washed and the lysates were centrifuged at $12000 \times g$ for 30 min at 4 °C. 30 μg of total cellular proteins was separated by 10% SDS–PAGE and blotted onto PVDF papers. Nucleophosmin/B23 (B23), c-Myc and β-actin were detected by Western blot using respective specific antibodies and ECL reaction.

3. Results

3.1. Effect of RA on the levels of nucleophosmin/B23 and c-Myc proteins

Total cellular lysates (containing equal amounts of protein) from control untreated HL-60 cells or the HL-60 cells treated with 1 or 5 μM RA for various times (24–96 h, 4–24 h) were separated by 10% SDS–PAGE and subsequently analyzed by Western blot immunoassay. The results showed that there was a decrease in the level of total cellular nucleophosmin/B23 protein during RA-induced differentiation (Fig. 1A). After 72 h of 5 μM RA treatment, nucleophosmin/B23 protein level decreased to about 20%. Co-treatment of HL-60 cells with p38 map kinase inhibitor SB203580 (SB) enhanced the RA-induced differentiation (Fig. 2), and the decrease of nucleophosmin/B23 (Fig. 1A) and c-Myc proteins (Fig. 1B). Culturing the cells for 72 h with 5 μM RA caused a marked increase in differentiation marker, CD11b. In addition, there was significantly elevated CD11b expression in cells treated with SB and RA as compared to the cells treated with RA alone (Fig. 2). Nucleophosmin/B23 protein level in SB co-treated cells further decreased to less than 10% after 72 h of 5 μM RA treatment (Fig. 1A). In parallel, c-Myc protein level changed in similar manner during RA-induced differentiation. The reduction of c-Myc occurred in larger extent and within shorter times (4–24 h) during RA-induced differentiation in the presence of SB (Fig. 1B) as compared with RA treatment alone without SB.

3.2. Downregulation of nucleophosmin/B23 promoter by RA

To further determine the effect of RA on nucleophosmin/B23 gene expression, we tested the responsiveness of nucleophosmin/B23 promoter-reporter construct (pGL3-B23I1) to RA treatment. Fig. 3A shows the schematic diagram of nucleophosmin/B23 promoter and its c-myc binding sites (11). Nucleophosmin/B23 promoter activity was decreased after RA treatment (1–5 μM, 24 h) (Fig. 3B). There was about 22 ± 5% and 48 ± 3% decrease of nucleophosmin/B23 promoter activity in HL-60 cells at 12 h after 1 and 5 μM RA treatment, respectively (Fig. 3B). Co-treatment of HL-60 cells with SB potentiated RA-induced downregulation of nucleophosmin/B23 promoter. About 50.0 ± 2% decrease of nucleophosmin/B23 promoter activity was observed in HL-60 cells treated with 1 μM RA in the presence of 10 μM SB (Fig. 3B). We have also performed the promoter activity analysis for 48 and 72 h of RA treatment. There was still only about 50% decrease of nucleophosmin/B23 promoter activity in HL-60 cells at 48 or 72 h after 5 μM RA treatment (Fig. 3C). In the reporter assay with construct having deletion of intron 1 sequences (−744/+97) where the c-myc-binding sites are located, virtually no reduction of nucleophosmin/B23 promoter activity was observed during RA treatment (1–5 μM, 24 h) (Fig. 3D).

3.3. c-Myc regulates nucleophosmin/B23 promoter activity

Since there was a strong correlation of nucleophosmin/B23 and c-Myc expressions in cells upon RA treatment, we next sought to determine whether expression of nucleophosmin/B23 was targeted by c-Myc during RA-induced differentiation. We first analyzed the changes of nucleophosmin/B23 promoter activity upon antisense-mediated reduction of the intracellular amount of c-Myc. Western blot analysis (Fig. 4A) showed that there were decreases in protein levels of c-Myc in the cells treated with c-Myc antisense oligomer as compared to the control untreated cells. In addition, c-Myc antisense oligomer treatment significantly deactivated nucleophosmin/B23 promoter (Fig. 4B). There was virtually no effect of c-Myc reverse oligomer treatment on the nucleophosmin/B23 promoter activity (Fig. 4B). Next, we examined whether c-Myc bound and directly regulated nucleophosmin/B23 gene during RA-induced differentiation. Chromatin was collected after RA treatment (5 μM, 24 h) and CHIP analysis was performed using an antibody against c-Myc (Fig. 4C). Our results demonstrated that c-Myc binding to the nucleophosmin/B23 promoter site was

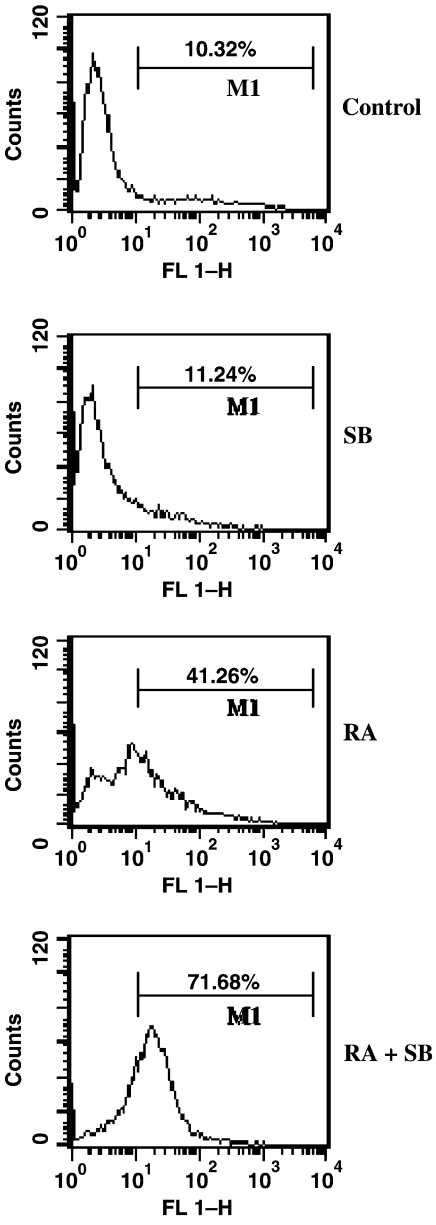

Fig. 2. SB potentiated RA-induced differentiation as measured by the expression of differentiation marker. HL-60 cells were pretreated without or with 20 μM SB203580 (SB) for 1 h. The cells were then treated with 5 μM RA for 72 h. After the cells were harvested, expressions of the CD11b were evaluated by cytofluorimetry using specific anti-CD11b FITC-conjugated MAb. The figure is a representative of the results obtained from at least three independent experiments.

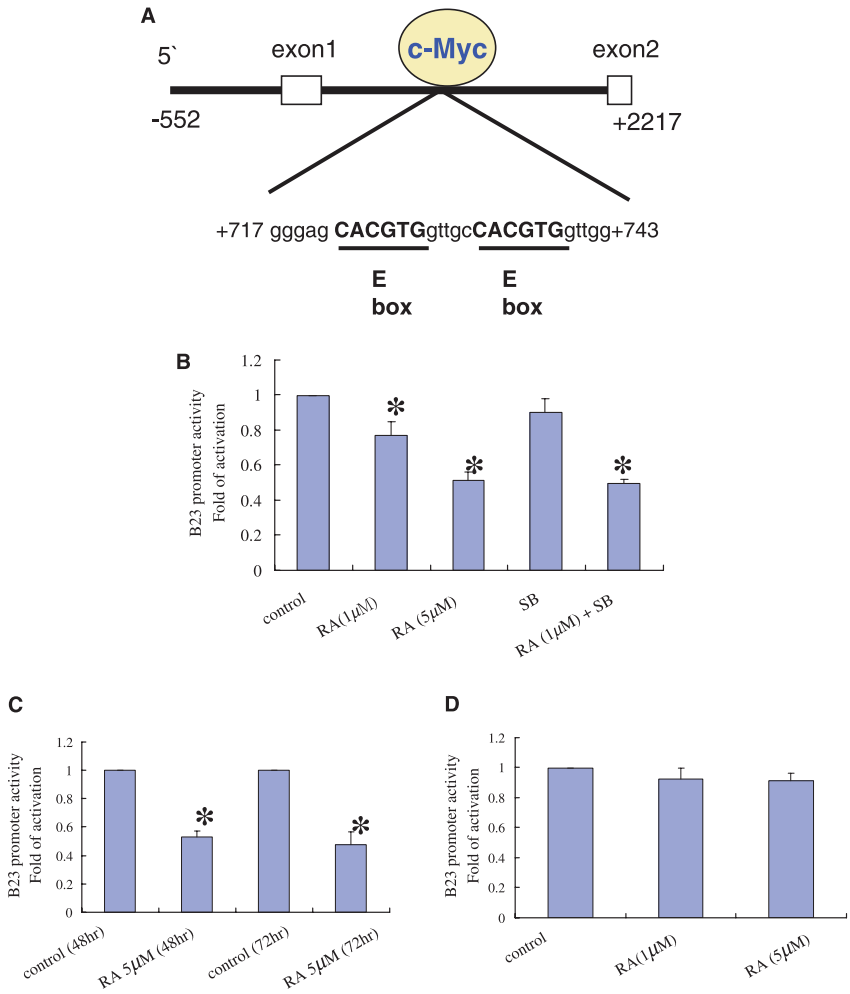

Fig. 3. Effect of RA on nucleophosmin/B23 promoter activity. (A) The schematic diagram of nucleophosmin/B23 promoter containing the c-myc binding site. (B) HL-60 cells were transiently co-transfected with nucleophosmin/B23 promoter plasmid (pGL3-b23I1) and SV40-β-galactosidase (as an internal control). Twenty-four hours after transfection, cells were subcultured and then were pre-treated with or without 10 μM SB203580 (SB) for 1 h. Cells were then treated with 1 or 5 μM RA for 12 h before being harvested and lysed by reporter lysis buffer. The cell extracts containing equal amounts of proteins were analyzed for luciferase activity and β-galactosidase activities. Luciferase activity was normalized to the β-galactosidase activity of the same sample. Fold activation was determined by dividing the luciferase activity of each sample by basal luciferase activity of control without any drug treatment. Bars represent means of triplicates ± S.D. $*P < 0.05$, as compared with nucleophosmin/B23 promoter activity in control cells. (C) HL-cells were treated with 5 μM RA for 48 and 72 h before promoter activity was analyzed. $*P < 0.05$, as compared with nucleophosmin/B23 promoter activity in control cells. (D) The reporter assay was carried out with construct having deletion of intron 1 sequences (−744/+97) where the c-myc-binding sites are located. HL-cells were treated with 5 μM RA for 12 h before promoter activity was analyzed.

significantly decreased in RA-treated cells as compared to control untreated cells (Fig. 4C).

4. Discussion

Nucleophosmin/B23 is enriched in proliferating cells and thought to be involved in ribosome assembly and transport due to its localization to the granular region of the nucleolus [12]. Nucleophosmin/B23 has been ascribed a number of diverse properties, including a potential role in proliferation due to its rapid increase in synthesis during mitogenic stimulation [13]; a role as a cytoplasmic/nuclear shuttle protein [14]; relieves transcription repression by YY1 [15]; binds the HIV Type 1 Rev protein [16], the human T-cell leukemia virus-1-Rex protein [17], and another cell cycle-regulated nucleolar oncoprotein p120 [18]; is a substrate of protein kinase C [19], mitotic cdc2 kinase [20], and casein kinase II [21]; and can be ADP-ribosylated [22]. Nucleophosmin/B23 is of potential interest because it is involved in at least three distinct forms of hematologic malignancy. The N-terminal region of nucleophosmin/B23 is fused to ALK tyrosine kinase in anaplastic lymphoma with t(2;5) [23], to RA receptor α in acute promyelocytic leukemia with t(5;17) [24], and to a novel gene called MLF1 in myelodyplastic syndrome with t(3;5) [25].

In light of many potential roles of nucleophosmin/B23, it is conceivable that it plays important role in the regulation of cell growth. In our previous studies, HL-60 cultures induced to differentiate with RA are shown to contain markedly reduced levels of nucleophosmin/B23 mRNA when compared with control HL-60 cells. The nucleophosmin/B23 protein levels, on the other hand, persist after 24 h of negligible transcript [10]. Nucleophosmin/B23 has a protein half-life of 18–20 h [26]. The stabilities of the nucleophosmin/B23 transcripts of

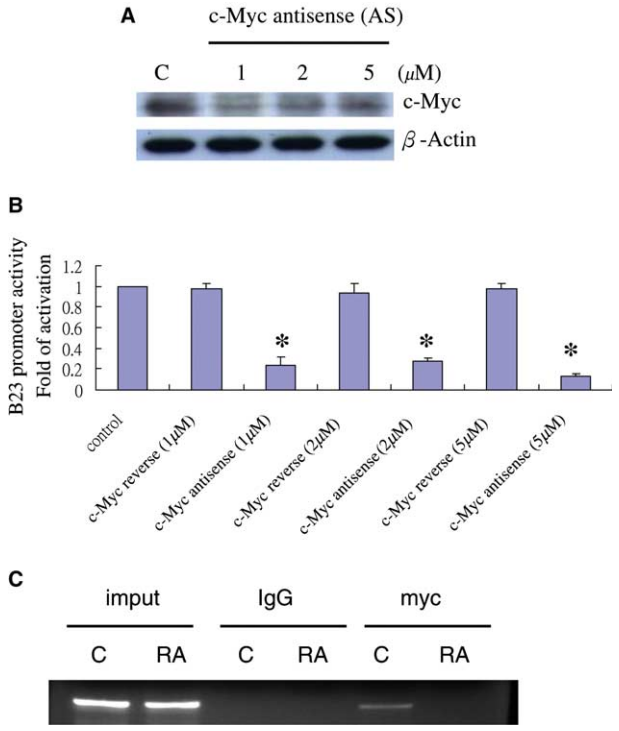

Fig. 4. c-Myc binding and transcriptional activation of nucleophosmin/B23 promoter decreased during RA-induced differentiation. (A, B) Effect of c-Myc antisense oligonucleotide on nucleophosmin/B23 promoter activity. HL-60 cells were transiently co-transfected with nucleophosmin/B23 promoter plasmid (pGL3-b23I1) and SV40-β-galactosidase (as an internal control). Six hours after transfection, cells were subcultured and were treated without (control, C), or with 1, 2 or 5 μM c-Myc antisense oligomer (AS) for 24 h. Cells were then harvested for Western blot (A). (B) Cells were treated with 1, 2 or 5 μM c-Myc antisense oligomer (AS) or c-myc reverse oligomer for 24 h before luciferase activity was measured and normalized to the β-galactosidase activity of the same sample (B). Fold activation was determined and represented as in Fig. 3. $*P < 0.05$, as compared with nucleophosmin/B23 promoter activity in control cells. (C) effect of RA on c-Myc binding to nucleophosmin/B23 promoter. HL-60 cells were treated with 5 μM RA for 24 h. Cells were then harvested, chromatin was collected and CHIP analysis was performed using antibody against c-Myc (C). The figure is a representative of the results obtained from at least three independent experiments.

uninduced and RA-induced cells are similar. Measurements of the relative rates of synthesis of nucleophosmin/B23 gene indicate that, subsequent to differentiation, a decrease in nucleophosmin/B23-specific transcription occurs. These studies demonstrate that transcriptional regulation is a major component of the control of the nucleophosmin/B23 gene during differentiation. Recently, we have shown that nucleophosmin/B23 can be rapidly induced in response to UV [27]. The immediate-early induction of nucleophosmin/B23 indicates the existence of a new regulatory pathway that might enable cells to react rapidly upon induction of DNA damage. This is an important evidence for the involvement of nucleophosmin/B23 in an acute response of mammalian cells to environmental stress.

Previous studies have shown that expression of c-Myc correlates with nucleophosmin/B23 expression [28–31]. Wild-type Rat1 fibroblasts maintain a nucleophosmin/B23 transcript level 3.5-fold higher than fibroblasts bearing deletion of myc [28]. In myc-overexpressing avian bursal neoplasia, nucleophosmin/B23 transcript levels are 3.5-fold higher than in normal bursa [29]. Adenoviral transfection of c-Myc into mice leads to dramatic increase in liver nucleophosmin/B23 mRNA, which correlates with increasing myc protein levels 3–5 days post-infection [30]. These studies indicate that increased myc expression results in elevated nucleophosmin/B23 transcript level. Furthermore, myc binding site has been identified and serum stimulation of nucleophosmin/B23 expression is shown to be myc-dependent [11]. C-myc binds nucleophosmin/B23 promoter in vivo and in vitro. C-myc could activate transcription of nucleophosmin/B23 promoter [11]. In our present study, CHIP was utilized to demonstrate the effect of RA on binding of c-Myc to nucleophosmin/B23 promoter, thus providing evidence that c-Myc directly regulates nucleophosmin/B23 transcription in cellular differentiation. Downregulation of nucleophosmin/B23 transcription during RA-induced differentiation is a consequence of the decrease of c-Myc binding to the nucleophosmin/B23 promoter.

Interferon factor 1 (IRF-1) suppresses tumorigenicity of cells transformed by c-Myc [32]. IRF-1 plays a role in cell growth control and surveillance against cancer development [33]. Nucleophosmin/B23 interferes with IRF-1 binding to target DNA sequences and attenuates the in vitro transcriptional activity of IRF-1 [34]. Furthermore, the IRF-1 transcriptional activity is attenuated in nucleophosmin/B23 overexpressed cells. Thus, nucleophosmin/B23 through interacting with IRF-1 plays an important role in the control of the susceptibility of cells to RA-induced differentiation [35]. We thus speculate that decrease of c-Myc binding to nucleophosmin/B23 promoter leads to downregulated expression of nucleophosmin/B23. Without interference by nucleophosmin/B23, IRF-1 would then be free to transcriptionally activate the genes for differentiation.

In conclusion, we have elucidated the mechanism for the control of nucleophosmin/B23 expression. c-Myc is one of the factors that binds and regulates nucleophosmin/B23 promoter. The binding of c-Myc to nucleophosmin/B23 promoter decreases in RA-induced differentiation. These results provide the novel mechanism for transcriptional downregulation of nucleophosmin/B23 and the functional role of c-myc during RA-induced differentiation.

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