ATRA-regulated *Asb-2* gene induced in differentiation of HL-60 leukemia cells

Junya Kohroki[a], Sayaka Fujita[a], Norio Itoh[a], Yukiko Yamada[a], Harue Imai[a], Noboru Yumoto[b], Tsuyoshi Nakanishi[a], Keiichi Tanaka[a,*]

[a] *Graduate School of Pharmaceutical Science, Osaka University, Yamada-oka 1-6, Suita, Osaka 565-0871, Japan*
[b] *National Institute of Advanced Industrial Science and Technology, Midori-oka 1-8-31, Ikeda, Osaka 563-8577, Japan*

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Abstract Suppressors of cytokine signaling (SOCS) proteins possess common structures, a SOCS box at the C-terminus and a SH2 domain at their center. These suppressors are inducible in response to cytokines and act as negative regulators of cytokine signaling. The ASB proteins also contain the SOCS box and the ankyrin repeat sequence at the N-terminus, but do not have the SH2 domain. Although *Socs* genes are directly induced by several cytokines, no *Asb* gene inducers have been identified. In this study, we screened the specific genes expressed in the course of differentiation of HL-60 cells, and demonstrated that ASB-2, one of the ASB proteins, was rapidly induced by all-*trans* retinoic acid (ATRA). Typical retinoid receptors (RARs) or retinoid X receptors (RXRs) binding element (RARE/RXRE) were presented in the promoter of the *Asb-2* gene. We showed that RARα, one of the RARs, binds to the RARE/RXRE in the *Asb-2* promoter. In addition, we demonstrated by luciferase reporter assay that this element was a functional RARE/RXRE. These findings indicate that ASB-2 is directly induced by ATRA and may act as a significant regulator, underlying such physiological processes as cell differentiation.

Key words: ASB-2; All-*trans* retinoic acid; Retinoid receptor α; Leukemia; Differentiation

1. Introduction

Suppressors of cytokine signaling (SOCS) proteins comprise a family of intracellular inhibitors of cytokine signal transduction [1–3]. SOCS proteins are characterized by a central SH2 domain and a C-terminal motif termed the SOCS box [4]. SOCS proteins inhibit cytokine signaling via direct interactions with JAK kinases or cytokine receptors [3,5]. The SH2 and the N-terminal domains of SOCS proteins are important for this activity. The SOCS box functions to mediate interaction with the elongin B and C complex, and acts as an adapter interacting with the proteasomal pathway [6]. Other proteins containing the SOCS box but differing from SOCS proteins in the combination domain have also been identified [4]. The ASB, WSB and SSB proteins contain ankyrin repeats, WD-40 repeat and SPRY domain, respectively, in their N-terminals and possess the SOCS box at the C-terminal. Although it is likely that these proteins may also potentially interact to specific proteins via their N-terminal domains, the target proteins and physiological effects of these remain unclear. In addition, while the expression of *Socs* genes is induced by several cytokines and growth factors, the primary regulator for other SOCS box family genes is unknown.

Human myelocytic leukemia HL-60 cells have been extensively studied as an experimental model of the molecular mechanisms of leukemic and myelocytic cell differentiation. In response to treatment with all-*trans* retinoic acid (ATRA), HL-60 cells differentiate into a granulocytic lineage in vitro [7]. In addition, 1α,25(OH)$_2$D$_3$ or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) are capable of inducing HL-60 cells into monocytes [8,9]. In ATRA-stimulated differentiation, ATRA binds to retinoid receptor (RAR) α, which forms a heterodimer with retinoid X receptor (RXR) [10]. This complex in turn binds to a specific DNA responsive element (RARE/RXRE) present in the regulatory region of the target gene. In general, this element consists of a direct repeat of the core consensus hexamer, AGGTCA [11,12]. C/EBP ε and p21*WAF1/CIP1* have been reported to be the target gene of ATRA, and to be related to leukemia differentiation [13,14]. Despite numerous findings regarding ATRA action, ATRA target genes aside from these and its downstream mechanisms are poorly understood.

To identify novel differentiation-related genes, we developed a novel approach that analyzes differentially expressed genes [15]. Using this method, we identified several genes differentially expressed in ATRA-treated HL-60 cells. We found that the expression of ASB-2, one of the ASB proteins, was rapidly induced by ATRA. Here we report the first evidence that *Asb-2* is a target gene of ATRA that is induced during differentiation of HL-60 cells through the activation of its RARE/RXRE.

2. Materials and methods

2.1. Cells and treatment

Human myeloblastic leukemia HL-60 cells and mouse F9 cells were cultured with a medium containing 10% heat-inactivated fetal calf serum in a humidified atmosphere of 5% CO_2–95% air. We used RPMI 1640 medium (Nissui, Tokyo, Japan) for HL-60 cells and Dulbecco's modified Eagle's medium (Nissui) for F9 cells. For the differentiation-inducing experiment, HL-60 cells ($1–2\times10^5$ cells/ml, 9 ml/10 cm dish) were precultured for 16 h, then differentiation inducers were added (1 ml of inducer-containing medium/10 cm dish). To induce granulocytic differentiation, HL-60 cells were treated with 1 μM

*Corresponding author. Fax: (81)-6-6879 8234.
E-mail address: k-tanaka@phs.osaka-u.ac.jp (K. Tanaka).

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ATRA for the indicated period. To induce monocytic differentiation, HL-60 cells were treated with 100 nM 1α,25(OH)$_2$D or 10 ng/ml TPA for the indicated period. The differentiation response was monitored by morphological change, increase in NBT-reducing activity and expression of surface markers.

2.2. Improved differential display (DD) analysis

Improved DD was performed as described previously [15]. In brief, HL-60 cells were treated with 1 μM ATRA for 0, 4, 8 and 12 h. 10 μg of total RNAs from differentiation-induced HL-60 cells was reverse-transcribed. Double-stranded cDNA was then synthesized and digested using *Sau*3AI. Fragments were ligated to an adapter composed of long (5′-CGGGTAAGGGGTCGACATGAGTGT-3′) and short (5′-GATCACACTCATGT-3′) oligonucleotides. The ligated 3′-end fragments from the cDNA pool were selectively amplified by PCR using an FITC-labeled oligo dT extended primer and an adapter extended primer. PCR products were loaded onto 6% denaturing polyacrylamide gel and visualized using a FluorImager (Amersham Pharmacia, CA, USA).

2.3. Northern blot analysis

10 μg of total RNAs was electrophoresed on formaldehyde-MOPS 1.5% agarose gel. RNAs separated in the gel were capillary-transferred to a nylon membrane (Hybond N$^+$; Amersham Pharmacia) and crosslinked by UV irradiation. The blot was probed with ^{32}P-labeled specific DNA probe for ASB-2, GAPDH or 18S rRNA. The mRNA signals were detected and analyzed using a BAS1500 Mac radio imaging system (Fuji Film, Tokyo, Japan).

2.4. Preparation of antibody and Western blot analysis

For antibody production in rabbit, a synthetic peptide, MIKEGKNLAEPNKEGC, was conjugated to keyhole limpets hemocyanin using 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester reagent. Cell lysates of HL-60 cells treated by 1 μM ATRA for the indicated periods were prepared by using lysis buffer composed of 50 mM Tris–HCl, 150 mM NaCl, 1% NP40, 0.5 mM dithiothreitol (DTT), 1 μg/ml pepstatin, 2 μg/ml leupeptin and 0.5 mM PMSF, pH 8.0. SDS–PAGE was carried out by the method of Laemmli, and the proteins in the gel were transferred to a polyvinyl difluoride (PVDF) membrane. The membrane was probed with the anti-ASB-2 IgG followed by peroxidase-conjugated second antibody. Immunoreactive bands were visualized using the ECL system (Amersham Pharmacia).

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method of Schreiber et al. [16]. For EMSA, binding reactions were carried out in a reaction buffer composed of 12 mM HEPES, 5 mM $MgCl_2$, 60 mM KCl, 0.5 mM DTT, 2 μg of poly(dI-dC), pH 7.9 and 10 fmol of labeled probe, with 10 or 20 μg of nuclear extract in a volume of 20 μl. After incubation for 30 min on ice, protein–DNA complexes were separated electrophoretically at 4°C. The gel was dried and labeled complexes were detected by a radio imaging system. To observe competition of probe–protein binding or supershift of probe–protein bands, cold DNA fragment or antibodies against transcriptional factors were used.

2.6. Transfection and luciferase reporter assay

The plasmids used for transfection were derived from a SV40 promoter-bearing vector (PGV-P2; Toyo Inc. Co., Tokyo, Japan). RARE/RXRE-like DNA fragment from the *Asb-2* promoter was cloned upstream from the SV40 promoter. All constructs were verified by sequencing the relevant portions. Transient transfection with reporter DNA to F9 cells was performed in 12-well culture plates by the calcium phosphate method. After 24 h treatment with ATRA, cell lysates were prepared, and luciferase activity in the lysates was measured on a Luminometer (EG and G Berthold, Bed Wildbad, Germany). The transfection experiments were performed in triplicate using different preparations of DNA. Luciferase activity of the cells was normalized by their protein contents.

3. Results

3.1. Newly identified Asb-2 gene differentially expressed in ATRA-treated human leukemia HL-60 cells

We identified several novel genes differentially expressed in ATRA-treated human leukemia HL-60 cells using our newly developed method (Table 1). We focused on the *Asb-2* gene, originally identified by Kile et al. [17], as it showed the most rapid induction in response to ATRA treatment. The length of the original cDNA sequence for human ASB-2 (accession no. AF159164) was 1640 bp, but the mRNA signal detected in our Northern blot analysis suggested it to be longer (about 3 kb). Therefore, we cloned human ASB-2 cDNA containing the full length of the coding region, and defined its sequence (Fig. 1A). The open reading frame was composed of 1758 bp, which encoded 585 amino acids (Fig. 1B). The deduced structure contains nine ankyrin repeats and a SOCS box.

3.2. ASB-2 mRNA expression in HL-60 cells treated by differentiation inducer

Next, we analyzed the change in expression of ASB-2 mRNA in response to treatment with several differentiation

Table 1
Identification of the differentially expressed transcripts in HL-60 cells after ATRA treatment

Clone	Similar sequence	Northern blot
1124A	Human mRNA for five-lipoxygenase activating protein mRNA	*
1132A	Novel transcription factor (termed ATF9, unpublished)	Down
1133A	Human mRNA for granulin	Up
1323A	*Homo sapiens* basement membrane induced gene (ICB-I) mRNA	Up
1323B	*Homo sapiens* phK II-1 (hHb1 gene)	Down
1431A	*Homo sapiens* CGI-96 protein mRNA	Up
1442A	*Homo sapiens* Src-like adapter protein mRNA	*
3141A	Human mRNA for lymphocyte-specific protein 1	*
3211B	*Homo sapiens* monocyte chemotactic protein-3 mRNA	Down
3241A	Human laminin receptor mRNA	*
3241D	*Homo sapiens* DAP12 mRNA	Up
3323A	*Homo sapiens* DKFZp762B195 mRNA	Down
4131A	*Homo sapiens* amyloid β (A4) precursor-like protein 2 mRNA	Up
4134A	Human eosinophil granule major basic protein mRNA	Down
4144A	*Homo sapiens* ankyrin repeat-containing protein ASB-2 mRNA	Up
4224A	Human mRNA for 90 kDa heat-shock protein	*
4231A	Unknown	Up
4232A	Human mRNA for CLP	Up
4242B	Human mRNA for testis enhanced gene transcript protein	Up
4312A	Human mRNA for properdin	Up

*These transcripts of which differential expression (up-regulation) was already reported in HL-60 cells by ATRA.

A

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   1  aggttactatagtgtctgctcctaccccctgggacaagatcccctaatgagctggcaggtg  60
  61  ggcgtgggggggcacaggtacagcttggcttctcagctttgctcccctatcctctgctttg 120
 121  ccatgaatctctcatccagccccgcagggatctgcctggccagcagtgagcctgagcccc  180
 181  tttcatcctgggacctgccgaccacggctcacatcctcctgggtgaagaatgtcaccttg  240
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2341  gtgacagatgaggctgcaggctgcctccctctcagcctggacagctaccaggatctcact 2400
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2461  atttgtttacaaactgatgagcagatcccagaccttctctaccttcaggaatggcagaaa 2520
2521  cctctattcctggggccagggcagagcttgaggtgttctggggaaggtggtgctcagagc 2580
2581  cttccctgtgcccctccacttgttctggaaaactcaccacttgacttcagagctttctct 2640
2641  ccaaagactaagatgaagacgtggcccaaggtaggggggtaggggggagcctgggtcttgga 2700
2701  gggctttgttaagtattaatataataaatgttacacatgtgaaaaaaaaaaaaaaaaa   2758
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B

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MTRFSYAEYFSLFHSCSAPSRSTAPPESSARAPMGLFQGVMQKYSSSLFKTSQLAPADPLI
KAIKDGDEEALKTMIKEGKNLAEPNKEGWLPLHEAAYYGQVGCLKVLQRAYPGTIDQRTLQ
EETAVYLATCRGHLDCLLSLLQAGAEPDISNKSRETPLYKACERKNAEAVKILVQHNADTN
HRCNRGWTALHESVSRNDLEVMQILVSGGAKVESKNAYGITPLFVAAQSGQLEALRFLAKY
GADINTQASDNASALYEACKNEHEEVVEFLLSQGADANKTNKDGLLPLHIASKKGNYRIVQ
MLLPVTSRTRIRRSGVSPLHLAAERNHDEVLEALLSARFDVNTPLAPERARLYEDRRSSAL
YFAVVNNNVYATELLLQHGADPNRDVISPLLVAIRHGCLRTMQLLLDHGANIDAYIATHPT
AFPATIMFAMKCLSLLKFLMDLGCDGEPCFSCLYGNGPHPPAPQPSSRFNDAPAADKEPSV
VQFCEFVSAPEVSRWAGPIIDVLLDYVGNVQLCSRLKEHIDSFEDWAVIKEKAEPPRPLAH
LCRLRVRKAIGKYRIKLLDTLPLPGRLIRYLKYENTQ
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Fig. 1. Nucleotide sequence of ASB-2 cDNA and its deduced amino acid sequence. A: Nucleotide sequence of ASB-2 cDNA (accession no. AB056723). Initiation and termination codons are underlined. B: Deduced amino acid sequences of ASB-2. Ankyrin repeat is indicated by a single underline and the SOCS box is indicated by a double underline.

inducers. When HL-60 cells were treated with ATRA, ASB-2 mRNA was induced 4 h after treatment and continued to increase until at least 72 h later (Fig. 2, upper panel). TPA, a monocytic inducer of HL-60 cells, also induced ASB-2 mRNA as well as ATRA, but the induction potency was weaker than that of ATRA (Fig. 2, lower panel). However, ASB-2 mRNA was not induced by $1\alpha,25(OH)_2D_3$, one of the monocytic inducers of HL-60 (data not shown). We next examined the tissue specificity of ASB-2 expression. In skeletal muscle, but not in the heart, brain, kidney, liver, lung, pancreas and spleen, a signal from ASB-2 mRNA was shown (data not shown).

3.3. Identification of ASB-2 protein in HL-60

To confirm the expression of the ASB-2 protein, an antibody against a synthetic peptide (MIKEGKNLAEPNKEGC) from the amino acid sequence of ASB-2 was developed. Western blotting of the extract from HL-60 cells with anti-ASB-2 IgG was performed, and an immunoreactive 65 kDa band was detected, as shown in Fig. 3. The estimated size of the immunoreactive band was close to the value (65 kDa) estimated based on the nucleotide sequence of ASB-2 cDNA. Immunoreactivity in the HL-60 cells was time-dependently increased upon ATRA treatment.

3.4. Direct regulation of ASB-2 gene expression by ATRA

We found that cycloheximide blocks TPA-induced expression of ASB-2 mRNA, but does not block ATRA-induced expression (data not shown). We thus examined whether ATRA directly regulates expression of the *Asb-2* gene. By

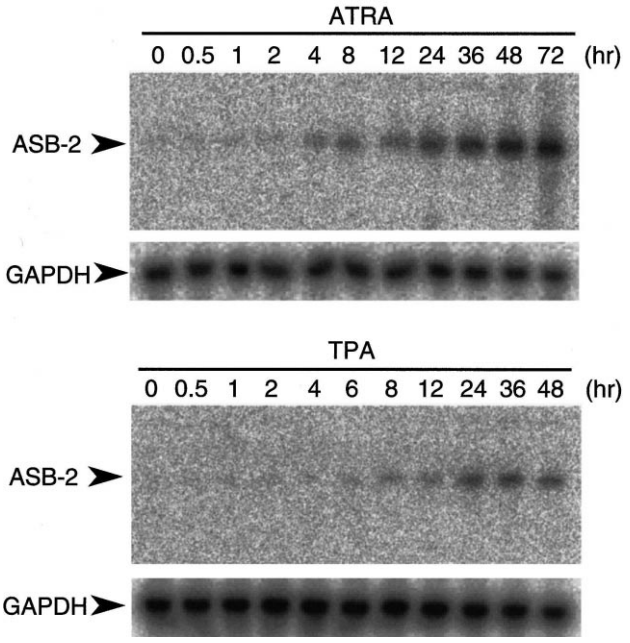

Fig. 2. Expression of ASB-2 mRNA in ATRA-treated HL-60 cells. HL-60 cells were treated with 1 μM ATRA or 10 ng/ml TPA for the indicated periods. Total RNAs (10 μg/lane) were subjected to Northern blot analysis. GAPDH was used as internal control.

searching for a RARE/RXRE consensus sequence (DR5) in the *Asb-2* promoter, we found a RARE/RXRE-like sequence at −132/−116 (Fig. 4A). The half site of this element consists of 5′-AGGACA-3′ instead of 5′-AGGTCA-3′, which is the consensus sequence of RARE/RXRE. Thus, we analyzed whether the RARE/RXRE-like sequence in the *Asb-2* promoter was recognized by nuclear receptors. As shown in Fig. 4B, nuclear extracts from HL-60 cells form specific shifted bands bound with a RARE/RXRE-like sequence in the *Asb-2* promoter. The formation of the shifted bands (indicated by arrow heads) was competitively inhibited by addition of cold probe and cold DR5. Supershift analysis using anti-RARα (Fig. 4C) showed that the shifted band (band B) contained RARα. These results showed that RARα binding to ATRA acts at the RARE/RXRE-like sequence in the *Asb-2* promoter.

3.5. Response of RARE-like sequence in the ASB-2 promoter to ATRA

To determine whether the RARE/RXRE-like sequence in the *Asb-2* promoter is functional, we inserted four copies of this element upstream of the SV40 promoter linked to the luciferase gene. The construct was introduced into F9 cells, and then response to ATRA was analyzed. The RARE/RXRE-like sequence in the *Asb-2* promoter showed significant response to ATRA (Fig. 5). A control vector without the RARE/RXRE-like sequence did not respond to ATRA. These findings indicate that ASB-2 is directly induced by ATRA.

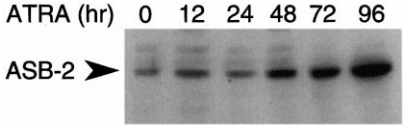

Fig. 3. Identification of ASB-2 protein in HL-60 cells by Western blotting. HL-60 cell lysates were prepared and subjected to SDS–PAGE (7.5% gel, 6 μg protein/lane), blotted onto PVDF membrane. Immunochemical detection was carried out with anti-ASB-2 IgG and ECL system.

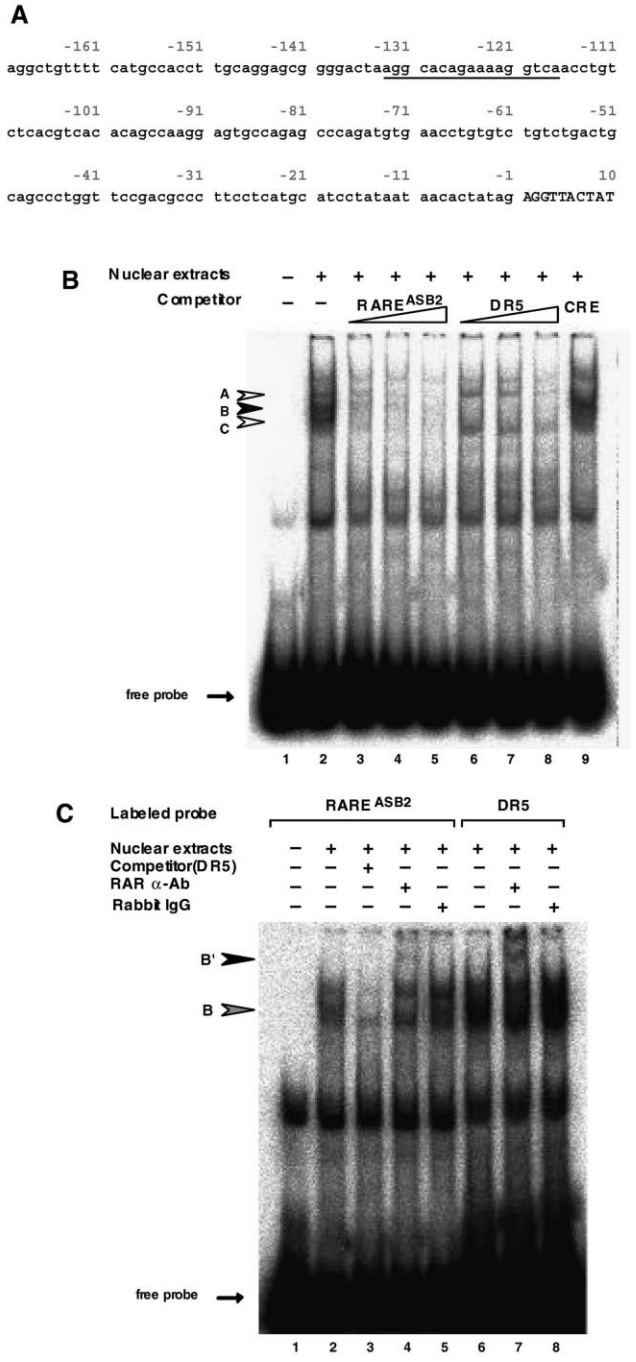

Fig. 4. EMSA of a RARE-like sequence in the ASB-2 promoter. A: Deduced ASB-2 promoter sequence derived from the human genomic working-draft sequence is shown. Potential RARE/RXRE is underlined. The 5′-untranslated region of ASB-2 is capitalized. B and C: Nuclear extract was prepared from HL-60 cells. RARE/RXRE-like sequence in the *Asb-2* promoter was used as a probe in the double strand form. ^{32}P-labeled probe was incubated with 10 (C) or 20 (B) μg of nuclear extract for 30 min on ice. Competition binding assays (B) were performed by 20-, 40- or 80-fold molar excess of indicated cold DNA. In supershift analysis (C), nuclear extracts were preincubated with 2 μg of control or anti-RARα IgG overnight on ice.

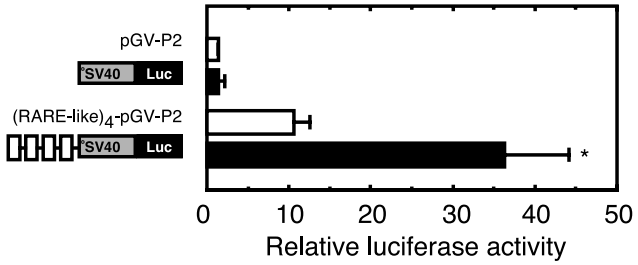

Fig. 5. Response of RARE-like sequence to ATRA. RARE/RXRE-like sequence in the *Asb-2* promoter (4×RARE/RXRE-like) was connected to a SV40 promoter-driven luciferase reporter gene. F9 cells were transiently transfected with 2 μg/well of luciferase reporter plasmid and recovered for 5 h. The cells were then treated with vehicle (open column) or 1 μM ATRA (closed column) for 24 h. Cell lysates were prepared and luciferase activities in the lysates were assayed. Data are shown as the relative values of luciferase activities against the activities in RARE/RXRE-less vector-transfected cells. Data are expressed as means ± S.D. of three independent experiments. *Significantly different from control group ($P < 0.05$).

4. Discussion

In the present study, we isolated a number of genes differentially expressed in ATRA-induced differentiation of HL-60 cells, and showed that the transcript encoding ASB-2 was rapidly induced after ATRA treatment. We also showed that *Asb-2* was the target gene of ATRA.

ATRA has been shown to induce differentiation of normal myeloid and myeloid leukemia cells into mature cells through the activation of the RARα nuclear receptor, which also acts as a transcription factor [7,18]. In most cases of acute promyelocytic leukemia (APL), the RARα gene is altered by chromosomal translocations leading to the synthesis of chimeric proteins, e.g. PML/RARα or PLZF/RARα [19,20]. These chimeras strongly suppress the activation of RARα, by which the leukemic phenotype might be triggered [21]. Despite accumulating data regarding chimeric proteins, relatively little is known about the target genes of RARα or how RARα induces myeloid cell differentiation. Here, we found a functional RARE/RXRE in the *Asb-2* gene promoter and showed that RARα acts at this element. These findings indicate that the *Asb-2* gene is a novel RARα target gene. Thus, we believe that ASB-2 proteins participate in RARα-mediated myeloid cell differentiation. More detailed analysis is needed to clarify this finding.

Due to prominent induction of ASB-2 mRNA by ATRA, we expected that ASB-1 and ASB-3 would also be induced by the same stimulation. However, reverse transcription PCR analysis revealed that the mRNAs of ASB-1 and ASB-3 were not induced by ATRA treatment (data not shown), indicating that the regulation of ASB proteins by ATRA is restricted to ASB-2. It remains to be determined what the different inducers of ASB-1 and ASB-3 are, or alternatively, whether a common inducer of ASB proteins exists, as with cytokines for SOCS proteins. Our study demonstrated that TPA also induced the mRNA of ASB-2. Terstegen et al. reported that TPA induced SOCS-3 mRNA within 45 min after treatment [22]. They showed that this expression was triggered by extracellular signal-regulated kinases 1 and 2. However, because of delayed induction of ASB-2 mRNA compared with SOCS-3, we believe that the mechanism of ASB-2 mRNA induction by TPA differs from that of SOCS-3.

It has been demonstrated that the tumor suppressor gene product of von Hipple–Lindau disease, which also possesses the SOCS box, forms a complex with both elongin B/C complex and cullin-2, both of which function as ubiquitin ligases [6,23,24]. As the SOCS box of ASB and SOCS proteins are capable of interacting with the elongin B/C complex, the ASB and SOCS proteins have been hypothesized to function as part of the ubiquitin ligase complex contributing to ubiquitin transfer to target proteins. TEL–Jak2 fusion oncoprotein is known to possess constitutive tyrosine kinase activity and transforming properties in hematopoietic cells [25]. Recently, three groups independently reported that TEL–Jak-mediated transformation of hematopoietic cells was inhibited by SOCS-1 proteins through the induction of proteasome-mediated degradation [26–28]. Moreover, Yoshikawa et al. indicated that human hepatocellular carcinomas, in which interleukin-6 stimulates malignant progression via JAK/STAT activation, are caused by the aberrant methylation of *Socs-1* CpG, and constitutive activation of the JAK/STAT pathway is associated with silencing of *Socs-1* [29]. These results suggest that SOCS-1 proteins play a tumor suppressor role. Based on their structural similarity to SOCS proteins and the expression profile on differentiation of HL-60 cells, it is likely that ASB proteins also interact with specific proteins via their ankyrin repeats, and that they could play a significant role, such as tumor suppression. Therefore, the next important step in this line of research is to identify ASB-2-regulated ankyrin repeat binding factor(s).

In conclusion, we found that ASB-2 mRNA is induced by ATRA, and that it is directly regulated by RARα and RARE/RXRE in the promoter region of the *Asb-2* gene. Its inducing profile, function and characteristic structure suggest that ASB-2 might be an important regulator of differentiation and/or proliferation of myeloid cells.

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