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# Positive auto-regulation of *MYCN* in human neuroblastoma

Yusuke Suenaga [a,b], Yoshiki Kaneko [a,b], Daisuke Matsumoto [a,c], Mohammad Shamim Hossain [a], Toshinori Ozaki [b,d], Akira Nakagawara [a,b,*]

[a] Division of Biochemistry and Innovative Cancer Therapeutics, Chiba Cancer Center Research Institute, Nitona, Chuoh-ku, Chiba 260-8717, Japan
[b] Department of Molecular Biology and Oncology, Graduate School of Medicine, Chiba University, Nitona, Chuoh-ku, Chiba 260-8717, Japan
[c] Department of Medical Engineering, Suzuka University of Medical Science, Kishioka, Suzuka 1001-1, Japan
[d] Laboratory of Anti-tumor Research, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuoh-ku, Chiba 260-8717, Japan

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ABSTRACT

*MYCN* oncogene is one of the most important regulators affecting the prognosis of neuroblastoma and is frequently amplified in the high-risk subsets. Despite its clinical significance, it remains unclear how the *MYCN* expression is regulated in human neuroblastomas. Here, we found the presence of a positive auto-regulatory mechanism of *MYCN*. Enforced expression of MYCN induced endogenous *MYCN* mRNA expression in SK-N-AS neuroblastoma cells with a single copy of *MYCN* gene. Luciferase reporter assay revealed that MYCN protein activates its own promoter activity in a dose-dependent manner and the downstream region relative to the transcription start sites is responsible for the activation. Furthermore, ChIP analysis showed that MYCN is directly recruited onto the intron 1 region of *MYCN* gene which contains two putative E-box sites. Intriguingly, in response to all-*trans*-retinoic acid (ATRA), MYCN was down-regulated in *MYCN*-amplified SK-N-BE neuroblastoma cells, and the recruitment of MYCN protein onto its own intron 1 region was reduced in association with an induction of neuronal differentiation. Collectively, our present results suggest that MYCN contributes to its own expression by forming a positive auto-regulatory loop in neuroblastoma cells.



## Introduction

Neuroblastoma is one of the most common solid tumors in children and originates from the embryonic neural crest cells [1,2]. It accounts for about 15% of childhood cancer deaths, and at least 40% of all neuroblastomas are designated as high-risk tumors which often occur in patients over one year of age and show characteristic genomic abnormalities including allelic loss of the distal part of chromosome 1 and gain of chromosome 2p [1–3]. *MYCN* is an oncogene mapped to chromosome 2p, and its amplification is a strong indicator for poor outcome in patients' survival [4–6]. Transgenic mice which overexpress *MYCN* driven by the tyrosine hydroxylase promoter in sympathetic neurons develop aggressive neuroblastoma, indicating that *MYCN* has an intrinsic oncogenic potential *in vivo* [7]. MYCN protein activates transcription of the genes that are involved in diverse cellular function, such as cell growth, apoptosis, and differentiation [8,9]. Recently, by using computational analysis of gene expression profile, Fredlund et al. showed that high transcriptional activity of MYC family pathway in primary neuroblastomas predicts poor outcome of the patients and is correlated with low grade of neuronal differentiation in tumors [10]. These results are consistent with the notion that MYCN promotes tumor progression via transcriptional activation of the target genes, and that down-regulation of MYCN may be a critical step for the process of neuronal differentiation. Indeed, in response to differentiation stimuli like NGF or retinoic acids (RAs), endogenous expression of MYCN is suppressed [11,12] and enforced expression of MYCN inhibits NGF- or retinoic acid-mediated neuronal differentiation [13,14]. Moreover, MYCN silencing alone is enough to induce neuronal differentiation in several *MYCN*-amplified neuroblastoma cell lines [15,16].

Retinoic acids (RAs) are now being used as one of the tools in the standard treatment protocols for high risk neuroblastomas, and demonstrated significant therapeutic effects on event-free survival [1,17,18]. Although the down-regulation of MYCN by RA was first reported in 1985 [12], the precise mechanism of its regulation is still elusive [19]. Previously, E2F family and Sp1/Sp3 were reported as the transcription factors which regulate basal expression of MYCN [20–22]. In response to retinoic acid treatment, binding of E2F-2, -3 and -4 to the core promoter of *MYCN* gene was decreased after 12 days of RA treatment [20], whereas Sp1/Sp3 binding was not affected by RA [23]. However, since RA treatment represses *MYCN* transcription at more early stage [12], there might

* Corresponding author. Address: Division of Biochemistry and Innovative Cancer Therapeutics, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuoh-ku, Chiba 260-8717, Japan. Fax: +81 43 265 4459.
*E-mail address:* akiranak@chiba-cc.jp (A. Nakagawara).

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be alternative mechanisms which accelerate a decline of *MYCN* mRNA in response to RA treatment.

In the present study, we have found the presence of a positive auto-regulation in *MYCN* transcription. MYCN protein enhances its own promoter activity through its direct recruitment onto the intron 1 region of *MYCN* gene. Treatment with all-*trans*-retinoic acid (ATRA) significantly represses *MYCN* mRNA expression accompanied by a marked decrease of the amount of MYCN protein recruited onto the intron 1 region. These results suggest that the positive auto-regulation of MYCN is repressed by ATRA, resulting in the further down-regulation of *MYCN* mRNA expression.

## Materials and methods

*Cell culture and transfection.* SK-N-AS neuroblastoma cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and antibiotic mixture in a humidified atmosphere of 5% $CO_2$ in air at 37 °C. SK-N-BE neuroblastoma cells were cultured in a mixture of minimal essential medium (MEM) and Hanks F12 medium supplemented with 15% heat-inactivated FBS and antibiotics. For transfection, cells were transfected with the indicated expression plasmids using Lipofect-AMINE 2000 according to the manufacturer's instructions (Invitrogen).

*Construction of luciferase reporter plasmids*. A luciferase reporter plasmid containing the region of *MYCN* promoter encompassing from −221 to +1312 (where +1 represents the transcription initiation site) was generated by PCR-based amplification using genomic DNA prepared from human placenta as a template. Oligonucleotide primers used were as follows: 5′-GAGCTCCAGCTTTGCAGCCTTCTC-3′ (forward) and 5′-AACCAGGTTCCCCAATCTTC-3′ (reverse). An underlined sequence in the forward primer indicate the SacI restriction site. PCR products were subcloned into pGEMT Easy plasmid (Promega) according to the manufacturer's protocol. After sequencing, PCR products were digested with SacI and subcloned into SacI restriction sites of pGL3 basic plasmid (Promega) to give MYCN(−221/+1312).

*RT-PCR and quantitative real-time RT-PCR*. Total RNA was prepared using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. cDNA was synthesized using SuperScript II with random primers (Invitrogen). Quantitative real-time RT-PCR using an ABI PRISM 7500 System (Perkin-Elmer Applied Biosystems) was carried out according to the manufacturer's protocol. Following were primers used for this analysis: human *MYCN* 5′-TCCATGACAGCGCT AAACGTT-3′ (forward) and 5′-GGAACACACAAGGTGACTTCAACA-3′ (reverse). All the reactions were performed in triplicate. The mRNA levels of each of the genes were standardized by β-actin.

*Immunoblotting.* Cells were washed with ice-cold PBS and lysed with SDS-sample buffer. After a brief sonication, cell lysates were boiled for 5 min, resolved by 15% SDS–PAGE, and electrotransferred onto Immobilon-P membranes (Millipore). The membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% nonfat dry milk, and then incubated with monoclonal anti-MYCN (AB1, Oncogene Research Products), monoclonal anti-tTG (AB-3, NeoMarker), anti-TUBBIII (TuJ1, Covance), or with polyclonal anti-actin (20–33, Sigma) antibody for 1 h at room temperature, followed by an incubation with an appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The chemiluminescence reaction was performed using the ECL reagent (Amersham Biosciences).

*Luciferase reporter assay.* SK-N-AS cells were co-transfected with the indicated *MYCN* promoter luciferase reporters, pRL-TK *Renilla* luciferase cDNA together with or without the increasing amounts of the expression plasmid for MYCN. Total DNA per transfection was kept constant (510 ng) with pcDNA3 (Invitrogen). Forty-eight hours after transfection, firefly and *Renilla* luciferase activities were measured with Dual-luciferase reporter assay system according to the manufacturer's instructions (Promega).

*Cell counting.* Cells were seeded at a density of 10,000 cells/well in 12-well tissue culture plates. After allowing the attachment of cells overnight, culture medium was replaced with the fresh medium containing with or without 5 μM of ATRA. At the indicated time periods after ATRA treatment, the numbers of viable cells were measured in triplicate under microscopic observation.

*Chromatin immunoprecipitation (ChIP) assay.* ChIP assay was performed according to the protocol provided by Upstate Biotechnology. In brief, SK-N-BE cells were treated with or without 5 μM of ATRA for 3 days, and cells were cross-linked with 1% formaldehyde in medium at 37 °C for 8 min. Cells were then washed in ice-cold PBS and resuspended in 200 μl of SDS lysis buffer containing protease inhibitor mixture. The suspension was sonicated to an average length of 200–600 nucleotides, and pre-cleared with protein G-agarose beads for 30 min at 4 °C. The beads were removed by centrifugation and the chromatin solution was immunoprecipitated with normal mouse serum (NMS) or with monoclonal anti-MYCN (AB1, Oncogene Research Products) antibody at 4 °C overnight, followed by incubation with protein G-agarose beads for an additional 1 h at 4 °C. The immune complexes were eluted with 100 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$) and formaldehyde cross-links were reversed by heating at 65 °C for 6 h. Proteinase K was added to the reaction mixtures and incubated at 45 °C for 1 h. DNA of the immunoprecipitates and control input DNA were purified and then analyzed by standard PCR. Primers used were as follows: *MYCN*: forward 5′-CTGTCGTAGACAGCTTGTAC-3′, reverse 5′-AACCAGGTTCCCCAATCTTC-3′; *NLRR1*: forward 5′-AAGTT GGATTTGATGACTGATACG-3′, reverse 5′-AGGCAAGAGACCATGTGC AGGAG-3′. *NLRR1* was used as a positive control [24].

## Results

### *MYCN enhances its own promoter activity*

To examine whether MYCN could directly regulate its own expression in neuroblastoma cells, human neuroblastoma-derived SK-N-AS cells bearing a single copy of *MYCN*, were transiently transfected with MYCN expression plasmid and the expression levels of endogenous *MYCN* mRNA were measured by semi-quantitative RT-PCR. The primer set used in this study was designed to detect the 3′UTR region of *MYCN* mRNA. Therefore, only the endogenous *MYCN* mRNA was detectable. As shown in Fig. 1A, enforced expression of MYCN significantly induced the endogenous *MYCN* mRNA. Lower panel of Fig. 1A showed the results obtained from immunoblotting experiments. Under our experimental conditions, our antibody against MYCN detected both the endogenous and exogenous MYCN. Similar results were also obtained from the quantitative real-time RT-PCR (Fig. 1B), suggesting that MYCN has an ability to transactivate its own promoter.

To identify the MYCN-responsive region within human *MYCN* genomic sequence, we generated a luciferase reporter plasmid containing *MYCN* genomic fragment encompassing from −221 to +1312, where +1 represents the transcriptional initiation site, termed MYCN(−221/+1312). SK-N-AS cells were transiently co-transfected with the constant amount of MYCN(−221/+1312), *Renilla* luciferase reporter plasmid together with or without the increasing amounts of the expression plasmid for MYCN. As shown in Fig. 1C, enforced expression of MYCN resulted in a significant enhancement of the luciferase activity driven by *MYCN* promoter in a dose-dependent manner. These results strongly suggest that the genomic fragment of *MYCN* (at positions −221 to +1312) contains a MYCN-responsive region(s).

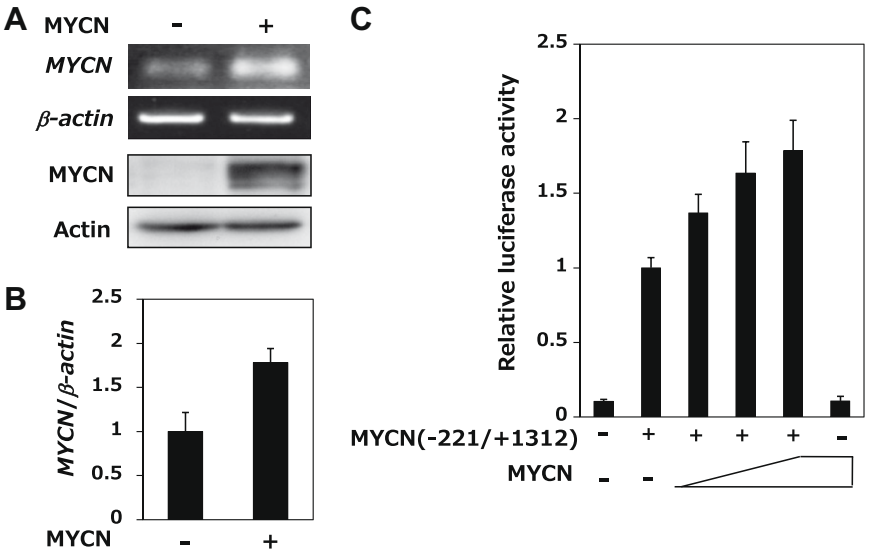


**Fig. 1.** Enforced expression of MYCN induces the endogenous *MYCN* mRNA. (A) Expression levels of the endogenous *MYCN* mRNA. SK-N-AS neuroblastoma cells were transiently transfected with pcDNA3 or with MYCN expression plasmid. Forty-eight hours after transfection, total RNA and whole cell lysates were prepared and subjected to semi-quantitative RT-PCR and immunoblotting, respectively. For RT-PCR, β-actin was used as an internal control. For immunoblotting, actin was used as a loading control. (B) Quantitative real-time RT-PCR. Total RNA were prepared as in (A) and subjected to quantitative real-time RT-PCR to examine the expression levels of the endogenous *MYCN* mRNA. (C) Luciferase reporter assay. SK-N-AS cells were transiently co-transfected with the constant amount of the luciferase reporter plasmid termed MYCN(−221/+1312) (100 ng) and *Renilla* luciferase reporter plasmid (pRL-TK) (10 ng) along with or without the increasing amounts of MYCN expression plasmid (100, 200, or 400 ng). Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured. Firefly luminescence signal was standardized by the *Renilla* luminescence signal. Results are shown as fold induction of the firefly luciferase activity compared with control cells transfected with the empty plasmid.

*MYCN is recruited onto the putative E-boxes located within intron 1 of MYCN to enhance its own promoter activity*

During an extensive search for the 5′-upstream region and intron 1 of human *MYCN*, we have found out two canonical E-boxes within intron 1. We then generated two kinds of 3′-trucated *MYCN* promoter luciferase reporter constructs termed MYCN(−1030/+21) and MYCN(−221/+21) and determined their luciferase activities in response to ectopic MYCN. As shown in Fig. 2A, the luciferase reporter assay demonstrated that both of those luciferase reporter constructs do not respond to the increasing amounts of MYCN, suggesting that the genomic fragment (at positions +22 to +1312) containing the putative E-boxes but not the 5′-upstream region of *MYCN* is required for MYCN-dependent transcriptional activation of *MYCN*.

To further confirm this notion, we performed a chromatin immunoprecipitation (ChIP) assay. The cross-linked genomic DNA prepared from *MYCN*-amplified human neuroblastoma-derived SK-N-BE cells was subjected to ChIP assay using the indicated primer set (Fig. 2B). *NLRR1* which is one of MYCN-target gene [24], was employed as a positive control for this experiment. As clearly shown in Fig. 2B, DNA fragment containing the putative E-boxes was specifically amplified, indicating that the endogenous MYCN directly binds to the canonical E-boxes.

*ATRA induces neuronal differentiation in SK-N-BE cells*

All-*trans*-retinoic acid (ATRA) is one of the well-established inducers for neuronal differentiation and/or apoptosis in neuroblastoma cells. In response to ATRA, a marked reduction in the expression level of *MYCN* is detectable in neuroblastoma-derived cell lines [8]. Consistent with those observations, ATRA treatment resulted in a significant decrease in growth rate of SK-N-BE cells (Fig. 3A) in association with their remarkable morphological changes (Fig. 3B). Close inspection of cell shapes demonstrated that

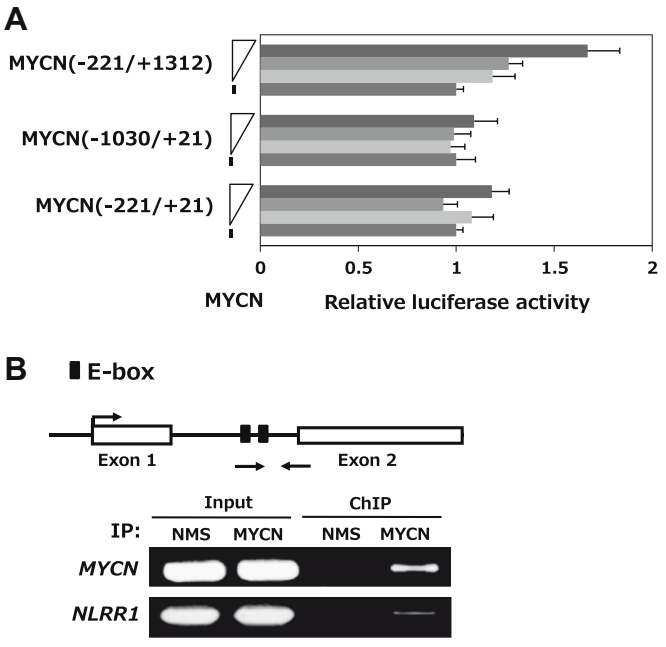


**Fig. 2.** MYCN has an ability to enhance its own promoter activity. (A) Luciferase reporter assay. SK-N-AS cells were transiently co-transfected with the constant amount of the indicated luciferase reporter constructs (100 ng) and *Renilla* luciferase reporter plasmid (pRL-TK) (10 ng) together with the empty plasmid (pcDNA3) or with the expression plasmid for MYCN. Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured as in Fig. 1C. (B) ChIP assay. SK-N-BE neuroblastoma cells were cross-linked with formaldehyde and the cross-linked chromatin was sonicated followed by immunoprecipitation with normal mouse serum (NMS) or with monoclonal anti-MYCN antibody. Genomic DNA was purified from the immunoprecipitates and subjected to PCR using the indicated primer set. The positions of the putative E-boxes and primer set are also shown. The anti-NLRR1 immunoprecipitates were used as a positive control.

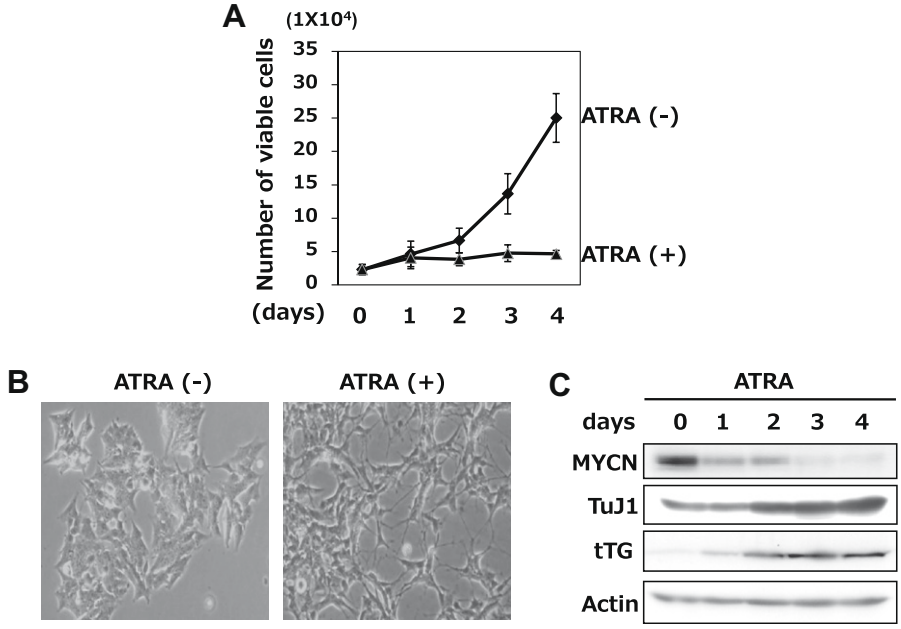


**Fig. 3.** ATRA induces neuronal differentiation in SK-N-BE cells. (A) Growth curves of SK-N-BE cells in the presence (solid diamond) or absence (solid triangle) of ATRA. Cells were grown in the standard culture medium and treated with 5 μM of ATRA or left untreated. At the indicated time points after the treatment with ATRA, number of viable cells was measured in triplicate. (B) ATRA-mediated neuronal differentiation in SK-N-BE cells. Cells were exposed to ATRA at a final concentration of 5 μM or left untreated. Four days after the treatment with ATRA, cells were examined by phase-contrast microscopy. (C) Immunoblotting. SK-N-BE cells were exposed to 5 μM of ATRA. At the indicated time periods after ATRA treatment, whole cell lysates were prepared and processed for immunoblotting with the indicated antibodies. Actin was used as a loading control.

ATRA treatment induces neurite outgrowth, suggesting that SK-N-BE cells undergo neuronal differentiation in response to ATRA. Additionally, ATRA-mediated down-regulation of the endogenous MYCN and concomitant up-regulation of neuronal markers such as neuron specific class III β-tubulin (TuJ1) [25] as well as transglutaminase II (tTG) [26] were detected as examined by immunoblotting (Fig. 3C).

*A significant decrease in the amounts of MYCN recruited onto the genomic region containing the putative E-boxes in response to ATRA*

We then examined the expression levels of *MYCN* mRNA in ATRA-treated SK-N-BE cells. To this end, SK-N-BE cells were exposed to 5 μM of ATRA. At the indicated time points after ATRA treatment, total RNA was prepared and processed for quantitative real-time RT-PCR. In accordance with previous observations [12], the expression levels of the endogenous *MYCN* mRNA significantly decreased in ATRA-treated SK-N-BE cells (Fig. 4A, upper panel). Similar results were also obtained from semi-quantitative RT-PCR (Fig. 4A, lower panel). These observations prompted us to examine whether MYCN could be involved in a decrease in *MYCN* mRNA levels in response to ATRA. For this purpose, we performed ChIP assays using ATRA-treated SK-N-BE cells. As shown in Fig. 4B, ATRA treatment remarkably reduced the amounts of the endogenous MYCN recruited onto the genomic region containing the putative E-boxes. Since MYCN enhanced its own promoter activity, our present findings indicate that ATRA-mediated decrease in the expression level of the endogenous MYCN leads to the repression of the positive auto-regulation of MYCN, and thereby promoting neuronal differentiation.

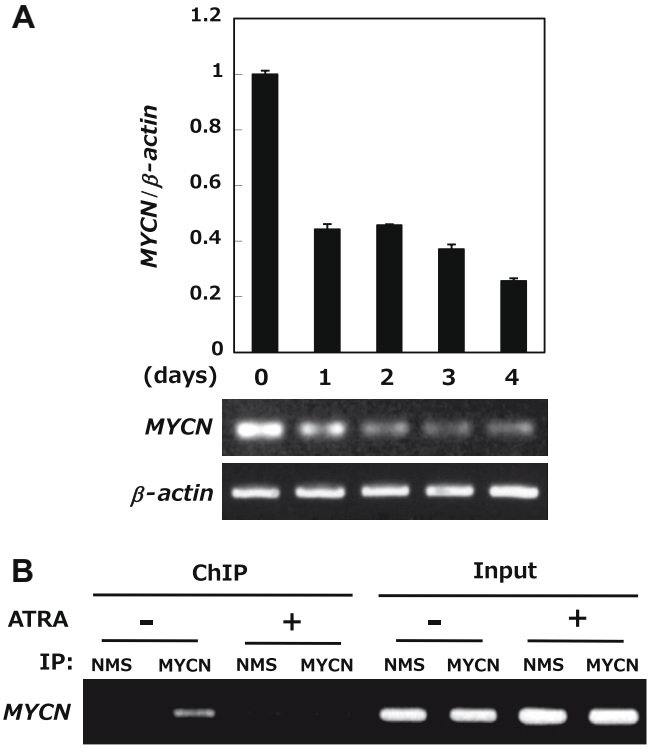


**Fig. 4.** ATRA-mediated down-regulation of *MYCN* mRNA. (A) Quantitative real-time and semi-quantitative RT-PCR. SK-N-BE cells were treated with 5 μM ATRA. At the indicated time periods after the treatment with ATRA, total RNA were prepared and subjected to quantitative real-time RT-PCR (upper panel) and semi-quantitative RT-PCR (lower panel). *β-actin* was used as an internal control. (B) ChIP assay. SK-N-BE cells were treated with 5 μM of ATRA or left untreated. Three days after ATRA treatment, cells were cross-linked with formaldehyde. The cross-linked chromatin was sonicated and immunoprecipitated with normal mouse serum (NMS) or with monoclonal anti-MYCN antibody. Genomic DNA was then purified from the immunoprecipitates and subjected to PCR-based amplification by using the primer set as shown in Fig. 2B.

## Discussion

Similarly to other MYC family members, c-*MYC* and *MYCL*, the *MYCN* gene is frequently amplified in many types of human cancers including neuroblastoma, and its overexpression is significantly associated with aggressiveness of the tumors [1,27]. In

neuroblastoma, *MYCN* is amplified and closely linked to poor survival probability of patients [4–6]. However, expression levels of *MYCN* in individual tumors are regulated by not only the gene copy number but also the transcriptional regulation [28,29]. Indeed, *MYCN* mRNA is expressed at significantly high levels even in some subsets of neuroblastoma with a single copy number of *MYCN* gene. In the present study, we have demonstrated that MYCN protein enhances its own promoter activity through direct binding to the intron 1 region. To our knowledge, this is the first evidence that MYCN forms a positive auto-regulatory loop through its own transcriptional activation. Since the amplified genomic DNA at *MYCN* gene contains the responsive MYCN binding sites (within intron 1), the identified mechanism may explain the reason why transcription of each *MYCN* gene is activated both in *MYCN*-amplified and non-amplified neuroblastoma cells.

In a sharp contrast to *c-MYC*, the expression of *MYCN* is strictly restricted in both human and mouse adult tissues [9,19,30]. To delineate the essential region of *MYCN* promoter for the tissue-specific expression, the previous studies using transgenic mice showed that the human transgene containing 3.5 kb of upstream and 3 kb of downstream sequences were, at least in part, responsible for the expression pattern of the endogenous *MYCN* gene [31,32]. Further studies revealed that the downstream region including exon 1 and intron 1 is required for tissue-specific promoter activity of *MYCN*, whereas the upstream region regulates basal promoter activity [19]. In support with this notion, the latter region includes a sequence with high homology to the second promoter of *c-MYC*. Furthermore, E2F family proteins bind to enhance basal transcription activity through these regions in both of the two genes [19,20]. Our present results have also revealed that ectopically expressed MYCN activates its own promoter activity through the intron 1 region but not the upstream region, suggesting that the positive auto-regulation of *MYCN* may contribute to maintain tissue-specific expression of *MYCN* rather than basal transcription of the gene.

We have previously found that endogenous expression level of Bcl-2 is one of the keys to determine responsiveness to ATRA for inducing neuronal differentiation or apoptosis in neuroblastoma cells [33]. Indeed, ATRA treatment repressed *MYCN* expression and thereby cells underwent differentiation or apoptotic cell death [12,19]. However, it has been shown that *MYCN* mRNA is transiently down-regulated in response to RA and begins to increase 3–4 days after the administration of RA in several RA-resistant neuroblastoma cell lines [34 and our unpublished observations]. Therefore, it is likely that there could exist at least two distinct molecular mechanisms behind *MYCN* expression in response to RA. The first one is that *MYCN* expression is rapidly down-regulated in response to RA in RA-sensitive neuroblastoma cells. The second one is that RA-mediated repression of *MYCN* is recovered in RA-resistant neuroblastoma cells. Considering that MYCN has an ability to transactivate *MYCN* gene, it is conceivable that this positive auto-regulatory mechanism of *MYCN* expression might be at least in part involved in both cases. Thus, the disruption of this positive auto-regulatory mechanism of *MYCN* expression might provide a novel strategy for developing anti-cancer treatment. To date, it remains unclear how ATRA treatment could cause the down-regulation of *MYCN* in ATRA-sensitive neuroblastoma cells. Further studies should be required to address this issue.

## Acknowledgments

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