High Mobility Group A1 Is a Molecular Target for MYCN in Human Neuroblastoma

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Abstract

High mobility group A1 (HMGA1) is an architectural transcription factor and a putative protooncogene. Deregulation of its expression has been shown in most human cancers. We have previously shown that the expression of the HMGA family members is deregulated in neuroblastoma cell lines and primary tumors. On retinoic acid (RA) treatment of MYCN-amplified neuroblastoma cell lines, HMGA1 decreases with a kinetics that strictly follows MYCN repression. In addition, MYCN constitutive expression abolishes HMGA1 repression by RA. Here we explored the possibility that HMGA1 expression might be sustained by MYCN in amplified cells. Indeed, MYCN transfection induced HMGA1 expression in several neuroblastoma cell lines. HMGA1 expression increased in a transgene dose–dependent fashion in neuroblastoma-like tumors of MYCN transgenic mice. In addition, it was significantly more expressed in MYCN-amplified compared with MYCN single-copy primary human neuroblastomas. MYCN cotransfection activated a promoter/luciferase reporter containing a 1,600 bp region surrounding the first three transcription start sites of the human HMGA1 and eight imperfect E-boxes. By heterodimerizing with its partner MAX, MYCN could bind to multiple DNA fragments within the 1,600 bp. Either 5′ or 3′ deletion variants of the 1,600 bp promoter/luciferase reporter strongly decreased luciferase activity, suggesting that, more than a single site, the cooperative function of multiple cis-acting elements mediates direct HMGA1 transactivation by MYCN. Finally, HMGA1 repression by RNA interference reduced neuroblastoma cell proliferation, indicating that HMGA1 is a novel MYCN target gene relevant for neuroblastoma tumorigenesis. (Cancer Res 2005; 65(18): 8308-16)

Introduction

The high mobility group (HMG) proteins of the A type belong to a larger family of nonhistone DNA binding factors that play important architectural functions in the organization of active chromatin (1). The HMGA family is composed of three members: HMGA1a and HMGA1b, encoded by the same gene through an alternative splicing of a short exon, and HMGA2 (2). By means of specific DNA binding domains, called AT-hooks, HMGA proteins bind to AT-rich sequences in the minor groove of the DNA helix (3) and coordinate the assembly of higher-order multiprotein complexes (called enhanceosomes; refs. 4–6) involved in the regulation of the expression of a growing number of genes (reviewed in ref. 7). HMGA proteins are strongly and almost ubiquitously expressed in early mammalian development (8). Their expression declines through development and is scant or completely absent in adult differentiated tissues (9, 10). They are thought to be important regulators of cell growth and differentiation. Indeed, the HMGA2 knockout mouse shows a "pigmby" phenotype, characterized by reduced size and weight of most body organs, reduced body fat, and a cell-autonomous defect in cell growth (8). Conversely, a mouse overexpressing a shortened HMGA2 develops a "giant" phenotype with diffused lipomatosis, underlying the role of this molecule in the regulation of (adipocytic) cell growth and differentiation (11, 12). Adipocytic differentiation also requires HMGA1 up-regulation (13). Apart from their role in physiologic processes, deregulated HMGA1 and/or HMGA2 expression has been described in most tumors of epithelial and mesenchymal origin and is considered a hallmark of cancer (14). In particular, HMGA1 expression was proposed to be a diagnostic indicator of carcinoma in thyroid and colorectal cancer, and higher levels of expression associate with more malignant and metastatic phenotypes in epithelial cancers (15–17). In experimental models, HMGA1 behaves as a transforming protein and has been indicated as a putative oncogene. Consistently, its repression strongly impairs the malignant phenotype of Burkitt’s lymphoma cells (18) and the tumorigenicity of several human cancer cell lines (19). Despite its established role in tumor biology, very little is known about the molecular mechanisms involved in its deregulation in cancer cells.

We have reported on the expression of HMGA genes in neuroblastoma (20, 21). Neuroblastoma is a heterogeneous neoplasm in which different clinical, molecular, and genetic variables may contribute to prognostic stratification of the patients. Indeed, the expression of TRK receptor family members, gain of chromosome 17q, and loss of 1p and 11q were shown to affect tumor behavior (reviewed in ref. 22). The most powerful prognostic factor of neuroblastoma is MYCN amplification, which occurs in 20% to 25% of the tumors and predicts a negative outcome (23–25). Transgenic mice with the neural crest targeted MYCN oncogene develop a "giant" phenotype with diffused lipomatosis, organ reduction, loss of body fat, and a cell-autonomous defect in cell growth and differentiation (26). MYCN inactivation via an antisense strategy leads to decreased neuroblastoma proliferation and reduced anchorage-independent growth in vitro (27, 28) and decreased mouse neuroblastoma tumorigenesis in vivo (29), suggesting that it also plays an important pathogenic role. MYCN belongs to the large family of helix-loop-helix proteins and operates in a complex network of transcription factors, including MAX and MAD. In an oversimplified view, MYC proteins (including c-MYC, MYCN, and L-MYC) might be recruited in transcriptionally active or inhibitory complexes by binding to MAX and MAD, respectively. Binding of these molecules to specific elements, called E-boxes, regulates...
transcription on target genes (reviewed in ref. 30). Although the list of c-MYC targets has grown exponentially in the last few years, only a few biologically relevant MYCN target genes have been described thus far. This clearly contrasts with the outstanding relevance of MYCN in neuroblastoma tumor biology. We have previously shown that HMGA1 decreases with a kinetics that strictly follows MYCN repression on retinoic acid (RA) treatment of MYCN-amplified neuroblastoma cell lines (20). In addition, MYCN constitutive expression abolished HMGA1 repression by RA (20), suggesting that HMGA1 regulation might be controlled by MYCN. We report here that MYCN up-regulates HMGA1 expression in neuroblastoma cells and in neuroblastoma-like tumors arising in MYCN transgenic mice. Higher HMGA1 expression is associated with MYCN amplification in primary human neuroblastomas. Our studies indicate that multiple cis-acting elements mediate HMGA1 transactivation by MYCN and that HMGA1 is a direct MYCN transcriptional target. Because HMGA1 repression by RNA interference was associated with reduced cell proliferation, we propose that HMGA1 is a new and biologically relevant MYCN target gene.

Materials and Methods

Neuroblastoma tumor samples. Tumor samples from primary site were obtained from 16 children with previously untreated neuroblastoma admitted at the Department of Pediatrics, La Sapienza University. Institutional written informed consent was obtained from the patient’s parents or legal guardians. Each sample was characterized for MYCN amplification by Southern blot as previously described (31).

DNA constructs. The HMGA1-5\#1 luciferase/reporter construct was obtained by PCR amplification and cloning of a 1,600 bp fragment of the human HMGA1 promoter containing the three major transcription start sites into the pGL3 basic vector (Promega Corporation, Madison, WI). 5’ and 3’ progressive deletions were generated using Erase-a-Base System (Promega). Cloning of the 1,982 bp EcoRI fragment containing the –1,353 canonical E-box of the human HMGA1 promoter 5’ of the HMGA1-5\#1 construct generated the HMGA1-3LE-box luciferase/reporter construct. The QE-9 vector and the QE-10 vector (Qiagen, Hilden, Germany) expressing the MYC and MAX protein, respectively, were kindly furnished by E.V. Prochownik (Section of Hematology/Oncology, Children’s Hospital of Pittsburgh, Pittsburgh, PA) (32).

Cell lines and culture condition. Human neuroblastoma and Hek-293 cell lines were grown in standard conditions. To obtain pools of SK-N-AS cells stably expressing MYCN and c-MYC, after liposomal transfer with the TransFast reagent (Promega), cells were selected in the presence of G418 (800 \(\mu\)g/mL, Sigma Chemical Co., St. Louis, MO) or puromycin (1 \(\mu\)g/mL, Sigma). SK-MYC and Tet21/N cell lines were cultured as reported (33, 34).

RNA preparation and Northern blot analysis. Total RNA was extracted using the RNeasy system (Qiagen) and analyzed by Northern blot, as described (35). Total RNA extraction from human and mouse tissues was done with TRIzol reagent (Invitrogen, San Diego, CA).

Real-time quantitative PCR analysis. For quantitative reverse transcription-PCR (RT-PCR) analysis, total RNA (1 \(\mu\)g) was reverse transcribed using M-MLV reverse transcriptase (Invitrogen). One-tenth of the reaction was used for PCR amplification using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom) for the analysis of HMGA1 and MYCN expression in transgenic mice tumor tissues kindly provided by Dr. W. Weiss (Department of Neurology, University of California-San Francisco, San Francisco, CA) (26). Primers were as follows: mGAPDH forward, 5’-TTGTGGAGGCTGATGACC-3; mGAPDH reverse, 5’-GATG-CAGGGATGATGTTTCTGG-3; mHMGA1 forward, 5’-GGGACACAACAAGAACTTGA-3; mHMGA1 reverse, 5’-CAGTGCTATGAGCAAGGC-3; hNMYC forward, 5’-GGAGAGGACACCTGAGCG-3; hMYCN reverse, 5’-GGAGGAGGAGCCGGC-3. Gene expression analysis in the primary human neuroblastomas and cell lines was carried out by quantitative RT-PCR employing commercially available TaqMan Assay reagents for MYCN, \(\beta\)-actin, and GAPDH, and the following primers for HMGA1: A1-7 forward, 5’-GGAGACAAAACAAGGTTG-3; A1-7 reverse, 5’-CTCTCTCTGAGGTGTGGT-3; A1 probe, 5’-FAM-TGCCAAGAGCCGGAMA-MGB. In all cases, samples underwent 35 amplification steps (95°C, 30 seconds; 58°C, 1 minute) monitored by an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). All amplification reactions were done in triplicate and the averages of the threshold cycles were used to interpolate standard curves and to calculate the transcript amount in samples using SDS version 1.7a software (Applied Biosystems, Warrington, United Kingdom). Quantification results for HMGA1 and MYCN were normalized on two endogenous controls, GAPDH and \(\beta\)-actin, with similar results.

Transfection and luciferase reporter assay. Plasmid vectors were transfected in SK-N-SH and Hek-293 by liposomal transfer with the Lipofectamine Plus reagent (Promega). Cells were cotransfected with the HMGA1 promoter/luciferase reporter constructs and the pRL-TK vector expressing the Renilla luciferase for normalization of the transfection efficiency (Promega). Stimulation experiments were done by cotransfecting the MYCN and/or MAD, HMGA2, and c-MYC expressing vectors or the control empty vector. Sixty to sixty-eight hours after transfection, cells were lysed and luciferase activity determined using a TD-20/20 automatic dual injector luminometer (Turner Designs, Sunnyvale, CA) and the Dual-Luciferase Reporter Assay System (Promega).

Purification of recombinant MYCN and MAX protein and electrophoretic mobility shift assays. Recombinant proteins expressed in bacteria were purified simultaneously in nondenaturing conditions by nickel-agarose affinity chromatography system (Qiagen). DNA probes, each containing at least two of the putative E-boxes described in Table 1, were obtained by restriction digestion of the luciferase/reporter constructs. Specifically, probe 1 spanned nucleotides (nt) –429 to –175, probe 2 from nt +153 to +330, and probe 3 from nt +697 to +891. The double-strand

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*As indicated by the MatInspector software report.
control oligonucleotide (CM1) or the DNA fragments were end-labeled with [γ-32P]ATP (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Recombinant purified MYCN and MAX proteins (10 μL, 250 ng each) were mixed with an equal volume of 32P-labeled DNA fragment (200,000 cpm) and poly(deoxyinosinic-deoxycytidyl acid; 2 μg, Sigma), incubated for 30 minutes at room temperature, and electrophoresed through a 5% polyacrylamide gel.

Nuclear protein extraction and Western blot. For the analysis of c-MYC and MYCN, protein nuclear extracts (20 μg) were separated on 15% SDS-PAGE gel, blotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and probed with rabbit anti-MYCN and anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibodies and anti–c-MYC mouse monoclonal antibody. Immunoreactive bands were visualized by enhanced chemoluminescence (Pierce Chemical Co., Rockford, IL). HMGA1 protein expression was analyzed as previously described (21).

RNA interference and cell proliferation assays. The sequence of the short interfering RNA (siRNA) targeting HMGA1 was as follows: open reading frame (ORF), ACCACCACAACCTCCAGGAA; untranslated region (UTR), ACTACCTCTGGACAGTTGT. A nonspecific siRNA with a similar GC content was used as control (NS-IX, Dharmacon Res., Inc., Lafayette, CO). SK-N-Be cells were plated in multiwell plates and transfected with either the siRNA duplex mix (ORF + UTR) against HMGA1, nonspecific siRNA, or vehicle only by using the Lipofectamine 2000 reagent (Invitrogen). Three days after plating, cells were detached by trypsinization and split in three replicate wells or plated in chamber slides for 5'-bromo-2'-deoxyuridine (BrdUrd) labeling assay. The following day, cells were retransfected as above. At days 3 and 6 after initial plating, cells were either counted or processed for RNA and protein extraction. For BrdUrd incorporation assay, cells at days 1 and 4 from initial plating were transfected with a mix of siRNA duplexes and enhanced green fluorescent protein plasmid (Clontech, Palo Alto, CA). At days 3 and 6 after initial plating and after a 10-hour pulse with BrdUrd, cells were processed as previously described (36). For each sample, BrdUrd incorporation was specifically assessed in transfected (green fluorescent protein–positive) cells.

Results

HMGA1 expression in MYCN-transfected cells. We previously reported that MYCN repression by RA precedes HMGA1 reduction in MYCN-amplified cells and its constitutive expression abolished HMGA1 repression by RA in SK-N-BE cells (20). This suggests that HMGA1 levels might be controlled by MYCN expression in MYCN-amplified cells. To test whether MYCN could regulate HMGA1 expression, we explored the effects of the exogenous MYCN

Figure 1. Regulation of HMGA1 expression by MYCN. A, SK-N-AS neuroblastoma cells were transfected with either MYCN or c-MYC and subjected to G418 or puromycin selection for 10 to 20 days and then analyzed for HMGA1 expression via Northern blot (NB). Western blot analysis (WB) shows the expression of c-MYC and MYCN transgenes in SK-N-AS cells. B, graphical representation of the HMGA1 expression in MYCN- and c-MYC–transfected SK-N-AS cells after normalization on GAPDH expression. C and D, quantitative RT-PCR analysis of HMGA1 (black columns) and MYCN (white columns) expression on SK-N-SH, SK-MYC (C), and Tet21/N (D) cell lines. E, Hek-293 cells were transiently transfected with MYCN and analyzed for HMGA1 expression by Northern blot. GAPDH, α-actin, or ribosomal 28S RNA was used for normalization as indicated.
expression in different neuroblastoma cell lines. In pools of transfected SK-N-AS cells, the basal level of HMGA1 increased by 2.8-fold on transfection of MYCN (Fig. 1A and B). In addition, c-MYC, which has been shown to regulate its expression in mouse cells (18), increased HMGA1 expression by 2-fold. We then tested the SK-MYC cells that constitutively expressed transfected MYCN (33) and compared them with untransfected SK-N-SH cells. Also in this case, we observed a 3-fold increase in HMGA1 expression (Fig. 1C). In Tet21/N, a SH-EP cell clone where MYCN expression can be regulated through a tetracycline sensitive promoter (34), HMGA1 expression increased on tetracycline removal and MYCN expression (Fig. 1D). The transient transfection of a human MYCN expression vector induced an increase in HMGA1 expression even in the nonneuroblastic Hek-293 cells (Fig. 1E). Thus, MYCN regulates HMGA1 expression in neuroblastic and nonneuroblastic environments.

**HMGA1 promoter/luciferase reporter analysis.** In the mouse HMGA1 promoter, a typical E-box was shown to be responsive to either c-MYC or growth factors (18). A similar element is also present at position −1,353 from the first transcription start site in the human HMGA1 promoter (Fig. 2A; ref. 37), but its activity has not been investigated thus far. We noticed the presence of eight putative and imperfect MYCN binding sites distributed around the first three transcription starts (Fig. 2A). Their different degree of homology to the canonical E-box is reported in Table 1. The first three putative E-boxes are highly conserved in the mouse HMGA1 promoter (not shown). To test MYCN activity on the HMGA1 promoter, we generated two different luciferase reporter constructs
either containing all E-boxes (HMGA1-5LE-box) or missing the −1,353 element, but containing the eight novel putative E-boxes (HMGA1-5L#1), schematically represented in Fig. 2A. MYCN, but not c-MYC, was able to increase luciferase activity from the HMGA1-5LE-box and, to an even larger extent, from the HMGA1-5L#1 construct, when cotransfected in the SK-N-SH cells (Fig. 2B).

We obtained a more prominent activation of the HMGA1 promoter by MYCN in Hek-293 cells (Fig. 2C). However, in these cells, c-MYC induced the activity of the HMGA1-5LE-box, but not of HMGA1-5L1, suggesting that the canonical −1,353 E-box is necessary for c-MYC–dependent, but not for MYCN-dependent, activation of the HMGA1 human promoter. Further luciferase reporter experiments were done in Hek-293 to better define the HMGA1 promoter responsiveness to MYCN. Increasing amounts of MYCN DNA, but not its control vector, raised the levels of luciferase activity when cotransfected with constant amounts of HMGA1 promoter/luciferase reporter construct (Fig. 2D). Cotransfection of MYCN with equal amounts of MAD, an inhibitory partner for MYC proteins, strongly reduced MYCN-dependent luciferase activity (Fig. 2E), providing an indication for a specific and direct transcriptional activation of this reporter construct by MYCN.

Definition of the cis-acting elements relevant for HMGA1 activation by MYCN. To test which of the promoter elements are required for an optimal response to MYCN, we generated deletion mutants of the HMGA1-5L#1 by either shortening its 5’ or 3’ regions. Deletion of the first 387 bp containing the first three putative E-boxes resulted in a reproducible drop in MYCN-dependent luciferase activity (Fig. 3A, 5L#1-387 and 5L#1-473), which was not increased by the subsequent loss of the first transcription start site (Fig. 3A, 5L#1-473). Further shortening of the 132 bp containing the putative E-box 4 resulted in a reduction of MYCN-dependent activity (Fig. 3A, 5L#1-605). Deletion of the second transcription start site resulted in a strong decrease in basal promoter activity (not shown), in keeping with previous indication that this site is required for transcriptional activity (38). A 3’-end deletion of the last two E-boxes also caused a significant reduction in MYCN-dependent luciferase activity (Fig. 3A, 5L#1-453). A reporter construct missing the third transcription start and the last three E-boxes (3L#1-730) showed a further drop in MYCN-dependent luciferase activity, but not in the basal promoter activity (data not shown and Fig. 3).

Figure 3. MYCN response regions in the HMGA1 promoter and MYCN/MAX binding to HMGA1 promoter. A, schematic representation of the HMGA1-5L#1 deletion mutants generated for this study and their relative activity on MYCN cotransfection in HEK-293 cells. *, P < 0.01; **, P < 0.05. A dual luciferase reporter assay was used to normalize for transfection efficiency. Columns, average fold induction normalized on the activity of the empty vector; bars, SD. B, radiolabeled probes (probe 1, probe 2, and probe 3) or the positive (CM1) and negative (NC) control double-stranded oligonucleotides were incubated with the in vitro translated MYCN and/or MAX proteins as indicated and subjected to electrophoretic mobility shift assay.
To test whether MYCN could directly bind to the HMGA1 promoter, we generated three different DNA fragments, each of which contained multiple putative E-boxes (see Materials and Methods), and used them in a gel retardation assay. Each of the DNA fragments could bind the \textit{in vitro} translated MYCN/MAX heterodimer (Fig. 3B), thus indicating that MYCN might directly interact with the human HMGA1 promoter. In particular, probe 1, containing a region deleted in the reporter construct 5L\#1-387 and highly conserved in the mouse sequence, showed the highest binding efficiency (Fig. 3B). Confirming the specificity of the interaction, neither MYCN nor MAX proteins were able to shift any of the three DNA fragments or the CM1 oligo/positive control. These results suggest that multiple regions of the HMGA1-5L\#1 fragment are requested for optimal MYCN-dependent promoter activity.

**HMGA1 expression in neuroblastoma-like tumors from MYCN transgenic mice.** To study whether increased HMGA1 expression could be associated with MYCN deregulation \textit{in vivo}, we analyzed neuroectodermal tumors that developed in transgenic mice with the neural crest targeted expression of the human MYCN (26). We detected scant HMGA1 expression in the adrenal gland of a control mouse as well as in the liver of the transgenic mice. However, HMGA1 expression was much higher in the three tumor samples obtained from the transgenic animals (Fig. 4A), whereas we detected no modification of the other family member, HMGA2 (not shown). As shown in Fig. 4B, we noticed a strong correlation between MYCN transgene and HMGA1 expression levels.

**HMGA1 expression in primary human neuroblastomas.** We then searched for an association between HMGA1 expression and MYCN amplification in 16 primary human neuroblastoma samples. In keeping with previous results (20), we observed scant HMGA1 expression in an adrenal medulla mRNA sample and higher levels in most of the primary neuroblastomas examined. In particular, all MYCN-amplified samples showed more than 1,000 times higher HMGA1 expression compared with adrenal medulla (Fig. 5A). They also showed higher HMGA1 expression if compared with the average level of expression in MYCN single-copy samples (= 4.8), with values ranging from 17 to 86 (Fig. 5A). Indeed, the average HMGA1 expression among MYCN-amplified tumors was about 10-fold higher than the average of the single-copy tumors (Fig. 5A, inset). Thus, very high HMGA1 expression was associated with MYCN amplification. This was also confirmed at the protein level. Indeed, we found higher HMGA1 and MYCN expression in one MYCN-amplified neuroblastoma sample compared with MYCN single-copy samples (Fig. 5B). Interestingly, 3 of the 12 (25%) single-copy neuroblastomas also had rather high HMGA1 expression (Fig. 5A). In one case, this was associated with increased MYCN expression.

![Figure 4](https://example.com/figure4.png) **Figure 4.** HMGA1 expression in MYCN transgenic mice tumor tissues. Quantitative RT-PCR analysis of the expression of the mouse HMGA1 (A) and MYCN human transgene (B) in normal mouse adrenals, three different tumor tissues (T2849, T1834, and T3217), and two liver tissues obtained from the same MYCN transgenic mice (L2849 and L3217). Results were normalized on GAPDH expression.

![Figure 5](https://example.com/figure5.png) **Figure 5.** HMGA1 expression in primary human neuroblastoma. A, a human adrenal medulla (Adr) and 16 primary neuroblastomas were analyzed for HMGA1 expression by quantitative RT-PCR. Results were normalized on \(\beta\)-actin expression. Inset, average HMGA1 expression levels (bars, SE). Black columns, MYCN single-copy samples; white columns, MYCN-amplified samples. B, Western blot analysis of the expression of HMGA1 and MYCN proteins in primary neuroblastoma samples; A, MYCN-amplified tumor; SC, MYCN single-copy tumor.
In the remaining two, we found no significant MYCN overexpression, suggesting that other oncogenic pathways might be leading to HMGA1 deregulation in human neuroblastomas.

**HMGA1 knockdown in SK-N-BE cells via RNA interference.**
MYCN is known to affect many aspects of neuroblastoma tumorigenesis, including cell proliferation, survival, and drug resistance. Increased HMGA1 expression was also shown to be involved in the regulation of cell proliferation, survival, adhesion, and transformation (13, 18, 19, 39, 40). To test whether MYCN-dependent enhancement of HMGA1 expression is involved in supporting neuroblastoma cell growth, we used specific siRNA duplexes against HMGA1 transcript. Whereas we accomplished a significant reduction in HMGA1 mRNA expression after either 3 or 6 days of treatment in the MYCN-amplified SK-N-BE cell line, HMGA1 protein only decreased after 6 days (Fig. 6A and B). HMGA1 knockdown was associated with a consistent reduction in cell number, which reached about 35% after 6 days of culture in the presence of the HMGA1 siRNAs compared with cells treated with the nonspecific siRNA duplex (Fig. 6C, NS-si). We also monitored BrdUrd incorporation in S-phase cells as a measure of cell proliferation. We observed more than 20% reduction in the rate of BrdUrd incorporation after treatment with HMGA1 siRNA compared with those treated with nonspecific siRNA (Fig. 6D), suggesting that HMGA1 knockdown directly affects cell proliferation. In contrast, we failed to observe significant modification in the number of Hoechst-stained picnotic nuclei, indicative of the absence of massive apoptotic events (not shown).

**Discussion**
MYCN amplification represents the first recognized and one of the most important genetic alteration in human neuroblastoma, but the molecular mechanisms through which it contributes to tumor development and/or progression are far from being truly understood. Despite large-scale and genome-wide approaches that have been applied to the search for MYCN regulated genes (41–43), the number of MYCN targets relevant for neuroblastoma tumor biology remains rather small. Among the few known genes, there are some potentially involved in cell cycle progression (α-prothymosin, ODC, MCM7, ID2, and MDM2), cell differentiation (PAX-3), protein translation, drug resistance (such as MRP1), and cell-matrix interactions (33, 34, 41, 44–48). We have previously shown that HMGA1 decreases with a kinetics that strictly follows MYCN repression on RA treatment in MYCN-amplified neuroblastoma cell lines and that a constitutive MYCN overexpression abolishes HMGA1 repression by RA (20). HMGA1 increase also seems to follow MYCN induction in hypoxic SK-N-SH cells (49). Here we provide evidence that HMGA1 is a novel MYCN target both in cultured cells and in vivo, and this is likely to be dependent on a direct transcriptional activation involving previously undescribed HMGA1 promoter elements. Indeed, MYCN overexpression induces increased HMGA1 expression in HEK-293, SK-N-AS, SK-N-SH, and SH-EP cells. In MYCN transgenic mice, HMGA1 expression increased in a transgene dose-dependent manner in the neuroblastoma-like tumors, suggesting that human MYCN might indeed induce HMGA1 expression also in vivo. As we previously observed (20, 21), also the present analysis confirmed that HMGA1 expression is increased in most neuroblastoma tumors compared
with adrenal gland levels. However, we also observed a significant association between a very high HMGA1 expression and MYCN amplification. Cotransfected MYCN transactivated the HMGA1-5L.1 reporter in a dose-dependent fashion. Consistent with a specific and direct transcriptional activation of the HMGA1 promoter by MYCN, we also showed that MAD inhibits MYCN stimulatory activity. Indeed, MAD is a member of the same network known to compete with MYC proteins for binding to MAX and E-boxes (50) and to inhibit transcription via recruitment of corepressors (reviewed in refs. 30, 51).

The activation of the mouse HMGA1 promoter by another member of the myc family has been previously reported. A typical CACGGG E-box located 1,337 bp upstream the major transcription start site was shown to be the unique c-MYC response element (18). A similar site is present at position −1,353 from the major transcription start site in the human HMGA1 promoter (37). The HMGA1-5L-E-box construct containing this site seems to be inducible by either c-MYC or MYCN in HeK-293 cells. However, the shorter HMGA1-5L.1 construct, which strongly responds to MYCN but not to c-MYC, does not contain such an element. Thus, the transactivating function of MYCN on human HMGA1 promoter we reported here involves previously unidentified MYC-responsive elements. Whether MYCN, in addition to the more downstream sites, might also bind to the −1,353 canonical E-box for an optimal regulation of the HMGA1 promoter in vivo remains to be determined. Although no perfectly matching E-box is present within the HMGA1-5L.1, we showed that MYCN/MAX heterodimers could directly bind to distinct DNA fragments within this region. Eight imperfect E-boxes are present within the construct. Deletion of the regions containing either the first three or the last three of those putative E-boxes caused a significant drop in luciferase activity dependent on MYCN. Consistently, DNA probes obtained from similar regions could bind to MYC/MAX heterodimers. Overall, these results suggest that multiple elements of the 1,600 bp fragment may cooperate for an optimal response to MYCN. Interestingly, human and mouse HMGA1 genomic sequences seem strikingly similar around the first three putative E-boxes, suggesting that relevant and conserved cis-acting elements might operate on these regions for the binding of specific transcriptional regulators. In keeping with this, we have shown increased HMGA1 expression in tumors arising in MYCN transgenic mice.

HMGA1 is a bona fide oncogene of which overexpression promotes transformation of Rat1a and CB33 cells and induces a more malignant phenotype in breast cancer cells (18, 39, 40). Conversely, HMGA1 repression strongly impairs the malignant phenotype of Burkitt’s lymphoma cells (18) and the tumorigenicity of several other human cancer cell lines (19). Deregulated HMGA1 expression is also one of the most consistent features in primary human cancer. Higher levels of this protein are most often associated with higher-grade, more invasive, and/or metastatic phenotypes (15–17). Thus, it seems that increased HMGA1 expression confers a more aggressive phenotype to many tumors both in vitro and in vivo. We showed that HMGA1 knockdown in MYCN-amplified SK-N-BE cells reduced their proliferation, suggesting that its expression may provide an advantage to neuroblastoma cells, too. Of course, the effect of HMGA1 repression on the malignant phenotype of neuroblastoma cells needs to be more thoroughly characterized. Indeed, we are looking at the modification of gene expression and tumorigenicity of HMGA1 knockdown SK-N-BE cells.

The widespread overexpression of HMGA1 in human cancer indicates that multiple oncogenic pathways may converge on its activation, thus recruiting its oncogenic potential. Consistent with this, HMGA1 not only is induced by a number of growth factors (52) but also is activated by c-MYC (18) and activator protein 1 (38), two well-known oncogenic transcription factors. Here we have shown that another oncogene, MYCN, sustains HMGA1 overexpression in MYCN-amplified neuroblastomas. However, increased levels of HMGA1 expression have also been observed in MYCN single-copy neuroblastoma cells and primary tumors (20), as well as a number of other tumor cells without c-MYC translocations. This indicates that additional oncogenic transcription factors might also deregulate HMGA1 expression and that further work will be required to understand the signals supporting high HMGA1 expression in tumors not bearing c-MYC or MYCN genetic aberrations.

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