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Expression and DNA-Binding Activity of MYCN/Max and Mnt/Max During Induced Differentiation of Human Neuroblastoma Cells

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Abstract Amplification of *MYCN* is one of the most important prognostic markers for neuroblastoma and is correlated with rapid tumor progression and poor prognosis. MYCN belongs to the Myc/Max/Mad/Mnt network of proteins that regulate proliferation, apoptosis, and differentiation. It is well established that *MYCN* is downregulated during induced differentiation of neuroblastoma cells carrying an amplified *MYCN* gene, but very little is known about other components of the network, i.e., the Max, Mad, and Mnt proteins, during this process. In this study we show that *Mad and Mnt expression* was only modestly regulated in differentiating SK-N-BE(2) neuroblastoma cells, while *MYCN* was rapidly downregulated. This downregulation was reflected in a decreased MYCN/Max DNA-binding activity while the Mnt/Max binding did not change during differentiation. In parallel experiments we also analyzed the Myc/Max/Mad expression and DNA binding capacity during induced differentiation in the *MYCN* single copy neuroblastoma cell line SH-SY5Y. In this cell line only modest changes in expression of the components of the MYCN/Max/Mad/Mnt network was detected, but since the cell line expresses relatively low levels of MYCN and c-Myc, these changes might be of functional significance. Cell cycle analyses of SK-N-BE(2) demonstrated an increase in the G1-phase fraction after RA-treatment. These data show that the decreased *MYCN* expression and MYCN DNA-binding is correlated with retarded cell cycle progression. Furthermore, when *Mad1 or Mnt* was overexpressed in SK-N-BE(2) cells they retained the capacity to differentiate, underscoring the notion that MYCN downregulation, and not changes in *Mad/Mnt* expression, is essential for neuroblastoma cell differentiation. J. Cell. Biochem. 92: 1282–1295, 2004.

Key words: neuroblastoma; differentiation; Mad; MYCN; Mnt; expression; DNA-binding activity

Neuroblastoma is the most common extracranial solid childhood cancer and is derived from the sympathetic nervous system. It is highly heterogeneous and is lethal in approximately 50% of the patients. The malignancy can be staged into five different groups (1–4 and 4S), where stage 4 tumors are most malignant. Amplification of the *MYCN* gene is correlated to advanced stages of the disease [Brodeur et al., 1984] and is found in 40–50% of the tumors with a poor prognosis [Seeger et al., 1985; Brodeur and Seeger, 1986], thereby making it one of the most important prognostic markers for neuroblastoma.

MYCN belongs to the Myc/Max/Mad network that plays a key role in the regulation of cell growth, differentiation, and apoptosis [Henriksson and Lüscher, 1996; Facchini and Penn, 1998; Eilers, 1999; Johnston et al., 1999; Grandori et al., 2000; Amati et al., 2001]. *Myc* expression is tightly linked to cell proliferation and Myc proteins are positive regulators of cell growth. *Constitutive c-myc* expression enforces cell cycle progression, blocks differentiation of various cell types, and sensitizes to apoptosis in

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the absence of mitogens [Henriksson and Lüscher, 1996; Grandori et al., 2000]. Whereas *c-Myc* is present in most proliferating cells, the expression of *L-Myc* and *MYCN* is restricted to certain tissues and developmental stages. The importance of *MYCN* for proper development of several organs and tissues has been demonstrated in *MYCN* knockout mice [Charron et al., 1992; Sawai et al., 1993]. Here, a number of tissues display a lack of cellularity, including the peripheral and central nervous system, indicating that MYCN is necessary for the proliferative capacity in several cell types. Furthermore, a murine *MYCN* "knockin" model, where the overexpression was targeted to neural crest cells, has demonstrated that MYCN regulates migratory potential and promotes neuronal differentiation [Wakamatsu et al., 1997], further confirming the idea of MYCN playing a crucial role during the development of the nervous system. Furthermore, in an elegant murine neuroblastoma model transgenic mice was generated by overexpressing *MYCN* in neural crest cells using the *tyrosine hydroxylase* promoter, thereby demonstrating the central role of MYCN in the genesis of neuroblastoma [Weiss et al., 1997]. The role of MYCN in neuroblastoma cell differentiation and proliferation has been studied in several cell lines. In vitro studies have shown that inhibition of *MYCN* translation in LA-N-5 neuroblastoma cells results in a decreased proliferation rate and a more differentiated phenotype [Negroni et al., 1991]. In addition, *MYCN* overexpression stimulates proliferation and inhibits induced differentiation in neuroblastoma cell lines [Thiele and Israel, 1988; Lutz et al., 1996; Peverali et al., 1996]. It has also been shown that *MYCN* mRNA levels decrease during neuroblastoma cell differentiation in vitro [Amatruda et al., 1985; Thiele and Israel, 1988; Thiele et al., 1988]. Taken together, these data show that proliferating and immature neuroblastoma cells often have high *MYCN* levels, in contrast to differentiated cells.

In contrast to the *Myc* genes, *Mad* genes are expressed primarily in differentiated, nonproliferating tissues, and Mad proteins are negative regulators of cell growth [Ayer and Eisenman, 1993; Zervos et al., 1993; Larsson et al., 1994; Chin et al., 1995; Hurlin et al., 1995a,b; Västrik et al., 1995; Quéva et al., 1998]. *Mnt* is expressed in differentiated cells but in contrast to *Mad1*, also in proliferating cells together with *Myc* [Hurlin et al., 1997]. Mnt has a repressive effect on Myc-dependent transcriptional activation and transformation in vitro [Hurlin et al., 1997; Meroni et al., 1997]. A large body of evidence shows that the repressive activity of Mad and Mnt is mediated through remodeling of chromatin and modulation of gene expression, as reviewed [Wolffe, 1997]. The finding that *Mnt* is expressed in parallel with *Myc* in proliferating cells and can affect the same promoters, albeit with the opposite effect, has led to the suggestion that Mnt is a modulator of Myc function. In a recent study *Mnt* knockout mice were generated and fibroblasts isolated from these animals were analyzed for expression of Myc-target proteins, apoptotic response, and in transformation assays [Hurlin et al., 2003]. It was found that these cells expressed the Myc target genes *cdk4* and *cyclin E* at higher levels than wt fibroblasts. Importantly, the *Mnt* knockout cells were prone to apoptosis and could be transformed with oncogenic *ras* alone. Interestingly, the *Mnt*−/− cells lost *Myc* expression after several passages in cell culture [Hurlin et al., 2003]. These results strengthen the hypothesis that there is a functional interplay between Myc and Mnt.

The Myc/Max/Mad/Mnt network may constitute a molecular switch where the abundance and activity of Myc- versus Mad containing heterodimers determines whether a cell enters a differentiation pathway or remains in a proliferative state [Ayer and Eisenman, 1993]. This view has been further supported by the observations that ectopic expression of *Mad1* stimulates differentiation of MEL cells, and that targeted disruption of *Mad1* inhibits cell cycle exit and delays terminal differentiation of granulocytic precursor cells [Cultraro et al., 1997; Foley et al., 1998]. Also, as discussed above, MYCN levels can determine the proliferation and differentiation state of neuroblastoma cells. These observations in conjunction with the fact that *MYCN* amplification is one of the most important prognostic markers for neuroblastoma, makes it interesting to clarify the dynamics of the MYCN/Max/Mad/Mnt network in this malignancy. Furthermore, studies of the MYCN/Max/Mad/Mnt network during neuronal differentiation have so far only been performed in murine systems. We have therefore investigated the expression patterns and the DNA-binding activities of the MYCN/Max/Mad/Mnt network during neuroblastoma cell

differentiation in SK-N-BE(2), that has a 85-fold *MYCN* amplification [Squire et al., 1995], and in SH-SY5Y, that lacks *MYCN* amplification [Schwab et al., 1983]. For induction of differentiation the SK-N-BE(2) cells were induced with RA, while SH-SY5Y cells were induced by TPA. This experimental set-up was chosen due the mature and partly similar phenotypes that were induced in the respective cell lines based on the following arguments: (i) TPA induces a well-characterized sympathetic neuronal phenotype of SH-SY5Y cells with, e.g., increased GAP-43, NPY and noradrenaline levels and neurite outgrowth [Påhlman et al., 1981; Andersson et al., 1994] while RA-treatment causes downregulation of *NPY* [Grynfeld et al., 2000; Magni et al., 2000] and fails to induce *GAP-43* upregulation [Grynfeld et al., 2000]. (ii) The SK-N-BE(2) cell line fails to grow long neurites when treated with TPA, but responds efficiently to RA in this respect ([Grynfeld et al., 2000] and AGS, unpublished observations). Furthermore, in this study we show that the treatment of SK-N-BE(2) cells with 10 μM RA not only induces neurite outgrowth, but also *NPY* upregulation. The set-up of using specific inducers for the different cell lines thus enabled a study of MYCN/Max/Mad/Mnt network regulation during neuronal differentiation in both cell lines.

MATERIALS AND METHODS

Cell Cultures and Transfections

The neuroblastoma cell lines SH-SY5Y [Biedler et al., 1978] and SK-N-BE(2) [Ciccarone et al., 1989] (kindly provided by Dr. J. Biedler, Sloan Kettering Institute, New York, NY) were grown in Eagle's MEM supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in 5% CO_2. The cells were induced to differentiate using 16 nM of TPA or 10 μM RA (Sigma Chemical Co., St. Louis, MO). COS-7 cells were maintained in Iscove's Modified Eagle's Medium (IMDM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 5% CO_2. Transfections of COS-7 cells were performed using the calcium phosphate precipitation method as described [Chen and Okayama, 1987].

Northern Blot Hybridizations

Total RNA and Northern blots were prepared as described previously [Grynfeld et al., 2000]. The blots were hybridized to the following cDNA probes; *Mad1* was derived from NotI/EcoRI-digested pVZ1-Mad and kindly supplied by Dr. D. Ayer. Mad3 (AC: AA278224) and Mad4 (AC: AA019251) were kindly supplied by the Resource Centre of the German Human Genome Project at the Max Planck-Institut for Molecular Genetics, Berlin, Germany. *Glyceraldehyde-3-phosphate dehydrogenase* (AC: M17851) [Tso et al., 1985] was used as an internal loading control. The *NPY* and *MYCN* cDNA probes were prepared as described [Kohl et al., 1983; Andersson et al., 1994]. cDNA probes were labeled with ^{32}P using a oligonucleotide labeling kit (Amersham Pharmacia Biotech, San Francisco, CA). Hybridizing mRNA was visualized by autoradiography using Curix Blue film (AGFA-Gevaert AG, Leverkusen, Germany).

RT-PCR Analyses

cDNA synthesis was performed using the SuperScript Preamplification System according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). The amplifications consisted of 15–25 cycles with an annealing temperature of as follows: 94°C 15″, 55°C 30″, 72°C 45″. The optimal cycle number for a given primer pair was established for PCR reactions to be in a linear range. The primers used for RT-PCR amplified bases 253–483 of *Mad1*, bases 251–457 of *β-actin*, bases 218–546 of *GAPDH*, and for *Mnt* primers flanking the regions encoding the bHLHZip (bases 625–915) and the SID (bases 1–132) were employed. All PCR reactions were performed in separate amplification reactions and repeated at least 3-times. The PCR products were separated on 1.5% agarose gels. For densitometric analyses, the PCR products were visualized using SYBR Green (Roche Molecular Biochemicals, Indianapolis, IN) and analyzed using Image Gauge V3.3 and Fuji LAS-1000 equipment (Fujifilm, Tokyo, Japan).

Electrophoretic Mobility Shift Assay (EMSA)

Whole cell extracts were prepared in buffer containing 10 mM Tris-HCl, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $ZnCl_2$, and 1% Triton X-100, pH 7.07 and protease inhibitors. For EMSAs, 0.2 ng of radiolabeled double-stranded oligonucleotide CMD (5′-TCAGACCACGTGGTCGGG) or NCC (5′-TCAGACCACGCGGTCGGG) were incubated with 2.5 μg of cellular extracts at 25°C for 30 min. The

binding reactions were performed in 20 mM Tris-HCl, 50 mM NaCl, 0.5 mg/ml BSA, 1 mM EDTA, 20% glycerol, and 200 ng of unrelated double-stranded oligonucleotide as carrier DNA. For antibody supershifts, 0.05 μg of anti-Max (C-17, Santa-Cruz Biotechnology Inc., Santa Cruz, CA), 1 μg of anti-Mnt (N-18 or M-132, Santa-Cruz), or 0.2 μg of anti-MYCN antibody (C-19, Santa-Cruz) were added to the binding reactions and incubated for 30 min at room temperature prior to the addition of labeled probe. Protein/DNA complexes were resolved on 5% nondenaturing polyacrylamide gel in GS-buffer (25 mM Tris, 25 mM boric acid, 0.5 mM EDTA). Following electrophoresis, gels were dried and developed using PhosphorImager technology (Molecular Dynamics Inc., Sunnyvale, CA).

FACS Analyses

SK-N-BE(2) and SH-SY5Y cells (approximately 1×10^6) were grown in the presence of 10 μM RA or 16 nM TPA for 96 h. Cells were trypsinised, collected by centrifugation, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol overnight. Fixed cells were pelleted and incubated in PBS containing 50 μg/ml propidium iodine (Sigma Chemical Co.) and 100 μg/ml RNAse A (Sigma Chemical Co.) for 1 h at room temperature. Cell cycle data acquisition and analyses were performed on a Becton Dickinson flow cytometer using CellQuest and ModFit software.

Overexpression Studies

SK-N-BE(2) cells were seeded as previously described [Grynfeld et al., 2000] and cotransfected with pEGFP-C1 (Clontech, Palo Alto, CA) and pEQ176P2-Mad1 or pRCMnt1A (kind gifts from Dr. B. Lüscher and Dr. R.N. Eisenman, respectively). Mad1 function was confirmed by EMSA (data not shown) and previously by Mad1-associated-HDAC activity [Sommer et al., 1997] and Mnt function was confirmed by EMSA (Fig. 2C and data not shown). The cotransfection was performed at a ratio of 1:3 using Fugene according to the manufacturer's instruction (Roche Molecular Biochemicals). After 5 h of transfection, the cells were induced to differentiate in 10 μM RA for 96 h and then fixed in 4% paraformaldehyde in PBS and mounted on microscope slides. The transfected cells were identified by presence of green fluorescent protein using a fluorescence microscope. Cells were considered morphologically differentiated when the neurites were at least twice as long as the cell body diameter.

Western Blotting

Cells were collected in PBS, boiled in SDS loading buffer, and sonicated. Samples containing approximately 30 μg of total protein were separated on 10% SDS–polyacrylamide gels, transferred to nitrocellulose and visualized with the following antibodies: Mnt (M-132, Santa-Cruz), c-Myc (9E10, Santa-Cruz), MYCN (C-19, Santa-Cruz), and β-actin (AC-15, Sigma Chemical Co.).

RESULTS

The *Mad* Genes Exhibit Different Expression Patterns in Differentiating Neuroblastoma Cells

Since MYCN and the Mad proteins often display opposite roles during development, we were interested to see whether this was reflected at the expression level in differentiating neuroblastoma cells. We, therefore, performed Northern hybridization analyses and RT-PCR experiments on total RNA prepared from SK-N-BE(2) and SH-SY5Y cells treated with RA and TPA, respectively. The neuronal differentiation was confirmed by *NPY* hybridization, showing an increased *NPY* expression over time (Fig. 1A–C). The analyses showed that the *Mad* genes give rise to a complex transcript pattern, in agreement with a previous report on neuronal differentiation of the murine cell line P19 [Quéva et al., 1998]. We also found that the expression of the different *Mad* genes varied in the two neuroblastoma cell lines. For example, we detected only one *Mad4* transcript in SK-N-BE(2) cells, while two transcripts were observed in SH-SY5Y (see Fig. 1A,B). In SK-N-BE(2), the expression of *Mad4* peaked transiently after 24 h of treatment with RA (Fig. 1A). *Mad1* expression was detected in control RNA from SK-N-BE(2) cells and after 2 h of RA-treatment. To study this expression pattern in further detail, we employed the more sensitive RT-PCR technique. These experiments revealed a biphasic expression pattern of *Mad1* with a peak at 2 h, followed by a down-regulation at 8 h, and a moderate increase after 96 h of differentiation (Fig. 1C). In contrast, the expression of *Mad3* in SH-SY5Y cells and SK-N-BE(2) cells seemed almost unaffected during differentiation, although a moderate transient downregulation

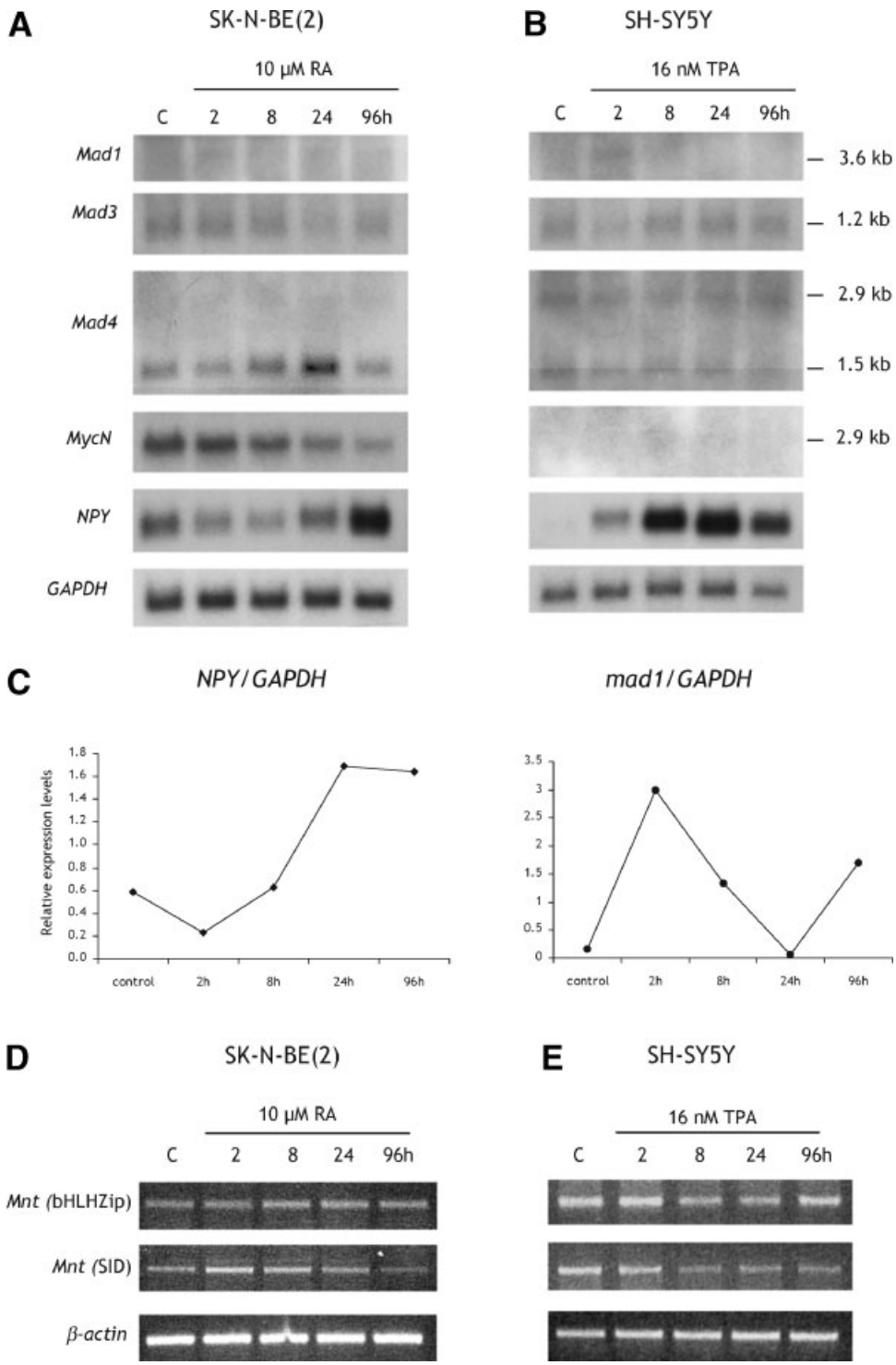

Fig. 1. Expression patterns of *MYCN*, *mnt*, and *mad* network genes during neuroblastoma cell differentiation. Northern blot and RT-PCR analyses of SK-N-BE(2) and SH-SY5Y cells at different time-points after addition of RA and TPA, respectively. Fifteen microgram of total RNA per sample was separated electrophoretically and transferred to a nylon membrane. The membrane was sequentially hybridized with ^{32}P-labeled cDNA fragments encoding *Mad1, Mad3, Mad4*, *NPY*, and *MYCN*. *GAPDH* served as RNA loading control. **A**: Northern blot with RNA from SK-N-BE(2) cells treated with 10 μM RA for 2–96 h. **B**: Northern blot with RNA from SH-SY5Y cells treated with 16 nM TPA for 2–96 h. **C**: Graphs presenting *Mad1 and NPY* expression levels in SK-N-BE(2) cells treated with 10 μM RA for 2–96 h as measured by RT-PCR. *NPY* was used as a control for confirming induced differentiation and *GAPDH* was used as reference for cDNA input. Expression levels for *Mad1* (at cycle 35), *NPY* (at cycle 26), and *GAPDH* (at cycle 22) were measured by densitometric analyses. In each graph, one representative experiment out of three is shown RT-PCR analysis of *Mnt* expression in SK-N-BE(2) cells treated with 10 μM RA for 2–96 h (**D**) and in SH-SY5Y cells treated with 16 nM TPA for 2–96 h (**E**). Amplification of *β-actin* verified that the material contained equal amounts of cDNA. The PCR-products are indicated on the left. One representative experiment out of three is shown.

was noted at 2 and 24 h, respectively. Unfortunately, we could not investigate the expression pattern of *Mxi1*, since we did not achieve any specific signal with our probe (derived from pBS-Mxi1, a generous gift from Dr. Zervos, data not shown). The *MYCN* expression decreased considerably during differentiation of SK-N-BE(2) (Fig. 1A) in agreement with previous reports on *MYCN* expression in other differentiating neuroblastoma cell lines [Thiele et al., 1985; Hammerling et al., 1987]. In the non-*MYCN* amplified SH-SY5Y cells, the *MYCN* level was below the threshold of detection when using 15 μg total RNA and Northern blot hybridizations. In these cells *Mad1* is transiently upregulated after 2 h of differentiation. The expression of both *Mad4* transcripts and *Mad3* decreased transiently at 2 h of differentiation, after which a moderate increase could be observed (Fig. 1B). We conclude that the expression of the *Mad* genes are differently regulated in SK-N-BE(2) and SH-SY5Y cells.

Mnt Expression in Differentiating Neuroblastoma Cells

Expression of the *Mnt* gene in neuroblastoma cells was analyzed by RT-PCR, since Northern hybridization was not sensitive enough to detect the *Mnt* transcript (data not shown). SK-N-BE(2) cells were treated with RA and SH-SY5Y cells with TPA (Fig. 1D) to induce neuronal differentiation. Total RNA was isolated from cells at different time-points of treatment and used to generate cDNA for RT-PCR analyses. *β-actin* primers were employed to verify that equal amounts of cDNA were used in the reactions (Fig. 1D). To confirm the presence of the full-length *Mnt* mRNA in the cells, two different primer pairs were used to amplify regions critical to Mnt function [Hurlin et al., 1997; Meroni et al., 1997]. One primer pair amplified the region encoding the SID and the other pair amplified the sequences encoding the bHLHZip region. We observed that *Mnt* mRNA was expressed at relatively steady levels in both cell lines. In SH-SY5Y line the mRNA levels seemed to peak at 2 h of differentiation (Fig. 1E).

Expression and DNA-Binding Activities of MYCN/Max/Mad/Mnt Network Proteins in Differentiating Neuroblastoma Cells

We analyzed expression of MYCN, c-Myc, and Mnt proteins by Western blotting during differentiation of SK-N-BE(2) and SH-SY5Y cells. MYCN expression was high in untreated SK-N-BE(2) cells, increased at 8 h after treatment and declined at later stages of differentiation (Fig. 2A). Expression of MYCN was extremely weak in the SH-SY5Y cell line and was detectable only at 2 and 8 h of treatment (Fig. 2A). Interestingly, we were able to detect expression of c-Myc protein in SH-SY5Y but not in the SK-N-BE(2) cells (Fig. 2A). Expression of c-Myc was slightly increased at 2 h of treatment and decreased at later time points. This observation may indicate that Myc proteins are essential for proliferation of neuroblastoma cells, and that c-Myc may be upregulated in neuroblastoma cells without MYCN amplification. Downregulation of both MYCN and c-Myc proteins is apparent at later stages of differentiation, while their expression slightly increases as an early event (2–8 h) after differentiation stimuli. Expression of Mnt was fairly constant in both cell lines with a slight increase at 2 h after treatment, which well paralleled the RT-PCR data (Fig. 2A).

To assess DNA-binding activity of E-box binding complexes during differentiation we performed EMSA with double-stranded oligonucleotides containing the Myc/Max/Mad consensus (CACGTG) E-box sequence (CMD). In order to identify the protein complexes binding to this oligonucleotide, a series of control experiments were performed. Three specific bands were observed in extracts from SK-N-BE(2) cells (Fig. 2B,C and data not shown). Antibodies against Max, MYCN, Mnt, and USF, a ubiquitously expressed bHLHZip factor known to bind E-box sequences [Gregor et al., 1990], were used for identification of the respective complexes (Fig. 2C, lanes 1–5, and data not shown). By adding the Max antibody, two of the complexes were shifted indicating that they contained the Max protein (Fig. 2C, lane 2). The faster migrating complex represented the MYCN/Max heterodimer since it disappeared upon incubation with the MYCN antibody while a new supershifted band appeared (Fig. 2C lane 3). Addition of Mnt antibodies affected the slower migrating complex (Fig. 2C, lanes 4 and 5). The third specific band present in all lanes represented USF as identified by antibody supershift experiments (data not shown) and by comparison with the band observed in extracts from COS-7 cells transiently transfected with a USF-expressing plasmid (Fig. 2C, lane 7). The migration of the Mnt/Max complex was also

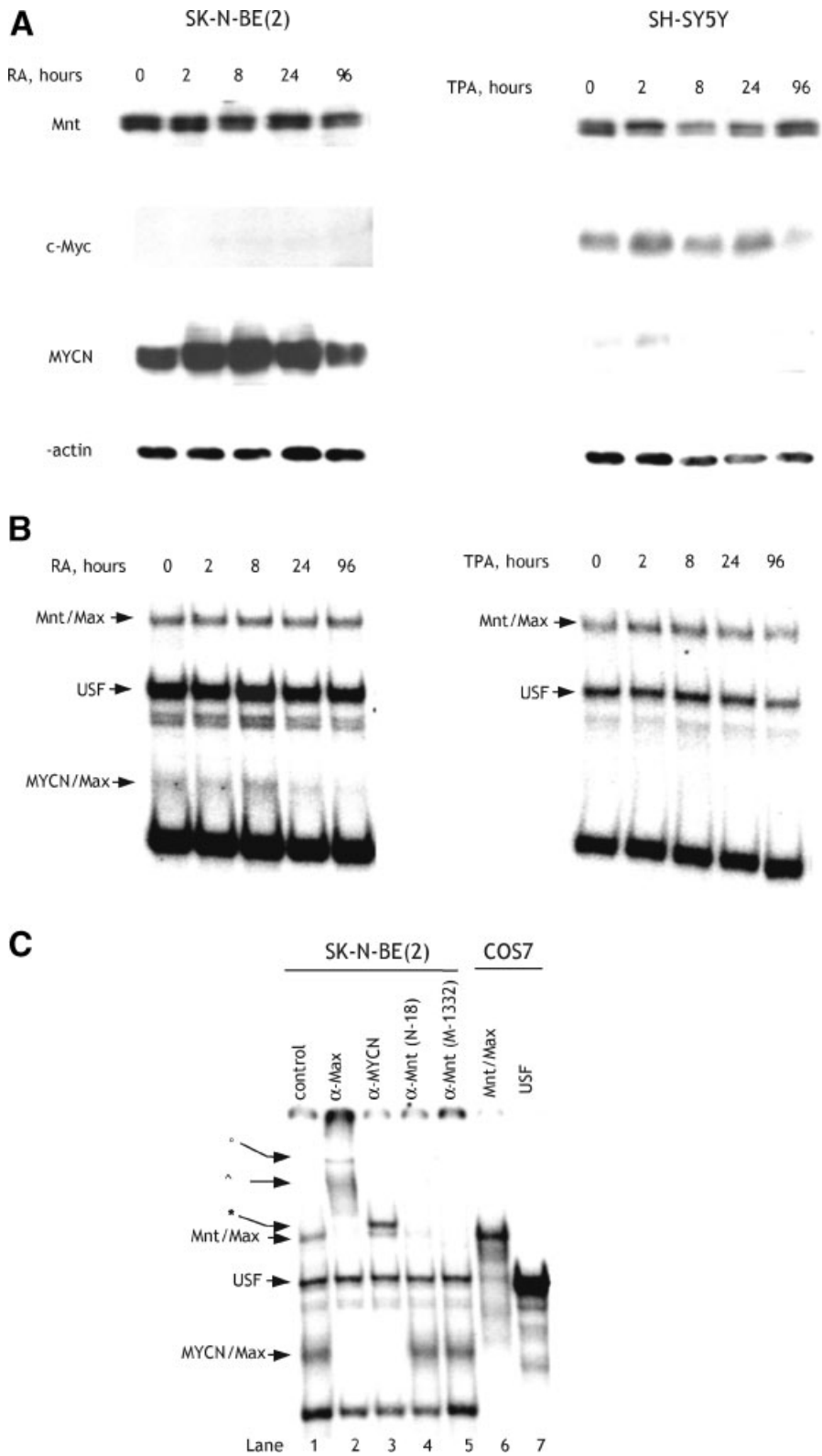

Fig. 2. Expression and E-box binding activity of Myc network proteins during differentiation of human neuroblastoma cells. **A**: Western blotting of total cell extracts with Mnt, c-Myc, MYCN, and β-actin antibodies. Extracts were prepared from SK-N-BE(2) cells treated with 10 μM RA for 2–96 h, and SH-SY5Y cells treated with 16 nM TPA for 2–96 h, as well as the untreated cells. **B**: EMSAs performed with extracts from undifferentiated and differentiated neuroblastoma cells using the CACGTG-containing CMD oligonucleotide. The Mnt/Max, MYCN/Max, and USF complexes are indicated by arrows. **C**: Extracts from undifferentiated SK-N-BE(2) cells (**lanes 1–5**) and from COS-7 cells transfected with expression plasmids encoding USF or Mnt and Max (**lanes 6–7**). Antibodies against Max, MYCN, and Mnt were added as shown. The supershifted complexes are indicated with ° and ^ (lane 2) and * (lane 3).

corroborated using this approach (Fig. 2C, lane 6).

The DNA-binding activities of the identified complexes were then analyzed using extracts from the SK-N-BE(2) and SH-SY5Y cell lines induced to differentiate using RA and TPA, respectively. The MYCN/Max complexes decreased after 24 h of RA treatment of SK-N-BE(2) cells (Fig. 2B). This complex was undetectable in the SH-SY5Y line, probably due to the low MYCN expression level in these cells (Fig. 1B and 2A,B). Mnt/Max complexes were present at all time-points of differentiation in both cell lines and no significant changes in binding were observed, although there was a modest transient increase at 2–8 h in TPA-treated SH-SY5Y cells (Fig. 2B). No significant change in USF binding could be observed during differentiation in neither SK-N-BE(2) nor SH-SY5Y cells (Fig. 2B). The Mnt/Max dimer was the most prominent Max containing DNA-binding complex present in neuroblastoma cells, whereas we did not observe any Mad/Max complexes (Fig. 2 and data not shown). We have also analyzed Mnt/Max and MycN/Max DNA binding to the non-consensus E-box, CACGCG, shown to bind Mnt/Max with higher affinity compared to the canonical CACGTG sequence [Meroni et al., 1997]. We observed that Mnt/Max bound weaker to the CACGCG E-box oligonucleotide than to the consensus E-box, and that binding did not vary during differentiation. Furthermore, we were unable to detect MycN/Max binding to this non-consensus sequence (data not shown). These results are in agreement with previous studies in medullablastoma and HL60 cells showing that Mnt/Max is the strongest Max-binding complex whereas no Mad/Max complexes could be detected [Sommer et al., 1998, 1999].

Cell Cycle Distribution in Differentiating Neuroblastoma Cells

In order to monitor cell cycle distribution in SK-N-BE(2) and SH-SY5Y cells during RA- and TPA-induced differentiation, we performed FACS analyses of propidium iodine-stained cells. As seen in Figure 3A, the percentage of SK-N-BE(2) cells in S-phase did not change significantly (from 20 to 18%) during differentiation. However, we observed an increase in the percentage of cells in G0-G1-phase (from 49 to 63%) and a concomitant reduction of cells in G2-M-phase (from 31 to 19%). Thus, these results suggest that the SK-N-BE(2) cells continue to cycle 4 days after RA-treatment albeit at a slower rate. Changes in cell cycle distribution were more pronounced in SH-SY5Y with a significant decrease in the proportion of cells in S-phase (from 15 to 5%) after differentiation and a concomitant increase of cells in G0/G1 (from 68 to 80%; Fig. 3B). These results were consistent with the morphological observation that at 96 h of treatment the SH-SY5Y cells virtually stop growing and differentiate as evidenced by extensive neurite outgrowth. This is also accompanied by a complete downregulation of c-Myc in these cells after differentiation (Fig. 2A).

Morphology of Undifferentiated and Differentiated Neuroblastoma Cells Overexpressing Mad1 and Mnt

The finding of a complex expression pattern of the *Mad/Mnt* genes in differentiating neuroblastoma cells (Fig. 1) incited us to study the consequence of ectopic *Mad1* and *Mnt* expression. Since we previously have shown that transient overexpression of constitutively active *Notch1* inhibits RA-induced differentiation in neuroblastoma cells [Grynfeld et al., 2000], we used a similar approach to study the effect of high levels of Mad1. SK-N-BE(2) cells were cotransfected with EGFP- and *Mad1* expressing plasmids at a 1:3 ratio and were induced to differentiate by addition of 10 μM RA. Transfected cells were identified by expression of green fluorescent protein and the morphology of transfected cells was analyzed by fluorescence microscopy. The experiments suggested that the elevated *Mad1* levels did not affect the morphology of unstimulated SK-N-BE(2) cells (Fig. 3C,D). Neither did they appear to affect RA-induced differentiation (Fig. 3E–G). The identical experiments were performed with SK-N-BE(2) cells transfected with a *Mnt*-expressing plasmid. Similar to the results for *Mad1*, overexpression of *Mnt* did not affect morphology of either control or RA-differentiated cells (data not shown). We also performed BrdU-incorporation analyses on *Mad1*-transfected SK-N-BE(2) cells that indicated that transient ectopic *Mad1* expression does not affect the proliferation rate in these cells in our experimental set-up (data not shown).

DISCUSSION

We have assessed expression and DNA-binding activity of members of Myc/Max/Mad/Mnt

network in differentiating neuroblastoma cells. The notion of *Mad* genes being involved in differentiation processes has been supported by findings showing that *Mad* transcripts generally are expressed in differentiating tissues while often being absent from proliferating cells during mouse development [Quéva et al., 1998]. In differentiating SH-SY5Y cells, *Mad4* expression was slightly decreased during differentiation (Fig. 1B) and *Mad1* expression increased after 2 h of TPA-treatment, but was not detected at later time-points. Furthermore, *Mad3* seemed to be regulated differently than both of the other *mad* genes investigated. The expression

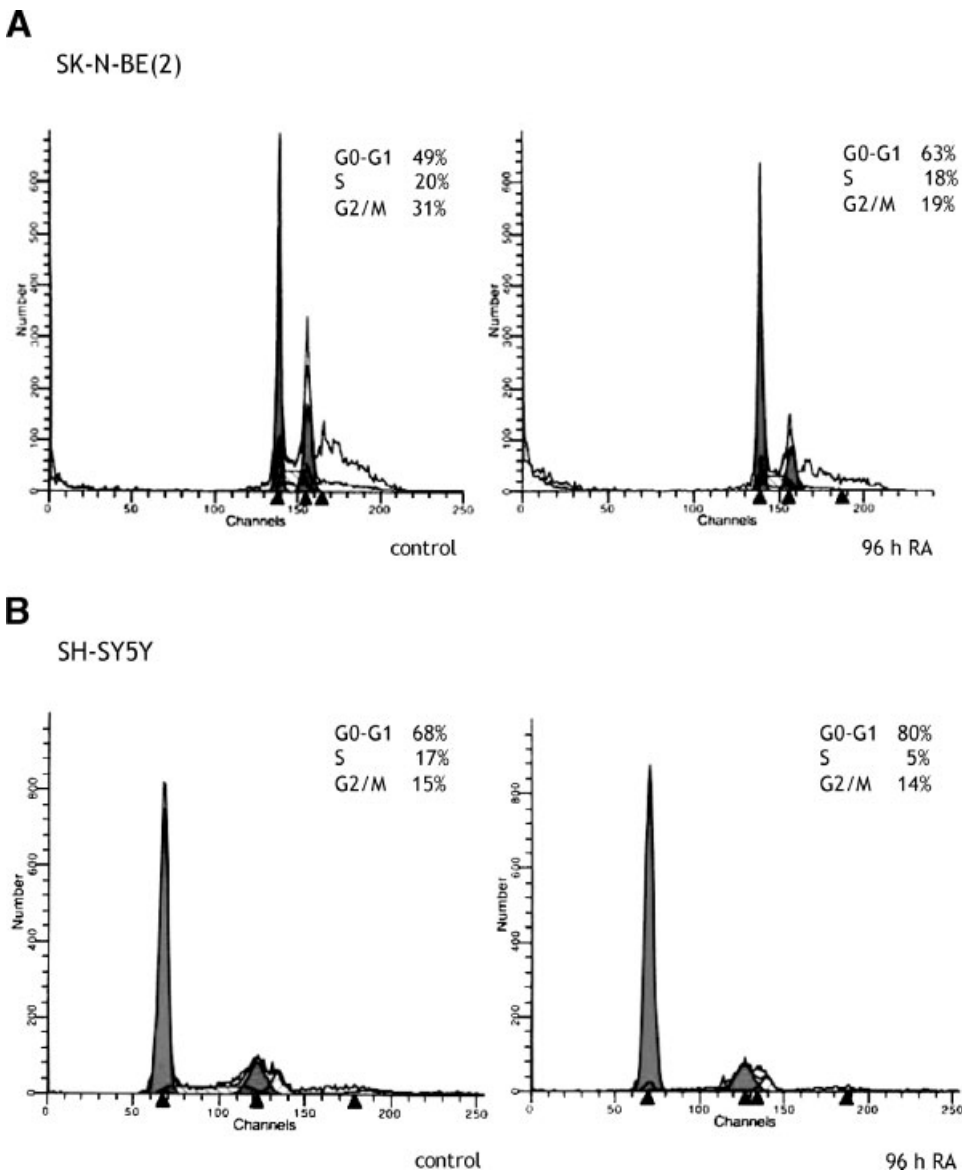

Fig. 3. Cell cycle distribution of undifferentiated and differentiated SK-N-BE(2) and SH-SY5Y cells and morphological analysis of *Mad1*-overexpressing cells. **A**: FACS analyses of propidium iodine-stained untreated (**left panel**) and treated with 10 μM RA for 96 h (**right panel**) SK-N-BE(2) cells. **B**: FACS analyses of propidium iodine-stained untreated (**left panel**) and treated with 16 nM TPA for 96 h (**right panel**) SH-SY5Y cells. The experiment was performed twice and resulted in similar data. To study the effect of high Mad1 levels, SK-N-BE(2) cells were cotransfected with pEGFP-C1 and pEQ176P2 or pEQ176P2-Mad1. After 96 h the transfected cells were identified by presence of green fluorescent protein and the morphology was analyzed by fluorescence microscopy. **C**: Uninduced control and (**D**) uninduced *Mad1*-overexpressing cells. **E**: RA-induced control and (**F**) RA-induced *Mad1*-overexpressing cells. **G**: Graph showing the percentage of morphologically differentiated transfected cells. Approximately 200 cells were counted per experiment and each experiment was performed 3-times. Error bars indicate standard deviations.

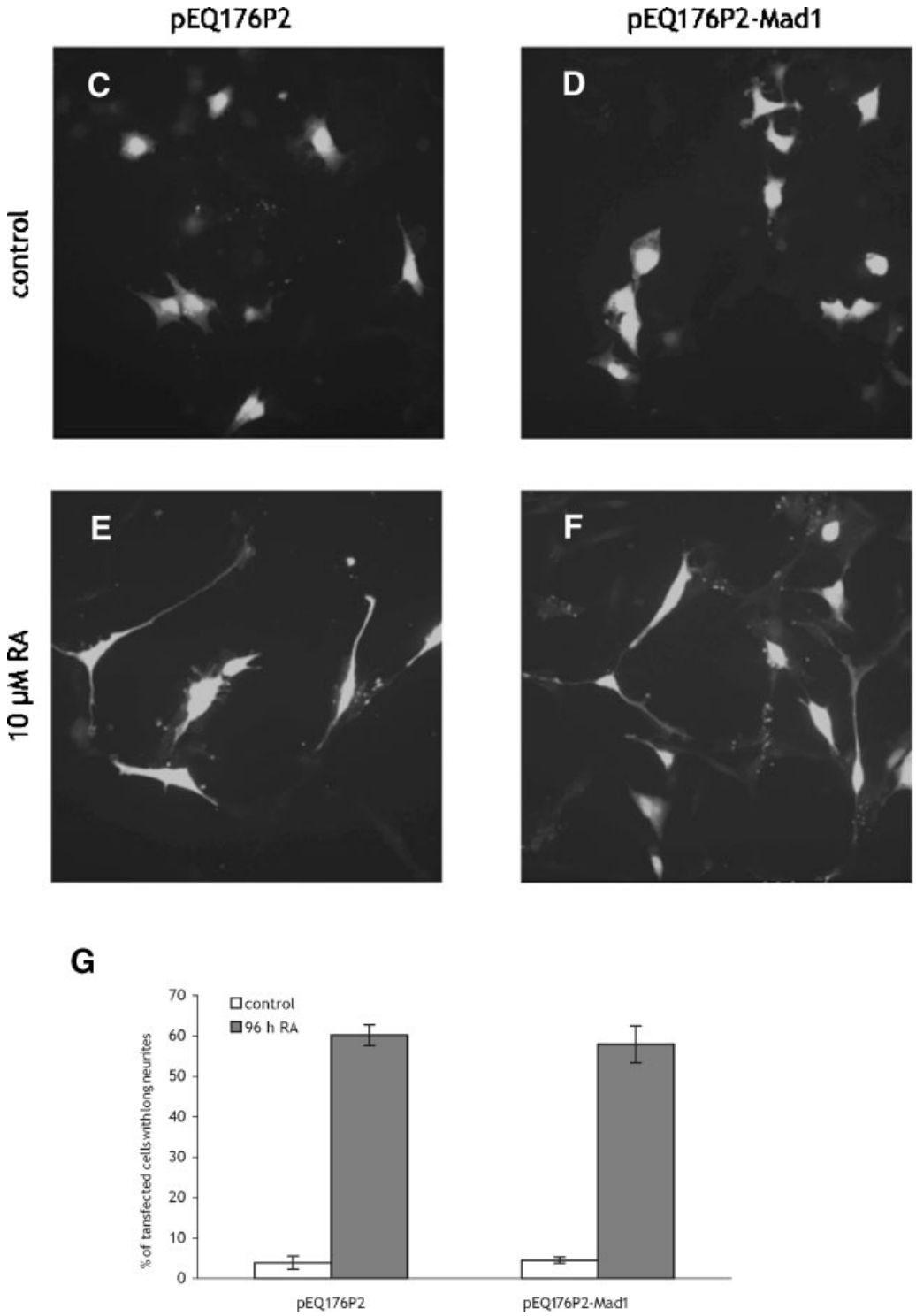

Fig. 3. (*Continued*)

pattern of *MYCN* in these cells, which lack *MYCN* amplification, has been shown to be complex with an early transient increase followed by a sharp decline. However, after 4 days of induction, the *MYCN* expression is almost back to the level observed in uninduced cells [Hammerling et al., 1987]. We found that c-Myc protein is expressed in a similar fashion in SH-SY5Y cells, transiently increasing at 2 h after TPA induction, and declining afterwards

which is consistent with previous observations on *c-Myc* RNA in these cells [Hammerling et al., 1987]. In differentiating SK-N-BE(2) cells, we found that mad4 increased (Fig. 1A), which is in line with the pattern seen in neuronally differentiating murine P19 cells [Quéva et al., 1998]. Furthermore we observed a biphasic expression pattern of *Mad1*, and an expression pattern of *Mad3* that was distinct from that of the other *Mad* genes. In line with previous studies on neuroblastoma cell lines carrying an amplified *MYCN* gene [Amatruda et al., 1985; Thiele et al., 1985, 1988; Thiele and Israel, 1988], we show that the *MYCN* expression was downregulated during RA-induced differentiation. Importantly, we also show that this decrease in expression level is paralleled by a decrease in DNA-binding activity.

In order to further illustrate the RA- and TPA-induced changes in the two cell lines, we performed analyses of cell cycle distribution. FACS analysis of propidium iodine-stained SH-SY5Y cells showed significant decrease in S-phase cells. This finding was consistent with morphological observations of cells undergoing differentiation, which indicated that cells nearly stopped dividing after 96 h of TPA treatment. It was also in line with our data showing that c-Myc protein levels were sharply downregulated after 24 h of differentiation (Fig. 2A). In the SK-N-BE(2) cell line propidium iodine staining revealed that the percentage of cells in the G0-G1 phase increased with approximately 30%, corroborating previous analyses that showed a 20% increase after 48 h of RA-treatment [Di Martino et al., 1990]. The percentage of cells in S-phase was however constant, which is consistent with the fact that the cells continued to divide even after 96 h of treatment. Nevertheless, the cells undergo G1-S transition slower when receiving a differentiation stimuli than when grown under normal conditions which probably is related to the decreasing *MYCN* expression and DNA-binding upon differentiation (as shown in Figs. 1A and 2B). Hence, a decrease in MYCN activity might be important to allow neuronal differentiation to proceed in neuroblastoma cells. This reduction in MYCN DNA-binding could be attained in two ways, either by downregulation of *MYCN* expression and/or by upregulation of Mad-like proteins. In cells carrying *MYCN* amplification such as SK-N-BE(2), and thereby expressing profuse levels of MYCN, a reduction in *MYCN* expression probably is a prerequisite for changes in the balance in the Myc/Mad/Max/Mnt network. As judged from the expression profiles of *Mad*/*Mnt* described in this study, the decrease in MYCN DNA binding activity in SK-N-BE(2) seems primarily to be a consequence of decreased MYCN levels, and not of increased Mad/Mnt levels. This is in contrast to the situation in non-*MYCN* amplified cell lines, such as SH-SY5Y. Both *c-Myc* and *MYCN* expression is low in these cells [Hammerling et al., 1987] and the DNA-binding activities are below the threshold of detection in gel shift experiments (Fig. 2B). It is therefore reasonable to assume that minor changes in the expression of Mad-like proteins will have more pronounced effects on the activity of the Myc proteins in SH-SY5Y cells than in SK-N-BE(2) cells.

Concerning the role of Mad1 in differentiation processes, it has been demonstrated to not only promote differentiation in MEL cells [Cultraro et al., 1997], but also to inhibit differentiation of adipocytes [Pulverer et al., 2000]. In adipocytes, it has been suggested that exogenous expression of *Mad1* inhibits the proliferative burst that is necessary for the differentiation process to proceed [Pulverer et al., 2000]. In addition, cell cycle exit of granulocyte precursors from mice with targeted deletion of *Mad1* is inhibited leading to delayed terminal differentiation [Foley et al., 1998]. The different outcomes might reflect differences in the differentiation processes in the various cell types or stages of differentiation. Studies on ectopic expression of *Mad1* in fibroblasts, adipocytes, and different tumor cells have demonstrated that Mad1 inhibits proliferation [Roy and Reisman, 1995; Roussel et al., 1996; Sommer et al., 1997; Bejarano et al., 2000; Gagandeep et al., 2000; Gehring et al., 2000; Pulverer et al., 2000; Cerni et al., 2002]. Furthermore, it has been shown that *Mad1* transgenic mice have a lack of cellularity and are smaller than normal mice [Quéva et al., 1999]. We could not detect any effects of ectopic *Mad1* expression on induced differentiation in the SK-N-BE(2) cell line, indicating that Mad1 does not play a central role in the differentiation process in these cells.

In contrast to the *Mad* genes, *Mnt* is often coexpressed with c-myc in proliferating cells [Hurlin et al., 1997] and might, therefore, have a function that is distinct from the *Mad* genes. In SH-SY5Y cells, *Mnt* expression was transiently increased upon TPA-treatment (Fig. 1E).

Peaking at 2 h, this expression pattern is similar to the *Mnt* expression pattern in TPA-stimulated U937 cells [Meroni et al., 1997]. The transient increase and decrease in *Mnt* expression observed in SH-SY5Y cells (Fig. 1D), correlated to the Mnt protein expression (Fig. 2A), which, however, was not reflected by the DNA-binding activity of the Mnt/Max complex neither to the consensus nor to the non-consensus E-Box (Fig. 2B and data not shown). In the SK-N-BE(2) cell line, *Mnt* mRNA and Mnt protein were expressed at a very steady levels and the DNA-binding activity of Mnt/Max also remained unchanged during differentiation. In summary, we could not detect any significant alteration of the Mnt/Max E-box-binding activity during differentiation in neuroblastoma cells. Moreover, overexpression of *Mnt* did not have any obvious effect on morphological differentiation of SK-N-BE(2) cells (data not shown).

We have observed distinct *Mad*/*Mnt* gene expression patterns during differentiation of two neuroblastoma cell lines. Furthermore, we have demonstrated E-box-binding activity of both the Mnt/Max and MYCN/Max complexes in undifferentiated neuroblastoma cells. Since these complexes both bind the E-box CACGTG, Mnt might compete with Myc in forming complexes with Max. It should be noted that Mad/Max heterodimers bind to the same target sequence but were not detectable in our gel shift experiments, probably due to the low *Mad* expression levels (Fig. 1A,B). Another possibility is that the Mad proteins are distributed into many distinct subcomplexes that individually may not be detectable by EMSA as suggested by Sommer et al. [1998]. Conditions used for cell lysis or binding reactions might also be too stringent for Mad/Max to bind DNA sufficiently well. The same reasoning applies to the Myc DNA binding. Mad/Max and Myc/Max complexes nearly always display weaker DNA binding then the Mnt/Max complexes, even when *c-Myc* is expressed at high levels compared to *Mnt* (for instance, in HL60 cells [Xu et al., 2001]). Other groups have experienced similar difficulties obtaining Myc/Max and Mad/Max signals in gelshift experiments with endogenous proteins [Larsson et al., 1997]. As judged from the gel shift data presented in this study one can speculate that the high *MYCN* expression levels frequently found in high stage neuroblastoma might be required to overcome the inhibiting effect of the Mnt/Max complex on target genes. In neuroblastoma cells lacking *MYCN* amplification other mechanisms should be considered. Hence, by analyzing the function of the Mnt/Max complex, and other MYCN/Max/Mad/Mnt network components in neuroblastoma cells, we might gain a deeper understanding of the role of *MYCN* amplification in the genesis of this tumor.

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