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# Cyclin D1-dependent regulation of B-myb activity in early stages of neuroblastoma differentiation

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## Abstract

**Levels of the transcription factor B-myb must be down-regulated to allow terminal differentiation of neuroectodermal cells and yet its constitutive expression induces early markers of neural differentiation. Thus, we investigated potential mechanisms of enhanced B-myb activity in early stages of neural differentiation. We report here that B-myb expression does not decrease, cyclin A and Sp1 levels remain constant while p21 levels increase continuously upon retinoic acid-induced differentiation of the LAN-5 neuroblastoma cell line. In contrast, cyclin D1 expression is down-regulated at the onset of the differentiative process by protein destabilization. Luciferase assays of promoter activity indicate that B-myb-dependent transactivation is enhanced in LAN-5 cells treated with retinoic acid (RA) for 24 h. The enhancement is independent from cyclin A but is suppressed by a degradation-resistant mutant form of cyclin D1. The importance of cyclin D1 in controlling B-myb activity is further suggested by co-immunoprecipitation experiments, showing that the amount of cyclin D1 co-immunoprecipitated with B-myb decreased after RA treatment. Thus, B-myb may play an active role in the early stages of differentiation when its transactivation activity is enhanced as a consequence of cyclin D1 down-modulation.**
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**Keywords:** B-myb; cyclin A; cyclin D1; neuroblastoma; differentiation

**Abbreviations:** DTT, dithiothreitol; GSK-3$\beta$, glycogen synthase kinase 3 beta; NB, neuroblastoma; RT–PCR, reverse transcriptase- polymerase chain reaction; SDS–PAGE, polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate.

## Introduction

Neuroblastoma is a tumor of the sympathetic nervous system which retains the ability to differentiate *in vitro* along pathways reminiscent of its embryonic origin.[1] Three differentiation lineages, neuronal, schwann-like and melanocytic have been described.[2] *In vivo* spontaneous tumor regression by differentiation or apoptosis is not uncommon.[3] 1p deletion and MYCN amplification are the most characterized genetic alterations in neuroblastoma and are markers of poor prognosis.[4] Among other prognostic factors, B-myb over-expression also correlates with poor outcome.[5]

B-myb is a ubiquitously expressed transcription factor; it belongs to the Myb family which includes A-*myb* and c-*myb*, two genes required for spermatogenesis and hematopoiesis respectively.[6] Myb proteins possess three functional domains: a highly conserved and almost identical NH2-terminal DNA-binding domain recognizing the consensus sequence PyAACG/TG, a central acidic trascriptional activation domain only partially conserved among the family members, and a COOH-terminal negative regulatory domain.[7] B-myb activity is required in proliferating cells in cooperation with cyclins to allow the G1/S transition.[8] B-myb expression is down-modulated toward the end of retinoic acid-induced differentiation of neuroblastoma cells, and its ectopic expression prevents the completion of the differentiative process.[9] In post-mitotic neural cells, B-myb appears to enhance programmed cell death, probably up-regulating yet unknown pro-apoptotic genes.[10]

Cell cycle control and proliferation are tightly regulated by expression of cyclin-dependent kinases.[11] The onset of differentiation is marked by the inhibition of some cyclins which induces cell cycle withdrawal before terminal differentiation.[12] The cyclin A/cdk2 complex phosphorylates B-myb promoting an increase of its transactivation ability.[13] The same kinase is active during the transition from G1- to S-phase before its activity is shut down by proteolytic degradation of cyclin A.[14] In addition to enhancing B-myb activity, cyclin A/cdk2 phosphorylation also promotes B-myb ubiquitin-dependent and proteasome-mediated degradation, suggesting the existence of a regulatory loop by which B-myb is first activated and then is degraded.[15] Cyclin D1/cdk4 or /cdk6 complexes and unbound cyclin D1 levels are equally essential for cell cycle progression into S-phase. 26S proteasome-mediated degradation primed by GSK-3$\beta$-mediated phosphorylation of tyrosine 286 is a key process in the regulation of cyclin D1.[16] In homogeneous neuronal-type (N-type) cell lines the neuronal differentiation program is completed after an early proliferative phase.[17] Up-regulation of cyclin D1 and p21 expression after RA treatment has been described in these cell lines. Recently, it has been demonstrated that cyclin D1 can bind B-myb independently from cdk4 and cdk6 causing inhibition of B-myb-mediated trancriptional transactivation.[18] Of interest,

retinoic acid causes cyclin D1 down-regulation in bronchial epithelial cells[19] and in the human embryonal carcinoma cell line NT2/D1[20] by enhancing proteasome mediated degradation. In different cell types, distinct receptors of the RAR family mediate the activity of retinoic acid on cyclin D1.[19,20] For example, RAR$\beta$ mediates the effect of RA on cyclin D1 stability in bronchial epithelial cells[19] while the same effect is mediated by RAR$\gamma$ in the embryonic carcinoma cell line NT2/D1.[20]

In this report we studied the pattern of B-myb expression and the cyclin-mediated regulation of B-myb activity during early differentiation of neuroblastoma cells. Our data suggest the existence of a complex and tightly regulated network whereby B-myb activity is controlled through functional interactions with cyclin A and cyclin D1.

## Results

### Expression of B-myb, cyclin A, Sp1, p21 and cyclin D1 during early phases of neuroblastoma differentiation

Human neuroblastoma cell line LAN-5 was induced to differentiate by treatment with 5 $\mu$M *all trans*-retinoic acid (RA). Cells were harvested at different times and cellular extracts were prepared for Western blot analysis using specific antibodies against B-myb, cyclin A, Sp1 and p21. B-myb levels fluctuated during the first three days of differentiation to decline at later stages (10 days) (Figure 1a). Of interest, a transient increase in B-myb expression was detected between 4 and 8 h of RA treatment but B-myb levels were essentially identical to those of proliferating cells during the early stages of RA-induced differentiation. Levels of cyclin A, which enhances B-myb transactivation,[13] were stable during the first 24 h but declined thereafter. At 10 days cyclin A became undetectable. A similar pattern was observed for the transcription factor Sp1 which has been shown to cooperate with B-myb in transactivation.[21] In addition an increase in p21 expression was detected during the entire course of the differentiation process. To assess proliferation in LAN-5 cells during the initial stages of differentiation we carried out a flow cytometric analysis. As shown in Table 1, proliferation was stable up to 1 day in RA (compare the per cent S-phase of uninduced cells with that of cells treated with RA for 1 day) but declined after 3 days of treatment. Next, we analyzed the expression of cyclin D1 during the early phases of LAN-5 differentiation (Figure 1) since cyclin D1 reportedly binds B-myb and inhibits its transactivation potential.[18] Cyclin D1 levels decreased to 33% of the initial value after 4 h of RA treatment, but returned to approximately 59% of the starting level after 24 h (Figure 1B).

**Table 1** Flow cytometric[a] analysis of neuroblastoma cell line LAN-5

| | G0/G1 % | S % | G2 % |
|---|---|---|---|
| LAN-5 uninduced | 59.7 | 22.0 | 18.3 |
| LAN-5 +RA (1 day) | 54.9 | 20.9 | 24.2 |
| LAN-5 +RA (3 days) | 64.6 | 7.3 | 28.1 |

[a]Each measurement refers to a sample of 20 000 cells

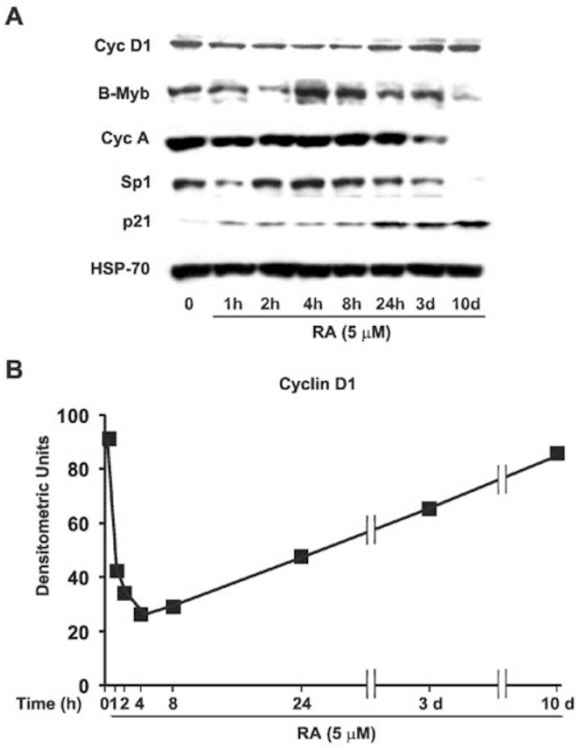


**Figure 1** (**A**) Western blot analysis of Cyclin D1 (Cyc D1), B-myb, Cyclin A (Cyc A), Sp1 and p21 during Retinoic Acid (RA)-induced (5 $\mu$M) differentiation of LAN-5 cells; h=hours, d=days. (**B**) densitometric analysis of Cyc D1 during differentiation of LAN-5 cells

To assess if the half lives of B-myb and cyclin D1 were markedly affected by RA treatment, inhibition of protein synthesis by cycloheximide (20 $\mu$g/ml) was carried out (from 0–4 h) in LAN-5 cells 2, 8 and 24 h after RA treatment (Figure 2A). Cellular extracts at different time points were prepared and B-myb and cyclin D1 were detected by Western blotting. Up to 4 h, the stability of B-myb protein did not appear to be shortened by RA treatment. In agreement with data in other cell types,[19,20] the stability of cyclin D1 decreased from $\sim$65 min in untreated cells to $\sim$40 min 24 h after RA treatment, as calculated by densitometric reading and using an algorithm to estimate proteins' half life[22] (Figure 2B). Thus, the decrease of cyclin D1 expression in the early stages of neuroblastoma differentiation is, at least in part, caused by protein destabilization.

### RA induction increases B-myb transactivation

To evaluate the transactivation activity of B-myb, luciferase assays were carried out in LAN-5 cells co-transfected with the B-myb-responsive reporter vector pG1-MIM[21] and the wild-type (wt) B-myb expression vector (CMV-B-myb) in the presence or in the absence of the expression vector encoding cyclin A (CMV-cycA). After transfection, cells were left untreated or treated with RA (5 $\mu$M) for 24 h before the assay. Similar experiments were carried out using a mutant form of B-myb in which the carboxy-terminus, where the interaction

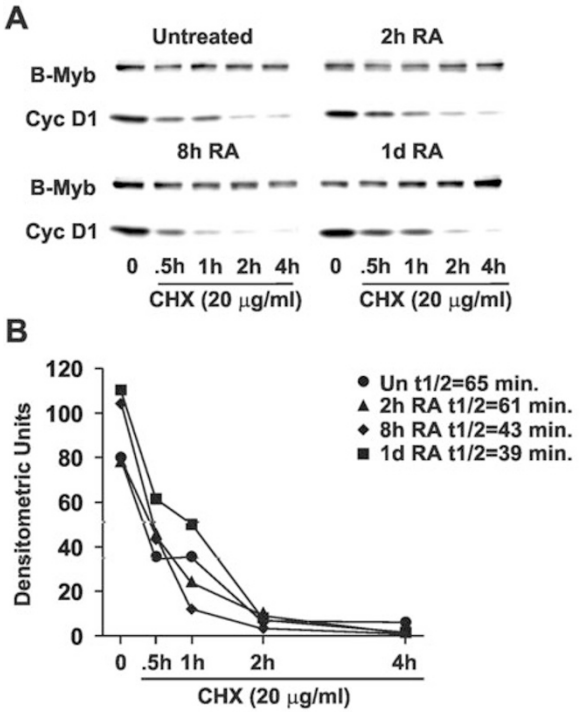


**Figure 2** (**A**) Western blot analysis of B-myb, and Cyc D1 after treatment with protein synthesis inhibitor cycloheximide (20 $\mu$g/ml) for the indicated time during Retinoic Acid (RA)-induced (5 $\mu$M) differentiation of LAN-5 cells. (**B**) Graphic representation of the densitometric analysis of the blots in (**A**). An algorithm discussed in the text was used to calculate Cyc D1 half life at each considered time point

with cyclin A occurs,[13] was removed by digesting the full-length cDNA with the BspE1 restriction enzyme. As shown in Figure 3A, the combination of wt B-myb and cyclin A was more efficient than wt B-myb alone in transactivation of the B-myb responsive promoter. On the contrary, cyclin A did not cooperate with mut B-myb which was a stronger transactivator than wt B-myb. Compared to untreated cells, B-myb-dependent transactivation always increased in cells treated with RA. The increase in transactivation obtained with the combination of wt B-myb, cyclin A and RA was particularly striking (65-fold of activation compared to 13 in absence of RA). The increase in transactivation after RA treatment was detectable independently from the presence of the carboxy-terminal region of B-myb (compare mut B-myb with mut B-myb plus RA, Figure 3A) and the mutant B-myb was not able to promote a further increase in transactivation upon coexpression with cyclin A.

## Cyclin D1 abolishes the RA- mediated increase in B-myb transactivation

The increase in B-myb transactivation in the presence of RA could be due to the release of cyclin D1-dependent inhibition,[18] since a decrease in cyclin D1 expression occurs at the onset of neuronal differentiation (Figure 1B). To test this hypothesis, we generated a T286A cyclin D1 mutant in which threonine 286 was substituted with alanine. Upon GSK-3$\beta$-dependent phosphorylation of threonine 286 cyclin D1 is primed for degradation by the proteasome while the threonine to alanine mutation renders cyclin D1 more stable.[16] We carried out luciferase assays by co-transfecting LAN-5 cells with the reporter vector pG1-MIM and wt B-myb with or without mut T286A cyclin D1 in presence or absence of RA. Expression of T286A cyclin D1 abolished the transactivation by B-myb even in the presence of RA (Figure 3B). The effect of T286A cyclin D1 was dose-dependent since increasing amounts of this plasmid brought about a progressive decrease in B-myb transactivation (Figure 3C). Thus, the increase in B-myb transactivation induced by RA can be efficiently counteracted by cyclin D1. To assess why the transactivation activity of B-myb correlates with a decrease in the B-myb/Cyclin D1 interaction, we carried out co-immunoprecipitation experiments in untreated LAN-5 or after 1 day of RA-induced differentiation (Figure 4). While B-myb-immunoprecipitated levels were comparable in both treated and untreated cells, the levels of cyclin D1 co-immunoprecipitated using an anti-B-myb antibody were higher in untreated cells, demonstrating that the induction of RA-induced differentiation leads to a decrease in the B-myb/cyclin D1 direct interaction.

## Constitutive B-myb expression is associated with the expression of early but not late neuroectodermal differentiation markers

We have previously shown that constitutive expression of B-myb in neuroblastoma cells is associated with the appearance of typical features of neuroectodermal differentiation along different lineages.[23] Thus, we tested the expression of early (BM 88)[24] and late (GAP 43)[25] neuronal differentiation markers in a LAN-5 cell line stably expressing B-myb (LAN-5-B-myb).[9] Total RNA was extracted from LAN-5 – B-myb and parental cells in basal and differentiating conditions (5 $\mu$M RA) and a semi-quantitative RT – PCR analysis was carried out to detect BM 88 and GAP 43 transcripts. The early differentiation marker BM 88 was expressed in LAN-5-B-myb at intermediate levels of those in parental cells maintained in basal growth conditions *versus* the same cells after 1 day of RA treatment (Figure 5). On the contrary, the late differentiation marker GAP 43 was inhibited by the ectopic expression of B-myb. Thus, constitutive expression of B-myb is associated with increased levels of transcripts for an early neuronal differentiation marker while those of a late marker are diminished.

## Cyclin D1 and B-Myb have opposite effects on the expression of the early neural differentiation marker BM 88

To confirm the functional link between cyclin D1 level and the expression of B-myb-regulated early markers during RA-induced differentiation, an expression vector coding for the T286A cyclin D1 mutant was stably transfected in LAN-5 by standard calcium-phosphate precipitation and the levels of BM 88 and GAP 43 were measured by semi-quantitative RT – PCR in a mix population expressing high levels of T286A cyclin D1. GAP 43 expression was essentially unchanged in parental and cyclin D1-transfected LAN-5 cells in basal and differentiating conditions. Basal BM 88 levels were comparable in parental and T286A cyclin D1-expressing LAN-5

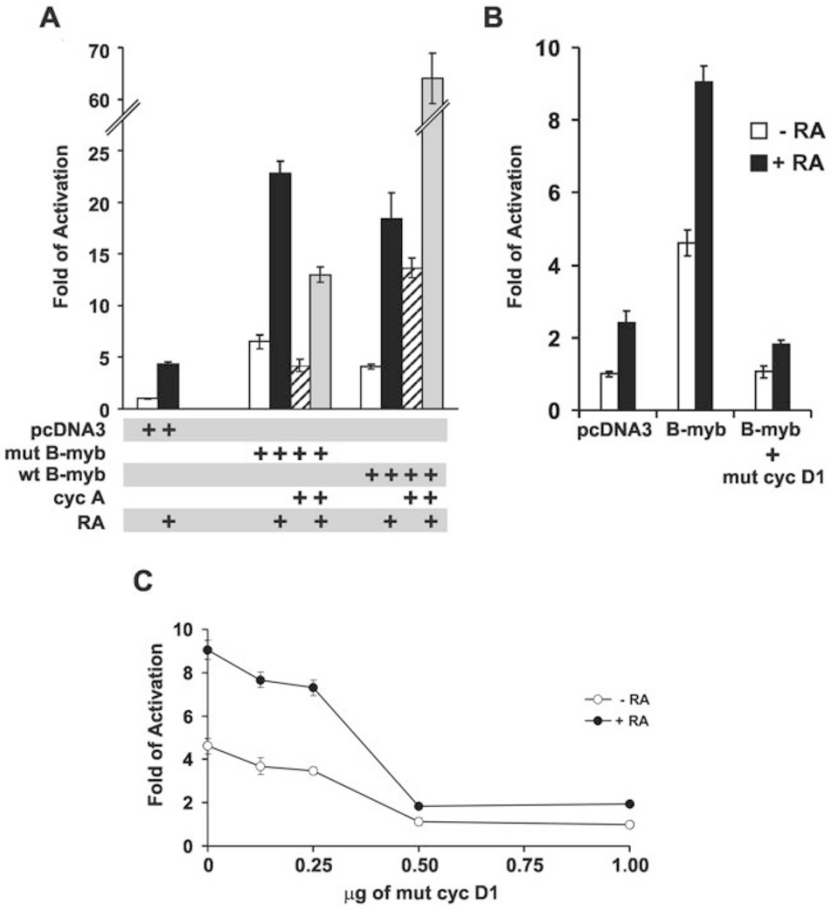


**Figure 3** (**A**) Luciferase assays of wt and C-terminal deleted B-myb (mut B-myb) in the presence or absence of cyc A in basal conditions and after RA-induced differentiation. (**B**) Luciferase assays of wt B-myb in the presence or absence of T286A cyc D1 (mut cyc D1) in basal conditions and after RA-induced differentiation. (**C**) Luciferase assays of wt B-myb in the presence of increasing amounts of mut cyc D1. RA was always added to cultures after transfection for 20 h before the assays. Experiments were carried out in triplicate

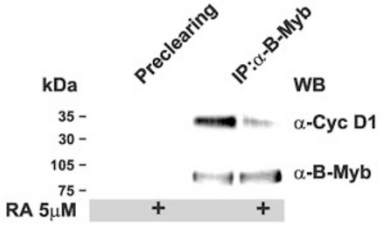


**Figure 4** Co-immunoprecipitation of B-myb and cyclin D1 from untreated and RA-treated (5 $\mu$M for 1 day) LAN-5 cells. 500 $\mu$g of cellular extracts for each experimental point were used to immunoprecipitate B-myb with a specific antibody. Immunoprecipitated material was separated on SDS–PAGE and Western blot analysis was carried out using anti cyclin D1 and B-myb specific antibodies. Preclearing was obtained from protein A-sepharose beads exposed to cellular extracts in absence of specific antibodies

(Figure 5); however, 1 day of RA treatment induced an increase in the levels of BM 88 in parental but not in T286A cyclin D1-transfected LAN-5 cells. Together with the results described above, this further suggests that the activity of B-myb during the early stages of differentiation correlates with the decrease of cyclin D1 levels. Thus, we carried out antisense oligonucleotides experiments to reduce the expression of endogenous cyclin D1 and monitored the effects on the expression of the BM 88 differentiation marker. Upon incubation of LAN-5 cells with sense or antisense cyclin D1 oligonucleotides, there was a $\sim$30% decrease of cyclin D1 levels in the antisense treated cells (Figure 6A and B). This decrease in cyclin D1 levels correlated with a $\sim$46% increase of BM 88 expression.

## Discussion

Ectopic expression of B-myb, a nuclear transcription factor whose activity is required for transition from the G1- to the S-phase of the cell cycle,[8] inhibits terminal differentiation in neuroblastoma[9] and other cell types.[22] In this study, we analyzed the expression of endogenous B-myb in the early phases of neuroblastoma differentiation. In neuroblastoma LAN-5 cells which differentiate along a neuronal pathway,[9] B-myb expression is up-regulated early after RA treatment and remains at the level of uninduced cells for the first three days. The differentiation process of N-type neuroblastoma cells is associated with a proliferative phase which precedes overt

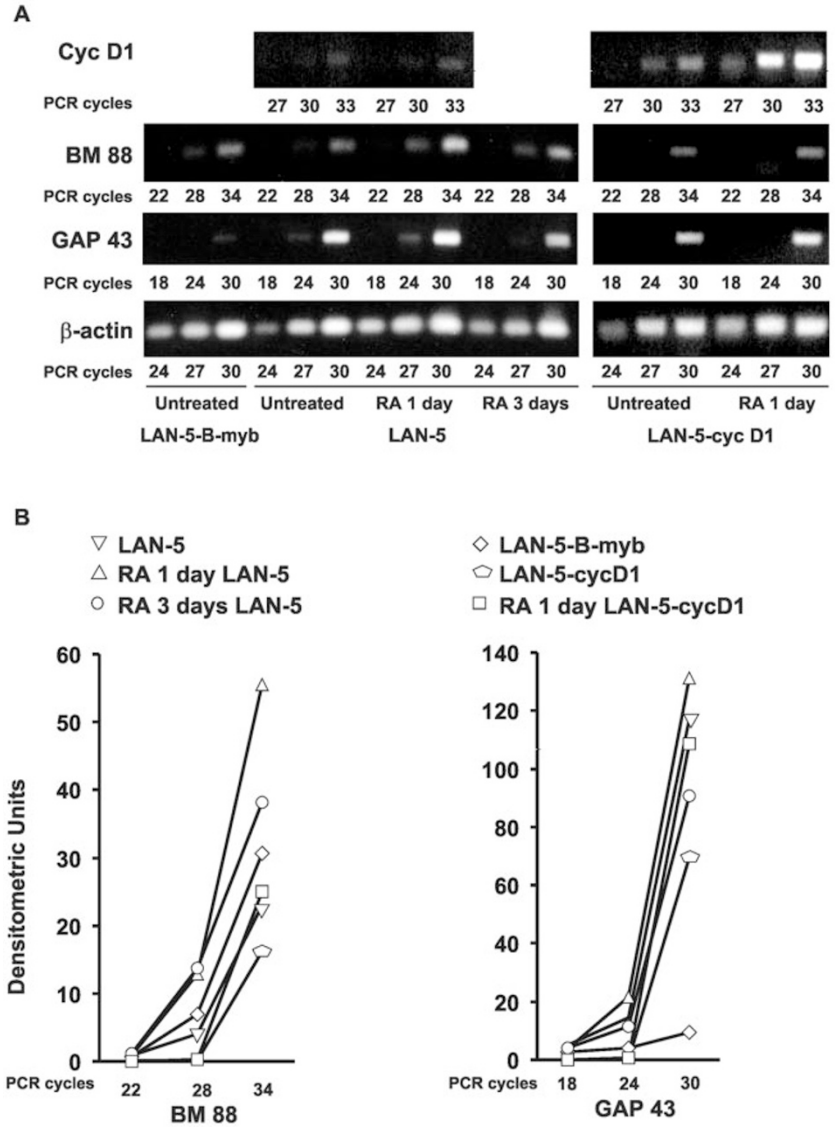


**Figure 5** (**A**) Reverse transcriptase (RT)–PCR analysis of cyclin D1, BM 88 and GAP 43 expression in B-myb-overexpressing LAN-5 cells (LAN-5-B-myb), parental and T286A cyclin D1-overexpressing LAN-5 (LAN-5-cyc D1) grown in basal conditions and after RA-induced differentiation. PCR cycles are indicated at the bottom of the ethidium bromide-stained gels. $\beta$-actin was used as control to normalize the amount of input cDNA. (**B**) Densitometric analysis of BM 88 and GAP 43 expression levels shown in (**A**) after normalization for their $\beta$-actin content. Data are representative of four different experiments

differentiation.[17] At late stages of differentiation, there is a parallel increase of cyclin D1 and p21 expression that is consistent with the model of N-type differentiation described by Wainwright *et al*.,[17] except that the increase in cyclin D1 levels is detected at late but not early stages of differentiation (3 to 10 days compared to 18 to 24 h). In fact, our analysis of cyclin D1 expression at very early times of LAN-5 differentiation (1–24 h) revealed a significant decrease which was not detected in the SH-N model.[17] These discrepancies could be due to differences in the differentiation kinetics of SH-N, an homogeneous N-type subline of the SK-N-SH neuroblastoma cell line, and LAN-5 cells which, although prevalently N-type, are a more heterogeneous.[26]

Our data indicate that B-myb is expressed at fairly high levels in a temporal window overlapping the proliferative period at the beginning of neuronal differentiation. Expression of cyclin A and Sp1, which by different mechanisms can positively regulate the transactivation activity of B-myb,[13,21] remained also unchanged during the first three days of RA induction. By contrast, expression of cyclin D1 which has been recently demonstrated to physically interact with B-Myb and to inhibit its transactivation activity,[18] was rapidly down-regulated after RA induction. In neuroblastoma (our data ) as well as in other cell types,[19,20] cyclin D1 down-regulation depends, at least in part, on a decrease of its stability. Of interest, the decrease of cyclin D1 expression levels starts immediately after differentiation induction (1 h) when a decrease in cyclin D1 half life is not yet detectable. Since a decrease in mRNA levels of cyclin D1 was not detected 1 h after RA treatment, a difference in

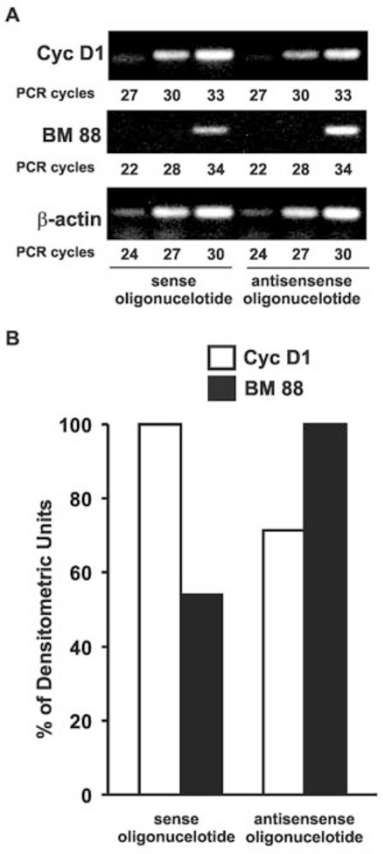


**Figure 6** (**A**) RT–PCR analysis of cyclin D1 and BM 88 in untreated and cyclin D1 oligonucleotide-treated LAN-5 cell. (**B**) Percentage of densitometric units of cyclin D1 and BM 88 expression levels shown in (**A**) after normalization based on $\beta$-actin levels. The higher Cyc D1 and BM 88 densitometric readings were taken as 100

translational efficiency might be the post-trancriptional mechanism explaining the decreased cyclin D1 levels. In this regard, control of translational efficiency has already been described as important during differentiation. For example, the 60S ribosomal subunit could be recruited with a decreased affinity at the onset of differentiation,[27] or specific RNA binding proteins could inhibit translation efficiency, contributing to a differentiation-specific translational attenuation.[28] The pattern of gene expression we observed (elevated levels of B-myb and cyclin A and down-regulation of cyclin D1) suggests that the activity of B-myb can be up-regulated in the early phases of neuronal differentiation. In fact, functional assays carried out in the presence of RA demonstrated that B-myb transactivation was strongly increased as compared to parallel assays done in basal growth conditions. The RA-induced increase was particularly striking upon co-transfection of wt B-myb and cyclin A. Nevertheless, transactivation driven by a mutant B-myb with a deletion of the C-terminal domain phosphorylated by cyclin A was still enhanced by the addition of RA suggesting that the RA effect is independent from cyclin A. The enhancing effect of RA on B-myb transactivation was abolished in a dose-dependent manner by expression of a degradation-resistant cyclin D1 mutant (Figure 3C). This suggests that the decrease in the expression of cyclin D1 at the onset of neuronal differentiation (Figure 1) alleviates its inhibitory effect on B-myb transactivation which depends on its physical interaction with B-myb.[18]

Consistent with this hypothesis, there was a decrease in the amount of cyclin D1 in complex with B-myb during early stages of LAN-5 differentiation. Constitutive expression of B-myb allowed the expression of BM 88, an early neuronal differentiation marker,[24] but not of GAP 43 which accumulates in mature neuronal cells.[25] Likewise, down-regulation of cyclin D1 by antisense oligodeoxynucleotides was accompanied by an increase of BM 88 transcripts. Of interest, constitutive expression of B-myb mimics the effect of RA by increasing the levels of BM 88 in comparison to those found in parental cells maintained in basal growth conditions (Figure 5). These latter data are in agreement with our previous observation of a simultaneous accumulation of intracellular structures typical of neuronal, schwann-like and melanocytic lineages in neuroblastoma cells constitutively expressing B-myb.[29] Based on this effect, it is tempting to speculate that during RA-induced differentiation there is a switch in B-myb targets from those involved in proliferation control to those associated with the early differentiation phase.

In conclusion, prior to B-myb down-modulation, which is necessary to complete the differentiation program of neuroblastoma cells, there is a temporal window during which B-myb plays an active role as indicated by induction of an early neuronal marker. The down-regulation of cyclin D1 appears to facilitate the effect of B-myb as indicated by the increase of BM 88 transcripts in cyclin D1 antisense oligodeoxynucleotide-treated cells and the suppression of B-myb-dependent transactivation of a responsive promoter by a degradation-resistant cyclin D1 mutant.

## Materials and Methods

### Cell cultures and transfections

LAN-5 and stably transfected LAN-5-B-myb[9] were cultured in RPMI 1640 medium (Euroclone) supplemented with 10% fetal calf serum (Hyclone), penicillin and streptomycin (Euroclone) (100 $\mu$g/ml each), 2 mM L-glutamine (Euroclone) at 37°C, 5% $CO_2$. Stable transfections were performed using standard calcium-phosphate methods, in 100 mm Petri dishes at ~50% of confluence. Culture medium was changed 16 h after transfection. G418 (Sigma) (400 $\mu$g/ml) was added 24 h later to select transfected cells.

### Flow cytometric analysis

Flow cytometric analysis was carried out using a FACS-STAR-plus cytometer (Beckton-Dickinson), after propidium-iodide staining.

### Protein analysis and co-immunoprecipitation

Cellular pellets were lysed in hypertonic buffer (20 mM HEPES pH 7.2, 400 mM NaCl, 1% NP40) supplemented with protease and phosphatase inhibitors. Protein analyses were performed as follows: (a) lysate was centrifuged at 14 000 $\times g$ at 4°C for 10 min, the

supernatant was collected and protein concentration was determined using a colorimetric assay (BioRad) and (b) 100 $\mu$g of total extract per lane were separated on SDS–PAGE and Western blot analysis using specific antibodies was carried out as previously described.[30]

Immunoprecipitation was carried out in 20 mM HEPES pH 7.9, 150 mM NaCl, 1% Nonidet P-40 (ICN Biomedical Inc.), Pefablock SC (Roche) and Complete Protease Inhibitor Cocktail (Roche) at the manufacturer's indicated concentrations. Five hundred micrograms of cellular extracts were pre-incubated with Protein A-Sepharose beads (Amersham-Pharmacia Biotech.) for 45 min at 4°C and supernatant was incubated with Protein A-Sepharose beads coated with B-myb specific antibody for 2 h at 4°C. Beads were washed seven times with the immunoprecipitation buffer. 2× SDS gel-loading buffer (100 mM Tris HCl pH 6,8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added directly on the Protein A-Sepharose beads, that were boiled for 5 min. Supernatants were separated on SDS–PAGE and Western blot analysis using specific antibodies was carried out as previously described.[30]

Antibodies used were: $\alpha$-B-myb (sc-725), $\alpha$-cyclin A (sc-751), $\alpha$-cyclin D1 (sc-717), $\alpha$-Sp1 (sc-59) from Santa Cruz Biotechnology, Inc., $\alpha$-HSP-70 (SPA-820) Stress Gen Biotechnology Corp., $\alpha$-HSP-90 (H38220), $\alpha$-p21 (C24420) BD Transduction Laboratories.

### Luciferase assays

Luciferase assays were carried out as previously described[31] in LAN-5 co-transfected with the pG1-MIM reporter vector and pcDNA3-B-myb, pcDNA3 cyclin A,[21] pcDNA3-B-myb-Bsp E1, pcDNA3 T286A cyclin D1 as needed.

pcDNA3 B-myb-Bsp EI was obtained by sub-cloning the blunt-ended *Sma*I/*Bsp*EI fragment from pUHD 10-3 B-myb (pTRE B-myb, containing the full length cDNA of B-myb) into the *Eco*RV cut and dephosphorylated pcDNA3 (Invitrogen), using standard cloning procedures. T286A cyclin D1 mutant was obtained by site-directed mutagenesis (Quick-Change Site-Directed Mutagenesis kit, Stratagene), using the wt cyclin D1 as template, according to the manufacturer instructions and using the following pair of mutated primers:
Strand +5′-GGCTTGCACGCCCACCGACG-3′,
Strand −5′-CGTCGGTGGGCGTGCAAGCC-3′

### RNA extraction and RT–PCR

Total RNA was prepared by TRIzol extraction (Gibco–BRL). Carry over DNA contamination was eliminated by treatment of the total RNA with DNA free kit (Ambion) according to the manufacturer extraction. RNA was reverse transcribed with the first strand cDNA synthesis kit for RT–PCR (Roche) using an input of 500 ng for each reaction.

Subsequent PCR were carried out for the indicated number of cycles at the appropriate annealing temperature for each of the following pair of primers:
$\beta$-actin: Up 5′-TCATCACCATTGGCAATGAG-3′
Down 5′- CACTGTGTTGGCGTACAGGT-3′
BM 88: Up 5′-CGATGGGAAAGCCCCCTTGACCAAGC-3′
Down 5′-GGGGGTTGAAGTTCTCACAGGACCAGG-3′
GAP 43: Up 5′-GAAGGATGATGTCCAAGCTGCTGAGGC-3′
Down 5′-CATCGGCTTGTTTAGGCTCCTCCTTGG-3′

### Antisense experiments

Antisense experiments were carried out as previously described[32] with some modifications: a 20-bp oligonucleotide (5′-AGCTGGTGTTCCATGGCTGG-3′) complementary to the translation initiation codon (underlined) and flanking sequences of human cyclin D1 mRNA, was synthesized as the antisense oligonucleotide. An oligonucleotide matching the same region in sense orientation was also synthesized as control (sense oligonucleotide) (5′-CCAGCCATG-GAACACCAGCT-3′). The first and the last three nucleotides were phosphorothioate. Five times $10^5$ LAN-5 cells were plated on 60 mm Petri dishes and after 24 h 10 $\mu$M single-stranded 20 bp oligonucleotides were added to the culture media. Twenty-four hours later cells were harvested, RNA extracted and an RT–PCR performed as described above.

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