Direct Recruitment of N-myc to Target Gene Promoters

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The *N-myc* gene is amplified in 20–25% of human neuroblastomas, and this amplification serves as a poor prognostic factor. However, few genes have been determined to be direct targets of N-myc. Our current studies focused on identifying N-myc target genes, especially those affected in cells such as neuroblastomas that have high levels of N-myc protein. To pursue this goal, we performed differential expression screens with cell-culture systems containing high versus low levels of N-myc. The design of our experiments was such that we should identify genes both upregulated and downregulated by N-myc. Accordingly, we identified 22 genes upregulated by N-myc and one gene downregulated by N-myc. However, only five of these genes responded to increased N-myc levels in more than one system. Further analysis of the regulation of these genes required determining whether they were direct or indirect targets of N-myc. Therefore, we used a formaldehyde crosslinking and immunoprecipitation procedure to determine whether N-myc was bound to the promoters of these putative target genes in living cells. We found that low levels of N-myc were bound to the promoters of the telomerase and prothymosin genes in neuroblastoma cells having low amounts of N-myc but that the amounts of N-myc bound to these promoters greatly increased with overexpression of N-myc. However, the amount of max bound to the promoters was high before and after induction of N-myc. Therefore, our studies suggest that N-myc competes with other max partners for binding to target promoters. Our use of the chromatin immunoprecipitation assay suggests a molecular explanation for the consequences of amplification of the N-myc gene in neuroblastomas. *Mol. Carcinog. 29:76–86, 2000.*

Key words: transcription; chromatin; nuclear oncogene; formaldehyde crosslinking; neuroblastoma

INTRODUCTION

Deregulation of members of the myc family of transcriptional regulators (i.e., c-myc, N-myc, and L-myc) is found in a variety of human cancers [1]. The association of N-myc with human neoplasia is most striking in neuroblastoma, a tumor of neural crest origin, in which the wild-type *N-myc* gene can be found to be amplified over a hundredfold [2]. The resulting N-myc overexpression is associated with a poor clinical outcome [3]. The *N-myc* gene is also altered by amplification in retinoblastoma, astrocytoma, small cell lung carcinoma, glioma, rhabdomyosarcoma, and Wilms' tumor [4–6]. Evidence in support of the hypothesis that deregulation of N-myc is causative in the formation of neuroblastoma comes from transgenic mice studies. When N-myc is expressed under the control of a neural promoter, tumors arise in neural tissues of the transgenic mice [7]. Although c-myc, not N-myc, is normally associated with human lymphomas, N-myc is able to cause tumor development to a similar degree as c-myc when lymphoid-specific promoters are used in other transgenic mice studies [8]. However, the latency before tumor development seen in both systems, the clonal nature of the lymphomas, and the additional chromosomal changes seen in the neural tumors all suggest that N-myc acts in concert with other factors to cause neoplasia.

Although it is clear that N-myc deregulation is associated with cancer, the mechanisms by which N-myc contributes to a neoplastic phenotype are still unclear, and elucidating the function of N-myc has presented a challenge to both basic and clinical researchers. Overexpression of N-myc can decrease the time it takes for a cell to complete a cell cycle and can decrease attachment of cells to the extracellular matrix [9–11]. Although these two properties of N-myc provide insight into how N-myc functions on a cellular level, the molecular mechanisms by which N-myc mediates these effects are not known. The amino terminus of N-myc contains a transactivation domain, whereas the carboxyl terminus contains the helix-loop-helix and leucine zipper domains, which mediate heterodimerization with max, and the basic region, which is required for

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Abreviations: ODC, ornithine decarboxylase; RDA, representational difference analysis; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; SSC, sodium chloride–sodium citrate; SDS, sodium dodecyl sulfate; CAD, carbamoyl phosphate synthase, aspartate transcarbamylase, dihydroorotase.

binding of N-myc/max heterodimers to consensus sites known as E boxes [12]. The expression of a few genes has been shown to correlate with levels of N-myc in certain cells, including ornithine decarboxylase (*ODC*), prothymosin-α, *RCC1*, *ECA39*, insulin-like growth factor-1 receptor, and the catalytic subunit of telomerase, all of which have E boxes in or near the promoter [10,13–16]. The expression of other mRNAs, e.g., neural cell adhesion molecule, multidrug resistance–associated protein, several major histocompatibility class I genes, and several integrins, has been shown to be influenced by increased levels of N-myc. However, it is not known whether any of these genes is directly regulated by N-myc binding to the promoter region [17–20].

To better understand the molecular mechanisms by which N-myc mediates neoplasia, we have pursued a multifaceted approach toward the identification of N-myc target genes. We began by using cDNA filter arrays to determine what genes N-myc can regulate in both neuroblastoma cells and skin cells harboring transfected N-myc; we also used the technique of representational difference analysis (RDA) to clone mRNAs from the skin cell line. Further, we used a formaldehyde crosslinking procedure to demonstrate that several of the genes that respond to changes in levels of N-myc are directly bound by N-myc in living cells. Our studies support a model of N-myc function in neuroblastomas that is characterized by a limited ability of N-myc to compete with other factors for binding to cellular promoters.

MATERIALS AND METHODS

Cell Culture

Tet21 and Tet21N cell cultures were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 4 mM L-glutamine. Tetracycline was added to a final concentration of 1 μg/mL to repress N-*myc* transcription. C11 and C11N cells were maintained in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Both cell types were grown in incubators at 37°C, 5% CO_2, with humidity. C11 and C11N cells are derived from a skin fibroblast cell line from a patient with Li-Fraumeni syndrome [21], and Tet21 and Tet21N cells were derived from the SH-EP cell line, a human neuroblastoma cell line containing single-copy *N-myc* [10].

Clontech Atlas cDNA Expression Arrays

Poly(A)+ mRNA was prepared by using the Qiagen Oligotex mRNA kit (Qiagen, Valencia, CA); the protocols used to hybridize to Clontech Atlas cDNA Expression Arrays are detailed in the user's manual (Clontech, Palo Alto, CA). Briefly, the poly(A)+ mRNA was reverse transcribed, and [α-^{32}P] dATP was incorporated into the resulting cDNA. These radiolabeled cDNAs were column purified and then hybridized to either the Human, Human Cancer, or Human Apoptosis Atlas Arrays (the list of genes present on each filter type can be obtained at www.clontech.com/atlas/genelists/index.html). Tet21 and Tet21N mRNA were compared using the Human and Human Cancer Clontech filters; mRNA from Tet21N cells grown in the presence or absence of tetracycline was compared using the Human and Human Apoptosis Clontech filters; and mRNA from C11 and C11N cells was compared using the Human and Human Cancer Clontech filters. After an overnight incubation at 68°C and washes as described in the manual, the arrays were analyzed by a phosphorimager. Phosphorimage analysis was performed using the ImageQuant verson 1.2 computer program. The signal for each gene on an array, represented by duplicate cDNA dots, was determined by subtracting a local background value. Any gene whose corrected signal was not at least as strong as the background was excluded from further consideration. For the remaining genes, the relative difference in signals for a particular gene between a pair of filters was normalized to any difference seen in signals on each filter for the housekeeping gene ubiquitin. Genes determined by this method to be differentially expressed were then evaluated by reverse transcriptase (RT)–polymerase chain reaction (PCR).

RT-PCR

RT-PCR was performed by using the Perkin Elmer rTth DNA Polymerase and EZ Buffer Pack, optimizing for the amount of starting RNA and number of cycles of PCR (25–40). RT-PCR was performed two or more times for each gene, starting with two or more amounts of RNA, e.g., 10 and 50 ng of cytoplasmic RNA, to verify that increased input resulted in increased signal. If the gene in question was abundant enough, it was coamplified with glyceraldehyde phosphate dehydrogenase (*GAPDH*), which served as an internal control for the amount of RNA. All products were quantitated on ethidium bromide–stained gels by densitometry. For genes confirmed as differentially expressed, PCRs were Southern blotted to more accurately quantitate any difference. For these experiments, RT-PCR was performed for a target gene and *GAPDH* simultaneously by using 2–250 ng of mRNA (depending on target gene expression levels) for either 15 or 20 cycles. The RT-PCR products were then electrophoresed on a 1% agarose gel and transferred to a Zeta probe filter in 0.4 M NaOH buffer. After transfer, the filter was rinsed for 10 min in 2× sodium chloride sodium citrate (SSC) at room temperature and then incubated at 42°C in a prehybridization solution (50% formamide, 0.12 M Na_2HPO_4, 0.25 M NaCl, 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA)

for 15–30 min at 42°C before addition of a radioactive probe containing either nick-translated target gene or *GAPDH* RT-PCR product. Incubation continued for 4 h to overnight at 42°C in a hybridization oven. The filter was then rinsed for 10 min each at room temperature in (i) 2 × SSC/0.1% SDS, (ii) 0.5 × SSC/0.1% SDS, (iii) 0.1 × SSC/0.1% SDS, and (iv) 0.1 × SSC/0.1% SDS at 65°C, before exposure to a Phosphorimages. Subsequent analysis was performed with ImageQuant IQMac version 1.2. and Excel verson 4.0 computer programs.

Nuclear Extract Preparation and Western Blotting

Nuclei were pelleted in a microfuge and sheared several times by passage through a 26-gauge needle, and the resulting nuclear extract preparation was either directly loaded on to a 10% acrylamide gel, Tris pH 8.5, with a 5% stacking gel or frozen for later use. After protein samples were separated on the gel, the gel was transferred to a nitrocellulose filter with the BioRad transfer system. The filter was incubated with α-N-myc antibody sc-791 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 5 μg/mL and then secondary antibody. For secondary antibody linked to horseradish peroxidase, proteins were detected by chemiluminescense with the Amersham Life Sciences ECL kit (Amersham, Piscataway, NJ).

RDA

RDA was performed with C11 or C11N poly(A)+ mRNA by using the method described previously [22]. After three rounds of subtractive hybridization, the products were cloned and sequenced. Primers were designed for all inserts that were cloned more than once, and expression of these inserts in the C11 versus C11N cells was examined by RT-PCR. Also, inserts from all clones were produced by using vector primers; the inserts were spotted onto duplicate filters (according to directions from Clontech's PCR-select Differential Screening Kit User Manual), and the filters were probed with radiolabeled difference products obtained from the third subtractive hybridization using C11 versus C11N cells as the tester. The arrays were exposed with Phosphorimager for 18 h, and analysis was performed with ImageQuant IQMac version 1.2.

Formaldehyde Crosslinking and Chromatin Immunoprecipitation

Tet21N cells were formaldehyde crosslinked essentially as described previously [23]. In brief, formaldehyde (Fisher Scientific, Pittsburgh, PA) was added directly to tissue culture medium to a final concentration of 1%. Crosslinking was allowed to proceed for 10 min at room temperature and was then stopped by the addition of glycine to a final concentration of 0.125 M. Crosslinked cells were trypsinized, scraped, washed with 1× phosphate-buffered saline, and swelled in 3 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris-Cl (pH7.4), and 0.1% IGEPAL CA-330 (Sigma, St. Louis, MO). Nuclei were pelleted by microcentrifugation and lysed by incubation in nuclei lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.1, 0.5 mM phenylmethylsulfonyl fluoride, 100 ng/mL leupeptin, and 100 ng/mL aprotinin). The resulting chromatin solution was sonicated for three 30-s pulses at power setting 7. After microcentrifugation, the supernatant was precleared with blocked protein A–positive *Staphylococcus aureus* cells (Boehringer-Mannheim, Indianapolis, IN), diluted 1:5 with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 167 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 100 ng/mL leupeptin, and 100 ng/mL aprotinin), and divided into aliquots. One microgram of antibody was added to each aliquot of chromatin and incubated on a rotating platform for 12–16 h at 4°C. Antibodies against N-myc and max were purchased from Santa Cruz Biotechnologies. Antibody-protein-DNA complexes were isolated by immunoprecipitation with blocked protein A–positive *S.aureus* cells. After extensive washing, bound DNA fragments were eluted, and the immunoprecipitates were dissolved in 30 μL of water (except for input samples, which were diluted 1:100). Each reaction contained 3 μL of immunoprecipitated chromatin, 1 × Taq reaction buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 50 ng of each primer, 1.7 U of Taq polymerase (Promega), 200 μM each dNTP (Boehringer Mannheim), and 1 M betaine (Sigma) in a final reaction volume of 20 μL. PCRs were amplified for one cycle at 95°C for 5 min, at the annealing temperature of the primers for 5 min, and at 72°C for 3 min and 34–36 cycles at 95°C for 1 min, at the annealing temperature of the primers for 2 min, and at 72°C for 1.5 min. PCR products were separated by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide intercalation. The sequences of the primers used (each indicated in the 5′ to 3′ direction) and the appropriate annealing temperatures are as follows: for telomerase (64°C), ccttcacgtccggcattcgtgg and aaggtgaaggggcaggacgggt; and for prothymosin (63°C), atcttgtgtgtggcacaggt and tcgtctgtggagccagttgg. All primers were synthesized at the University of Wisconsin Biotechnology Center.

RESULTS

Identifying N-myc Target Genes by Using DNA Filter Arrays

Although many target genes have been suggested to be regulated by c-myc, relatively few studies have focused on N-myc target genes. Rather than begin by assaying known c-myc target genes for responsiveness to N-myc, we began with a more unbiased approach that would allow us to detect genes previously unknown to be regulated by the myc

family. It is possible that a single cell line may not express all N-myc target genes. Therefore, we used two cell systems in which N-myc can be experimentally overexpressed [10,21]. The first cell system consists of a parental cell type having low amounts of N-myc and derived from a patient with Li-Fraumeni syndrome (C11 cells) and the same cells containing a stably transfected N-myc expression construct (C11N cells). The second cell system consists of a human neuroblastoma cell line (Tet21) and the same cells stably transfected with a tetracycline-regulated N-myc expression construct (Tet21N cells). As described below, we used these two cell systems in combination with cDNA filter screening, RDA, and a candidate gene approach to identify N-myc target genes.

To determine what genes are differentially regulated between the low N-myc and high N-myc cell types, we used Clontech Atlas Expression Arrays, which contain a wide variety of human cDNAs. Poly(A)+ mRNA from each cell type was reverse transcribed to make a radiolabeled cDNA probe, which was then hybridized to a cDNA filter array. We compared expression patterns seen with different pairs of cell types on several different human array filters. To determine gene expression changes resulting from long-term overexpression of N-myc in the neuroblastoma cells, we compared mRNAs from the parental Tet21 cells (which had low N-myc expression) with mRNAs from Tet21N cells that had been maintained in the absence of tetracycline (and therefore had long-term exposure to very high N-myc levels). To detect those genes whose expression is affected by a short-term change in N-myc expression, we employed the tetracycline-controllable aspects of the N-myc plasmid. We compared mRNAs from Tet21N cells maintained in the absence of tetracycline (high N-myc) with mRNA from Tet21N cells grown in the presence of tetracycline for 7 d (causing a rapid decrease in N-myc levels). For the skin-derived cells, we compared mRNAs from parental C11 cells (low N-myc levels) with mRNAs from C11N cells (high N-myc levels).

To determine whether expression of mRNA was altered by N-myc levels, the signal from the filter hybridized with a probe from cells expressing high N-myc levels was compared with the signal from the filter hybridized with a probe from cells expressing low N-myc. Because we did not expect variation in expression of housekeeping genes in our pairwise comparisons, we normalized the signal for each gene to the signal for ubiquitin, one of the several housekeeping genes on the filters. This allowed us to control for possible differences in probe quality. In general, we found that very few mRNAs were differentially regulated in response to changes in N-myc abundance. However, we did detect differential expression of several genes, including both up- and downregulated genes. Next, RT-PCR was used to confirm that the expression of the identified genes was truly influenced by N-myc. For these experiments, cytoplasmic RNA was used and positive results were confirmed using RNAs from the same cell type comparison used in the filter analysis (e.g., if a gene was identified to be deregulated by filter comparisons of Tet21 with Tet21N, the confirmation was performed using these same two cell types but with a different preparation of RNA). These RT-PCR assays confirmed a qualitative deregulation of nine different genes, eight upregulated and one downregulated. However, the differences seen by both the filter hybridizations and RT-PCR

Table 1. N-myc Target Genes Identified by Filter Arrays*

Cell comparison	Name	Fold-change	
		Filter	RT-PCT
Tet21 vs. Tet21N	*CRABPII* (M68867)	0.3	0.25
	Prothymosin-α (M26708)	7	3
	Puf/nm23 (L16785)	4	2
	Y box–binding protein (M83234)	6	2
	Bax (L22474)	4	3
	HGF (D14012)	3	3
Tet21N vs. Tet21N+tet	Prothymosin-α (M26708)	5	3
	Replication factor C 38 (L07541)	NQ(+)	2
	Wee1hu (U10564)	*4*	*2*
C11 vs. C11N	c-myc–binding protein MM1 (D89667)	3	2

*The pairwise comparisons of mRNAs are indicated in the first column; Tet21N+tet indicates Tet21n cells in which expression of N-myc has been repressed by tetracycline for 7 d. The relative change in expression detected when N-myc levels are increased is indicated; genes activated by N-myc have fold changes greater than 1 and genes repressed by N-myc have fold changes less than 1. For the filter studies, the fold change was calculated from Phosphorimage analysis, with subtraction of local background and normalization to the ubiquitin signal. NQ(+) indicates increased expression that was not quantitated because of an extremely low signal in the absence of high amounts of N-myc. The RT-PCR column indicates the fold difference observed after quantitation of PCR products using ethidium bromide–stained gels. All numbers are an average of at least two experiments. *CRABPII*, cellular retinoic acid binding protein II; *HGF*, hepatoma-derived growth factor. GenBank accession numbers are provided in parentheses next to the name of each target gene.

analyses were fairly modest, ranging from twofold to sevenfold (Table 1).

Expression of N-myc Target Genes in Three Different Systems

We confirmed that nine genes identified in the filter hybridization experiments were regulated by increased levels of N-myc. However, we did not know whether these nine genes were only regulated by N-myc in the cell type used in the original hybridization experiment (i.e., neuroblastoma cells vs. skin-derived fibroblasts) or were influenced by N-myc levels in more than one cell type. It also seemed possible that short-term and long-term overexpression of N-myc might have different effects on gene expression. To address these possibilities, we next determined whether any of the genes confirmed to be altered in a single pairwise comparison were differentially expressed in all three of our pairwise comparisons: C11 versus C11N, Tet21 versus Tet21N, and Tet21N versus Tet21N+tet (cultures in which tetracycline has been added for 7 d to repress N-myc expression). We also assayed expression of several previously identified c-myc target genes [12,24]. Because we found that one gene that was regulated by N-myc was *E2F2*, we expanded our studies to include other E2Fs. RT-PCR was performed with cytoplasmic RNA from cells from all three pairwise comparisons, and the amount of signal of each candidate gene was normalized to the amount of *GAPDH* in each sample (Table 2).

We found that several mRNAs were influenced by levels of N-myc only in the original cell type and/or comparison and that levels of *E2F4* did not respond to N-myc levels under any experimental conditions. However, five mRNAs (prothymosin-α, replication factor C, *ODC*, telomerase, and *E2F5*) were upregulated in all three comparisons. Similarly, a recent study investigating c-myc target genes found that only 60% of the genes identified using one cell type also responded to c-myc in a second cell type [25]. It is likely that cell-type–specific coactivators can influence the ability of myc family members to

Table 2. Analysis of N-myc Target Genes in Multiple Systems*

	Fold change		
	Tet21N/tet	Tet21N/Tet21	C11N/C11
Genes identified by filter arrays			
Prothymosin-α	3†	3	2
Replication factor C 38	2†	3	2
WEE1hu	2	3	1
bax	1	3	1
CRABPII	1	0.25	3
Hepatoma-derived growth factor	1	3	1
Puf/nm23-H2	1	2	1
Y box–binding protein	1	2	1
c-Myc BP MM-1	1	2	2
Candidate genes			
CAD	1	3	3 (p)
EIF-2a	1	2	1
EIF-4E	1	3	2
ODC	2†	3	2
p53	1	2	1
RCC1	2	3	1
Telomerase	3†	3	3
Topoisomerase IIa	1	3	2
E2F1	1	2	2
E2F2	2	3	NE
E2F3	1	2	1
E2F4	1	1	1
E2F5	2†	3	3
E2F6	1	2	1

*The expression of genes was measured in three pairwise comparisons, with difference expressed as the fold change seen with increased N-myc levels. Tet21N/tet indicates the difference between Tet21N mRNA and mRNA from cells with tetracycline-repressed N-myc; Tet21N/Tet21 indicates the difference between Tet21N and Tet21 mRNAs; and C11N/C11 indicates the difference between C11 and C11N mRNAs. p, the differential regulation was detected using an RNAse protection assay and not RT-PCR; NE, not expressed at detectable levels.

†Genes upregulated in all three comparisons. Literature citations for genes previously suggested to be regulated by c-myc are as follows: *CAD* [23], *EIF-2a* and *EIF-4e* [49], *ODC* [29], p53 [31], *RCC1* [34], telomerase [27,35–39], and *E2F2* [33]. *CRABPII*, cellular retionic acid binding protein II.

activate specific promoters. We found that certain mRNAs (e.g., carbamoyl phosphate synthase, aspartate transcarbamylase, dihydroorotase (CAD), topoisomerase IIa, *EIF-4E*, c-myc–binding protein MM-1, and *E2F1*) were only increased if N-myc was overexpressed in a long-term assay (i.e., Tet21N vs. Tet21 and C11N vs. C11). Although the expression of these genes does not change when N-myc levels are transiently decreased, we believe the changes seen in the long-term overexpression assays do indicate a true difference in expression due to N-myc and not an artifact of clonal variation because these changes in expression are seen in both cell systems. It is possible that a short-term reduction in N-myc levels may not result in a large decrease in mRNAs with very long half-lives and that a longer time course of tetracycline addition may have resulted in greater changes in gene expression.

We detected relatively few genes when using commercially available cDNA filter arrays. We thought that additional N-myc target genes may not yet have been cloned and thus would not be present on commercial filter arrays. To expand our analyses to include all possible N-myc target genes, including previously unknown genes, we next performed RDA. Briefly, to find mRNAs upregulated by N-myc, mRNA from cells having high amounts of N-myc served as the tester, and mRNA from cells expressing low amounts of N-myc served as the driver. To identify mRNAs downregulated by N-myc, the tester and driver samples were reversed. Three successive subtractive hybridizations were performed in each direction and, as expected, we saw an enrichment for different mRNA fragments, depending on whether N-myc–expressing cells were used as the driver or as the tester (data not shown). Overall, we sequenced 103 cDNA fragments corresponding to 65 different mRNAs and 11 sequences not found in any sequence database. Of the known genes, the most striking was *N-myc*, which we cloned 22 times when N-myc–expressing cells were used as the tester. Because the number of times a mRNA is cloned should correlate with the fold difference between expression of that gene in the tester versus that in the driver population, obtaining these *N-myc* clones served as a control in demonstrating that our technique functioned well. Unfortunately, we could not confirm differential regulation of any of the other cloned mRNAs with either RT-PCR or dot blots (data not shown). RDA, although having the advantage of being able to identify novel genes, has the disadvantage of not detecting small differences in expression levels. Therefore, inserts corresponding to mRNAs that differ about twofold to threefold in the two mRNA populations are usually not cloned. Accordingly, we did not detect genes such as *ODC*, which was found to be twofold upregulated in the C11 N cells when using RT-PCR assays (Table 2).

PCR Blotting of Candidate Target Genes

We found five genes regulated by N-myc in three different comparisons. To more quantitatively measure the difference in expression of these target genes in response to changes in N-myc levels, we performed PCR blotting assays. This method provided two advantages over straightforward ''cold'' RT-PCR, which was used to determine the inductions shown in Table 2. First, only 15–20 versus 25–40 cycles of PCR are required to detect a signal, so there is less chance of expression differences being misrepresented. Second, each target gene can be coamplified with *GAPDH*, which allows both for RNA quantitation and possible variation from experiment to experiment or from sample to sample. With ethidium-stained gels, coamplification is not possible because of the large differences in expression levels between the target genes and *GAPDH*.

Briefly, RT-PCR was performed for each target gene, coamplifying for *GAPDH*, which we chose as our normalization standard. For each experiment, two different amounts of RNA were analyzed to demonstrate that the signal was proportional to the amount of template RNA. The PCR products were then electrophoresed and transferred to a filter, which was probed first for the target gene and then for *GAPDH*. The resulting signals were quantitated by a phosphorimager; each blot was performed in duplicate and the averaged results of these blots are shown in Figure 1. As a positive control, we measured the expression of *N-myc* mRNA (Figure 1A) and observed a 50-fold difference in the mRNA from Tet21N cells grown in the presence versus in the absence of tetracycline. Dramatic difference in N-myc protein levels between these two cell populations are also seen (Figure 1B). It is difficult to determine exactly how much more N-myc protein is made in the cells expressing high amounts of N-myc because of the very low signal in the tetracycline-repressed cells. However, dilution of the extract indicates that the increase in N-myc protein is greater than 10-fold (data not shown). Using the PCR blotting technique, three mRNAs (telomerase, *E2F5*, and prothymosin) were found to be greater than twofold increased in this experiment in the presence of increased levels of N-myc (Figure 1C). Two other mRNAs (replication factor C38 and ODC) showed less than a twofold increase in this particuar experiment. A recent study of c-myc target genes also reported that certain mRNAs show a greater than twofold increase in response to c-myc in some, but not all, experiments [25].

Analysis of N-myc Binding to Target Promoters in Living Cells

Transiently changing the amount of N-myc can reproducibly influence the expression of telomerase, *E2F5*, and prothymosin. However, it is never

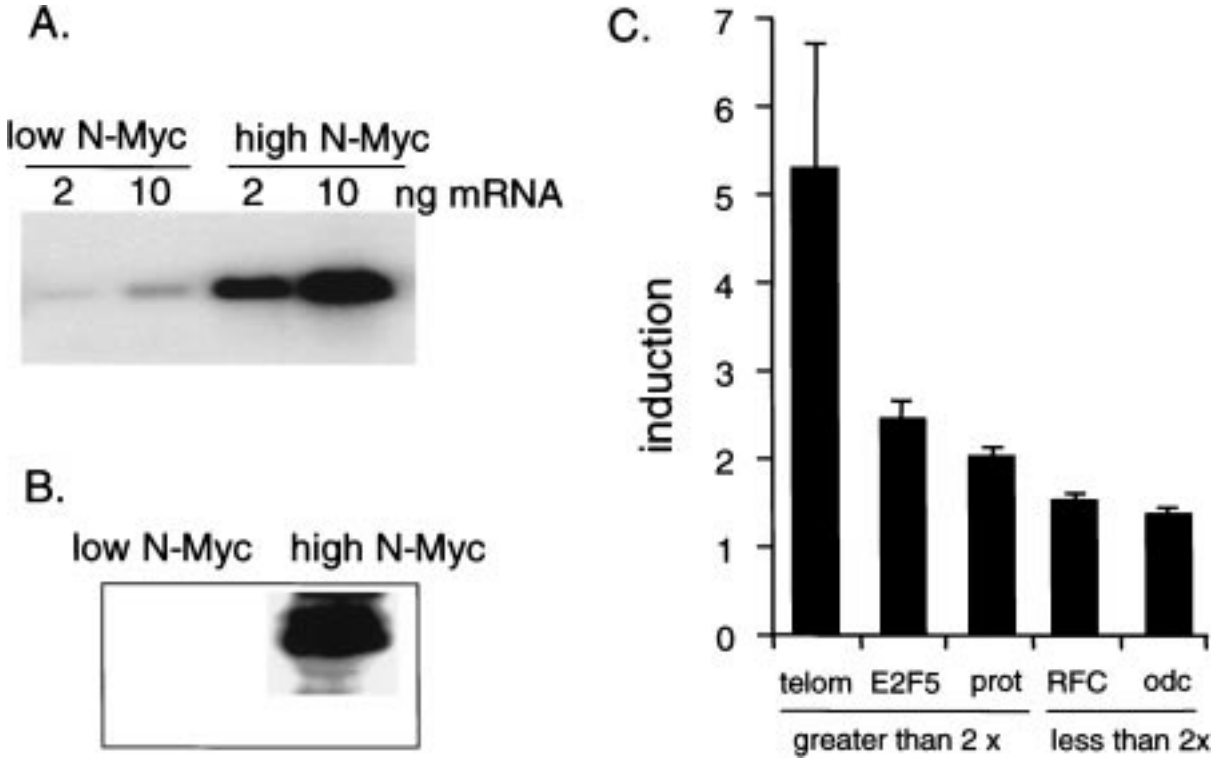

Figure 1. PCR blotting of expression of N-myc target genes. Tet21N cells were grown in the presence or absence of tetracycline and RNA, and protein was prepared. (A) PCR blotting assay performed for N-*myc* using 2 and 10 ng of each mRNA. (B) Western blot indicating the differences in levels of N-myc protein in cells grown in the presence or absence of tetracycline. (C) RT-PCR of N-myc target genes. For each reaction, a simultaneous analysis of a target gene and *GAPDH* was performed. Products were separated on an agarose gel and transferred to a nitrocellulose filter. The filter was probed sequentially for both genes, and the signal for the N-myc target gene was normalized to *GAPDH* expression. For each gene, the experiment was performed at least twice with two different amounts of mRNA. The average fold increases seen in Tet21N mRNA versus Tet21N+tet mRNA were calculated and are shown with the standard error. Gene names are shortened as follows: telom, telomerase; prot, prothymosin-α; RFC, replication factor C38; odc, ornithine decarboxylase.

certain whether a gene that is shown to respond to a particular transcription factor is directly regulated by that factor or whether it is downstream of the factor in a signal transduction pathway. Because the increases in mRNA levels that occurred in response to changes in N-myc levels were modest, we decided to determine whether these genes are indeed direct targets of N-myc. For these experiments, we used a formaldehyde crosslinking and chromatin immunopreciptiation protocol that we previously used to detect binding of c-myc to target genes in mouse 3T3 cells [23,26]. Using this technique, transcription factors are crosslinked to target gene promoters in living cells. Antibodies to the transcription factors of interest are then used to immunoprecipitate the chromatin fragments bound by the factors; preimmune serum rather than a primary antibody is used as a negative control. The immunoprecipitated chromatin is then analyzed in PCR reactions using primers specific to the target promoters. The amount of the PCR product provides an indication of the degree of occupancy of the promoter by the transcription factor recognized by the antibody. The promoter regions of telomerase and prothymosin have been cloned; therefore, we made primers that spanned consensus E boxes (N-myc–binding sites) in these two promoters [27,28]. Unfortunately, there is no genomc sequence available for the *E2F5* promoter; only the cDNA has been cloned. Therefore, we could not analyze binding of N-myc to the *E2F5* promoter.

Tet21N cells were grown in the absence of tetracycline or for 14 d in the presence of tetracycline. We expected that, after 14 d of tetracycline treatment (which shuts off the promoter driving N-myc expression), most of the N-myc should be gone from the cell. Accordingly, we found that N-*myc* mRNA could not be detected by RT-PCR in cells grown in the presence of tetracycline (Figure 2A). We also showed that levels of prothymosin and telomerase mRNAs were higher in the cells containing high amounts of N-myc than in the cells containing low amounts of N-myc. The 14-d tetracycline treatment resulted in a greater difference in the expression of these two mRNAs than did the 7-d treatment (Figure 1), suggesting that the longer time course of treatment was important to see maximal changes in N-myc target gene expression. To deter-

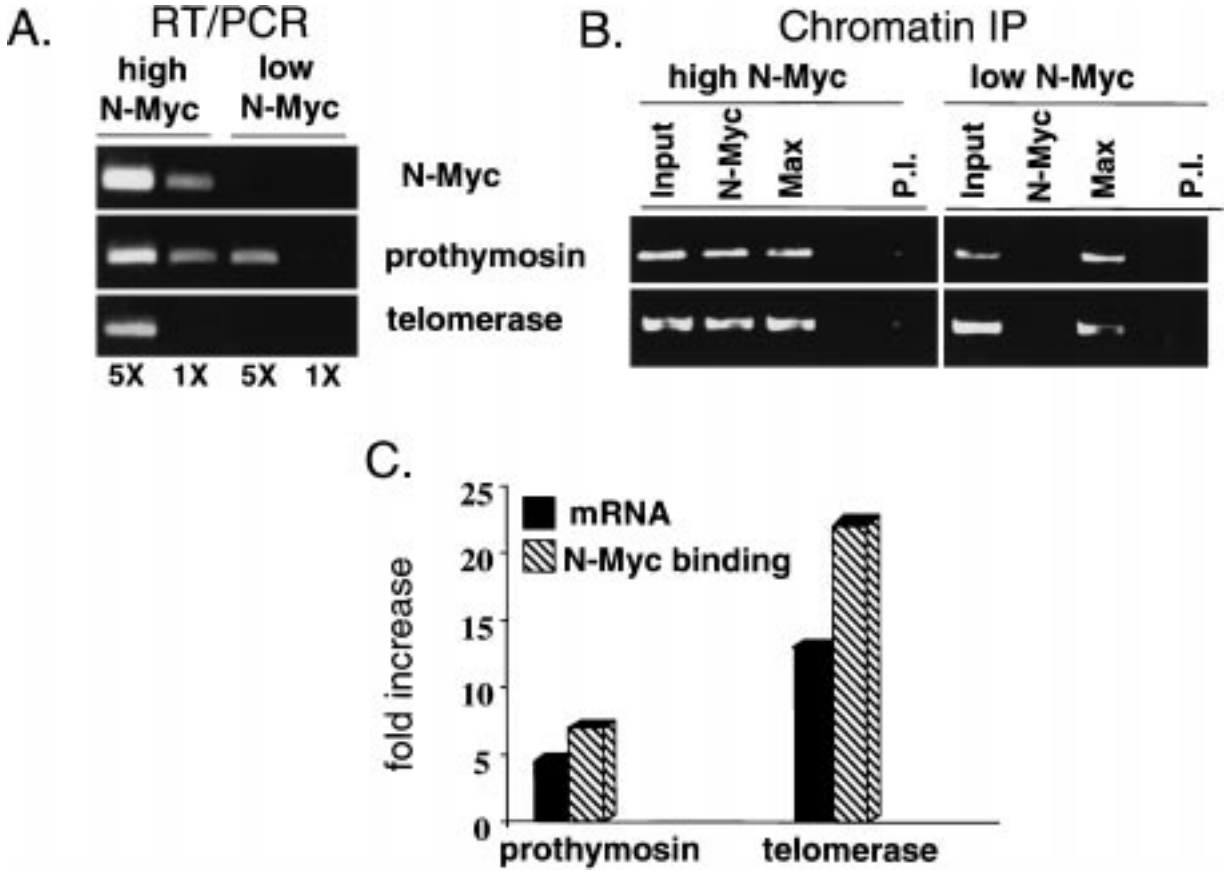

Figure 2. Analysis of N-myc binding to promoter DNA in living cells. (A) Primers specific for N-*myc*, telomerase, and prothymosin mRNAs were used in RT-PCR reactions with two different concentrations of mRNA from cells containing high or low amounts of N-myc protein. (B) Crosslinking analysis of N-myc and max binding at target promoters in cells containing high amounts versus low amounts of N-myc; preimmune rabbit antisera (P.I.) was used as a negative control. Immunprecipitates were analyzed using primers that flank the high-affinity myc/max binding site in the telomerase and prothymosin promoters. The input sample contains 0.02% of the total input chromatin as PCR template. The amount of max bound to each promoter remains relatively constant independent of the levels of N-myc. (C) Graphic representation of the RT-PCR and chromatin immunoprecipitation results. The fold increases in mRNA levels are calculated from the 1× lanes shown in the RT-PCR results. The fold increases in N-myc binding from the crosslinking experiment are calculated from the amount of promoter fragment, relative to the amount of input chromatin, bound by N-myc in the cells expressing high versus low amounts of N-myc. The experiment shown in this figure was performed three times; each experiment produced results very similar to those shown here.

mine whether the genes that responded to transient changes in N-myc levels are directly bound by N-myc in living cells, the two same two cell populations used for the RNA analysis were treated with formaldehyde to crosslink the transcription factors to the chromatin; the chromatin was precipitated using antibodies against N-myc and max, and the immunoprecipitates were analyzed by PCR reactions by using primers specific for the target genes (Figure 2B). We found that low amounts of N-myc were bound to the promoters of the prothymosin and telomerase genes in the cells containing a single copy of the N-*myc* gene. However, binding of N-myc was greatly increased in the cells containing high amounts of N-myc protein. Interestingly, we found that the amount of max, the heterodimeric partner of N-myc, did not greatly change with overexpression of N-myc. These results suggested that the E boxes are occupied by either max homodimers or, more likely, max in a heterodimeric complex with another protein before the induction of high levels of N-myc. We found that the N-myc–mediated increase of both the telomerase and prothymosin mRNAs was very similar to the increased amount of N-myc bound to the endogenous promoter regions (Figure 2C), indicating a good correlation between gene expression and N-myc binding. The immunoprecipitation experiment was repeated several times using chromatin from cells that were independently treated with tetracycline and crosslinked, and very similar results were obtained, i.e., the amount of N-myc bound to the prothymosin and telomerase promoters was higher in the cells with high amounts of N-myc and the fold increase in binding of N-myc to the telomerase promoter was greater than the fold increase of N-myc binding to the prothymosin promoter (data not shown).

DISCUSSION

We have sought to gain insight into the molecular mechanisms by which N-myc mediates neoplasia by identifying genes activated by high levels of N-myc protein. Using filter arrays, RDA, and a candidate gene approach, we identified 22 genes whose expression was increased as a result of increased levels of N-myc in a certain cell type and one gene that was downregulated by N-myc levels in one cell type and upregulated by N-myc in a different cell type. This is not a large number of mRNAs to be regulated by a site-specific transcription factor. However, a recent study focused on the identification of c-myc target genes also showed very few genes to be regulated by c-myc [25]. In fact, approximately 5% of the genes tested using either filter arrays (present study) or DNA microchips [25] responded to overexpression of an myc family member. Although myc family members have been shown to function as both positive and negative regulators of transcription, we (present study) and others [25] found many more genes to be upregulated than to be downregulated by myc family members. Several of the N-myc target genes we characterized, e.g., *CAD*, *eIF-2α*, *eIF-4E*, *E2F2*, *ODC*, prothymosin-α, *p53*, *RCC1*, and telomerase, have been shown to be regulated by c-myc as well [23,27,29–39]. In addition, we discovered some genes upregulated by N-myc that had not previously been implicated as myc family target genes, such as *E2F5*, replication factor C38, and *WEE1*. It is interesting that myc family members can upregulate *E2F2* and *E2F5* because members of the E2F family of transcription factors has been shown to regulate expression of c-myc and N-myc [40,41]. This crosstalk between the E2F and myc families may be partly responsible for the ability of single E2F or myc proteins to alter cell-growth parameters in overexpression assays [10,42–44].

Our results show that N-myc target genes do not display great changes in expression when increasing the amount of N-myc in the cell. A recent study of c-myc target genes [25], using DNA microchips to test more than 6000 mRNAs, also found very modest changes in gene expression (i.e., most genes that responded to increased levels of c-myc changed by only twofold to fivefold). Small changes in the levels of myc target genes such as *EIF-4E*, the mRNA cap binding protein that is rate limiting for translation, could affect the cell on a more global basis if translation of a large number of proteins was accelerated. Evidence in support of the hypothesis that overexpression of myc family members leads to increased protein synthesis has been recently reported in a B-lymphocyte model system [45]. Other myc target genes are involved in DNA synthesis and maintenance; CAD is the rate-limiting enzyme for pyrimidine biosynthesis, replication factor C38 is involved in DNA synthesis and repair, and telomerase is required to maintain DNA integrity. Slight increases in such enzymes may also have significant effects on cell growth or survival.

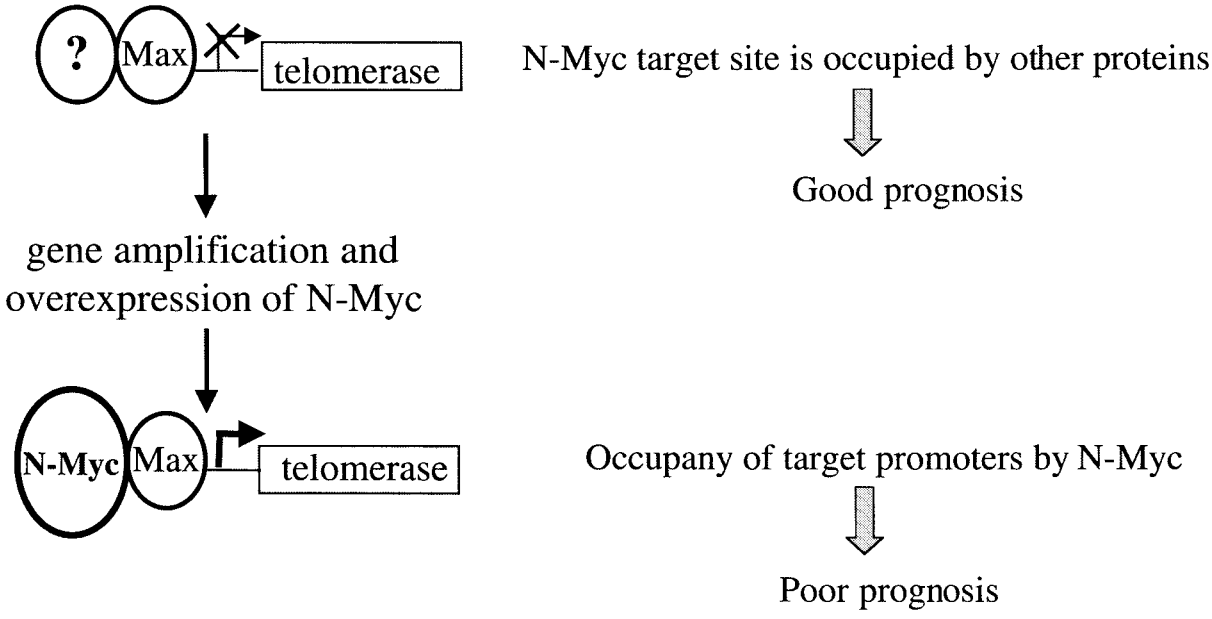

Figure 3. Model depicting a possible mechanism by which progressive amplification of the *N-myc* gene can lead to a poor prognosis. In cells containing a single copy of the *N-myc* gene, the N-myc protein must compete for binding to target promoters, such as telomerase, with other max heterodimers. Progressive amplification of the *N-myc* gene changes the ratio of N-myc/max heterodimers versus other max-containing heterodimers, and the target promoters become occupied by N-myc/max complexes. As a result of promoter occupancy by N-myc/max heterodimers, transcription is increased and the products of the N-myc target genes (e.g., telomerase) enhance the neoplastic characteristics of the tumor, resulting in a poorer prognosis.

We attempted to mimic the progressive amplification of N-myc that occurs in late-stage poor-prognosis neuroblastomas by overexpressing N-myc in a neuroblastoma cell line that has a single copy of the endogenous N-*myc* gene. Using this model system, we found that the amount of N-myc bound to the prothymosin-α and telomerase promoters increased with induction of N-myc protein. This relative increase in promoter occupancy corresponded well with the relative difference in mRNA levels of these target genes seen when N-myc levels were changed, i.e., telomerase showed the largest increase in binding of N-myc and the largest mRNA induction. Previous reports [16,46,47] have shown that telomerase mRNA is upregulated in neuroblastoma cells. The present studies show that an increase in telomerase mRNA can be a direct result of increased N-myc bound to the telomerase promoter. Thus, we suggest that antineoplastic therapies that target telomerase activity or the telomerase promoter may be clinically relevant for the treatment of neuroblastomas with amplified N-*myc*. We have shown that max, the heterodimeric partner of N-myc, at high levels is bound to N-myc target promoters regardless of the amount of N-myc in the cells. Interestingly, previous studies have shown a threshold effect for N-*myc* amplification in human tumors; patients with more than 10 copies of the N-*myc* gene have a much worse prognosis than do patients with fewer than 10 copies [48]. We suggest that our use of the formaldehyde crosslinking and immunoprecipitation assay may have provided a molecular explanation for these clinical observations. Amplification of the N-*myc* gene may be a risk factor because of the production of enough N-myc protein to efficiently compete with other max partners for binding to target genes (Figure 3).

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