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N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis

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The *myc* oncogenes are frequently activated in human tumors, but there is no comprehensive insight into the target genes and downstream cellular pathways of these transcription factors. We applied serial analysis of gene expression (SAGE) to identify targets of N-myc in neuroblastomas. Analysis of 42 000 mRNA transcript tags in SAGE libraries of N-*myc*-transfected and control neuroblastoma cells revealed 114 up-regulated genes. The majority of these genes have a role in ribosome assembly and activity. Northern blot analysis confirmed up-regulation of all tested transcripts. Induction was complete within 4 h after N-myc expression. The large majority of the ribosomal proteins were induced, as well as genes controlling rRNA maturation. Cellular rRNA content was 45% induced. SAGE libraries and northern blot analysis confirmed up-regulation of many of these genes in N-*myc*-amplified neuroblastomas. As N-*myc* can functionally replace c-*myc*, we analyzed whether N-myc targets were induced by c-myc as well. Approximately 40% of these N-myc targets were up-regulated in a c-*myc*-transfected melanoma cell line. These data suggest that *myc* genes function as major regulators of the protein synthesis machinery.

Keywords: c-myc/N-myc/neuroblastoma/protein synthesis/ribosomes

Introduction

The members of the *myc* oncogene family play a prominent role in cancer. N-*myc*, c-*myc* and L-*myc* are rearranged, amplified, mutated and/or overexpressed in many human tumor types. The c-*myc* gene is expressed in a wide variety of tissues and tumors, while N-*myc* expression is largely restricted to embryonic tissues and neuroendocrine tumors. Approximately 20% of neuroblastomas have N-*myc* amplification, and these tumors follow a very aggressive course (Schwab *et al*., 1983; Seeger *et al*., 1985). Overexpression of transfected N-*myc* genes in neuroblastoma cell lines strongly increased proliferation rates (Bernards *et al*., 1986; Lutz *et al*., 1996). Transgenic mice overexpressing N-*myc* in neural crest-derived tissues showed frequent development of neuroblastomas (Weiss *et al*., 1997). Numerous comparable observations have implicated c-*myc* and L-*myc* in the pathogenesis of many other tumor types (Cole, 1986; Henriksson and Luscher, 1996). While c-*myc* and N-*myc* homozygous knockout mice are embryonic lethal, transgenic mice in which N-*myc* replaced c-*myc* showed a gross normal development, indicating that both proteins have largely overlapping functions (Malynn *et al*., 2000).

Many experiments have suggested a role for *myc* genes in cell cycle control, metastasis, blocking of differentiation, apoptosis and proliferation rate (Henriksson and Luscher, 1996; Dang, 1999; Schmidt, 1999). The myc family members are transcription factors with a basic/helix–loop–helix/leucine zipper (bHLHzip) domain. They form heterodimers with Max proteins and bind to the E-box motif CACGTG to activate target gene transcription (Alex *et al*., 1992; Blackwood *et al*., 1992). The limited number of initially identified targets of c-myc gave little insight into the mechanism of how c-myc induces the variety of phenotypes. Examples of myc target genes are prothymosin α, ornithine decarboxylase, CAD and the DEAD-box gene MrDb (Eilers *et al*., 1991; Bello-Fernandez *et al*., 1993; Rosenwald *et al*., 1993; Grandori *et al*., 1996; Jones *et al*., 1996; Boyd and Farnham, 1997). Induction of cyclins D1, E and A and cdc25A were found in some but not all model systems (reviewed in Obaya *et al*., 1999). Induction of cyclin D2 was consistently found and provides a direct link to the cell cycle (Bouchard *et al*., 1999; Perez-Roger *et al*., 1999; Coller *et al*., 2000). Furthermore, c-myc and N-myc also induce Id2 transcription, thus stimulating Rb inactivation and cell cycle progression (Lasorella *et al*., 2000). However, in addition to these direct effects on proliferation, several findings suggest that c-myc also promotes the growth of cells, thereby inducing an increase in cell mass that may be a prerequisite for rapid cell proliferation. Impaired *in vivo* expression of *Drosophila* d*myc* results in adult flies half the normal size (Johnston *et al*., 1999). Both the volume and proliferation rate of their cells are reduced, and could be restored by overexpression of d*myc*. B cells of transgenic mice with overexpression of c-*myc* show for all differentiation stages an increased cell size and increased protein synthesis rate (Iritani and Eisenman, 1999). Furthermore, fibroblasts with a homozygous inactivation of c-*myc* have a reduced proliferation rate as well as a reduced protein synthesis rate (Mateyak *et al*., 1997). Conversely, activation of c-*myc* in fibroblasts activates protein synthesis (Schmidt, 1999). Furthermore,

a role for c-*myc* in protein synthesis is suggested by the finding that c-*myc* induces the translation initiation factors elF-4E, eIF-2-α (Rosenwald *et al*., 1993; Jones *et al*., 1996), eIF5A and eIF4G (Coller *et al*., 2000). Very recently, ectopic *in vivo* expression of c-*myc* was found to result in increased expression of six ribosomal protein genes in mouse liver (Kim *et al*., 2000). A role for *myc* genes in growth regulation is in line with their effect on the cell cycle. Inactivation of c-*myc* in fibroblasts prolonged the G_1 and G_2 phases of the cell cycle, but not the S phase, while high expression of *myc* genes accelerated transition through G_1 (Steiner *et al*., 1995; Lutz *et al*., 1996). A similar effect was found for *Drosophila* d*myc* (Johnston *et al*., 1999).

Here we describe the use of SAGE (serial analysis of gene expression; Velculescu *et al*., 1995) to identify the downstream genes that are activated by N-*myc* in human neuroblastoma. To date, only prothymosin α, ornithine decarboxylase and Id2 have been identified as targets of N-myc (Lutz *et al*., 1996; Lasorella *et al*., 2000). The analysis of the expression level of >40 000 transcripts identified 114 genes up-regulated in N-myc-expressing cells. Our results indicate that N-myc functions as a regulator of cell growth by stimulating genes functioning in ribosome biogenesis and protein synthesis. Several of the identified genes are induced by N-*myc* as well as by c-*myc*.

Results

SAGE libraries of N-myc-transfected neuroblastoma cell lines

To identify the downstream target genes of N-myc, we applied the SAGE technique to an N-*myc*-transfected neuroblastoma cell line. The SHEP cell line has no N-*myc* amplification and expression, or c-*myc* expression. A tetracycline-dependent N-*myc* expression vector has been introduced into these cells, resulting in the SHEP-21N clone (Lutz *et al*., 1996). The SHEP-21N cells have constitutive exogenous N-*myc* expression that can be switched off by tetracycline. N-*myc* expression in the SHEP-21N cells was shown to increase the rate of cell division, shorten the G_1 phase of the cell cycle and render the cells more susceptible to apoptotic triggers (Lutz *et al*., 1996; Fulda *et al*., 1999). SAGE libraries were constructed from SHEP-21N cells expressing N-*myc* and from SHEP-2 control cells. The SHEP-2 clone was transfected with the empty expression vector. From each library, we sequenced ~21 000 transcript tags, corresponding to 8566 (SHEP-21N) and 10 154 (SHEP-2) different transcripts (Table I). A tag for the transfected N-*myc* construct has a frequency of 0 and 8 in SHEP-2 and SHEP-21N, respectively. Comparison of the SAGE libraries yielded 114 significantly (P <0.01) up-regulated tags in N-*myc*-expressing cells, with induction levels of up to 37-fold (Tables II–IV). Another 70 tags were significantly down-regulated. Here we focused on the analysis of a series of tags induced in the N-*myc*-transfected cells. The transcripts corresponding to these tags were identified using the SAGEmap database (Lal *et al*., 1999) and our own tag assignment program (Caron *et al*., 2001) and checked by mRNA and expressed sequence tag (EST) sequence analyses. A comprehensive set of up-regulated genes functions in ribosome biogenesis and activity and in later steps of the protein synthesis and protein turnover machinery.

Table I. Summary of neuroblastoma SAGE libraries

	Total tags	Different transcripts
Neuroblastoma cell lines		
SHEP-2	20 950	10 154
SHEP-21N (N-myc transfected)	20 938	8566
Neuroblastoma tumors		
N52	19 597	9356
N159 (N-myc amplified)	20 001	10 262
Total	81 486	

Induction of genes involved in ribosome biogenesis and protein synthesis

The first functional group consists of 56 ribosomal protein genes, which were induced up to 37-fold (P <0.01; Table II). Tags for 10 more ribosomal protein genes were less strongly up-regulated (0.01> P >0.05), with induction levels from 1.4- to 8-fold (Table II, lower part). These 66 tags correspond to 82% of the human ribosomal proteins (Wool and Chan, 1996). Northern blot analysis of SHEP-2 and SHEP-21N with probes for seven ribosomal protein genes (S12, S27, Fau-S30, L8, S6, S19 and PPARP0) confirmed their up-regulation (Figure 1). The fraction of tags for ribosomal protein mRNAs was increased from 4.1% in SHEP-2 to 12.6% in SHEP-21N. The level of induction of individual ribosomal protein genes is a function of their basal expression levels in SHEP-2. Highly expressed genes are less induced than genes with a low basic expression in SHEP-2 (Figure 2).

A second functional group of genes up-regulated in SHEP-21N consists of two nucleolar protein genes (Table III). Nucleophosmin (or B23) is induced from 26 to 55 tags (P <0.001), which was confirmed by northern blot analysis (Figure 1). Nucleophosmin is an abundant nucleolar protein that processes rRNA by cleavage of the 5′ end of the 5.8S pre-rRNA (Zhang *et al*., 1997) and functions in assembly and nuclear–cytoplasmic shuttling of pre-ribosomal particles (Borer *et al*., 1989; Olson, 1991). Transcription of another nucleolar protein is also induced: nucleolin, which has two tags due to alternative transcripts, is induced from 4 to 12 tags in total (P = 0.035). This induction was confirmed by northern blot analysis (Figure 1). Nucleolin processes pre-rRNA to mature 18S rRNA (Ginisty *et al*., 1998, 1999). It binds to nucleophosmin and is also involved in the assembly of pre-ribosomal particles and their nucleo-cytoplasmic transport. The induction of nucleolin and nucleophosmin in SHEP-21N suggests that rRNA and ribosome biosynthesis are targets of N-*myc* stimulation.

Additionally, tags corresponding to nine translation initiation and elongation factors were induced (Table III). They are eukaryotic translation initiation factors eIF3s8, eIF4A and eIF5A, and the subunits α, β, γ and δ of translation elongation factor 1 (EEF1). Furthermore, elongation factor 2 and the mitochondrial elongation factor Tu (tuFM) are up-regulated. Northern blot analysis of SHEP-21N and SHEP-2 confirmed the induction of

Table II. Downstream targets induced by N-*myc*: ribosomal proteins

Tag sequence	SHEP-2	SHEP-21N	Fold induction	*P* value	Unigene Hs.	Gene
GCCGAGGAAG	1	37	37.0	<0.001	82148	ribosomal protein S12
GCTTTTAAGG	1	29	29.0	<0.001	8102	ribosomal protein S20
CCCATCCGAA	1	23	23.0	<0.001	91379	ribosomal protein L26
GGCCGCGTTC	0	23	>23	<0.001	5174	ribosomal protein S17
CCAGTGGCCC	0	21	>21	<0.001	180920	ribosomal protein S9
GTGTTGCACA	1	16	16.0	<0.001	165590	ribosomal protein S13
GATGCTGCCA	1	16	16.0	<0.001	99914	ribosomal protein L22
CCGTCCAAGG	2	31	15.5	<0.001	80617	ribosomal protein S16
GGAGTGGACA	1	14	14.0	<0.001	75458	ribosomal protein L18
GCCTGTATGA	2	27	13.5	<0.001	180450	ribosomal protein S24
GTTCCCTGGC	2	26	13.0	<0.001	177415	ribosomal protein Fau-S30
ATGGCTGGTA	6	72	12.0	<0.001	182426	ribosomal protein S2
GTGTTAACCA	1	11	11.0	<0.001	74267	ribosomal protein L10
CACAAACGGT	4	43	10.8	<0.001	195453	ribosomal protein S27
CTCAACATCT	3	32	10.7	<0.001	73742	ribosomal protein, large, P0
GTTCGTGCCA	2	18	9.0	<0.001	179666	ribosomal protein L35a
GACGACACGA	4	30	7.5	<0.001	153177	ribosomal protein S28
TCGTCTTTAT	3	21	7.0	<0.001	75538	ribosomal protein S7
GGACCACTGA	5	34	6.8	<0.001	119598	ribosomal protein L3
CCTCGGAAAA	4	24	6.0	<0.001	2017	ribosomal protein L38
AATCCTGTGG	8	48	6.0	<0.001	178551	ribosomal protein L8
ATCAAGGGTG	4	21	5.3	<0.001	157850	ribosomal protein L9
GGGCTGGGGT	20	101	5.1	<0.001	183698	ribosomal protein L29
AAGGAGATGG	5	25	5.0	<0.001	184014	ribosomal protein L31/tag matches mitochondrial sequences
AAGGTGGAGG	11	55	5.0	<0.001	163593	ribosomal protein L18a
TTACCATATC	10	49	4.9	<0.001	177461	ribosomal protein L39
GTGAAGGCAG	6	27	4.5	<0.001	77039	ribosomal protein S3A
GAACACATCC	4	18	4.5	0.002	75879	ribosomal protein L19
CGCCGCCGGC	10	40	4.0	<0.001	182825	human ribosomal protein L35 mRNA
GCCGTGTCCG	5	20	4.0	0.002	119213	ribosomal protein S6
AGGAAAGCTG	13	52	4.0	<0.001	76437	ESTs, highly similar to 60S rpL36 (*Rattus norvegicus*)
CCCCAGCCAG	7	27	3.9	<0.001	75459	ribosomal protein S3
GCAGCCATCC	13	48	3.7	<0.001	4437	ribosomal protein L28
GGCAAGAAGA	7	25	3.6	<0.001	111611	ribosomal protein L27
CCCGTCCGGA	19	65	3.4	<0.001	180842	ribosomal protein L13
CGCTGGTTCC	15	51	3.4	<0.001	179943	ribosomal protein L11
TAAGGAGCTG	9	30	3.3	<0.001	77904	ribosomal protein S26
CCTTCGAGAT	8	26	3.3	0.001	76194	ribosomal protein S5
GGATTTGGCC	33	103	3.1	<0.001	119500	ribosomal protein, large, P2
AGGCTACGGA	20	63	3.2	<0.001	119122	60S ribosomal protein L13A
CTGCTATACG	7	22	3.1	<0.004	180946	ribosomal protein L5
TGTGCTAAAT	12	35	2.9	<0.001	179779	ribosomal protein L37
GAGGGAGTTT	34	97	2.9	<0.001	76064	ribosomal protein L27a
AAGAAGATAG	8	22	2.8	0.007	184776	ribosomal protein L23a
ACATCATCGA	17	46	2.7	<0.001	182979	ribosomal protein L12
CTGTTGGTGA	12	31	2.6	0.003	3463	ribosomal protein S23
AAGACAGTGG	26	63	2.4	<0.001	184109	ribosomal protein L37a
TTGGTCCTCT	46	108	2.3	<0.001	108124	ribosomal protein L41
CTCCTCACCT	12	28	2.3	0.008	119122	60S ribosomal protein L13A
AATAGGTCCA	22	50	2.3	<0.001	113029	ribosomal protein S25
ACTCCAAAAA	23	46	2.0	0.004	133230	ribosomal protein S15
CTGGGTTAAT	47	87	1.9	<0.001	126701	ribosomal protein S19
TCAGATCTTT	38	74	1.9	<0.001	75344	ribosomal protein S4, X-linked
AGCTCTCCCT	38	56	1.9	0.003	82202	ribosomal protein L17
TAATAAAGGT	45	78	1.7	0.002	118690	ribosomal protein S8
TTCAATAAAA	56	89	1.6	0.004	177592	ribosomal protein, large, P1
Additional tags with *P* values >0.01 and ⩽0.05						
AAGGTCGAGC	1	8	8.0	0.022	184582	ribosomal protein L24
CTCGAGGAGG	0	6	>6	0.016	3254	ribosomal protein L23-like
GCTCCGAGCG	0	5	>5	0.028	80617	ribosomal protein S16
TACAAGAGGA	5	16	3.2	0.014	174131	ribosomal protein L6
CCATTGCACT	7	17	2.4	0.032	53798	ESTs, highly similar to 60S RP L18A
CGCCGGAACA	12	27	2.3	0.012	286	ribosomal protein L4
ATTATTTTTC	8	18	2.3	0.037	153	ribosomal protein L7
CAATAAATGT	34	53	1.6	0.027	179779	ribosomal protein L37
CCAGAACAGA	40	60	1.5	0.030	111222	ribosomal protein L30
GCATAATAGG	38	55	1.4	0.048	184108	ribosomal protein L21

Data obtained from the comparison of the SAGE libraries of the transfected neuroblastoma cell lines (SHEP-2 versus SHEP-21N). The transcripts are ordered by fold induction. *P* values were calculated by Monte Carlo simulations according to the SAGE 300 software package (see Materials and methods; Velculescu *et al*., 1995). Tag frequencies are given for the total SAGE libraries of SHEP-2 and SHEP-21N, ~21 000 tags each.

Table III. Downstream targets induced by N-*myc*: protein synthesis, protein degradation and ribosome biogenesis

Tag sequence	SHEP-2	SHEP-21N	Fold induction	*P* value	Unigene Hs.	Gene
GCATAGGCTG	0	12	>12	<0.001	198304	Tu translation elongation factor, mitochondrial (tufM)
GCCCAGCTGG	1	11	11.0	0.003	223241	translation elongation factor 1δ (EEF1δ)
TGTGTTGAGA	12	111	9.3	<0.001	181165	eukaryotic translation elongation factor 1α1 (EEF1α1)
TGGGCAAAGC	5	39	7.8	<0.001	2186	eukaryotic translation elongation factor 1γ (EEF1γ)
CAGTCTAAAA	0	8	>8	0.004	76118	ubiquitin C-terminal esterase L1 (ubiquitin thiolesterase)
GAGCGGGATG	0	8	>8	0.004	77060	proteasome subunit 6 (β type)
GGCTCCCACT	3	16	5.3	0.002	74335	90-kDa heat-shock protein (HSP90)
GCATTTAAAT	86	138	1.6	<0.001	261802	eukaryotic translation elongation factor 1β (EEF1β)
AGCACCTCCA	22	66	3.0	<0.001	75309	eukaryotic translation elongation factor 2
TGAAATAAAA	26	55	2.1	<0.001	173205	nucleophosmin (B23)
Additional tags with *P* values > 0.01 and ≤0.05						
CTGGCGAGCG	1	9	9.0	0.011	174070	human ubiquitin carrier protein (E2-EPF)
GGGGCAGGGC	1	8	8.0	0.022	119140	eukaryotic translation initiation factor 5A
GGCCCTGAGC	2	11	5.5	0.012	71618	human RNA polymerase II subunit (hsRPB10)
TACCAGTGTA	0	5	>5	0.028	79037	60-kDa heat-shock protein 1 (HSP60)
TGGCTAGTGT	2	10	>5	0.019	118065	proteasome subunit, β type, 7
TACAAAACCA	1	4	4.0	0.202	79110	nucleolin[a]
GTTTTTGCTT	3	8	2.7	0.110	79110	nucleolin[a]
CAGATCTTTG	3	11	3.7	0.029	119502	proteasome subunit, α type, 7
TCACAAGCAA	4	15	3.8	0.010	146763	αNAC mRNA
CGCCGCGGTG	5	16	3.2	0.014	4835	eukaryotic translation initiation factor 3, subunit 8 (eIFs8)
GTGACAGAAG	5	13	2.6	0.047	129673	eukaryotic translation initiation factor 4A, isoform 1
CCATTGCACT	7	17	2.4	0.032	173694	ESTs, highly similar to probable ubiquitin C-terminal hydrolase
AACTAAAAAA	71	97	1.4	0.028	3297	ubiquitin

Genes are listed by fold induction.
[a]Two reliable tags were found for this gene due to alternative polyadenylation.

eIF3s8, EEF1α1 and tuFM (Figure 1). These data further support a role for N-*myc* as a regulator of protein synthesis.

Genes involved in routing, folding and degradation of proteins were also up-regulated. The nascent polypeptide-associated complex α (NAC) mRNA was induced (Table III, lower part). NAC protects nascent cytosolic proteins from translocation to the endoplasmatic reticulum (Wiedmann *et al*., 1994). Induction of the chaperones HSP60 (from 0 to 5 tags) and HSP90 (from 3 to 12 tags) further suggested an increased cellular capacity for protein folding and maturation. Additionally, the cellular capacity for protein degradation was possibly induced. Three ubiquitin pathway proteins (ubiquitin, ubiquitin C-terminal esterase L1 and ubiquitin carrier protein) and three proteasome subunits (β type 6, β type 7 and α type 7) showed increased tag frequencies. Northern blot analysis confirmed induction of HSP60, proteasome subunit β type 6 and ubiquitin in SHEP-21N (Figure 1).

Up-regulation of glycolysis genes

Another functional group of N-*myc*-induced genes encoded key enzymes in the glycolytic pathway (Table IV, upper part). Tags for aldolase A fructose-bisphosphate (ALDOA), triosephosphate isomerase 1 (TPI1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase are all increased (Table IV). A series of other metabolic enzymes was also induced. Northern blot analysis confirmed the mRNA induction of ALDOA, pyruvate kinase, TPI1 and GAPDH (Figure 1).

N-myc activates downstream targets within 4 h

In several systems it was observed that c-myc can induce cell growth and cell mass. The finding that many genes with a role in protein synthesis are induced in SHEP-21N therefore raises the question of whether their up-regulation is an indirect and late effect consequent on N-myc-induced growth or whether these genes are early targets of induction by N-myc and the cause of myc-mediated cell growth. We therefore tested in a time course experiment whether the genes of the protein synthesis machinery are early or late targets of N-myc-mediated induction. N-*myc* expression can be switched off reversibly in SHEP-21N cells by tetracycline. SHEP-21N cells were treated for 24 h with tetracycline. Northern blot analysis showed that the N-*myc* mRNA expression is switched off within 8 h of tetracycline treatment (Figure 3A, lanes 1–3). After 24 h, cells were washed and grown for an additional 2–36 h without tetracycline. N-*myc* mRNA expression is restored between 2 and 4 h after tetracycline removal (Figure 3A, lanes 5 and 6). Western blot analysis showed that N-myc protein expression closely follows N-*myc* mRNA expression (Figure 3B). The northern blot filter was hybridized with probes for the N-myc downstream targets nucleolin, nucleophosmin and the ribosomal protein genes RPS6 and RPS12 (Figure 3A). After repression of N-*myc* by tetracycline, the mRNA levels of these genes were unaffected at 0 and 8 h, but were reduced to low basic levels at 24 h. Within 2–4 h after re-expression of N-*myc* mRNA and protein, the mRNA expression of all four genes was strongly re-induced (Figure 3B, lanes 6 and 7). Similar results were obtained for EEF1A1, TPI1 and eIF3s8 (data not shown). The expression level of cofilin that we used as a control does not change significantly during the time course. To exclude a direct effect of tetracycline on nucleolin or nucleophosmin expression, we conducted the same experiment with SHEP-2 cells, but no

Table IV. Downstream targets induced by N-*myc*: glycolysis

Tag sequence	SHEP-2	SHEP-21N	Fold induction	*P* value	Unigene Hs.	Gene
GCGACCGTCA	1	14	14.0	<0.001	183760	aldolase A fructose-bisphosphate (ALDOA)
TAGCTTCTTC	0	7	>7	0.008	76392	aldehyde dehydrogenase 1, soluble[a]
TCTGCTTGTC	0	5	>5	0.028	76392	aldehyde dehydrogenase 1, soluble[a]
TGGCCCCACC	3	18	6.0	<0.001	198281	pyruvate kinase
TGAGGGAATA	4	21	5.3	<0.001	83848	triosephosphate isomerase 1 (TPI1)
TACCATCAAT	17	59	3.5	<0.001	195188	glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Additional tags with *P* value >0.01 and ⩽0.05						
TGACTGAAGC	0	5	>5	0.028	3343	3-phosphoglycerate dehydrogenase mRNA
CGGCTGAATT	0	5	>5	0.028	75888	ESTs, highly similar to 6-P-gluconate dehydrogenase, decarboxylating
ACCTTGTGCC	0	5	>5	0.028	878	sorbitol dehydrogenase

Transcripts are listed by fold induction.
[a]Two reliable tags were found for this gene.

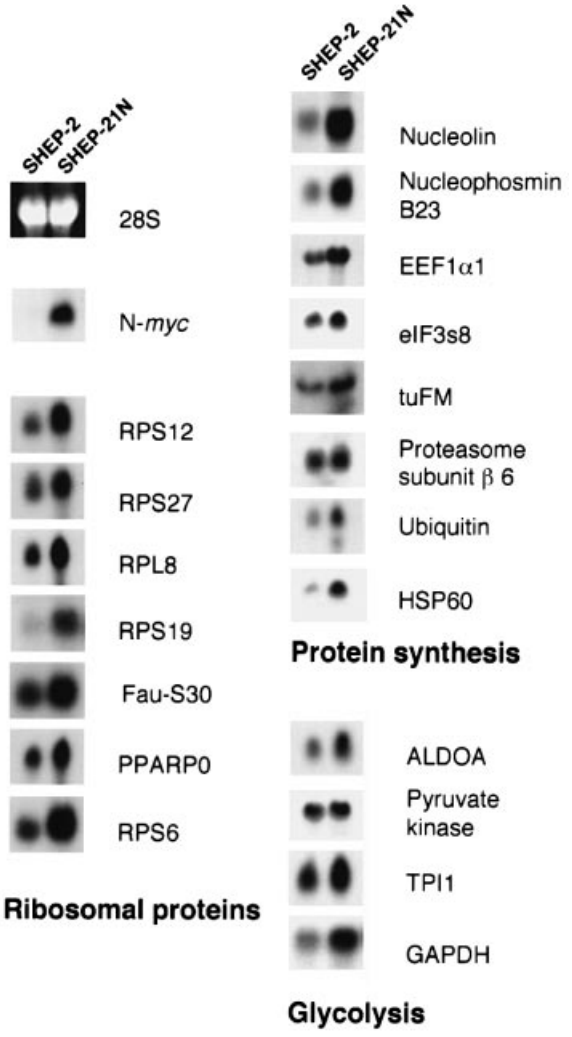

Fig. 1. Northern blot analysis of N-myc downstream target genes. Equal amounts of total RNA from exponentially growing SHEP-2 and SHEP-21N cells were loaded. Northern blots were hybridized with probes for the 19 indicated N-myc targets. RNA quantification was checked by ethidium bromide staining; the 28S band is shown.

effect on gene expression was observed (data not shown). Fluorescence activated cell sorting (FACS) analysis of SHEP-21N at 0 and 24 h of tetracycline treatment and at 7.5 h after tetracycline removal showed no change in cell volume, formally excluding the possibility that induction of the genes of the protein synthesis machinery is a result of increased cell volume (data not shown). These results show that the genes of the protein synthesis machinery are early targets in the N-myc downstream pathway, although not necessarily direct targets of N-myc. Induction of these genes by N-myc is highly versatile: expression drops after N-myc abrogation and is restored swiftly after N-myc re-expression.

Effect at the protein level and induction of rRNA synthesis

To analyze further the effect of N-myc on the protein synthesis machinery, we analyzed the protein levels of nucleolin, nucleophosmin, EEF1γ and EEF1β by western blotting. All four proteins are more strongly expressed in SHEP-21N cells compared with SHEP-2 (Figure 4, lanes 1 and 2). As a further test that these expression levels are controlled by N-myc, we treated the SHEP-21N cells for 1–8 days with tetracycline, which suppressed N-*myc* expression (Figure 4, lanes 3–8). After 2–3 days, the high protein expression levels of the four N-myc-induced genes dropped to the basic expression level observed in SHEP-2 cells. Regulation of the mRNA level of these genes by N-myc is therefore effective at the protein level. Finally, when tetracycline was washed away after 8 days and cells were cultured for 1 or 7 days without tetracycline, N-myc expression was restored and expression levels of the four target proteins were increased (Figure 4, lanes 9 and 10).

The induction by N-*myc* of nucleolin and nucleophosmin, two genes with a key role in rRNA processing and ribosome biogenesis, urged us to analyze whether SHEP-21N cells have a higher rRNA content than SHEP-2 cells. Total RNA was isolated from 10 samples of 10^6 cells of SHEP-2 and SHEP-21N. Spectrophotometric analysis showed that SHEP-21N cells on average have a 45% higher yield than SHEP-2 cells (P <0.001, Student's t-test for independent samples; Figure 4B). Triplicate experiments on independently cultured cells gave the same results. Densitometric quantification of the 18S and 28S rRNA bands fractionated on agarose gels confirmed that this increase is caused by rRNA (data not shown).

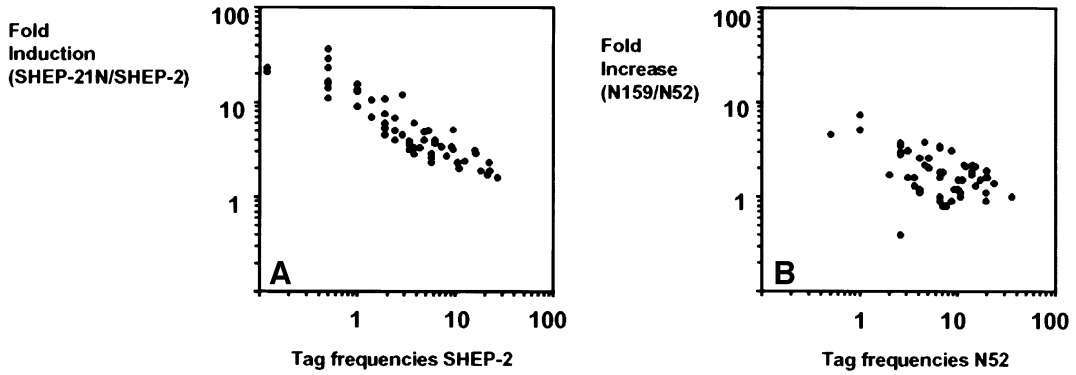

Fig. 2. Level of induction of the 56 ribosomal protein genes identified as N-myc targets (P <0.01) in SHEP-21N cells. (**A**) Fold induction by N-myc in SHEP-21N cells as a function of the basic expression levels in SHEP-2. *x*-axis, basic expression level in SHEP-2 cells normalized per 10 000 tags; *y*-axis, fold induction in SHEP-21N cells. (**B**) Increase in the same 56 ribosomal protein genes in the N-*myc*-amplified neuroblastoma N159 as a function of the basic expression level in N-*myc* single-copy neuroblastoma N52. *x*-axis, expression level in N52 normalized per 10 000 tags; *y*-axis, fold increase in N159 relative to N52.

We also measured protein content and the rate of protein synthesis. Lysates of 10^6 SHEP-2 and SHEP-21N cells contained equivalent amounts of total protein (data not shown). Protein synthesis rates were analyzed by [^{35}S]methionine incorporation, but no differences were observed between SHEP-2 and SHEP-21N cells. Also, when N-*myc* expression was switched off by a 48 h tetracycline treatment, no differences in incorporation could be observed (data not shown). N-myc therefore strongly induces the rRNA content of SHEP-21N cells, but not the protein synthesis rate. The protein synthesis in SHEP-21N cells may be limited by a factor not induced by N-myc, or may have been maximal already in the SHEP-2 cells and beyond a level that can be boosted by N-myc.

SAGE libraries of neuroblastomas with and without amplification of endogenous N-myc

To analyze whether genes of the protein synthesis machinery are also induced in neuroblastomas with N-*myc* amplification, we generated SAGE libraries of two neuroblastomas. Neuroblastoma N159 has N-*myc* amplification and expression, and neuroblastoma N52 is an N-*myc* single-copy tumor without N-*myc* expression (Figure 5B, lanes 9 and 10). We sequenced 39 598 tags of the two libraries (Table I). The tag frequencies were normalized per 20 000 tags and compared. N-*myc* was represented by 16 tags in N159 and 0 tags in N52. There are 52 tags differentially expressed (P <0.01) in the libraries. We analyzed which of the N-myc target genes identified in the SHEP cells correlated with N-myc in the two tumors. The 56 significantly (P <0.01) induced ribosomal protein genes detected in SHEP-21N cells produce a total of 988 tags in N52 and 1600 tags in N159 (per 20 000 tags). The N-*myc*-amplified N159 tumor therefore has 62% higher ribosomal protein gene expression (Figure 2B). This strongly suggests that N-myc induces ribosomal protein gene expression *in vivo*. Other genes functioning in protein synthesis are also up-regulated. Increased expression in N159 compared with N52 is seen for nucleophosmin (from 4 to 19 tags), nucleolin (from 3 to 9 tags), eukaryotic translation initiation factor 4A, isoform 1 (from 4 to 8 tags) and the translation elongation factors EEF1α1 (from 50 to 98 tags) and EEF1γ (from 18.4 to 31 tags). There is almost no induction of the genes involved in glycolysis. The expression levels of nucleolin, nucleophosmin and ribosomal protein S6 were confirmed by hybridization of northern blots with total RNA from N159 and N52 (Figure 5B). These results show that the expression levels of many of the N-myc target genes identified in SHEP-21N cells also correlate *in vivo* with N-*myc* amplification and overexpression.

N-myc target gene expression in neuroblastoma cell lines and tumors

We further analyzed the expression of N-myc downstream genes in a panel of neuroblastoma cell lines and tumors. Hybridization of a northern blot of total RNA from 21 neuroblastoma cell lines showed a fair correlation between expression of N-*myc*, nucleolin, nucleophosmin and the ribosomal protein PPARP0 (Figure 5A). Cell line SJNB12 shows high expression of the N-myc target genes (Figure 5A, lane 7). This cell line has no N-*myc* expression, but has c-*myc* amplification and overexpression (Cheng *et al.*, 1995), suggesting that c-myc may induce the same target genes as N-myc (see below).

As cell lines are not fully representative of neuroblastoma tumors *in vivo*, we analyzed 16 fresh neuroblastomas of all stages. A northern blot analysis showed a rather good overall correlation between expression of N-*myc*, nucleolin and nucleophosmin (Figure 5B). There are some exceptions, but the results suggest that nucleolin and nucleophosmin are also *in vivo* targets of N-myc induction. Ribosomal protein S6 (RPS6) expression showed a less consistent relationship with N-*myc*, indicating that besides N-myc, other factors may also modulate its expression.

Several N-myc target genes are also induced by c-myc

N-*myc* belongs to the same family of proto-oncogenes as c-*myc*. Since N-*myc* can replace c-*myc* in transgenic mice without inducing gross phenotypic defects (Malynn *et al.*, 2000), and since both myc proteins share the same target recognition sequence, we analyzed whether the N-myc downstream targets identified in this study are also

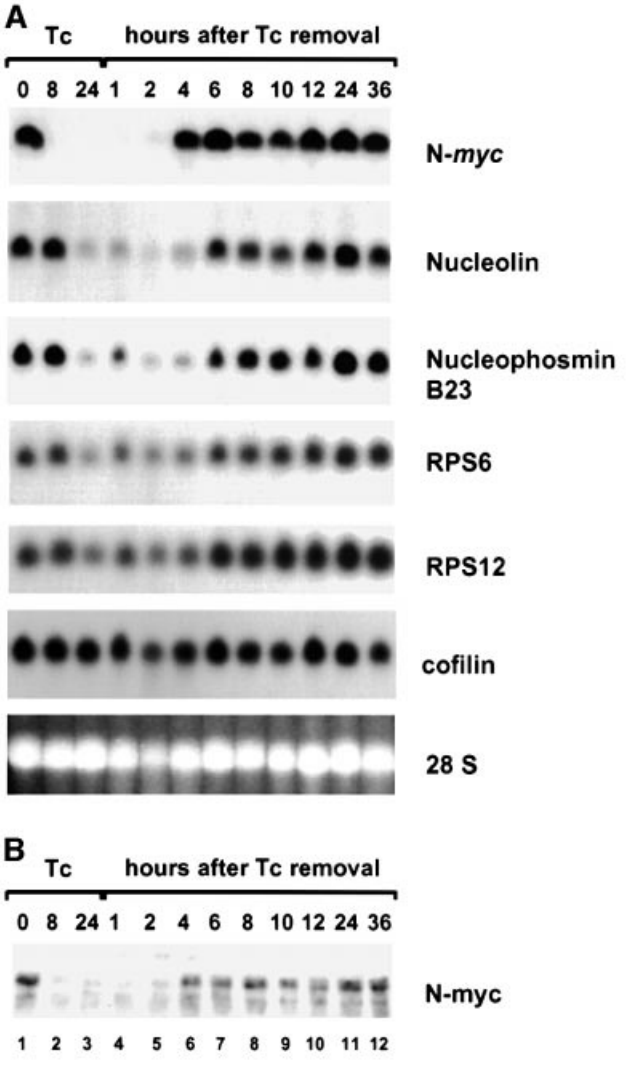

Fig. 3. Time course analysis of N-*myc* and downstream target gene induction in SHEP-21N cells. SHEP-21N cells were treated for 24 h with 10 ng/ml tetracycline, washed and grown for an additional 36 h without tetracycline. Cells were harvested at 0, 8 and 24 h of tetracycline treatment. Subsequent samples were taken at 1, 2, 4, 8, 10, 12, 24 and 36 h after removal of the antibiotic. (**A**) Northern blot analysis of total RNA at the indicated time points. (**B**) Western blot analysis of N-myc protein at the indicated time points. A 10 µg aliquot of total protein samples of the time course experiment were fractionated through a 10% SDS–polyacrylamide gel, blotted on an Immobilon membrane and probed with a monoclonal anti-N-myc antibody.

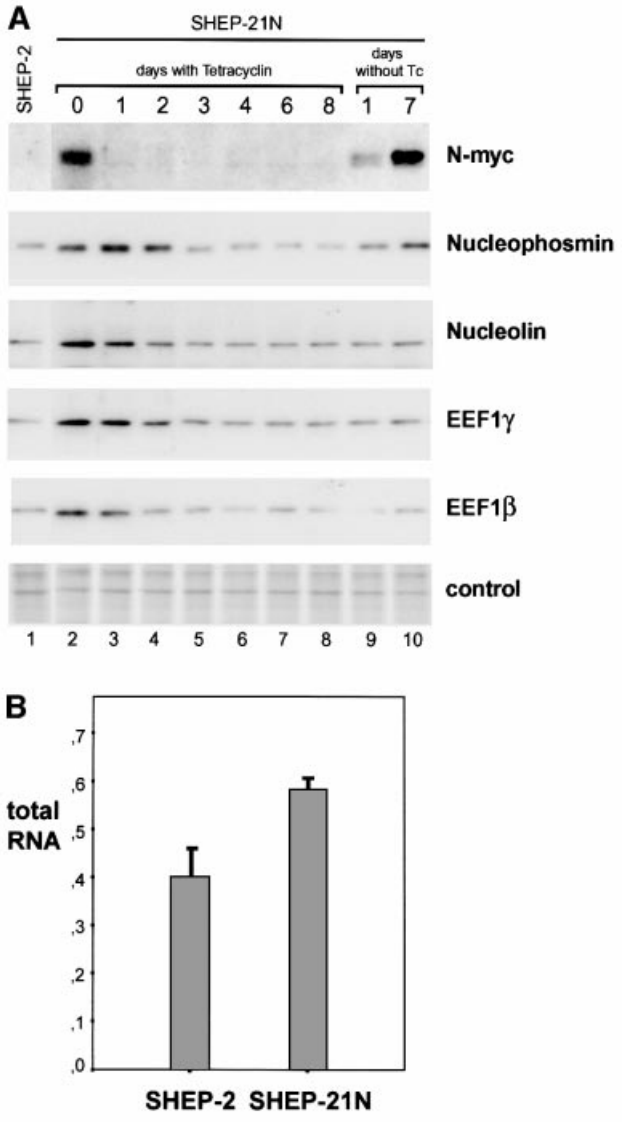

Fig. 4. Expression of N-myc, nucleolin, nucleophosmin, translation elongation factors EEF1γ and EEF1β and total RNA content of SHEP-2 and SHEP-21N cells. (**A**) Western blot analysis. Total cell extracts (10 µg) were fractionated through an acrylamide gel, blotted and probed with monoclonal antibodies against N-myc and nucleophosmin, and with polyclonal antibodies against nucleolin, EEF1γ and EEF1β. SHEP-21N cells were treated for 0–8 days with tetracycline (lanes 2–8) and subsequently cultured for 1 or 7 days without tetracycline (lanes 9 and 10). A Coomassie Blue staining is shown as control for loading. (**B**) Total RNA content of SHEP-2 and SHEP-21N cells. RNA was isolated from 10 samples of 10^6 cells of each cell line and analyzed spectrophotomerically. Error bars give the SD.

induced by c-*myc*. We analyzed the melanoma cell line IGR39D and a c-*myc*-transfected clone of this cell line (clone 3; Versteeg *et al*., 1988). Northern blots with total RNA of these cell lines were hybridized with the 19 probes tested on the SHEP-2 and SHEP-21N cells. Eight of the N-*myc* targets appeared to be induced by c-*myc* as well (Figure 6). They are the ribosomal protein genes S12, S27, S19 and S6, and nucleolin, nucleophosmin, ubiquitin and GAPDH. The remaining 11 genes showed no induction by c-*myc*. Therefore, c-*myc* and N-*myc* share >40% of their target genes in the cell systems tested here. Interestingly, nucleophosmin, nucleolin and most ribosomal protein genes are among them.

Discussion

One of the surprising aspects of *myc* oncogenes is their multitude of phenotypic effects. They are known to induce growth, cell division, metastasis and apoptosis. A series of target genes of myc transcription factors has been identified, some of which can be related to specific phenotypes. However, our knowledge of myc target genes is probably still fragmentary and insufficient to explain the full range of phenotypes. As a step towards a complete inventory of the myc downstream pathway, we applied the

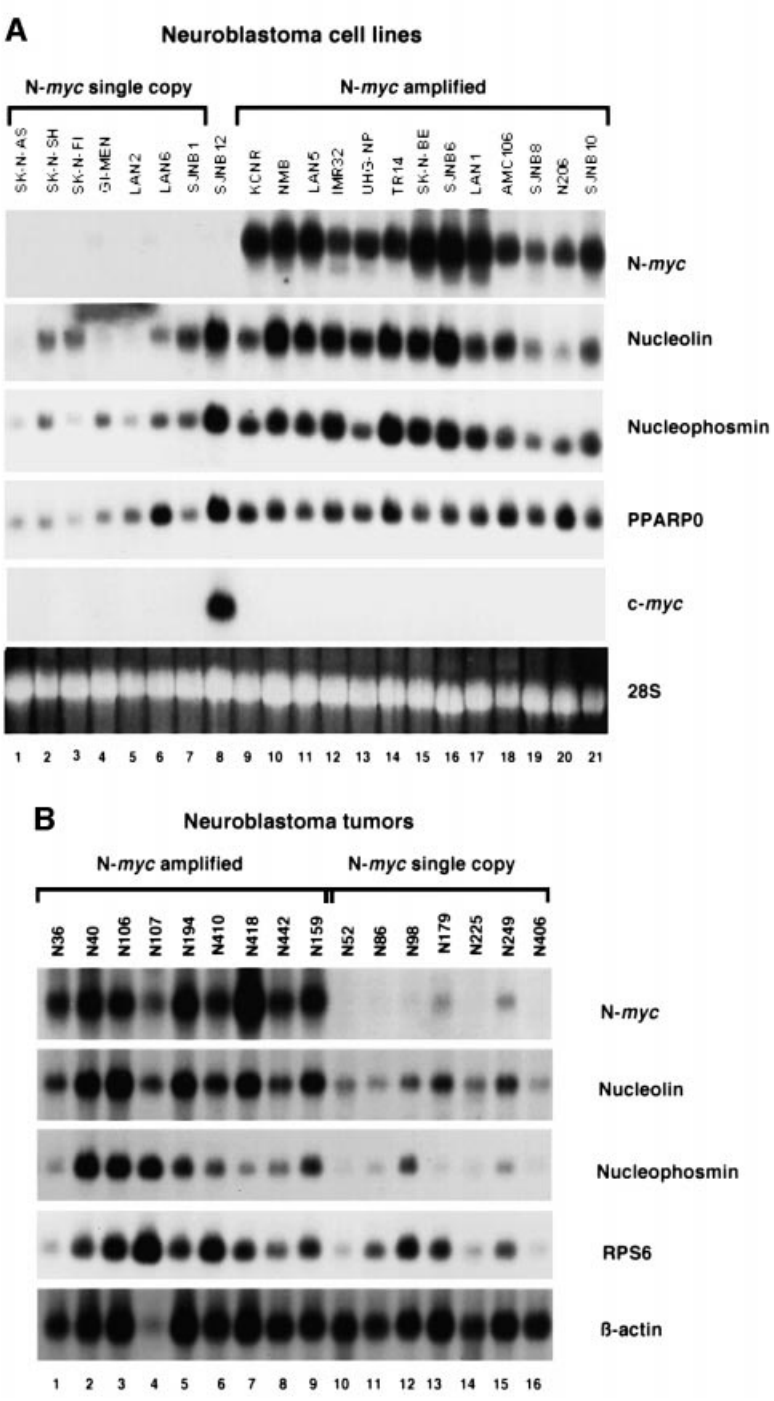

Fig. 5. Northern blot analysis of total RNA from neuroblastoma cell lines and tumors. Filters were hybridized with the indicated probes. RNA quantification was checked by ethidium bromide staining; the 28S band is shown. (**A**) Panel of 21 neuroblastoma cell lines. (**B**) Panel of 16 fresh tumors. Tumors in lanes 1–9 are N-myc amplified.

SAGE technology to N-*myc*-transfected cells. We have chosen to compare SHEP-21N with SHEP-2 cells, as we aimed to identify all genes that are up-regulated in a situation of stable and enduring N-myc expression, rather than in a transition period after induction of N-myc. SAGE provides an integral gene expression profile of a tissue or cell line. Comparison of the SAGE libraries of the N-myc-expressing SHEP-21N and control SHEP-2 cells identified 114 genes significantly (P <0.01) induced in SHEP-21N. Moreover, since SAGE is quantitative, the libraries permit the analysis of the full myc-induced transcription shift. N-myc turns out to have a massive effect on genes with a role in protein synthesis. Approximately 80% of ribosomal protein genes turned out to be enhanced, as well as some key genes in rRNA maturation and ribosome assembly. Furthermore, expression of many translation initiation and elongation factors is considerably enhanced. We detected induction of 89 genes involved in protein synthesis (Tables II and III). Together, they produced 1119 of the 20 950 sequenced tags in SHEP-2. These 89 genes

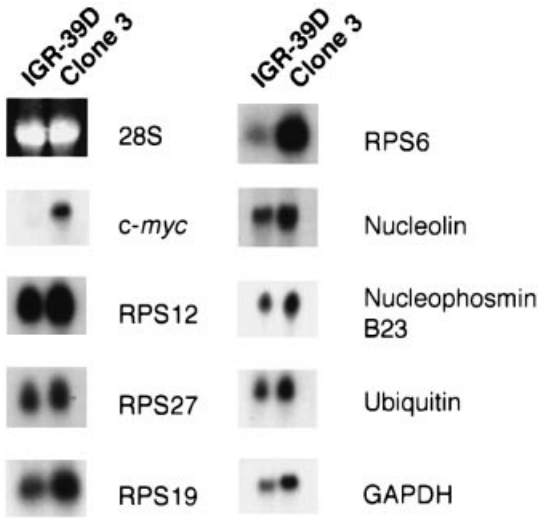

Fig. 6. Northern blot analysis of induction of N-myc target genes in a c-myc-transfected melanoma cell line. Clone 3 is a c-myc-transfected clone of the IGR39D melanoma cell line. Equal amounts of total RNA of IGR39D and clone 3 were loaded. Filters were hybridized with the indicated probes.

therefore contributed 5.3% of the total number of transcripts in SHEP-2. In SHEP-21N, these genes give rise to 3327 transcript tags, or 15.9% of all transcripts. Two of the up-regulated genes, nucleolin and nucleophosmin, function in rRNA maturation and ribosome assembly (reviewed in Ginisty *et al*., 1999). We therefore analyzed whether N-myc expression results in higher rRNA levels. We found a striking 45% higher rRNA content in SHEP-21N than in SHEP-2 cells on a per cell basis. Somewhat surprisingly, there was no overall increase in the rate of protein synthesis in SHEP-21N cells. One interpretation is that some rate-limiting components of the protein synthesis machinery are not induced in SHEP-21N cells. Alternatively, protein synthesis may already have been maximal in SHEP-2, beyond a level that can be boosted further.

Crucial to the interpretation of these data is the finding that genes of the protein synthesis machinery are early targets of the N-myc pathway. Activation of N-*myc* results within 2–4 h in full induction of expression of the tested target genes, amongst which were nucleolin, nucleophosmin, two ribosomal protein genes, a translation initiation factor and a translation elongation factor. This leads us to the conclusion that the massive induction of genes of the protein synthesis pathway is an early effect of N-myc. However, we have not addressed the question of whether these genes are direct targets of N-myc or, in contrast, part of a hierarchical pathway with myc at the top. Although nucleolin was identified previously as a direct target of c-myc (Greasley *et al*., 2000), the time course experiments do not exclude the possibility that other genes of the protein synthesis machinery are induced by an intermediary transcription factor that is up-regulated by N-myc. The quantitative character of SAGE enabled a further analysis of the induction. All N-myc downstream targets have a fair basal expression level in SHEP-2 cells, which is not surprising in view of their essential role in protein synthesis. Interestingly, the ribosomal protein genes do not show an equal overall induction by, for example, a factor of two or three, but induction appears to be related to the basic level of expression in SHEP-2. The strongest induction is observed for genes with the weakest basal expression level (Figure 2). This could suggest that the ribosomal protein genes in SHEP-2 cells are restricted in their expression to a variable extent and that N-myc can relieve this restriction.

Induction of genes of the protein synthesis machinery is likely to be a general effect of N-myc in neuroblastomas. Comparison of SAGE libraries of neuroblastoma cell lines with and without N-*myc* amplification shows a 62% increase of ribosomal protein gene transcripts in N-myc-expressing cells. Moreover, northern blot analysis of 37 neuroblastomas and neuroblastoma cell lines showed an overall induced expression level of nucleolin, nucleophosmin and ribosomal protein genes in N-*myc*-amplified cases (Figure 5). In addition, c-myc was found to induce a series of N-myc target genes as well. Of the 19 targets of N-myc that we tested on northern blots, eight were induced in melanoma cells with ectopic expression of c-myc. Amongst them are the ribosomal protein genes S12, S27, S19 and S6, and nucleolin and nucleophosmin. These data suggest that induction of the protein synthesis machinery is a major function of both c-myc and N-myc.

Myc genes are general inducers of the protein synthesis machinery

Our data are well in line with some of the recently identified target genes of c-myc and with phenotypic effects of myc observed *in vivo* and *in vitro*. Early analyses identified two translation initiation factors as targets of c-myc (Rosenwald *et al*., 1993; Jones *et al*., 1996), while recent microarray analyses revealed induction by c-myc of two more translation initiation factors, nucleolin and one ribosomal protein gene (RPS11) (Coller *et al*., 2000). Nucleolin was also identified as a c-myc target by Greasley *et al*. (2000). The microarray analyses of Coller *et al*. (2000) do not reveal induction of 32 other ribosomal protein genes that were represented on their chips. However, it was observed recently that 4 days after *in vivo* transduction of a c-myc-expressing retrovirus in mouse, liver cells expressing ectopic c-myc are greatly enlarged and have increased expression of six ribosomal protein genes as well as nucleolin and nucleophosmin (Kim *et al*., 2000). Together with our SAGE analyses in which the qualitative and quantitative induction of genes of the protein synthesis machinery were established, these data implicate the protein synthesis machinery as a major target of induction by myc proteins. These findings are in agreement with phenotypic effects of *myc* genes observed in several experiments (Schmidt, 1999). Rat fibroblasts with inactivated c-*myc* alleles showed a slower growth rate and a reduced protein synthesis rate (Mateyak *et al*., 1997), while induction of protein synthesis in fibroblasts was observed after c-*myc* activation (Schmidt, 1999). B cells with ectopic c-*myc* expression in transgenic mice are larger at any stage of differentiation and have an increased protein synthesis rate (Iritani and Eisenman, 1999). *Drosophila* with a mutated d*myc* grow more slowly and only attain a tiny body volume (Johnston *et al*., 1999). The data in animal model systems, in normal fibroblasts and in neuroblastoma tumor cells all suggest that induction of the

protein synthesis machinery is a major function of *myc* genes. In addition to their direct effect on the cell cycle by inducing cyclin D2 and Id2, this induction of the protein synthesis machinery may provide the increase in cell mass required to keep the cell volume in step with proliferation.

Materials and methods

Cell lines

Neuroblastoma cell lines and culture conditions were as described before (Cheng *et al*., 1995). The melanoma cell lines IGR39D and clone 3 were described earlier (Versteeg *et al*., 1988). The SHEP cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Lutz *et al*., 1996). Tetracycline (Sigma) was used at a concentration of 10 ng/ml to inhibit N-*myc* expression.

Generation of SAGE libraries

SAGE was performed as described (Velculescu *et al*., 1995) with minor adaptations. Total RNA was extracted by guanidium thiocyanate (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated using the MessageMaker kit (Gibco-BRL) according to the manufacturer's instructions. SAGE libraries were generated using minimally 4 μg of poly(A)$^+$ RNA. The cDNA was synthesized according to the Superscript Choice System (Gibco-BRL), digested with *Nla*III and bound to streptavidin-coated magnetic beads (Dynal). Linkers containing recognition sites for *Bsm*FI were ligated to the cDNA. Linker tags including a cDNA tag were released by *Bsm*FI digestion, ligated to one another and amplified. The PCR products were heated for 5 min at 65°C before preparative analysis on a polyacrylamide gel. After the ligation into concatamers, a second heating step was included (15 min at 65°C) and fragments between 800 and 1500 bp were purified and cloned in pZero-1 (Invitrogen). Colonies were screened with PCR using M13 forward and reverse primers. Inserts >300 bp were sequenced with a BigDye terminator kit and analyzed on a 377 ABI automated sequencer (Perkin Elmer).

Analysis of the SAGE database

The SAGE libraries were analyzed using the SAGE 300 program software package (Velculescu *et al*., 1997). *P* values were calculated using Monte Carlo simulations. Transcripts were identified by comparison of the tags in the database with the 'tag to gene map' (SAGEmap) from the Cancer Genome Anatomy Project at the NCBI (http://www.ncbi.nlm.nih.gov/SAGE). This database links Unigene clusters to SAGE tags (Lal *et al*., 1999). The gene assignments were subsequently checked by hand for sequencing errors causing incorrect tags and for erroneous gene assignments based on hybrid Unigene clusters. Other database analyses and generation of specific primers utilized the Wisconsin GCG package software.

Northern blot analysis

Total RNA (20 μg per lane) was electrophoresed through a 0.8% agarose gel in the presence of 6.7% formaldehyde and blotted on Hybond N membranes (Amersham) in 10× SSC. Hybridization was carried out overnight in 0.5 M $NaHPO_4$ pH 7.0, 7% SDS, 1 mM EDTA at 65°C. Filters were washed in 40 mM $NaHPO_4$, 1% SDS at 65°C. Probes were labeled by random priming of sequence-verified PCR products. A complete list of all the primers used in RT–PCRs is available on request.

Total protein content

Exponentially growing cells were harvested and cell number was determined using a Coulter counter. Cells (1×10^6) were lysed in 20 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40 and protease inhibitors (protease cocktail, Roche). Samples were assayed with the Bio-Rad Protein assay. Assays were performed at least in duplicate.

Western blots

Cell lysates were separated on an SDS–polyacrylamide gel and electroblotted onto Immobilon-P transfer membrane (Millipore). Blocking of the membrane and incubation with antibodies involved standard procedures. Proteins were visualized using the ECL detection system (Amersham). Anti-nucleophosmin monoclonal antibody was a gift of Dr P.K.Chan (Baylor College of Medicine). The antibody against nucleolin was a gift of Dr P.Bouvet (CNRS, IPBS, Toulouse, France). Anti-N-myc was obtained from PharmIngen (Clone B8.4.B). Rabbit anti-human EEF1γ and anti-human EEF1β antibodies (Sanders *et al*., 1996) were a gift of Dr J.Dijk (Sylvius Laboratories, LUMC, Leiden, The Netherlands).

Total rRNA content

Total RNA of 1×10^6 exponentially growing cells was extracted by guanidium isothiocyanate (Chomczynski and Sacchi, 1987) and quantified spectrophotometrically. Results of 10 isolations of each of the cell lines SHEP-2 and SHEP-21N were statistically analyzed with the Student's *t*-test for independent samples. Aliquots on a per cell basis were subjected to agarose gel electrophoresis and stained with ethidium bromide. The relative fluorescence of the rRNA bands was quantified using the Kodak Digital Science 1D Image Analysis Software package (EDAS 120).

FACS analysis

SHEP-21N cells treated or not treated with tetracycline were trypsinized, stained with propidium iodine and analyzed on a Beckman FACScan flow cytometer. Forward scatter (FSC) was used as a means for cell mass. FSC was measured for the total cell population or for the G_0/G_1 fraction, and did not differ for SHEP-21N cells treated for 0 or 24 h with tetracycline and for cells subsequently cultured for 7.5 h without tetracycline.

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