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The transcriptional program of a human B cell line in response to Myc

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ABSTRACT

The proto-oncogene c-*myc* (*myc*) encodes a transcription factor (Myc) that promotes growth, proliferation and apoptosis. Myc has been suggested to induce these effects by induction/repression of downstream genes. Here we report the identification of potential Myc target genes in a human B cell line that grows and proliferates depending on conditional *myc* expression. Oligonucleotide microarrays were applied to identify downstream genes of Myc at the level of cytoplasmic mRNA. In addition, we identified potential Myc target genes in nuclear run-on experiments by changes in their transcription rate. The identified genes belong to gene classes whose products are involved in amino acid/protein synthesis, lipid metabolism, protein turnover/folding, nucleotide/DNA synthesis, transport, nucleolus function/RNA binding, transcription and splicing, oxidative stress and signal transduction. The identified targets support our current view that *myc* acts as a master gene for growth control and increases transcription of a large variety of genes.

INTRODUCTION

The proto-oncogene c-*myc* (*myc*) plays a key role in the regulation of cell proliferation, differentiation and apoptosis (1). *myc* is activated in response to mitogenic factors and repressed after exposure to anti-proliferative signals. Expression of *myc* is sufficient to induce proliferation in quiescent mouse fibroblasts (2). The *myc* protein (Myc) contains a basic region, a helix–loop–helix/leucine zipper motif that allows dimerization with the Max protein. Myc/Max heterodimers bind DNA at the E-box sequence motif CACGTG and related sequences, thereby modulating the transcriptional activity of genes (1,3). The detailed mechanisms of how Myc can activate or repress gene activity are not yet understood (4–6).

Previous screens in mouse and human fibroblasts expressing a fusion protein of Myc and the hormone binding domain of the estrogen receptor have identified a number of Myc target genes (reviewed in 7). Many of these genes are involved in regulation of cell metabolism and cell growth. In addition, genes important for signaling, cell cycle and cell adhesion were also identified. Among the identified genes are cdk4 (8), p21 (9) and cyclin D2 (10) for cell cycle activation, dehydrofolate reductase (dhfr) (11), ornithine decarboxylase (odc) (12) and carbamoyl-phosphate synthetase-aspartate transcarbamoylase-dihydroorotase (cad) (13) required for nucleotide/DNA synthesis, the inititaion factor eIF4E for translational control (14,15), eIF5A for nuclear export (9), nucleolin for nucleolus function (16) and genes for extracellular matrix proteins (9). Several of these genes have also been found in a more global screen using oligonucleotide microarrays for human fibroblasts (9). The wide spectrum and large number of identified genes strengthens the notion that Myc acts by directly controlling a large variety of genes involved in proliferation and growth.

As a model for cell cycle activation by *myc* in Burkitt lymphoma cells, we established the B cell line P493-6 carrying a conditional, tetracycline-regulated *myc*. In the absence of serum, Myc induces cell growth (increase of cell size) in these cells without activating the cell cycle (17). In the presence of serum, Myc activates the cell cycle in P493-6 cells with features similar to cell cycle activation by Myc in fibroblasts, however, without inducing apoptosis (17,18). In contrast to P493-6 cells, rat *myc* knock-out fibroblasts proliferate at a reduced rate in the presence of high serum (19). This indicates that growth and proliferation of fibroblasts may be less dependent on Myc function than P493-6 cells.

We have used two techniques to identify potential Myc target genes in P493-6 cells. Changes in mRNA levels upon *myc* expression were measured using high density oligonucleotide arrays. To assess changes in transcription rates, nuclear run-on RNAs were analyzed on cDNA arrays.

MATERIALS AND METHODS

Cell culture

The cell line P493-6 was established by stable transfection of EREB2-5 cells with the construct p*myc*-tet (17,18). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Life Technologies). For repression of *myc*,

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0.1 µg/ml tetracycline was added to culture medium. For reinduction of *myc*, cells were washed three times with tetracycline-free, prewarmed phosphate-buffered saline (PBS) containing 10% FCS. Uninduced cells (0 h), which were arrested by tetracycline for 72 h, served as a control for the screens and northern blots. The control cells were washed similarly to the induced cells, except that tetracycline was present in all washing solutions. All experiments were performed in the absence of EBNA2 function (without estrogen) (18).

Northern blots

Northern blot analysis was performed as described elsewhere (20). Total RNA was isolated using the RNeasy Midi Kit (Qiagen) and 10 µg were loaded per lane. Probes were generated as follows: Myc, cDNA of exons 2 and 3; Nm23-H1, accession no. X17620, PCR fragment from position 101–327; JTV-1, U24169, 185–425; KiAA0035 (p130), D21262, 504–634; EIF3, U78525, 1512–1739; RFC4, M87339, 98–329; MCM4, X74794, 1948–2192; C-ERBA-1, M24898, 1676–2070; HDGF, D16431, 359–597; DP-1, L23959, 935–1162; control, Jnk cDNA. Filters were exposed to Kodak X-Omat AR film at –80°C with intensifying screens.

High density oligonucleotide array expression analysis

The techniques for array analysis have been described elsewhere (9). In brief, total RNA was prepared using the RNeasy Midi Kit (Qiagen). Total RNA (20 µg) was used to synthesize cDNA using a T7-polyT primer and the reverse transcriptase Superscript II (Gibco BRL) according to the Gibco protocols. The cDNA was treated with 1.25 µg/µl RNase A (Roche) at 37°C for 30 min and then with 2 µg/µl proteinase K (Roche) and 0.4% SDS (Sigma) at 37°C for 30 min; subsequently a phenol extraction was performed. Approximately 1 µg of cDNA was used for an *in vitro* transcription reaction (Amersham Pharmacia, according to the provided protocols) in the presence of biotinylated UTP. Sonificated cRNA (15 µg) was used to prepare 300 µl of hybridization solution (containing final concentrations of control oligonucleotides 0.05 µg/µl; control cRNAs 1.5, 5, 25 and 100 pM; sonicated herring sperm DNA 0.1 µg/µl; acetylated BSA 0.5 µg/µl; MES sodium salt 75 mM and MES free acid 27.5 mM). The solution was hybridized for 16 h at 40°C to the oligonucleotide array Hu6800 (Affymetrix), which contains oligonucleotides representing 6800 different human genes. The arrays were washed with a non-stringent buffer (MES sodium salt 75 mM, MES free acid 27.5 mM, NaCl 26 mM and 0.01% Tween-20) at 25°C and with a stringent buffer [12× SSPE (0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA); 0.02 % Tween-20 and 0.1 g/l Antifoam O-30 (Sigma)] at 50°C according to Affymetrix protocols. The arrays were stained with streptavidin, then with biotinylated antibody followed by incubating with streptavidin phycoerythrin (SAPE) to stain the arrays according to Affymetrix protocols. The fluorescence intensities were captured with a laser confocal scanner (Hewlett Packard) and were analyzed with the GENECHIP software (Affymetrix). Genes were analyzed which were scored as 'present' and 'induced' and with a reasonable fluorescent signal (average difference >200). Detailed protocols are provided by the corresponding author.

Nuclear run-on analysis

Isolation of nuclei and nuclear run-on reactions were carried out as previously described (21) with slight modifications. Briefly, cells were spun down, washed once with PBS (4°C), resuspended in 10 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM NaCl, 0.5% (v/v) NP-40 (4°C) and incubated on ice for 5 min. The nuclei were spun down at 500 *g*, resuspended in storage buffer [50 mM Tris–HCl pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA–NaOH pH 8.0, 40% (v/v) glycerin], frozen in liquid nitrogen in portions of 100 µl corresponding to 2×10^7 nuclei and stored at –80°C. Nuclei (100 µl) were thawed on ice and then mixed with 100 µl of reaction buffer (300 mM KCl, 5 mM $MgCl_2$, 0.5 mM of each ATP, UTP, GTP) and 100 µCi of [α-^{32}P]CTP (800 Ci/mmol, 10 mCi/ml; Amersham Pharmacia Biotech); 10 mM Tris–HCl pH 8.0; 1.2% (w/v) sarcosyl and incubated for 15 min at 28°C. DNase I (5 µl, 50 U, RNase free; Roche Diagnostics) was added and the incubation continued for 10 min at room temperature. DNase treatment was repeated for 10 min and followed by treatment with proteinase K (0.65 µg/µl) for 60 min at 37°C. After isolation of nuclear transcripts by Sephadex G-50 column filtration, labeled RNA was hybridized to an ATLAS human 1.2 or a human cancer 1.2 array (Clontech, http://www.clontech.com/atlas/genelists/index.html) at 68°C for 36 h in 5 ml ExpressHyb (Clontech). ATLAS arrays were extensively washed in succession with 1% SDS, 2× SSC; 0.5% SDS, 0.1× SSC; and 1× SSC, 1 mM EDTA at 45°C. Subsequently filters were treated for 15 min with 2× SSC, 1 mM EDTA, 2 µg/ml RNase A at 30°C. RNase A treatment ensures a high specificity of signals on the arrays, because only specific, double-stranded DNA–RNA hybrids are protected from RNase A digestion. The filters were finally washed with 0.5% SDS, 0.1× SSC at 45°C. Thereafter, membranes were exposed to Kodak X-Omat AR film at –80°C with intensifying screens. Signal intensities were determined with a Phosphoimager (Fuji-BAS 1000) and by visual inspection. The ATLAS Human 1.2 Array was used in three independent experiments, the ATLAS Human Cancer 1.2 Array was used in two independent experiments, which are shown in Figures 1 and 2.

RESULTS

Screen for potential Myc target genes using oligonucleotide microarrays

The B cell line P493-6 expressing a conditional *myc* gene has been described before (17,18). P493-6 cells switch off *myc* expression in the presence of tetracycline and arrest in the G_0/G_1 phase of the cell cycle. Induction of *myc* expression by removal of tetracycline induces hyperphosphorylation of Rb, activation of cyclin-dependent kinase 2 (cdk2) and cell cycle entry of P493-6 cells without inducing apoptosis (17,18). Significant Myc protein levels are detectable after 40 min and reach a maximum 4 h after the removal of tetracycline. An increase in mRNA levels of known Myc target genes such as ODC, nucleolin and LDH-A, is observed between 4 and 8 h after Myc induction (17).

Therefore we harvested total RNA from uninduced cells (0 h) and from cells induced for 4, 8 and 24 h for microarray analysis. We reasoned that direct Myc target genes should be induced or repressed between 4 and 8 h after Myc induction.

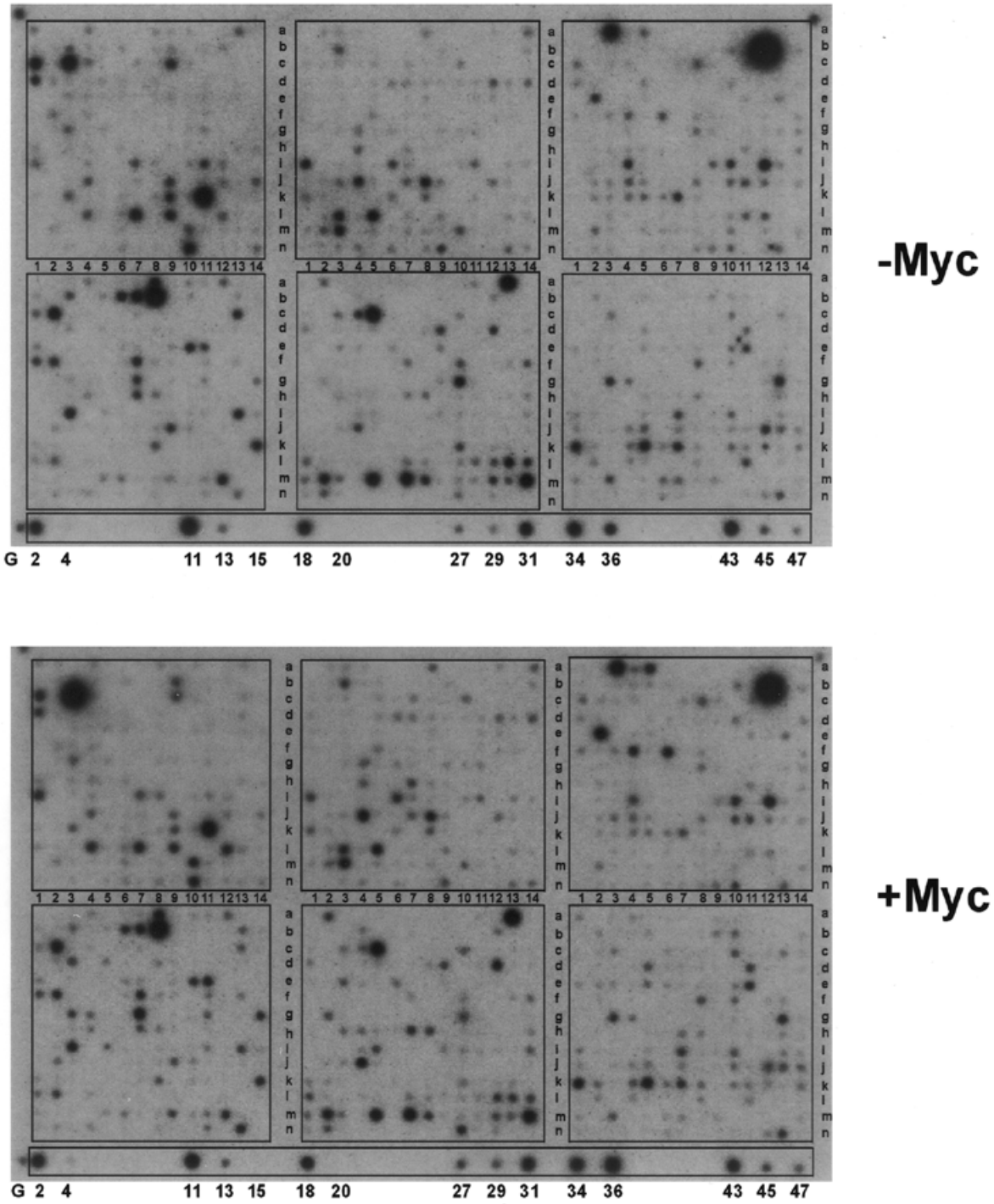

Figure 1. Transcriptional activity of Myc target genes in nuclear run-on experiments. Nuclei were prepared from arrested P493-6 cells (–Myc) and cells 4 h after induction (+Myc). Nuclear run-on reactions were performed. Labeled RNAs were purified and hybridized to ATLAS Human Cancer 1.2 arrays (Clontech). Filters were washed, RNase treated and exposed to Kodak XAR-5 films. The arrays are divided into six sections : A, B, C (upper row) and D, E, F (lower row), from left to right. The position of a gene is given with a capital letter for the section and a small letter and number for the coordinates (for example A3c for *myc*). The G row represents housekeeping genes and control DNA. A complete list of all the genes on the array with a clickable array image is given at the Clontech home page at http://www.clontech.com/atlas/genelists/index.html. Reproducible candidate genes are listed in Table 2.

Table 1 represents the results of two independent experiments. The fold changes in experiment 1 were generally a little higher compared with experiment 2 (Table 1). Therefore, we included genes in Table 1 which showed a fold induction of at least factor 3 in experiment 1 and a fold change of factor 2 in experiment 2.

Table 1. Myc regulated genes identified by oligonucleotide microarrays

Accession	Fold change exp1/exp2	Group Gene description
		Amino acid-/ protein-synthesis
D00723	11.8 / 6.9	Hydrogen carrier protein
U24169	4.3 / 2	JTV-1 [b]
D28473	3.4 / 3.5	Isoleucyl-tRNA synthetase [a]
M77836	3.2 / 4.7	Pyrroline 5-carboxylate reductase
X59303	2.8 / 3.1	Valyl-tRNA synthetase
X06323	2.7 / 3	Ribosomal protein L3 (MRL3)
		Lipid metabolism
M94856	12.4 / 5.1	Fatty acid binding protein homologue [a]
D50678	7 / 3.9	Apolipoprotein E receptor 2
D4204	3.9 / 2.9	KIAA0089 (Gpdh)
X75252	3 / 2.1	Phosphatidylethanolamine bp
S80437	3.4 / 4.9	Fatty acid synthase
U23942	3.5 / 1.8	Lanosterol 12-demethylase cytochrome P450 (CYP51)
D10040	3.4 / 2.4	Long-chain acyl-CoA synthetase
X79888	3 / 5.4	AUH (RNA binding protein with intrinsic enoyl-CoA hydratase activity
		Protein-folding /-turnover
U07550	3.1 / 2.3	Chaperonin 10
X87212	3.7 / 2.2	Cathepsin C
M30496	3.8 / 2.3	Ubiquitin carboxyl-terminal hydrolase (PGP9.5)
		Nucleotide-/ DNA-synthesis
D78586	2.8 / 3.6	CAD [a]
U00238	27.3 / 23.3	GPAT (Gln PRPP amidotransferase)
Y00971	3.8 / 7.5	PRPP synthetase subunit II
HG4157	3.1 / 2.1	Glycinamide ribonucleotide synthetase
D78335	9.4 / 34.8	UMK (UMP kinase)
L35035	9.4 / 3.2	Ribose 5-phosphate isomerase (RPI)
X52142	5.8 / 4.2	CTP synthetase
X60673	5.6 / 3.8	AK3 (adenylate kinase)
X17620	4.6 / 4.1	Nm23-H1
J04031	3.6 / 2.1	MTHFD
M94065	3.3 / 1.9	Dihydroorotate dehydrogenase
X53793	9.1 / 9.7	ADE2H1 (AIR carboxylase)
U21090	3.6 / 3.4	DNA polymerase δ small subunit
AB000449	3.2 / 2	VRK1
		Nucleolus / RNA-binding proteins
U86602	4.8 / 3	Nucleolar protein p40
D21262	7 / 4	KIAA0035 [b] (p130)
U41387	6.1 / 4	Gu protein [b]
U38847	2.5 / 3.6	TAR RNA loop binding protein (TRP-185)
U28042	4.3 / 4.2	DEAD box RNA helicase-like protein [a]
U07231	3.6 / 2.5	GRSF-1 (G-rich sequence factor-1)
X74987	4.9 / 5.8	2-5 oligoadenylate binding protein
		Transport
M83416	3.3 / 2.7	E16 mRNA for amino acid permease 4F2hc/CD98
Z97074	5.4 / 5.2	Rab9 effector p40
D00591	5.2 / 3.4	RCC1 [a]
U51478	4.4 / 2	Na, K –ATPase β3 subunit
L31801	3.3 / 2.2	SLC16A1 Monocarboxylate transporter [a]
L20859	3.1 / 2.5	Leukemia virus receptor 1 (GLVR1)
		Antioxidative enzymes
U25182	5.1 / 3.6	AOE37-2 (Thioredoxin peroxidase)
L29008	4.7 / 5.3	L-iditol-2 dehydrogenase
U46499	4.6 / 3.7	Glutathione transferase (GST12)
		Transcription / splicing
U09564	4.2 / 4.4	SRPK1 (splicing factor serine kinase)
M37197	3.9 / 2	CCAAT-box-binding factor (CBF) [a]
L07648	3.2 / 2.4	MXI1
U37689	3.1 / 2.2	RNA polymerase II subunit (hsRPB8) [a]
U07563	2.4 / 3	c-abl
M88279	5.4 / 3.3	Immunophilin (FKBP52) [a]
		Miscellaneous
L00058	15.1 / 22.8	c-myc
X15306	5.4 / 3.3	NF-H
X97335	4.3 / 3.9	Kinase A anchor protein [b]
M34338	3.2 / 3.1	Spermidine synthase
U12595	6 / 5	TRAP1 (TNF 1 associated protein) [a]
M22877	3.5 / 3.1	Somatic cytochrome c (HCS)
U43747	3.7 / 2.4	Frataxin (FRDA)
U37022	3.2 / 1.9	CDK4
		Unknown function
U02609	20.5 / 28.2	SazD (Transducin like protein)
D78611	2.8 / 3.2	MEST
L19183	5.3 / 3.4	MAC30
L48692	2.4 / 4	Homo sapiens clone p5-23-3 mRNA
X82125	5.5 / 6.8	HOK-2
U41060	3.8 / 2.5	Breast cancer, estrogen regulated LIV-1 protein
D13645	3.7 / 3.3	KIAA0020
D31887	7.4 / 3.9	KIAA0062
D25218	3.3 / 2.2	KIAA0112
D29958	4.3 / 2.3	KIAA0116
D50914	4.1 / 4.6	KIAA0124
D28589	3.1 / 2.7	KIAA0167
D80001	5.4 / 4.7	KIAA0179
D87466	2.4 / 3	KIAA0276
		Known Myc targets, less than 3 fold
U02020	2.7 / 2.4	Pre-B cell enhancing factor (PBEF) [a]
M33764	1.4 / 1.7	Ornithine decarboxylase (ODC) [a]
X02152	1.5 / 1.8	Lactate DH-A (LDH-A) [a]
D38076	2.5 / 2.2	RanBP1 (Ran binding protein 1) [a]
X01060	1.6 / 2.1	Transferrin receptor [a]
M61832	nd / 2.5	S-adenosylhomocysteine hydrolase (AHCY) [a]
L33842	2.5 / 2.6	Type II inosine monophosphate dh (IMPDH2) [a]
		Repressed genes
M54992	0.37 / 0.45	B cell differentiation antigen
AF006087	0.2 / 0.5	Arp2/3 protein complex subunit p20 (ARC20)
M83652	0.38 / 0.36	Complement component properdin
M97796	0.5 / 0.4	Id-2 helix-loop-helix protein
U40282	0.5 / 0.5	Intregin-linked kinase (ILK)
J04430	0.5 / 0.45	Tartrate-resistant acid phosphatase 5
U72882	0.5 / 0.3	Interferon-induced leucine zipper protein (IFP35)
D29642	0.5 / 0.4	KIAA0053

Genes are shown that were regulated 8 h after c-*myc* induction.
Induced genes have been considered that showed 3-fold induction at least in one experiment, repressed genes show at least 2-fold change.
[a]Genes that have been described as Myc targets in other screens.
[b]Genes that were also induced after 4 h: KIAA0035 (factor 3.5/1.7), Gu (2.4/2.1), Kinase A anchor protein (4.6/1.7), JTV-1 (1.7/1.5).

Approximately 50% of the 6800 gene probes on the array produced a signal that was considered positive for expression in both experiments. Four hours after *myc* induction, a significant increase or decrease in steady-state RNA levels was not

detectable and none of the genes, except *myc* itself, met the criteria described above. After 8 h, 74 genes were found to be induced by our criteria. Ten of these genes (CAD, isoleucyl-tRNA synthetase, fatty acid binding protein, FKBP52, RCC1, SLC16A1, DEAD box RNA helicase-like protein, CBF, RPB8 and TRAP1) have been described as Myc targets in other screens before (Table 1). Seven further known Myc targets were detected in our screen, but did not fulfil our selection criteria, these include ODC, LDH-A and the transferrin receptor (Table 1). Sixty-four genes were identified as new potential Myc target genes. The average induction rates of these genes were in the range of 3- to 5-fold and a few RNAs were induced more than 10-fold. The upregulated genes were grouped in 10 different classes, corresponding to amino acid/protein synthesis, lipid metabolism, protein folding/turnover, nucleotide/DNA synthesis, nucleolus/RNA binding proteins, transport, antioxidative enzymes, transcription/splicing, miscellaneous and unknown function (Table 1). We also identified eight genes whose steady-state RNA levels were 2- to 3-fold down-regulated 8 h after Myc activation (Table 1).

Identification of potential Myc target genes in nuclear run-on experiments

Myc acts as a transcriptional activator. However, it cannot be ruled out that Myc might also act at a post-transcriptional level, e.g. by modifying and/or stabilizing transcripts. To address the question of the transcriptional activity of Myc target genes directly, we performed nuclear run-on experiments with nuclei of P493-6 cells 4 h after Myc activation. Only nascent RNA, associated with the transcription complex is radioactively labeled with this technique. Therefore it allows measurement of the increase or decrease in transcription rates of a gene. We also reasoned that this technique might identify genes that express unstable, low abundance mRNAs, by using a high number of isolated nuclei for labeling experiments.

Run-on RNAs prepared from uninduced cells and cells induced for 4 h were hybridized to cDNA arrays. We used two different filters, the ATLAS Human 1.2 Array (used in three independent experiments) and the Human cancer 1.2 DNA array (two independent experiments, which are shown in Figs 1 and 2). Together, both arrays represented 1891 different gene probes and 15 controls. In all experiments, ~30–40% of the gene probes generated a positive and reproducible transcription signal (Fig. 1). The remaining probes produced either no signal, or the signal was too faint and not reproducible in all experiments. We have shown previously that these signals are specific for RNA polymerase II transcription and do not appear in run-on experiments, performed in the presence of α-amanitin (22; data not shown).

The transcription signal for *myc* served as an internal control and increased strongly after removal of tetracycline (array position A3c, Fig. 1). The arrays were evaluated by phosphoimaging. Weak signals turned out to be difficult to evaluate by this technique and were therefore evaluated by visual inspection of the autoradiographs. Only reproducibly induced or repressed genes were included. Altogether, we found 33 of 1891 genes were transcriptionally induced 4 h after *myc* induction, while eight genes were found to be repressed (Table 2). The induced genes encode proteins required for protein synthesis, nucleotide/DNA synthesis, cell cycle control, transcription/splicing, signal transduction, cell adhesion and

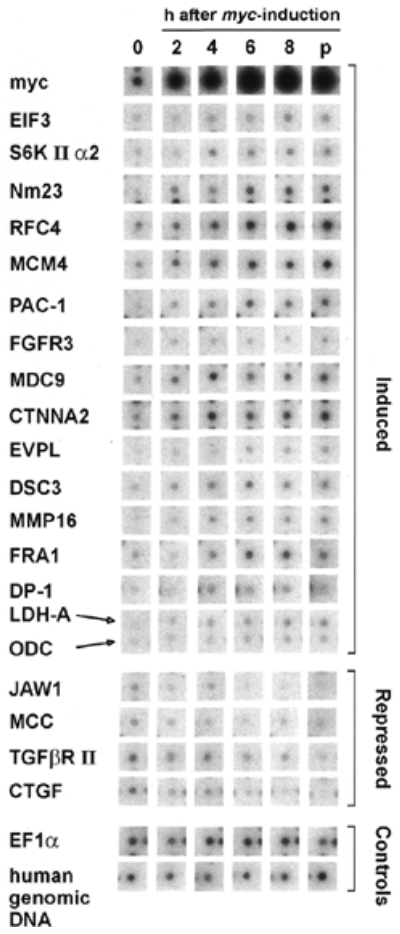

Figure 2. Candidate genes in nuclear run-on kinetics. Nuclei were prepared from arrested cells (0) and 2, 4, 6 and 8 h after *myc* induction. P indicates proliferating cells. Labeled nuclear run-on RNAs were hybridized to ATLAS Human cancer arrays 1.2. A selection of candidate genes listed in Table 2 is shown. Some candidate genes already showed an increase in transcription after 2 h (nm23-H2, RFC4, MCM4, PAC-1, FGFR3, MDC9 and LDH-A).

others. Three of the identified genes, lactate dehydrogenase (LDH), ornithine decarboxylase (ODC) and hepatoma-derived growth factor (HDGF) have been described as Myc regulated genes before. Of the 33 detected genes in the run-on experiment, 30 were not induced on the oligonucleotide microarrays. Possible explanations will be discussed below. Four of the Myc-repressed genes have been described as involved in the regulation of growth arrest before. Two of them, *gadd*45 and CTGF have already been described as Myc targets (9,23).

Induction kinetics of potential Myc target genes in nuclear run-on experiments

One important criterion for a direct Myc target gene is its transcriptional activation in the presence of protein synthesis inhibitors. Since *myc* is regulated transcriptionally in P493-6 cells, inhibitors of protein synthesis cannot be applied. A further important criterion is a correlation between the time course of the induction of *myc* and Myc target genes. Kinetic experiments indicate that the *myc* transcription rate (Fig. 2) and

Table 2. Myc regulated genes identified in nuclear run-on screens

Array Position [b] (Human cancer 1.2)	Accession number	Group Gene description	Regulation
		Protein-synthesis	
B14d	X85106	Ribosomal protein S6 kinase alpha 2 (S6KII-α2)	induced
F 8k	U78525	Eukaryotic translation initiation factor 3 beta subunit (EIF3)	Induced
F13j	X51466	Elongation factor 2 (EEF-2)	Induced
F14j	M60854	40S ribosomal protein S16	Induced
F 2k	U14970	40S ribosomal protein S5	Induced
		Nucleotide-/ DNA-synthesis	
A 9b	L16785	Nucleoside Diphosphat Kinase B (Nm23-H2)	Induced
C 4f	M87339	Replication factor C 37-kDa subunit (RFC4)	Induced
C 6f	X74794	DNA replication licensing factor (MCM4)	Induced
F10b	J05272	Inosine-5'-monophosphate dehydrogenase 1 (IMPDH1)	induced
F11d	X91247	Thioredoxin reductase	Induced
		Cell cycle control	
A12l	M34065	CDC25C	Induced
A 8i	M92287	CyclinD3	Induced
		Transcription / splicing	
A13c	X16707	Fos-related antigen (FRA-1)	Induced
F10i	L22253	Splicing factor, arginine/serine rich 7 (SFRS7)	Induced
A13l	L23959	E2F-related transcription factor DP-1	Induced
		Signal transduction	
A10d [b]	M24898	Thyroid hormone receptor (THRA1, c-ERBA-1)	Induced
B 7h	L11329	Dual-specificity protein phosphatase (PAC-1)	Induced
D 5i	M58051	Fibroblast growth factor receptor 3 (FGFR3)	Induced
E12d	X14445	Fibroblast growth factor-3 (FGF-3)	Induced
E 4b	D16431	Hepatoma-derived growth factor (HDGF) [a]	Induced
E 4j	U41766	Metalloprotease ADAM9 (MDC9)	Induced
E 6e	L36034	Pre-B cell stimulating factor (PBSF)	Induced
F 8l	D63485	KIAA0151 (putative serine/threonine kinase)	Induced
D 4l [b]	M59371	Ephrin type-A receptor 2 (EPHA2)	Induced
D10h [b]	L41939	Ephrin type-B receptor 2 (EPHB2)	Induced
E12b	M60278	Heparin-binding EGF-like growth factor (HB-EGF)	Induced
		Cell adhesion	
D 3g	L34059	Retinal-cadherin (CDH4)	Induced
D 7g	M94151	Alpha-catenin related protein (CTNNA2)	Induced
D11g	U53786	Envoplakin (EVPL)	induced
D14g	X83929	Desmocollin type 3 (DSC3)	Induced
E14i	D50477	Membrane-type matrix metalloproteinase 3 (MMP16)	Induced
		Miscellaneous	
F 5d	X02152	Lactate dehydrogenase A (LDH-A) [a]	Induced
F 5e	X16277	Ornithine decarboxylase (ODC) [a]	Induced
		Repressed genes	
D13c	U10485	Lymphoid-restricted membrane protein (JAW1)	Repressed
A14n	M20681	Brain glucose transporter 3 (GLUT3)	Repressed
F 6m	X03557	56-kDa protein induced by interferon (IFI56)	Repressed
A11i	U11791	Cyclin H	Repressed
		Growth arrest	
A13a	M62397	Colorectal mutant cancer protein (MCC)	Repressed
C 9i	M60974	Growth arrest & DNA-damage-inducible protein (GADD45) [a]	Repressed
D13i	D50683	Transforming growth factor, beta receptor II (TGFR-2)	Repressed
E 4c	M92934	Connective tissue growth factor (CTGF) [a]	Repressed

Genes are shown that were regulated in the run-on 4 h after c-*myc* induction.
[a]Genes that have been described as Myc targets in other screens.
[b]Genes only located on the ATLAS Human 1.2 array.

Myc protein levels (17; data not shown) are significantly increased 4 h after removal of tetracycline. All induced genes listed in Table 2 already showed an increased transcription rate 4 h after removal of tetracycline. For a number of these genes, the transcription rate had already increased 2 h after removal of tetracycline (nm23-H2, RFC4, MCM4, PAC-1, FGFR3, MDC9 and LDH-A), at a time point when Myc levels had not yet reached their maximum (Fig. 2). This observation makes it very likely that Myc targets and transcriptionally activates these genes directly. All induced genes in Figure 2 are also expressed in proliferating cells, indicating that they are not induced transiently by Myc. Repression of JAW1, MCC and

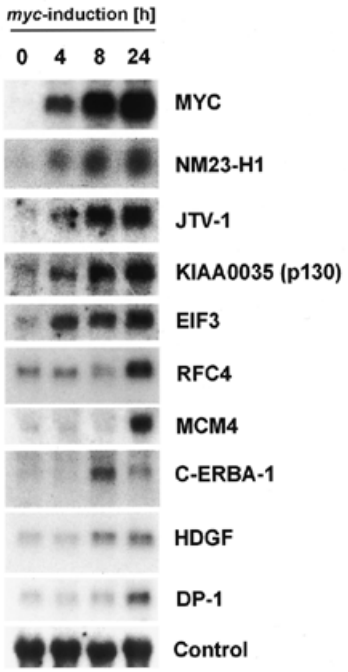

Figure 3. Evaluation of results by northern blot analysis. Total RNA was prepared from arrested P493-6 cells 0, 4, 8 and 24 h after *myc* induction and subjected to northern analysis. RNAs were hybridized with probes specific for the indicated genes. Nm23-H1, JTV-1 and p130 were identified in the cDNA screen, the other genes in the run-on screen. Genes differ in the kinetics of induction. RFC4, MCM4 and DP-1 mRNA levels do not accumulate at 8 h, although transcriptional activity is observed already after 4 h.

CTGF transcription by Myc is also observed already 2 h after removal of tetracycline, and is maintained in proliferating cells, suggesting that these genes are likely direct targets of Myc.

Evaluation of results in northern blot analysis

Screens with oligonucleotide microarrays have been reported to produce reliable results. Nevertheless, we tested induction of several genes by northern blot analysis. *myc* served as an internal control, which was induced 15.1- and 22.8-fold in two experiments 8 h after removal of tetracycline (Table 1). An ~20-fold induction of *myc* RNA levels could be confirmed in northern analysis (Fig. 3). Array analysis of JTV-1, Nm23-H1 and KIAA0035 (p130) showed induction rates of 4.3/2.0, 4.6/4.1 and 7.0/4.0, respectively (Table 1). The induction rates of these genes in the northern blot analysis were in a similar range 8 h after removal of tetracycline (Fig. 3), confirming the results of the microarray experiments by a different technique.

We also checked a number of genes by northern analysis which were upregulated in nuclear run-on experiments after 4 h. Only the mRNA levels of EIF3 were found to be increased after 4 h. The steady-state RNA levels of two other genes, C-ERBA-1 and HDGF, increased after 8 h. RFC4, MCM4 and DP1 were induced 24 h after *myc* induction (Fig. 3). This indicates that genes such as RFC4 and MCM4 are likely to be direct targets of Myc, but that post-transcriptional mechanisms may contribute to accumulation of mRNA at later time points.

DISCUSSION

Identification of putative Myc regulated genes by analyzing cellular mRNA levels and transcription rates

We have identified genes that are induced and repressed in a human B cell line in response to Myc. Two different methods were applied: (i) analysis of mRNA levels with oligonucleotide microarrays, and (ii) analysis of nuclear run-on RNAs with cDNA arrays. The first technique led to the identification of 74 genes, whose mRNA levels were upregulated 8 h after *myc* induction. The nuclear run-on experiments identified 33 genes induced 4 h after *myc* induction. For a number of genes, the induction was also confirmed by northern analysis.

The analyses summarized in Tables 1 and 2 show that there is only a small overlap in the results obtained by the two different techniques. Probes for 18 genes that were identified as potential Myc target genes with oligonucleotide microarrays (Table 1) were also spotted on the cDNA arrays, but only three of these genes (nm23, ODC and LDH-A) showed an increased transcription rate. The other 15 genes produced very faint or undetectable transcription signals in the run-on, which could not be interpreted. This does not exclude that these 15 genes are also regulated transcriptionally by Myc. However, this may indicate that the run-on assay is not sensitive enough to detect transcription of these genes.

Interestingly, only 3 of the 33 genes identified with run-ons were also found induced on the oligonucleotide microarrays, although all 33 genes were represented on the chip. One explanation for this small overlap is given by the northern analysis. Transcriptional activation of a gene does not necessarily lead to an increase of the corresponding mRNA levels. For example, the RFC4 and MCM4 genes are already transcriptionally induced 2 h after *myc* induction, the corresponding mRNAs, however, accumulate between 8 and 24 h. This suggests that Myc can set up the transcription rate for genes, but that additional mechanisms control the accumulation of mRNA in the cytoplasm. Another example is cdc25C. The mRNA of cdc25C does not accumulate in significant amounts in the cytoplasm of P493-6 cells, even though a strong transcription signal is produced in nuclear run-on experiments. Thus, the two applied methods can complement each other in a sensible manner, with nuclear run-ons being superior for strongly transcribed genes and cDNA analysis being ideal for genes with abundant mRNA levels.

The candidate genes that were identified in this study are good candidates to be direct targets of Myc. The time course of their induction, either by a prompt transcriptional induction as shown in Figures 1 and 2, or the fast accumulation of mRNA by northern blot analysis (Fig. 3) correlates with the induction of *myc*. Nevertheless, the formal proof that these genes are direct targets of Myc will have to be performed for every single gene, e.g. by promoter studies and induction of RNA in the presence of protein synthesis inhibitors in a suitable B cell system.

Myc regulated genes in growth control

We have previously shown that induction of *myc* expression in P493-6 cells activates the cell cycle (18). Myc also induces cell growth (increase in cell mass) independently of cell cycle activation (17,24,25). The latter observation suggested that Myc could activate genes required for growth control in P493-6 cells. This idea has already been suggested for other cell lines (26). The global screen for target genes in this study further strengthens this assumption.

Myc in protein synthesis

It has been shown recently that Myc target genes are involved in the regulation of protein synthesis (reviewed in 27). In fact, among the Myc regulated genes we found the translation factors EIF3, EEF-2 and a subunit of the S6 kinase, which is important for initiation of translation (28) and crucial for cell size control (29). Another group of genes is involved in the synthesis of tRNAs, molecules which transport the most basic material for protein synthesis, the amino acids. Among them are isoleucyl- and valyl-tRNA synthetase. A number of further tRNA synthetases were found to be upregulated in the screen, but did not fulfil our selection criteria (data not shown). We further found upregulation of JTV-1, a homolog to a common subunit of all aminoacyl-tRNA synthetases. Finally, it has been suggested that Myc regulated genes are also involved in ribosome biogenesis (16). In addition to this we found genes that encode factors present in the nucleolus, the location of ribosome formation. Among these are p130, which might be involved in nucleologenesis (30). Finally, we found RNA binding proteins and RNA helicases that are located in the nucleolus and are possibly involved in RNA transport (9), RNA processing and turnover, like the Gu (31) or MrDb (26) genes.

Myc in DNA synthesis

The CAD gene was among the first described Myc targets (13), which suggested that Myc might be involved in the regulation of DNA synthesis. We can now extend the list with new candidate genes, which allow the notion that Myc is involved in providing the nucleotides for DNA synthesis. One is the thioredoxin reductase (Table 2), which is critical for the activity of the ribonucleotide reductase. Many other genes listed in Table 1 are enzymes involved in pyrimidine or purine synthesis, like PRPP synthetase (whereby PRPP is also an important component for tryptophane and histidine synthesis). In addition we found the DNA replication factors RFC4, MDM4 (Table 2) and DNA polymerase δ (Table 1).

The E2F transcription factor also regulates genes that are important for DNA synthesis (32). In P493-6 cells, we could not detect regulation of the E2F protein (18). Interestingly, we found that Myc regulates transcription of DP-1, the dimerization partner of E2F. Therefore, in P493-6 cells, E2F activity might be regulated by Rb-phosphorylation and the abundance of DP-1.

Other growth related gene groups

Many other genes listed in Table 1 and 2 can contribute to growth regulation. Genes for regulation of lipid metabolism, like glycerol-3-phosphate dehydrogenase or phosphatidylethanolamine binding protein (Table 1) are probably involved in synthesis of membrane lipids.

It is known that rapidly proliferating cells need to be protected from oxidative stress. It has been suggested that pyruvate serves as a scavenger for oxygen radicals (33). In many tumor cells pyruvate is produced in anaerobic glycolysis by LDH-A, known as the Warburg effect. The contribution of Myc in the Warburg effect via induction of LDH-A has already been shown (7,34). In addition to LDH-A, we found other enzymes that protect from oxidative stress e.g., thioredoxin peroxidase and gluthatione transferase. It is also reasonable that transport has to be increased during growth. Remarkably, Myc regulates genes encoding for transporters of metabolic compounds and intermediates (Table 1), e.g. h4F2hc for amino acid transport (35), SLC16A1 for pyruvate and lactate transport (36) or GLVR1 for transport of inorganic phosphate (37). A number of additional genes listed in Tables 1 and 2, which are not discussed here, are involved in metabolic pathways and thereby contribute to cell growth.

Myc and signal transduction

Previous observations suggested that Myc might be involved in autocrine stimulation of P493-6 cells. Cycling cells have a doubling time of ~30 h (data not shown). Quiescent cells, however, require 4 days for the first round of doubling and then accelerate proliferation (17,18). This suggests that cells at a higher density were able to stimulate the culture by an autocrine loop. Listed in Table 2, we could identify increased transcription of growth factor and growth factor receptor genes. HDGF and CTGF have recently been described as being Myc regulated (9). In addition we found upregulation of HB-EGF and the metalloprotease ADAM9. ADAM9 is required for activation of HB-EGF (38) and has been suggested to be involved in activation of the EGF receptor via HB-EGF (39). A scenario in which Myc induces growth factors, growth factor receptors and the required metalloproteinase for activation of growth factors, favors the idea that Myc can facilitate progression of P493-6 through G_1 by an autocrine loop.

In addition, the thyroid hormone receptor gene (c-ERBA/TRα/EAR-1) was upregulated by Myc (Fig. 3). C-ERBA overexpression has been reported to stimulate proliferation and block differentiation (40). Mutated forms of c-ERBA block transcriptional activation and are oncogenic (41). Moreover, c-ERBA can induce apoptosis (42,43), and in that respect phenotypes caused by c-ERBA are similar to phenotypes caused by Myc. Therefore, it will be interesting to study whether c-ERBA is an important downstream target of Myc in tumor formation.

Myc, a gas pedal for G_1 progression?

The list of the reported genes in this study provides a view of how quiescent B cells may be stimulated after *myc* induction. We conclude that Myc increases transcription of a large variety of genes that are necessary for acceleration of G_1 progression. Some of the Myc target genes have also been shown to be regulated by serum in wild-type fibroblasts and in fibroblasts with deleted *myc* alleles (44). This suggests that Myc is not the only factor that regulates transcription of these genes in response to growth factors. Expression of Myc target genes in quiescent cells also appears reasonable, as many of the genes are essential, and minimum levels of protein synthesis and energy also have to be provided in resting cells. Indeed, the

genes that have been reported as Myc targets are, as a rule, not completely downregulated in the absence of Myc. Therefore it is conceivable that inducing Myc is more like treading the gas pedal (and for most of the genes by not more than a factor 4) to accelerate progression through G_1 and to increase the accumulation of cell mass. Indeed, previous studies reported that cells expressing high levels of Myc constitutively spend less time in G_1 (reviewed in 1). This is in line with observations in *myc* null cells, which exhibit prolonged G_1 and G_2 phases (19). Whether Myc has a role in G_2 is not yet clear. CDC25C is an important regulator for entry into mitosis and regulation of *cdc25*C expression by Myc (Table 2) could contribute to explain the extended G_2 phase in *myc* null cells.

The progression through G_1 is regulated by two paths. One is cell growth, which leads to an increase in cell mass to provide enough material for two daughter cells. The other is cell cycle regulation, which ensures a controlled order of events. Both paths have to be coordinated to avoid cell division before cells have reached a critical size (45,46). Despite its growth function, Myc is also involved in regulation of the abundance and activity of cell cycle components in G_1 (47,48). Therefore it is very likely that Myc is a central regulator for the coordination of cell cycle regulation and cell growth in G_1.

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