Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion

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MYC affects normal and neoplastic cell proliferation by altering gene expression, but the precise pathways remain unclear. We used oligonucleotide microarray analysis of 6,416 genes and expressed sequence tags to determine changes in gene expression caused by activation of c-MYC in primary human fibroblasts. In these experiments, 27 genes were consistently induced, and 9 genes were repressed. The identity of the genes revealed that MYC may affect many aspects of cell physiology altered in transformed cells: cell growth, cell cycle, adhesion, and cytoskeletal organization. Identified targets possibly linked to MYC's effects on cell growth include the nucleolar proteins nucleolin and fibrillarin, as well as the eukaryotic initiation factor 5A. Among the cell cycle genes identified as targets, the G1 cyclin D2 and the cyclin-dependent kinase binding protein CksHs2 were induced whereas the cyclin-dependent kinase inhibitor $p21^{Cip1}$ was repressed. A role for MYC in regulating cell adhesion and structure is suggested by repression of genes encoding the extracellular matrix proteins fibronectin and collagen, and the cytoskeletal protein tropomyosin. A possible mechanism for MYC-mediated apoptosis was revealed by identification of the tumor necrosis factor receptor associated protein TRAP1 as a MYC target. Finally, two immunophilins, peptidyl-prolyl *cis-trans* isomerase F and FKBP52, the latter of which plays a role in cell division in *Arabidopsis*, were up-regulated by MYC. We also explored pattern-matching methods as an alternative approach for identifying MYC target genes. The genes that displayed an expression profile most similar to endogenous *Myc* in microarray-based expression profiling of myeloid differentiation models were highly enriched for MYC target genes.

The c-*Myc* protooncogene plays a key role in cell proliferation, differentiation, and apoptosis. *Myc* transcripts are rapidly induced upon mitogenic stimulation and are down-regulated during cellular differentiation (1). Consistent with MYC's role in promoting cell proliferation, genetic alterations resulting in deregulation of *Myc* expression are common to a wide range of tumor types (2).

The MYC protein possesses a basic helix-loop-helix/leucine zipper domain that mediates dimerization with its partner MAX. MYC-MAX heterodimers bind DNA at the E-box sequence CACGTG and other related sequences, and activate transcription (1). MYC has also been reported to repress transcription at specific initiator elements, although the mechanism involved has not been clarified (3, 4).

Many previously reported MYC target genes are involved in metabolism and growth (ref. 5 and references therein). The MYC-induced genes ornithine decarboxylase, carbamoylphosphate synthetase-aspartate transcarbamoylase-dihydroorotase (CAD), and dihydrofolate reductase suggest a role for MYC in DNA metabolism whereas the targets ferritin and iron regulatory protein 2 suggest MYC may affect iron metabolism (6). Previously reported targets involved with protein synthesis include the translation initiation factors eIF4E and 2A and the RNA helicase MrDb (DDX18). A role for MYC in cell adhesion has previously been suggested by the observation that MYC down-regulates expression of LFA-1 ($\alpha_L\beta_2$ integrin) in lymphoblastoid cells. Reported MYC target genes that may be critical for its effects on cell proliferation and immortalization include the phosphatase cdc25A and the catalytic subunit of telomerase.

Because MYC overexpression has such a profound impact on cell behavior, we hypothesized that many other, as yet undiscovered, targets may exist. However, identifying additional MYC target genes by conventional methods has proven difficult. MYC-MAX heterodimers induce only a modest increase in transcription in mammalian cells (7), and the short target recognition sequences provide little guidance. Other available approaches for identifying MYC target genes to date, for example, cDNA subtraction or isolation of MYC-MAX bound chromatin (8), have been time consuming or cumbersome. Most known MYC candidates were identified by testing specific *a priori* hypotheses.

A systematic approach for identifying MYC targets would allow us to answer several outstanding issues about MYC's function as a transcription factor. For instance, although MYC has been reported to function as both an activator and repressor, a global view of MYC's transcriptional activity has not been possible. It is also unknown whether the targets activated in the context of proliferation are the same, overlapping, or distinct from targets affected in another context, for instance, during differentiation.

We used hybridization to microarrays (9) to assess changes in RNA expression upon c-MYC activation as a strategy for identifying MYC target genes. A conditional MYC-estrogen receptor (MYC-ER) fusion protein comprising MYC and the estrogen receptor ligand binding domain (10, 11) was used to induce MYC transcriptional activity. The steroid receptor fusion molecule is inactive unless stimulated with the estrogen analog 4-hydroxytamoxifen (OHT), thus permitting conditional activation of MYC. In primary human fibroblasts used for these experiments, MYC activation results in an increase in the S phase fraction (C. G., S. K. Hirst, M. McMurray, and R.N.E., unpublished work). Hybridization to high density oligonucleotide arrays allowed us to monitor 6,416 human genes and unnamed expressed sequence tags (ESTs) as potential MYC targets. The specific changes in gene expression observed suggest new mechanisms for the biological functions of MYC.

Materials and Methods

Retroviral Vectors and Cell Culture. Amphotropic viral stocks were generated by co-transfection of pBabe-puro plasmid containing

Abbreviations: OHT, 4-hydroxy-tamoxifen; MYC-ER, MYC-estrogen receptor; ODC, ornithine decarboxylase; PPIF, peptidyl-prolyl *cis-trans* isomerase F; eIF5A, eukaryotic initiation factor 5A; cdk, cyclin-dependent kinase; EST, expressed sequence tag.

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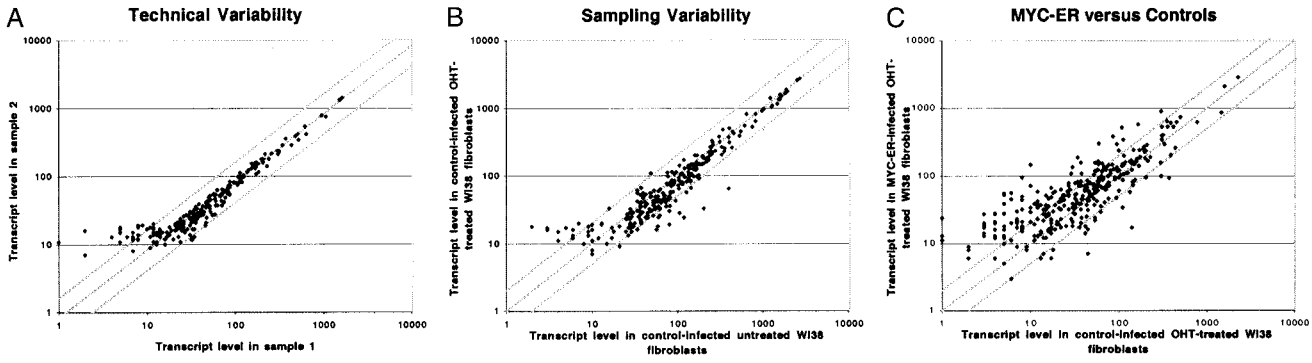

Fig. 1. Comparison of MYC-ER induction with technical and biological variability. For each gene, the RNA expression level in one sample is given on the *x* axis and the expression level for the same gene in the other sample is plotted on the *y* axis. Two-fold changes are indicated. (*A*) RNA from human leukemia cell line CCRF-CEM (a gift of U. Sherf and J. Weinstein, National Cancer Institute) was subjected to one or two rounds of poly(A) selection, converted into target, and hybridized to oligonucleotide arrays. (*B*) RNA from WI38 fibroblasts was infected with control vector and then was either induced with OHT or left untreated. (*C*) RNA from WI38 fibroblasts was MYC-ER infected, OHT-treated or empty vector-infected, OHT-treated.

MYC-ER™ or Δ-MYC-ER™ (11) together with Psi⁻ helper construct (12) in 293 T cells. Subconfluent WI38 cells (American Type Culture Collections catalog no. CCL75) grown in DMEM with 10% FCS were infected with viral supernatant on 2 consecutive days. The next day, cells were plated at $\approx 10^4$cells/cm^2 in phenol-red free DME medium with 10% FCS and were selected in the presence of puromycin for pBABE vectors. Cells were grown to confluence, for 7–8 days, without medium changes. Density arrested cells were induced with 200 nM OHT (4-hydroxy-tamoxifen) or were serum starved (0.1% FCS) for 48 hr (only experiment 3) and then were induced. Where specified, cells were exposed to cycloheximide (10 μg/ml) for 30 min before addition of OHT.

High Density Oligonucleotide Array Expression Analysis. A complete protocol for converting RNA into "target" suitable for hybridization to microarrays is available at our web site (http://www.genome.wi.mit.edu/MPR). In brief, poly(A) mRNA was selected with oligo(dT) beads (Promega) from total RNA extracted with Trizol reagent (Life Technologies, Gaithersburg, MD) and was used to create cDNA with a T7-polyT primer and the reverse transcriptase Superscript II (GIBCO/BRL). Approximately 1 μg of cDNA was subjected to *in vitro* transcription (Ambion, Austin, TX) in the presence of biotinylated UTP and CTP (Enzo Diagnostics). Target for hybridization was prepared by combining 40 μg of fragmented transcripts with sonicated herring sperm DNA (0.1 mg/ml) and 5 nM control oligonucleotide in a buffer containing 1.0 M NaCl, 10 mM Tris·HCl (pH 7.6), and 0.005% Triton X-100. Target was hybridized for 16 hr at 40°C to a set of four oligonucleotide arrays (HUM6000–1, HUM6000–2, HUM6000–3, HUM6000–4; Affymetrix, Santa Clara, CA) containing probes for 6,416 human genes (5,223 known human genes and 1,193 unnamed ESTs). Arrays were washed at 50°C with 6× SSPET (0.9 M NaCl/60 mM NaH_2PO_4/6 mM EDTA/0.005% Triton X-100, pH 7.6), then at 40°C with 0.5× SSPET. Arrays were then stained with streptavidin-phycoerythrein (Molecular Probes). Fluorescence intensities were captured with a laser confocal scanner (Hewlett–Packard) and were analyzed with the GENECHIP software (Affymetrix). Expression data were analyzed as described (13), including thresholding small and negative expression values to 20. Genes most similar to MYC were identified in the myeloid differentiation experiments based on a Euclidean distance metric, after eliminating genes that failed to vary in expression level within an experiment by a factor of three and an absolute value of 100 and normalizing within experiments to a mean of zero and a standard deviation of 1.

Analysis of RNA by Northern Blots. Northern blots were performed according to standard procedures (14). For cyclin D2 and p21, complete cDNA was used as probes. For FKBP52 (a 52-kDa FK506 binding protein), a PCR amplicon of bp 1,215–1,767 (GenBank accession no. M88279) was used; for FABP5 (PA-FABP), bp 60–481 (M94856); for ornithine decarboxylase 1 (ODC1), bp 1,198–1,984 (X55362); for peptidyl-prolyl *cis-trans* isomerase F (PPIF) (hCyP3), bp 404–803 (M80254); and for eukaryotic initiation factor 5A (eIF5A), bp 46–512 (U17969). To assess the relative amounts of RNA loaded into each lane, the same filter was stripped and hybridized with a PCR product for GAPDH or MAX, genes that remain essentially constant among samples. Hybridized filters were exposed sequentially to x-ray films and PhosphorImager screens (Fuji).

Results

MYC Targets Identified with MYC-ER. We introduced the MYC-ER gene by retroviral transduction of primary human fibroblasts. When quiescent, infected cells are treated with OHT, 20% enter the cell cycle by 17 hr whereas only 1–6% of OHT-treated non-expressing controls ever enter S phase (C.G., S. K. Hirst, M. McMurray, and R.N.E., unpublished work). Hyperphosphorylation of Rb, activation of cyclin-dependent kinase 2 (cdk2), and increases in transcript levels of three known MYC target genes—MrDb (DDX18), ODC, and cdc25A—are observed within 5 hr after OHT treatment. MYC-ER-stimulated cells eventually undergo apoptosis 48–72 hr after serum withdrawal. For microarray analysis, we harvested RNA from these cells 9 hr after OHT treatment, reasoning that direct MYC targets would have increased or decreased in expression by this time, yet secondary changes in RNA levels that occur as cells enter S phase at 17 hr would be minimized.

We first assessed whether the "signal," in terms of changes in RNA levels caused by MYC induction, was greater than the background "noise" of fluctuations in gene expression expected from experimental variables. MYC activation of fibroblasts, as depicted in Fig. 1*C*, resulted in a larger number of genes showing a given change in expression level as compared with the variability observed from target preparation and independent samplings of the same cell line (compare Fig. 1 *A*, *B*, and *C*). Based on our observation that few genes changed expression level by more than two-fold in the control experiments ($\approx$2 per 1,000 for technical variability and $\approx$20 per 1,000 for biological variability), we applied a threshold of a two-fold change in expression level between MYC-ER infected, OHT-stimulated samples, and empty virus-infected, OHT-treated controls for identifying putative MYC targets.

Conditional MYC induction was performed in triplicate exper-

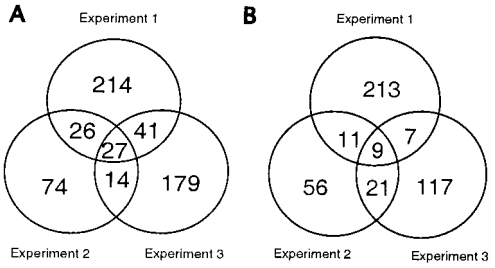

Fig. 2. Venn diagram of the number of genes altered in each of three independent MYC-ER experiments. (*A*) The criteria for increased gene expression were the following: (*i*) the gene was called "present" in the MYC-ER + OHT sample; (*ii*) the ratio of the expression level in the MYC-ER + OHT sample to the expression level in the control + OHT sample was greater than 2; and (*iii*) the ratio of control + OHT to control was not greater than two. (*B*) The criteria for decreased gene expression were the following: (*i*) the gene was called "present" in the control + OHT sample; (*ii*) the ratio of expression level in the MYC-ER + OHT sample to the expression level in the control + OHT was less than 0.5; and (*iii*) the ratio of control + OHT to control was not less than 0.5.

iments. Shown in Fig. 2 are Venn Diagrams representing the number of genes that changed expression levels by at least two-fold in each of the three independent experiments, and the overlap among experiments. Twenty-seven genes were up-regulated and nine genes were down-regulated in all three experiments (listed in Table 1), significantly more genes than would be expected based exclusively on fluctuations due to technical and biological variability (fewer than one gene expected). Of the genes in Table 1, only two were previously reported as MYC targets—ODC (5), which increased 5- to 7.5-fold in all three experiments, and nucleolin (15). Performing repeat experiments was crucial: If we had performed this experiment only once, we would have detected increased expression of 75–200 genes that would not have replicated in further experiments. Some of this variability may result from sampling ($\approx 20/1{,}000$ genes $\times$ 6,416 genes $= \approx 130$ genes). Several other previously reported MYC targets showed some evidence of regulation but did not meet our strict criterion of 2-fold induction in all three experiments. A table listing the changes in expression observed in our system for previously reported MYC targets along with the complete data set for all of the experiments reported herein is available on our web site (http://www.genome.wi.mit.edu/MPR).

Direct Versus Indirect Targets of MYC. To discriminate between direct and indirect MYC targets, we activated MYC-ER in the presence of cycloheximide (16). By inhibiting protein synthesis, cycloheximide eliminated the possibility that MYC-induced proteins would subsequently modulate a secondary set of genes. Of the 27 genes consistently induced by MYC-ER, 18 genes (68%) were also up-regulated in the presence of cycloheximide whereas almost all of the repressed genes (8/9) were also down-regulated under these conditions. Based on the cycloheximide experiments, we conclude that the genes indicated with an asterisk in Table 1 are direct targets of MYC.

Target Verification by Northern Blot Analysis. To verify induction by an independent method, we chose six induced target genes from Table 1 for Northern blot analysis. In all cases, the Northern blots confirmed the microarray results indicating up-regulation by MYC-ER. For four genes, we examined the same RNA employed for the microarray measurements, and for two genes we used RNA from an independent MYC-ER induction. As shown in Fig. 3 *A* and *B*, FKBP52, FABP5, PPIF, eIF5A, and cyclin D2 follow a similar pattern of expression to that of the known target gene ODC. Our Northern blot data demonstrate an increase in expression in the same range as expected from the microarray results for all of the genes tested (see Fig. 3 legend). In addition, we selected p21 as an example of a repressed MYC target (Fig. 3*C*). Within 2 hr after OHT stimulation, levels of p21 had decreased.

To ensure that the transcriptional activity of MYC is required for the observed changes in target gene expression, we also tested a MYC-ER fusion protein in which an internal deletion (bp 106–143) renders the protein transcriptionally inactive (17). As shown in Fig. 3*A*, the four MYC target genes tested by Northern blot analysis were not induced by this transcriptionally inactive fusion protein.

Altered Expression of MYC Targets During Differentiation. To determine whether the targets we identified in the MYC-ER experiments are influenced by changes in MYC levels under physiologically relevant conditions, we asked whether these targets are also affected during the shut-off of endogenous MYC which accompanies hematopoietic differentiation (1). We therefore investigated previously published experiments performed on the same microarrays in which HL60 cells were induced to differentiate into macrophages by treatment with TPA, a process during which endogenous MYC levels decline substantially (13). Within 24 hr of treatment, essentially all of the cells become adherent and exit the cell cycle. In Table 1, ratios of gene expression in differentiated and undifferentiated HL60 cells are given for each of the genes identified as a candidate MYC target in the MYC-ER experiments. Seventeen of the twenty-seven genes consistently induced in the MYC-ER experiments showed a greater than 2-fold decline in expression as HL-60 cells differentiated whereas four of the nine genes repressed by MYC-ER increased in abundance more than 2-fold. The small number of genes identified in the MYC-ER system that were not also regulated during differentiation may reflect differences in the cell type monitored, or between MYC targets that are affected during proliferation versus differentiation. Nevertheless, many of the genes identified with the MYC-ER system were also regulated in a physiological context.

Identifying Candidate MYC Targets in the Myeloid Differentiation Data Alone. Previous reports have suggested that specific transcriptional networks may be identifiable based on coordinate changes in gene expression under a variety of conditions (for instance, ref. 18). Although this approach has yielded success in yeast models, mammalian systems have proven more difficult to decipher. We tested whether a strategy of defining genes with expression profiles similar to *Myc* in myeloid differentiation experiments (13) would have identified the same genes as the conditional MYC model system. The data set we examined included TPA-induced differentiation of HL60 and U937 cells into macrophages and all-trans-retinoic acid induced differentiation of NB4 cells into neutrophils (see ref. 13 for full data set). Five of the top ten genes that showed an expression pattern most similar to MYC in these differentiation experiments were independently discovered as MYC targets in the MYC-ER experiments. Given that there were 27 genes that were reproducibly induced in the MYC-ER experiments, the probability of selecting five or more of them among the ten top genes is less than 2×10^{-8}. These five genes are FKBP52, nucleolin, cyclin D2, TRAP1, and CksHs2. The complete analysis is available on our web site. This approach was less successful for genes repressed in the MYC-ER experiments probably because the genes that increased during cell differentiation were more likely to be cell-type specific.

Discussion

Using oligonucleotide microarrays to monitor the effects of MYC, we addressed some fundamental questions about MYC's transcriptional activity. Based on changes in expression in the presence of cycloheximide, we discovered that most MYC target genes detected at 9 hr after induction (18/27 for induced, 8/9 for repressed) were direct targets. This finding argues against the

Table 1. Summary of Genes Regulated by MYC

	Accession Number	MYC-ER1 MYC-ER + OHT/ control + OHT	MYC-ER2 MYC-ER + OHT/ control + OHT	MYC-ER3 MYC-ER + OHT/ control + OHT	Cycloheximide MYC-ER + OHT + cx/ control + OHT + cx	HL60 0 hr/24 hr
Induced genes						
Ornithine decarboxylase 1*	X55362	5.0	5.2	7.5	2.5	3.1
AHCY, *S*-adenosylhomocysteine hydrolase*	M61832	2.3	2.4	7.3	6.8	3.4
CCND2, cyclin D2*	D13639	4.8	2.2	5.7	4.4	5.6
ASS, argininosuccinate synthetase*	T51288	2.7	2.1	5.5	2.9	0.5
FKBP52, 52-kDa FK506 binding protein*	T70920	14.7	4.3	5.4	2.4	4.2
Pre-B cell enhancing factor (PBEF)	U02020	2.5	4.0	5.2	1.9	0.4
Tumor necrosis factor receptor associated protein (TRAP1)*	R61502	4.3	4.5	5.0	2.8	2.9
FABP5, psoriasis-associated fatty acid binding protein*	H73758	8.3	13.6	4.7	2.7	10.0
Nucleolin*	H17434	2.4	2.7	4.5	4.0	2.2
GOS2, lymphocyte G0/G1 switch gene 2*	M69199	7.3	4.0	4.4	6.1	1.0
PPIF (hCyp3), peptidyl-prolyl *cis-trans* isomerase F*	H55916	3.8	3.9	4.3	3.6	0.7
RNA polymerase II subunit (hsRPB8)	Z49199	2.8	2.4	4.0	1.4	1.3
Fibrillarin*	T57468	3.9	4.4	3.9	3.5	2.0
TFRC, transferrin receptor (p90, CD71)*	R23889	2.3	2.4	3.9	2.4	9.1
CksHs2*	X54942	2.4	2.1	3.3	3.7	3.2
SLC16A1, solute carrier family 16*	L31801	11.1	2.7	2.9	2.0	5.6
IARS, isoleucine-tRNA synthetase*	U04953	5.9	2.1	2.9	2.6	1.3
HLA-DRB1, major histocompatibility complex, DR beta 5	T62633	3.4	8.9	2.9	0.4	0.5
EST highly similar to GRPE protein homolog precursor*	T51856	9.7	3.1	2.8	2.0	7.1
GPI, glucose phosphate isomerase	R49964	3.1	2.5	2.7	1.6	0.5
HSPD1, heat shock 60-kD protein 1 (chaperonin)	M22382	2.7	2.3	2.7	1.8	2.2
Hepatoma-derived growth factor*	D16431	2.2	2.3	2.6	2.6	2.5
Splicing factor SF2	R60749	4.3	3.6	2.5	1.6	6.3
Coup transcription factor	M37197	3.2	2.9	2.5	1.1	2.4
RPS11, ribosomal protein S11	X60673	7.3	2.6	2.4	1.6	1.3
EIF5A, eukaryotic translation initiation factor 5A*	M23419	3.0	2.3	2.3	2.3	4.8
EIF4G, eukaryotic translation initiation factor 4 gamma	R39681	2.4	3.8	2.1	0.7	1.1
Repressed genes						
p311 (neuronal protein 3.1)*	U30521	0.29	0.38	0.15	0.13	0.43
A2M, alpha-2-macroglobulin*	T69425	0.10	0.22	0.18	0.22	0.22
TPM1, tropomyosin alpha chain (skeletal muscle)*	Z24727	0.33	0.31	0.20	0.14	1.10
PDGFRA, platelet-derived growth factor receptor alpha*	H23235	0.43	0.30	0.30	0.42	1.00
FN1, fibronectin 1*	M76378	0.48	0.39	0.30	0.35	0.53
CTGF, connective tissue growth factor*	X78947	0.32	0.33	0.31	0.24	1.00
COL3A1, alpha-1 type 3 collagen*	X06700	0.34	0.38	0.39	0.33	1.00
CDKN1A, cyclin-dependent kinase inhibitor 1A (p21, Clp1)*	U03106	0.24	0.48	0.41	0.33	0.04
EST moderately similar to dithiolethione-inducible gene-2	R73450	0.22	0.38	0.44	0.93	0.28

Genes are listed in order of fold induction in experiment 3. The following genes were not present on the microarrays: EIF2a, CAD, ECA-39, MrDb, telomerase, LAF-1a, HLA-A2, gadd45, C/EBPα, and iron regulatory protein 2.

*Regulated by MYC-ER in the presence of cycloheximide.

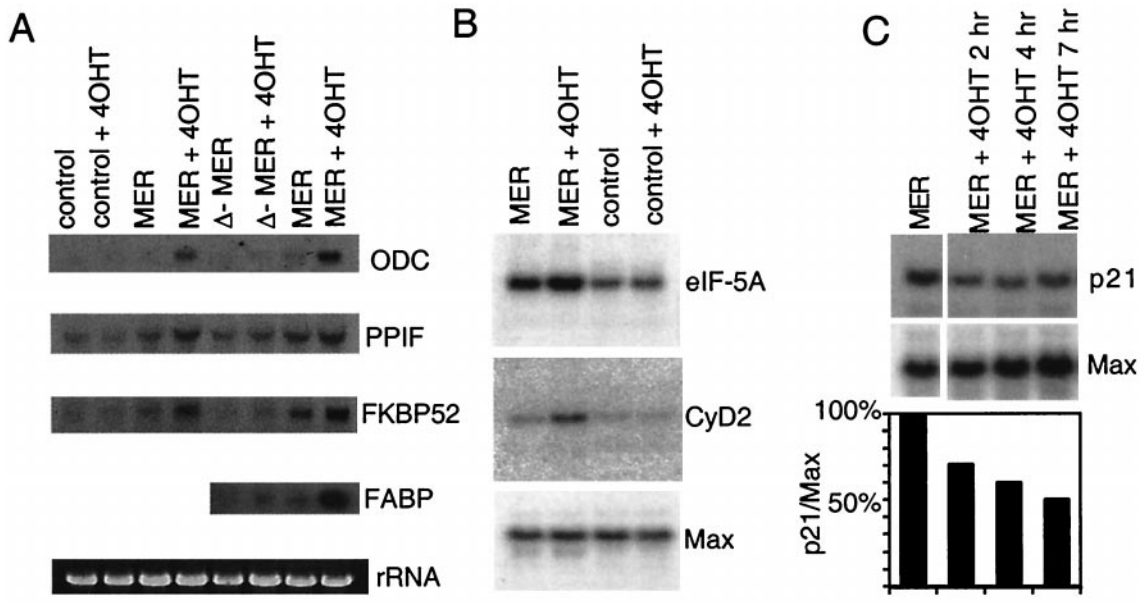

Fig. 3. Northern blots of target genes. (*A*) RNA harvested from the indicated MYC-ER samples was used for Northern blots. Also depicted are Northern blot analysis results for RNA samples harvested from fibroblasts infected with a deletion mutant of the MYC-ER fusion protein incapable of transactivating MYC-responsive genes. Ethidium bromide-stained rRNA levels demonstrates similar loading in each lane. Fold inductions comparing MYC-ER infected cells with and without OHT treatment are 2.3 (Northern blot, experiment 1)/2.3 (microarray, experiment 1) and 2.2 (Northern blot, experiment 2)/2.1 (microarray, experiment 2) for FKBP52; 1.8/2.0 and 1.4/2.1 for PPIF; 4.1/3.6 for FABP5. (*B*) Northern blot analysis results for a fourth MYC-ER experiment are shown for eIF5A and cyclin D2. Fold inductions (MYC-ER+OHT/MYC-ER) are 1.8 (Northern blot)/2.3–3.0 (microarrays) for eIF5A and 3.5/2.2–5.7 for cyclin D2 (*C*). Northern blot analysis results for p21 showing repression of transcript levels after MYC-ER induction. *Max* was used as a loading control because its levels are not altered by MYC activation.

idea that MYC's role is to activate a transcriptional cascade by inducing a small number of transcription factors. Indeed, few of the MYC targets identified are involved in transcription *per se*.

We also discovered that MYC does not have a large (>10-fold) effect on the induction or repression of any of the 6,416 human genes and ESTs monitored. We note, however, that these estimates may be lower limits considering that only 20% of the infected cells enter S phase and that transcript induction may have been maximal at a different time point. Nevertheless, the modest levels of change previously observed are likely to reflect not the particular targets examined, but rather the relatively weak transcriptional activity of the MYC protein itself (7).

Whether MYC exerts its effects on cells via transcriptional activation or repression has been debated (see, for example, refs. 3 and 19). Our results showed that MYC consistently induces more targets than it represses (27 vs. 9). However, our data are limited to the ≈6% of the genome tested and do not permit us to assess the relative importance of the induced versus repressed genes on cellular functions.

Another previously unanswered question is whether MYC activates the same, overlapping, or distinct sets of targets in different physiological contexts. We discovered that ≈60% of the target genes that were identified using conditional MYC expressing fibroblasts were also regulated during macrophage differentiation. These findings support a model in which these sets of targets overlap. In fact, by monitoring only the differentiation data, we could have identified many of the induced target genes discovered by our directed approach.

Cell transformation is characterized by increases in cell size, cell division even in the absence of mitogenic stimuli, alterations in cell adhesion, and changes in cell shape and the organization of the cytoskeleton. Although overexpression of MYC alone is not sufficient to transform cells, the genes we identified suggest that MYC plays a role in all of these processes (see diagrammatic representation in Fig. 4). Recent experiments have shown that an important effect of MYC on both *Drosophila* and mammalian cells is to increase the accumulation of cell mass (i.e., growth rate) (20–22). Our data provide support for the view that MYC directly influences protein synthesis. Earlier work had indicated that the rate-limiting translational initiation factor eIF4E is induced by MYC (5). Our findings indicate that MYC induces the initiation factors eIF4γ and eIF5A (Table 1; Fig. 3*A*), the latter of which is also thought to be involved in nucleocytoplasmic transport (23, 24). Interestingly, MYC also increases levels of the previously identified target ornithine decarboxylase (ref. 5; Table 1), which regulates a hypusine modification of eIF5A that is critical for its function (25). Other growth-associated genes identified as MYC targets in our studies include several involved in nucleolar rRNA processing, including the structural proteins fibrillarin and nucleolin, and the ribosomal protein rps11 (see Table 1).

MYC has also been implicated in cell cycle progression (26). Our results suggest several novel points of potential interaction

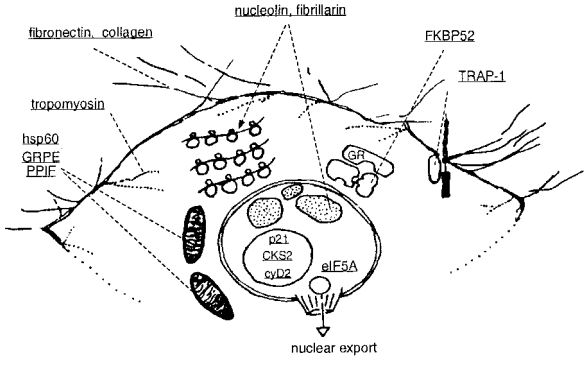

Fig. 4. Schematic representation of MYC target genes within a cell. Depicted is a selection of the MYC targets identified herein (underlined) along with their subcellular localization. GR, glucocorticoid receptor. See text for further discussion of the functions of the encoded proteins.

between c-MYC and the cell cycle machinery. Cyclin D2 was shown here to be a direct MYC target (Table 1; Fig. 3*B*), which is consistent with other recent reports (27, 28). Cyclin D2 may contribute to cell proliferation by directly increasing phosphorylation of the retinoblastoma protein via its association with cdk4, or by sequestering $p27^{KIP1}$. We also discovered that MYC induces CksHs2, a homologue of the yeast proteins CKS and $p13^{suc1}$, which bind tightly to some cdks and play a role in cell viability and proliferation (29). Finally, MYC was discovered to down-regulate the cdk inhibitor p21 (Table 1; Fig. 3*C*). Decreased p21 activity may represent another mechanism by which MYC increases cdk activity and cell proliferation.

A connection between MYC and cell adhesion is suggested by repression of the extracellular matrix proteins fibronectin and collagen. Repression of both of these molecules has been reported to accompany cell transformation, and their loss may contribute to the decreased adhesiveness and more rounded cell shape observed in transformed cells (30, 31), and MYC-overexpressing cells (C.G., unpublished observation).

Our discovery that MYC represses transcription of the actin-binding protein tropomyosin represents a potential link between MYC overexpression and cytoskeletal dysregulation observed in transformed cells. Tropomyosin suppression is a common biochemical change accompanying neoplastic transformation (32); overexpression of tropomyosin can abolish a transformed phenotype (33); and antisense-induced reduction in tropomyosin levels conferred anchorage-independent growth potential (34).

Another hallmark of MYC-overexpressing cells is a high level of apoptosis. TRAP1, which binds to the intracellular domain of the tumor necrosis factor receptor (35), was discovered to be a direct MYC target and may be part of a pathway leading to increased apoptosis in MYC-overexpressing cells. This target may also help to explain the elevated susceptibility of such cells to tumor necrosis factor α-mediated apoptosis (36).

We also discovered that MYC regulates a previously unsuspected class of proteins—the immunophilins. Two immunophilins, peptidyl-prolyl *cis-trans* isomerase F (PPIF) and the 52-kDa FK506 binding protein (FKBP52), were identified as direct MYC targets (see Table 1 and Fig. 3*A*). FKBP52 forms a multimeric complex with steroid receptors and has been localized to the mitotic spindles (37). Mutants of FKBP52 in *Arabidopsis* showed defects in cell proliferation in response to steroid signals (36). In addition to the two immunophilins, two other genes and ESTs involved in protein folding were identified as MYC targets: an EST homologous to the bacterial mitochondrial chaperone Grpe and the mitochondrial heat shock 60-kDa protein HSPD1.

Our analysis indicates that MYC target genes influence a variety of cellular processes including growth, metabolism, cell cycle progression, and signal transduction. MYC's complex physiological effects are therefore unlikely to be recapitulated by any single target. Our study represents an initial attempt to define these multiple interactions. Further systematic, genome-wide analyses should certainly provide new connections between MYC and cellular pathways that cannot be anticipated by our current limited knowledge of the genes controlling growth and proliferation.

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1. Henriksson, M. & Luscher, B. (1996) *Adv. Cancer Res.* **68,** 109–182.
2. Magrath, I. (1990) *Adv. Cancer Res.* **55,** 133–270.
3. Li, L., Nerlov, C., Prendergast, G., MacGregor, D. & Ziff, E. B. (1994) *EMBO J.* **13,** 4070–4079.
4. Bush, A., Mateyak, M., Dugan, K., Obaya, A., Adachi, S., Sedivy, J. & Cole, M. (1998) *Genes Dev.* **12,** 3797–3802.
5. Dang, C. V. (1999) *Mol. Cell. Biol.* **19**, 1–11.
6. Wu, K.-J., Polack, A. & Dalla-Favera, R. (1999) *Science* **283,** 676–679.
7. Kretzner, L., Blackwood, E. M. & Eisenman, R. N. (1992) *Nature (London)* **359,** 426–429.
8. Grandori C. & Eisenman R. N. (1997) *Trends Biochem. Sci.* **22,** 177–181.
9. Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H. & Brown, E. L. (1996) *Nat. Biotechnol.* **14,** 1675–1680.
10. Eilers, M., Picard, D., Yamamoto, K. R. & Bishop, J. M. (1989) *Nature (London)* **340,** 66–68.
11. Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G. & Evan, G. I. (1995) *Nucleic Acids Res.* **23,** 1686–1690.
12. Muller, A. J., Young, J. C., Prendergast, A. M., Pondel, M., Landau, N. R., Littman, D. R. & Witte, O. N. (1991) *Mol. Cell. Biol.* **11,** 1785–1792.
13. Tamayo, P., Slonim, D., Mesirov, J., Zhu, Q, Kitareewan, S., Dmitrovsky, E., Lander, E. S. & Golub, T. R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2907–2912.
14. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1990) *Current Protocols in Molecular Biology* (Green/Wiley-Interscience, New York).
15. Greasley, P. J., Bonnard, C. & Amati, B. (2000) *Nucleic Acids Res.* **28,** 446–453.
16. Eilers, M., Schirm, S. & Bishop, J M. (1991) *EMBO J.* **10,** 133–141.
17. Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J. M., Varmus, H. & Lee, W. (1987) *Mol. Cell. Biol.* **7,** 1697–1709.
18. Tavazoie, S., Hughes J. D., Campbell, M. J., Cho, R. J. & Church, G. M. (1999) *Nat. Genet.* **22,** 281–285.
19. Xiao, Q., Claassen, G., Shi, J., Adachi, S., Sedivy, J. & Hann, S. R. (1999) *Genes Dev.* **12,** 3803–3808.
20. Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N. & Gallant, P. (1999) *Cell* **98,** 779–790.
21. Iritani, B. M. & Eisenman, R. N. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 13180–13185.
22. Schuhmacher, M., Staege, M. S., Pajic, A., Polack, A., Weidle, U. H., Bornkamm, G. W., Eick, D. & Kohlhuber, F. (1999) *Curr. Biol.* **9,** 1255–1258.
23. Rosorius, O. Reichart, B., Kratzer, F., Heger, P., Dabauvalle, M. C. & Hauber, J. (1999) *J. Cell Sci.* **112,** 2369–2380.
24. Elfgang, C., Rosorius, O., Hofter, L., Jaksche, H., Hauber, J. & Bevec, D. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6229–6234.
25. Park, M. H., Joe, Y. A. & Kang, K. R. (1998) *J. Biol. Chem.* **273,** 1677–1683.
26. Amati, B., Alevizopoulos, K. & Vlach, J. (1998) *Front. Biosci.* **3,** D250–D268.
27. Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J. & Eilers, M. (1999) *EMBO J.* **18,** 5321–5333.
28. Perez-Roger, I., Kim, S. H., Griffiths, B., Sewing, A. & Land, H. (1999) *EMBO J.* **18,** 5310–5320.
29. Hadwiger, J. A., Wittenberg, C., Mendenhall, M. D. & Reed, S. I. (1989) *Mol. Cell. Biol.* **9,** 2034–2041.
30. Hynes, R. O. & Yamada, K. M. (1982) *J. Cell Biol.* **95,** 369–377.
31. Rowe, D. W., Moen, R. C., Davidson, J. M., Byers, P. H., Bornstein, P. & Palmiter, R. D. (1978) *Biochemistry* **17,** 1581–1590.
32. Cooper, H. L., Feuerstein, N., Noda, M. & Bassin, R. H. (1985) *Mol. Cell. Biol.* **5,** 972–983.
33. Prasad, G. L., Fuldner, R. A. & Cooper, H. L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7039–7043.
34. Boyd, J., Risinger, J. I., Wiseman, R. W., Merrick, B. A., Selkirk, J. K. & Barrett, J. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11534–11538.
35. Song, H. Y., Dunbar, J. D., Zhang, Y. X., Guo, D. & Donner, D. B. (1995) *J. Biol. Chem.* **270,** 3574–3581.
36. Klefstrom, J., Vastrik, I., Saksela, E., Valle, J., Eilers, M. & Alitalo, K. (1994) *EMBO J.* **13,** 5442–5450.
37. Perrot-Applanat, M., Cibert, C., Geraud, G., Renoir, J.-M. & Baulieu, E.-E. (1995) *J. Cell Sci.* **108,** 2037–2051.
38. Vittorioso, P., Cowling, R., Faure, J.-D., Caboche, M. & Bellinni, C. (1998) *Mol. Cell. Biol.* **18,** 3034–3043.