The c-myc proto-oncogene can direct a diverse array of biological activities, including cell cycle progression, apoptosis, and differentiation. It is believed that Myc can affect this wide variety of activities by functioning as a regulator of gene transcription, although few targets have been identified to date. To delineate the molecular program regulated downstream of Myc, we used a cDNA microarray approach and identified 52 putative targets out of >6000 cDNAs analyzed. To further distinguish the subset of genes whose regulation was dependent upon Myc per se from those regulated in response to activation of general mitogenic or apoptotic programs, the putative cDNA targets were then screened by a series of assays. By this approach 37 putative targets were ruled out and 15 Myc target genes were uncovered. Interestingly, comparing our results with other high throughput screens reveals that certain putative Myc targets previously reported are shown not to be regulated downstream of Myc (e.g. ribosomal proteins, HSP90β), whereas others are further supported by our analyses (e.g. pdgfβr, nucleolin). The identity of genes specifically regulated downstream of Myc provides the critical tools required to understand the role Myc holds in the transformation process and to delineate how Myc functions as a regulator of gene transcription.

The c-myc oncogene has been implicated in both the initiation and the progression of a wide variety of tumors. Indeed, it is estimated to contribute to one in seven cancer deaths, including breast, colon, and cervical carcinomas (reviewed in Ref. 1). Strikingly, 100% of Burkitt’s lymphoma patients harbor a deregulated c-myc allele as a result of a translocation that juxtaposes the c-myc gene with the regulatory region of an immunoglobulin gene (reviewed in Ref. 2). This genetic rearrangement leads to constitutively high expression levels of Myc and, when deregulated, promote cellular transformation (reviewed in Refs. 3–5). This diversity of functions has been attributed to Myc’s ability to activate or repress the transcription of different target genes that mediate these various activities. Myc activation of gene transcription has been studied in depth for many years, yet few targets have been discovered. These include ornithine decarboxylase, elongation initiation factor 4E (eIF-4E), cdc25A, and carbamoyl-phosphate synthase (glutamine hydrolyzing)/aspartate carbamoyltransferase/dihydroorotase (cad)1 (6–9). The current model of Myc activation involves Myc heterodimerization with its partner protein Max forming a DNA binding domain. This MycMax heterodimeric complex can then recognize and bind specific E-box elements associated with the target gene to directly activate transcription. It is hypothesized that Myc can activate genes through multiple regulatory events at the level of transcriptional initiation and elongation (3, 10–16). In contrast, Myc repression of gene expression is much less defined. Myc repressed genes include growth arrest and DNA damage-inducible gene (gadd45α), platelet-derived growth factor β receptor (pdgfβr), H-ferritin, p15INK4b, and c-myc itself (17–21). Evidence suggests that Myc may repress some genes through an initiator element in the core promoter (20, 22–26). However, Myc can also repress genes that lack initiator elements (17, 18), suggesting multiple mechanisms of Myc-mediated repression. It is thought that Myc’s ability to repress certain genes occurs via interference with transcription factors or enhancers that are required at these promoters for gene activation (3, 20, 27). Identifying Myc target genes provides the critical experimental tools to address both the biological role and molecular mechanism of Myc action.

Numerous reports have been published recently that have employed a variety of large scale gene expression approaches to uncover the subset of genes regulated by Myc in an effort to understand Myc’s biological role (28–36). Interestingly, very few overlapping targets have been identified in these array experiments, with the exception of many ribosomal genes. This may be due to the different profile of cDNAs analyzed in the various assays. However, it is likely that, in addition to the identification of Myc-specific targets, genes that are regulated as a consequence of Myc activity, but not dependent on Myc, may also be captured by the array approach, thus obscuring the isolation of true targets. This can lead to the study of false-positive gene targets as well as misconceptions regarding the nature of the genes that are regulated by Myc. For these reasons, the identification of target genes that are specifically regulated in a Myc-dependent manner is essential.
To this end, we have undertaken a microarray approach in combination with a series of subsequent screening steps to identify genes that are regulated downstream of Myc. By this approach we were able to rule out 37 of the original 52 putative target genes that were identified as differentially regulated by the microarray analysis. We report the identity of 15 genes that were regulated by Myc in each of our experimental systems and fulfilled our criteria of a downstream target of Myc.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Parental TGR-1 rat fibroblasts, Myc null HO15.19 rat fibroblasts, HO15.19 cells infected with green fluorescent protein (GFP) vector alone (HO15.19-GFP) and HO15.19 cells infected with GFP-myc retrovirus (HO15.19-myc) were described previously (18). They were maintained in 10% calf serum-Dulbecco’s modified Eagle’s medium-H21 (DMEM H21, Invitrogen). The media was supplemented with 100 µg of penicillin per milliliter and 100 µg of streptomycin sulfate per milliliter. Quiescence was achieved by maintaining the cells in 0.25% calf serum-DMEM H21 for 48 h, and serum stimulation was accomplished by replacing media with 10% calf serum-DMEM H21 for the indicated time periods. Rat1 MycER™ cells have been described previously (18). They were maintained in 10% fetal bovine serum-α-modified Eagle’s medium (αMEM) supplemented with 100 µg of penicillin per milliliter and 100 µg of streptomycin sulfate per milliliter. Quiescence was achieved by maintaining the cells in 0.25% fetal bovine serum-αMEM H21 for 48 h, and MycER™ was activated by adding hydroxytamoxifen (OH-T) to 100 nM (Sigma). Cycloheximide (CHX) was used at a concentration of 10 µg/ml. Primary rat embryo fibroblasts and mouse embryo fibroblasts were maintained in 10% fetal bovine serum-αMEM. The Phoenix Eco retroviral packaging cell line (American Type Culture Collection) was maintained in 10% fetal bovine serum-DMEM.

**Retroviral Production and Infection**—To produce infectious replication-deficient ecotropic retroviral particles, retroviral constructs were transfected by the calcium phosphate method into the Phoenix Eco packaging cell line, and viral supernatant was harvested 36–48 h later. This virus was then used immediately to infect target cells for 3–18 h in the presence of 8 µg/ml Polybrene or frozen at −70 °C for later use. Infected cells were isolated by fluorescence-activated cell sorting (FACS) for the GFP marker expressed from the bicistronic retroviral vector 2–3 days post-infection. GFP-positive cells were isolated with a Becton Dickinson FACStarPLUS cell sorter using a Coherent Enterprise laser emitting 175 milliwatts of light at 488 nm; GFP fluorescence (emission) was collected through a 530/30 band pass dichroic filter. BDIS CellQuest software was used for acquisition and analysis of data. GFP-positive cells from each infection were pooled.

**Microarray Analysis**—Approximately 4 × 10^6 subconfluent, proliferating HO15.19-GFP and HO15.19-myc cells were harvested and frozen, and the cell pellets were forwarded for microarray analysis in duplicate (Synteni, Fremont, CA) on a chip containing 6355 mouse cDNAs and expressed sequence tags. Differential expression values, calculated as P1/P2 where P1Signal > P2Signal or −P2/P1 where P1Signal < P2Signal, were compared between the duplicate experiments. Clones differentially regulated in both experiments that had a significant ratio of P1 to P2 (defined by us as greater than the value of 2) were then PCR-amplified and cloned into the TA cloning vector pCRII (Invitrogen).

**Northern Blotting**—Total RNA isolated from the cell lines using TRizol (Invitrogen) was resolved by electrophoresis on a formaldehyde-1.5% agarose gel, visualized by EtBr staining, photographed, transferred to a nylon membrane (GeneScreen Plus, Dupont), UV cross-linked, and baked. Blots were probed with gel-purified cDNAs random prime-labeled with [α-32P]dCTP (T7 Quick prime kit, Invitrogen). Northern blots were prehybridized for 2 h at 42 °C in 50% formamide, 5× saline/sodium phosphate/EDTA, 1% glycine, 5× Denhardt’s solution, and 100 µg/ml denatured, sheared salmon sperm DNA for at least 2 h. Blots were then hybridized in 50% formamide, 5× saline/sodium phosphate/EDTA, 1× Denhardt’s, 0.3% SDS, 100 mg/ml denatured, sheared salmon sperm DNA, 10% dextran sulfate, and 1 × 10^6 cpm/ml of denatured, 32P-radioabeled cDNA. Hybridization was carried out at 42 °C for 16–20 h. Blots were washed three times for 15 min at room temperature in 2× SSC, 0.1% SDS and then 2× at 60 °C in 0.2× SSC, 0.1% SDS. Bands were visualized by autoradiography on Biomax MS film (Kodak) using a BioMax Transcreen HE intensifying screen at −70 °C. Densitometry was accomplished using ImageQuaNT software and normalized to a 36B4 loading control (15).

**RNase Protection Assay**—RNase protection assays were conducted as previously described (17, 18, 21). Briefly, RNA harvested from cell
| Activated gene target identified by microarray analysis | Description | Regulated by exogenous Myc in the asynchronous H015.19 cell system | Regulated by exogenous Myc in primary cells | Regulated in response to MycER<sup>TM</sup> activation | Not mitogen-regulated in Myc-null cells | Previously observed as Myc-responsive (reference) | NCBI locus link<sup>b</sup> |
|-----------------------------------------------------|-------------|-------------------------------------------------|--------------------------------|-------------------------------------------------|--------------------------------|-------------------------------------------------|---------------------|
| Atpurinic/apyrimidinic exonuclease (APEX)            | Multifunctional DNA repair enzyme | ✓ | ✓ | ✓ | ✓ | ✓ | (31) 328 |
| BTF3                                                | General transcription factor 3; essential for transcription initiation | ✓ | ✓ | ✓ | ✓ | ✓ | 689 |
| CDA02                                               | Weakly similar to eukaryotic translation initiation factor 3 subunit 9 | ✓ | ✓ | ✓ | ✓ | ✓ | 83939 |
| CDT1                                                | Guanine nucleotide exchange protein | ✓ | ✓ | ✓ | ✓ | ✓ | 81620 |
| Eukaryotic elongation factor 1 δ (EEFID)           | Forms a nucleotide exchange complex with elongation factor 1 beta (EEFB2) | ✓ | ✓ | ✓ | ✓ | ✓ | 1936 |
| Eukaryotic elongation factor 1 γ (EEF1G)          | Heat-shock protein | ✓ | ✓ | ✓ | ✓ | ✓ | 1937 |
| HSP 90-β (HSPCB)                                   | Unknown | ✓ | ✓ | ✓ | ✓ | ✓ | 3326 |
| KIAA0664                                           | Microtubule-associated motor proteins; may function in intracellular transport and mitosis | ✓ | ✓ | ✓ | ✓ | ✓ | 23277 |
| Minichromosome maintenance deficient 6 (MCM6)       | Enables DNA replication after mitosis | ✓ | ✓ | ✓ | ✓ | ✓ | 4175 |
| Macrophage migration inhibitory factor (MIF)       | Glycosylation-inhibiting factor | ✓ | ✓ | ✓ | ✓ | ✓ | 4282 |
| Microphthalmin-associated transcription factor (MIF) | Basic helix-loop-helix and leucine zipper containing transcription factor | ✓ | ✓ | ✓ | ✓ | ✓ | 4286 |
| Nebulin (NEB)                                      | Component of the cytoskeletal matrix | ✓ | ✓ | ✓ | ✓ | ✓ | 4703 |
| NOP55                                              | Nuclear protein | ✓ | ✓ | ✓ | ✓ | ✓ | 10529 |
| Novel gene-clone 16                                | Unknown | ✓ | ✓ | ✓ | ✓ | ✓ | 6218 |
| Novel gene-clone 19                                | Unknown | ✓ | ✓ | ✓ | ✓ | ✓ | 6128 |
| Novel gene-clone 26                                | Unknown | ✓ | ✓ | ✓ | ✓ | ✓ | 6128 |
| Novel gene-clone 38                                | Unknown | ✓ | ✓ | ✓ | ✓ | ✓ | 6128 |
| Nucleolin (NCL)                                    | RNA-binding nucleolar phosphoprotein | ✓ | ✓ | ✓ | ✓ | ✓ | 4691 |
| Nucleophosmin (NPM1)                               | RNA-binding nucleolar phosphoprotein | ✓ | ✓ | ✓ | ✓ | ✓ | 4889 |
| RPS17                                              | Ribosomal protein S17 | ✓ | ✓ | ✓ | ✓ | ✓ | (32) 6218 |
| RPL4                                               | Ribosomal protein L4 | ✓ | ✓ | ✓ | ✓ | ✓ | 6128 |
| RPL6                                               | Ribosomal protein L6 | ✓ | ✓ | ✓ | ✓ | ✓ | 6128 |
| RPS23                                              | Ribosomal protein S23 | ✓ | ✓ | ✓ | ✓ | ✓ | 6128 |
| TOB3 (FLJ10709)                                    | Member of the AAA ATPase protein family | ✓ | ✓ | ✓ | ✓ | ✓ | 83858 |

<sup>a</sup> Key: ✓ = result is indicative of an Myc-regulated gene. ✓ = result is weakly indicative of an Myc-regulated gene, ✗ = result indicates gene is not Myc-regulated. NI—not informative.

<sup>b</sup> NCBI locus link refers to the human homologue of the mouse cDNAs identified.
cultures (TRIzol, Invitrogen) was hybridized to a radioactive 32P-labeled riboprobe specific for the gene of interest (pdgfrβ, c-myc exon 1, gadd45, cad, or glycerdehyde-3-phosphate dehydrogenase). RNases were added to digest single-stranded RNA molecules. Protected double-stranded RNA molecules were then electrophoresed in a 6% polyacrylamide denaturing gel and visualized by autoradiography.

RESULTS

To identify Myc target genes using a microarray approach, it was important to employ a cellular system that would maximize sensitivity of detection with respect to differential gene expression in the presence or absence of Myc protein. To this end, we used the Myc-null cell line HO15.19, established by homologous recombination of both c-myc alleles of the parental Rat1 TGR-1 cells thereby replacing the coding region with drug-selectable markers (37). HO15.19 cells were infected with control retrovirus containing the cDNA for green fluorescent protein (HO15.19-GFP) or retrovirus containing both human c-myc and GFP cDNAs (HO15.19-myc). The infected cells were isolated by fluorescence-activated cell sorting (FACS) using GFP as the selectable marker. Immunoblot analysis of the pooled populations confirmed Myc protein expression was restricted to the HO15.19-myc cells and not evident in the HO15.19-GFP cells (data not shown). To determine whether Myc-regulated gene expression was evident in this cell system, the expression of the known Myc-activated gene cad and known Myc-repressed genes gadd45α, pdgfβ, and c-myc were evaluated (Fig. 1A). Regulation of the endogenous c-myc promoter was determined by analyzing levels of c-myc exon 1, which remained intact after the knockout procedure. RNase protection analysis was carried out on RNA extracted from asynchronously growing TGR-1, HO15.19, HO15.19-GFP, and HO15.19-myc cells. All genes displayed the expected pattern of regulation in this system with the observed difference in signal intensity clearly evident when wild-type parental TGR-1 and Myc-null HO15.19 cells were compared, and strikingly evident between the HO15.19-GFP and HO15.19-myc cells. As expected, HO15.19 and the HO15.19-GFP control cell lines displayed a similar level of control gene expression suggesting that retroviral infection did not alter cellular gene expression. These results also indicate that these target genes were regulated by both the endogenous Myc in the parental TGR-1 cells and the exogenous constitutively expressed Myc in the reconstituted HO15.19 cells. Regulation of these target genes was more pronounced in the HO15.19-myc cells, which is consistent with previous observations showing Myc regulates gene expression in a dose-dependent manner (18, 21). Taken together, these data indicate that the HO15.19-GFP/HO15.19-myc cell system serves as a valuable tool for identify target gene regulation by Myc with maximum sensitivity of differential gene expression.

Identifying Differentially Expressed Genes Using a cDNA Microarray Approach—Fluorescently labeled cDNA representing mRNA from asynchronously growing HO15.19-GFP and HO15.19-myc cells was used to probe 6.3K cDNA microarrays in duplicate (Synteni). A cDNA was considered a putative Myc-regulated target if the hybridization signal was detected as a greater than 2-fold difference in signal intensity consistent across the two separate array experiments. These candidates were validated by visual confirmation of a corresponding signal pattern of uniform spots on the microarray. This stringent analysis identified 68 reproducible gene expression differences, representing 52 different genes of which 27 appeared to be activated and 25 were repressed. Of these genes, 5 were independently scored multiple times (ribosomal proteins S23 and S17, ef1α, HSP90β, and a novel gene). Importantly, a gene previously identified as a c-Myc target, pdgfβr (18), was also detected as differentially expressed, validating both the cell system and the microarray analysis. From our initial analysis, it was impossible to determine whether the identified differentially regulated genes were true Myc targets or if they were regulated as a consequence of Myc activity. To distinguish between these two groups, the 52 genes identified as differentially regulated by the microarray screen were subsequently investigated in four separate screens, each evaluating a specific aspect of Myc regulation. Our stringent criteria to identify genes that are regulated in a Myc-dependent manner include those that were regulated in both immortalized and primary cells and by both exogenous and endogenous Myc molecules. The results of these further screens are described below and summarized in Tables I and II.

Primary Selection Criteria: Confirmation of Differential Gene Expression by Northern Blot—To evaluate the results obtained from the microarray screen, the cDNAs spotted on the microarray were amplified by PCR, and their identities were verified by nucleotide sequence analysis. These cDNAs were radiolabeled and used as probes to analyze the expression patterns of the putative Myc-regulated genes in asynchronously growing TGR-1, HO15.19, HO15.19-GFP, and HO15.19-myc cells. Northern blot analysis confirmed that the expression of the majority of genes corresponded to that assessed by the microarray analysis. Of the original 52 putative target genes, 5 were eliminated due to their lack of regulation or peculiar expression patterns. Of the remaining 47 targets, 25 were induced (Table I) and 22 were repressed (Table II) in the presence of Myc. Representative blots of this first level screen of the microarray results are shown in Fig. 1B. This screen was not only useful in verifying the microarray results, it was also informative in determining whether the presence of Myc at low endogenous levels (TGR-1) and/or deregulated exogenous levels (HO15.19-myc) affected the basal level expression of these targets in asynchronous cells. Those 47 genes identified as potential Myc-regulated targets in this first level screen were then further investigated.

Secondary Selection Criteria: Regulation in Primary Cells in Response to Exogenous Myc—To evaluate whether the differential expression of the identified genes may have been a consequence of the immortalized TGR-1/HO15.19 cell system, target gene regulation was assessed in primary mouse or rat embryonic fibroblasts (EF). These early passage mouse or rat EFs were infected with control retrovirus containing GFP alone (−Myc) or with retrovirus containing both human c-myc and GFP (+Myc). Infected cells were then isolated by FACS and pooled, and RNA was isolated from asynchronously growing cultures and used for Northern blotting. In this second level screen, 12 of the 25 putative activated genes were not regulated in embryonic fibroblasts, 12 of the genes were up-regulated, and the data for 1 gene was not informative. 2 of the 22 putative Myc-repressed targets were not regulated in response to Myc expression in embryonic fibroblasts, whereas the remainder showed a down-regulation of expression (see Tables I and II). Representative results of this screen are shown in Fig. 2.

Tertiary Selection Criteria: Regulation by the Inducible MycER™ Protein in Serum-deprived Cells—To determine whether the putative target genes could be regulated in the absence of mitogen in response to Myc activation, we used the inducible MycER™ fusion protein system (38, 39) in Rat1 fibroblasts (18). This fusion protein, of Myc and the regulatory region of the estrogen receptor (ER), is constitutively expressed in the cells but maintained in an inactive conformation. Upon the addition of hydroxymatoxifen (OH-T) Myc is rapidly activated. Serum-deprived quiescent MycER™ cells that have been exposed to OH-T progress from the G0 to S phase of the cell cycle. In this third level screen, genes that are downstream targets of Myc should exhibit regulation upon Myc induction. However, it remains possible that a subset of Myc-regulated...
| Repressed gene target identified by microarray analysis | Description | Regulated by exogenous Myc in asynchromous H015.19 cell system | Regulated by exogenous Myc in primary cells | Regulated in response to MycER™ activation | Not mitogen regulated in Myc null cells | Previously observed as Myc responsive (reference) | NCBI locus link |
|------------------------------------------------------|-------------|---------------------------------------------------------------|-------------------------------------------|---------------------------------|------------------------------------------|---------------------------------------------|----------------|
| Cathepsin B (CTSB)                                   | Lysosomal cysteine protease                          | ✓                                          | ✓                                          | ✓                                  | ✓                                        | 1508                                        |                |
| DANCE                                                | Secreted protein; binds calcium and to vascular cell integrin receptors | ✓                                          | ✓                                          | ✓                                  | ✓                                        | 10516                                       |                |
| Δ6 fatty acid desaturase (FADS2)                     | Catalyzes production of polyenoic fatty acids        | ✓                                          | ✓                                          | ✓                                  | ✓                                        | 9415                                        |                |
| Fibronectin (FN1)                                   | Member of family of proteins found in plasma and extracellular matrix | ✓                                          | ✓                                          | ✓                                  | ✓                                        | (30)                                        |                |
| Gadd45γ (GADD45G)                                   | Growth arrest and DNA-damage-inducible gamma         | ✓                                          | ✓                                          | NI                                | x                                        | 10912                                       |                |
| Gene LL5 (KIA06318)                                 | Unknown                                              | NA                                         | ✓                                          | ✓                                  | x                                        | 23187                                       |                |
| LAMP2                                                | Glycoprotein that provides selectins with carbohydrate ligands | ✓                                          | ✓                                          | ✓                                  | ✓                                        | 3920                                        |                |
| Map kinase phosphatase (MKP-1/DUSP1)                | Inactivates mitogen-activated protein kinase         | NA                                         | ✓                                          | ✓                                  | x                                        | 1843                                        |                |
| Matrix GLA (MGP)                                    | Matrix GLA protein; calcium-binding component of the skeletal extracellular matrix | ✓(✓)                                      | ✓                                          | ✓                                  | x                                        | 4256                                        |                |
| Myosin regulatory light chain (MLC-B)                | May regulate myosin head ATPase activity in smooth muscle | ✓(✓)                                      | ✓                                          | ✓                                  | x                                        | 103910                                       |                |
| N-PAC                                                | Cytokine-like nuclear factor                         | ✓(✓)                                      | ✓                                          | ✓                                  | ✓                                        | 84656                                       |                |
| NIBAN                                                | Up-regulated in renal carcinogenesis                 | NA                                         | ✓                                          | ✓                                  | ✓                                        | 63911                                       |                |
| Novel gene-cloned 17                                | Unknown                                              | ✓                                          | x                                          | ✓                                  | x                                        | x                                           |                |
| Novel gene-cloned 22                                | Unknown                                              | ✓                                          | ✓                                          | x                                  | x                                        | x                                           |                |
| Novel gene-cloned IX                                | Unknown                                              | ✓                                          | x                                          | ✓                                  | x                                        | x                                           |                |
| Novel gene-cloned XIV                               | Unknown                                              | ✓                                          | ✓                                          | ✓                                  | x                                        | x                                           |                |
| Plasminogen activator inhibitor I (SERPINE1)         | Regulates fibrinolysis                               | ✓                                          | ✓                                          | x                                  | x                                        | 5054                                        |                |
| p120GFP (PDGFB)                                     | Platelet-derived growth factor receptor beta chain    | ✓                                          | ✓                                          | ✓                                  | ✓                                        | (18)                                        | 5159           |
| Similarity to serine carboxypeptidase I Thymosin β 4 | Lysosomal glycoprotein                               | ✓                                          | ✓                                          | ✓                                  | ✓                                        | 59342                                       |                |
| (TMSB4X)                                             | Sequesters actin monomers and inhibits actin polymerization | ✓                                          | ✓                                          | ✓                                  | ✓                                        | 7114                                        |                |
| TMS-1 (TDE1)                                        | Tumor differentially expressed                       | NA                                         | ✓                                          | ✓                                  | ✓                                        | 10955                                       |                |
| TRIP12                                               | May have ubiquitin-protein ligase activity           | ✓                                          | ✓                                          | ✓                                  | ✓                                        | 9320                                        |                |

Key: ✓ = result is indicative of an Myc-regulated gene. ✓ = result is weakly indicative of a Myc-regulated gene. x = result indicates gene is not Myc-regulated, NI = not informative, NA = not assayed.

NCBI locus link refers to the human homologue of the mouse cDNAs identified.
targets, which are regulated in collaboration with other mitogen-stimulated factors, may be ruled out by this criteria. RNA extracted from serum-deprived cells at various times following MycER™ induction was analyzed by Northern blot. Fig. 3 depicts the expression of representative activated and repressed targets and those not regulated by Myc in this system. Of the 25 putative activated targets, 12 were up-regulated in response to MycER™ activation with OH-T, 12 were not regulated, and the data for 1 gene were not informative (Table I). Of 22 potential down-regulated targets, 15 targets were repressed in this system, 6 were not regulated, and the data for 1 gene were not informative (Table II). This system also allows us to assess the kinetics of regulation upon Myc activation. Induction of the majority of the activated genes was first evident at 1, 3, or 6 h after MycER™ activation in response to OH-T. Interestingly, most genes were observed to respond maximally by 9 h as seen with many Myc-regulated genes (9, 15). The kinetics of repression were more variable, which may be due to the specific half-lives of the RNA in question.

Quaternary Selection Criteria: Lack of Regulation in Serum-stimulated HO15.19 Cells—To assess the Myc-dependent regulation of putative target genes, we compared gene expression in Myc-null HO15.19 cells and parental TGR-1 cells. Putative Myc target gene regulation was assessed following serum stimulation of quiescent HO15.19 and TGR-1 cells. RNA was harvested at specific time points after Myc activation and used for Northern blot analysis with probes generated from putative Myc-regulated cDNAs. Representative blots of “Activated,” “Repressed,” and “Not Myc Regulated” genes are shown.

very low in serum deprived cells and increased quickly after the addition of serum (data not shown). This comparison allows a fourth level screen to determine whether Myc is essential for target gene regulation in response to serum exposure. It is expected that those genes whose regulation is solely dependent on Myc would not be responsive to mitogen stimulation in the HO15.19 cells, but would be regulated in the parental TGR-1 cells, which express endogenous c-Myc upon serum stimulation. This stringent criterion may exclude a subset of genes whose regulation is not solely dependent on Myc and can also be modulated by serum-regulated factors in the presence or absence of Myc. In addition, genetic alterations may have occurred during the knock-out procedure that allow the cells to continue cycling in the absence of Myc and may mask the requirement for Myc in the regulation of some targets. Northern blots were prepared with RNA extracted from serum-deprived HO15.19 and TGR-1 cells at various times after serum stimulation and analyzed with probes generated from the cDNAs identified from the original microarray. Importantly, the length of time in which the HO15.19 cells were analyzed was extended to $\frac{1}{2}$ times that of the TGR-1 to reflect the differences in the cell-cycle time between these cell lines (37). The time points chosen represent equivalent stages in the cell cycle between these cell lines, as determined by DNA content assessed by flow cytometry (data not shown). In this system 5 of the 25 activated targets were dependent on Myc for their regulation, whereas 20 were not Myc-regulated (Table I). In addition, of the 22 Myc-repressed targets 11 were found to be dependent on Myc for their regulation in this cell system.

**Fig. 2.** Secondary screen of potential Myc-regulated targets in embryonic fibroblasts (EF) in response to exogenous Myc expression. RNA (10 µg) harvested from asynchronous EFs retrovirally infected with a control retrovirus expressing GFP alone (−) or a retrovirus expressing both Myc and GFP (+) was used for Northern blot analysis and probed with the cDNAs identified in the microarray screen. Representative blots of “Activated,” “Repressed,” and “Not Myc Regulated” clones are shown.

**Fig. 3.** Tertiary screen of putative Myc-regulated genes identified by microarray analysis in Rat1-MycER™ fibroblasts. Rat1-MycER™ cells were cultured in low serum conditions for 48 h, and then MycER™ was activated by the addition of hydroxytamoxifen (OH-T). RNA was harvested at specific time points after Myc activation and used for Northern blot analysis with probes generated from putative Myc-regulated cDNAs. Representative blots of “Activated,” “Repressed,” and “Not Myc Regulated” genes are shown.
whereas 11 genes did not appear to be regulated solely by Myc (Table II). Representative blots of Myc-activated targets, Myc-repressed targets, and targets not regulated by Myc are shown in Fig. 4. A representative blot probed with 36B4 as a loading control is also shown. In addition, quantification of signal is shown relative to the zero time points and normalized to 36B4 for nucleolin and cathepsin B, which possess expression patterns similar to that of the other activated and repressed targets, respectively, as well as CDT1, PAI-1, and Nop56, three targets that were not regulated by Myc and show distinct patterns of regulation. Those genes classified as "not Myc-regulated" in this system displayed one of two patterns of regulation. These include genes whose expression levels remained unchanged throughout the cell cycle and were not regulated as a result of serum stimulation (8 genes) as well as those that were serum-regulated in both the HO15.19 and TGR-1 cells (23 genes). Genes that were regulated by Myc in each of the previously described systems are strong candidates as Myc targets. These genes are listed in Table III and include 5 activated and 10 repressed targets.

**Regulation in the Absence of de Novo Protein Synthesis**—To distinguish whether the identified Myc target genes are regulated in a proximal or distal manner downstream of Myc, we evaluated whether de novo protein synthesis was required for regulation. To this end, the inducible MycER™ system and the translational inhibitor cycloheximide (CHX) were employed. It would be expected that, if de novo protein synthesis was not necessary, regulation of the gene by activated MycER™ would be observed in the presence of CHX. This would indicate that a gene was regulated proximally downstream of Myc. Serum-deprived Rat1-MycER™ cells were exposed to CHX, CHX plus OH-T, or OH-T alone for up to 12 h, and RNA was harvested for Northern blot analysis. From this analysis we determined that KIAA0664 is directly activated by Myc (Fig. 5A). KIAA0664 levels were up-regulated in response to OH-T in MycER cells, as expected. This up-regulation was not observed in cells...
**Identifying Myc-regulated Genes**

| Gene target | Description | Regulated in the presence of cycloheximide |
|-------------|-------------|------------------------------------------|
| **Activated** | Multifunctional DNA repair enzyme | NI |
| APEX | Unknown | NI |
| KIAA0664 | Unknown | NI |
| Nucleolin (NCL) | RNA-binding nuclear phosphoprotein | NI |
| Nucleophosmin (NPM1) | RNA-binding nuclear phosphoprotein | NI |
| TOB3 (FLJ10709) | Has low similarity to SPG7 (paraplegin); member of the AAA ATPase protein family | NI |
| **RepRESSED** | | |
| Cathepsin B (CTSB) | Lyosomal cysteine protease | NI |
| Δ6 fatty acid desaturase (FADS2) | Catalyzes production of polyenoic fatty acids | NI |
| Fibronectin (FN1) | Member of the family of proteins found in plasma and extracellular matrix | NI |
| LAMP2 | Glycoprotein that provides selectins with carbohydrate ligands | NI |
| N-PAC | Cytokine-like nuclear factor | NI |
| pdgfhr (PDGFB) | Platelet-derived growth factor receptor beta chain | NI |
| Similarity to serine carboxypeptidase 1 | | NI |
| Thymosin β 4 (TMSB4X) | Lysosomal glycoprotein | NI |
| TMS-1 (TDE1) | Sequesters actin monomers and inhibits actin polymerization | NI |
| TRIP12 | Tumor differentially expressed | NI |
| **FIG. 5. Regulation of target genes in the absence of de novo protein synthesis.** Serum-deprived Rat1-MycER<sup>35</sup> cells were treated with cycloheximide (CHX), CHX + OH-T, or OH-T alone, and RNA was harvested up to 12 h later. A, KIAA0664 is directly up-regulated by Myc. B, APEX RNA levels are effected by CHX addition, which precludes interpretation of the Myc regulation. treated with CHX alone but occurred when Myc was activated in the presence of CHX. This indicates that de novo protein synthesis is not necessary for the regulation of this gene. Similarly, a gene with similarity to serine carboxypeptidase 1 was directly repressed downstream of Myc (Table III). The results strongly suggest that these two genes are regulated immediately downstream of Myc. A caveat to the use of CHX to identify proximal gene regulatory events is that these experiments are not informative for the majority of genes, because exposure to CHX alone often effects target gene expression and precludes further interpretation of the results. This was observed in the analysis of the Myc-activated gene, APEX (Fig. 5B). The levels of APEX were up-regulated in response to Myc alone. However, RNA levels decreased in response to CHX alone and in the presence of both CHX and OH-T. Unfortunately, CHX had an effect on the expression of the majority of the genes that were assessed (Table III). This experiment was also accomplished with the cells under asynchronous growing conditions with similar results obtained (data not shown). |

**DISCUSSION**

Use of microarrays to identify genes regulated by various stimuli and/or transcriptional regulators is an efficient tool for examining thousands of potential target genes in a relatively short period of time. During the course of our work, various high throughput assays have been reported that identify several hundred putative Myc-regulated genes (28–35). Although some of these genes are undoubtedly downstream targets of Myc, many of the observed changes in gene regulation may be an indirect consequence of Myc action. It is of enormous importance to delineate the subset of genes whose regulation is dependent upon Myc and to prevent false positives as well as misconceptions regarding Myc’s true activities. These Myc-dependent targets are identified by additional experimentation to isolate genes that meet criteria consistent with genes regulated downstream of Myc. In this study a series of screens were designed to distinguish Myc-regulated genes from a panel of putative targets first identified by microarray analysis. This was accomplished using a variety of systems involving both primary and immortalized cells; inducible, constitutive, or endogenous Myc molecules; and asynchronous and synchronous cell growth conditions. We conducted each of these experiments in fibroblast cell systems to provide a homogeneous environment in which any additional variables may be introduced due to cell type differences would not confound the primary goal of identifying the gene targets that are regulated by Myc. Those genes that were regulated in each of these conditions are strong candidates as universally regulated Myc targets. The identification of these regulated targets will equip us to determine how Myc can elicit its many biological functions in a wide variety of systems and, in addition, provide the critical experimental tools to investigate the mechanism(s) by which Myc can activate and repress gene transcription. In our analysis, the genes that appear to be targets of c-Myc fall under a diverse array of functional categories (Table III). Many of the repressed targets that we identified had largely unknown functions, whereas others were involved in protein catabolism, growth responsiveness, gene regulation, integrin signaling, and cytoskeletal organization. The activated targets have functions pertaining to DNA repair, centrosome duplication, and/or ribosome assembly as well as unknown functions. This wide variety of targets is consistent with the ability of Myc to influence a wide variety of activities. Indeed, the Myc targets identified to date indicate that Myc regulates a diverse group of genes involved in many cellular processes (reviewed in Refs. 3 and 5). To determine the specific significance of Myc regulation of each of these genes, it will be imperative to study them further on a gene by gene basis in an Myc-specific manner. Ultimately the goal is to identify the functional consequences of...
these regulatory events and how they relate to the many biological activities of Myc.

Many of the genes identified by gene expression profiling by other groups were also identified by our initial microarray analysis. Interestingly, these genes were subsequently segregated on the basis of our selection criteria and either eliminated or verified as an Myc-regulated gene. Those eliminated included all of the ribosomal proteins that we analyzed (RPS17, RPS23, RPL4, and RPL6). These and other ribosomal genes have been identified in multiple screens for Myc target genes leading to the hypothesis that Myc may be involved in regulating protein synthesis at this level (28, 31, 32). Our results suggest ribosomal genes are not necessarily Myc targets; however, further evaluation to determine if the other identified ribosomal proteins are truly Myc-regulated genes is required. In addition to ribosomal proteins, eukaryotic elongation factor 1β and 1γ (32), HSP90β (31, 32, 35) MIF (31), and DANCE (31) have been identified by others as Myc targets but do not appear to be specifically regulated by Myc upon further investigation by our criteria. This indicates the extreme importance of further screening to identify Myc target genes identified by high throughput assays. Indeed, many of the genes that we identified as true Myc targets have also been identified in other screens or studies. These included p128 (18), nucleophosmin (31, 35, 40), nucleolin (30, 35, 41), fibronectin (30), and APEX (31). Thus, it is important to distinguish Myc-specific targets from the false positives that can be identified using a high throughput approach, and a thorough analysis of each putative target is necessary to determine whether it is truly a Myc-regulated gene. Indeed, one method to assess direct Myc targets is to examine gene regulation in response to Myc in the presence of the translational inhibitor cycloheximide (CHX). Unfortunately, in our analysis CHX had a dominant effect on the regulation of most gene targets on its own, thus we could not assess direct regulation by Myc using this approach (Table III).

An outstanding question that exists in the field of oncogenic research is whether an endogenous cellular proto-oncogene has the same or different function as the activated oncogene. This issue has been brought to light most recently by Guo et al. (31) in which a microarray analysis indicated that exogenous and endogenous Myc might regulate different subsets of target genes. Interestingly, although we employed a similar cellular system, we did not observe such a trend. Indeed, all 15 verified Myc downstream genes were regulated by constitutive and inducible exogenous Myc expression in both immortal and primary cell systems as well as in response to endogenous Myc expression following mitogen stimulation of TGR-1 cells. Importantly, the genes that were not regulated by endogenous Myc in the latter system were also not regulated by exogenous Myc in at least one of the cellular systems expressing ectopic Myc (HO15.19-myc, primary, and MycER™). These include BTF3, CDA02, CDT1, eukaryotic elongation factor 1γ, microphthalmia-associated transcription factor, and three novel genes clones 16, 38, and IX. Taken together, our results strongly suggest conclusions drawn directly from microarray screens must be verified by an independent measure. It will be important to further investigate each of the targets identified by Guo and colleagues (31) to determine if the observed differences are indeed the result of differential gene regulation by endogenous and exogenous Myc molecules. These additional levels of analyses show the Myc target genes identified and verified in the present report are regulated by both endogenous and exogenous Myc expression.

To identify true Myc target genes we systematically assessed the regulation of each gene identified as differentially regulated in our microarray screen. Our first criterion was to employ the same cell system in which we conducted our microarray experiment to verify these results. This primary screen ruled out 5 of 53 possible target genes, leaving 48 candidate up- or down-regulated genes (summarized in Tables I and II). Our secondary selection criteria was to evaluate whether Myc regulation of the target was intact in primary cells and to ensure that the observed regulatory events were not a consequence of cellular immortalization or unique to the TGR-1/HO15.19 system used in the initial microarray analysis. This screen identified 14 of 48 putative targets that were not regulated in response to ectopic Myc expression in asynchronous primary embryonic fibroblasts. Interestingly, these 14 clones also failed to meet other criteria (see below), and there were no instances in which a potential target was ruled out solely on the basis of this screen in primary cells. This indicates that genes regulated in the Myc-null HO15.19 immortalized cell system in response to exogenous Myc expression is similar to that in primary cells, and the former cell system can be successfully employed to identify Myc target genes. Our third and fourth selection criteria involved the Rat1-MycER™ cells and the HO15.19/TGR-1 system, respectively. The MycER™ system allows for the identification and elimination of those genes that are regulated as a result of full mitogenic stimulation and not in response to the induction of Myc expression alone. By analyzing gene expression in quiescent HO15.19 and TGR-1 cells following mitogen stimulation, we could address whether the regulation of the potential target gene was dependent or independent of c-Myc expression during the transition of the G0/G1 to S phase of the cell cycle. Analysis of the results of these two screens in combination was very instructive and lead to four patterns of response. The first pattern shows the putative target gene is serum responsive in the absence (HO15.19 cells) or presence (TGR-1 cells) of Myc expression and not regulated in response to MycER™. These genes are likely regulated as a consequence of cell cycle progression by factors other than Myc and represent 17 out of 48 genes. The second pattern reveals a gene that is serum-responsive in HO15.19 and TGR-1 cells as well as responsive in MycER™. This indicated that the target may be regulated by Myc, but its cell cycle regulation is not dependent upon Myc and can be orchestrated by other factors. Of the 48 genes entered into the screen, 11 showed this pattern of response. The third pattern of response is a target gene that is dependent on Myc expression in the HO15.19 and TGR-1 cells and is not regulated in MycER™. This gene is potentially dependent on Myc expression but requires other mitogen-stimulated factors for expression as well. 1 out of 48 genes exhibited this pattern of regulation. The fourth pattern is a target gene that is serum-regulated in the TGR-1 cells, but not in the HO15.19 cells, and is regulated as a result of Myc activation in the MycER™ cells. Clearly, the latter pattern reveals a target that is cell cycle-regulated in an Myc-dependent manner. Our last screen involving the HO15.19/TGR-1 cell system was by far the most stringent of our criteria; 31 out of 48 genes were eliminated based on these results. These combinations of analyses allowed us to identify 15 Myc-regulated targets that will be invaluable in identifying Myc's role within the cell.

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