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Identification of c-Myc Responsive Genes Using Rat cDNA Microarray

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Abstract

c-Myc functions through direct activation or repression of transcription. Using cDNA microarray analysis, we have identified c-Myc-responsive genes by comparing gene expression profiles between c-*myc* null and c-*myc* wild-type rat fibroblast cells and between c-*myc* null and c-*myc* null cells reconstituted with c-*myc*. From a panel of 4400 cDNA elements, we found 198 genes responsive to c-*myc* when comparing wild-type or reconstituted cells with the null cells. The plurality of the named c-Myc-responsive genes that were up-regulated, including 30 ribosomal protein genes, are involved in macromolecular synthesis and metabolism, suggesting a major role of c-Myc in the regulation of protein synthetic and metabolic pathways. When ectopically overexpressed, c-Myc induced a different and smaller set of c-Myc-responsive genes as compared with the physiologically expressed c-Myc condition. Thus, these results from expression profiling suggest a new primary function for c-Myc and raise the possibility that the physiological and transforming functions of c-*myc* may be separable.

Introduction

The proto-oncogene c-*myc* plays an important role in the control of cell proliferation, differentiation, and apoptosis, and its aberrant expression is a commonly found molecular defect in human cancers (1). c-Myc functions through the transcriptional activation or repression of its target genes. Using a variety of methods, such as subtractive hybridization, representational difference analysis, and differential display in systems with enforced c-Myc overexpression, only a small number of c-Myc target genes have been identified, although none has been shown to mediate a physiological function of Myc (2). Recently, the authenticity of some c-Myc target genes was re-evaluated in light of experiments in Rat1 fibroblasts bearing homozygous deletion of c-*myc* (3). The c-*myc* null rat fibroblasts continue to grow and show no detectable expression of N-*myc* or L-*myc*, which may compensate for the deficiency of c-*myc* (4). Using this system, Bush *et al.* (3) demonstrated that 9 of 11 previously reported c-Myc target genes appeared not differentially expressed between c-*myc* wild-type and c-*myc* null cells, suggesting that physiological expression of c-Myc may potentially regulate a different set of genes than overexpressed c-Myc. Given the limitations of the technologies used previously, we used cDNA microarray analyses to look for genes that are differentially expressed between c-*myc* wild-type and c-*myc* null cells. The advantage of this approach is the comprehensive nature of the technology and the ability to interrogate multiple cellular conditions so as to "triangulate" the true set of c-Myc-responsive genes. In this study, we report the identification of 245 genes that are responsive to the physiologically expressed or overexpressed c-Myc. Most of these c-Myc-responsive genes are involved in macromolecular synthesis and metabolism. Our findings are consistent with recent genetic studies of *Drosophila* c-*myc* (5), suggesting that c-Myc is an important regulator of protein synthetic and metabolic pathways.

Materials and Methods

EST[2] Clones and Cell Lines. Our rat microarray contained 4400 EST cDNAs clones selected from the Rat Gene Index of the Institute for Genomic Research. A small set of redundant clones (5%) were included for quality control. Selected clones were from 52,000 3′ ESTs generated from 12 different libraries constructed from 10 distinct tissues and two PC12 cell lines. All clones are available through the American Type Culture Collection. The clones used for our microarray were sequence-verified by generating both 5′ and 3′ ESTs. The EST sequences have been deposited into the GenBank database dbEST.[3]

The somatic c-*myc* null cells (Ho15.19) and its parental c-*myc* wild-type Rat1 cells (TGR-1) were from John Sedivy (Brown University, Providence, RI) and cultured as described (4). The log-phase cells were harvested at 60% confluence. For serum starvation condition, the cells were grown to confluence and then cultured without serum for 48 h. The cell lines overexpressing c-Myc were generated by cotransfection of pBabe-puro vector and CM19, which contains genomic c-*myc* gene under the control of a long terminal repeat promoter, into Ho15.19 or TGR-1 cells.

cDNA Microarray and Data Analysis. We fabricated our rat cDNA microarrays essentially as described previously (6). The plasmids of EST clones were purified using the QiaPrep Turbo kit (Qiagen). The cDNA inserts were amplified by PCR for 30 cycles with M13 froward and reverse primers. The PCR products were verified by gel electrophoresis, purified, and arrayed robotically onto polylysine-coated glass microscope slides with a GeneMachine arrayer (San Carlos, CA). The microarrays were then postprocessed to denature and immobilize the DNA (6).

The cDNA probes were made from total RNAs with cy5- or cy3-dUTP (Amersham Pharmacia) and Superscript II polymerase (Life Technologies, Inc.) as described (6). Microarrays were hybridized with probes, washed, and then scanned using an Axon scanner (Foster City, CA), with the sample intensities for cy5 and cy3 collected separately. The normalization of the sample intensities and calculation of the calibrated fluorescence ratios after background subtraction were as described (7, 8).

Northern Hybridization. The cDNA probes were made by random primer labeling. Fifteen μg of total RNA were separated on a formaldehyde 1.2% agarose gel, transferred to a Hybond-N nylon membrane (Amersham), and then immobilized by UV irradiation. The hybridization was carried out using ExpressHyb solution (Clontech), and bands were quantitated using a PhosphorImager (Molecular Dynamics).

Results and Discussion

Some Genes Are Differentially Expressed between c-*myc* Wild-Type and c-*myc* Null Cells. Because c-Myc is actively expressed in growing cells, we first compared gene expression profiles between c-*myc* null cells (Null) and its parental c-*myc* wild-type cells (WT) during

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[2] The abbreviation used is: EST, expressed sequence tag.

[3] Also accessible at www.tigr.org/tdb/ratarrays/index.html. A comprehensive listing of the genes matched, categories, and GenBank accession numbers of matched genes can be assessed at www.tigr.org/tdb/ratarrays.

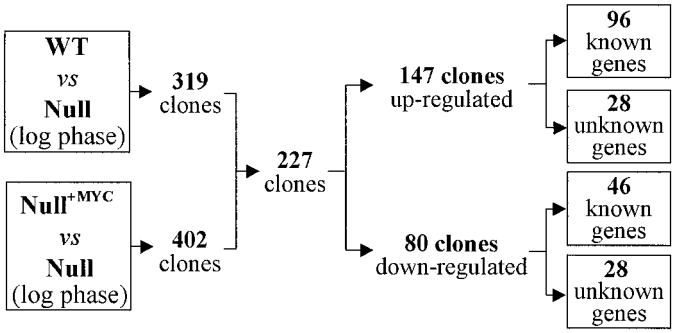

Fig. 1. Experimental scheme and summary of results. Some of the known genes were represented by more than one EST clone.

1 and 2, *A* and *B*). The correlation between the Null^{+MYC}/Null comparison and the WT/Null comparison ($r = 0.854$) is very similar to that between two repeated WT/Null comparisons ($r = 0.846$; Fig. 2, *C* and *D*). By comparing log-phase Null^{+MYC} cells and Null cells, we were also able to eliminate those gene expression differences attributable to potential clonal variations between log-phase WT and Null cells. Therefore, these 198 overlapping genes, which were differentially expressed between log-phase WT and Null cells, and also between Null^{+MYC} and Null cells, represent a stringent list of c-Myc-responsive genes in log-phase growth. Although all genes listed are responsive to the presence of c-Myc, we acknowledge that only some of these genes will be direct targets of c-Myc, and others will be indirect targets regulated by other c-Myc-responsive gene products. Our current experimental approach

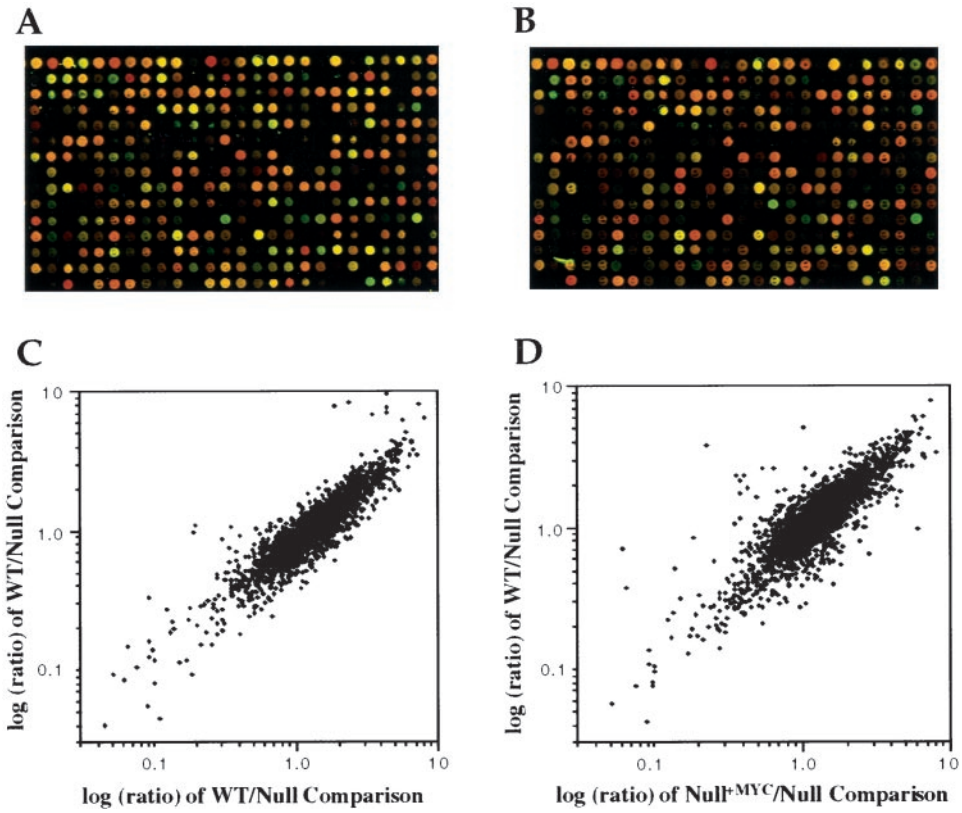

Fig. 2. Comparisons between WT and Null cells or between Null^{+MYC} and Null cells during log-phase growth. *A*, comparison of WT and Null cells at log phase. A section of the microarray is shown. Total RNA from WT cells was labeled with cy5-dUTP; total RNA from Null cells was labeled with cy3-dUTP. *B*, comparison of Null^{+MYC} and Null cells at log phase. The same section of the microarray as in *A* is shown. Total RNA from Null^{+MYC} cells was labeled with cy5-dUTP; total RNA from Null cells was labeled with cy3-dUTP. *C*, scatter plot of the calibrated ratios (log scale) in the two independent comparisons between WT cells and Null cells. *D*, scatter plot of the calibrated ratios (log scale) in the comparisons between WT cells and Null cells (*vertical axis*) and between Null^{+MYC} cells and Null cells (*horizontal axis*).

log-phase growth, as diagrammed in Fig. 1. Using the criteria of at least 2-fold difference in expression in two separate experiments, we found 319 reproducible gene expression differences, representing 283 distinct genes, between WT cells and Null cells during log-phase growth. One hundred eighty-eight of these genes were up-regulated, and 95 were down-regulated by 2.3–15-fold in WT cells. That 7.3% (319 of 4400) of the gene elements on the microarray were differentially regulated is highly significant, because the false-positive rate of our system is only 0.2% (8 of 4400) at an expression threshold of 2-fold difference.

Ectopically Expressed c-Myc Can Recover the Gene Expression Profiles in the c-*myc* Null Cells. To validate that the differential gene expression we found truly depends on the expression of c-Myc, we ectopically expressed c-Myc in the c-*myc* null cells (Null^{+MYC}) and compared the gene expression between Null^{+MYC} cells and Null cells (Fig. 1). We found that these c-*myc*-reconstituted cells behaved like their wild-type counterparts. As shown in Fig. 2, the gene expression profile of Null^{+MYC} cells resembled that of the WT cells, because they shared 198 differentially expressed genes (represented by 227 EST clones on the microarray) when each was compared with Null cells at log phase (Figs. cannot distinguish between the two mechanisms of induction but identifies the universe of RNAs whose levels are correlated with the expression of c-Myc.

Of these 198 c-Myc-responsive genes, 124 genes were up-regulated by c-Myc, which represent 96 known genes and 28 unnamed ESTs. There were 74 genes down-regulated by c-Myc, representing 46 known genes and 28 unknown genes. The known genes are listed in Tables 1 and 2.[4] Among the c-Myc-responsive genes we observed, *cyclin D1*, *Cdc2*, *LDH-A*, and *MHC-Iα* have been reported previously as c-Myc targets (2). *Cyclin D1* has been reported to be either up-regulated or down-regulated by c-Myc in different studies (9, 10). In our cDNA microarray study, *cyclin D1* was consistently down-regulated by c-Myc in WT cells compared with Null cells, and this was confirmed by Northern hybridization (Fig. 3). The Myc-mediated decrease of *cyclin D1* expression was also observed when Null cells were compared with Null^{+MYC} cells or WT cells were compared with wild-type cells overexpressing c-Myc

[4] Full lists are available at http://www-dcs.nci.nih.gov/research/labdata/Liulab. html.

Table 1 *Genes up-regulated by c-Myc*[a]

No.	Gene name	Function	Ratio[b]	-Sera[c]
	c-*myc*	Transcription	2.6	
1	*Cdc2*[d]	Cell cycle	3.6	
2	*cdk regulatory subunit 2 (CKS-2)*	Cell cycle	3.4	
3	*Proliferating cell nuclear antigen (PCNA)*	Cell cycle	3.2	
4	*AIM-1*	Cell cycle	2.7	
5	*Mitotic feedback protein control protein*	Cell cycle	4.2	
6	*Adenine nucleotide translocator*	Apoptosis	3.4	
7	*Proliferating cell-associated mRNA*	Cell growth	2.5	
8	*JTV-1 protein*	Cell growth	2.6	
9	*Lens epithelial protein*	Cell growth	3.4	
10	*rab GDP-dissociation inhibitor β*	Signaling	2.6	
11	*rhoB*	Signaling	4.0	
12	*Protein phosphatase-1 γ1 (pp-1γ1)*	Signaling	2.5	
13	*Macrophage migration inhibitory factor (MIF)*	Signaling	2.9	
14	*Hyaluronan synthase 2*	Signaling	2.6	
15	*Nucleosome assembly protein*	Chromosomal	3.0	2.7
16	*Histone H2a.z*	Chromosomal	3.4	
17	*HMG-17 non-histone chromosomal protein*	Chromosomal	2.8	
18	*APEX endonuclease*	DNA repair	4.7	
19	*DNA polymerase α*	DNA replication	3.8	2.9
20	*Rb-binding protein (RBP1)*	Transcription	3.7	3.1
21	*Transcriptional regulatory factor (α-NAC)*	Transcription	3.0	
22	*Transducin-like enhancer protein 4 (Grg-4)*	Transcription	2.5	
23	*C-1 putative transcription factor*	Transcription	2.6	
24	*Rat Y-Box binding protein α (RYB-α)*	Transcription	2.7	
25	*Glutamine-rich factor 1*	Transcription	2.7	
26	*Small nuclear ribonucleoprotein Sm D3*	RNA processing	2.9	
27	*Heterogeneous nuclear ribonucleoprotein G*	RNA processing	2.4	
28	*Splicing factor SC35*	RNA processing	2.9	
29	*Leucyl-tRNA synthetase homologue*	Protein synthesis	2.9	
30	*Ribosomal phosphoprotein P1*	Protein synthesis	3.2	
31	*Ribosomal protein L5*	Protein synthesis	4.7	2.7
32	*Ribosomal protein L6*	Protein synthesis	3.6	
33	*Ribosomal protein L7*	Protein synthesis	4.4	2.3
34	*Ribosomal protein L7a*	Protein synthesis	4.2	2.4
35	*Ribosomal protein L9*	Protein synthesis	3.4	
36	*Ribosomal protein L10*	Protein synthesis	4.0	2.2
37	*Ribosomal protein L12*	Protein synthesis	3.1	
38	*Ribosomal protein L19*	Protein synthesis	2.8	
39	*Ribosomal protein L21*	Protein synthesis	3.4	2.2
40	*Ribosomal protein L22*	Protein synthesis	3.3	
41	*Ribosomal protein L23*	Protein synthesis	4.6	2.2
42	*Ribosomal protein L26*	Protein synthesis	4.0	
43	*Ribosomal protein L27*	Protein synthesis	3.0	
44	*Ribosomal protein L30*	Protein synthesis	3.7	
45	*Ribosomal protein L32*	Protein synthesis	4.3	
46	*Ribosomal protein L35*	Protein synthesis	4.0	
47	*Ribosomal protein L41*	Protein synthesis	3.5	2.5
48	*Ribosomal protein L44*	Protein synthesis	5.4	2.4
49	*Ribosomal protein S4*	Protein synthesis	3.6	
50	*Ribosomal protein S7*	Protein synthesis	3.7	
51	*Ribosomal protein S9*	Protein synthesis	3.3	
52	*Ribosomal protein S12*	Protein synthesis	4.2	
53	*Ribosomal protein S13*	Protein synthesis	4.8	
54	*Ribosomal protein S15a*	Protein synthesis	3.1	
55	*Ribosomal protein S16*	Protein synthesis	2.8	
56	*Ribosomal protein S18*	Protein synthesis	4.1	3
57	*Ribosomal protein S19*	Protein synthesis	3.0	
58	*Ribosomal protein S20*	Protein synthesis	4.1	2.2
59	*Ribosomal protein S23*	Protein synthesis	3.5	
60	*Elongation factor 2 (EF-2)*	Protein synthesis	2.8	2.3
61	*Signal recognition particle subunit (SRP)*	Protein synthesis	4.9	2.2
62	*Breast basic conserved protein 1*	Protein synthesis	4.5	
63	*RNA helicase p68*	Protein synthesis	3.9	
64	*Nucleophosmin (NPM; B23)*	Protein synthesis	4.2	2.5
65	*Spermidine synthase*	Protein/RNA synthesis	3.7	
66	*Cyclophilin-40 (40 kD)*	Protein folding	2.8	
67	*Cyclophilin (18 kD)*	Protein folding	2.7	
68	*Ran/TC4 GTP-binding nuclear protein*	Protein/RNA transport	2.9	
69	*Ran/TC4 binding protein (RanBP1)*	Protein/RNA transport	3.2	
70	*Secretory carrier-associated protein 2*	Protein transport	3.2	
71	*Heat shock protein 60 (HSP60)*[d]	Protein folding	5.0	
72	*Heat shock protein 90 (HSP90)*	Protein folding	3.3	
73	*Chaperonin-containing TCP-1 zeta subunit*	Protein folding	2.9	
74	*E2-EPF ubiquitin-carrier protein*	Protein degradation	2.6	
75	*Ubiquitin-like protein SMT3B*	Protein degradation	2.3	
76	*Ubiquitin*	Protein degradation	4.2	
77	*R-IOTA (proteasome subunit)*	Protein degradation	2.9	
78	*Phosphorylation regulatory protein HP-10*	Protein phosphorylation	2.6	
79	*Lactate dehydrogenase (LDH-A)*[d]	Metabolism	3.1	
80	*Enolase-α*	Metabolism	2.6	
81	*Glycerate dehydrogenase (GDH)*	Metabolism	2.6	
82	*Thioredoxin*	Metabolism	2.3	

Table 1 *Continued*

No.	Gene name	Function	Ratio[b]	-Sera[c]
83	Thioredoxin-dependent peroxide reductase	Metabolism	2.3	
84	Cytochrome c	Metabolism	2.7	
85	Heme oxygenase-2	Metabolism	3.0	
86	β-1,4-Galactosyltransferase	Metabolism	3.3	
87	Sarcosine dehydrogenase	Metabolism	2.9	
88	Inosine 5′-monophosphate dehydrogenase	Metabolism	3.9	2.5
89	PEP carboxykinase	Metabolism	5.9	
90	Acyl-CoA-binding protein	Metabolism	2.5	
91	ATPase inhibitor protein IF1	Metabolism	3.0	
92	Microsomal glutathione-S-transferase 3	Metabolism	3.8	2.3
93	Cysteine-rich intestinal protein (CRIP)	Zinc absorption	3.3	
94	Microtubule-associated protein 4 (MAP4)	Cell structure	2.7	
95	Peripherin	Cell structure	2.9	
96	Voltage-dependent anion channel-like protein	Channel	2.3	

[a] A complete list of the differentially expressed genes, including unknown ESTs, can be found on our web site (http://www-dcs.nci.nih.gov/research/labdata/Liulab.html).
[b] The average of ratios in both WT/Null and Null^{+MYC}/Null comparisons during log-phase growth.
[c] The ratios in the comparison between WT cells and Null cells under the serum starvation condition.
[d] Gene reported previously to be a Myc target.

(WT^{+MYC}; Fig. 3). Although it has been reported that the expression of LDH-A is not changed between c-*myc* wild-type cells and c-*myc* null cells (3), we found that *LDH-A* is consistently induced in WT cells or Null^{+MYC} cells when they were compared with Null cells in our microarray analysis. This result was also confirmed by Northern hybridizations (Fig. 3). In addition to *cyclin D1* and *LDH-A*, we chose 10 other genes from Tables 1 and 2 and examined their expressions by Northern hybridizations in Null, Null^{+MYC}, WT, and WT^{+MYC} cells. Except for the two genes whose expression was not detectable by Northern, all others were differentially expressed, in concordance with our microarray

Table 2 *Genes down-regulated by c-Myc*[a]

No.	Gene name	Function	Ratio[b]	-Sera[c]
1	*Cyclin D1*[d]	Cell cycle	0.28	
2	*PINCH protein*	Apoptosis	0.35	0.29
3	*Protein phosphatase 2A regulatory subunit ε*	Signaling	0.22	
4	*Protein kinase C-binding protein (Enigma)*	Signaling	0.36	
5	*Mitogen activated protein kinase-1 (ERK1)*	Signaling	0.30	
6	*Protein kinase (MEKK)*	Signaling	0.40	
7	*RAC-α serine/threonine kinase (RAC-PK-α)*	Signaling	0.10	
8	*RAC-β serine/threonine kinase (RAC-PK-β)*	Signaling	0.20	
9	*Thy-1 glycoprotein*	Signaling	0.38	
10	*T-cell receptor, T3-epsilon glycoprotein*	Signaling	0.33	0.20
11	*unr protein (upstream of N-ras)*	DNA binding	0.10	
12	*Calcium-binding protein (nucleobindin, NUCB1)*	DNA binding	0.24	
13	*Vascular actin single-stranded DNA-binding factor*	Transcription	0.24	
14	*Retinoic acid receptor α2*	Transcription	0.40	
15	*Sp3 transcription factor*	Transcription	0.19	
16	*Ubiquitous Kruppel-like factor*	Transcription	0.26	
17	*Zinc finger X-linked protein ZXDB*	Transcription	0.34	
18	*Red-1 nuclear protein*	Transcription	0.16	
19	*Female sterile homeotic homolog RING3*	Transcription	0.32	
20	*Heterogeneous nuclear ribonucleoprotein H*	RNA processing	0.19	
21	*Nuclear ribonucleoparticle-associated protein*	RNA processing	0.23	
22	*Paraneoplastic encephalomyelitis antigen hud*	RNA processing	0.20	0.09
23	*GCN1 (translational activator)*	Protein synthesis	0.36	
24	*α-B-crystallin*	Protein folding	0.23	
25	*Ischemia-responsive 94 kDa protein*	Protein folding	0.11	
26	*Carboxypeptidase D (CPD)*	Protein processing	0.23	
27	*ATP-dependent metalloprotease FtsH1*	Protein degradation	0.09	
28	*Peptidylglycine α-amidating monooxygenase*	Protein modification	0.34	
29	*ARL-6 interacting protein-1 (Aip-1)*	Protein trafficking	0.24	
30	*Lysosomal membrane glycoprotein (LAMP-1)*	Metabolism	0.07	
31	*ATP-specific succinyl-CoA synthetase-β*	Metabolism	0.12	
32	*B-locus C-type lectin*	Metabolism	0.43	
33	*β-1,3-Galactosyltransferase*	Metabolism	0.37	
34	*Long chain fatty acid-CoA ligase*	Metabolism	0.26	
35	*Ganglioside GM2 activator protein*	Lipid degradation	0.23	0.36
36	*MHC class II RT1.B α chain*	Immune response	0.36	0.36
37	*MHC class II RT1.D β chain*	Immune response	0.23	0.17
38	*MHC Class I α-chain antigen*[d]	Immune response	0.31	
39	*NCAM-140/180 neural cell adhesion molecule*	Adhesion	0.28	0.36
40	*Peroxisomal farnesylated protein (PxF)*	Cell structure	0.36	
41	*Moesin*	Cytoskeletal	0.40	
42	*Tropomyosin α chain*[d]	Cytoskeletal	0.25	
43	*Na-K-Cl cotransporter*	Channel	0.16	
44	*Impact*	Paternally expressed	0.28	
45	*Tumor protein D52*	Expressed in tumor	0.40	
46	*Proline-rich protein*	Unknown	0.38	

[a] A complete list of the differentially expressed genes, including unknown ESTs, can be found on our web site (http://www-dcs.nci.nih.gov/research/labdata/Liulab.html).
[b] The average of ratios in both WT/Null and Null^{+MYC}/Null comparisons during log-phase growth.
[c] The ratios in the comparison between WT cells and Null cells under the serum starvation condition.
[d] Gene reported previously to be a Myc target.

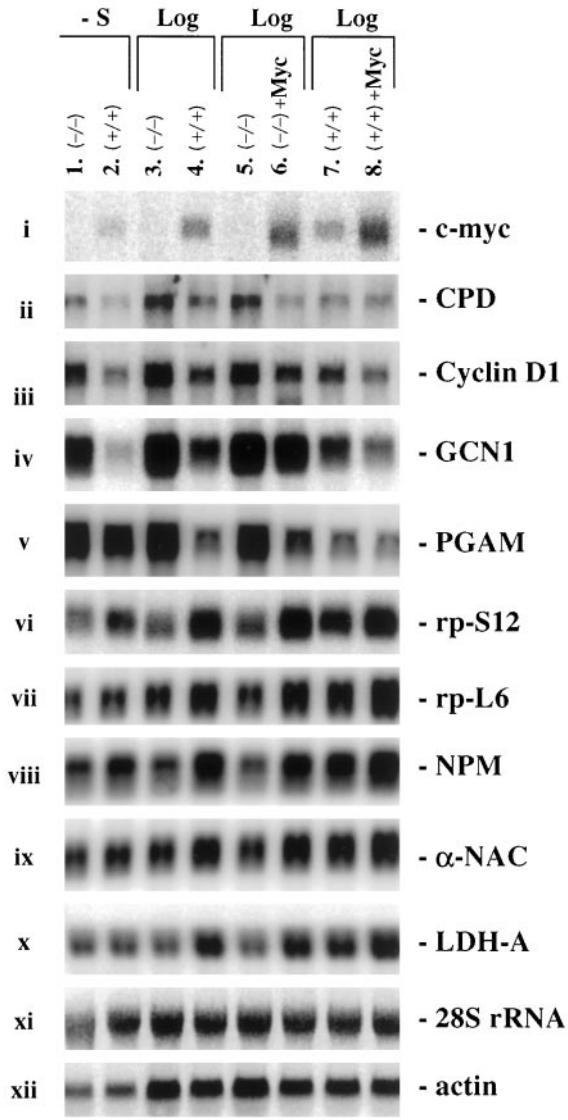

Fig. 3. Confirmation of differentially expressed genes in microarray analysis by Northern hybridizations. *(−/−)*, Null cells; *(+/+)*, WT cells; *(−/−)+Myc*, Null^{+MYC} cells; *(+/+)+Myc*, WT^{+MYC} cells. The cDNA probes were: *i*, c-myc; *ii*, CPD (carboxypeptidase D); *iii*, Cyclin D1; *iv*, GCN1; *v*, PGAM (peptidylglycine α-amidating monooxygenase); *vi*, rp-S12 (ribosomal protein S12); *vii*, rp-L6 (ribosomal protein L6); *viii*, NPM (nucleophosmin or nucleolar phosphoprotein B23); *ix*, α-NAC (nascent polypeptide associated complex); *x*, LDH-A; *xi*, 28S rRNA (inverted picture of agarose gel stained with ethidium bromide solution); *xii*, actin.

results (Fig. 3). In a limited number of instances, reported c-Myc target genes did not appear to be differentially expressed between WT and Null cells in our microarray analysis (*e.g.*, *RCC1*, *ODC*, and *Cad*). Several factors might explain these discrepancies:

(*a*) The criteria we set forth for defining c-Myc-responsive genes were very stringent. A greater than 2-fold difference in gene expression was required in repeated comparisons of WT and Null cells and also in the comparison between Null^{+MYC} cells and Null cells. These stringent inclusion criteria may exclude potentially real differences. For example, the gene expression differences for *Cad* in the three experiments were 2.81, 1.57, and 2.36 (ratios). Because the difference in one of the experiment is <2-fold cutoff (1.57), *Cad* was not considered positive.

(*b*) Some of the previously identified Myc target genes were found in different cellular systems and may not be regulated in the same manner as in the rat fibroblast cells.

(c) All of the previously reported c-Myc target genes were found in c-Myc overexpression systems. Cells may respond differently to overexpressed c-Myc than to physiologically expressed c-Myc. In fact, some of these discrepancies have been reported previously in other systems (3).

c-Myc Regulates the Expression of Genes Involved in Protein Synthesis and Metabolism. c-Myc has been considered an important regulator of the cell cycle machinery. We observed, however, that only a few cell cycle-associated genes were regulated by c-Myc. c-Myc up-regulates *CDC2*, *PCNA*, *CKS2*, *AIM1*, and *DNA polymerase α* but paradoxically down-regulates *cyclin D1*. Unexpectedly, however, we found that a majority of the c-Myc target genes (61 of 96 c-Myc-induced genes with known functions) are those involved in macromolecular synthesis, protein turnover, and metabolism (*e.g.*, *leucyl-tRNA synthetase*, *glycerate dehydrogenase*, *enolase*, *and lactate dehydrogenase*, *ubiquitin*, *E2-EPF ubiquitin-carrier protein*, and *heat shock proteins*). Interestingly, the largest category of c-Myc-induced genes were those involved in protein synthesis, including 30 of the 43 distinct ribosomal protein genes present on our microarray. In addition to the 43 ribosomal protein genes, there are 20 other genes represented on our microarray involved in protein synthesis. Taken together, 57% of genes involved in protein synthesis (36 of 63) were identified as overexpressed, despite of the fact that these protein synthesis genes represented only 1.4% of the cDNAs on the microarray. It is, therefore, very unlikely that the induction of ribosomal/protein synthesis-associated genes by c-Myc is attributable to their overrepresentation on our microarray.

Because ribosomal genes are known to be up-regulated by cell growth, we sought to minimize the general effects of growth by comparing WT and Null cells under serum-starved conditions. In this state, c-Myc is also expressed at low levels in WT cells, as confirmed by Northern hybridization (Fig. 3). Twenty-nine genes differentially expressed between WT and Null cells during serum starvation overlapped with the c-Myc-responsive genes found in log phase (Tables 1 and 2). Among these 29 genes, 9 of 19 c-Myc up-regulated genes were ribosomal protein genes. Supporting these findings, recent studies have identified other genes involved in protein synthesis not represented in our arrays that appear to be regulated by c-Myc: *eIF-2a*, *eIF4E*, *eIF5A*, *eIF4G*, *MrDb*, *nucleolin*, *isoleucine-tRNA synthetase*, and *ribosomal protein S11* (2, 11). Thus, an important function of c-Myc is in activating pathways of protein synthesis and metabolism in addition to cell cycle.

This conclusion is in concordance with results from recent genetic studies of c-*myc* in *Drosophila* [d-*myc*; (5)]. The phenotype of the three d-*myc* mutants is remarkably similar to a group of *Drosophila* mutants named *Minutes* (5). All molecularly characterized *Minute* mutants involve ribosomal protein genes (5). The only reported candidate d-Myc target gene *pit*, a *Drosophila* homologue of MrDb in mammalian cells, is also involved in ribosome assembly and protein synthesis (12). In addition to the evidence in *Drosophila*, the constitutively expressed c-*myc* transgene in mice and induced c-Myc expression in human B cells have also been found recently to increase protein synthesis (13, 14). These studies of c-Myc in *Drosophila*, mouse, and human would predict that the major c-Myc target genes involve protein synthesis and metabolism. Our findings support this prediction.

Overexpressed c-Myc Regulates Additional Set of Genes Compared with Physiologically Expressed c-Myc. Because c-Myc overexpression is very common in human and animal cancers, we ectopically expressed c-Myc in WT cells (WT^{+MYC}) and compared the gene expression between WT^{+MYC} cells and WT cells during log-phase growth (Table 3). At log phase, WT cells had a sustained c-Myc expression, and the c-Myc expression in WT^{+MYC} cells was ~4.5-

Table 3 *Genes regulated by overexpressed c-Myc*[a]

No.	Gene name	Function	Ratio[b]
	c-*myc*	Transcription	4.5
1	*PCAF associated factor-β*	Transcription	2.3
2	*DNA polymerase α*[c]	DNA synthesis	3.6
3	*Cyclin-dependent kinase-4 (cdk-4)*[d]	Cell cycle	2.9
4	*Centromere protein (27 kD)*	Cell cycle	2.5
5	*Phosphatidylinositol 4-phosphate 5-kinase Iα*	Signaling	3.5
6	*Sigma receptor type 1 (SigmaR1)*	Signaling	2.6
7	*Heparin-binding growth factor 8 (HBGF-8)*	Signaling	2.4
8	*Scavenger receptor class B type I (SR-B1)*	Metabolism	2.5
9	*Stearyl-CoA desaturase 2*	Metabolism	5.6
10	*Thioredoxin-dependent peroxide reductase*[c]	Metabolism	2.4
11	*Fibrillin 2*	Extracellular matrix	2.4
12	*PINCH protein*[c]	Apoptosis	0.32
13	*GADD45*[d]	DNA repair	0.45
14	*Histone H3.3B*	Chromosomal	0.37
15	*HSP27*	Protein folding	0.46
16	*Serine protease*	Protein degradation	0.41
17	*Ubiquitin-homology domain protein (PIC1)*	Protein degradation	0.35
18	*Peripheral-type benzodiazepine receptor*	Metabolism	0.44
19	*Drosophila frizzled gene homologue*	Signaling	0.47
20	*Nuclear dual-specificity phosphatase*	Signaling	0.30
21	*Prolactin receptor associated protein*	Signaling	0.42
22	*γ-PAK*	Signaling	0.21
23	*PAK-interacting exchange factor (β-PIX)*	Signaling	0.23
24	*PE31/TALLA*	Signaling	0.35
25	*Thy-I glycoprotein*[c]	Signaling	0.41
26	*Vascular endothelial growth factor D*	Signaling	0.44
27	*Channel integral membrane protein 28*	Water channel	0.37
28	*Osteonectin*	Cell growth	0.33
29	*UP50 (EGF-like serum-suppressed gene)*	Cell growth	0.34
30	*TGF-β responsive adhesion-inhibition gene*	Tumor suppression	0.29
31	*Thrombospondin*[d]	Metastasis	0.23
32	*Collagen α1(V)*	Cell structure	0.32

[a] A complete list of the differentially expressed genes, including unknown ESTs, can be found on our web site (http://www-dcs.nci.nih.gov/research/labdata/Liulab.html).
[b] The average of ratios in comparison between WT^{+MYC} and WT cells during log-phase growth. Values of more than 1 represent c-Myc up-regulated genes. Values of less than 1 represent the c-Myc down-regulated genes.
[c] Genes present in Tables 1 and 2.
[d] Gene reported previously to be a Myc target.

fold higher than that in WT cells (Fig. 3; Table 3). We found that 32 known genes and 19 unknown genes were differentially expressed between WT^{+MYC} cells and WT cells during log-phase growth. Forty-seven of these 51 genes uncovered here were not found in the comparison between log-phase WT cells and Null cells and may represent candidate genes responsive mainly to high levels of c-Myc expression. Among them, *Gadd45* and *thrombospondin* have been reported previously as target genes of overexpressed c-Myc (2). It is reasonable to hypothesize that the overexpressed c-Myc may turn on or off the expression of some genes not normally regulated by physiological levels of c-Myc either through direct effects on promoter occupancy or through the differential induction of other transcriptional activators.

Recently, using oligonucleotide microarray analysis, Coller *et al.* (11) found 36 genes regulated by ectopically overexpressed c-Myc in density arrested or serum-starved human fibroblasts. Of these 36 c-Myc-regulated genes, 28 were not present on our rat cDNA microarray. Thus, direct comparisons between array results cannot be made. Nevertheless, of the other 8 genes present on both microarrays, *tropomyosin*, *collagen*, and *HSP60* were also found in our study. Moreover, Coller *et al.* (11) also presented evidence for induction of some genes involved in protein synthesis and metabolism, although they did not show a predominance of ribosomal and protein synthesis gene induction as described herein. We suspect that the use of a primarily overexpression system involving a myc-ER construct, a different array system, and different growth conditions (arresting *versus* log-phase) may be the major reasons for the discrepancy.

That c-Myc up-regulates genes involved in protein synthesis may explain its role in carcinogenesis. In yeast, the ribosome assembly is precisely adjusted to the physiological demands of the cell, and the coordinated expression of ribosomal proteins is primarily regulated at the transcriptional level (15). In human, several ribosomal proteins (L5, L21, L27a, L28, S5, S9, S10, and S29) have been shown to be overexpressed in colorectal carcinoma (16). The increased expression of ribosomal protein S27 has been found in a wide variety of actively proliferating cells and tumor tissues, and its expression correlated with the degree of aggressiveness in prostate and colon malignancy (17, 18). Our results and the recent findings of others suggest that c-Myc may potentiate the sensitivity of cells to other mitogenic signals by preparing the protein synthesis machinery to meet the demand of cell proliferation.

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