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SHORT REPORT

A genetic screen to identify genes that rescue the slow growth phenotype of c-*myc* null fibroblasts

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The c-*myc* gene is frequently over-expressed in human cancers and is involved in regulation of proliferation, differentiation and apoptosis. c-Myc is a transcription factor that acts primarily by regulating the expression of other genes. However, it has been very difficult to identify *bona fide* c-Myc target genes that explain its diverse biological activities. The recent generation of c-*myc* deficient Rat1A fibroblasts with a profound and stable growth defect provides a new system to search for genes that can substitute for c-*myc* in proliferation. In this study, we have attempted to identify genes that rescue the slow growth phenotype of c-*myc* null cells through introduction of a series of potent cell cycle regulatory genes and several retroviral cDNA expression libraries. None of the candidate genes tested, including SV40 T-antigen and adenovirus E1A, caused reversal of the c-*myc* null growth defect. Furthermore, extensive screens with high-complexity retroviral cDNA libraries from three different tissue sources revealed that only c-*myc* and N-*myc* rescued the c-*myc* null slow-growth phenotype. Our data support the notion that there are no functional equivalents of the *myc* family of proto-oncogenes and also suggest that there are no c-Myc-activated genes that alone can substitute for c-Myc in control of cell proliferation. *Oncogene* (2000) **19,** 3330–3334.

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The c-*myc* proto-oncogene is deregulated in a variety of human malignancies and is implicated in the control of cellular proliferation, differentiation and apoptosis (Henriksson and Luscher, 1996). c-Myc expression is induced upon mitogenic signaling and peaks 3–4 h after growth factor stimulation (Kelly *et al*., 1983). In cycling cells, c-Myc levels remain invariant and decline after induction of differentiation (Einat *et al*., 1985; Pietenpol *et al*., 1990). Activation of c-*myc* is sufficient to induce cell cycle entry in quiescent cells (Eilers *et al*., 1991). Conversely, inhibition of c-*myc* expression using antisense methods leads to growth arrest and induction of differentiation (Biro *et al*., 1993; Heikkila *et al*., 1987; Prochownik *et al*., 1988).

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The c-Myc protein is a transcription factor of the helix–loop–helix–zipper class, which must dimerize with its partner Max, in order to bind DNA and transactivate from specific Myc sites (Blackwood and Eisenman, 1991; Kretzner *et al*., 1992). The prevailing model has been that the transactivation of c-Myc target genes is required for its growth-promoting properties. Many genes have been implicated as c-Myc targets, including genes involved in cell cycle regulation: *cdc25A*, *cyclin E*, *cyclin D2* (Bouchard *et al*., 1999; Galaktionov *et al*., 1996; Perez-Roger *et al*., 1997); apoptosis: *p53* (Reisman *et al*., 1993); DNA biosynthesis: *cad*, *ODC* (Bello *et al*., 1993; Miltenberger *et al*., 1995); macromolecular synthesis: *eIF-4E* and *MrDb* (Grandori *et al*., 1996; Rosenwald, 1996); and genes with unknown function: *rcl* and *α-prothymosin* (Eilers *et al*., 1991; Lewis *et al*., 1997). Myc proteins have also been implicated in the repression of several genes including MHC class I antigens, c-*myc*, *gadd45* and *C/EBP* (Bernards *et al*., 1986; Li *et al*., 1994; Marhin *et al*., 1997; Penn *et al*., 1990). For many genes it is still unclear whether they are direct or indirect targets and none of them appears to explain the diverse biological activities of c-Myc.

The generation of c-*myc* deficient Rat1 fibroblasts provides a new experimental system to search for genes that can substitute for c-Myc in proliferation. These c-$myc^{-/-}$ cells are viable but proliferate approximately three times slower than their wild type counterparts, a phenotype that can be rescued by ectopic expression of c-*myc* (Mateyak *et al*., 1997). It was shown that c-$myc^{-/-}$ cells have a reduced activity of all cyclin/CDK complexes, and CDK7 and p27 were implicated as downstream effectors of c-Myc (Mateyak *et al*., 1999). Interestingly, when these cells were analysed for expression of several putative c-Myc-regulated genes, only the expression of *cad* and *gadd45* appeared to be affected, suggesting that many of the proposed c-Myc-activated genes do not contribute to the growth defect of the c-*myc* null cells (Bush *et al*., 1998). Although the mechanism of c-Myc-induced repression is still unclear, accumulating evidence suggests that gene repression may be sufficient for many aspects of c-Myc function. For example, a mutated c-Myc lacking Myc Box II (amino acid 106–143), which can still transactivate from Myc sites in reporter assays but has lost its repression function, is inactive in transformation assays (Kato *et al*., 1990; Li *et al*., 1994; Marhin *et al*., 1997; Stone *et al*., 1987). Furthermore, recent studies with the transactivation defective c-MycS protein showed that it retains the ability to stimulate proliferation and

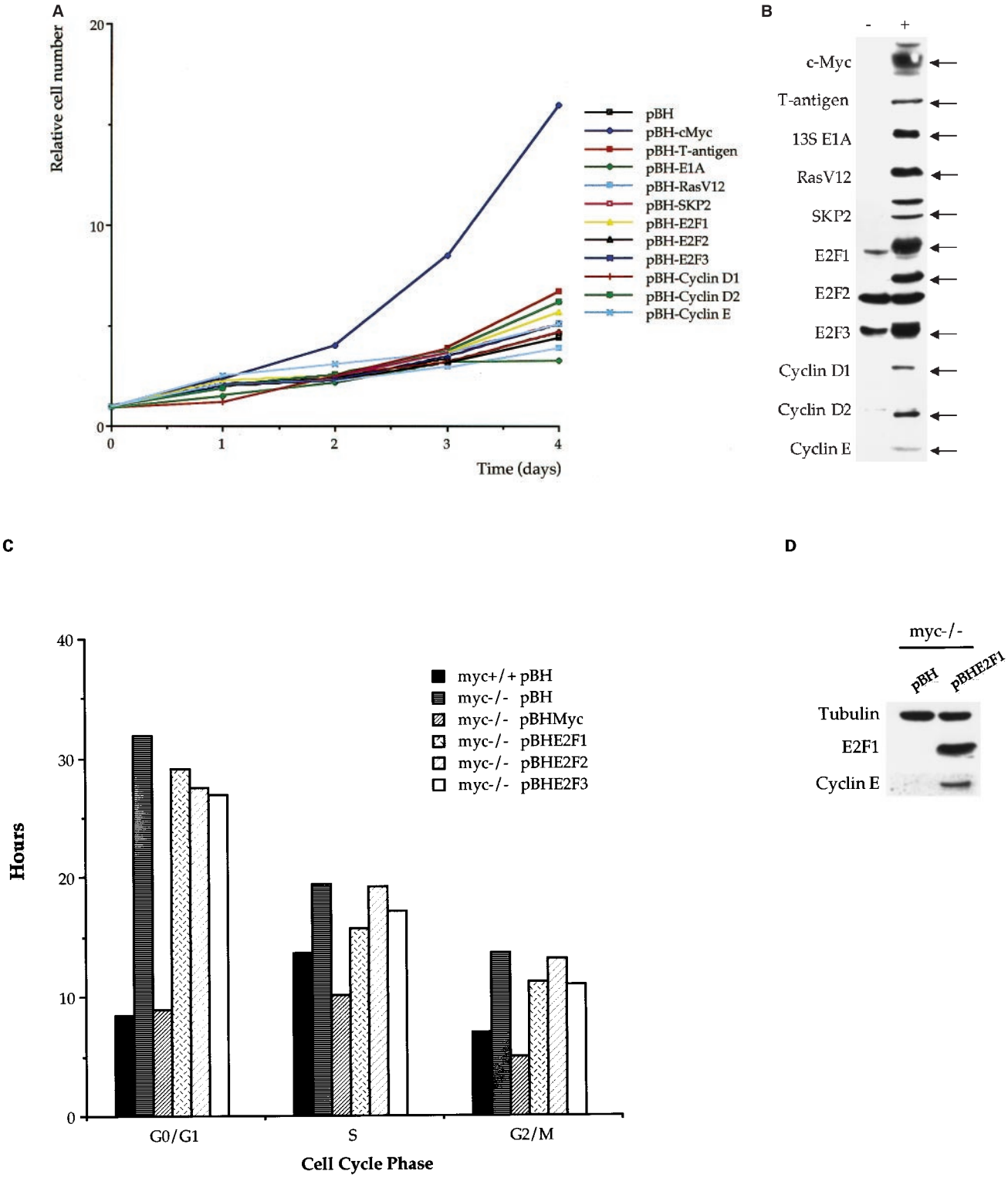

Figure 1 Rescue of slow-growth phenotype of c-*myc* null cells with known cell cycle regulators. (**a**) Growth rates of *myc* null cells over-expressing known cell cycle regulators. The pBabe-hygro based retroviruses (Morgenstern and Land, 1990) were packaged in the Phoenix producer cell line. The c-*myc* null Rat1A cells (Mateyak *et al*., 1997) and wild type Rat1A cells were infected with the indicated retroviruses and polyclonal cell lines were generated by hygromycin selection (100 μg/ml; Calbiochem) for 2 weeks. The selected cells were seeded in six well plates (5.10^4 cells per well) and the relative cell numbers were calculated at the indicated time points by staining the cells with crystal violet (Sigma) and determining the optical density at 590 nm (Serrano *et al*., 1995). The growth rates were determined in multiple independent experiments from which one representative experiment is shown. (**b**) Immunoblot analysis of the retrovirally infected polyclonal cell lines, (−): control pBabe-hygro infected Rat1A cells; (+): Rat 1A cells infected with the indicated cDNA in the pBabe-hygro vector, was performed as described (Berns *et al*., 1997). The following antibodies were used: c-Myc: PAN-Myc (Genosys Biotechnologies, OA-11-802); cyclin D1: H-285 (Santa Cruz, SC-753); cyclin D2: C-17 (SC-181); cyclin E: C-19 (SC-198); E2F-1: C-20 (SC-193); E2F-2: C-20 (SC-633); E2F-3: C-18 (SC-878); Ras: (Transduction laboratories, R 02120); SV40 T antigen: monoclonal 419 (kindly provided by Dr AJ van der Eb); SKP2: affinity purified rabbit anti-p45 (a kind gift from Dr W Krek). (**c**) Duration of the cell cycle phases. The times spent in the G0/G1, S and G2/M phases were calculated from cell cycle distribution as determined by flow cytometry of asynchronously growing populations (data not shown) and the doubling times as determined in **a**. One representative experiment is shown. (**d**) Target gene activation by E2F1. Western analysis of the E2F1 overexpressing c-*myc* null cell line shows that E2F1 is functional in the induction of one of its targets, cyclin E

apoptosis and can substitute for full-length c-Myc in c-*myc* null cells (Xiao *et al*., 1998). To understand the molecular mechanism by which c-Myc exerts its growth control, the identification of functional equivalents of c-Myc may be very helpful. In this study we describe functional genetic screens in c-$myc^{-/-}$ cells to search for genes that can revert their slow growth phenotype.

First, we investigated whether a defect in a known cell cycle pathway could account for the slow growth phenotype of the c-*myc* null cells. To test this, we infected the c-*myc* null cells with retroviral vectors expressing either c-Myc (as a positive control), SV40 T-antigen, adenovirus E1A (13S), RasV12, SKP2, E2F1 (which activates the c-Myc target gene *CDC25A* (Vigo *et al*., 1999)), E2F2, E2F3, cyclin D1, cyclin D2 or cyclin E. Cell lines were generated by hygromycin selection for 2 weeks and analysed for reversion of the slow growth phenotype. All cell lines were tested by Western analysis for the expression of the introduced cell cycle proteins (Figure 1b). Only in the case of stable c-Myc expression, the growth rate of the c-*myc* null cells was restored to wild type levels (Figure 1a). Interestingly, ectopic expression of SV40 T-antigen, which affects both the pRb and p53 pathways had no effect on the growth properties of the c-*myc* nulls. Moreover, it is striking that expression of adenovirus 5 E1A had no effect on the growth rate since c-Myc and E1A behave similar in many growth assays. Perhaps even more importantly, c-Myc-E1A chimeric proteins have immortalizing activity, suggesting that growth regulation by c-*myc* and E1A involves interactions with common substrates (Ralston, 1991). The cDNAs used to establish the polyclonal cell lines depicted in Figure 1a have all been tested in functional assays in our lab and by others. Although none of the cell cycle genes tested rescued the c-*myc* null defect in long term growth, it remained possible that some of the defects in the c-*myc* null cells, such as the G1 or S phase delay, could be rescued. Therefore, we used flow cytometry on exponentially growing E2F-overexpressing c-*myc* null cell lines (data not shown). Together with the cell doubling times as determined from the growth curves shown in Figure 1a, the number of hours spent in each of the cell cycle phases was determined. Figure 1c shows that ectopic expression of E2F1, 2 or 3 had no effect on the duration of the cell cycle phases, whereas c-*myc* restored the hours spent in each phase to wild type levels. Nevertheless, ectopic expression of E2F1 caused an increase in cyclin E expression (Figure 1d), indicating that E2F1 is functionally active in these cells.

The growth curves presented here extend earlier work of others (Mateyak *et al*., 1999), which showed that ectopic expression of cyclin D1, cyclin E and cyclin A did not revert the c-*myc* null phenotype. Our data indicate that many of the genes that act in G1 to S phase transition are not responsible for the growth defect of the c-*myc* null cells.

To perform an unbiased search for genes that rescue the growth defect of c-*myc* null cells we performed a functional screen with retroviral cDNA expression libraries. As was previously reported, the slow growth phenotype of the c-*myc* null cells is very stable and in all the screens described here, no spontaneous rescue of the slow growth phenotype was observed in the control infections with GFP virus (Table 1). In these screens, $1-2.10^6$ cDNA library-infected cells (which carried on average 1–3 proviruses per cell) were subdivided in pools and subsequently cultured as depicted in Figure 2a. Fast-growing colonies became visible 4 to 5 weeks after infection (Figure 2b). In almost all cases, the rescued cells had undergone a morphological change in that they were smaller and grew to higher density, which resembles the changes evoked by reintroduction of c-*myc*.

The first rescue screen was performed with a retroviral expression library made from size-fractionated K562 human erythroleukemia cDNA. Rescued colonies were only identified in the small size fraction (1–3 kb cDNA size) of the K562 library (Table 1). Multiple rescued colonies from each pool were isolated. Since c-*myc* is a likely candidate to be cloned in this

Table 1 Summary of retroviral cDNA rescue screens in c-*myc* null cells

Retroviral cDNA library used	*Complexity of retroviral cDNA library*	*Number of virus particles used for infection*	*Number of cells used for infection*	*Number of subdivided pools*	*Number of pools with rescue activity*	*c*-myc *cDNA*	*N*-myc *cDNA*
GFP			1.10^6	5	0	–	–
K562(1–3)	$1.4.10^6$	$>3.10^7$	2.10^6	10	7	7/7	–
K562(>3)	$0.7.10^6$	$>3.10^7$	2.10^6	10	0	–	–
JEG3(1–3)	$3.0.10^6$	$>3.10^7$	2.10^6	10	9	6/9	3/9
JEG3(>3)	$1.5.10^6$	$>3.10^7$	2.10^6	10	5	2/5	3/5
JEG3(1–3)II	$3.0.10^6$	$>3.10^7$	1.10^6	15	6	6/6	–
JEG3(>3)II	$1.5.10^6$	$>3.10^7$	1.10^6	15	0	–	–
Mouse embryo	$2-3.10^6$	$>3.10^7$	2.10^6	30	7	7/7	–

Construction of the K562 and JEG3 retroviral cDNA libraries in the pMXsubF retroviral vector will be described elsewhere. The whole mouse embryo retroviral cDNA expression library was purchased from Clontech (ML8000BB). High titer library retroviral stocks were generated by transfecting 15 cm plates of Phoenix cells with 50 μg of cDNA library plasmid, 2.5 μg of pEGFP-N1 (Clontech) and 5 ng of pMXsubF-GFP. Virus supernatants were only collected when transfection efficiencies were higher than 60% (as determined by scoring GFP-positive cells using a fluorescence microscope). Rough estimates of the library virus titers were obtained through the use of the 1:10 000 diluted GFP virus present in the library stocks. For the PCR analysis of the JEG3 and K562 rescuing cDNAs, the primers gag:AAAGGACCTTACACAGTCCTGCTGA and subF:CCACAGGTAATGCTTTTACTGGCCT were used. For the mouse embryo retroviral cDNA library from Clontech we generated the primers pLibFW1:CCCCCTTGAACCTCCTCGTTCGAC and pLibREV2:TGGCGTTACTTAAGCTAGCTTGCCAAA. Genomic DNA was isolated with the use of DNAzol (Gibco–BRL), and 100 ng DNA was used per PCR reaction. For the PCR, Expand Long Template (Boehringer Mannheim) was combined with Taqstart+dilution buffer (Clontech) in a 1:1:4 ratio. The PCR reaction conditions were: 94°C (2 min); 94°C (20 s); 65°C (30 s); 68°C (5 min); 35 cycles. For Southern blot analysis of rescued colonies, 15 μg of genomic DNA was digested overnight and the restriction fragments were separated on a 0.8% agarose gel. The following ^{32}P-labeled probes were generated by PCR labeling: c-*myc*, using pRC/CMV-cmyc (Berns *et al*., 1997); N-*myc*, using a plasmid harboring a full-length mouse N-*myc* cDNA

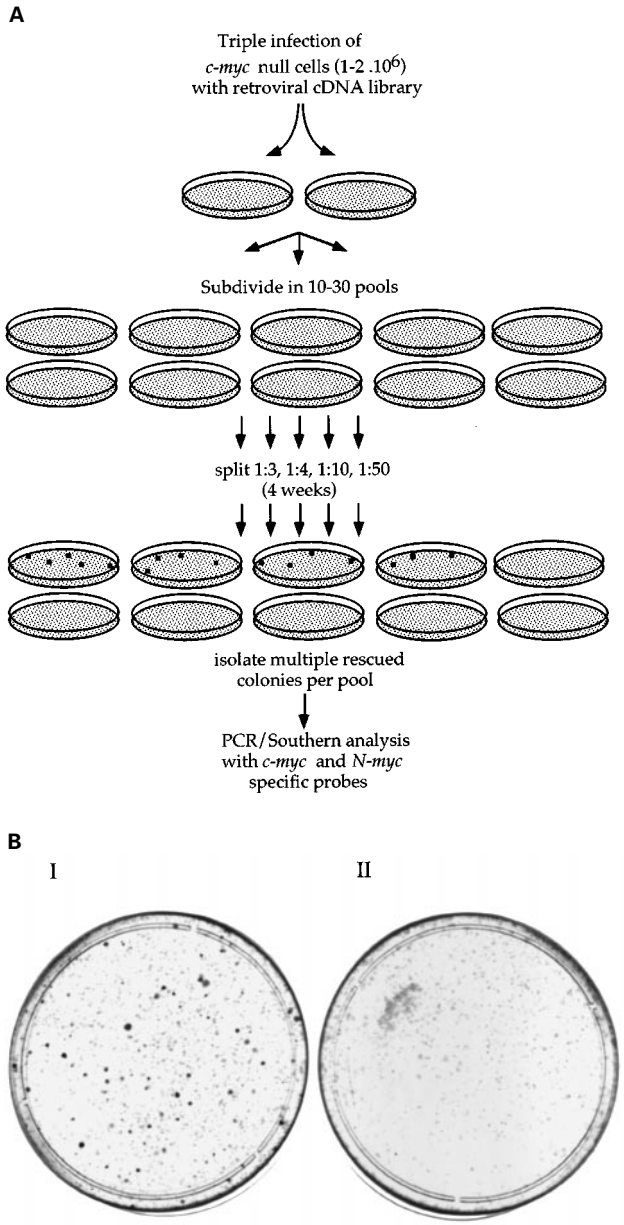

Figure 2 Schematic representation of a retroviral cDNA library rescue screen. (**a**) c-*myc* null cells were infected three consecutive rounds with high titer library virus resulting in 25 to 80% infected cells. Two days after infection, cells were divided into 10–30 pools and plated at low density. When plates became confluent, cells were split initially 1 : 3, later 1 : 4, 1 : 10 and finally at a dilution of 1 : 50. The last plating at very low density allowed the identification of fast-growing single colonies in a background of slow-growing colonies, an example of which is shown in **b**. Rescued colonies are readily detectable within the non-rescued population (I) since multiple colonies arise from a single rescue event in the culture protocol used and they are denser and bigger

type of screen, we first focused on the identification of c-*myc*-positive colonies. We performed PCR reactions using retroviral vector-specific primers (gag and SupF). The amplified cDNA inserts were separated on an agarose gel and analysed by Southern blotting with a c-*myc* specific probe. This assay indicated that all the fast-growing variants derived from the K562 cDNA library infection contained a (near) full-length (2.0–2.2 kb) c-*myc* cDNA.

Next, the rescue screen was performed twice with a size fractionated human JEG3 choriocarcinoma-derived cDNA library. In these experiments, fast-growing variants were found both in pools of cells infected with the 1–3 kb and >3 kb cDNA size fractions of the library (Table 1). Subsequent PCR amplification of the retroviral cDNA inserts followed by hybridization of the PCR products with both c-*myc* and N-*myc* specific probes suggested that a subset of the fast-growing colonies did not contain a c-*myc* or N-*myc* cDNA insert. The morphology of the fast-growing non-*myc* containing clones was nevertheless very similar to those that did contain *myc*. We therefore performed a Southern blot analysis on genomic DNA isolated from these colonies. Genomic DNA was digested with *Xba*I (which releases the cDNA insert and the retroviral vector-derived SupF sequence) and probed with an N-*myc* specific probe. This experiment revealed that all the non-c-*myc* rescued colonies contained N-*myc* cDNA library inserts of 3.0–3.3 kb (data not shown). Apparently, under the conditions used, the PCR reaction did not amplify the N-myc cDNA inserts from the retroviral vectors, yielding a false-negative result.

Finally, we infected the c-*myc* null cells with a whole mouse embryo-derived cDNA library and subdivided the infected cells in 30 pools. After 4–5 weeks, rescued colonies were identified in seven pools. Again, multiple clones were isolated and genomic DNA from rescued clones was subjected to Southern analysis. Also in this screen, only c-*myc* cDNA inserts were cloned (Table 1).

In summary, despite extensive efforts we have been unable to identify genes, other than Myc family members, that rescue the slow growth phenotype of c-*myc* null fibroblasts. It is unlikely that this is due to inadequate screening efficiency, complexity, or size representation of the retroviral cDNA libraries, as full length c-*myc* (2.2 kb) and N-*myc* (3.5 kb) cDNAs were each independently isolated several times. However, it remains possible that such genes indeed exist, but were not present in the cDNA libraries used. The observation that potent cell cycle regulators, such as SV40 T-antigen and adenovirus E1A, did not rescue the slow growth phenotype of the c-*myc* null cells suggests that the cell cycle components that are deregulated by these viral proteins are not among the critical targets of c-Myc. Recently, the cyclin-dependent kinase CDK4 was identified as a critical target of c-Myc since it could partially alleviate the c-*myc* null growth defect (Hermeking *et al*., 2000). However, in our hands a c-*myc* null derivative that over-expressed the tumor-derived CDK4/R24C mutant (Wolfel *et al*., 1995) did not even show a partial rescue of the growth defect (data not shown). This apparent discrepancy may be caused by the fact that the CDK4/R24C mutant has a reduced ability to bind the CDK4/6 inhibitor $p16^{INK4A}$ and therefore more experiments are required to clarify this issue.

Our results are compatible with a model in which c-Myc is a pleiotropic factor that controls many aspects of cell growth through a number of distinct target genes, some of which may be activated by c-Myc, others repressed. The fact that spontaneous fast-growing revertants of the c-*myc* null phenotype were never recovered also favors this multiple target model. Since E1A is likely to regulate at least some of the c-Myc target genes required for cell proliferation, a

retroviral cDNA library screen in E1A overexpressing *myc* null cells may allow us to identify genes that collaborate with E1A in rescue of the c-*myc* null growth defect.

In conclusion, our data suggest that the human and murine genomes do not harbor functional equivalents of Myc family proteins and also suggest that there are no Myc-activated genes that can substitute for c-*Myc* in stimulation of cell proliferation.

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