c-Myc-Mediated Regulation of Telomerase Activity is Disabled in Immortalized Cells*

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Myc overexpression is a hallmark of human cancer and promotes transformation by facilitating immortalization. This function has been linked to c-Myc’s ability to induce the expression of the catalytic subunit of telomerase, Telomerase Reverse Transcriptase (TERT)\(^1\), as ectopic expression of TERT immortalizes some primary human cell types. c-Myc up-regulates telomerase activity in primary mouse embryonic fibroblasts (MEFs) and myeloid cells. Paradoxically, Myc overexpression also triggers the ARF-p53 apoptotic program, which is activated when MEFs undergo replicative crisis following culture \textit{ex vivo}. The rare immortal variants that arise from these cultures generally suffer mutations in p53 or delete \textit{Ink4a/ARF} and Myc greatly increases the frequency of these events. ARF- and p53-null MEFs have increased telomerase activity, as do variant immortal clones that bypass replicative crisis. Similarly, immortal murine NIH-3T3 fibroblasts and myeloid 32D.3 and FDC-P1.2 cells do not express ARF and have robust telomerase activity. However, Myc overexpression in these immortal cells results in remarkably discordant regulation of TERT and telomerase activity. Furthermore, in MEFs and 32D.3 cells TERT expression and telomerase activity are regulated independently of endogenous c-Myc. Thus, the regulation of TERT and telomerase activity is complex and is also regulated by factors other than Myc, ARF or p53.

\(^1\)The abbreviations used are: IL-3, interleukin-3; MEF, mouse embryonic fibroblast; TERT, Telomerase Reverse Transcriptase; TR, telomerase RNA; TRAP, telomeric repeat amplification protocol; MSCV, Murine Stem Cell Virus; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; PCR, Polymerase chain reaction; IRES, internal ribosome entry site; GFP, green fluorescence protein; ER, estrogen receptor; 4-HT, 4-hydroxytamoxifen; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.
Transformation of normal cells to cancer requires the bypass of several hurdles that monitor the ability of the cell to proliferate, differentiate and survive. The most common target in human tumors is the ARF-p53 tumor suppressor pathway, which is inactivated in up to 70% of all tumors (1,2). p53 functions as a critical sensor and transcriptional regulator of the DNA damage pathway, yet is also activated by other signals that stress the cell (3). The latter includes hyper-proliferative signals that are induced by dominant-acting oncogenes such as c-Myc that promote continuous but dysregulated cell proliferation (1,4). Activation of p53 in this scenario requires the p19ARF nucleolar tumor suppressor protein (5), which is expressed from an alternative reading frame of the INK4a locus (6), which also encodes the p16INK4a inhibitor of the cyclin D-dependent kinases (cdk) cdk4 and cdk6 (7). ARF induces p53 transcriptional activity in part by nucleolar sequestration of p53’s natural inhibitor Mdm2 (1,8). Mdm2 is a transcription target induced by p53 (9), yet serves to harness the p53 response by ubiquitinating p53 (10) and shuttling it to the cytosol for destruction by the 26S proteasome (11), and by inhibiting its transactivation functions (9). ARF overexpression alone results in a rapid and p53-dependent G1-G2 cell cycle arrest (6), yet can also induce a protracted form of G1 arrest in the absence of p53 and Mdm2 (12). However, in the presence of collateral signals emanating from oncogenes such as Myc the robust activation of ARF and p53 results in apoptosis (1,4).

A second obstacle that must be overcome for a cell to become transformed is the bypass of the erosion of the telomere, a cap structure present on the termini of mammalian chromosomes consisting of the repetitive series of the DNA sequence TTAGGG (13). The semi-conservative nature of DNA replication results in a failure to replicate the most terminal portion of the telomeric repeat, and thus unless this telomeric repeat is synthesized it is progressively lost at
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each cell division leading to replicative senescence (14-18, 60). To handle this end replication problem, eukaryotes express an enzyme complex called telomerase. This utilizes an essential telomerase RNA (mTR) as a template to prime the reverse transcription of the telomeric sequence, by a reverse transcriptase coined Telomerase Reverse Transcriptase (TERT). In addition, the telomerase complex contains several other factors that serve to tether the complex to the telomere, and to regulate its activity (13, 16, 19-21).

In most normal human cells TERT expression is silent and, as a net result, the telomere shortens with each cell division. After a finite number of cell divisions the cells then undergo replicative senescence (16, 22-26). Bypass of this hurdle in human cells can occur through the inactivation of both the retinoblastoma and p53 tumor suppressor pathways as well as oncogenic signals from Ras (27,28), yet cell division leads to a further shortening of the telomere (23). Ultimately a second blockade, termed crisis, ensues that is usually bypassed by the activation of TERT expression or by ALT (alternative lengthening of telomeres), and this leads to some recovery in telomere length (23). In some human cell types bypass of both replicative senescence and crisis can occur by ectopic expression of TERT alone, which can immortalize some human cell types, but not others (27,29).

By contrast, in rodent cells telomeres can be extremely long (up to 150 Kbp in Mus musculus) and TERT is expressed in several tissues in adult mouse (30); thus events required to immortalize cells are much less stringent. Like human fibroblasts (23), when passaged on a scheduled protocol mouse embryonic fibroblasts (MEFs) display a finite number of cell divisions before undergoing replicative senescence (31). This is not due to rapid erosion of the telomere but rather appears a consequence of the stress of ex vivo culture conditions that somehow activate the ARF-p53 tumor suppressor pathway (28). Thus, ARF- or p53-null cells behave as
immortal cells and rare immortal variants that arise from the culture of wild-type MEFs typically have inactivating mutations in p53 or deletions of the INK4a/ARF locus (4, 5, 32).

Myc overexpression is a common denominator of many cancers and appears to promote transformation by providing signals that allow continuous entry of cells into S phase (33-35) and promote angiogenesis (36). Myc cooperates with Ras to transform primary rodent fibroblasts and provides immortalizing functions. In large part this has been attributed to Myc’s ability to trigger the ARF-Mdm2-p53 apoptotic program, which selects for immortal variants having p53 mutations or deletion of ARF (4). Indeed, greater than 80% of the lymphomas that arise in Eµ-myctransgenic mice harbor alterations in the ARF-Mdm2-p53 pathway (37). However, Myc also induces the expression of TERT in primary human cells (38) and this also been linked to Myc’s immortalizing functions.

We addressed the role that the c-Myc-ARF-p53 axis may play in regulating TERT expression and telomerase activity in primary and established murine fibroblasts and myeloid cells. Here we report that telomerase activity is indeed induced by Myc activation in primary murine cells, or by ARF or p53 loss in MEFs. However, in immortal cell lines Myc’s ability to regulate TERT expression and telomerase activity is superceded by other events associated with immortalization, adaptation to ex vivo culture, and/or cell context. Furthermore, TERT expression and telomerase activity were independent of mitogen withdrawal, which results in the marked down-regulation of endogenous c-Myc expression. Thus, the relationships between Myc, TERT and telomerase are complex and are likely influenced by many events that participate in tumorigenesis.
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EXPERIMENTAL PROCEDURES

**Cell Culture** - MEFs derived from wild-type, ARF-, p53- and p21CIP1-null day E13.5 embryos were explanted and cultured as described previously (4). To compare levels of telomerase activity, all MEFs were harvested at exponential growth phase (70-85% confluence). To assess the effects of serum on the regulation of telomerase activity, MEFs were washed twice in medium containing 0.1% serum and cultured for 48 hours in this medium. NIH-3T3 cells were grown in DMEM medium supplemented with 10% fetal calf serum and L-glutamine (39). The murine 32D.3 and FDC-P1.2 cell lines were derived from long term bone marrow cultures, are diploid and IL-3-dependent (40, 41) and were maintained as previously described (34, 42). c-Myc overexpressing 32D.3, FDC-P1.2 and NIH-3T3 cells have been previously described (34, 39, 42). To ensure that parental and c-Myc overexpressing myeloid cells were at similar phases of growth prior to analysis, the cells were sequentially passaged for two consecutive days at 0.3 x 10^6 cells per ml (FDC-P1.2) or at 0.5 x 10^6 cells per ml (32D.3), and on the third day they were assessed for levels of mTR and mTERT and telomerase activity. To assess the effects of IL-3 withdrawal on telomerase activity, exponentially growing cultures of 32D.3 cells were washed three times in RPMI-1640/10% FCS media lacking IL-3 and then suspended at 0.5 x 10^6 cells per ml in the same medium. Primary myeloid cells were isolated from long term liquid cultures of day E15.5 fetal liver cells cultured in RPMI-1640/10% FCS medium supplemented with IL-3, IL-6 and SCF (all at 10 ng/ml) as previously described (43).

**Virus infection** - Exponentially growing cultures of MEFs were infected with recombinant murine retroviruses as previously described (4). Human c-Myc was expressed in
MEFs using the pSRα retroviral vector that expresses the lymphoid-specific cell surface marker CD8 from an internal herpes simplex virus thymidine kinase promoter (4). Human c-Myc was expressed in myeloid cells from the MSCV retrovirus that expresses the gene for green fluorescence protein (GFP) in cis by virtue of an internal ribosome entry site (37). A conditional form of human c-Myc, Myc-ER™, which consists of a fusion of Myc with the estrogen binding domain of the estrogen receptor (ER) modified to bind to estrogen agonist tamoxifen (44), was used in some experiments. As a control, cells were also infected with the MSCV-GFP virus. Infection efficiencies of MEFs, as assessed by GFP positivity by FACS, were >90%, whereas those of primary myeloid cells ranged from 10-30% and infected myeloid cells were sterile sorted by FACS fluorescence for GFP and then expanded in RPMI-1640 supplemented with IL-3, IL-6 and SCF as described (37).

**Telomerase Assays** - Cell extracts were prepared according to protocols provided by the manufacturers. Telomerase activity was measured using the TRAPEze Telomerase detection kit (Oncor, Gaithersburg, MD, USA) and Telomerase polymerase chain reaction (PCR) ELISA (ROCHE, Mannheim, Germany). The Telomerase PCR ELISA is an extension of the original described TRAP protocol (45) and allows for the highly specific amplification of Telomerase mediated elongation products, combined with nonradioactive detection following an ELISA protocol. Absorbance values are reported as the A$_{450}$ nm reading against blank control (reference wavelength A$_{655}$ nm). For the PCR ELISA, telomerase elongation conditions were 25°C for 30 min, and 94°C for 5 min. PCR cycling conditions were for 30 cycles (94°C for 30 sec, 50°C for 30 sec, 72°C for 90 sec), followed by 1 cycle of 72°C for 10 min. For TRAPEze, telomerase elongation conditions were 30°C for 30 min and PCR cycling conditions were for 27 cycles (94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec). The two methods showed very similar
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results; therefore for some experiments only the results from the ELISA are shown. All telomerase assays were performed in duplicate and repeated at least twice. The values shown are the mean of the combined experiments, with standard deviations.

RT-PCR Analysis - Total RNA was extracted with RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). cDNA synthesis and PCR amplification were performed using a Perkin Elmer Kit (Roche, Branchburg, New Jersey, USA). To generate cDNA, a 20 µl reaction containing 1 µg of total RNA, 5 mM MgCl₂, 1X PCR Buffer II, 1 mM of dGTP, dATP, dTTP, and dCTP, 1 U/µl of RNase inhibitor, 2.5 U/µl of MuLV reverse transcriptase and 2.5 µM of random hexamers were incubated for 10 min at room temperature. The samples were then incubated for 30 min at 42°C, 5 min at 99°C and then 5 min at 5°C. The total volume used for PCR amplifications, except for mTR cDNA, was 10 µl. PCR amplification of the 160 bp of mTERT mRNA (Telomerase Reverse Transcriptase) was performed with primers coined LT7 (5’-CACATTCCAGAAGAACAGG-3’) and LT8 (5’-CAGATGGGCATGGCTAG-3’), based on sequence from GenBank Accession AF 029235, for 38 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min) and extension (72°C, 2 min). PCR amplification of mTR transcripts was performed using Qiagen Taq PCR Core Kit (Qiagen Inc., Valencia, CA, USA), with primer LT13 (5’-GCTGTGGGTTCTGTCTTTG-3’) and primer mTR-3 (5’GTGCACCTCCACAG CTCAGG-3’) for 35 cycles of denaturation (94°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 1 min) and one cycle of 10 min at 72°C. PCR amplification of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) mRNA was performed with the primers K138 (5’-GGTGAAGGTCGGTGTCGGACCGG-3’) and K139 (5’-GTGGTGCCAGGTGACATGCTCGT-3’) for 20 cycles of denaturation (95°C, 1 min), annealing (64°C, 1 min), and extension (72°C, 2
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... Amplification products were separated by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide and photography.

*Western blot analyses* - 50 µg of total protein extracts were separated on 8% SDS-PAGE and electroblotted onto nitrocellulose membranes. The membrane was incubated with a rabbit serum containing polyclonal antibody raised against the C-terminal peptide sequence (SRKLPGTTLTALEAAAPAL) corresponding to amino acids 1105-1124 from hTERT sequence (GenBank Accession Number NM_003219). This antibody recognizes both hTERT and mTERT proteins.

**RESULTS**

c-Myc induces TERT expression and telomerase activity in primary mouse embryonic fibroblasts and myeloid cells - Activation of Myc in primary human cells results in the induction of TERT expression and a marked increase in telomerase activity (38). To confirm that c-Myc also induced TERT expression and telomerase activity in primary murine cells, we infected early passage MEFs and fetal liver-derived myeloid cells (4, 43) with retroviruses that expressed either human c-Myc, or a conditionally inducible form of human c-Myc, Myc-ER\textsuperscript{TM}. This form has c-Myc coding sequences fused in-frame with those of the estrogen binding domain of the Estrogen Receptor (ER) modified so it can only bind the ER agonist 4-hydroxytamoxifen (4-HT). In the absence of tamoxifen this chimeric protein is sequestered into inactive heat shock complexes in the cytoplasm, but following the addition of 4-HT Myc-ER\textsuperscript{TM} rapidly re-localizes to the nucleus and performs all the known functions of c-Myc (44). MEFs were infected with the pSRα...
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retrovirus which expresses the T-lymphocyte-specific marker CD8 in *cis* from an internal thymidine kinase promoter (4). Primary myeloid cells were infected with the MSCV-IRES-GFP vector (37), which expresses the gene for green fluorescence protein (GFP) by virtue of an internal ribosome entry site (IRES). Infected fibroblasts or GFP FACS-sorted myeloid cells were expanded in culture and then examined for telomerase activity. Both MEFs (Fig. 1A) and primary myeloid cells (Fig. 1B) infected with the Myc-expressing retrovirus had marked increases in telomerase activity (>10-fold with MEFs) relative to cultures infected with the parental retroviral vector. Furthermore, the activation of Myc-ER<sup>TM</sup> by the addition of 4-HT led to a marked induction of telomerase activity relative to telomerase activity present in vector-only infected cultures treated with 4-HT (Fig. 1, C and D). As expected (38), the increases in telomerase activity in the Myc-expressing cultures were associated with increases in TERT transcripts as detected by semi-quantitative PCR (data not shown and see below). Therefore, similar to primary human cells, Myc overexpression or activation up-regulates TERT expression and telomerase activity in primary murine cells.

*Induction of TERT by c-Myc is p53- and ARF-independent* - Myc overexpression accelerates replicative crisis in primary cells and induces apoptosis at least in part by triggering the ARF-p53 pathway. In turn, this selects for variants that have p53 mutations or deletions of the *Ink4a/ARF* locus and these are resistant to Myc-induced apoptosis in a fashion similar to *ARF* or *p53*-null cells (4). Since Myc activation induces ARF and p53 expression, we assessed whether the induction of telomerase activity by Myc was dependent upon ARF or p53 function. Early passage MEFs derived from *ARF*- or *p53*-null embryos were infected with the pSRα-Myc-ER<sup>TM</sup>-tk-CD8 virus and these cells were compared to those infected with the pSRα-tk-CD8 vector control. Myc activation by 4-HT resulted in comparable levels of telomerase activity in
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MEFs of all three genotypes (Fig. 2A). Therefore, the induction of TERT by c-Myc is independent of ARF and p53 functions.

Loss of ARF or p53 function also augments telomerase activity – Although Myc induction of telomerase activity was independent of ARF or p53 status, the fact that loss of ARF or p53 is sufficient to immortalize MEFs suggested that these MEFs may have increased levels of telomerase activity. Indeed, analysis of the basal levels of telomerase activity demonstrated that ARF- and p53-null MEFs had increased (over 3-fold) basal levels of telomerase activity relative to those present in matched early passage MEFs derived from wild-type embryos. The effects of ARF or p53 loss on telomerase activity were not simply due the loss of any cell cycle regulator, as telomerase activity was not augmented in MEFs derived from embryos lacking the universal cdk inhibitor p21\textsuperscript{CIP1} (Fig. 2B).

Mutation of p53 is the most common alteration in variants that arise in MEFs that survive replicative crisis (4, 32). We therefore assessed whether MEF variants arising from long-term 3T3 cultures that have dominant-negative missense point mutations also displayed increases in telomerase activity, relative to levels expressed in these pre-senescent cultures. As expected, the levels of telomerase activity in these variant MEFs was significantly elevated (over 3-fold) over levels of telomerase activity present in these same MEFs prior to replicative crisis (Fig. 2C). Thus, the induction of telomerase activity is also associated with events that facilitate immortalization. Thus, Myc activation of telomerase activity does not strictly require either ARF or p53, but loss of either tumor suppressor results in up-regulation of telomerase activity.

Myc overexpression has disparate effects on TERT expression and telomerase activity in immortal cells - Myc overexpression in immortal NIH-3T3 fibroblasts or the murine IL-3 dependent myeloid 32D.3 and FDC-P1.2 cell lines augments the apoptotic program (34, 39, 42),
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despite the fact that all three cell lines fail to express ARF (6, 46). Furthermore, enforced Myc expression is sufficient to promote continuous entry of cells into S phase in the absence of mitogens in each of these cell lines (34, 39, 42). We therefore assessed the effects of Myc overexpression on TERT expression and telomerase activity in these three immortal cell lines. Initially we assessed the effects of Myc overexpression in 32D.3 cells, which have some of the hallmarks of committed myeloid progenitors (40). Exponentially growing cultures of 32D.3 cells overexpressing c-Myc and vector-only clones generated in parallel were initially assessed for telomerase activity. Surprisingly levels of telomerase activity were much lower in 32D.3 cells engineered to overexpress c-Myc (Fig. 3, A and B). The reduced levels of telomerase activity in c-Myc overexpressing cells was linear with respect to protein concentration (Fig. 3C). This was not due to the presence of an inhibitory activity in these cells, as mixing extracts from Myc-derived clones with those from 32D.3 cells failed to indicate any inhibitory activity specific to Myc overexpressing cells (Fig. 3D). Moreover, the levels of PCR amplification of the internal control (IC) with 32D.3 extracts were comparable to those with Myc extracts (Fig. 3B). The presence of high levels of endogenous RNase resulting in inactivation of telomerase upon lysis is unlikely to be the case, since inclusion of an RNase inhibitor in the lysis buffer prior to cell lysis did not affect telomerase activity (data not shown). Furthermore, boiling Myc extract before mixing with 32D.3 extract did not abrogate this inhibition (data not shown).

To determine if the reductions in telomerase activity in Myc-overexpressing cells were associated with alterations in TERT expression we performed semi-quantitative RT-PCR analyses. Levels of mTR were essentially equivalent in 32D.3 cells and Myc overexpressing cells, whereas there were marked reductions in mTERT RNA levels in all of the Myc-expressing clones relative to levels expressed in parental 32D.3 cells (Fig. 4A). To confirm that these
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changes were also manifest as changes in the level of mTERT protein, we performed immunoblot analyses. In agreement with the RNA expression analyses, Myc overexpressing 32D.3 cells expressed markedly reduced levels of mTERT protein (Fig. 4B). Thus, at least in these immortalized cells, Myc overexpression suppressed, rather than induced, TERT expression. Despite the low levels of telomerase activity in Myc-overexpressing 32D.3 cells, the mean telomere length in these cells was only slightly shorter than those present in parental 32D.3 cells (data not shown), suggesting that low levels of telomerase activity expressed in these cells is sufficient to maintain telomere length.

To determine the effects of c-Myc overexpression on TERT expression and telomerase activity in other immortal cell contexts, we also assessed the levels of TERT and telomerase activity in FDC-P1.2 myeloid cells and NIH-3T3 fibroblasts engineered to overexpress c-Myc (39, 42). FDC-P1.2 myeloid cells have intrinsically high levels of telomerase activity and this was not significantly different in FDC-P1.2 cells that overexpress c-Myc (Fig. 5). Similarly, levels of TERT and telomere length were essentially equivalent in parental FDC-P1.2 cells and FDC-P1.2 cells overexpressing c-Myc (data not shown). By contrast, NIH-3T3 fibroblasts engineered to overexpress murine c-Myc had modest increases in telomerase activity relative to parental NIH-3T3 cells (Fig. 6, A and B). Again, increases in telomerase activity were associated with increases in mTERT expression (Fig. 6, C and D). Thus, in immortal cells Myc overexpression results in a remarkable spectrum of changes in TERT expression and telomerase activity, and this response does not correlate with Myc’s proliferative or apoptotic functions.

TERT expression and telomerase activity are regulated independently of endogenous c-Myc - Gene targets ascribed to c-Myc have frequently been identified using overexpression screens (47-49). The expression of c-Myc is tightly regulated by mitogens and following the
withdrawal of growth factors c-myc transcription ceases (50) and c-Myc protein rapidly diminishes, as it has a short half-life (51). In all of the primary and immortal cells used in this study, c-Myc expression is strictly dependent upon mitogens and is induced as an immediate early gene following re-addition of mitogens to ligand starved cells (34, 39, 52). We therefore assessed whether TERT expression and telomerase activity was mitogen-dependent. Exponentially growing 32D.3 myeloid were deprived of IL-3 and at various intervals cells were collected for RNA and protein analyses and for assessing telomerase activity. Additionally samples were prepared from ligand-starved cells stimulated with IL-3. As expected (34), c-Myc levels rapidly diminished following IL-3 withdrawal, yet was highly stimulated following IL-3 stimulation of ligand starved cells (data not shown). By contrast, mTERT RNA and protein levels were essentially unaffected by IL-3 withdrawal and were not augmented by stimulating ligand-starved cells with IL-3 (Fig. 7A and data not shown). Similarly there were no significant changes in telomerase activity following IL-3 withdrawal or stimulation (Fig. 7B).

The failure of mTERT to be regulated following IL-3 withdrawal could reflect the long half-life of TERT protein or alterations in TERT regulation that occurred during establishment of the 32D cell line from long term bone marrow culture (40). We therefore assessed whether telomerase activity was regulated by serum in MEFs. Serum withdrawal led to a predictable arrest of MEFs in the G0-G1 phase of the cell cycle (Fig. 8A) and a loss of c-Myc expression (data not shown). Nonetheless there was only a modest reduction in telomerase activity in serum starved MEFs (Fig. 8B), despite a decrease in c-Myc protein to undetectable levels (data not shown). Therefore, telomerase activity is regulated independently of signals that regulate endogenous c-Myc.
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DISCUSSION

Myc’s ability to immortalize primary cells has been recognized for almost two decades (53), yet only recently have inroads been made into how this might occur. At least two pathways appear to be involved in the immortalization process and these are not necessarily mutually exclusive. First, Myc overexpression triggers the p53 apoptotic pathway through the induction of ARF (4). In turn this accelerates replicative crisis and selects for cells that can bypass this checkpoint, which generally have mutations in p53 or deletions of the Ink4a/ARF locus (4). On the other hand, Myc has been shown, as confirmed here, to activate the expression of TERT, and in some human cell types (but not others, ref. 27) ectopic hTERT expression is sufficient to immortalize cells (29). As shown here, there is some level of interplay between the ARF-p53 pathway and the regulation of telomerase activity, as ARF- and p53-null MEFs are immortal de novo and have augmented levels of telomerase activity. However, the relationships between c-Myc, ARF-p53 and TERT expression are complex, as c-Myc activation in primary cells activates all three, yet ARF or p53 loss also leads to increases in telomerase activity. Furthermore, c-Myc can clearly activate telomerase activity in MEFs lacking ARF or p53, and in immortal cells c-Myc has remarkably disparate effects on the regulation of TERT and telomerase activity. Overall these findings would suggest that a similar level of complexity is also likely to be found in human tumors, and that simple screens of tumors for telomerase activity may not be necessarily informative.

The disparate effects of c-Myc upon TERT expression and telomerase activity are, at this juncture, difficult to reconcile. A reasonable prediction based on findings in MEFs was that
c-Myc overexpression would lead to an induction of TERT and telomerase activity, yet in immortal cells this was only observed in NIH-3T3 cells, and here the effects of c-Myc were rather modest. In rapidly dividing FDC-P1.2 myeloid cells there was essentially no effect of c-Myc upon telomerase activity, despite the fact that c-Myc promotes mitogen–independent S phase entry and induces apoptosis in these cells (42). We have made similar observations using an inducible c-Myc overexpression system in human U937 leukemia cells, where c-Myc activation also leads to apoptosis, but no changes in TERT levels or telomerase activity were evident (data not shown). The most surprising finding was that in IL-3-dependent 32D.3 myeloid cells overexpression of c-Myc results in a marked reduction in mTERT levels and telomerase activity. Clones of 32D.3 cells engineered to overexpress the E2F-1 transcription factor are also augmented in their apoptotic program (54) and display a similar reduction in TERT expression and telomerase activity (data not shown). As we have failed to detect direct effects of c-Myc on E2F-1 or vice versa, this would suggest that somehow, at least in these cells, TERT expression is linked to signals that govern cell survival. Indeed it has been reported that at least in some cell types that telomerase activity is regulated by survival signals (55) and that TERT itself may have an anti-apoptotic role (56). However, these observations are clearly not generally applicable, as c-Myc augments the apoptotic program in all the cells used in this study, with widely varying effects on TERT and telomerase activity. Thus, c-Myc's ability to induce TERT levels and enhance telomerase activity are affected by immortalizing events and/or cell context, whereas Myc functions regulating its ability to activate apoptosis are less vulnerable to these alterations.

TERT has been proposed to be a direct transcription target induced by c-Myc (57, 58) and in primary cells this response is robust. As Myc activation also triggers the ARF-p53
pathway (4), one prediction was that the induction of TERT by Myc was linked to the activation of this tumor suppressor checkpoint. While loss of ARF or p53 indeed results in a modest up-regulation of telomerase activity in MEFs, the data also indicate that Myc activation induces telomerase activity in these null cells; therefore the induction of TERT is not strictly dependent upon ARF or p53 status. In 32D.3 and FDC-P1.2 cells the expression of ARF is silenced (46), whereas the Ink4a/ARF locus is deleted in NIH-3T3 cells (6). Nonetheless there were remarkably disparate effects of Myc on TERT expression and telomerase activity in these three cell types, indicating that other factors must modify the response.

TERT activation by c-Myc overexpression is certainly germane to events that occur in cancer, as Myc family oncoproteins are overexpressed in most human tumors. However, this does not mean that under physiological conditions that endogenous c-Myc regulates TERT transcription. Indeed under conditions in which endogenous c-Myc is down-regulated in primary and established murine cells, TERT expression is sustained, and similar findings have been reported in human cells (59). Furthermore, stimulation of ligand-starved 32D.3 myeloid cells with IL-3, which results in a substantial induction of c-Myc (34), has little effect on mTERT levels. Thus although one could argue that TERT regulation by c-Myc is compromised by immortalizing events and/or in vitro culture, an important experiment will be to evaluate TERT expression in c-Myc- and N-Myc-deficient embryos, to strictly evaluate whether TERT expression requires Myc oncoprotein functions.

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FIGURE LEGENDS

FIG.1. Myc induces telomerase activity in primary mouse embryonic fibroblasts and myeloid cells. A, primary mouse embryonic fibroblasts (MEFs) were infected with either a control (CD8) vector or the Myc-expressing retrovirus, and after 48 hours cell extracts were prepared. 0.03 µg of cell extracts were assayed for telomerase activity using telomerase PCR ELISA assay. RNAse indicates pre-treatment of the cell extract from Myc-overexpressing MEFs with RNAse A prior to the assay. B, primary mouse myeloid cells were infected with the control MSCV-GFP virus (vector) or MSCV-Myc-GFP virus, and 1 µg of cell extract was assayed for telomerase activity. C, primary MEFs infected with either the pSRα vector control or with pSRα-Myc-ERTM virus were treated with 4-TH for 24 hrs. 0.03 µg of the cell extracts was assayed for telomerase activity. D, primary mouse myeloid cells were infected with the control MSCV-GFP virus (vector) or MSCV-Myc-ERTM-GFP virus, and treated as in (C). 1 µg of cell extracts was assayed for telomerase activity. Results shown are the mean of duplicate telomerase assays and are representative of two independent experiments.

FIG.2. Loss of ARF or p53 augments telomerase activity. A, MEFs of the indicated genotypes infected with the pSRα-Myc-ERTM virus were treated with 4-TH for 24 hrs. 0.1 µg cell extract was used for the assay. B, 0.03 µg of the cell extracts from MEFs of indicated genotypes was assayed for telomerase activity, MEFs 1 and MEFs 2 are independently derived from individual wild-type embryos, MEFs/RNAse were extracts pretreated with RNAse before the telomerase assay. C, two different clones of primary wild-type MEFs (clones 10 and 14) were passaged on
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a 3T9 protocol (see experimental procedures). 10p7 and 10p36, are clone 10 at passage 7 and 36 respectively; 14p7 and 14p35 are clone 14 at passage 7 and 35, respectively. 0.03 µg cell extracts was assayed for telomerase activity, the legend (top right) indicates MEFs genotype. The values shown are the mean +/- standard deviation of two experiments performed in duplicate.

**FIG.3.** **c-Myc overexpressing 32D.3 myeloid cells have reduced telomerase activity.** A, cell extracts from exponentially growing cultures of 32D.3 and 32D.3 clones overexpressing c-Myc (myc.2, myc.4 and myc.12) pretreated (32D.3 and myc.2) or not with RNAse were assayed for telomerase activity. 0.3 µg of total protein extracts was used in the assays. B, inhibition of telomerase activity is not due to an inhibition of the PCR. The amount of total protein extract used in lanes 1 and 4: 0.075 µg, lanes 2 and 5: 0.15 µg, lanes 3 and 6: 0.3 µg; lane 7, positive telomerase extract control (supplied by the manufacturer), lanes 8 and 9 are heat inactivated extracts from 32D.3 and myc.2 cells, respectively. IC is the PCR amplification control, TSR8 is the telomerase quantitation control template both provided by the manufacturer. C, inhibition of telomerase activity in c-Myc overexpressing cells is due to limiting factor in the extract, as increasing amount of total protein assayed for telomerase activity correlates with telomerase activity in both cell types. 0.03, 0.3 and 3 µg of total protein extracts from 32D.3 and myc.2 cells were tested for telomerase activity with or without pretreatment with RNAse. D, c-Myc overexpressing cells do not contain an inhibitor of telomerase activity. 0.3 µg extract from myc.2 and 32D.3 cells was assayed for telomerase activity. myc.2 extract (0.3, 0.6, 1.2 µg; 1:1, 1:2, 1:4 respectively) was mixed with 0.3 µg 32D.3 extract before assaying telomerase activity. As controls, the 32D.3 extract was also assayed in the presence of equal volume of lysis buffer as
in 32D.3:myc.2 mixtures, or after RNAse treatment. Similar results were obtained with BSA (data not shown). Results shown are mean +/- standard deviation of three experiments performed in duplicate.

**FIG.4. c-Myc overexpressing 32D.3 myeloid cells have reduced levels of mTERT.** A, mTERT, mTR and mGAPDH mRNA levels were measured in 32D.3, myc.2, myc.4 and myc.12 cells by RT-PCR as described under "Experimental Procedures". GAPDH mRNA was used for normalization of RT-PCR efficiency. The number on the right margin indicates molecular weight markers. B, immunoblot analysis of mTERT expression. Extracts from 32D.3 and myc.2 cells were analyzed using anti-TERT antibody (see "Experimental Procedures"). 50 µg of total protein was loaded per lane and equal loading was confirmed by staining the blot. The position of mTERT is indicated.

**FIG.5. c-Myc overexpression has no effect on telomerase activity in FDC-P1 myeloid cells.** A, FDCP-P1 cells transfected with either empty vector (FDC-P1/Neo) or overexpressing c-Myc (FDC-P1/Myc, ref. 42) were assayed for telomerase activity following exposure to 1.5 µM dexamethasone (to induce c-Myc expression) for the indicated intervals. B, the same cells as in A were tested for telomerase activity using the Oncor telomerase detection kit after exposure to dexamethasone for 48 hrs. Lanes 1 and 3, prior to the assay extracts from FDC-P1/Neo and FDC-P1/Myc were heat inactivated, respectively; lanes 2 and 4, telomerase activity in FDC-P1/Neo and FDC-P1/Myc extracts, respectively; lane 5, telomerase activity positive control; lane 6, telomerase activity negative control (lysis buffer only, CHAPS); lane 7, telomerase quantitation control template; and IC, PCR internal control. 0.05 µg total protein was used in the
assay. Results shown in A are the mean of two experiments performed in duplicate. Those in B are representative of two separate experiments.

**FIG.6.** **c-Myc stimulates TERT expression and telomerase activity in NIH 3T3 cells.** A, 0.05 µg total protein lysates from parental NIH 3T3 cells or from two clones of NIH 3T3 cells overexpressing c-Myc (myc.2 and myc.3) was assayed for telomerase activity. B, Parental NIH-3T3 fibroblasts, and clones overexpressing c-Myc (myc.1, myc.2 and myc.3) were tested for telomerase activity (lanes 2, 4, 6 and 8, respectively) using the Oncor telomerase detection kit. As a control lysates were also heat inactivated prior to the assay (lanes 1, 3, 5 and 7). Control, CHAPS, IC and TSR8 same as in Fig.5, 0.05 µg total protein was used in this assay. C, mTR, mTERT and GAPDH levels were determined by RT-PCR using parental NIH 3T3 cells and clones overexpressing c-Myc, myc.2 and myc.3. D, Quantitation of mTERT-RT-PCR products normalized to GAPDH-RT-PCR products. The numbers on the left represent the density ratio of mTERT to GAPDH.

**FIG.7.** **mTERT protein expression and telomerase activity are independent of IL-3 in 32D.3 myeloid cells.** 32D.3 cells were passaged at 0.5 X 10^6 cells per ml for two consecutive days to ensure cells were in exponential growth phase. On the third day, cells were thoroughly washed in RPMI-1640/10% FCS medium lacking IL-3 and resuspended in this medium at 0.5 X 10^6 cells per ml for indicated intervals. After 16 hrs of incubation, a portion of the cells was stimulated with 100 Units of purified IL-3/ml and incubated for the indicated time. Cell viability and cell cycle analysis were monitored to confirm the effects of IL-3 withdrawal and IL-3 stimulation (data not shown). A, cells were harvested at the indicated time and lysates were prepared for
western blot analysis using anti-TERT antibody. 100 µg of total protein was loaded per lane. The arrow in (A) indicates the position of mTERT protein. B, lysates were also prepared at the indicated intervals for telomerase activity assays. 1 µg of total protein was tested in the assays. Assays shown are the mean +/- standard deviations from two experiments performed in duplicate.

**FIG.8. Effect of serum withdrawal on telomerase activity in MEFs.** 2 X 10^6 MEFs were washed twice and incubated in low serum medium (0.1%) for 48 hrs. As a control cells were cultured for the same period of time in complete medium. A, cells were harvested for FACS analysis. The histogram shows absence of cells in S phase and an accumulation of serum-starved MEFs in G₀/G₁. B, 0.3 µg of total protein from the cells in A was assayed for telomerase activity. Results shown are the mean of two experiments performed in duplicate.
Myc Regulation of TERT is Context Specific

FIGURES

Fig. 1
Fig. 2

A.  

B.  

C.  

Absorbance [A_{450 nm} - A_{655 nm}]

MEFs  
MycER  
MycER/p16Arf^{-/-}  
MycER/p53^{-/-}  

MEFs 1  
MEFs 2  
p16Arf^{-/-}  
p53^{-/-}  
P21^{-/-}  
MEFs/RNase

Absorbance [A_{450 nm} - A_{655 nm}]

ARF^{+/-}, p53^{+/-}  
ARF^{+/-}, p53 mutation
Myc Regulation of TERT is Context Specific

Fig. 3

A.

B.

C.

D.
Myc Regulation of TERT is Context Specific

Fig. 4

A.

<table>
<thead>
<tr>
<th>32D.3</th>
<th>myc.2</th>
<th>myc.4</th>
<th>myc.12</th>
<th>marker</th>
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</table>

- mTERT
- mTR
- GAPDH

200 bp
100 bp
400 bp
300 bp
500 bp
400 bp

B.

<table>
<thead>
<tr>
<th>32D.3</th>
<th>myc.2</th>
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mTERT
Fig. 5

A.

B. 

Incubation time in hrs after Dex induction

Absorbance [A450 nm - A655 nm]

FDC-P1/Neo

FDC-P1/Myc

16 48

ΔFDC-P1/Neo

ΔFDC-P1/Myc

FDC-P1/Myc

Control

CHAPS

TSR8

IC
Fig. 6

A. Absorbance [A_{450} - A_{885}] for NIH 3T3, myc.2, and myc.3 cells.

B. Gel electrophoresis of ΔNIH 3T3, NIH 3T3, Δmyc.1, myc.1, Δmyc.2, myc.2, Δmyc.3, myc.3, Control, CHAPS, and TSR8 samples, with IC indicated.

C. Southern blot analysis showing mTERT, mTR, and GAPDH bands with markers for 200, 100, 400, 300, 500, and 400 bp.

D. Relative amount of PCR product for NIH 3T3, myc.2, and myc.3 cells.
Fig. 7

A.

Hrs

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<tr>
<td>8</td>
<td>24</td>
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mTERT

B.

Absorbance [A_{450 nm} - A_{655 nm}]

Incubation Time in Hrs

<table>
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Fig. 8

A.

Control

G0/G1 = 41.1
S = 53.4
G2/M = 5.5

0.1% Serum

G0/G1 = 78.3
S = 6.2
G2/M = 15.5

B.

Absorbance [A450 nm - A655 nm]

Control

0.1% Serum
c-Myc mediated regulation of telomerase activity is disabled in immortalized cells
Rachid Drissi, Frederique Zindy, Martine F. Roussel and John L. Cleveland

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