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The role of c-*myc* in cellular growth control

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Cell division is coupled to cell growth. Since some c-*myc* target genes are regulators of cell growth while others function in cell division pathways, c-*myc* is apparently poised at the interface of these processes. Cell culture systems have shown specific *myc*-associated growth phenotypes. Increased cell growth precedes DNA synthesis after *myc* activation in cells expressing myc-estrogen receptor fuson constructs and cells lacking c-*myc* exhibit a marked loss of protein synthesis. A number of candidate c-*myc* target genes regulate processes required for cell growth including rRNA transcription and processing, ribosomal protein transcription and translation, and translation initiation. These interactions all have the potential to account for the growth phenotypes in c-*myc* mutant cells. The ability of translation initiation factors, including eIF4E, to transform cells makes them particularly interesting targets of c-*myc*. Further evaluation of these target genes will provide important insights into growth control and c-*myc*'s functions in cellular proliferation.

Keywords: translation initiation; rDNA transcription; eIF4E; translational control

Growth control and cellular proliferation

Cells proliferate by simultaneously doubling both their DNA and their mass. While synthesis of DNA is discontinuous, cells grow by continuously increasing synthesis of all their proteins and macromolecules. In general, DNA doubling and cell division are dependent on attainment of a critical growth rate in order to conserve cellular resources until daughter cell survival is assured. This principle was suggested by the function of START in yeast and the restriction point in mammalian cells. Most evidence suggests that normal cell proliferation is largely regulated by the length of the first gap phase of the cell cycle (G1) when this growth process is monitored (Figure 1a) (Pardee, 1989, 1974; Pardee *et al*., 1978; Sherr, 1994, 1996).

Cell cycle control has received considerable attention in the last ten years. In contrast, cell growth controls have been comparatively neglected. This neglect may be the result of the apparent lack of specificity in the enormous process of ribosomal synthesis, processing, and assembly which comprises up to 80% of the work of cell proliferation. The generation of 2×10^6 ribosomes per 15 h generation time guarantees that any growing cell will devote the majority of its metabolic energy to the construction of the protein synthetic apparatus leaving little room for obvious differences between normal and malignant cells (Sollner-Webb and Tower, 1986). However, recent progress in our understanding of the cell cycle might suggest areas worth exploring that have the potential to identify vulnerable differences in the response of cancer cells to perturbations of cell growth regulation. The cell cycle is controlled by cyclins, their dependent kinases, and their target genes, which function as key switches in the commitment to DNA synthesis (Sherr, 1993). Two features of the cyclin pathway emphasize the dependence of DNA synthesis on cell growth. First, acceleration of DNA synthesis by overexpression of positive cell cycle regulators (or loss of their inhibitors) makes cells divide at smaller sizes. Second, although the G1 phase is shortened by overexpression of the cell cycle machinery, the length of subsequent phases is delayed in compensation (Figure 1b). Loss of G1 control in cancer cells and altered cell size caused by perturbation of cell cycle regulation suggest potential vulnerabilities that might reveal interesting specificities in cancer cells.

Cells manufacturing DNA faster than they grow will ultimately become unstable (Figure 1d). Balanced proliferation requires equilibrium between growth and division. Some lines of evidence suggest that uncoordinated division indeed makes cells unstable. First, overexpression of known cell cycle regulators leads to apoptosis (Hiebert *et al*., 1995; Shan and Lee, 1994). The resistance of $Rb^{-/-}$ cells to cycloheximide emphasizes this point (Herrera *et al*., 1996). Though provocative, associations between this phenomenon and specific mechanisms coordinating growth remain largely unexplored. Second, leukemic cells are vulnerable to sudden loss of asparagine induced by L-asparaginase treatment (Broome, 1961). While its selective killing depends on the absence of asparagine synthetase, L-asparaginase causes a sudden loss of protein synthesis that drives leukemic cells into apoptotic pathways (Broome, 1981; Bussolati *et al*., 1995; Story *et al*., 1993).

Functions of c-*myc* in growth control

Cells expressing chimeric proteins that fuse c-*myc* with domains of the estradiol receptor provide a clear demonstration of c-*myc*'s role in cell proliferation (Eilers *et al*., 1989). Mycer cells enter S phase 24 h after addition of estradiol. Importantly, we found that

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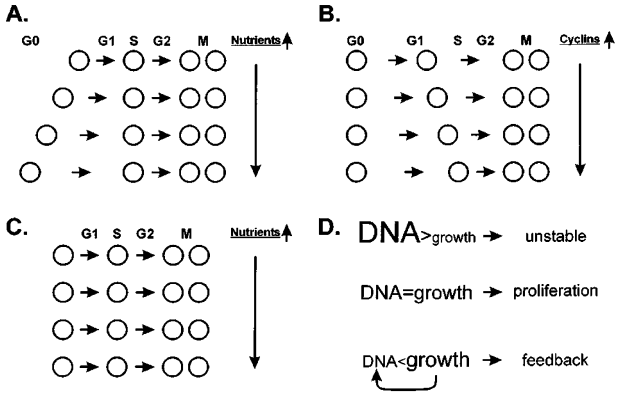

Figure 1 Schematic view of relationships between cell growth and cell division. (**a**) During normal growth, a decreased stimulus for growth (e.g. decreased nutrient supply) slows proliferation primarily by prolonging the G1 phase of the cell cycle (Pardee *et al*., 1978). (**b**) Changes in typical cell cycle controls (e.g. decreases in G1 cyclin function) provide incomplete controls for cell proliferation since shortening of the G1 phases results in a compensatory prolongation of S, G2 and M (Sherr, 1994). (**c**) Cancer cells have typically lost G1 controls and can no longer change their G1 regulation in response to their environment (Sherr, 1996). (**d**) Recent experiments confirm the general theme that growth and DNA synthesis are balanced by feedback controls with cell growth being upstream of cell division (Neufeld *et al*., 1998). In general, this suggests that signals driving cell division faster than cells can grow will lead to instability

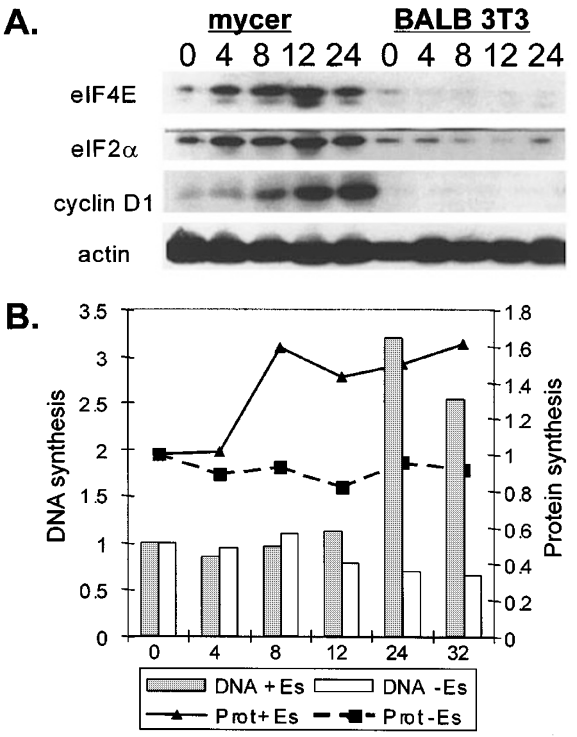

Figure 2 Cell growth precedes cell division in *mycer* cells. (**a**) Western blots of extracts from *mycer* and *BALB 3T3* control cells stimulated with estrogen reveal increased expression of eIF4E, eIF2α and cyclin D1 in response to activation of c-*myc* function by estradiol addition (data re-plotted: (Rosenwald, 1996; Rosenwald *et al*., 1993a,b)). (**b**) Protein synthesis increased in mycer cells 16 h before any increase in DNA synthesis was observed

increased protein synthesis actually preceded DNA synthesis in mycer cells (Figure 2); amino acid incorporation increased by 50% 4–8 h after addition of estradiol (Rosenwald, 1996; Rosenwald *et al*., 1993b). These data are consistent with the principle that increased protein synthesis is required to enter S phase. Moreover, homologous targeting that deleted the c-*myc* locus from rat fibroblasts revealed a strikingly similar result (Hanson *et al*., 1994; Mateyak *et al*., 1997; Prouty *et al*., 1993; Shichiri *et al*., 1993). Loss of a single myc allele in RAT1 fibroblasts cut Myc expression in half leading to a 3 h delay in S phase entry and a similar increase in doubling time. Homozygous deletion of both alleles abrogated c-*myc* expression with no compensatory increase in N- or L-*myc*. Under these conditions doubling time increased 2.5-fold, and *both* G1 and G2 phases of the cell cycle were delayed. Remarkably, net protein synthesis decreased 2.5-fold in these cells. Since cell size and ribosomal content were maintained at constant levels, protein turnover apparently decreased 2.5-fold to compensate for the decreased synthetic rate.

This growth defect in c-*myc* null cells is consistent with myc's genetic functions in organisms with less complex genomes. The recent identification of a Drosophila size mutant, *min*, as a mutation in c-*myc* supports the idea that c-*myc* has a primary function in growth control (Gallant *et al*., 1996). Moreover, defects in a specific target gene, *pitchoune*, lead to a similar phenotype. *Pitchoune* is a DEAD box RNA helicase of currently unknown function (Zaffran *et al*., 1998). Interestingly, the majority of RNA helicases in yeast are involved in ribosomal RNA processing, probably resulting from a critical need for unwinding of the large amounts of rRNA synthesized during growth (Daugeron and Linder, 1998; Kressler *et al*., 1998; Schmid and Linder, 1992). It will be interesting to learn more of the functions of this interesting *myc* target.

C-*myc* is also implicated as a direct regulator of the cell cycle machinery (Amati *et al*., 1998). Thus, it is poised at the intersection of growth and cell cycle control, potentially accounting for its oncogenic potency. Consistent with the speculation that loss of coordination of growth and DNA synthesis destabilizes cell proliferation, several alterations of growth control cause apoptosis in Myc-overexpressing cells (Evan *et al*., 1992). For example, removal of serum from growth media of myc-overexpressing cells induces apoptosis. While this has often been taken as a cell fate decision mediated by apoptotic signaling pathways, it could also simply reflect the loss of coordination of growth in a cell that cannot limit its DNA synthesis. This is suggested by the fact that loss of growth by glucose deprivation also induces apoptosis in mycer cells (Shim *et al*., 1998).

The finding that both G1 and G2 are delayed in c-myc null cells is most consistent with a primary defect in growth control. Its lack of specificity is not as consistent with a single cell cycle regulator functioning as myc's primary target. For example, the first genetic mutant identified in control of protein synthesis in yeast was *prt*-1; additional alleles of this mutant

(*cdc63*) are specific regulators of START (Hanic-Joyce *et al*., 1987). The *cdc63* version of *prt*-1 cells are mutant in the η component of the eukaryotic translation initiation complex 3 (eIF3η). Intriguingly, at different temperatures the *cdc63* arrest phenotype changes from a non-specific arrest at all stages of the cell cycle to a specific G1 arrest. Presumably, as the conformation of eIF3η changes with temperature, the number of proteins whose synthesis is limited by its effects on translation initiation steadily decrease until its effects become specific to the G1 stage of the cell cycle. These varying phenotypes of *cdc63* suggest that there are multiple points where growth limits the cell cycle, but G1 is the most sensitive target of its effects. This phenomenon is strikingly reminiscent of the G1 arrest seen with heterozygous loss of c-*myc* coupled with the generalized defect throughout the cell cycle found in c-*myc* null cells.

Elements of growth control

How is growth controlled? Since the list of c-*myc* target genes identified in transcriptional assays includes many genes involved in general cell growth and metabolism (Dang *et al*., 1997), a review of general growth control mechanisms may suggest areas of particular interest for the understanding of myc's function. Three different types of growth control can be distinguished: (1) 'Stringent' control during rapid changes in nutrient availability; (2) 'Growth rate controls' during continuous proliferation; and (3) Cell cycle variation in protein synthesis. The existence of the first two different sets of controls is best shown in bacteria. Rapid changes in protein synthesis following nutrient shifts, 'stringent' control, are primarily regulated by rDNA promoter elements in *E. coli* bacteria; these elements are changed to a different set of controls during continuous proliferation (Gourse *et al*., 1996). The most interesting mechanism of control is found during this continuous growth phase since a clear mechanism coupling nutrient availability to ribosomal synthesis has been found. During continuous proliferation, initiating NTP concentrations of the rRNA molecule itself (adenosine or guanosine triphosphate (ATP or GTP)) stabilize an open rDNA transcriptional complex thereby coupling ribosome synthesis to nutrients (Gaal *et al*., 1997). In contrast to the clear answers in *E. coli*, cell cycle controls of protein synthesis in eukaryotic cells remain somewhat obscure although varying rates of translation initiation may account for some of the decreased protein synthesis during late G2 and M phases in the cell cycle (Tarnowka and Baglioni, 1979).

To better understand potential genetic targets of c-*myc*, a review of the stages used in growth control may suggest areas of particular interest (Figure 3). The protein synthetic machinery is regulated at two stages – ribosomal synthesis and translation initiation (Figure 3a and b). Measurements of global protein synthesis seem trivial to interpret since they fundamentally assay the generation of ribosomes, which constitute up to 80% of the cell's material. However, this stage is critical since ribosomes then produce the remainder of the cell's proteins. Thus, control of ribosomal synthesis lies at the heart of growth control

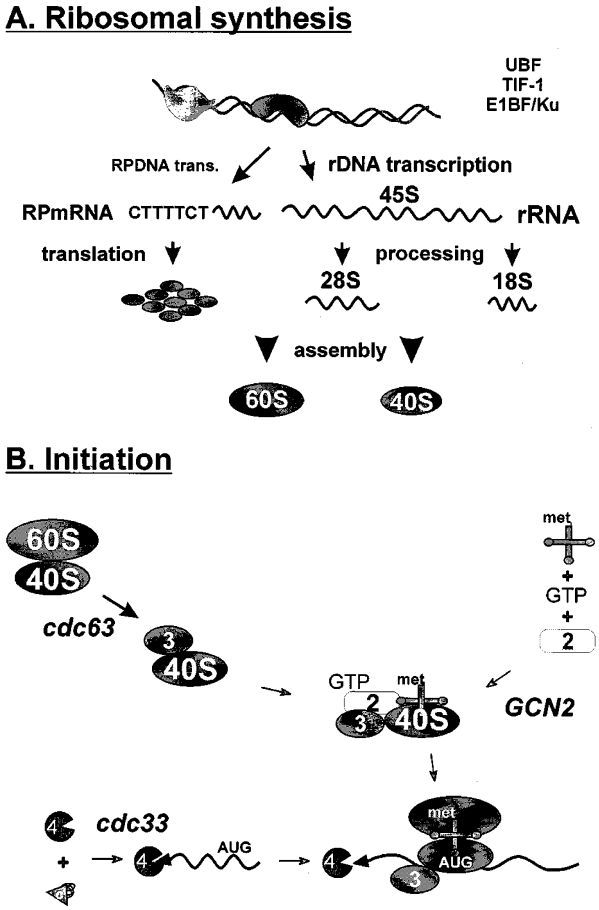

Figure 3 Major components of the protein synthetic machinery and critical contolr points that govern cell growth. (**b**) Ribosomal assembly requires complex coordination between rDNA transcription, rRNA processing, ribosomal protein transcription and translation, and final assembly into the 60S and 40S subunits. (**b**) Initiation of mRNA translation requires final assembly of a charged ribosome on an initiating AUG. Genetic mutants in yeast identify the eIF3 (*cdc63*), the eIF4 (*cdc33*) and eIF2 (*GCN2*) complexes as critical rate-limiting components of cell growth

and is regulated through several steps. Ribosomal biogenesis begins with rRNA transcription (Jacob, 1995), requires rRNA processing (Eichler and Craig, 1994), and is complemented by transcription and translation of ribosomal proteins. Different genetic elements control each of those processes. Subsequently, utilization of specific mRNAs depends on the abundance of a rate-limiting set of translation initiation factors (Hershey, 1991; Sonenberg, 1994). In general, translation initiation is thought to be the more significant rate-limiting step in translation. Ribosome content is not thought to be rate-limiting because translational elongation does not limit overall translation rates. Nevertheless, the details of which step is rate-limiting in various circumstances has not been clearly established and may vary.

The first step in ribosomal biogenesis is transcription of ribosomal DNA (rDNA). Half of the cell's transcriptional apparatus is devoted to rRNA synthesis, which is read from the 150 – 200 repeated rDNA copies present in mammalian genomes (Jacob, 1995; Sollner-Webb and Tower, 1986). At least some portion

of net ribosome biogenesis is limited by the rate of rRNA transcription. For example, amino acid starvation affects rRNA transcription rates (Grummt *et al*., 1976) and nucleolar RNA synthesis responds to NTP pools in the same manner as seen in bacteria (Grummt and Grummt, 1976). Furthermore, the rate of rRNA gene transcription decreases markedly in stationary phase cells (Sollner-Webb and Tower, 1986). The transcription factor primarily involved in this growth dependent regulation has been identified as TIF-IA (Buttgereit *et al*., 1985; Schnapp *et al*., 1990), which works in concert with other rDNA transcription factors including UBF and E_1BF/Ku (Datta *et al*., 1997; Jacob, 1995).

Though rRNA transcription may constitute one regulatory point, processing of rRNA has also been shown to be rate-limiting in some conditions (Eichler and Craig, 1994). First, loss of the DEAD-box helicase family members involved in rRNA processing in yeast cause severe slow growth phenotypes (Daugeron and Linder, 1998; de la Cruz *et al*., 1998). Furthermore, not all mature rRNA is incorporated into ribosomes in mammalian cells. As much as half of 18S rRNA is degraded continuously in resting cells; it is only stably incorporated into ribosomes when lymphocytes or liver cells are stimulated to grow (Cooper and Gibson, 1971; Dudov and Dabeva, 1983). This rapid turnover rate suggests that, in fact, excess rRNA is transcribed and subsequent steps are the key regulatory points in ribosomal biogenesis.

In contrast to rRNA regulation, translational controls regulate most of the growth response of mRNAs encoding ribosomal proteins (Meyuhas *et al*., 1996). This is most clearly demonstrated by the shift of ribosomal protein mRNAs (rpRNA) in polysomal profiles after growth stimulation (Avni *et al*., 1997; Shama *et al*., 1995). Polysomal profiles are performed by sucrose gradient centrifugation which separates highly translated mRNAs engaged by multiple ribosomes from poorly translated mRNAs migrating in less dense fractions. Growth stimulation moves the rpRNAs into dense, polysomal fractions of such gradients as evaluated by RNA blotting of the gradient fractions. An oligopyrimidine tract (CTTTTCT) conserved in the 5′ ends of these mRNAs accounts for much of this regulation (Perry and Meyuhas, 1990). Although this mechanism is thought to be the main regulator of ribosomal protein content in mammalian cells, several well characterized transcription factors can transactivate ribosomal protein promoters. The most prominent transcription factor involved in rpRNA transcription is YY1 (Delta factor) (Chung and Perry, 1993; Hariharan *et al*., 1991; Safrany and Perry, 1993).

Once a ribosome is made, it must be assembled into an active translation complex before protein synthesis begins (Figure 3b). This assembly is regulated by the translation initiation factors (Pain, 1996; Sonenberg and Gingras, 1998). Translation initiation factors have long been viewed as rate-limiting in protein synthesis because they are less abundant than ribosomal components themselves and because ribosomal elongation is rarely rate-limiting (Duncan and Hershey, 1983, 1985). Particular mutations in yeast translation initiation factors tend to confirm the importance of this step. The least abundant translation initiation factor is the mRNA cap binding protein which binds the 7-methyl guanosine at the 5′ end of all mRNAs. A yeast mutation in this factor (*cdc33*) exhibits a G1 arrest phenotype, similar to nutrient arrest (Brenner *et al*., 1988). In contrast, in mammalian cells overexpression of eIF4E transforms them to a malignant phenotype (Lazaris-Karatzas *et al*., 1990). The eIF3 complex acts at nearly all steps of translation initiation to stabilize interactions between all of the initiation factors. Yeast mutations in one of its components, eIF3η (*cdc63*), can exhibit a true START arrest (Hanic-Joyce, 1985; Hanic-Joyce *et al*., 1987). This mutation is of particular interest since *cdc63* cells continue to grow in size at the restrictive temperature although protein synthesis is generally decreased. Their arrest at START suggests that only those proteins essential to passage through START are particularly affected by *CDC63*. Like eIF4E, eIF3 components have also been found to be oncogenic in mammalian cells since MMTV insertions in the *int-6* locus up-regulate one specific component of eIF3 (Asano *et al*., 1997).

C-*myc* target genes and growth control

Which target gene or genes might account for the defect in global protein synthesis in c-*myc* null cells? Similarly, how might c-*myc* increase cell growth in *myc*-transformed cells? Several domains play significant roles in c-*myc*'s ability to transform cells. These include its DNA binding domain, its transrepression domain and its transactivation domain (Stone *et al*., 1987). Myc binds DNA through its basic, helix–loop–helix/leucine zipper domain which is essential to transformation. The promoters of at least some key target genes must therefore include a canonical E box or other known non-canonical binding sites, whether they are

Table 1 Potential target interactions connecting c-*myc* to growth control

Pathway	*Connections to myc-target genes*	*Reference*
Ribosomal availability		
rRNA transcription	USF site in spacer pRb interaction	(Ghosh *et al*., 1997) (Cavanaugh *et al*., 1995; Rustgi *et al*., 1991)
rRNA processing	DEAD box helicase (pitchoune)	(Grandori *et al*., 1996; Zaffran *et al*., 1998)
rRNA stability	Unknown	none
Ribosomal protein transcription	YY1 interacts with c-*myc* and ribosomal protein promoters	(Safrany and Perry, 1993; Shrivastava *et al*., 1993)
Ribosomal protein translation	Translation initiation factor effects on oligopyrimidine tract	(Meyuhas *et al*., 1996; Rosenwald *et al*., 1993b)
Translation initiation		
elF4E	E box transcription and potential interaction with 4ERFs	(Johnston *et al*., 1998; Jones *et al*., 1996)
elF2α	Non-canonical CGCATG site	(Rosenwald *et al*., 1993b; Shors *et al*., 1998)

transactivated or transrepressed (Blackwell *et al*., 1990, 1993; Prendergast and Ziff, 1991). Alternatively, c-*myc* could accomplish some of its functions by direct protein-protein interactions. Assuming those two possible criteria, the current list of candidate *myc* targets includes several genes whose regulation might easily explain its growth effects (Table 1).

Since net RNA synthesis is markedly decreased in c-*myc* null cells (Mateyak *et al*., 1997) and ribosomal RNA constitutes the bulk of cellular RNA, the c-*myc* null phenotype could be explained simply by a defect in Pol I transcription. This has not been formally tested but two lines of evidence suggest potential mechanisms. First, c-*myc* has been shown to interact with the retinoblastoma protein, pRb, in some experiments. C-*myc* directly interacts with specific domains on pRb *in vitro* and alters transcription of target genes (Hateboer *et al*., 1993; Maheswaran *et al*., 1991; Rustgi *et al*., 1991). Furthermore, pRb directly binds the UBF component of the *Pol*I activation apparatus and inhibits *Pol*I transcription *in vitro* (Cavanaugh *et al*., 1995; Voit *et al*., 1997). The combination of these two interactions might suggest a mechanism for c-Myc to regulate *Pol*I transcription. Although these functions have been shown in model systems, their significance *in vivo* is untested. Second, the core rDNA promoter also contains an E box which is both inhibited by USF and resembles c-*myc*'s binding motif (Ghosh *et al*., 1997). Since USF generally antagonizes transactivation by c-*myc* (Luo and Sawadogo, 1996), this site offers a potential site for c-*myc* to directly regulate rRNA transcription. Although both of these observations only suggest potential mechanisms, the decrease in bulk RNA synthesis in c-*myc* null cells offers an attractive area in which to explore potential mechanisms.

Immunoprecipitation of c-Myc-bound DNA provided a particularly attractive approach to the identification of c-*myc* target genes (Grandori *et al*., 1996). This approach revealed a DEAD box helicase of unknown function. Intriguingly, the DEAD box helicases comprise a family of proteins that catalyze unwinding of RNA. Members of this family include proteins involved in both translation initiation (eIF4A) and ribosomal RNA processing (Pause *et al*., 1994; Rozen *et al*., 1990). Although the biochemical function of this Myc target is currently unknown, its homologue in *Drosophila* has been named *pitchoune* as a consequence of the small size phenotype which results from its loss (Zaffran *et al*., 1998).

YY1 (Delta factor) is an additional c-*myc* interactor that suggests a potential mechanism for an interaction between c-*myc* and transcription of ribosomal proteins (Austen *et al*., 1997). The ribosomal protein rpS16 contains a downstream element in its promoter that is essential for its transcription (Hariharan and Perry, 1989). Characterization of this downstream element revealed a transcription factor first termed Delta factor, which was subsequently found to be identical to the transcription factor YY1 (Hariharan *et al*., 1991; Hariharan and Perry, 1990; Safrany and Perry, 1993). YY1 is one of a new class of factors that activate transcription at the initiation site of many genes; it also represses transcription of a variety of genes. Interestingly, YY1 binds directly to c-Myc in a novel region of the protein that differs from other known protein-protein interactions (Hariharan *et al*., 1991; Hariharan and Perry, 1990; Safrany and Perry, 1993). This interaction can repress or activate transcription, depending on Myc levels in the cell (Shrivastava *et al*., 1996). Once again, although not directly tested the interaction between YY1 and c-Myc has at least the potential to affect transcription of rpS16.

Reasoning that c-Myc levels peak after growth induction at the same moment that protein synthesis is rate-limiting for cell cycle progression, we originally examined the potential for c-Myc to stimulate transcription of specific translation initiation factors (Rosenwald *et al*., 1993b). Since translation initiation may be the critical control point in growth regulation, we examined translation initiation factors eIF4E and eIF2α. The kinetics of their increase in serum stimulated fibroblasts paralleled those of c-*myc* and both were markedly increased in myc-overexpressing rat embryo fibroblasts. We then examined mycer cells and found that translation initiation factor expression increased transcriptionally in run-on assays in response to activation of the Myc-chimeric molecule. The eIF2α promoter contained a non-canonical CGCATG site known to be a preferred *in vivo* target for c-*myc* (Blackwell *et al*., 1993; Humbelin *et al*., 1989; Rosenwald *et al*., 1993b). Furthermore, max binds to this site as a heterodimer with an interesting transcription factor called either α-PAL or NRF (Efiok *et al*., 1994; Shors *et al*., 1998; Virbasius *et al*., 1993). Moreover, this factor, Nuclear Regulatory Factor (NRF) coordinates the transcription of nuclear mRNAs needed to make cellular mitochondrial proteins (Evans and Scarpulla, 1990). In contrast to the other candidate genes discussed above, we demonstrated that protein synthesis indeed increased in mycer cells in parallel with increases in these translation factors suggesting their potential functions in cell growth (Figure 2) (Rosenwald, 1996; Rosenwald *et al*., 1993b).

We then cloned eIF4E genomic sequences to provide convincing evidence that Myc directly interacts with the eIF4E promoter (Jones *et al*., 1996). The eIF4E promoter contained two canonical myc sites, CACGTG, that were essential for reporter gene activity. Dominant negative myc constructs down-regulated eIF4E-reporter fusions, suggesting that myc directly interacts with this promoter *in vivo*. The abundance of potential Myc-binding sites complicates all studies of c-*myc* target genes. The sequence CACGTG can be expected to appear an average of one to three times in every gene in mammalian cells. Thus, the presence of an E box is not sufficient to identify critical myc target. Presumably critical Myc target promoters must also contain associated transcription elements that work in concert with c-Myc to regulate only a subset of genes containing CACGTG. The absence of other known promoter elements known to function as collaborating basal elements in the eIF4E promoter led us to perform linker-scanning analyses. We identified two proteins with novel properties that we designated 4E regulatory factors (4ERFs) (Johnston *et al*., 1998). Fitting with our model that Myc functions in the balance of cell growth and cell division, we found that decreases in c-Myc expression in differentiating U937 and HL60 cells were accompanied by decreases in the 4ERFs, eIF4E,

protein synthesis and DNA synthesis. These experiments identified a second system where c-*myc* could interact with components of growth regulating pathways.

What target genes account for the defect in protein synthesis in c-*myc* null cells? The only candidate c-*myc* target genes down-regulated in c-myc null cells include cad and GADD45 (Bush *et al*., 1998). In contrast to the results in mycer cells, translation initiation factor eIF4E was not decreased in c-*myc* null cells. Disappointingly, although the *cad* gene is required for pyrimidine biosynthesis, addition of its pyrimidine products did not restore normal growth to myc-nulls. Moreover, over-expression of *cad* apparently has no effect in transformation assays (Bush *et al*., 1998). Thus, the current evaluation of c-*myc* null cells is incomplete. It is not surprising that myc target genes with essential functions and complex promoter regions can use alternative promoter elements to maintain their expression levels, even in the absence of c-*myc*. Indeed, the effects of *cdc33* in yeast suggests that loss of eIF4E is likely to have markedly deleterious effects and might not be tolerated in transfection models. Nevertheless, the broad implications of the c-*myc* null growth phenotype makes a strong argument that other growth regulators are down-regulated in them and must be important contributors to myc's functions.

Targets of c-*myc* target genes

The primacy of growth regulation over cell cycle control further implies that components of growth regulatory pathways must be able to specifically alter expression patterns of the known cell cycle regulators. Indeed, the small cell size phenotype found in yeast over-expressing G1 regulators holds true even in complex multicellular tissues (Cross, 1988; Nash *et al*., 1988; Neufeld *et al*., 1998). For example, increased numbers of small cells were found in the wing web of Rb-deficient and E2F-overexpressing *Drosophila* mutants, clearly demonstrating that growth signals fall upstream of cell cycle controls. Acting on the assumption that growth regulators should upregulate positive regulators of the cell cycle, we first evaluated cyclin D1 protein levels in mycer cells and found that cyclin D1 protein increased in response to myc-activation in the absence of any change in its mRNA (Figure 2) (Rosenwald *et al*., 1993a). These post-transcriptional increases could be attributed to increased eIF4E through complex mechanisms (Rosenwald *et al*., 1995; Rousseau *et al*., 1996). Moreover, similar mechanisms also increase cyclin D1 protein after serum stimulation (Muise-Helmericks *et al*., 1998).

Although the coupling of cell growth to cell division has been a fundamental tenet of cell cycle biology, specific mechanisms explaining this phenomenon have received less attention. The functions of yeast G1 cyclins are analogous to cyclin D1 and yeast deficient in *CLN1,2,3* are rescued by mammalian G1 cyclins in functional assays (Xiong *et al*., 1991). To examine the mechanisms coupling growth to division in an organism that could provide genetic insights, we therefore examined translational control of the G1 cyclin *CLN3* in yeast. The functional homologue of cyclin D1, *CLN3* functions upstream of all other G1 cyclins in yeast and its mRNA levels do not vary through the cell cycle. The absence of obvious transcriptional controls suggested that it might be under post-transcriptional controls like those we identified for cyclin D1. We therefore focused on regulation by an upstream open reading frame in the unusually long 5′ leader sequence of *CLN3* (Polymenis and Schmidt, 1997). Upstream open reading frames (uORF) act as translational repressors in most systems (Geballe, 1996). As expected, removal of the AUG codon in this uORF by a simple A>T mutation changed the translational efficiency of *CLN3* mRNA. The loss of its translational control caused increased S phase progression in poor growth conditions, while growth in rich conditions was inhibited by loss of the uORF. This mutation identifies translation control of *CLN3* as a key integrator of growth and cell cycle progression. Multiple growth regulating pathways appear to converge on this signal including the proliferative response to nitrogen deprivation, cAMP, and TOR signaling (Gallego *et al*., 1997; Hall *et al*., 1998; Polymenis and Schmidt, 1997). Indeed, we extended these studies and found that *CLN3* is the single rate-limiting G1 cyclin for cell proliferation in poor growth conditions (Polymenis and Schmidt, 1998). Moreover, any stimulus that results in limiting concentrations of ribosomes has the same effect on limitation of Cln3p synthesis. The importance of this signal was particularly emphasized by the ability of our ATG>TTG mutation to override the START arrest of the translation factor mutant, *cdc63*. Thus, *CLN3* appears to be the most critical target for the G1 coupling of growth to cell cycle progression. In

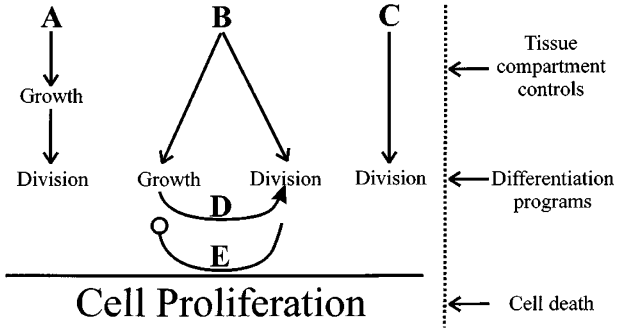

Figure 4 Conceptual framework for the coordination of cell growth with cell division. Cell growth refers to increased macromolecular synthesis exclusive of DNA. Cell division refers to the doubling of DNA, which is coupled to mitosis once DNA synthesis begins. Cell proliferation is the addition of new cells to a tissue or culture. Obviously, the total number of cells is determined not only by the rate they are generated, but also by the rate cells are dying, particularly in multicellular organisms. Based on experiments performed in budding yeast, flies and mammalian systems, regulatory molecules might affect cell division purely through their control of cell growth (**a**). Alternatively more stable controls might be provided by coordinating both growth and cell division simultaneously (**b**). Situations in which regulatory molecules limit cell proliferation through their control of the cell cycle are rarely observed (**c**). Since multiple overlapping signals might still drive cell division in preference to cell growth in (**b**), feedback controls should still drive DNA synthesis in response to growth (**d**). Cell division controls are generally ineffective in driving cell growth (**e**)

sum, the *CLN3*/cyclin D1 paradigm suggests that specific mechanisms can be identified that are key to the coupling of cell growth and cell division.

Translational control mechanisms offer the opportunity to regulate broad classes of mRNAs although they cannot fine-tune gene expression in the same manner as the combinatorial interactions inherent in transcriptional control. The classic model of translational control was developed to explain the equalization of α and β globins, despite inequality in the synthesis of their mRNAs. This model shows that translationally repressed mRNAs are differentially affected by changes in ribosomal availability when compared to translationally active mRNAs (Lodish, 1974). Thus, classes of translationally repressed mRNAs, perhaps including oncogene transcripts in general (Kozak, 1987), will be coordinately regulated by changes in the ribosomal content of the cell. Our analysis of CLN3 regulation is an important example of the power of this type of regulation to explain the interaction between seemingly non-specific events like changes in ribosome content and cell division control.

The loss of growth regulation in cancer cells

Cancer cells lose G1 control and have lost the capacity to respond to their environment (Figure 1c), which should make them vulnerable to manipulation of their growth control. It also implies that their growth control mechanisms are abnormal. Unfortunately, the genetic analysis of cancer cells has only revealed a few examples of mutations in regulators of cell growth. While manipulations of eIF4E, eIF2α and eIF3 can all cause malignant transformation in experimental systems, less evidence links them to specific cancers (Asano *et al*., 1997; Koromilas *et al*., 1992; Lazaris-Karatzas *et al*., 1990). While several surveys have found increased levels of eIF4E in breast adenocarcinomas (Kerekatte *et al*., 1995), mutations characteristic of oncogenic activation in any pure growth regulator have not been found. This leaves c-*myc* as a particularly important paradigm for studies of growth and cell cycle control. The general scheme of precedence (Figure 4) suggests that Myc should still increase cell growth even if cell division is arrested in Myc-expressing cells. Similarly, an arrest of cell growth should stop all proliferation in Myc-expressing cells. Experiments to clarify these interactions, together with identification of the c-*myc* target genes that account for its growth phenotypes should help clarify its role in overall cell proliferation.

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