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Myc: a single gene controls both proliferation and apoptosis in mammalian cells

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Abstract. c-*myc* was discovered as the cellular homologue of the transduced oncogene of several avian retroviruses. The gene encodes a transcription factor, which forms a heteromeric protein complex with a partner protein termed Max. In mammalian cells, Myc is a central regulator of cell proliferation and links external signals to the cell cycle machinery. Myc also induces cells to undergo apoptosis, unless specific signals provided either by cytokines or by oncogenes block the apoptotic pathway. Recent progress sheds light both on the factors regulating the function and expression of Myc and on the downstream targets in the cell cycle. Together, these findings suggest the existence of a novel signal transduction pathway regulating both apoptosis and proliferation.
Key words. c-*myc*; max; oncogene; transcription; cell cycle; apoptosis.

Introduction: tumorigenesis

The first *myc* gene was discovered as the transforming oncogene of the avian retrovirus, MC29, and named after the predominant disease caused by the virus (*my*elo*c*ytomatosis) (for review, see ref. 1). It soon became apparent that no less than four different retroviral isolates had transduced the same gene; this unique situation immediately pointed to an exceptional role for *myc* in the control of growth and proliferation and spurred a wave of interest in the function of *myc* that is still felt today.

Soon, a cellular homologue of viral *myc* was cloned from a number of organisms and termed c-*myc*. These organisms included many species of vertebrates. While for a number of years it was suspected that *myc* function might be restricted to these organisms, the recent identification of *myc* genes or genes encoding partner proteins of Myc (see below) in both *Drosophila* and *Caenorhabditis* demonstrates a much wider conservation of this protein (P. Gallant and B. Eisenman; M. Cole, personal communication). However, it appears clear that yeast does not contain genes closely related to *myc*, given that the entire genome has now been sequenced.

The central role for c-*myc* in tumorigenesis was further highlighted when this gene was shown to be involved in some of the most consistent chromosomal translocations found in human leukemias (for review, see ref. 2). In Burkitt lymphoma, c-*myc* genes are translocated to the loci of immunoglobulin genes; thus, the tight control of c-*myc* expression is overridden by the potent immunoglobulin enhancers. Cells carrying these translocated genes express strongly elevated levels of c-*myc* RNA. Transgenic mice engineered to express similar fusion genes (so-called *Eμ-myc* mice) show diseases that closely mimic Burkitt lymphoma, unequivocally demonstrating the causal role for Myc in the process [3, 4].

c-*myc* has now been shown to be part of a small multigene family, and at least three members of this family (c-*myc*, N-*myc* and L-*myc*) have been implicated in a large number of human and animal neoplasias, making *myc* genes some of the best documented human oncogenes (for review, see ref. 5). Experiments both in transgenic mice and in tissue culture have highlighted one characteristic feature of Myc's role in tumorigenesis: on its own, c-*myc* is a poorly transforming gene, and expression of c-*myc* alone often has little phenotype. However, in culture c-*myc* strongly synergizes with other oncogenes, for example activated alleles of ras, in transformation: this phenomenon is called oncogene co-operation [6]. Similarly, tumours arising in transgenic c-*myc* mice arise after long latency periods and are clonal: both findings strongly suggest that additional genetic lesions need to accumulate before tumours arise. Indeed, such lesions can be identified by a cloning method termed retroviral tagging; this method identifies a small number of genes that strongly accelerate tumorigenesis in transgenic mice carrying c-*myc* under the control of immunoglobulin enhancers. One of these genes is *pim-1*, a serine-threonine kinase that is localized in the cytosol [7].

Why do oncogenes co-operate with Myc (for review, see ref. 8)? Several models are conceivable which are not mutually exclusive. For example, ectopic expression of Myc provides a strong mitogenic stimulus (see below), and *Eμ-Myc* mice simply have more pre-B cells than their normal counterparts [9]. Thus, if pre-B cells are susceptible to transformation by pim-1, a strong synergy between Myc and pim-1 would result. DNA replication is also inherently

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mutagenic, and an increase in cell number of course reflects an increased number of S-phases. Thus, Myc might simply increase the risk of a given cell population to accumulate further mutations.

However, these arguments fail to explain oncogene co-operation in tissue culture, where cell numbers are essentially kept constant by experimental conditions. So how do oncogenes co-operate here? Two models will be discussed in this review. One model suggests that oncogenes co-operate because they control different rate-limiting steps in the assembly of an active cell cycle machinery. In this view, quiescent cells differ from proliferating cells because they have not executed a number of key transitions in the early cell cycle. Oncogenes are mitogenic because they control passage through these transitions, for example by controlling the synthesis of rate-limiting components. Oncogenes co-operate because many such rate-limiting steps exist, and only their co-ordinated activation results in (then uncontrollable) proliferation. In this view, oncogenes can be used to identify the critical rate-limiting steps involved in cell cycle progression. A second view of oncogene co-operation is based on the finding that cell number in mammalian organisms results from a balance between proliferation and active cell death, or apoptosis. Strikingly, activation of individual oncogenes often induces cells to undergo apoptosis, and other oncogenes exist that block the ability of a cell to undergo apoptosis [10]. Thus, oncogene co-operation may result from both a block in apoptosis and a stimulation to proliferation. Evidence exists to support both models.

The players

c-*myc* encodes a transcription factor of the helix-loop-helix family of proteins. Myc protein binds to specific DNA sequences (termed E-boxes) as part of a heteromeric complex with a partner protein termed Max (for review, see ref. 11). Myc is a highly unstable protein, and its synthesis is tightly controlled by growth factors; in contrast, Max is a very stable protein and is synthesized constitutively. Max can also form homodimers, whereas Myc forms obligatory heterodimers with Max. The heteromeric Myc/Max complex is a potent activator of transcription due to transcriptional activation domains located in the amino-terminus of Myc [12]. Max homodimers bind to the same DNA elements as Myc/Max heterodimers but fail to activate transcription [13]. Thus, the contrasting biochemical properties of Myc and Max together with their different patterns of transcription provide a model as to how the proliferation-dependent expression of Myc-regulated genes may be achieved. All mutants of Myc that impair its ability to bind to DNA or to Max also abolish its oncogenic, apoptotic and mitogenic properties, demonstrating that binding to DNA and complex formation with Max are necessary, but potentially not sufficient for Myc to transform cells [14, 15].

In differentiating cells, members of the Mad family of proteins accumulate [16–18]. Mad proteins form heterodimers with Max, but not with Myc. Mad/Max complexes actively repress transcription via recuritment of a co-repressor, sin3, and in culture Mad antagonizes Myc-mediated transformation [19, 20]. Evidence begins to accumulate that *mad* genes may be tumour-suppressor genes (D. Drechsler, personal communication). Thus, Mad, Max and Myc proteins form a network of interacting proteins that control proliferation and apoptosis. Other members of this network exist (B. Eisenman, personal communication); however, their biological function has not been elucidated.

Several genes are now known to be regulated by Myc/Max heterodimers. These include (in chronological order) the genes encoding prothymosin-α [21], an acidic chromatin protein of unknown function; ornithine decarboxylase [22], the rate-limiting enzyme in polyamine biosynthesis; bendless, a member of the family of ubiquitin-conjugating enzymes, an RNA helicase (C. Grandori and B. Eisenman, personal communication); and cdc25A, a phosphatase involved in regulation of cyclin-dependent kinases (K. Galaktionov and D. Beach, personal communication; see below). Other genes have been suggested to be regulated by Myc/Max complexes; however, their status is not as clear.

Two major open questions remain with the available model. The first stems from the fact that multiple transcription factors exist that bind to the same DNA element as Myc/Max heterodimers yet have very different biological functions. These include TFE-3 and TFE-B, factors involved in B-cell differentiation; microthalmia, which is involved in melanocyte differentiation; and USF, a widely expressed factor which has strong antimitogenic properties [23]. Thus, if gene activation via E-box elements is critically important for Myc function, then mechanisms must exist which discriminate between these closely related factors. Such mechanisms are only beginning to emerge [24]; one important factor may be the distance of the binding site from the transcriptional start site, as USF, by far the most abundant of this group, seems to be short-sighted in its activation potential. Also, many target genes identified so far have binding sites downstream of the transcriptional start site, and this polarity may also be important.

The second question is whether Myc can also act as a transcriptional repressor and, if so, how it might exert this function. There is no doubt that genes exist

that are repressed in Myc-transformed cells. The list is short but exciting, as it includes cell cycle regulators like c-*myc* itself, cyclin D1 and c/EBP, and cell surface proteins, the lack of which would significantly affect tumour cell adhesion and recognition by the immune system (for review, see ref. 25). However, whether the repression of these genes reflects an indirect effect of cell transformation or reflects a direct interaction of Myc with any protein binding to the promoter of any of these genes remains to be determined [26, 27]. There are several candidates for DNA-binding proteins other than Max that might interact with Myc; however, up to now, none has been convincingly implicated in gene repression by Myc [28, 29].

Upstream of Myc

Several lines of evidence have suggested that c-*myc* is part of a regulatory pathway that is necessary for and can be sufficient to induce proliferation and, potentially, apoptosis of mammalian cells. The first hint came from observations that expression of c-*myc* is tightly linked to cellular proliferation [e.g. ref. 30]. This finding is now so commonplace that expression of c-*myc* in turn has become a marker to assess the proliferative status of mammalian cells. c-*myc* belongs to the class of immediate early genes, genes that are induced by growth factors in the absence of de novo protein synthesis [31]. This group harbours a number of other proto-oncogenes, including the c-*fos*, c-*ets* and c-*jun* genes.

What regulates c-*myc* expression in response to growth factors? Even after many years of work on the c-*myc* gene, the *cis*-acting DNA elements of the c-*myc* promoter are not clearly defined. Indeed, our uncertainty about the factors that regulate both c-*myc* expression and Myc protein function has become one of the major roadblocks to understanding c-*myc* function. Recent work, however, suggests that progress has been made in several areas: First, the tyrosine kinase c-*src* has been identified as one component that is involved in activation of c-*myc* expression in response to addition of platelet-derived growth factor (PDGF) [32]. Addition of PDGF stimulates rapid expression of both c-*myc* and c-*fos*. Expression of dominant negative alleles of *ras* blocks induction of c-*fos*, but not c-*myc*, in response to PDGF; dominant negative alleles of s-*src* block induction of c-*myc*, but not c-*fos*. Ectopic expression of c-*myc* rescues the block imposed by dominant negative c-*src*. Taken together, the data suggest the existence of a signal transduction pathway by which c-*src* controls the activity of the c-*myc* promoter. But what are the intermediate factors? These are still unknown.

Second, there is accumulating evidence that not only is c-*myc* expression regulated, but the expression of *mad* genes is also regulated by external factors, such that the Myc/Mad balance is regulated on both sides (with Max being the constant hinge). For example, *mad-1* expression is strongly and rapidly induced by agents which cause differentiation of U937 cells, and this appears to be an immediate response as well [16, 33]. Growth factor signalling cascades have also been implicated in the downregulation of *mad-4* mRNA in proliferating cells (M. McMahon, personal communication).

Third, there is strong evidence that the function of Myc protein is regulated not only by the balance of Myc and Mad proteins but also by proteins interacting with the amino-terminus of Myc and regulating its transactivation function. Evidence to support this notion comes from the finding that virtually all tumour-derived or viral alleles of *myc* harbour mutations in a limited number of amino acids in the amino-terminus of the protein, and these mutations enhance the transforming potential of Myc (e.g. ref. 34). This form of regulation is also found for the E2F family of factors, which switch from being activators to being repressors by the binding of pocket proteins like the retinoblastoma protein or p107. Indeed, the similarity may go further, as p107 has also been suggested to interact with the amino-terminus of Myc [35, 36]. This is an important finding not only in itself but also because the function of p107 is in turn regulated by cyclin-dependent kinases. Thus, Myc function may be directly regulated by the cell cycle machinery. However, it should be stated as word of caution that several observations that support the presence of E2F/p107 complexes in vivo (such as the presence of p107 in E2F bandshifts) have not been documented for Myc. Also, several other proteins have been identified that interact with the amino-terminus of Myc; one of them, an SH 3 domain-containing protein (bin-1), may provide a direct link to signal transduction pathways (G. Prendergast, personal communication).

Downstream of Myc: proliferation

Activation of Myc exerts a strong mitogenic stimulus and can be sufficient to induce proliferation in resting cells. In culture, this behaviour is documented clearly by cells which express conditional alleles of c-*myc* (fusion proteins between Myc and the hormone-binding domain of estrogen receptors [37]). Induction of these alleles by the addition of the appropriate hormone causes resting cells to enter the cell cycle synchronously and undergo both S-phase (DNA replication) and mitosis [21]. This behaviour is essentially independent of the presence or absence

of growth factors in the cell environment. It reflects similar observations seen with cells that are constitutively transformed by Myc and which stay in the cycle essentially independent of whether growth factors are present or not [38].

Myc function may be not only sufficient but also necessary for cell proliferation. This is supported by antisense experiments in culture and knockout mice (where the mutation is lethal). More recently, expression of Mad proteins has been shown to arrest cell proliferation, further supporting the notion that the entire network of Myc-related proteins is involved in this process [39].

Several models are conceivable to explain how Myc might stimulate proliferation. Myc might stimulate growth in cell size by activating transcription of genes which encode rate-limiting metabolic enzymes. One potential example for such a target gene is ornithine decarboxylase. Polyamines stimulate a wide variety of biological processes; thus, induction of ornithine decarboxylase expression by Myc might contribute to cell growth and indirectly lead to cell proliferation. Alternatively, Myc might directly stimulate genes that encode components of the 'cell cycle machinery' and thus stimulate proliferation directly rather than indirectly via a possible effect on cell growth. This latter model predicts that Myc should activate downstream targets in the cell cycle independent of cell size and overt effects on cellular proliferation. Several observations suggest that this latter model may be correct and that Myc acts as an upstream regulator of cyclin-dependent kinases, most notably cyclin E/cdk2 kinase activity. This kinase has critical roles both in cell cycle-dependent transcription and in the induction of DNA replication; its activation could, therefore, account for the mitogenic properties of Myc. Evidence to support the notion that Myc regulates this kinase directly comes from four findings:

First, activation of Myc causes a strong and rapid increase in cyclin E/cdk2 kinase activity; this increase requires the ability of Myc to bind to DNA and to Max and to transactivate transcription, suggesting that Myc acts by transcriptionally inducing a set of target genes [40]. Second, activation of cyclin E/cdk2 is required for and is in part sufficient to account for the mitogenic properties of Myc; in particular, downstream target genes in the cell cycle are activated both by Myc and by cyclin E/cdk2 via the same response pathway [41]. Third, induction of cyclin E/cdk2 kinase activity by Myc occurs independently of the growth status and the size of cells, even under conditions in which Myc has no obvious effect on cell proliferation (Pusch et al., unpublished observations). Fourth, cdc25A has been suggested to act on cyclin E/cdk2 kinase, and its activation by Myc might contribute to the regulation of cyclin E/cdk2 kinase by Myc [42] (K. Galaktionov and D. Beach, personal communication).

How does Myc regulate cyclin E/cdk2 kinase activity? In resting cells and early in the cell cycle, cyclin E is held in complexes with an inhibitory protein, p27 [40]. Activation of Myc causes p27 to be degraded, and this correlates with and is required for activation of cyclin E/cdk2 by Myc. Thus, likely candidates for target genes of Myc include proteins that are involved in the degradation of p27, such as ubiquitin-conjugating enzymes.

cdc25A has also been suggested to act on cyclin E/cdk2 kinases [42]: the gene encodes a phosphatase, which removes an inhibitory phosphate on threonine-14 and tyrosine-15 in the ATP-binding site of cdk2. In vitro, complexes between cyclin E/cdk2 and p27 cannot be activated by cdc25A, apparently because the inhibitor blocks access of cdc25A to the cdk2 protein. A priori, therefore, it seems that cdc25A should not affect cyclin E/cdk2 kinase unless p27 has been removed.

However, the situation appears to be more complex for three reasons (B. Rudolph and D. Mueller, unpublished). First, it is not clear that all cyclin E/cdk2 complexes are bound to p27 in resting cells or whether a pool of inhibitor-free complexes also exists. Second, the stability of complexes with p27 may not be infinite, and p27 may well cycle between different cyclin E/cdk2 complexes. Third, overt degradation of the bulk of p27 is preceded by a specific loss of p27 from cyclin E/cdk2 complexes, whereas p27 held in other cyclin/cdk complexes is significantly more stable. The reason for this difference appears to be that p27 is not only an inhibitor but also a substrate for cyclin E/cdk2 and is hyperphosphorylated in cyclin E/cdk2 complexes. Inhibition of this phosphorylation stabilizes cyclin E/cdk2/p27 complexes.

Thus, not only is p27 an inhibitor of cyclin E/cdk2, the kinase also appears to inhibit the function of p27 by inducing its degradation. In such a system, any factor leading to activation of cyclin E/cdk2 kinase (such as cdc25A) may indirectly lead to a loss of p27 from cyclin E/cdk2 complexes, and it will take more work to show that components involved in p27 metabolism are indeed encoded by direct target genes of Myc.

How could such a view of Myc function explain oncogene co-operation? Activated alleles of *ras* lead to a strong transcriptional induction of the cyclin D1 promoter, and to a lesser induction of cyclin E expression. However, the resulting kinases remain largely inactive because they remain associated with the p27 inhibitor [43]. In contrast, in serum-deprived cells, Myc does not induce expression of cyclin D1

and can even lead to its repression (see above) [44]. Myc does, however, strongly antagonize the association of p27 with cyclin-dependent kinases (see above; J. Flach and B. Amati, unpublished). These findings suggest a view in which the co-ordinate activation of Ras and Myc leads to uncontrolled passage through G1 and – as a result – deregulated proliferation.

Downstream of Myc: apoptosis

Not only does Myc induce proliferation in cells, it also induces cells to undergo active cell death, or apoptosis [38]. The evolutionary rationale for this is very simple: apoptosis may kill cells in an organism that has suffered a mutation at a c-*myc* gene locus and would otherwise inevitably be bound for uncontrolled proliferation. Thus, induction of apoptosis by Myc appears as a safeguard protecting long-lived organisms such as humans from single-hit tumorigenesis. Whether or not Myc is also involved in forms of apoptosis that occur during normal development (such as during the development of the immune system) seems still a matter of debate: the only study we are aware of shows that antigen-mediated apoptosis of T-cell hybridomas depends on c-*myc* function [45].

So, whereas the 'why' provides few intellectual challenges, the 'how' has been more difficult to discover. Clearly, some of the players that appear in other forms of apoptosis are also involved in c-*myc*-induced apoptosis: for example, c-*myc*-induced apoptosis is blocked by ectopic expression of *bcl-2* (e.g. ref. 46) and it involves activation of interleukin-1β-converting enzyme-like proteases (G. Evan, personal communication). In culture, induction of apoptosis is obvious in serum-deprived cells: after induction of Myc, cells rapidly die, and after a few hours to days, all cells are dead [38]. Cells can be rescued by the addition of specific cytokines, most notably insulin-like growth factor [47]. Thus, specific signalling pathways can rescue cells from *myc*-induced apoptosis. Recent data suggest that growth-factor stimulated PI3-kinase activity is critically involved in this pathway, potentially signalling via the akt family of protein kinases [48–50].

The critical question appears to be how a cell knows that Myc has been activated in an unwanted manner. In other words, what does Myc induce in a cell that needs to be rescued? Two models exist, and unfortunately, evidence exists which at face value supports either model. First, a conflict model has been suggested in which Myc induces cells to enter the cell cycle inappropriately (for review, see ref. 10). Later in the cycle, cells somehow recognize the cycle as incomplete and respond to this abortive cycle by committing suicide. This has been termed the conflict model, as it proposes that apoptosis is a conflict between 'stop' (no growth factors) and 'go' (but c-*myc* is on) signals. One finding that supports this idea is the observation that loss of p53 impairs c-*myc*-mediated apoptosis [51]; as p53 recognizes damage to DNA, it is conceivable that inappropriate replication sets the trigger for c-*myc*-induced apoptosis. However, p53- and replication-independent forms of Myc-induced apoptosis exist, and it is unclear how the cycle might be recognized as inappropriate in these settings. Two sets of experiments have been performed that explicitly test the conflict model.

First, it was found that Myc-induced apoptosis is independent of the position of a cell in the cell cycle and that Myc-induced apoptosis both in the G1 and G2 phases of the cycle. However, G2 cells are committed to proliferation and do not generally respond to external signals; thus it is hard to see which conflict might be recognized. In addition, growth factors that rescue cells from apoptosis are not generally mitogenic and vice versa, again demonstrating a high degree of independence of both pathways [47].

Second, several inhibition experiments have been performed to test whether activation of cyclin-dependent kinases by Myc contributes to *myc*-induced apoptosis. Microinjection experiments using inhibitors of cyclin-dependent kinases support the notion that *myc*-induced apoptosis can occur when c-*myc*-induced cell cycle entry is blocked [41]. However, antisense experiments with cdc25A suggest that inhibition of this phosphatase (which is thought to be involved in cdk activation, see above) inhibits *myc*-induced apoptosis (K. Galaktionov and D. Beach, personal communication). Taken together, both sets of data are somewhat puzzling. However, several possibilities exist to explain the apparent paradox. First, cdc25A might contribute to the regulation of kinases other than cyclin E/cdk2, and these might be critical for *myc*-induced apoptosis. Alternatively, cdc25A by itself could send out a signal which is independent of its effect on cdk activity.

Third, experiments differ in the conditions used (stable cell lines with antisense constructs versus microinjection) and, while this sounds like a feeble argument, similar behaviour is seen with *bcl2* which blocks apoptosis effectively upon stable expression but poorly after microinjection. Potentially, cells arrested in serum-deprived medium are 'marked' in some way for apoptosis, and this marking is prevented by inhibition of cdc25A in the previous cycle.

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