There are two major lines of evidence which support the view that the function of the myc family of proto-oncogenes impinges on critical events in cell proliferation and differentiation. The first comes from findings that the c-myc gene is frequently rearranged in a wide variety of neoplasias over a broad range of species, including humans (for reviews see Cory 1986; Eisenman 1989; McGrath 1990) Rearrangements of the c-myc gene have been observed to occur through retroviral transduction, promoter/enhancer insertion, gene amplification, and chromosomal translocation. In addition the N- and L-myc genes have been found to be amplified in neuroblastomas and small cell lung carcinomas. These genetic alterations are sometimes accompanied by point mutations within the gene, but it is noteworthy that the major AUG-initiated protein coding region is not grossly altered by deletion or truncations as found during oncogenic activation of other oncogenes. Instead the common effect wrought by these diverse genetic alterations appears to the deregulation of myc expression, frequently accompanied by over-expression. Deregulation leads to overproduction of myc gene products and also to the inability to modulate myc expression in response to normal physiological signals. Studies using transgenic mice strongly support the notion that deregulation is likely to be an early event in the progression to frank neoplasia (Adams et al. 1985).
A second line of evidence in support of the view that myc function is involved in fundamental aspects of cell behavior comes from a large number of studies on the regulation of normal myc expression during cell growth and differentiation. This work has demonstrated that myc is continuously expressed in proliferating cells but is not expressed in most quiescent or terminally differentiated cells. Importantly, when resting cells are induced to enter the cell cycle following treatment with mitogens, c-myc expression transiently increases and then falls during G1 to the continuous basal level of expression characteristic of proliferating cells (Kelly et al. 1983; Hann et al. 1985; Dean et al. 1987). A number of recent studies have demonstrated that this initial burst of c-myc expression is likely to be a critical event for cell cycle entry (Eilers et al. 1991; Roussel et al. 1991). The levels of c-myc gene products have also been found to change upon induction of terminal differentiation where down-regulation of c-myc expression occurs. Several laboratories have demonstrated that ectopic expression of exogenous c-myc leads to a block in terminal differentiation in several cell types, despite the fact that the endogenous c-myc gene is normally down-regulated (Coppola and Cole 1986; Langdon et al. 1986; Freytag 1988; Kume et al. 1988; Miner and Wold 1991). Taken together these results argue that the regulation of c-myc expression is important in both cell cycle entry and exit.

The Myc protein

Given the importance of myc gene expression in the regulation of cell proliferation, and the number of studies focused on myc, it is surprising that we know so little concerning the molecular function of the myc-encoded protein (Myc). To a certain extent this is due to the difficulty in working with Myc and the low levels at which it is present in most cell types. Nonetheless a substantial amount of information has accumulated on the nature and the biochemical properties of the Myc protein (for review see Dang 1991). It is now clear that all Myc family proteins are number-localized phosphoproteins that are characterized by an extraordinarily short half-life ($t_{1/2} = 20-40$ min).

Fig. 1. Organization of the human c-Myc protein (439 residues). Shown are the relative locations of phosphorylation sites (P); acidic (AR) and basic (BR) regions; nuclear localization signal (NL); helix-loop-helix (HLH) and zipper (LZ) domains. The phosphorylation sites in regions (i) and (iii) are targeted by casein kinase II (CKII) (Lüscher et al. 1989) while MAP kinase appears to be involved in phosphorylation at the site indicated in region (ii) (Alvarez et al. 1991).
The organization of the Myc protein is outlined in Fig. 1. Myc can be roughly divided into three regions: (i) The C-terminal 100 amino acids which comprises a basic region contiguous with a helix-loop-helix domain immediately followed by a leucine zipper motif (b-HLH-Zip). This region has substantial similarity with the DNA binding and protein-protein interaction domains of a large class of transcription factors, including MyoD, E12, and achaete-scute, which are thought to be involved in cell determination and proliferation (for review see Jones 1990). The presence of a highly conserved b-HLH-Zip region in all Myc family proteins strongly suggests that Myc should also possess specific DNA binding and dimerization activities. (ii) The N-terminal 150 amino acids has phosphorylation sites for the mitogen activated protein kinase (MAP kinase) (Alvarez et al. 1991) and for glycogen synthase kinase III (GSKIII). In addition this region has been demonstrated to possess moderate transcriptional activation activity when linked to a heterologous promoter (Kato et al. 1990). (iii) The central region contains a highly acidic segment with several CKII phosphorylation sites (Lüscher et al. 1989) and a nuclear localization signal (Dang and Lee 1988). Although deletions in region (ii) have some influence on the biological properties of Myc, deletions in regions (i) and (ii) profoundly depress Myc's ability to cotransform cells and to inhibit cell differentiation (for review see Dang 1991).

**Myc associates with Max both in vitro and in vivo**

The realization that Myc family proteins possess highly conserved bHLH regions prompted experiments to determine whether or not Myc might form dimers with itself or with other members of the b-HLH, the b-Zip, or the b-HLH-Zip group. While c-Myc could be shown to form dimers and tetramers it could only do so at concentrations considerably higher than physiological (Dang et al. 1989). Furthermore c-Myc did not appear to interact with a large number of other HLH or Zip class proteins. This raised the possibility that Myc is associating in vivo

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**Fig. 2.** Organization of p22 and p21 Max proteins. The regions of p22 (160 residues) and p21 (151 residues) are designated at in Fig. 1. Note that all phosphorylation sites indicated are potential CKII sites.
with an unknown HLH protein. To identify such a protein the b-HLH-Zip region of Myc (region i in Fig. 1) was expressed as a bacterial fusion protein, iodinated, and used to screen a λgt11 protein expression library. The two positive plaques identified out of >10^6 screened were found to encode the same novel protein which was termed Max (Blackwood and Eisenman 1991). The deduced amino acid sequence of human Max suggested that it too possessed a basic region and an HLH-Zip domain. However, it appeared to be quite unrelated to Myc in terms of both sequence and organization (Fig. 2). Also identified was a cDNA encoding an alternatively spliced form of Max with a nine amino acid insertion upstream of the basic region. In vitro translation demonstrated that these two cDNAs were translated as 21 kd and 22 kd proteins (Blackwood and Eisenman 1991). A murine homolog of Max was also identified and found to have a nearly identical amino acid sequence (Prendergast et al. 1991).

In vitro binding experiments were used to define the interactions between Myc and Max. The results demonstrated that these two proteins interact through their HLH-Zip regions and that both regions are required for association (Blackwood and Eisenman 1991; Prendergast et al. 1991). Max, in contrast to Myc, is capable of forming homodimers but will preferentially bind to Myc. In addition Max will associate with the N-, and L-Myc proteins as well as with c-Myc, but was not found to interact with a series of other HLH, Zip, or HLH-Zip proteins tested. Furthermore the affinity of Myc : Max for an oligonucleotide containing CAC-GTG, a sequence originally shown to be specifically recognized by the c-Myc bHLH-Zip region (Blackwell et al. 1990), was greater than for either Myc or Max alone. Considered together these experiments demonstrated that Myc was capable of forming a sequence-specific DNA binding complex with Max.

Once the interaction between Myc and Max was established in vitro we turned to the problem of defining the properties of Max in cells and determining whether Myc and Max associate in vivo. Using an antiserum specific for human Max, the p21 and p22 Max proteins could be identified in human, murine, and avian cells as nuclear phosphoprotein (Blackwood et al. 1992). In contrast to the highly unstable Myc family proteins (Hann and Eisenman 1984), Max was found to be an extremely stable protein with a half-life >18 hr.

To determine whether Myc and Max proteins are associated in vivo, immunoprecipitates were prepared from different cell types using either anti-Myc or anti-Max antisera under conditions designed to keep potential complexes intact. Analysis of such immunocomplexes revealed that Myc : Max heterodimers are present in the cell lysate. Such complexes probably preexist in the cell, rather than being formed subsequent to lysis, because the conditions for lysis are such that subunit exchange is unlikely to occur. An example of the strong and preferential interactions between Myc and Max as well as evidence that the two proteins are associated in the cell is shown in Fig. 3. In this experiment unlabeled anti-Myc and anti-Max immunocomplexes were prepared under different
conditions from Manca cells, fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. In Western blot such filters are usually probed with antibodies. However in the experiment shown in Fig. 3 the filters were probed with [125I]-labeled fragments of Myc and Max containing their HLH-Zip regions. The figure shows that when iodinated Myc and Max were both used as probes both Max and Myc could both be detected in each of the immunocomplexes. Note that both proteins are present only when the precipitation was carried out in buffer lacking the detergents SDS and deoxycholate (low stringency conditions; lanes LS). Antibody in the absence of any cell lysate was used as a control (lanes Ab). The immunoprecipitates were electrophoresed on SDS polyacrylamide gels and the proteins transferred to nitrocellulose filters. The filters were probed with bacterially expressed proteins fused to the carboxy-terminus of glutathione-S-transferase (GST). GST-MycC92: the 92 carboxy-terminal amino acids of Myc; GST-MaxC124: the 124 carboxy-terminal amino acids of Max.

Further studies on the biosynthesis of Max have determined that Max protein is present in quiescent cells and that its levels are unchanged after serum stimulation and entry into G1 and S phase (Blackwood et al. 1992). In addition the levels of the Myc: Max complex do not appear to change during the cell cycle of
proliferating cells. These studies suggest that Max is not an immediate early gene as is Myc, but is rather expressed in a proliferation independent manner in the cell.

**Myc may be rate-limiting step for complex formation**

The ability to detect Myc: Max complexes in cells permitted us to examine the stability and stoichiometry of the complex. Kinetic analysis produced two interesting findings. First that essentially all of the newly synthesized Myc protein was present in a complex with Max. Second that the short half-life of Myc ($t_{1/2} = 20-40$ min) was not changed in the complex with Max. Thus Myc is not stabilizing only a fraction of the Myc but rather nearly all of the Myc protein is "passing through" a complex with Max (Blackwood et al. 1992).

The experiments described above have led us to propose a model for Myc function. The studies on Max expression demonstrated that Max, unlike Myc, is not subject to regulation during cell cycle entry. Therefore we posit that in resting cells Max is primarily homodimeric. Since Max preferentially associates with Myc, and since Myc does not homodimerize, the synthesis of new Myc protein during cell cycle entry and maintenance results in a shift in a fraction of Max homodimers to Myc: Max heterodimers. The amount of Max shifted the heterodimers should be dependent on the concentration of Myc protein. Since Myc protein turns over so rapidly the concentration of heterocomplexes is likely to be highly dependent on the rate of synthesis of Myc.

Many studies have demonstrated that Myc expression in the cell is tightly controlled at both transcriptional and post-transcriptional levels (see Spencer and Groudine 1991 for review). Such tight control over Myc expression may reflect the need to maintain the relative amounts of Myc and Max homo- and heterodimers. If so then we would expect that Max homodimers and Myc: Max heterodimers would have distinct functions. Experiments using reporter gene constructs with Myc: Max binding sites proximal to the promoter suggest that Max may act as a repressor while Myc is capable of relieving Max repression (L. Kretzner et al. manuscript in preparation). Bearing in mind that as yet unidentified in vivo target genes for Myc and Max may behave differently than artificial reporter constructs, these results nonetheless are consistent with the possibility of distinct functions for the two complexes. The notion that complexes, functionally for cell proliferation are dependent on the level of Myc is intriguing in light of evidence, discussed above, that the myc gene rearrangements in a wide variety of neoplasms so frequently involve deregulation myc expression.

The identification of Max has opened up new ways of thinking about Myc function and has more clearly defined questions that will be most important in elucidating Myc's role in cell behavior. These include identification of target genes for Myc: Max and Max: Max, and a search for other proteins that may bind to the complex. Answers to these questions are likely to reveal details of the circuitry driving cell proliferation and differentiation.
References


