Abstract

The members of the Myc/Max/Mad network function as transcriptional regulators. Substantial evidence has been accumulated over the last years that support the model that Myc/Max/Mad proteins affect different aspects of cell behavior, including proliferation, differentiation, and apoptosis, by modulating distinct target genes. The unbalanced expression of these genes, e.g., in response to deregulated Myc expression, is most likely an important aspect of Myc’s ability to stimulate tumor formation. Myc and Mad proteins affect target gene expression by recruiting chromatin remodeling activities. In particular Myc interacts with a SWI/SNF-like complex that may contain ATPase activity. In addition Myc binds to TRRAP complexes that possess histone acetyl transferase activity. Mad proteins, that antagonize Myc function, recruit an mSin3 repressor complex with histone deacetylase activity. Thus the antagonism of Myc and Mad proteins is explained at the molecular level by the recruitment of opposing chromatin remodeling activities.

Keywords: Cell cycle; Chromatin; Differentiation; Oncogene; Signaling; transformation

1. Introduction

The broad and lasting interest in myc genes and Myc proteins is based on the realization that they regulate decisively various aspects of cell behavior. Foremost is the large body of information that has been accumulated over the last twenty years which demonstrates a strong involvement of myc in tumorigenesis (Marcu et al., 1992; Henriksson and Lüscher, 1996; Nesbit et al., 1999). Soon after the initial identification of v-myc in several transforming chicken retroviruses, genetic alterations of myc genes were found in many different human malignancies, e.g., translocations of the c-myc gene in Burkitt’s lymphoma and amplification of the N-myc gene in neuroblastoma. This results in general in a deregulated, enhanced expression of myc genes, a condition found in many if not most human tumors. In addition studies in animal models and in tissue culture systems have provided compelling evidence for a tumor promoting activity of Myc proteins (Morgenbesser and DePinho, 1994; Pelengaris et al., 2000). This appears to be the result of Myc’s ability to stimulate cell proliferation and at the same time to inhibit cells to enter a resting state or to terminally differentiate. Thus a consequence of the constitutive presence of Myc is an increase in the respective cellular compartment which is thought to provide the base for additional genetic alterations that cooperate with Myc in tumor formation (Berns, 1991; Adams and Cory, 1992). Recently a more direct role of Myc in transforming cells has also been suggested. This is based on findings that overexpression of Myc causes genomic destabilization potentially due to its ability to induce endoreplication (Mai et al., 1999; Taylor and Mai, 1998; Felsher and Bishop, 1999; Felsher et al., 2000). Knock-out experiments provide further strong evidence for a critical role of Myc for normal cell behavior (Stanton et al., 1992; Charron et al., 1992; Davis et al., 1993; Moreno de Alboran et al., 2001). In addition Myc proteins can stimulate apoptosis, a finding that on first view does not seem to be compatible with the other functions mentioned above (Evan et al., 1992; Thompson, 1998; Prendergast, 1999). However it is now thought that the induction of apoptosis by Myc is part of a safeguard mechanism that helps restrict the dominant action of Myc proteins on cell proliferation. In support tumor cells with elevated Myc levels are frequently defective in apoptotic pathways.
Another activity of Myc that has been demonstrated recently is the ability to induce cell growth independent of cell cycle progression in some systems (Johnston et al., 1999; Iritani and Eisenman, 1999; Schuhmacher et al., 1999). Together these findings define Myc as a highly versatile factor influencing many aspects of normal cell behavior.

For many years it was unclear how Myc proteins achieve the diverse biological effects summarized above. From various studies, including the nuclear localization of these proteins, it was inferred that Myc could play roles in nuclear architecture, replication, splicing, and/or transcription (Lüscher and Eisenman, 1990). A hypothesis supporting the latter was formulated towards the end of the 1980s mainly on the basis of sequence homologies to other transcription factors. The motif found in common is referred to as the basic region/helix-loop-helix/leucine zipper (bHLHZip) domain and functions as a DNA binding and protein-protein interaction domain (Lüscher and Larsson, 1999). A break-through observation in defining a role of Myc in transcription was the identification of the bHLHZip protein Max as a Myc dimerization partner 10 years ago (Blackwood and Eisenman, 1991; Prendergast et al., 1991; Blackwood et al., 1992). Max is the essential heterodimerization partner of Myc proteins for various biological activities, including transformation, apoptosis, and transcriptional activation (Amati et al., 1992, 1993a,b). In agreement with these findings Max is essential for normal mouse development (Shen-Li et al., 2000). However whether Max is required for all molecular functions of Myc is presently not known. Nevertheless the identification of Max and the finding that Myc/Max heterodimers bind specifically to E box DNA sequences with the consensus core 5'–CACGTG and through such elements activate the expression of reporter genes supported the hypothesis that Myc functions as a transcriptional regulator (Dang, 1999; Grandori et al., 2000). In addition a transactivation domain (TAD) was identified near the N-terminus of Myc (Facchini and Penn, 1998; Dang, 1999). Both the bHLHZip and the TAD are important for Myc function in the control of cell behavior. Together these findings provide support for a role of Myc in gene transcription. Other activities, however, cannot be excluded.

How can Myc affect so profoundly different aspects of cell behavior? To answer this question it will be important to define the genes that are targeted by Myc and to understand how the expression and function of Myc is regulated. Of particular interest is the question how Myc causes cell transformation in view of identifying targets that might be accessible to therapeutic strategies. During the early days of the search for Myc target genes it was hoped, somewhat naively from toady’s point of view, that one could identify ‘the Myc target gene for transformation’. It seems more realistic now that Myc will modulate the expression of many genes to control cell behavior. A continuously increasing number of target genes are being proposed some of which are thought to be relevant for transformation (Grandori and Eisenman, 1997; Facchini and Penn, 1998; Boyd and Farnham, 1999). In particular the use of DNA microarray technology has made it possible to probe a large number of genes and to define a set of potential target genes that reflect Myc’s broad role in the control of cell behavior (Coller et al., 2000; Guo et al., 2000; O’Hagan et al., 2000b; Schuhmacher et al., 2001). The targets include genes involved in the control of the cell cycle, protein and DNA biosynthesis, cell growth, cell adhesion, apoptosis, and immortality (Grandori et al., 2000). It will now be challenging to sort out the hierarchy of the importance of these genes for specific Myc functions, particularly for transformation.

The regulation of Myc expression and function is still far from being understood. In particular the expression of myc genes appears highly complicated. While a large number of transcription factors have been defined that can regulate the c-myc promoter, no unifying concept exists that explains myc expression during the cell cycle and during the transitions from resting to cycling and from cycling to differentiating cells (Spencer and Groudine, 1991; Marcu et al., 1997). Recent findings however provide some insight into c-myc regulation during the transition from G0 into G1 (Nasi et al., 2001). The tyrosine kinase Src has been implicated in mediating growth factor signal-induced c-myc expression (Barone and Courtneidge, 1991; Blake et al., 2000). Src appears to signal through the Rho GTPases rather than Ras or mitogen-activated protein kinases to activate the c-myc promoter (Chiariello et al., 2001). Although the transcription factor targets of Src/Rho signaling remain undefined, these findings provide a starting point to unravel growth factor-dependent c-myc expression.

Regarding the regulation of Myc proteins, recent progress has been made in understanding the signals that impinge on Myc. In particular phosphorylation has been shown to affect the stability of Myc, a protein with a short half life in the order of 20–30 min. Furthermore numerous Myc interaction partners have been identified, the functions of some support a role of Myc in the regulation of chromatin structure (Dang, 1999; Grandori et al., 2000; Amati et al., 2001). One task for the coming years will be to determine the role of these different proteins for specific aspects of Myc function and to connect these interactions to signaling pathways. In addition the essential Myc dimerization partner Max binds also to several other bHLHZip factors, forming what is referred to as the Myc/Max/Mad network of transcriptional regulators (Fig. 1). To understand the biological functions of Myc proteins requires also that the other components of this network are studied and their regulation and function determined since they impinge in one way or another on Myc function.

Numerous excellent reviews have been published in recent years that describe various aspects of the function and the biology of the Myc/Max/Mad network. Several of these reviews are cited to summarize work that can not be discussed in detail here. This review concentrates on novel
aspects regarding the molecular functions and regulations of Myc and its network partners in gene transcription and the control of cell proliferation.

2. The Myc/Max/Mad network: components and their basic functions

The identification of Max as binding partner of different Myc proteins, including c-Myc, N-Myc, and L-Myc, initiated a search for additional bHLHZip dimerization partners. This led to the description of the Mad proteins Mad1, Mxi1, Mad3, and Mad4, as interaction partners of Max and together these factors define the Myc/Max/Mad network (Fig. 1) (Henriksson and Lüscher, 1996; Grandori et al., 2000). Later two additional Max partners, Mnt and Mga, were found (Hurlin et al., 1997, 1999). Recently the identification of Mlx, a Max-like protein that can heterodimerize with Mad1, Mad4, and Mnt, but neither with the other Mad proteins nor with Myc or Max, has been reported (Billin et al., 1999; Meroni et al., 2000). The most recent extensions of the network are the cloning of MondoA as an Mlx binding partner (Billin et al., 2000) and the identification of WBSCR14 that also interacts with Mlx (Cairo et al., 2001). The gene encoding WBSCR14 lies in the commonly deleted region associated with Williams-Beuren syndrome (Cairo et al., 2001). Thus both Max and Mlx are the central components of network subdomains that are interconnected by common interaction partners (Fig. 1). It is possible that not all members of the network have been defined yet and it will be of interest to detail additional components that may further expand the network laterally. Together with the many proteins found associated with the Myc/Max/Mad network (see also Sections 3 and 4), an assemblage of factors is being defined that is rapidly growing not only in number of components but also in functional complexity.

Comparing the structure of the different network components reveals similarities but also differences (Fig. 2). As expected from the identification of these factors as dimerization partners, all components possess a bHLHZip motif as DNA binding/dimerization interphase. Max and Mlx are capable of forming homodimers but none of the other components. All these dimers function as DNA binding competent complexes that recognize E box DNA sequences and have been suggested to function at least in part as transcriptional regulators (Dang, 1999; Lüscher and Larsson, 1999; Grandori et al., 2000). The importance of the bHLHZip domains of both Myc and Mad proteins is supported by mutational analysis, demonstrating an essential role of this domain for all functions tested. Interestingly as far as analyzed, only Myc proteins, MondoA, and WBSCR14 carry a TAD (Fig. 2) and Myc proteins and MondoA can transactivate reporter genes through E box elements (Dang, 1999; Billin et al., 2000; Cairo et al., 2001).

In addition to gene activation, Myc can also function as a repressor (Claassen and Hann, 1999; Grandori et al., 2000; Amati et al., 2001). This activity of Myc has remained, however, largely unexplained at the molecular level until recently. One mechanism of Myc-dependent repression functions through blocking of the positive acting, initiator (INR) binding factor Miz-1 by Myc (Section 4) (Staller et al., 2001; Seoane et al., 2001). From many different studies
it appears that both repression and activation of transcription are essential activities of Myc proteins (for a recent discussion see Amati et al., 2001).

In contrast to Myc, Mad proteins and Mnt possess an mSin3 interaction domain (SID) which mediates transcriptional repression (Kiermaier and Eilers, 1997; Schreiber-Agus and DePinho, 1998; Knoepfler and Eisenman, 1999). Besides bHLHZip, the SID is critical for the function of Mad proteins. This is also true for Mnt, however this protein has most likely additional domains relevant for function. The former is suggested by the findings that Mad proteins with an impaired SID show little biological activity. In support of the latter is that MntD with impaired SID gains transforming activity in cooperation with an activated Ras oncoprotein, whereas Mnt and Mad proteins with functional SIDs interfere with transformation (Grandori et al., 2000). The findings summarized above, which are based mainly on overexpression studies, are supported by the analysis of mad knock-out and mad transgenic mice (Schreiber-Agus et al., 1998; Henriksson and Lüscher, 1996; Grandori et al., 2000).

3. The Myc–Mad antagonism: opposing regulation of chromatin structure and gene transcription

All the studies that have been reported today provide evidence for a functional antagonism of Myc and Mad proteins (Fig. 3). This has been proposed first from findings showing that Mad proteins are expressed preferentially in non-proliferating cells as opposed to Myc proteins which are present almost exclusively in proliferating cells. Furthermore Mad proteins inhibit reporter genes that are activated by Myc, interfere with transformation of rat embryo fibroblasts, block cell growth, and prevent apoptosis (Henriksson and Lüscher, 1996; Grandori et al., 2000). The findings summarized above, which are based mainly on overexpression studies, are supported by the analysis of mad knock-out and mad transgenic mice (Schreiber-Agus et al., 1998; Henriksson and Lüscher, 1996; Grandori et al., 2000).
Foley et al., 1998; Queva et al., 1999, 2001). However, in particular the knock-out studies have not provided the strong phenotypes originally expected, most likely due to overlapping and redundant functions of Mad proteins in different cell types. Indeed in all cells analyzed more than one Mad protein is expressed (Grandori et al., 2000).

Transcriptional regulators are thought to affect the transcription of specific genes at several different levels (Goodrich et al., 1996; Struhl, 1996). Early findings suggested that activators are important for the efficient recruitment and activation of the polymerase complex. In addition activators may regulate the processivity of the polymerase complex. More recently it has become evident that these activities are most likely downstream of effects on the chromatin structure of gene loci and more specifically of promoter regions. The structure of the chromatin appears to be a major determinant whether promoters are accessible to the polymerase complex and thus whether a gene can be transcribed or not.

Fig. 3. Model for the control of promoter activity by Myc/Max/Mad network members. Distinct dimeric complexes, i.e. Myc/Max, Max/Max, and Mad/Max, are thought to recruit different cofactors to promoters through specific interaction with E box DNA elements. Myc binds to a component of the SWI/SNF complex that contains an ATPase activity involved in nucleosome remodeling. In addition Myc interact with TRRAP complexes that possess HAT activity important for modulating the acetylation status of core histones and possibly other components associated with promoters, including transcription factors and subunits of the PolII complex. Max has not been reported to bind transcriptional cofactors. Thus Max/Max homodimers will most likely not influence directly promoter activity. Nevertheless Max/Max dimers are potentially able to compete for DNA binding with other E box binding factors. Mad proteins recruit an mSin3-dependent repressor complex that is associated with HDAC activity. This complex will deacetylate core histones and possibly other acetylated proteins. The results of binding of Myc/Max, Max/Max, or Mad/Max dimers to a promoter are summarized and a possible order of events is indicated. Myc/Max binding and recruitment of SWI/SNF may lead to alterations of a promoter’s chromatin structure by modulating nucleosomes. This may make binding sites available for other transcriptional regulators that may or may not cooperate with Myc in promoter modulation. The recruitment of a cofactor complex with HAT activity will further enhance promoter accessibility and in addition also provide acetylation-dependent binding sites on histone tails. It is not clear whether SWI/SNF and TRRAP complexes work on the same promoters and in which order. Myc interacts with components of the PolII complex and may therefore be involved in PolII recruitment. The binding of Mad/Max and recruitment of the mSin3 repressor complex will lead to histone deacetylation and increased chromatin compaction. This is thought to result in decreased accessibility of transcription factors to their binding sites and of the PolII complex to the core promoter region. Besides HDAC other components of the mSin3 complex contribute to repression. This suggests that other functions, e.g. an activity that antagonizes SWI/SNF function, may be associated with this repressor. E box: refers to a DNA element that is recognized by Myc/Max/Mad network members; RE: response element for transcriptional regulators; Ac: acetylation of histones; TF: transcription factor; HAT: histone acetyl transferase; HDAC: histone deacetylase;
Evidence is accumulating that the antagonism of Myc and Mad proteins at the molecular level works at least in part by affecting chromatin structure (Fig. 3). The first evidence that Myc/Max/Mad network proteins may indeed function in chromatin remodeling came from the findings that Mad proteins recruit through the SID a repressor complex that contains histone deacetylase (HDAC) activity (Hassig et al., 1997; Alland et al., 1997; Sommer et al., 1997; Lathery et al., 1997). Several lines of evidence support the notion that the recruitment of the repressor complex is essential for the biological effects of Mad1 described above. Deletion of the SID impairs the activity of Mad proteins in transactivation, transactivation, cell growth inhibition, and apoptosis (Grandori et al., 2000; Baudino and Cleveland, 2001). The bHLHZip is also important for these functions, it is likely that these are, at least in part, the result of specific target gene regulation. Although the widely expressed Mnt also possesses a SID domain that binds mSin3 in vitro and its deletion affects Mnt function, it remains to be determined whether in vivo the SID of Mnt serves to recruit an HDAC-containing repressor complex. It is possible that the interaction of Mnt with the mSin3 complex is regulated in an as yet unknown manner. Since Mnt is, in contrast to Mad proteins, a stable protein expressed ubiquitously such a regulation might be desirable to modulate its function.

The findings that Mad proteins recruit a repressor complex with HDAC activity led to the suggestion that Myc proteins might recruit histone acetyl transferase (HAT) activity, thus providing a molecular explanation for the antagonism between Myc and Mad proteins. Indeed recent findings demonstrated that Myc interacts with a large protein, TRRAP, that appears to function as coactivator (McMahon et al., 1998; Saleh et al., 1998). Like mSin3 for Mad proteins, TRRAP can function as a platform for additional proteins involved in gene transcription. TRRAP is part of at least two large complexes that possess HAT activity, one containing GCN5/PCAF the other Tip60/NuA4 (Vassilev et al., 1998; Grant et al., 1998; Ikura et al., 2000). Indeed Myc recruits GCN5 and HAT activity, most likely through TRRAP (McMahon et al., 2000; Park et al., 2001). A potential involvement of Tip60 is also indicated by the identification of Tip48 and Tip49, both are part of the Tip60 complex, as Myc interaction partners (Wood et al., 2000). TRRAP interacts with the TAD of Myc, more precisely it requires a conserved element in the TAD, the so called Myc box II (MBII) (McMahon et al., 1998). MBII is a small element that is essential for all biological activities of Myc proteins. Although a role of MBII in gene transcription has been questioned (Xiao et al., 1998), the binding of this Myc domain to HAT complexes suggests that MBII is involved in the regulation of gene transcription. Indeed recent studies using chromatin immunoprecipitation (ChIP) assays, in which transcription factors and their cofactors are crosslinked in vivo to their DNA binding sites and subsequently the associated DNA is analyzed by gene-specific PCR, have demonstrated that Myc recruits TRRAP activity in a MBII-dependent manner to target genes. This results in histone acetylation of responsive promoters and subsequently in gene expression (Bouchard et al., 2001; Frank et al., 2001). The apparent conflict in regard of a role of MBII in transcription reflects most likely the difference between the analysis of transiently transfected reporter genes and endogenous, chromosome-embedded genes (Amati et al., 2001). The latter are dependent for expression on chromatin remodeling and histone modification activities, while reporter genes have more relaxed requirements. Together these findings define an important role for MBII in the control of gene transcription by Myc and thus support the concept that regulation of gene transcription is an essential aspect of Myc biology.

Early on after the identification of Mad1 it was suggested that Myc/Max and Mad/Max complexes form a molecular switch involved in the regulation of transitions between different cell states, in which Myc/Max complexes are exchanged by antagonizing Mad/Max complexes, and vice versa, on responsive E box DNA elements (Ayer et al., 1993; Ayer and Eisenman, 1993). In support of this model Myc/Max and Mad/Max complexes are expressed mainly in growing and in resting and differentiated cells, respectively. More recently the exchange of Myc/Max to Mad/Max complexes could be observed on two responsive promoters. Using ChIP assays it has been demonstrated that Myc/Max complexes bound to the promoters of hTERT and human cyclin D2 genes in exponentially growing HL-60 myeloblast cells are replaced by Mad/Max complexes during differentiation (Xu et al., 2001; Bouchard et al., 2001). Furthermore this switch from Myc/Max to Mad/Max complexes on cyclin D2 is associated with recruitment of HDAC1, a decrease in histone acetylation, and a loss of polymerase II binding to the cyclin D2 promoter (Bouchard et al., 2001). Thus the findings discussed above provide evidence for and define one consequence of the proposed...
switch, i.e. the alternate recruitment of HAT and HDAC activity to target genes. This suggest that the distinct regulation of histone acetylation in response to Myc/Max/Mad network members represents one level of the functional antagonism of Myc and Mad proteins.

While at present there is little evidence for additional Mad-interacting proteins that are involved in the regulation of gene transcription, several other factors implicated in the regulation of transcription have been identified that bind to Myc. The C-terminal domain of Myc interacts with INII1, hSNF5, a component of the multiprotein SWI/SNF complex, that is involved in chromatin remodeling in an ATP-dependent manner (Cheng et al., 1999; Kingston and Narlikar, 1999; Peterson and Workman, 2000). Interestingly INII1 has been suggested to function as a tumor suppressor and the two SWI2/SNF2 homologues, BRG1 and hBRM, can induce cell cycle arrest (Versteeg et al., 1998; Muchardt and Yaniv, 1999). Thus it appears, somewhat puzzling, that the proto- Oncoprotein c-Myc interacts with a complex that has growth inhibitory activity. Although it has been reported that recruitment of the SWI/SNF complex is necessary for Myc transactivation function in reporter gene assays, it is not yet clear what the role of this complex is for the regulation of chromosome embedded target genes. It will now be of interest to determine the relative contribution of the different complexes, including TRRAP and SWI/SNF complexes, for Myc-specific gene regulation.

4. Myc and gene repression through initiator elements

While evidence has steadily accumulated over the last years that Myc can function as transcriptional activator, the suggestion that Myc can also function as a transcriptional repressor has been ill-defined at the molecular level until recently (Facchini and Penn, 1998; Claassen and Hann, 1999; Grandori et al., 2000; Amati et al., 2001). A main problem has been the difficulty in identifying response elements that mediate Myc repression and in defining the mode of Myc function on such elements. Recent findings however have shed light on Myc-mediated repression. Early on it has been suggested that the initiator element (INR), which is part of some core promoters, responds to Myc (Li et al., 1994; Philipp et al., 1994; Mai and Martensson, 1995; Lee et al., 1996). At least three DNA binding proteins that can bind to INR sequences, TFII-I, YY1, and Miz-1, are also interacting with Myc, leading to the proposal that these factors mediate Myc repression (Peukert et al., 1997; Hop et al., 1993; Harihu et al., 1991; Seto et al., 1991; Shrivastava et al., 1993). While a role for TFII-I and YY1 in Myc-dependent repression has not been firmly established, recent evidence suggests that Miz-1 mediates Myc repression. Miz-1 binds to and activates transcription from INR elements and inhibits cell cycle progression, both functions are antagonized by Myc (Peukert et al., 1997; Staller et al., 2001; Seoane et al., 2001). Since the cell cycle arrest by Miz-1 depends on the retinoblastoma tumor suppressor, the expression of the Ink4 cyclin-dependent kinase inhibitors was analyzed. Miz-1 binds to the Ink4b INR and activates the expression of this gene. Importantly Myc is also associated with the INR thus providing the first direct evidence for the physical interaction of Myc with a negatively regulated promoter. Several genes are repressed by Myc through core promoter and INR elements, including c-myc, p21CIP1, C/EBPα, p27, and H-ferritin (Antonson et al., 1995; Facchini et al., 1997; Wu et al., 1999; Claassen and Hann, 2000; Yang et al., 2001) suggesting an involvement of Miz-1. Other genes, including gadd45 and pdgf-β receptor, are repressed in an INR-independent manner (Marlin et al., 1997; Oster et al., 2000; Izumi et al., 2001). Repression of the pdgf-β receptor is mediated by NF-Y, a factor that is inhibited by Myc (Izumi et al., 2001).

How does Myc inhibit the activation of the Ink4b promoter by Miz-1? Miz-1 recruits the coactivator p300, an interaction that is competed for by Myc (Staller et al., 2001). Since the C-terminal HLHZip region of Myc is sufficient for this inhibition, one would expect that Myc brings a functional TAD into Miz-1/Myc complex. However the Myc TAD appears not to function under these conditions, either coactivators cannot be recruited efficiently or the Ink4a promoter is not responsive. Another possibility is that additional domains in Myc are relevant for repression, including MBII and the region spanning amino acids 96–106 (region R in Fig. 2) as suggested previously (Philipp et al., 1994; Li et al., 1994; Lee et al., 1997). As indicated above it will be important to define whether other Myc-repressed promoters are regulated by a Miz-1 dependent mechanism or whether Myc can regulate INR elements through additional means.

5. Myc and cell cycle control

The products of a substantial number of the proposed Myc target genes are involved in the control of cell proliferation, i.e. in the regulation of cell-matrix interaction, of protein and DNA synthesis and of the transition from G1 into S phase of the cell cycle (Coller et al., 2000; Guo et al., 2000; O’Hagan et al., 2000b; Schuhmacher et al., 2001). These genes and their products provide a mechanistic base to evaluate Myc’s role in stimulating cell proliferation, particularly the G1-S transition and the inactivation of the G1-S checkpoint (or restriction point) (Fig. 4). Overcoming the latter is essential for cell proliferation. This requires the inactivation of pocket proteins, in particular the retinoblastoma tumor suppressor protein (RB), and the activation of the cyclin E/cyclin-dependent kinase (CDK) 2 complex (Adams, 2001; Sherr and Roberts, 1999; Ekholm and Reed, 2000). Early on it was shown that activation of Myc results in the activation of cyclin/CDK complexes (Jansen-Dürr et al., 1993; Steiner et al., 1995; Perez-Roger et al., 1997; Müller et al., 1997), leading to the suggestion that one important activity of Myc in regulating cell proliferation is...
stimulating the G1–S transition. This transition is also a key target during oncogenesis (Bartek et al., 1997; Reed, 1997). Most if not all tumor cells have mutations that result in altered control of the G1–S checkpoint and thus facilitate the entry into the DNA replication mode. Once cells have progressed into S phase, the remainder of the cell cycle proceeds independent of the presence of growth factors. Thus under limiting amounts of such factors, advancing beyond the G1–S checkpoint is critical for proliferation. Myc is sufficient to overcome the restriction point and to induce S phase in the absence of growth factors (Eilers et al., 1991). Therefore it is attractive to suggest that the activation of cyclin/CDK complexes is critical for Myc’s ability to induce S phase.

With the identification of Myc as a regulator of the expression and activity of cyclin/CDKs, one focus of subsequent research was the analysis of the mechanism of this regulation. While some components of cyclin/CDK complexes are direct targets of the Myc/Max/Mad network, including cyclin D2 and possibly cyclin D1 and CDK4 (Bouchard et al., 1999; Bouchard et al., 2001; Perez-Roger et al., 1999; Hermeking et al., 2000; Coller et al., 2000), indirect effects of Myc also contribute to the activation of cyclin/CDK. Together the identification of these target genes will now allow to define in more detail how Myc proteins affect the activity of different cyclin D/CDK and cyclin E/CDK2 complexes (Fig. 4). Both these cyclin/CDK complexes are required to enter S phase (Sherr and Roberts, 1999; Ekholm and Reed, 2000). Cyclin D/CDK complexes are built from several different subunits including cyclin D1, D2, and D3 and the two catalytic subunits CDK4 and CDK6. The activation of cyclin D1 and D2 by Myc is of particular importance since their expression not only can positively influence cyclin D/CDK complexes but these two cyclins have also been shown to sequester p27KIP1, an inhibitor of cyclin E/CDK2 (Bouchard et al., 1999; Bouchard et al., 2001; Perez-Roger et al., 1999; Coller et al., 2000; Dey et al., 2000). Furthermore the Ink4b gene, encoding the cyclin-dependent kinase inhibitor (CDK) p15INK4b, is repressed by Myc as summarized above (Staller et al., 2001; Seoane et al., 2001). The activation of different Ink4 genes has been recognized as an important mechanism to negatively regulate cell proliferation by many different agents (Vidal and Koff, 2000). In particular the Ink4b gene is activated in response to TGFβ, a function that is antagonized by Myc (Massague et al., 2000; Amati, 2001). p15INK4b is an inhibitor of cyclin D/CDK4/6 function. Together these findings demonstrate that Myc can stimulate, through the regulation of Ink4b, cyclin D2, and CDK4, cyclin D/CDK function.

The targets of cyclin D/CDK4/6 are pocket proteins. In particular the inactivation of the RB protein, a frequently mutated and by this inactivated tumor suppressor, is critical for the regulation of the G1-S checkpoint (Bartek et al., 1997; Reed, 1997). Besides directly impinging on the activity of cyclin D/CDK complexes, Myc has also been shown to stimulate the expression of Id2, which encodes an HLH protein that inhibits RB function (Lasorella et al., 2000). This provides an additional mechanism to release E2F transcription factor activity, which is negatively regulated by RB, and thus to promote S phase progression (Harbour and Dean, 2000). The findings that Myc can interfere at multiple levels with the function of RB proteins and the frequent mutations in RB and in the upstream regulatory components in human tumors suggest that this is an important function of Myc in tumorigenesis. Both gene activation and repression are important for this activity of Myc.

The second cyclin/CDK activity that is required for entering S phase is cyclin E/CDK2. Although neither of the two genes appear to be direct targets of Myc, multiple ways have
evolved how Myc can influence cyclin E/CDK2 activity. The cdc25A gene has been proposed to be a Myc target (Galaktionov et al., 1996). Activation of Cdc25A, a dual specificity phosphatase, stimulates cyclin E/CDK2 activity (Blomberg and Hoffmann, 1999; Sexl et al., 1999). In addition several pathways target the cyclin E/CDK2 inhibitor p27KIP1. This CKI is an important regulator of cell proliferation that is induced when growth factor levels are insufficient to sustain proliferation. Consistently p27KIP1 is highly expressed in resting cells and downregulated upon serum induction (Sherr and Roberts, 1999). Importantly p27KIP1 is also repressed in response to Myc activation. First p27KIP1 is sequestered by Myc-induced cyclin D2 and subsequently degraded in response to cyclin E/CDK2-dependent phosphorylation by a ubiquitin/proteasome dependent pathway (Bouchard et al., 1999; Bouchard et al., 2001; Perez-Roger et al., 1999; Pagano et al., 1995). Also the latter process is activated by Myc (Steiner et al., 1995). A recently identified target gene of Myc is cull1 that is involved in p27KIP1 degradation (O’Hagan et al., 2000a). Last but not least Myc can also directly repress the expression of the p27KIP1 gene (Yang et al., 2001). These findings are consistent with the observation that immortal myc−/− cells have increased p27 levels (Moreno de Alboran et al., 2001). A third CKI gene that is targeted by Myc is p21CIP1 (Claassen and Hann, 2000). This molecule inhibits cyclin E/CDK2 and is upregulated in response to the activation of the tumor suppressor p53 and in differentiating as well as aging cells as part of an antiproliferative program (Sherr and Roberts, 1999).

Together these studies have defined the cyclin D and cyclin E kinase complexes, that are essential regulators of the decision process whether or not a cell proceeds through the G1-S checkpoint and into a new round of cell division, as targets of Myc. The identification of multiple Myc regulated genes whose products impinge on these kinases provide strong support for the notion that these two cyclin/CDK complexes are critical targets for Myc’s function in the regulation of cell proliferation.

6. Myc and signal transduction

The conclusions that in normal cells the expression of Myc is highly regulated and that deregulated expression of Myc is important for tumor progression are well supported by experimental evidence. The regulation of Myc at the posttranslational level has become more transparent over the last several years. In particular regulation by phosphorylation and protein stability have obtained considerable interest, also in view of their relevance for tumorogenesis. Nevertheless some of the findings that have been reported are still controversial and will require more detailed analysis.

c-Myc is a highly phosphorylated protein with three clusters of phosphorylation sites. Two of these clusters, one in the central acidic domain and one near the basic region, are phosphorylated by protein kinase CK2 (Lüscher et al., 1989). Little is known about the regulation and function of these phosphorylation sites. The third cluster is located in the transactivation domain, more specifically at and near MBI (Seth et al., 1992, 1993; Pulverer et al., 1994; Henriksson et al., 1993; Lutterbach and Hann, 1994). Of particular importance are two sites, Thr-58 and Ser-62, because this region is a mutational hot spot (Bhatia et al., 1993, 1994; Yano et al., 1993; Albert et al., 1994; Axelson et al., 1995). These mutations that are prevalent in Burkitt’s lymphomas, a tumor characterized by a translocated c-myc gene, affect predominantly phosphorylation of Thr-58. This site is phosphorylated in vitro by glycogen synthase kinase (GSK) 3 dependent on phosphorylation of the adjacent Ser-62 ( Henriksson et al., 1993; Sears et al., 2000). This latter site is targeted by MAP kinases and by cyclin/CDKs (Seth et al., 1992; Hoang et al., 1995; Pulverer et al., 1994; Lutterbach and Hann, 1994; Henriksson et al., 1993; J. Vervoorts and B. Lüscher, unpublished findings). For some time the functional relevance of these two phosphorylation sites and the observed mutations in this region remained unresolved. Early on it was suggested that phosphorylation at Ser-62 stimulated the transcriptional potential of Myc (Seth et al., 1992, 1993). However this finding is controversial ( Henriksson et al., 1993; Lutterbach and Hann, 1994). In in vitro transformation assays small differences between wt c-Myc and Thr-58 and Ser-62 mutants were detected (Pulverer et al., 1994; Henriksson et al., 1993). Thus these findings did not allow to make strong conclusions regarding the function of the N-terminal phosphorylation sites.

However more recently, evidence has been provided indicating that these phosphorylation sites are regulating Myc stability. Myc is degraded by ubiquitin-mediated proteolysis (Salghetti et al., 1999; Bahram et al., 2000; Gregory and Hann, 2000), a process that involves covalent attachment of ubiquitin to target proteins and subsequent proteolysis by the proteasome (Varshavsky, 1997). In Myc the TAD determines the short half life, in particular MBI and II have been implicated in degradation (Flinn et al., 1998; Salghetti et al., 1999; Gregory and Hann, 2000; Chen et al., 2000). The mutations at and around Thr-58 and Ser-62 found in Burkitt’s lymphoma have been shown to result in increased stability of Myc (Salghetti et al., 1999; Bahram et al., 2000; Gregory and Hann, 2000). Consistent with this finding the analysis of signaling that emanates from Ras and targets Myc has been shown to result in increased Myc stability ( Sears et al., 1999, 2000). This appears to be the consequence of Ser-62 phosphorylation and at the same time inhibition of Thr-58 phosphorylation by inhibiting a PI3K/GSK3-dependent mechanism (Sears et al., 2000). This increase in Myc stability may be relevant during the early phases of the cell cycle in which Ras-dependent signaling contributes to entry of cells into G1 from a resting state. Since these findings have been obtained from Myc overexpression studies, it will now be important to demonstrate
an increase in stability of endogenous Myc in response to growth factors. Ser-62 phosphorylation is not only triggered by Ras signaling but also by cyclin/CDK complexes and possibly other kinases suggesting that changes in Myc turnover may also occur in other phases of the cell cycle. However such effects may be difficult to document since at any given point in time only a subtraction of Myc may respond. Indeed the half life of the total Myc pool changes little through the cell cycle with the exception of mitosis (Lüscher and Eisenman, 1987; Gregory and Hann, 2000). It has also been suggested recently that transactivation and degradation may be coupled (Thomas and Tyers, 2000; Tansey, 2001). Thus this may provide yet another signal for Myc turnover, potentially through the same phosphorylation sites. The emerging picture indicates that Myc protein turnover is a highly dynamic process we are just beginning to understand.

While more evidence regarding the regulation of Myc is becoming available (see above), there is still almost nothing known about the regulation of Mad proteins. Nevertheless the short half life of Mad and the fact that these proteins are phosphorylated in vivo offers possibilities for regulation that will have to be explored in the future. In addition Max has been considered as the inert interaction partner for Myc and Mad proteins. Max is ubiquitously expressed and constitutively phosphorylated by CK2 and little information regarding its regulation has been obtained (Henriksen and Lüscher, 1996; Grandori et al., 2000). However recent evidence indicates that during apoptosis several pathways target Max resulting in its dephosphorylation and cleavage by caspases (Krippner-Heidenreich et al., 2001). These findings suggest that the central component of the Myc/Max/Mad network is regulated under specific circumstances. Further studies are required to define whether that affects the function of other components of the network, in particular Myc and Mad regarding their involvement in apoptosis.

7. Outlook

Recent years have brought to light novel aspects of Myc function and regulation. In particular a number of new interaction partners have been identified that suggest an important role of Myc in the control of chromatin remodeling. Both SWI/SNF and TRRAP complexes can be recruited by Myc, however, the order of events and many of the molecular details remain to be determined. As one example it is unclear which HAT activity is recruited to mediate the acetylation of core histones of Myc responsive promoters. A more fundamental question that has not been addressed yet is whether Myc has the ability to open silent promoters, i.e. whether Myc can function as the initial chromatin remodeling activity that is required to make promoters permissive for transcription factors. Or is Myc a factor that can only function when promoters are preactivated? From the decisive role Myc plays in the control of cell proliferation one would suspect the former possibility.

The expression of the c-myc gene and the regulation of the protein are highly regulated. Recent progress implicates the Ras pathway in the control of the half life of Myc. Also mutations in the TAD of c-Myc are associated with increased protein stability. These findings suggest that the short half life is one way to interfere with Myc function by limiting its presence. The regulation of protein stability appears to be connected to signaling pathways that are controlled by Ras. Since the majority of studies have been performed by using overexpression systems, it will be critical in the future to establish whether similar events occur in cells that are not manipulated. This will be important since overexpression of both, Myc and Ras, can have adverse effects on cell behavior. Thus indirect effects will have to be excluded.

Our knowledge regarding other Myc/Max/Mad network members is still patchy. The prediction that Mad proteins would function as tumor suppressors did not come true, although the overexpression studies have indicated that these proteins are potent inhibitors of cell proliferation. However the analysis of knock out mice did not support these original findings. This may be due to overlapping expression patterns and functions of different Mad proteins. Thus combinations of knock outs will hopeful provide more insight into the function of these proteins.

We are witnessing an ever increasing list of Myc target genes, mainly due to the use of DNA microarray screens. It will be a challenge to understand which of these target genes are critical for tumor formation and thus may provide targets for therapy. In this respect it will also be interesting to see whether Myc that is overexpressed, as in many human tumors, regulates additional genes as compared to normal physiological levels of Myc, or whether the difference is merely quantitative. Defining these differences will require the input and work, preferentially concerted, of many laboratories.

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References


proteins are phosphorylated by casein kinase II. EMBO J. 8, 1111–1119.


