Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 516–519, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00060-7

The mycN/max Protein Complex in Neuroblastoma. Short Review

A. Wenzel and M. Schwab

The oncogenic activation by amplification of the *MYCN* gene is frequently observed in human neuroblastomas and occasionally in other tumours with neuronal qualities. As a consequence of amplification, elevated levels of the mycN protein are expressed. mycN contains a C-terminal basic region (BR) that can bind to DNA, and a helix-loop-helix (HLH)–leucine zipper (Zip) domain, which is responsible for the physical interaction with another HLH–Zip protein, max. This principle structure is conserved among all members of the *MYC* gene family. The resulting dimers can bind to the DNA sequence CACGTG. The mycN protein, but not max, contains, near the N-terminus, a region conferring the ability to activate the transcription of genes. mycN/max heterodimers probably activate and max/max homodimers repress transcription of, as yet, unidentified target genes. In neuroblastoma cells, where mycN is deregulated, the balanced interaction of BR–HLH–Zip proteins is probably perturbed, and, therefore, genes controlled by mycN might be abnormally expressed and thereby alter normal cell growth with the consequence of tumorigenesis.

Key words: max, amplification, helix-loop-helix–leucine zipper, basic region heterodimers, transcription activation, CACGTG, myc-boxes
Eur J Cancer, Vol. 31A, No. 4, pp. 516–519, 1995

INTRODUCTION

MYCN IS A proto-oncogene which is expressed in cells of primarily neuronal lineages during embryogenesis and, when deregulated, can participate in tumorigenesis. The oncogenic activation of the *MYCN* gene has been found to take various molecular routes. The most frequently observed alteration is amplification originally found in human neuroblastomas [1], and subsequently also uncovered in other types of neuroectodermal tumours, such as retinoblastomas, astrocytomas, small cell lung cancers and gliomas [2–5]. *MYCN* activation has also been observed to occur through integration of a Moloney murine leukaemia provirus in lymphomas of the rat [6], of papilloma virus in human genital tumours [7] and by hepadnavirus insertion in woodchuck liver tumours [8]. Regardless of the mechanism, activation of *MYCN* results in enhanced expression of both mRNA and protein.

FEATURES OF THE MYCN PROTEIN

mycN, like other proteins of the *MYC* gene family, is a short-lived nuclear protein [9] that is phosphorylated by casein kinase II (CKII) [10], and binds DNA [9]. Human *MYCN* encodes two polypeptides, 456 and 464 amino acids long, resulting from two translation start sites [11]. The differently phosphorylated mycN proteins migrate in SDS-polyacrylamide gels with apparent molecular weights of 58 and 64 kDa. Inspection of the amino acid sequence, deduced from the nucleotide sequence [12]; reveals two highly conserved regions that are of significance for mycN function. The carboxy-terminus (Figure 1) contains a nuclear localisation signal (N) [13]; a basic region (BR) responsible for DNA binding [14]; helix-loop-helix (HLH) [15] and a leucine zipper domain (Zip) [16]. The HLH–Zip domain is a critical determinant for protein–protein interactions [15]. Phosphorylation at a CKII site [10], adjacent to the basic region of mycN, could modulate the α-helical structure. This principle structure of the C-terminus is shared among myc proteins and is similar to that of various transcription factors [17–20]. The central part of mycN contains clusters of acidic amino acids as well as several serine and threonine residues and is phosphorylated by CKII [10].

Another region of functional significance is located at the N-terminus and has been referred to as "myc-boxes" [21]. The myc-boxes represent two domains, 19 and 12 amino acids long that share close to 90% sequence conservation among myc proteins including the retroviral v-myc and the human mycN-mycI and myc [21]. The myc-boxes most likely have activity related to transcription. Transfection studies allowed the identification of an internal region within the amino terminal portion of the mycN protein capable of transactivating reporter gene transcription [22]. A major proportion of the transactivating activity maps to the highly conserved myc-box 1. Interestingly, this is also part of the region to which binding of the protein Rb1, encoded by retinoblastoma suppressor gene was observed in an *in vitro* assay [22, 23]. However, the significance of Rb1 binding is unclear, as transactivation of reporter gene transcription is maintained in the absence of Rb1 [22]. It also remains to be shown whether Rb1 protein associates with myc proteins *in vivo*. The myc-box1 of mycN protein, like that of myc, contains a putative MAP kinase (mitogen-activated protein

Correspondence to M. Schwab.
Both authors are at the Department of Cytogenetics, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.

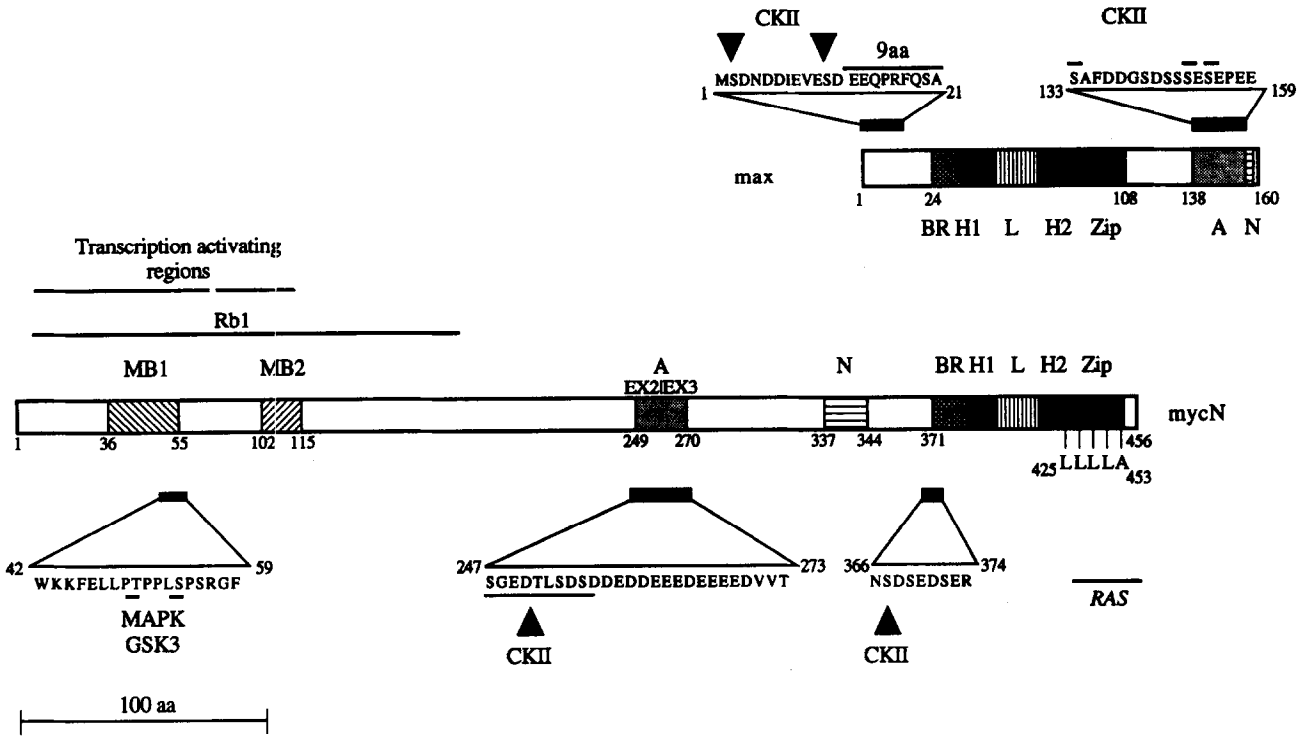

Figure 1. Functional domains of the mycN and max proteins. MBI, MB2, "myc-boxes"; A, acidic region; EX2, EX3, boundary of exon 2 and 3; N, nuclear localisation signal; BR, basic region responsible for DNA binding; H1-L-H2, helix1-loop-helix2; Zip, leucine zipper, CKII, casein kinase II; MAPK, mitogen-activated protein kinase; GSK3; glycogen synthase kinase 3. Shown is the 160 amino acid form of max ($p22^{max}$ with the amino acid (9aa) insert at the N-terminus. Numbers refer to positions of amino acids according to the sequences of mycN [12] and max [25]. Small characters highlight the amino acids of segments of the proteins. Arrowheads point to amino acids which are phosphorylated by CKII. Amino acids denoted with short lines are putative targets of the kinases shown in the figure. Regions which are either responsible for *RAS*-cooperation, Rb1 protein association or for transcription activation are indicated. A solid line marks a major site in the acidic region (A) of mycN phosphorylated by CKII.

kinase) and GSK3 (glycogen synthase kinase 3) site [24]. Phosphorylation in this region could influence mycN function by modulating transcription activation or interaction with other proteins.

FEATURES OF MAX PROTEINS

Max, like myc, is a BR–HLH–Zip protein (Figure 1), originally identified by screening a human cDNA expression library with a hybrid protein containing the C-terminal dimerisation region of the myc protein [25]. p21-/22^{max} proteins consist of 151 or 160 amino acids, respectively. The 160 amino acid form contains a 9 amino acid insert at the amino terminus, probably generated by alternative splicing [25, 26]. The 9 amino acid insert contains a proline introduced in a predominantly α-helical region which might change the α-helical structure in such a manner that DNA binding and dimerisation of the adjacent BR–HLH–Zip region might be modulated. DNA binding studies showed that heterodimeric complexes containing $p21^{max}$ were kinetically less stable than complexes with $p22^{max}$ [27]. In max proteins, the BR–HLH–Zip region is located at the N-terminus. In contrast to myc proteins, max lacks a transcriptional activation domain [28] and is relatively stable, with a half-life of more than 12 h [29]. The p21- and $p22^{max}$ are the major variants that are expressed in a wide variety of species and cell types [29]. Δmax proteins have molecular weights of approximately 16 and 17 kDa, and appear to represent a minority of the total cellular max protein [26]. They are presumably the result of alternative splicing, possess a BR–HLH–Zip region, but lack the carboxy terminus, including the nuclear localisation signal, and therefore remain in the cytoplasm [25, 26, 30]. Expression of Δmax enhances the rate of transformation in *MYC–RAS* co-transformation assay [30].

Max has also been shown to be a phosphoprotein [25, 31]. The N-terminus contains two CKII sites that are phosphorylated by CKII *in vitro* and *in vivo* [29, 32]. CKII phosphorylation of these particular two sites alters DNA binding of both max homo- and max/myc heterodimers, apparently as the result of an increase in both the on- and off rates of the phosphorylated proteins [27]. Max mutants, deficient in the N-terminal phosphorylation, inhibit both the basal and myc induced transcription of a reporter gene, more efficiently than the wild type protein, indicating an important role of N-terminal phosphorylation in transcription activation [32]. In line with this, the ability of *MYC–RAS* induced transformation was also suppressed more efficiently by the mutant [32]. These effects can be explained by a possible modulation of the structure important for dimerisation or DNA binding. The C-terminal region of max contains several charged amino acids and a nuclear localisation signal, as well as several putative CKII sites [25, 28, 29]. The significance of these CKII sites for max function is still unknown.

p21-/22^{MAX} DIMERISATION WITH MYCN IN HUMAN NEUROBLASTOMA

By employing immunoprecipitation with monoclonal antibodies against N-terminal epitopes of mycN, two nuclear phosphoproteins with relative molecular weights of 21 and 22 kDa which associate with the mycN protein *in vivo*, were detected in extracts of the neuroblastoma line, Kelly [31]. The p21/22 proteins from neuroblastoma cells turned out to be identical to max [31]. Using a series of carboxy terminally mutated proteins,

it was shown, by transfection assays, that max dimerises with mycN *via* the HLH–Zip region. Helix 2 and the leucine zipper can probably form a large α-helical domain which is responsible for the coiled-coil interaction of mycN with max. Expression of max proteins is not restricted to cells with high levels of mycN, but was also observed in neuroblastoma cells without detectable mycN expression. The p21/22 proteins are probably the predominantly expressed forms of max in neuroblastoma cells, no other variants, including △max could be detected. An open question is whether heterodimeric complexes of mycN with $p21^{max}$ or with $p22^{max}$ have different functions.

DIMERISATION AND SEQUENCE-SPECIFIC DNA BINDING ACTIVITY OF THE MYCN/MAX COMPLEX

Specific DNA binding was shown for the first time with a fragment of the myc protein containing the BR–HLH–Zip region. Using polymerase chain reaction (PCR)-based binding site selection technique (SAAB), the hexamer CACGTG was identified as the core binding region [33]. This technique was also used to study the DNA binding characteristics of both the mycN and mycN/max complexes [34]. In electrophoretic mobility shift assays (EMSA), a GST–mycN fusion protein, containing the terminal 100 amino acids CACGTG, was identified as the specific sequence, but binding to CATGTG was also observed for mycN homodimers. When mycN and max proteins were analysed simultaneously in EMSA, heteromeric complexes bound preferentially to DNA. Mutational analysis of the mycN basic region (BR), the helix-loop-helix (HLH) and the leucine zipper (Zip) regions revealed that all three regions are necessary for DNA binding by mycN/max complexes. Dimerisation requires both HLH and LZ motifs and contact with DNA is mediated by the BR [31, 34]. Binding of mycN/max complexes to CACGTG was also observed when extracts from neuroblastoma cells were used [35]. Although CACGTG is the best binding site for mycN and max *in vitro*, it is possible that flanking sequences may contribute to site-specific recognition of a physiologically relevant target *in vivo*. No striking difference in the DNA binding activities of mycN/max and myc/max complexes was observed. CKII phosphorylation negatively influences the DNA binding of max homodimers [27, 36]. The effect of CKII phosphorylation on DNA binding by mycN/max heterodimers has not yet been adequately assessed.

REGULATION OF THE MYCN/MAX COMPLEX

Currently, it is believed that mycN exerts its biological activity only in a heterodimer with max. In this complex, mycN contributes to activation of transcription when mycN/max, the heterodimer, is attached to a DNA sequence containing CACGTG. Mutational analysis revealed that the C-terminal dimerisation region of mycN is essential for the *in vitro* transforming activity [37], and at least also for binding with max [31]. These results clearly underline the significance of an intact C-terminal for the biological function of the mycN/max complex.

In continuously growing neuroblastoma cells, where *MYCN* is overexpressed as the result of amplification, the presence of mycN forces max into abnormal amounts of mycN/max heterodimeric complexes, which could perturb the fine-tuned expression of, as yet, unidentified genes (Figure 2). Max/max homodimeric complexes would repress transcription due to the lack of a transcription activation domain. This model must also consider the fact that other BR–HLH–Zip proteins like mad [38] or mxi [39], which bind max, but not myc or mycN, probably also contribute to transcriptional control in neuroblastoma. The essence of the model is that in the normal neuroblast both homodimeric and heterodimeric populations of BR–HLH–Zip proteins exist at a level that is strictly regulated. It is obvious that, if the level of one member of this system is altered as the result of genetic change, regulation is lost with the consequence of neoplastic cell growth. As compared to myc, there are fewer data on the function of mycN, but basic features, e.g. biochemical properties, dimerisation, and DNA binding appear to be similar between the two proteins. The preferential expression of mycN *versus* the almost ubiquitous expression of myc appears to indicate different functions, nevertheless. This functional difference could result from association of other proteins with mycN that could target and direct this complex to specific promoters. The abnormal expression of genes controlled by mycN could, therefore, perturb normal cellular growth of neuroectodermal cells with the consequence of progression of neuroblastoma.

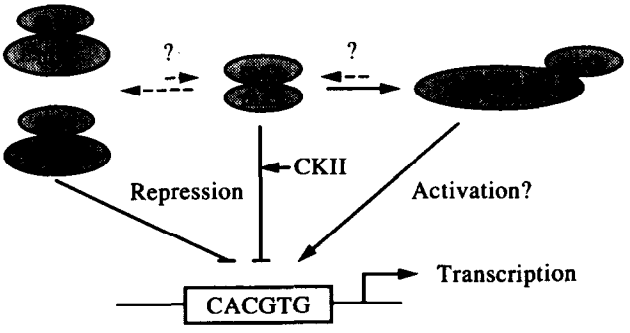

Figure 2. A speculative model (modified according to [37]) for interaction and regulation of BR–HLH–Zip proteins in neuroblastoma. It is thought that max homodimers, mad/max or mxi/max heterodimers function as repressors, and mycN/max heterodimers as activators of targets containing CACGTG sequences in their promoter elements. In neuroblastoma cells with overexpression of mycN the formation of mycN/max heterodimers is probably favoured. The existence of mxi/max and mad/max complexes in neuroblastoma has not been demonstrated. mad/max heterodimers formed during the differentation of cells of myeloid lineages are thought to be more effective repressors than max homodimers because of their higher DNA binding activity [40]. DNA binding activity and repressor function of max homodimers are probably regulated by phosphorylation by CKII.

CONCLUSIONS AND FUTURE ASPECTS

The information presented here strongly suggests that mycN is a transcription factor whose activity might be regulated, at least in part, through interaction with other proteins. Final proof for such a function has yet to come with the identification of a natural promoter linked to a target that is modulated through binding of the mycN/max complex. Any model of mycN function in tumorigenesis, however, must accommodate the enhanced level of mycN proteins as the consequence of amplification or viral insertion. Normal activity of mycN probably requires a fine tuned interplay with at least more than two proteins. Enhanced levels of mycN could result in disturbance of this balanced interaction. mycN and max work together in binding DNA, and probably enhanced levels of mycN lead to a greater number of transcription activating complexes. Altogether, the molecular mechanisms by which deregulated expression of mycN contribute to tumorigenesis are still unclear. Currently, the view prevails that mycN participates in the formation of a nucleo-protein complex that has a function related to transcription. The question of whether this is the function that mycN has under physiological conditions can only be

answered positively when a eukaryotic promoter has been identified that is a *bona fide* target of mycN. Approaches related to this question should have priority towards an understanding of mycN function.

1. Schwab M, Alitalo K, Klempnauer KH, *et al.* Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. *Nature* 1983, **305**, 245–248.
2. Schwab M. Amplification of N-*myc* in human neuroblastomas. *TIG* 1985, **1**; 271–275.
3. Schwab M, Varmus HE, Bishop JM. Human N-*myc* gene contributes to neoplastic transformation of mammalian cells in culture. *Nature* 1985, **316**, 160–162.
4. Schwab M. Molecular cytogenetics of human neuroblastoma. *Biochim biophys Acta* 1992, **1114**, 43–50.
5. Schwab M, Amler L. Amplification of cellular oncogenes: a predictor of clinical outcome in human cancer. *Genes Chrom Cancer* 1990, **1**, 181–193.
6. Van Lohuizen M, Breuer M, Berns A. N-*myc* is frequently activated by proviral insertion in MuLV-induced T cell lymphomas. *EMBO J* 1989, **8**, 133–136.
7. Couturier J, Sastre-Garau X, Schneider-Maunoury S, Labib A, Orth G. Integration of papillomavirus DNA near *myc* genes in genital carcinomas and its consequence for protooncogene expression. *J Virol* 1991, **65**, 4534–4538.
8. Fourel G, Trepo C, Bougueleret L, *et al.* Frequent activation of N-*myc* genes by hepadnavirus insertion in woodchuck liver tumors. *Nature* 1990, **347**, 294–298.
9. Ramsay G, Stanton L, Schwab M, Bishop JM. Human proto-oncogene N-*myc* encodes nuclear proteins that bind DNA. *Mol Cell Biol* 1986, **6**, 4450–4457.
10 Hamann U, Wenzel A, Frank R, Schwab M. The MYCN protein of human neuroblastoma cells is phosphorylated by casein kinase II in the central region and at serine 367. *Oncogene* 1991, **6**, 1745–1751.
11. Mäkelä TP, Saksela K, Alitalo K. Two N-*myc* polypeptides with distinct amino termini encoded by the second and third exons of the gene. *Mol Cell Biol* 1981, **9**, 1545–1552.
12. Stanton LW, Schwab M, Bishop JM. Nucleotide sequence of the human N-*myc* gene. *Proc Natl Acad Sci USA* 1986, **83**, 1772–1776.
13. Dang CV, Lee WMF. Nuclear and nucleolar targeting sequences of c-*erb*, c-*myc*, N-*myc*, p53, HSP70 and HIV *tat* proteins. *J biol Chem* 1989, **30**, 1809–1823.
14. Kouzarides T, Ziff E. The role of the leucine zipper in the fos–jun interaction. *Nature* 1988, **336**, 646–651.
15. Murre C, Schonleber McCaw P, Baltimore D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 1989, **6**, 777–783.
16. Landschulz WH, Johnson PF, McKnight S. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 1988, **240**, 1759–1764.
17. Lüscher B, Eisenman RN. New light on Myc and Myb Part I. Myc. *Genes Devel* 1990, **4**, 2025–2035.
18. Beckmann H, Su LK, Kadesch T. TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer μE3 motif. *Genes Devel* 1990, **4**, 167–179.
19. Carr CS, Sharp PA. A helix-loop-helix protein related to the immunoglobulin E-box binding proteins. *Mol Cell Biol* 1990, **10**, 4384–4388.
20. Hu YF, Lüscher B, Admon A, Mermod N, Tjian R. Transcription factor AP-4 contains multiple dimerization domains that regulate dimer specificity. *Genes Devel* 1990, **4**, 1741–1752.
21. Schwab M. The *myc*-box oncogenes. In Reddy EP, Skalka AM, Curran T, eds. *The Oncogene Handbook*. Amsterdam, Elsevier, 1988.
22. Cziepluch C, Wenzel A, Schürmann J, Schwab M. Activation of gene transcription by the amino terminus of the N-myc protein does not require association with the protein encoded by the retinoblastoma suppressor gene *RB1*. *Oncogene* 1993, **8**, 2833–2838.
23. Rustgi AK, Dyson N, Bernards R. Amino-terminal domains of c-*myc* and N-*myc* proteins mediate binding to the retinoblastoma gene product. *Nature* 1991, **352**, 541–544.
24. Henriksson M, Bakardjev A, Klein G, Lüscher B. Phosphorylation sites mapping in the N-terminal domain of c-Myc modulate its transforming potential. *Oncogene* 1993, **8**, 3199–3209.
25. Blackwood EM, Eisenmann RN. max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with myc. *Science* 1991, **251**, 1211–1217.
26. Västrik I, Koskinen PJ, Alitalo K, Mäkelä TP. Alternative mRNA forms and open reading frames of the *max* gene. *Oncogene* 1993, **8**, 503–507.
27. Bousset K, Henriksson M, Lüscher-Firzlaff JM, Litchfield DW, Lüscher B. Identification of casein kinase II phosphorylation sites in Max: effects on DNA-binding kinetics of Max homo- and Myc/Max heterodimers. *Oncogene* 1993, **8**, 3211–3220.
28. Kato GJ, Lee WMF, Chen L, Dang CV. Max: functional domains and interaction with c-Myc. *Genes Devel* 1992, **6**, 81–92.
29. Blackwood EM, Lüscher B, Eisenman RN. Myc and Max associate *in vivo*. *Genes Devel* 1992, **6**, 71–80.
30. Mäkelä TP, Koskinen P, Västrik I, Alitalo K. Alternative forms of Max as enhancers or suppressors of Myc-Ras cotransformation. *Science* 1992, **256**, 373–376.
31. Wenzel A, Cziepluch C, Hamann U, Schürmann J, Schwab M. The mycN oncoprotein is associated *in vivo* with the phosphoprotein Max (p21/22) in human neuroblastoma cells. *EMBO J* 1991, **10**, 3703–3712.
32. Koskinen PJ, Västrik J, Mäkelä TP, Eisenman RN, Alitalo K. Max activity is affected by phosphorylation at two NH_2-. terminal sites. *Cell Growth Different* 1994, **5**, 313–320.
33. Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN, Weintraub H. Sequence-specific DNA-binding of c-Myc protein. *Science* 1990, **250**, 1149–1151.
34. Ma A, Moroy T, Collum R, Weintraub H, Alt FW, Blackwell TK. DNA binding by N-and L-myc proteins. *Oncogene* 1993, **8**, 1093–1098.
35. Raschella G, Romeo A, Negroni A, *et al.* Lack of correlation between N-*myc* and max expression in neuroblastoma tumours and in cell lines: implication for N-*myc*-max complex formation. *Cancer Res* 1994, **54**, 2251–2255.
36. Berberich SJ, Cole MD. Casein kinase II inhibits the DNA-binding activity of Max homodimers but not Myc/Max heterodimers. *Genes Devel* 1992, **6**, 166–176.
37. Nakajima H, Ikeda M, Tsucida N, Nishimura S, Taya Y. Inactivation of the N-*myc* gene product by single amino acid substitution of leucine residues located in the leucine-zipper region. *Oncogene* 1989, **4**, 991–1002.
38. Ayer DE, Kretzner L, Eisenman RN. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. *Cell* 1993, **72**, 1–20.
39. Zervos A, Gyuris J, Brent R. Mxi, a protein that specifically interacts with Max to bind Myc–Max recognition sites. *Cell* 1993, **72**, 21–40.
40. Ayer DE, Eisenman RN. A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Devel* 1993, **7**, 2110–2119.

Acknowledgements—Work in our laboratory was supported by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 229, the NCRD (Israel)-DKFZ Cooperation Program, and the Deutsche Krebshilfe.