The cellular proto-oncogene c-myc is involved in cell proliferation and transformation but is also implicated in the induction of programmed cell death (apoptosis). The c-Myc protein is a transcriptional activator with a carboxy-terminal basic region/helix-loop-helix (HLH)/leucine zipper (LZ) domain. It forms heterodimers with the HLH/LZ protein Max and transactivates gene expression after binding DNA E-box elements. We have studied the phenotype of dominant-negative mutants of c-Myc and Max in microinjection experiments. Max mutants with a deleted or mutated basic region inhibited DNA synthesis in serum-stimulated 3T3-L1 mouse fibroblasts. In contrast, mutants of c-Myc expressing only the basic region/HLH/LZ or HLH/LZ domains rapidly induced apoptosis at low and high serum levels. Co-expression of the HLH/LZ domains of c-Myc and Max failed to do so. We suggest that the c-Myc HLH/LZ domain induces apoptosis by specific interaction with cellular factors different to Max.

Programmed cell death is an intrinsic death program operating to eliminate unwanted cells during normal development. It also has been suggested to kill cells after acquisition of growth-factor-independent growth properties due to genetic alterations (Ellis et al., 1991; Evan and Littlewood, 1993). Common morphological features of programmed cell death are blebbing of the cytoplasmic membrane, chromatin condensation, and breaking of the dead cell into apoptotic bodies (Wyllie, 1980, 1987). This kind of cell death, often termed apoptosis, does not elicit an inflammatory response in the tissue and can therefore clearly be distinguished from cell necrosis in which cells die as the result of acute injury (Kerr et al., 1972).

The apoptotic program appears to be installed in all animal cells and can operate in the presence of inhibitors of RNA and protein synthesis (Ellis et al., 1991). In some cellular systems it is even induced by these inhibitors, indicating that short-lived proteins or RNAs may negatively control the apoptotic machinery. Jacobson et al. (1994) recently proposed a model in which the process leading to apoptosis is divided into three phases: (i) an activation phase in which control systems of apoptosis are activated or derepressed (this phase can be sensitive to inhibitors of RNA and protein synthesis), (ii) an effector phase in which the activated control system acts on multiple targets in the cell, and (iii) a degradation phase in which the dying cell is broken down. In the latter two phases, inhibitors of RNA and protein synthesis are not effective to block the apoptotic program.

The cellular proto-oncogene c-myc has been implicated in the control of proliferation and apoptosis. Expression of c-myc is tightly linked to mitogenic stimuli and is a prerequisite for cell growth (for reviews, see Lüscher and Eisenman (1990) and Marcu et al., 1992). Moreover, post-translational activation of a c-Myc estrogen receptor chimera in resting cells is sufficient to induce entry into the cell cycle (Eilers et al., 1989, 1991). Expression of an exogenous c-myc gene renders hematopoietic cells and fibroblasts unable to exit from the cell cycle upon withdrawal of growth factors or serum. Instead, these cells continue cycling and concomitantly undergo apoptosis (Askew et al., 1991; Evan et al., 1992; Hermeking and Eick, 1994). Expression of c-myc is also required for activation-induced apoptosis of T-cell hybridomas (Shi et al., 1992). These observations suggest a model in which proliferation and cell death are processes that are co-induced by c-Myc and which are subsequently modulated by cytokine action (Evan and Littlewood, 1993; Harrington et al., 1994).

The c-Myc protein has features of a transcription factor with a transcriptional activation domain in the amino-terminal region (Kato et al., 1990). The carboxy-terminal region contains a basic region (BR), helix-loop-helix (HLH), and leucine zipper (LZ) domain (Murre et al., 1990). The carboxy-terminal region is contiguous array essential for specific DNA binding (BR) and dimerization (HLH/LZ) of c-Myc with the BR/HLH/LZ protein Max (Blackwood and Eisenman, 1991; Blackwood et al., 1992; Kato et al., 1992). c-Myc/Max heterodimers and Max homodimers bind specifically to the E-box motif CACGTG (Blackwell et al., 1990). Homodimers of c-Myc are not found in vivo. Since Max lacks a transcriptional activation domain, c-Myc/Max heterodimers have been suggested to act as transcriptional activators and Max homodimers as repressors (Kretzner et al., 1992; Amati et al., 1992). The biological functions of c-Myc reported so far including cell transformation (Stone et al., 1987, Amati et al., 1993a), transcriptional activation (Kretzner et al., 1992; Amati et al., 1993b), and induction of proliferation and apoptosis in quiescent cells (Evan et al., 1992; Amati et al., 1993b) require dimerization of c-Myc with Max and sequence-specific binding of the heterodimer to DNA. Hopewell and Ziff (1995) recently reported proliferation of the nerve growth factor responsive PC12 cell line in the absence of
c-Myc HLH/LZ Domain Induces Apoptosis

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RESULTS

Construction of Dominant-Negative Mutants of c-Myc and Max—We have used the approach of dominant-negative mutants to interfere with the function of c-Myc in vivo. Two types of mutants were constructed to inhibit the formation of functional c-Myc/Max heterodimers in vivo (Fig. 1). Dominant-negative mutants of Max lacking the BR (MaxBR2) or containing point mutations in the BR (MaxBRmut) can still dimerize with c-Myc (Reddy et al., 1992; Billaud et al., 1993). Overexpression of the Max mutants under the control of the CMV enhancer/promoter should disrupt c-Myc/Max and other c-Myc or Max-containing complexes and shift the equilibrium from DNA binding complexes to non-DNA binding complexes. As a consequence, the c-Myc-Max-specific E-box motifs remain unoccupied.

A similar mode of action is expected for a dominant-negative c-Myc mutant lacking the transcriptional activation domain and BR (ctMycBR2). Overexpression of ctMycBR2 should sequester Max protein, which is then no longer available for dimerization with wild-type c-Myc. A second c-Myc mutant, ctMyc, differs from the previous mutant by having an intact BR. Heterodimers of ctMyc/Max have lost their transactivation potential but should be able to bind specifically to DNA.

To ensure nuclear transport of all mutant c-Myc proteins, the NLS M1 of c-Myc (Dang and Lee, 1988), which maps outside of the BR/HLH/LZ region, was cloned 3′ to the BR/HLH/LZ region, was cloned.
c-Myc HLH/LZ Domain Induces Apoptosis

MaxCKII*  
MaxBRmut  
MaxBR*  
ctMyc  
ctMycBR*  
MycHLH  
MycLZ

Fig. 1. Schematic survey of c-Myc and Max mutants used in this work. Wild-type c-Myc and Max proteins are shown (stippled box). Dimerization domains are indicated by arrows. The mutant MaxCKII has replaced Ser-11 by Ala in a known CKII phosphorylation site. MaxBRmut has replaced amino acids Glu-32 and Lys-34 by Ile and Glu in the DNA-binding domain, respectively. MaxBR* has deleted the DNA-binding domain (positions 1–35). The mutant c-Myc has deleted the amino-terminal region up to the basic domain at position 354, and ctMycBR* has additionally deleted 13 amino acids with the basic region. MycHLH and MycLZ consist of either the HLH domain (amino acids 368–409) or the LZ domain (amino acids 410–439) of c-Myc. NLS indicates the nuclear localization signal M1 of c-Myc, the cross-hatched boxes indicate the viral epitopes recognized by the 12CA5 or C3 antibody. For details of construction of all mutants see "Materials and Methods."

Properties of the mutant proteins were examined in gelshift experiments. Two functions were tested: (i) formation of ctMycMax dimers and specific binding to an oligonucleotide that contains the E-box recognition motif and (ii) the ability of MaxBR*, MaxBRmut, ctMycBR*, MycHLH, and MycLZ mutants to compete for the formation of ctMycMax complexes. Unprogrammed reticulocyte lysate extract (translation reaction without addition of exogenous RNA) already revealed a binding activity (Fig. 2A, lane 2, marked with an asterisk). This shift was competed by oligonucleotides CM1 containing the CACGTG motif (Blackwell et al., 1990) and CM1mut with an inversion of the middle CG of the E-box motif (Fig. 2A, lanes 4 and 5), indicating that this activity is not specific for the CACGTG-binding motif. Additionally, this complex could not be disrupted or supershifted by the addition of antibodies directed against Max (Fig. 2B, lane 2) and c-Myc (data not shown) and therefore contains no Max-Max homodimers or c-MycMax heterodimers. The amount of this endogenous shift activity varied considerably depending on the batch of the reticulocyte lysate.

When ctMyc was added to the binding reaction, an additional shift appeared that most likely is produced by a heterodimer of c-Myc and endogenous Max, which is already present in the extract (Fig. 2A, lane 3). This shift can be competed with the oligonucleotide CM1 but not with CM1mut (Fig. 2A, lanes 4 and 5). We observed that the Max protein alone did not produce a specific shift (Fig. 2A, lane 6). This was not unexpected since phosphorylation of Max at an amino-terminal casein kinase II (CKII) site in the reticulocyte lysate inhibits DNA-binding of Max-Max homodimers but not c-MycMax heterodimers (Berberich and Cole, 1992). We could confirm this observation by using the mutant MaxCKII*, which has replaced Ser-11 by Ala (Fig. 2A, lanes 7–9). Mixing of ctMyc and Max generated a strong shift that can be explained by synergistic action of both proteins (Fig. 2A, lanes 10–12).

The specificity of the observed shifts was also analyzed by using antibodies. The α-Max antibodies reduced and/or supershifted ctMycMax complexes (Fig. 2B, lanes 1–4). Antibodies specific for the c-Myc LZ domain or the viral tag of Max only inhibited formation of ctMycMax specific shifts (lanes 6 and 7).

We next tested whether the presence of the mutant Max and c-Myc proteins in the binding reaction can compete for the formation of the ctMycMax shift. Addition of a 2-fold excess of MaxBR*, MaxBRmut, or ctMycBR* protein to the binding reaction resulted in a clear reduction of the ctMycMax-specific shift (Fig. 2C, lanes 2, 3, and 6), whereas addition of unprogrammed reticulocyte lysate (data not shown) or MycHLH and MycLZ had no effect (Fig. 2C, lanes 4 and 5). In summary, ctMycBR*, MaxBR*, and MaxBRmut were able to disrupt a ctMycMax complex in vitro and therefore act in a dominant-negative manner as expected.

Dominant-Negative Mutants of Max Block DNA Synthesis in Serum-stimulated Mouse 3T3-L1 Fibroblasts—To test whether dominant-negative mutants of c-Myc and Max could inhibit c-Myc function in vivo, we used a combined microinjection/proliferation assay (Fig. 3a). Mouse 3T3-L1 fibroblasts were serum-starved for 48 h and microinjected with expression plasmids (Graessmann and Graessmann, 1983) coding for MaxBR* and MaxBRmut. Subsequently, cells were stimulated with 10% fetal calf serum, and BrdUrd was added to visualize DNA synthesis. The cells were fixed 20 h after serum stimulation and stained with antibodies directed against the viral epitope and BrdUrd. Stimulation of quiescent fibroblasts with serum consistently induced DNA synthesis in about 70% of the un.injected cells. This induction was almost completely blocked in cells expressing MaxBR* (Fig. 3b). A similar but less strong block was observed in cells expressing MaxBRmut.

Expression of MaxBR* and MaxBRmut differed in regard to their cellular distribution. MaxBRmut was almost exclusively demonstrable in the nucleus, whereas MaxBR* was also observed in the cytoplasm in a considerable portion of cells. As a control, an expression plasmid coding for β-galactosidase was injected. Expression of β-galactosidase had no significant effect on induction of DNA synthesis (Fig. 3b). A quantification of the proliferation assay is shown in Fig. 3c.

Dominant-Negative Mutants of c-myc Induce Apoptosis in Mouse 3T3-L1 Fibroblasts—Examination of the dominant-negative c-Myc mutants in the proliferation assay turned out to be impossible. 6 h after injection of the expression plasmids, many of the cells expressing ctMyc or ctMycBR* showed morphological changes with characteristic features of apoptosis. These changes included cytoplasmic blebbing, chromatin condensation, and nuclear fragmentation (Fig. 4). Nuclei of uninjected nonapoptotic cells exhibit a regular blue chromatin staining. Chromatin staining of nuclei of apoptotic cells is irregular with bright areas (Fig. 4, arrow). 20 h after microinjection, the microinjected fields on the coverslip were almost free of cells, and the few remaining cells staining positive for the c-Myc
**Fig. 2.** Inhibition of sequence-specific DNA binding of ctMyc-Max heterodimers by dominant-negative mutants. Binding reactions contained in vitro translated proteins as indicated, the radiolabeled oligonucleotide CM1 (Blackwell et al., 1990) with the consensus c-Myc-binding site, and binding mix. A, Lanes 2–9, 1.5 μl of the indicated lysate was incubated at 37 °C for 10 min; lanes 10–12, 0.75 μl of ctMyc lysate was mixed with 0.75 μl of Max lysate and incubated at 37 °C for 10 min. ctMyc and Max generate a specific gel shift (arrow). Labeled CM1 oligonucleotide alone (lane 1) and unprogrammed reticulocyte lysate (UL) (lane 2) served as controls. The endogenous unspecific complex in the unprogrammed lysate is marked with an asterisk. Competition experiments were performed by addition of a 200-fold molar excess of unlabeled oligonucleotide CM1 (200xCM1) or CM1mut (200xCM1mut). B, lanes 1, 3, and 5, 1.5 μl of ctMyc lysate (lane 1) or 0.75 μl of ctMyc lysate mixed with 0.75 μl of Max lysate

|        | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|--------|---|---|---|---|---|---|---|---|---|----|----|----|
| ctMyc  | + | + | + | + | + | + | + |   |    |    |    |    |
| Max    | - | - | - | + | + | + | + | + | + | + | + | + |
| MaxCKII-| - | - | - | - | + | + | + |   |    |    |    |    |
| UL     | - | - | - | - | - | - | - | - | - | - | - | - |
| 200xCM1| - | - | - | + | + | + | + |   |    |    |    |    |
| 200xCM1mut | - | - | - | - | - | - | - | + | + | + | + | + |

|        | 1 | 2 | 3 | 4 | 5 | 6 |
|--------|---|---|---|---|---|---|
| ctMyc  | + | + | + | + | + | + |
| Max    | + | + | + | + | + | + |
| MaxBR- | - | - | - | - | - | - |
| MaxBRmut | - | - | + | + | - | - |
| MycLZ  | - | - | - | + | - | - |
| MycHLH | - | - | - | - | + | - |
| ctMycBR- | - | - | - | - | - | + |
mutants were detached and stuck loosely at the surface.

We performed kinetic experiments to determine the onset of apoptosis in cells expressing ctMyc and ctMycBR. For this purpose, the injected cells were inspected and photographed in intervals of 2 h after microinjection (Fig. 5). Expression of mutant c-Myc proteins could be detected in the nucleus and in the cytoplasm 2 h after microinjection (data not shown). The rate of expressing cells was >70% of injected cells and thus in the range as observed for other proteins (MaxBR, MaxBRmut, β-galactosidase). At this time, cells expressing ctMyc and ctMycBR showed a normal shape. However, after 4 h, the cells changed their morphology, and bright spots became apparent in the field of injected cells. The number of affected cells consistently increased between 6 to 10 h, while at the same time many of the affected cells disintegrated. After 12 h, cells with a normal shape expressing ctMyc or ctMycBR were no longer detectable. Apoptosis was not observed in cells expressing β-galactosidase, MaxBRmut (see Fig. 3), and MaxBR (Fig. 5).

Dominant-negative mutants of c-Myc have been successfully used in earlier studies to inhibit transformation (Dang et al., 1989; Sawyers et al., 1992) and induction of DNA-synthesis (Hermeking et al., 1994). These c-Myc mutants carried deletions in the transactivation domain between amino acids 40 and 178 and therefore differ markedly from the mutants employed in this study, which have deleted the amino terminus up to amino acids 354 and 367. Notably, the mutant with the deletion in the transactivation domain did not induce apoptosis in quiescent 3T3-L1 fibroblasts in microinjection experiments (Hermeking et al., 1994; data not shown).

Induction of apoptosis by wild-type c-Myc in serum-starved mouse fibroblasts can be inhibited by refeeding the cells with medium containing high serum levels (Evan et al., 1992). Therefore, similar kinetic experiments as carried out with serum-starved 3T3-L1 fibroblasts were performed with proliferating 3T3-L1 cells in the presence of 10% FCS. Since proliferating fibroblasts showed a high mobility on the surface of the coverslip with a permanent change of positions, a similar documentation of the results as shown for quiescent fibroblasts in Fig. 5 was impossible. Despite this problem, plasmids coding for ctMycBR and ctMyc were microinjected in proliferating cells. Both mutants showed a normal expression rate of 70% after 2 h. Similar to quiescent cells, many of the ctMycBR and ctMyc expressing cells showed an apoptotic morphology after 6–8 h. 12 h after microinjection, cells with a normal morphology expressing the c-Myc mutants were no longer detectable (data not shown).

Max Mutants Inhibit Apoptosis Induced by ctMyc and ctMycBR. — The mutant ctMycBR induces apoptosis without binding to DNA. However, this mutant can still dimerize with Max. Therefore, we tested whether co-expression of MaxBR could modulate ctMycBR-induced apoptosis (Fig. 6). Plasmids encoding ctMycBR or ctMyc were mixed with a plasmid coding for MaxBR and injected in quiescent 3T3-L1 cells. 12 and 24 h after microinjection, the cells showed a normal morphology, and the rate of ctMycBR-expressing cells was in the range of 50–60% of injected cells. Thus, co-expression of MaxBR suppressed ctMycBR-induced apoptosis. Apoptosis was also suppressed by MaxBRmut, but less efficiently (Table I).

Separate Expression of MycHLH and MycLZ Does Not Induce Apoptosis. — We next studied whether expression of either the HLH (MycHLH) or the LZ domain (MycLZ) of c-Myc is sufficient to induce apoptosis. Therefore, expression plasmids were constructed encoding either the HLH or LZ domain of c-Myc (Fig. 1). Both mutants separately or in combination could not compete for the formation of the ctMycMax complex in gel-shift experiments (Fig. 2c, lanes 4 and 5; data not shown). Despite high expression, each mutant failed to induce apoptosis in quiescent mouse 3T3-L1 fibroblasts after 12 h (Fig. 7). Cells expressing MycHLH showed an increased rate of vacuolization after 12 h, which might be an indicator of lack of cell health. However, the rate of apoptotic cells was not increased when these cells were examined after 24 h (data not shown). Interestingly, co-expression of MycHLH and MycLZ restored induction of apoptosis with similar kinetics as observed for ctMycBR (Table I). A survey of the apoptotic activity of all mutants used in this work is shown in Table I.

**DISCUSSION**

Expression of the c-myc gene in serum-starved mouse fibroblasts induces DNA synthesis and at the same time triggers apoptosis. Both events have been shown to require heterodimerization of c-Myc and Max. Here we have studied dominant-negative mutants inhibiting specifically DNA binding of c-MycMax heterodimers. The phenotypes of c-Myc and Max mutants after expression in 3T3-L1 mouse fibroblasts differed markedly. While Max mutants inhibited serum-induced DNA synthesis, c-Myc mutants rapidly induced apoptosis.

Phenotypes of Max and c-Myc Mutants. — The mutants MaxBR and MaxBRmut specifically inhibited binding of cMycMax heterodimers to DNA in gel-shift experiments. Both mutants also inhibited DNA synthesis in serum-stimulated 3T3-L1 mouse fibroblasts, indicating that they could act as dominant-negative mutants in vivo. This confirms earlier results showing that induction of cell cycle progression depends on binding of the c-MycMax heterodimers to DNA (Cogliati et al. 1993; Amati, 1993b). Several c-Myc target genes have been identified so far (Eilers et al., 1991; Benvenisty et al., 1992; Bello-Fernandez et al., 1993; Jansen-Dürr et al., 1993; Reisman et al., 1993; Yang et al., 1993; Gaubatz et al., 1994). Modulation of the activity of these and yet unidentified genes by the Max mutants may be responsible for the inhibition of cell cycle progression. Since Max mutants also affect homodimerization of wild-type Max and heterodimerization of Max with Mad and Mxi1 (Ayer et al., 1993; Zervas et al., 1993), the inhibitory effect of Max mutants could be even more complex and rely not only on the inhibition of DNA binding of c-MycMax heterodimers. The mutant ctMycBR inhibited binding of the cMycMax heterodimer to the cognate E-box to a similar extent as the
mutants MaxBR mut and MaxBR - did. From this observation, we expected an inhibitory effect on DNA synthesis also for the mutant ctMycBR mut. However, this mutant induced apoptosis in serum-stimulated cells before the onset of DNA synthesis could be measured. Apoptosis was induced by this mutant at low and high serum levels and also observed after expression of ctMyc.

Induction of apoptosis by wild-type c-Myc requires dimerization of the HLH/LZ domains of c-Myc and Max (Amati et al. 1993b). The following reasons argue against dimerization of Max and the c-Myc mutants as requirement for induction of apoptosis by these mutants.
apoptosis. (i) The presence of Max does not confer DNA binding activity to the ctMycBR−Max heterodimer. Alternatively, ct-MycBR− may act by sequestering Max from other DNA binding complexes. However, Max is also sequestered by MaxBR and MaxBRmut, which do not induce apoptosis. (ii) Instead, the mutants MaxBR and MaxBRmut suppress ctMycBR− induced apoptosis, indicating that complexes of ctMycBR−MaxBR− and ctMycBR−MaxBRmut are unable to induce apoptosis. We therefore conclude that ctMycBR− and ctMyc interact probably with other factor(s) than Max to induce apoptosis in 3T3-L1 cells.

Induction of Apoptosis by the HLH/LZ Domain of c-Myc—Additional factors interacting with c-Myc have recently been identified including the transcription factors TFII-I (Roy et al., 1993), YY1 (Shrivastava et al., 1993), and AP2 (Gaubatz et al., 1995). The interaction with all three factors has been shown to involve the HLH/LZ domain of c-Myc. Ternary complexes of c-MycMaxTFII-I or c-MycMaxYY1 could not be demonstrated, indicating that binding of TFII-I or YY1 to c-Myc excludes Max binding. In an attempt to define the carboxy-terminal domain of c-Myc responsible for inducing of apoptosis more precisely, the HLH and LZ domains were expressed separately. Both domains could be expressed at high levels in 3T3-L1 cells without any sign of apoptosis. However, co-expression of the HLH and LZ domains restored induction of apoptosis, indicating that both domains are important for induction of apoptosis in our system but that the contiguous arrangement of the HLH and LZ domains is not necessary. Additionally, the results indicate that apoptosis is induced by the specific expression of the c-Myc HLH/LZ domain and not by the experimental approach and expression of high levels of proteins. The data suggest that the c-Myc HLH/LZ domain induces apoptosis by its specific interaction with other cellular factors containing HLH and/or LZ motifs.

Induction of apoptosis by wild-type c-Myc depends on the presence of the amino-terminal transcriptional activation domain (Evan et al., 1992) and dimerization of c-Myc with Max (Amati et al., 1993b). This suggests specific gene-regulatory activity of the c-MycMax complex during induction of apoptosis. In this respect, induction of apoptosis by the c-Myc mutants differs markedly from wild-type c-Myc-induced apoptosis. It is very unlikely that the c-Myc mutants can still regulate target
genes of wild-type c-Myc. However, since the mutants ctMyc and ctMycBR 
and c-Myc probably can still interact with other transcription factors, both mutants may also induce apoptosis by altering gene expression. The changes in gene expression are probably severe and induce apoptosis even in the presence of high serum. Although we are far from understanding how the mutants ctMyc and ctMycBR act, the presented data are of importance for our understanding of c-Myc protein function.

A c-Myc mutant with a deleted transactivation domain (amino acids 40–178) is unable to induce apoptosis in quiescent 3T3-L1 fibroblasts but has been shown to inhibit serum-induced DNA synthesis (Hermeking et al., 1994). This implicates that the central part of the c-Myc protein (amino acids 179–354) either by itself or by binding of other proteins, controls the interaction of cellular proteins with the carboxyl-terminal HLH/LZ domain of c-Myc. Deletion of the central part of the c-Myc protein renders the HLH/LZ domain highly promiscuous for otherwise tightly controlled interactions with cellular factors. For this reason, c-Myc mutants with a deleted transactivation-domain are more useful as dominant-negative mutants than mutants consisting only of the HLH/LZ domain (Mukherjee et al., 1992; Hermeking et al., 1994).

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