Opposite regulation of gene transcription and cell proliferation by c-Myc and Max

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ABSTRACT   c-Myc and Max are nuclear phosphoproteins capable of forming DNA-binding, homo- and heteropolymeric complexes *in vitro* and *in vivo*. Using a transient cotransfection assay involving c-Myc and Max expression vectors and a reporter gene plasmid containing the Myc/Max binding site, we find that Max represses transcription, whereas a significant stimulation is obtained when Max is coexpressed with c-Myc. Analysis of specific mutants indicates that transcriptional activation requires both the c-Myc and the Max dimerization and DNA-binding domains, as well as the c-Myc transactivation function; transcriptional repression by Max requires both DNA binding and dimerization. Analogously, in stably transfected human B-lymphoblastoid cell lines, overexpressed c-Myc and Max synergize to cause malignant transformation, whereas overexpression of Max alone leads to growth inhibition. These results indicate that the c-Myc and Max are transcriptional regulators with the ability to oppositely regulate target-gene expression and cell proliferation, most likely as the result of the opposite effects of heterodimeric c-Myc–Max (positive) versus homodimeric Max (negative) complexes.

The c-*myc* protooncogene codes for an evolutionarily conserved nuclear phosphoprotein which is ubiquitously expressed in somatic cells, where it is involved in the control of proliferation and differentiation (1, 2). Alterations in c-*myc* gene structure and expression caused by retroviral insertion, amplification, and chromosomal translocation are associated with tumorigenesis in different species (1–3). However, the precise function of the c-Myc protein in normal and in neoplastic cells is unknown.

Several observations have supported the hypothesis that c-Myc may be a transcription factor. c-Myc can bind DNA *in vitro*, and a specific sequence (CACGTG) has been identified as its specific binding site (4–6). In addition, c-Myc contains domains which are typical of several types of transcription factors. These include carboxyl-terminal basic, helix–loop–helix, and leucine-zipper (b–HLH–LZ) domains which can mediate the formation of oligomeric complexes capable of specific DNA binding (7) and an amino-terminal domain capable of transcriptional transactivation when assayed in experimental chimeric constructs (8, 9). Further, a second b–HLH–LZ protein, Max, has been identified which can specifically associate with c-Myc *in vitro* and *in vivo* to form heterodimeric complexes capable of specific DNA binding (10–12). However, no direct evidence is available for the function of c-Myc and Max as transcriptional regulators or on their specific role within heterodimeric or homodimeric complexes.

In this study, we demonstrate a direct role of c-Myc and Max as transcriptional regulators by showing that they can regulate the transcription of a reporter gene linked to a c-Myc/Max binding site. The results indicate that opposite regulation of transcription can be obtained by varying the

c-Myc/Max ratio, strongly suggesting that c-Myc/Max heterodimers activate, whereas Max represses, transcription. To confirm a biological role for this differential regulatory activity, we show that varying the c-Myc/Max ratio can cause opposite effects on cell proliferation.

MATERIALS AND METHODS

Plasmid Construction and Transient Transfection. The construction of the pHeBo-CMV-Myc2.3 plasmid was previously described (13). To construct the pHeBo-CMV-Max plasmid, we first generated a full-length coding region of human Max cDNA by reverse transcription/PCR amplification of RNA to generate a fragment spanning bp −6 to 465 of the published human Max cDNA sequence (10). The amplified product was cloned into the pGEM-3 plasmid (Promega), completely sequenced to confirm that it matched the published sequence coding for 151 amino acids (10), and then transferred into the pHeBo-CMV expression vector by blunt-end ligation into the filled-in *Not* I site. The p-MMBS-SV1-LUC plasmid was constructed by cloning into the *Mlu* I site of the pGL2-promoter vector (catalogue no. E1631, Promega) the palindromic self-annealed synthetic double-stranded oligodeoxynucleotide 5′-CGCGGGAAGCAGACCACGTG-GTCTGCTTCC-3′, which includes the Myc/Max binding site flanked by the *Mlu* I site (underlined). Transient transfection of HeLa cells was performed by a calcium phosphate procedure. Luciferase assays were performed according to standard protocols (Promega) using a luminometer.

Lymphoblastoid Cell Line (LCL) Transfection and Analysis. LCLs were transfected by electroporation (14). For clonogenicity analysis, transfected LCLs were plated in triplicate agar plates (0.3% in Iscove's modified Dulbecco's medium plus 20% fetal bovine serum) at 5×10^3, 1×10^4, and 2×10^4 cells per ml over a feeder layer (3×10^4 cells) of irradiated (6000 rads; 1 rad = 0.01 Gy) human lung fibroblasts.

Immunoprecipitation and Immunoblot Analysis. For immunoprecipitation analysis of Max in transfected cells (see Fig. 4A), 5×10^5 cells were metabolically labeled with L-[^{35}S]methionine (0.3 mCi, 11.1 MBq) for 2 hr. After labeling, the cells were processed for immunoprecipitation with 5 ml of rabbit anti-Max serum (a gift from R. Eisenman; ref. 12). The immunoprecipitates were analyzed by SDS/12% PAGE followed by autoradiography overnight at −70°C.

For immunoprecipitation/immunoblot analysis of Myc-Max heterodimers (see Fig. 4B), 2.5×10^7 cells were collected, washed twice with cold phosphate-buffered saline, lysed in 1 ml of phosphate-buffered saline containing 1% (vol/vol) Triton X-100 and 1 mM phenylmethanesulfonyl fluoride, and subjected to mild sonication. The cell extracts were then centrifuged for 20 min at 12,000 × g at 4°C, and the anti-Max immunoprecipitations were performed. The immunoprecipitates were washed three times with the same lysis buffer, solubilized by Laemmli sample buffer, and electro-

Abbreviations: b, basic; HLH, helix–loop–helix; LZ, leucine zipper; LCL, lymphoblastoid cell line.

phoresed through an SDS/10% polyacrylamide gel. After electrophoresis, the separated proteins were electroblotted onto a nitrocellulose filter. After transfer, the filter was saturated in blocking buffer (3% bovine serum albumin/10 mM Tris, pH 8.0/100 mM NaCl/0.05% Tween 20) for 1 hr at 37°C, incubated with mouse monoclonal anti-human c-Myc antibody 9E10 (1 μg/ml) in blocking buffer for 2 hr at room temperature, and then stained with alkaline phosphatase-conjugated goat anti-mouse antibody.

Northern Blot Analysis. RNA samples (15 μg) were analyzed as described (15) and hybridized to probes ^{32}P-labeled by the random priming method (16).

RESULTS

c-Myc and Max Can Oppositely Regulate the Transcription of a Reporter Gene Linked to Their Specific DNA Binding Site. To investigate whether c-Myc and Max are capable of transcriptional regulation of gene expression in live cells, we first used a transient-transfection assay in which c-Myc (pHeBoCMVMyc2.3; for brevity, CMVMyc) and/or Max (pHeBoCMVMax; CMVMax) expression vectors were cotransfected with a reporter plasmid (pMMBS-SV1-LUC) in which a single copy of the Myc/Max binding site (5) is linked upstream of a minimal promoter sequence (simian virus 40) and a reporter gene (luciferase) (Fig. 1). Various combinations of effector CMVMyc or CMVMax plasmids, or corresponding control plasmids not expressing c-Myc or Max (see Fig. 1 legend), were cotransfected with target plasmids into HeLa cells and the activities of c-Myc and Max were measured as changes in the expression of the reporter gene in effector- versus control-transfected cells.

Moderate levels of reporter gene expression (baseline in Fig. 2) were detectable when the target plasmid was transfected with control expression vectors which do not express c-Myc or Max, consistent with the fact that, as for all proliferating cells, HeLa cells contain endogenous c-Myc, Max, and other CACGTG-binding proteins. Transfection of the Max vector led to a significant, dose-dependent repression of target gene expression, and high concentrations of transfected Max vector nearly abolished transcription from the reporter gene. This repression was specifically mediated by Max expression and was not due to competition for transcription factors, since the total concentration of promoter sequences was held constant in all transfections (see Fig. 2 legend). However, this repression was alleviated when c-Myc was coexpressed with Max, and a significant stimulatory activity (3- to 8-fold above baseline; 10- to 25-fold above repressed levels induced by Max) was detected when c-Myc was transfected in excess of Max. The stimulatory activity could be titrated by varying the amounts of c-Myc versus Max, indicating that transcriptional activity was de-
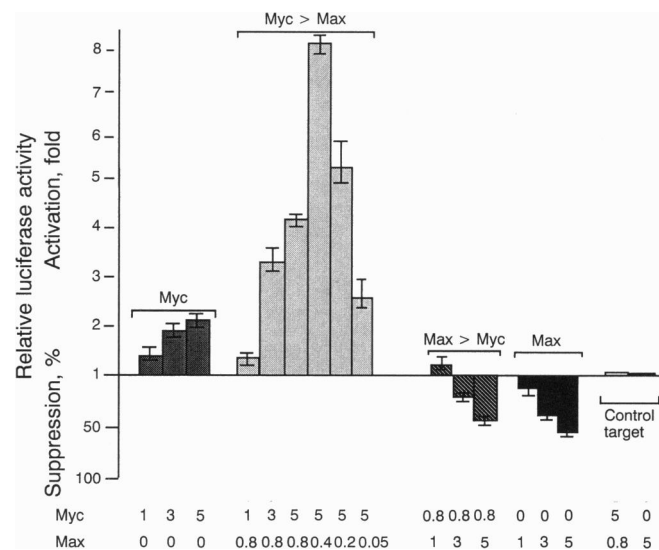

FIG. 2. Analysis of c-Myc and Max transcriptional regulatory activity in transient-cotransfection assays in HeLa cells. The indicated amounts (picomoles) of pHeBoCMVMyc2.3 (indicated as Myc at the bottom) and/or pHeBoCMVMax (Max) vector (or corresponding controls, see Fig. 1 legend and below) were cotransfected into HeLa cells with 3 μg (0.8 pmol) of the p-MMBS-SV1-LUC plasmid by calcium phosphate precipitation. The total amount of transfected DNA was kept constant (50 μg) in each experiment by adding pHeBoCMV DNA. Two micrograms of a plasmid containing the bacterial β-galactosidase gene was also cotransfected in each experiment to serve as an internal control for transfection efficiency (see below). At 48 hr after transfection, cells were harvested and transcriptional activation was assayed as luciferase activity. The values are expressed as fold increase (or percent decrease) of luciferase activity relative to the baseline value obtained by cotransfecting the target plasmid with control effector only (pHeBo-CMV and p-SV1-LUC). Each value is expressed after normalization for (*i*) nonspecific competition for endogenous transcriptional factors, using the values obtained by transfection of corresponding amounts of control plasmids, and (*ii*) efficiency of transfection, using β-galactosidase as an internal control. Each transfection was performed in triplicate and standard deviation bars are shown. "Control target" values correspond to the activity of c-Myc and Max plasmids on the control target plasmid (p-SV1-LUC) lacking the Myc/Max binding site. The results are from one representative assay; average maximum induction ranged between 5- and 22-fold over baseline in different experiments.

pendent upon the c-Myc/Max ratio. Cotransfection of the target plasmid with the c-Myc vector alone produced a modest (2- to 3-fold), although reproducible and dose-dependent, increase in target gene expression. We interpret this result as most likely due to the recruitment of limiting amounts of endogenous Max or Max-like factors. No differ-
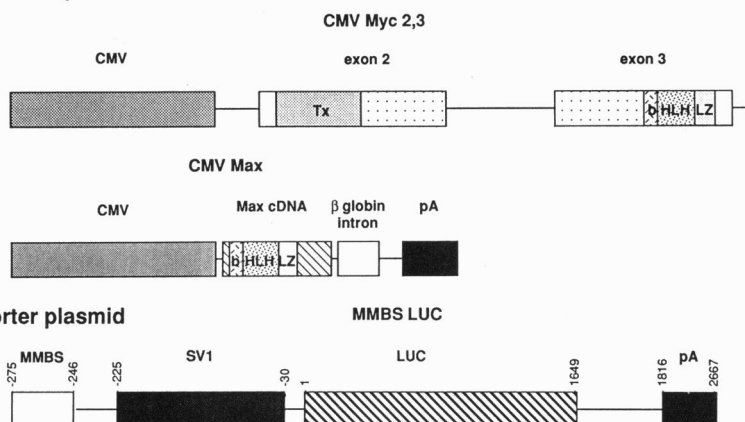
Expression plasmids

FIG. 1. Plasmid vectors. All constructs were made in pHeBo vectors (17). Only relevant regions of the plasmids are shown: CMV, cytomegalovirus promoter; exons 2 and 3 of the human c-*myc* gene; Tx, transactivation domain; β-globin intron, intron with splicing sequences from rabbit β-globin gene; pA, polyadenylylation signal of the simian virus 40 virus; Max, Max cDNA; LUC, luciferase gene; SV1, simian virus 40 promoter; MMBS, single copy of the Myc/Max binding site. Control plasmids (not shown) included a plasmid lacking c-Myc or Max sequences (pHeBo-CMV) and a control target plasmid lacking the MMBS site (pSV1-LUC).

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ence in target gene expression was observed when c-Myc or Max was cotransfected with a reporter plasmid lacking the Myc/Max binding site (control target in Fig. 2). Analogous results were obtained when similar transfections were performed with the B-cell lymphoma cell line P3HR1 (data not shown).

Both DNA Binding and Dimerization Are Required for c-Myc and Max Activity; Transcriptional Activation Is Dependent upon an Intact c-Myc Transactivation Domain. Fig. 2 indicates that transcriptional activation is dependent upon an appropriate c-Myc/Max ratio and suggests that this may be due to stoichiometric requirement for heterodimerization. To directly demonstrate that c-Myc heterodimerization is required for transcriptional activation, we tested the activity of c-Myc mutants (Fig. 3B) which have deletions within the HLH (CMVMycΔ371–412) or LZ (CMVMycΔ414–433) domain, preventing dimerization, or an insertion at the border of b–HLH domains (CMVMycIn370), preventing DNA binding (10, 18). In addition, we tested whether the transcriptional activity of c-Myc–Max complexes was dependent upon the transactivation domain present within the N-terminal portion of c-Myc by using two c-Myc mutants (Δ7–91 and Δ41–178 in Fig. 3B) which can dimerize and bind DNA but cannot transactivate, due to specific deletions (8, 18). These mutants were compared with wild-type c-Myc for their ability to stimulate reporter gene transcription when cotransfected with Max as described above for Fig. 2. The results (Fig. 3A) indicate that all five of the mutants are essentially devoid of transcriptional activity.

The transcriptional activity of Max was also further investigated by determining whether dimerization and DNA binding are required for transcriptional activation or repression. Toward this end, we tested the activity of Max mutants (Fig. 3D) which have deletions within the HLH domain (Δ83–90), preventing dimerization, or within the basic region (Δ15–28), preventing DNA binding. In addition, we tested the activity of a physiological alternatively spliced form of Max (MAX9; see Fig. 3D) containing a 9-residue insertion amino-terminal to the basic region (10). These mutants were compared with wild-type Max for their ability to repress or activate reporter gene transcription when used alone or when cotransfected with c-Myc, respectively. The results (Fig. 3C) indicate that MAX9 represents a slightly weaker effector than Max and that both the repressor and activator functions of Max require dimerization and DNA binding. Taken together, these findings indicate that transcriptional activity by both c-Myc and Max requires specific DNA binding and dimerization. Since transcriptional activation is dependent upon the c-Myc transactivation function, yet c-Myc is virtually inactive alone, the results strongly suggest that transcriptional activation is in fact mediated by c-Myc–Max heterodimers.

Opposite Effects on Cell Proliferation Caused by Varying the c-Myc/Max Ratio. To determine the biological significance of the distinct transcriptional activity of c-Myc–Max and Max complexes, we tested the consequences of their overexpression on the growth phenotype of appropriate target cells. Transfection of vectors constitutively overexpressing c-Myc under the control of heterologous promoters is known to cause the transformation (acquisition of clonogenicity and tumorigenicity) of Epstein–Barr virus-immortalized human B LCLs (13, 14). In our present study, the transfection of plasmids constitutively expressing Max (CMVMax) in LCLs (CB33 or UH1-10.1) previously transformed by c-Myc (SVMyc-CMVMax) led to a further, 10-fold increase in clonogenicity. This result is consistent with growth stimulation being dependent upon increased c-Myc–Max levels, which were presumably limited by Max in cells overexpressing only c-Myc. Since Max overexpression alone (CMVMax) did not induce any effect in the clonogenicity assay (Fig. 4A), we explored the possibility that Max overexpression had a
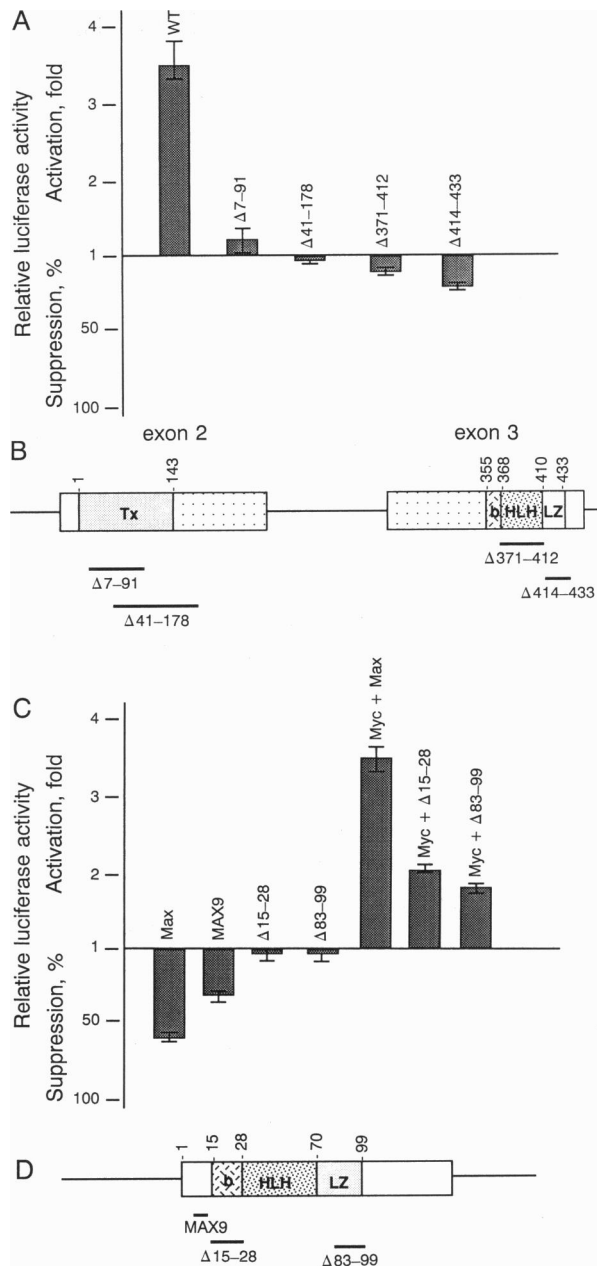

FIG. 3. Analysis of transcriptional activity of c-Myc and Max mutants in DNA-binding, heterodimerization, and transactivation domains. (A and B) Analysis of c-Myc mutants. Five picomoles of the wild-type (WT) or c-Myc mutant constructs (see B; numbers refer to amino acid positions) was cotransfected with 0.4 picomole of Max vector and 0.8 picomole of the pMMBS-SV1-LUC reporter plasmid as described in Fig. 2. (C and D) In addition to pMMBS-SV1-LUC 5 pmol of wild-type (Max), alternatively spliced (MAX9), or deletion mutant (Δ) Max constructs (see D) was transfected to test transcriptional suppression or 0.4 pmol of wild-type or Δ mutant was cotransfected with 5 pmol of c-Myc to test transcriptional activation.

negative effect on growth and studied the proliferation rate of transfected LCLs in suspension cultures. As expected, c-Myc and Max overexpression synergized in accelerating proliferation; conversely, Max overexpression in LCLs (CMVMax) containing only endogenous, normally expressed c-myc genes led to inhibition of growth (Fig. 4B). All the observed changes in the growth curves reflected changes in cell doubling times; no change in the rate of cell death was observed (data not shown).

To confirm that the enhanced growth and clonogenicity of cells overexpressing c-Myc and Max were in fact due to
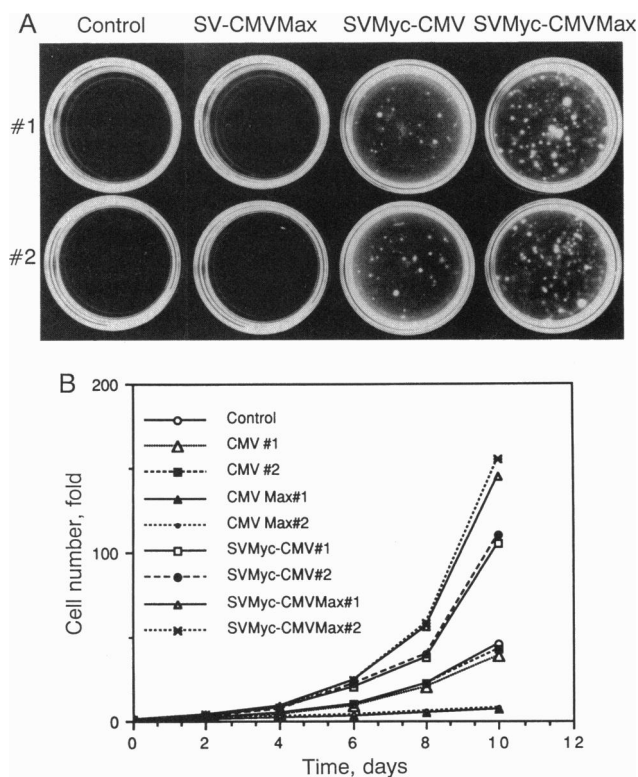
FIG. 4. Differential effect of c-Myc and/or Max overexpression on the growth phenotype of LCLs. (*A*) Max overexpression increases the *in vitro* clonogenicity of LCLs overexpressing c-Myc. Cloned CB33 LCL cells were transfected sequentially in duplicate by electroporation with one or two of the following episomally replicating pHeBo plasmids: SV, CMV, SVMyc, CMVMax, SVMyc, and CMVMax. Each plasmid is available in two forms, carrying either hygromycin- or neomycin-resistance genes, allowing for double selection of two different plasmids. After selection in the appropriate antibiotic(s), mass cultures of transfected cells were characterized for expression of exogenous c-Myc and Max RNA (data not shown) and proteins (Fig. 5). Duplicate transfectants were then plated in triplicate agar plates. Shown here is a photograph of representative plates seeded with 10^4 of the indicated cells at 14 days (nos. 1 and 2 refer to duplicate transfectants). Similar results were obtained with analogous transfectants of the UH1-10.1 LCL. (*B*) Max overexpression partially inhibits the growth of LCL cells containing normally expressed c-Myc genes. The same cell types used in *A* were tested for growth rate in suspension cultures (Iscove's modified Dulbecco's medium with 10% fetal bovine serum). Results are expressed as fold increase in cell number during time (days) in culture. Analogous transfectants of the UH1-10.1 LCL behaved similarly except that CMV-Max transfected cells displayed a virtually complete growth arrest.

increased levels of c-Myc–Max heterodimers, we directly tested whether increased levels of c-Myc were bound to Max in c-Myc-transfected cells. Increased levels of 20-kDa Max were detected in all Max-transfected LCLs by immunoprecipitation analysis using an anti-Max antiserum under stringent conditions (Fig. 5*A*). To semiquantitatively determine the amounts of c-Myc bound to Max, extracts from the same cells were first immunoprecipitated at low stringency with the anti-Max antiserum, and the resulting immunocomplexes were then immunoblotted with an anti-c-Myc monoclonal antibody (12). The levels of Max-bound c-Myc were below the threshold of detectability in LCLs expressing normal levels of c-Myc (LCLs express very low levels of endogenous c-Myc) (Fig. 5*B*). These levels, however, become clearly detectable in either c-Myc- or c-Myc/Max-transfected LCLs (no difference was detectable in c-Myc- versus c-Myc/Max-transfected cells, presumably because of the poor quantita-
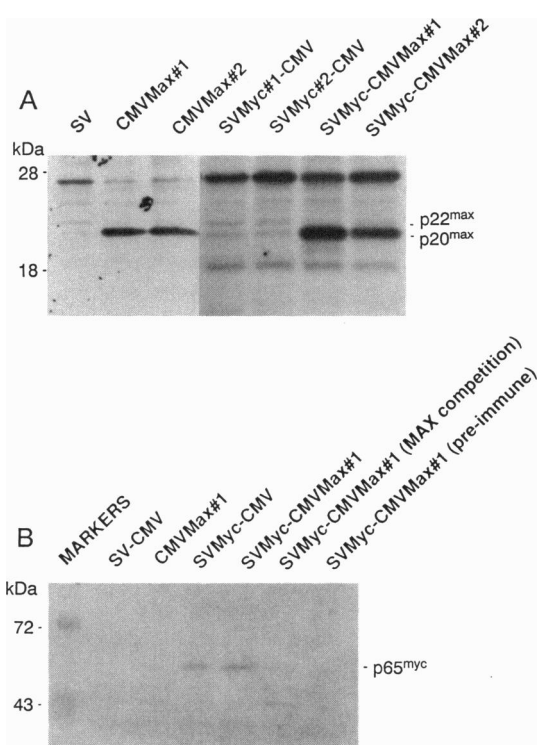

FIG. 5. Immunoprecipitation/immunoblot analysis of Max and c-Myc–Max heterodimers in transfected LCLs. Cell lines are indicated as in Fig. 4. (*A*) Immunoprecipitation analysis of Max proteins in transfected cells with an anti-Max serum; 65-kDa c-Myc (data not shown; ref. 15) and 20-kDa Max molecules were detected by immunoprecipitation analysis, consistent with the coding capacity of the respective expression vectors. (*B*) Immunoblot analysis using anti-Myc antibodies of proteins obtained by anti-Max immunoprecipitation (see text). Controls for the specificity of the anti-Max immunoprecipitation included immunoprecipitation in the presence of excess Max synthetic peptide (MAX competition) and immunoblot with normal rabbit serum (pre-immune).

tive nature of the assay). These results indicate that increased levels of c-Myc drive increased heterodimerization with Max and that this is associated with growth stimulation.

DISCUSSION

c-Myc–Max and Max as Opposite Regulators of Transcription. The results of the transfection assays shown in Fig. 2 indicate that initiation of transcription adjacent to a c-Myc/Max binding site can be oppositely regulated by varying the c-Myc/Max ratio. While this work was under review, analogous results and conclusions were obtained by others studying the ability of c-Myc and/or Max to regulate transcription from a reporter gene in mammalian cells or in yeast (19, 20). These results, including the analysis of dimerization and transactivation mutants, strongly suggest that c-Myc–Max heterodimers are positive regulators of transcription, whereas Max or Max–Max homodimers are repressors. This notion is entirely consistent with several structural/functional features of both the c-Myc and Max molecules: c-Myc contains a transcriptional activation domain (8, 9) and is capable of only weak DNA binding (10–12), whereas Max apparently lacks a transcriptional activator domain (9) but is capable of strong DNA binding, further enhanced by the association with c-Myc (10–12). Thus, c-Myc–Max heterodimerization, which may occur at higher affinity than either c-Myc or Max homodimerization, could represent the way to form a complex capable of both high-affinity DNA binding and transcriptional activation.

While the results shown here suggest that c-Myc homodimers may be functionally inactive (in fact, these may never occur *in vivo*, since Max is constitutively expressed), the function of Max as a repressor needs further analysis. Max-dependent negative regulation of transcription may occur through simple stoichiometric displacement of c-Myc–Max heterodimers or by an intrinsic transcriptional repressor activity. In addition, other heterodimeric partners may exist for Max and contribute to its function. Finally, the activity of the c-Myc/Max system is likely to be subject to additional multiple regulatory levels *in vivo*, including regulation by binding of the retinoblastoma protein to the c-Myc amino-terminal domain (21) and by casein kinase II-dependent phosphorylation of Max (22). The transcriptional assay used in this study provides an experimental framework which may be helpful in integrating the effects of all these regulatory mechanisms on c-Myc/Max transcriptional activity.

Implications for the Role of c-Myc and Max in the Control of Cell Proliferation and Differentiation. The second main finding of this study is that, consistent with its ability to oppositely regulate transcription, the c-Myc/Max system is capable of oppositely regulating cell proliferation. Consistent with the results shown here, previous work had shown that Max overexpression could synergize with c-Myc overexpression in a Ras cotransformation assay in fibroblasts (11). The same assay has been used to show that transformation can be suppressed by overexpression of a small, differentially spliced form of Max gene (ΔMax) (23). However, the corresponding ΔMax protein is naturally present at levels too low to play that role physiologically (unpublished results). Conversely, the present results show that the major physiologic form of Max can suppress cell proliferation when present in vast excess of c-Myc, suggesting that it may be physiologically involved in growth suppression in cells lacking c-Myc, such as quiescent and terminally differentiated cells.

In fact, the opposite transcriptional and biological activities of c-Myc–Max heterodimers versus Max homodimers, together with the pattern of regulation of c-Myc and Max gene expression, have precise implications for the role of these factors in controlling cell proliferation and differentiation. Through continued gene expression and due to the extremely long half-life of the protein, Max is constitutively expressed during cell quiescence, proliferation, and terminal differentiation (12); conversely, through a specific pattern of gene transcription and synthesis of an extremely labile RNA and protein, c-Myc expression is tightly regulated during proliferation and differentiation, being absent from quiescent cells, rapidly induced by mitogenic stimulation (G_0 to G_1 transition), and rapidly suppressed after induction of differentiation (1, 2). Thus, the results of these studies strongly suggest a model in which resting cells are under the effects of the transcriptional and growth suppressive activities of Max, whereas mitogenically stimulated cells contain high levels of the oppositely acting c-Myc/Max heterodimers. Cell proliferation and differentiation may be induced *in vivo* by varying c-Myc levels, which, in turn, would control the levels of the differentially acting c-Myc–Max and Max transcriptional complexes.

Implications for the Role of c-*myc* Oncogenes in Tumorigenesis. Although c-*myc* activation can occur by several distinct mechanisms, including retroviral insertion, amplification, and chromosomal translocation, the common functional consequence of all these alterations appears to be the deregulation of c-*myc* gene expression leading to the synthesis of constitutively high levels of c-Myc (1–3). The results of this study strongly suggest that the pathogenetic action of such deregulation may be to unbalance the c-Myc/Max system by sequestering most of the available Max into c-Myc–Max heterodimers. This would lead to constitutive activation of gene expression programs associated with proliferation.

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