Identification and Characterization of Specific DNA-binding Complexes Containing Members of the Myc/Max/Mad Network of Transcriptional Regulators*

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In the past, eukaryotic cell-derived complexes of the Myc/Max/Mad network of transcriptional regulators have largely been refractory to DNA binding studies. We have developed electrophoretic mobility shift assay conditions to measure specific DNA binding of Myc/Max/Mad network complexes using a COS7 cell-based overexpression system. With the established protocol, we have measured on- and off-rates of c-Myc/Max, Max/Max, and Mad1/Max complexes and determined relative affinities. All three complexes appeared to bind with comparable affinity to a Myc E-box sequence. Furthermore, our data derived from competition experiments suggested that the Mad3/Max and Mad4/Max complexes also possess comparable DNA binding affinities. The conditions established for COS7 cell-overexpressed proteins were then used to identify c-Myc/Max, Max/Max, and Mnt/Max complexes in HL-60 cells. However, no Mad1/Max could be detected, despite the induction of Mad1 expression during differentiation. Whereas the DNA binding activity of c-Myc/Max complexes was downregulated, Max/Max binding increased, and Mnt/Max binding remained unchanged. In addition, we have also tested for upstream stimulatory factor (USF) binding and observed that, in agreement with published data, USF comprises a major Myc E-box-binding factor that is more abundant than any of the Myc/Max/Mad network complexes. Similar to the Mnt/Max complex, the binding activity of USF remains constant during HL-60 differentiation. Our findings establish conditions for the analysis of DNA binding of Myc/Max/Mad complexes and indicate posttranslational regulation of the Max/Max complex.

The c-Myc proto-oncoprotein plays a pivotal role in cell growth control (for review, see Refs. 1–3). The forced expression of this protein promotes progression into S phase (4) and inhibits differentiation and entry into a quiescent state (5–8). In addition, c-Myc can stimulate apoptosis in certain cell types (9, 10). The importance of c-Myc is further supported by the finding that this protein is essential for normal murine embryonic development (11). To perform these functions, c-Myc, and most likely its close relatives N-Myc and L-Myc, have to heterodimerize with Max (12–15). In addition to c-Myc, Max also heterodimerizes with Mad proteins that negatively regulate cell growth (for review, see Ref. 3). Therefore, Max is the central component of the Myc/Max/Mad network. All members of this network belong to a class of DNA-binding molecules characterized by a dimerization domain composed of a helix-loop-helix, a leucine zipper motif (HLHZip), and a basic DNA-binding region (b).

To date, four different Mad proteins (Mad1, Mxi1, Mad3, and Mad4) have been identified (16–18). These Mad proteins appear to perform similar functions; i.e., they inhibit transformation, repress transcription, and interfere with cell cycle progression (19–22). In addition, the expression of the Mad proteins is mainly confined to noncycling cells (18, 21, 23, 24). Collectively, these results suggest that Mad proteins are potential tumor suppressors. More recently, a novel protein that interacts with Max and also belongs to the bHLHZip class of transcriptional regulators has been identified independently by two groups and has been designated either Mnt in mice (25) or Rox in humans (26). Similar to Mad, Mnt acts as a transcriptional repressor and suppresses Myc/Ras cotransformation. Taken together, it appears that the Myc/Max/Mad network plays a role in both positive and negative cell growth control with the potential to either promote or inhibit cell transformation.

The central component of the Myc/Max/Mad network, Max, is very stable, and little regulation of its expression has been reported (13, 27–29). In contrast, both the Myc and Mad proteins are highly regulated. The myc genes are transcribed in growing cells, but little or no message or protein can be detected in resting or differentiated cells. By comparison, the mad genes are generally expressed in resting, differentiating, or terminally differentiated cells, with minimal transcription in proliferating cells (16–18, 24, 28). An exception appears to be mad3, which has been suggested to be expressed in S phase cells in the developing neural tube (18). In addition, c-Myc and Mad proteins are short lived. Whereas the expression of myc and mad mRNAs and proteins are generally mutually exclusive or overlap only during transition periods (e.g., G0 to G1 and cycling to differentiating cells), mnt mRNA and protein are coexpressed with c-Myc in a number of proliferating cell types (25, 26). This latter finding suggests that Mnt is functionally distinct from the Mad proteins. Overall, it is apparent that the levels of these factors are tightly controlled and that their relative concentrations are likely to be critical parameters in the regulation of cell growth.

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The different Max-containing dimeric protein complexes of the Myc/Max/Mad network bind specifically to DNA (for a review, see Ref. 30). Using either bacterially expressed (27, 31, 32), in vitro transcribed/translated proteins (12, 33–35) or c-Myc/Max complexes precipitated from mammalian cells (13, 36), a DNA consensus sequence for c-Myc/Max and Max/Max complexes was obtained. The same consensus sequence is also bound by Mad/Max. This sequence with a CACGTG core is referred to as a Myc E-box. More detailed analyses using binding site selection assays revealed some flexibility regarding the central two bases as well as preferred bases flanking the core sequence (27, 35, 37). This E-box can confer transcriptional responsiveness to the proteins of the Myc/Max/Mad network (14, 38–40) and is found in a number of Myc-regulated genes including prothymosin α (41), ornithine decarboxylase (42), p53 (43), elongation initiation factors 2α and 4E (44, 45), CAD (46), ECA39 (47), MrDb (48), and Cdc25a (49).

Despite the identification of consensus DNA-binding sequences, it has been very difficult to analyze the DNA binding capacities of cell-derived Myc/Max/Mad complexes. The so far observed in vivo derived Max/Max complexes showed rather weak real DNA binding capacity, which did not allow further characterization, whereas little evidence for c-Myc/Max DNA binding was obtained (50, 51). One reason thought to be important for the difficulties encountered is that Myc proteins appear poorly soluble under conditions typically used to extract nuclear proteins (52). Furthermore, renaturation of c-Myc/Max complexes prepared under denaturing conditions is inefficient (53). Another reason is the low abundance of both Myc and Mad proteins in many cells and tissues (54). In addition, several other factors including USF1 (55, 56), which is an abundant other factor binding to specific DNA sequences and probably also contribute to the difficulties encountered in analyzing DNA binding of Myc/Max/Mad network complexes.

To define conditions under which cell-derived c-Myc/Max, Max/Max, or Mad/Max complexes bind to specific DNA sequences, we made use of the finding that whole cell extracts prepared with mild detergents preserved these heterodimeric complexes. Such extraction conditions were employed on COS7 cells overexpressing the relevant proteins. The resulting whole cell extracts revealed significant DNA binding activity for Max/Max, c-Myc/Max, or Mad/Max complexes. The DNA binding activities of these cell-derived complexes were compared regarding their binding specificity and apparent affinity. In addition, we used the methods established for COS7-derived complexes to analyze the DNA binding of endogenous c-Myc/Max and Max/Max dimers and other Myc E-box-binding proteins in human myeloid HL-60 cells and followed their DNA binding during induced differentiation.

**EXPERIMENTAL PROCEDURES**

**Cells, Plasmids, and Transient Transfections**—COS7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 100 units of penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. HL-60 cells were grown in RPMI 1640, penicillin/streptomycin, 10% fetal calf serum. For differentiation, HL-60 cells were grown in flasks coated with 2% agarose M (Pharmacia Biotech Inc.) to prevent human myeloid HL-60 cells and followed their DNA binding activity. To define conditions under which cell-derived c-Myc/Max, Max/Max, or Mad/Max complexes bind to specific DNA sequences, we made use of the finding that whole cell extracts prepared with mild detergents preserved these heterodimeric complexes. Such extraction conditions were employed on COS7 cells overexpressing the relevant proteins. The resulting whole cell extracts revealed significant DNA binding activity for Max/Max, c-Myc/Max, or Mad/Max complexes. The DNA binding activities of these cell-derived complexes were compared regarding their binding specificity and apparent affinity. In addition, we used the methods established for COS7-derived complexes to analyze the DNA binding of endogenous c-Myc/Max and Max/Max dimers and other Myc E-box-binding proteins in human myeloid HL-60 cells and followed their DNA binding during induced differentiation.

**DNA Binding of Myc/Max/Mad Complexes**

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**SEQUENCES 1–6**

The mutations in CMM, CMF, CMD-M1, and CMD-M2 compared with CMD are indicated. The oligonucleotides were 32P-end-labeled with T4-polynucleotide kinase.

1–3 μl of COS7 lysate (1/300 to 1/100 of a 10-cm dish) or 2–5 μl of HL-60 cell extract (standardized for protein content) roughly corresponding to 40,000–100,000 cells were incubated with 0.1–0.5 ng of labeled oligonucleotide for 15 μl of GS buffer (20 mM HEPES, pH 7.3, 50 mM KCl, 3 mM MgCl2, 1 mM EDTA, 8% glycerol, 1 mM β-mercaptoethanol, 10 mM dithiothreitol) in the presence of 1 μg of sonicated salmon sperm DNA (Sigma) at 25 °C for 30 min. The off-rate experiments were done as described previously (63). Briefly, aliquots from F-buffer lyses were bound to labeled CMD probe for 30 min at 30 °C and placed on ice. Then the 0-min time point was taken. After the addition of a 200-fold excess of unlabeled CMD, the incubation was continued at 30 °C, and at the 0-min time point was taken. After the addition of a 200-fold excess of unlabeled CMD, the incubation was continued at 30 °C, and at the 0-min time point was taken. After the addition of a 200-fold excess of unlabeled CMD, the incubation was continued at 30 °C, and at the 0-min time point was taken. After the addition of a 200-fold excess of unlabeled CMD, the incubation was continued at 30 °C, and at the 0-min time point was taken. After the addition of a 200-fold excess of unlabeled CMD, the incubation was continued at 30 °C, and at the 0-min time point was taken.
were from Santa Cruz Biotechnology. Anti-USF1 polyclonal serum was kindly provided by M. Sawadogo (66).

For immunoblot analysis, transiently transfected cells were lysed in 300 μl of F-buffer, and the proteins were separated by SDS-PAGE and blotted to nitrocellulose membranes and manipulated as described previously (67). The membranes were incubated with primary antibodies as indicated and with appropriate secondary antibodies (goat anti-mouse IgG alkaline phosphatase-coupled (Jackson ImmunoResearch Laboratories), sheep anti-rabbit IgG alkaline phosphatase-coupled (Dia-nova), and sheep anti-rat IgG horse-radish peroxidase-coupled (Amer- sham Life Sciences)). Detection was carried out with nitro blue tetrazo- lium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) in the case of the alkaline phosphatase-coupled secondary Ab (68) or using the enhanced chemiluminescence system (ECL) with the horse-radish peroxidase-coupled Ab (Amer sham Corp.) according to the manufacturer’s instructions.

**Northern Blotting**—Logarithmically growing HL-60 cells were treated with or without TPA or G-CSF for the times indicated. RNA was extracted with the RNeasy total RNA kit (Qiagen). 15 μg of RNA was separated on 1% formaldehyde-agarose gels. The RNA was blotted onto GeneScreen™ membrane and hybridized with [32P]-random prime-labeled probes in 0.25 M NaPi, 7% SDS, 1 mM EDTA at 65 °C. The membrane was washed with 50 mM NaPi, 0.5% SDS. As probes, an EcoRI fragment of pSP-hu-c-myc and a NotI/EcoRI fragment from pVZI-hu-mad1 were used.

**Metabolic Labeling and Immunoprecipitations**—For metabolic labeling, 1 × 10⁷ HL-60 cells/time point were resuspended in 1 ml of methi- onine/cysteine-free Dulbecco’s modified Eagle’s medium supplemented with 2% dialyzed fetal calf serum and labeled with 150 μCi of [35S]me- thionine/cysteine (ICN) for 30 min at 37 °C. The cells were washed once with medium and once with phosphate-buffered saline, lysed either in F-buffer or in AB-buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.5% SDS, 0.5% aproti- nin, 0.5% phenylmethylsulfonyl fluoride), and sonicated, and the lysates were cleared by centrifugation. c-Myc proteins were precipitated with polyclonal serum IG13 and Mad1 with a 1:1 mix of monoclonal antibodies 5C9 and 5F4 as described (69). An equal number of trichlo- roacetic acid-precipitable counts were used for each sample. The protein complexes were collected with protein A- or protein G-Sepharose and roacetic acid-precipitable counts were used for each sample. The protein

**RESULTS**

**Specific DNA Binding of in Vivo Derived c-Myc/Max and Max/Max Complexes**—One limitation in studying the DNA binding activities of eukaryotic cell-derived c-Myc/Max complexes is their low expression and poor extraction from cells. Therefore, we chose to overexpress c-Myc and Max in COS7 cells using vectors containing SV40 origins of replication in combination with either a CMV or an SV40 promoter. The expressed proteins of the Myc/Max/Mad network were exclusively nuclear as determined by immunofluorescence (data not shown). Whole cell extracts were generated using L-buffer, which preserves the c-Myc/Max complexes and solubilizes more than half of both proteins (Fig. 1a and data not shown; see also Ref. 13). The extraction of both c-Myc and Max was slightly increased by the addition of salt (L-100 and L-200, respectively) or by using F-buffer (Fig. 1a). Under all of these conditions, the different dimers were preserved (data not shown). The c-Myc/ Max/Max complexes in different cell extracts were tested for DNA binding activity to a c-Myc E-box consensus oligonucleotide (CMD; for sequences see “Experimental Proce- dures”) in electrophoretic mobility shift assays (EMSA) (Fig. 1b). In all lysates, DNA binding of Max/Max homodimers was readily detectable, whereas c-Myc/Max binding was most efficient in F-buffer with little binding in L-buffer lysates (Fig. 1b). We concluded that extractability did not strictly correlate with DNA binding activity and therefore used F-buffer lysates for all following experiments.

To determine the identity and specificity of c-Myc/Max and Max/Max complexes, we performed antibody supershift and competition experiments. Both complexes were supershifted with a Max-specific antiserum and the c-Myc/Max complex with a c-Myc-specific mAb (Fig. 2). Neither complex was super- shifted using Mad-specific antibodies. Furthermore, both the c-Myc/Max and Max/Max complexes were readily competed with unlabeled specific probe (CMD) but not with CMM, which contains a mutated core sequence, and only weakly with CMF, which has disfavored flanking sequences with two base changes on both sides of the core sequence (Figs. 1a and 2a (35)). Competition with CMD-M1 or CMD-M2, which contain only one base change on each side of the core sequence, was intermediate compared with CMD and CMF (data not shown), thus confirming the importance of the flanking sequences (35). In addition, two endogenous complexes with slower mobility were competed in an identical pattern. The faster complex of the two could be supershifted by an anti-USF serum (Fig. 2a, asterisk). The latter complex is likely to represent Mnt/Max. This is compatible with its reactivity with different Max-specific antisera, its apparent size, and its comigration with the complex obtained after overexpression of Mnt and
Whereas the endogenous Max/Max, Mnt/Max, and USF complexes could readily be detected in control extracts (Fig. 3C), the endogenous c-Myc/Max complexes were only weakly detectable. Thus, the overexpression of c-Myc and Max resulted in a significant increase in binding of c-Myc/Max and Max/Max complexes. Together, these analyses identify specific DNA binding of four different protein complexes to the Myc E-box consensus sequence. An additional complex (indicated with an open circle) was evident, which appeared to be nonspecific since it was also competed with the CMM oligonucleotide (Fig. 2) and with 250 ng of poly(dI-dC) (data not shown). In summary, we have established conditions to obtain significant amounts of c-Myc/Max and Max/Max DNA binding activity, which will allow us to study these complexes in more detail.

To further confirm the identity of the c-Myc/Max complex, we tested three different monoclonal antibodies in supershift experiments. The mAb 9E10 (64), which recognizes an epitope in the HHLZip domain of human c-Myc, did not affect the mobility of the c-Myc/Max complex despite its ability to stain c-Myc on Western blots (Figs. 1a, 2b, and 2c). This is consistent with our finding that the mAb 9E10 inhibits dimerization of bacterially expressed Max and Myc92 (data not shown), suggesting that mAb 9E10 and Max binding to c-Myc are mutually exclusive. In support of this finding, the first leucine residue of the c-Myc Zip domain is part of the mAb 9E10 epitope and thus buried in the c-Myc-Max interphase (70). In contrast, the mAbs 6A10 and 4H3, which both recognize epitopes in the N-terminal transactivation domain of c-Myc (see below), induced a supershift (Fig. 2, a and b), demonstrating that c-Myc is a component of this complex. The regions recognized by the mAbs 6A10 and 4H3 were mapped by Western blot analysis (Fig. 2c). COS7 cells were transfected with plasmids expressing murine c-Myc, human c-Myc, or mutants as indicated. mAb 9E10 recognized all different human c-Myc proteins. mAb 6A10 immunoreacted neither with MycD100 nor MycD7–38, indicating that the recognized epitope is at least in part contained between amino acids 7 and 38. mAb 4H3 recognized an epitope between amino acids 55 and 92. The latter antibody also cross-reacted weakly with mouse c-Myc.

Binding of in Vivo Derived USF to Different E-box-containing Oligonucleotides—To define in more detail the identity of other factors binding to the CMD oligonucleotide, we overexpressed USF1 in COS7 cells. This resulted in the appearance of a complex that comigrated with an endogenous complex, both of which were supershifted with an anti-USF1 serum (Fig. 3). Again, these findings clearly identify USF as a component of a major CMD-binding complex. Since USF is generally more abundant than c-Myc, it was of interest to identify binding sites that could potentially distinguish between c-Myc/Max and USF complexes. Binding site selection studies indicated the existence of a binding site for USF in the CMD oligonucleotide.
ence of such a site, which is represented in the M36 oligonucleotide (36). Using the in vivo-derived protein complexes, we therefore tested for binding to M36. USF and Max/Max complexes bound very poorly and c-Myc/Max complexes bound weakly to this oligonucleotide in comparison with CMD (Fig. 4). Thus, the previously observed selectivity could be reproduced only in part using native protein complexes. This interpretation was also compatible with the observation that M36 could not compete for binding of either Max- or USF-containing complexes to an optimal Myc E-box consensus oligonucleotide (Fig. 4).

Specific DNA Binding of Mad-containing Protein Complexes—To expand our observations regarding the DNA-binding complexes to other members of the Myc/Max/Mad network, we cotransfected expression plasmids for Max with plasmids for Mad proteins into COS7 cells. Similar to the c-Myc/Max and Max/Max complexes described above, we detected specific complexes containing Mad1/Max, Mad3/Max, and Mad4/Max in whole cell extracts (Fig. 5). These complexes could not be seen in untransfected cells and were supershifted with the respective specific antisera but not with control antisera. Binding of the different Mad/Max complexes to CMD and their competition by specific and nonspecific oligonucleotides was very similar to c-Myc/Max and Max/Max complexes, suggesting comparable relative specificities and affinities of all these complexes. Together, these data demonstrate that the different dimeric complexes of the Myc/Max/Mad network can be detected in COS7 cell lysates, which makes this a suitable system to test and compare DNA binding activities of eukaryotic cell-derived wild-type or mutant proteins.

Relative DNA Binding Affinities of Myc/Max/Mad Network Complexes—It has been suggested previously that Max/Max homodimers bind DNA less efficiently than c-Myc/Max heterodimers or Mad/Max heterodimers (e.g. see Ref. 16). To address whether the relative affinities of different cell-derived native complexes differed, we performed titration, on-rate experiments, and off-rate experiments (Fig. 6) (71, 72). Titrating the labeled probe and determining the bound fraction revealed a similar increase in binding of the different complexes (Fig. 6a). Furthermore, the c-Myc/Max complex displayed a faster on- and off-rate compared with the Max/Max complex (Fig. 6, b and c). By comparison, the Mad1/Max complex showed a slightly decreased on- and off-rate from that observed for Max/ Max. From the on- and off-rate experiments, we determined the relative association constants of the different complexes. This analysis indicated that the c-Myc/Max complexes bound only slightly better than the other two (Table I). Collectively, these findings suggest that the affinities of the different Myc/Max/Mad complexes are similar.

Analysis of Endogenous Myc/Max/Mad Network Complexes—The expression of c-Myc, Max, and Mad proteins in COS7 cells allows the production of high protein levels from the transfected expression plasmids normally not found in cell lines. However, as already indicated (Fig. 2a), we could observe endogenous c-Myc/Max, Max/Max, and Mnt/Max complexes from COS7 cells. To analyze endogenous proteins, HL-60 cells were utilized as a model to study DNA binding of endogenous proteins. HL-60 is a human promyelocytic leukemia cell line that has an amplified c-myc locus and overexpresses c-Myc (73). In addition, during induced differentiation of HL-60 cells the expression of c-Myc and Mad1 is regulated (Refs. 28, 74; also see below). Several complexes binding to the CMD oligonucleotide could be detected with whole cell extracts of exo-
Max, and Mad/Max complexes are indicated. The circle CMF. The supershifts are marked with competed with a 10-, 50-, and 250-fold excess of cold CMD, CMM, or 6A10, or Mad1-, Mad3-, and Mad4-specific antibodies (Santa Cruz) and the Mad/Max extracts were preincubated with anti-Max serum, mAb pCMV-mu-mad3 (a), or pCMV-mu-mad4 (b) or with PBS as control (C). The Mad/Max extracts were preincubated with anti-Max serum, mAb pCMV-mu-mad3 (a), or pCMV-mu-mad4 (b) or with PBS as control (C). The Mad/Max extracts were preincubated with anti-Max serum, mAb pCMV-mu-mad3 (a), or pCMV-mu-mad4 (b) or with PBS as control (C).

**FIG. 5.** Analysis of DNA binding by different Mad/Max complexes after transient expression in COS7 cells. COS7 cells were transiently co-transfected with pSP-max p22 and pCMV-hu-mad1 (a), pCMV-mu-mad3 (b), or pCMV-mu-mad4 (c) or with PBS as control (C). The Mad/Max extracts were preincubated with anti-Max serum, mAb 6A10, or Mad1-, Mad3-, and Mad4-specific antibodies (Santa Cruz) and used in EMSA experiments. Binding to the CMD oligonucleotide was competed with a 10-, 50-, and 250-fold excess of cold CMD, CMM, or CMF. The supershifts are marked with arrows heads. The USF, Max/Max, and Mad/Max complexes are indicated. The circle marks a nonspecific complex, and the asterisk indicates the Mnt/Max complex.

nentially growing HL-60 cells. By comparing the migration pattern of COS7- and HL-60-derived protein complexes and by using specific antisera, we could define four of these complexes (Fig. 7). A Max/Max complex was consistently, albeit weakly, observed in HL-60 cell lysates, which was supershifted with several different Max-specific antisera (Fig. 7 and data not shown). The c-Myc/Max complex comigrated with the complex from transfected COS7 cells and was retarded by both c-Myc and Max antibodies. Two complexes were supershifted with the anti-USF1 serum, one had the same mobility as USF1 overexpressed in COS7 cells, the other most likely contains USF2 (75). USF was the major E-box binding activity in HL-60 whole cell lysates. The complex with the slowest mobility was also supershifted with anti-Max serum and, as discussed above, consists of Mnt/Max (Fig. 7, indicated by an asterisk). The nonspecific complex (open circle) was seen both in HL-60 and in COS7 cell lysates. Its abundance appeared to vary depending on the extract (see e.g. Fig. 8c). We also tested whether endogenous TFE-3 from HL-60 cells was detectable in EMSA. Comparison of COS7-derived TFE-3 bound to the CMD oligonucleotide with HL-60 whole cell lysates did not result in a comigrating complex (data not shown). Therefore, we concluded that TFE-3 is not a major E-box-binding factor in HL-60 lysates. Thus, by comigration and supershift experiments, we were able to define three distinct Max-containing complexes i.e. Max/Max, c-Myc/Max, and Mnt/Max and USF complexes in cell lysates from exponentially growing HL-60 cells.

**DNA Binding of Myc/Max/Mad Network Complexes during Differentiation of HL-60 Cells**—During the differentiation of hematopoietic cells, the expression of c-Myc and Mad1 is precisely regulated. Therefore, we analyzed the DNA binding activity of Max/Max, c-Myc/Max, Mnt/Max, and USF complexes in HL-60 cells induced to differentiate along the monoblast/macrophage lineage with TPA (73). In addition, we were interested in studying possible Mad/Max complexes. Upon induction of the differentiation program, c-myc mRNA transcription rapidly decreased, whereas the expression of the two mad1 transcripts was induced (Fig. 8a) as shown previously (28). On the other hand, treatment of HL-60 cells with G-CSF, which induces alterations in the expression of surface markers but does not trigger terminal differentiation (76), did not dramatically alter the expression of the two genes. To analyze whether the mRNA expression data correlated with protein expression, HL-60 cells were differentiated with TPA and processed for Western blotting or metabolically labeled with [35S]methionine/cysteine. Whereas c-Myc protein levels rapidly decreased in response to TPA, the two Mad1-interacting proteins Max (p21/p22) and Sin3B remained unchanged (Fig. 8b). We were not able to detect Mad1 on Western blots using either polyclonal antisera or a panel of mAbs (data not shown). To further evaluate Mad1 expression, this protein and c-Myc were immunoprecipitated from lysates of metabolically labeled cells, separated on SDS-PAGE, and visualized using a phosphoimaging. c-Myc was down-regulated and Mad1 was up-regulated within 6 h of TPA treatment (Fig. 8c). Thus, the expression of Mad1 is induced in differentiating HL-60 cells, albeit to low levels.

We next examined DNA binding to the CMD oligonucleotide. To increase the visibility of the different Max-containing complexes, the following experiment was performed in the presence of an anti-USF serum to displace USF-DNA complexes (Fig. 8d). c-Myc/Max complexes were readily detectable in undifferentiated HL-60 cells. In TPA-induced HL-60 cells, DNA binding activity quickly decreased within 2 h and was no longer measurable after 24 h. By comparison, DNA binding-competent c-Myc/Max complexes were still present 24 h after G-CSF treatment (Fig. 8d). In contrast, the DNA binding activity of Max/Max complexes increased with differentiation (Fig. 8d), and these complexes were supershifted with Max-specific antisera (data not shown). This was not due to an increase in Max levels as shown above (Fig. 8b) and was more substantial than expected from the release of Max from the c-Myc/Max complexes. The DNA binding activity of the Mnt/Max complex and of the USF complexes did not change during TPA-induced differentiation (Fig. 8d and data not shown). In summary, the DNA binding activity of c-Myc/Max complexes closely parallels the RNA and protein expression data. However, Max/Max DNA binding increases after TPA-induced differentiation of HL-60 cells. We have not been able to detect a Mad1/Max complex despite the observation that the expression of Mad1 is up-regulated in response to TPA.

**DISCUSSION**

A large body of data has accumulated that describes the dimerization properties of Myc/Max/Mad network proteins and their DNA binding specificities. In addition, several Myc-regulated genes that contain Myc E-boxes have been identified. Despite these findings, it has proven extremely difficult to show DNA binding of in vivo derived complexes in EMSA. A number of attempts have been made, but only Max/Max complexes could be faithfully detected (50, 51, 77). By comparison,
a large number of studies demonstrated that in vitro transcribed/translated, bacterially derived, or vaccinia virus-overexpressed and purified proteins or protein fragments of c-Myc and Max bind DNA (12, 13, 27, 31–36, 78, 79). Discrepancies between the studies addressing DNA binding of in vivo versus in vitro derived complexes are most likely related to the difficulties encountered in extracting native c-Myc proteins. Early studies indicated that c-Myc is tightly associated with the nuclear matrix (52). However, this tight association appears to result from the preparation of nuclei in low salt buffers or as a consequence of other forms of stress (69). Also, c-Myc seems to denature relatively quickly and cannot be renatured very efficiently as evidenced by the large amount of protein that has to be used to detect DNA binding in various systems (53, 78).

Since DNA binding is an essential aspect of transcription factor biology and since a number of cases have been described in which the ability to bind DNA is regulated (80), we were interested in developing assay conditions that would allow us to study DNA binding of native, in vivo derived Myc/Max/Mad network complexes. Using physiological salt conditions in the presence of nonionic detergents, c-Myc can be readily solubilized, and c-Myc/Max complexes are preserved (13, 74, 81). Therefore, we have developed conditions based on these observations and on our own experience using whole cell extracts to detect c-Myb-specific DNA binding activity (67). These conditions, in combination with overexpression of the different relevant proteins in COS7 cells, as well as competition experiments and the use of specific antibodies, allowed us to detect DNA-bound c-Myc/Max, Max/Max, Mad1/Max, Mad3/Max, Mad4/Max, Mnt/Max, and USF complexes. The determination of relative DNA binding affinities revealed that c-Myc/Max, Max/Max, and Mad1/Max bound DNA with comparable strength. The identification of these different, COS7-derived complexes was subsequently used to analyze endogenous com-

### TABLE I
**Similar DNA binding affinities of the c-Myc/Max, Max/Max, and Mad1/Max complexes**

| Complex         | Slope$_{on}$ | Slope$_{off}$ | Relative $K_a$ | Relative affinity |
|-----------------|--------------|---------------|----------------|-------------------|
| c-Myc/Max       | 0.122        | 0.250         | 0.490          | 1                 |
| Max/Max         | 0.067        | 0.170         | 0.394          | 0.8               |
| Mad1/Max        | 0.050        | 0.135         | 0.370          | 0.76              |

From the on- and off-rate experiments, the slope of DNA bound/time was measured. The relative $K_a$ was determined as $K_a = \frac{\text{slope}_{on}}{\text{slope}_{off}}$. For comparison of affinities, the relative $K_a$ of the c-Myc/Max complex was set as 1.
c-Myc/Max complexes are indicated by specific serum or mAb 6A10 as indicated. The USF, Max/Max, and supershift experiments were carried out using a USF1- or a Max-rithmically growing HL-60 cells were used in an EMSA. Antibody with pSP-max p22 and pCMV-hu-c-myc or F-buffer lysates from loga-

plexes from HL-60 cells. During differentiation, we observed a decrease in c-Myc/Max and an increase in Max/Max complexes, whereas little change in the level of USF and Mnt/Max complexes was seen. Despite the presence of Mad1 in differenti-

FIG. 7. Identification of endogenous USF, c-Myc/Max, and Max/Max complexes in HL-60 lysates by EMSA. F-buffer lysates from COS7 cells transiently transfected with pCDNAI-USF1 or cotransfected with pSP-max p22 and pCMV-hu-c-myc or F-buffer lysates from loga-

plexes have different relative DNA binding affinities, we performed titration, and on- and off-rate experiments. Collect-

Interestingly, the Max/Max complex was readily detected in HeLa cell extracts, whereas the complex from NIH3T3 cells overexpressing Max p21 bound DNA only after dephosphorylation, suggesting that phosphorylation of Max may regulate its DNA binding activity. Max is phosphorylated at protein kinase CK2 (formerly casein kinase II) sites in vivo (65). We have recently found that COS7-derived Max p21 binds DNA with lower affinity compared with Max p22 (which was used exclus-

regions of the Myc/Max/Mad family members are highly re-

comprising DNA binding capacities of different Myc E-box-

Max binds DNA with a lower affinity than c-Myc/Max com-

phorylation sites will be of interest, since both c-Myc and

Max is the central and essential component of the Myc/Max/

Max/Mad network complexes were competed equally well

phosphorylation at the CK2 sites or whether other as yet

whether this is the result of altered Max phosphorylation at the CK2 sites or whether other as yet undefined mechanisms are responsible for the observed effect is presently unclear and will be the subject of future studies.

By comparing DNA binding of in vitro transcribed/translated or bacterially expressed proteins using in part N-termi-

Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant. Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant. Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant. Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant. Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant.

DNA binding of Myc/Max/Mad complexes

...DNA binding affinities, we performed titration, and on- and off-rate experiments. Collectively, the data indicate that the relative affinity of c-Myc/Max, Max/Max, and Mad1/Max were very similar. Since the basic regions of the Myc/Max/Mad family members are highly related, this finding is not unexpected. Furthermore, the amino acids that were identified to make base contacts in the Max/Max-DNA co-crystal are identical in all members of the Myc/Max/Mad network (for a review, see Ref. 30). Binding site selection studies have shown that in addition to the six-nucleotide core sequence, the flanking sequences are also relevant. Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant. Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant. Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant. Recent analysis of a full-length Max-DNA crystal revealed a contact to a base flanking the core sequence, the flanking sequences are also relevant.

Mad/Max complexes obtained after overexpression in COS7 cells bound readily to Myc E-box sequences with comparable affinity to c-Myc/Max or Max/Max (Figs. 2, 5, and 6). However, we were not able to detect Mad1/Max complexes in whole cell extracts of HL-60 cells, whereas c-Myc/Max complexes could be identified in extracts of exponentially growing cells, which disappeared upon differentiation (Figs. 7 and 8). It is unlikely that the Mad1/Max complexes are unable to bind to DNA, since we observed specific DNA binding using a solid phase DNA bind-
ing assay (84). This assay involves the generation of specific immunocomplexes bound to a support, which can then be tested for their DNA binding activity (84, 85). This assay is more sensitive than an EMSA but does not allow us to determine the composition of the analyzed complexes. Consequently, other explanations have to be considered. An obvious explanation is that the levels of Mad1 are too low to be detected in EMSA, whereas in the solid phase assay the activity of a much larger number of cell equivalents can be analyzed. In support of this explanation, we have not been able to detect Mad1 in HL-60 cell lysates by Western blotting. Another possibility is that the Mad1/Max complexes are distributed into many distinct subcomplexes. This suggestion is based on the findings that Mad1 interacts with Sin3 proteins in vivo and Sin3 binds to several additional proteins including histone deacetylases and N-CoR (65, 86–92). Thus, Mad1/Max complexes may be associated with a variable number of distinct proteins, which would result in many different complexes that individually may not be detected. In contrast, the solid phase DNA binding assay would not distinguish between these complexes.

FIG. 8. TPA down-regulates c-Myc and up-regulates Mad1 expression and affects Max/Max DNA binding in HL-60 cells. a, HL-60 cells were treated with TPA or G-CSF for 0, 2, 6, and 24 h, and total RNA was extracted. RNA samples (15 μg/lane) were separated on a formaldehyde-agarose gel and blotted. The ethidiumbromide staining showed equal loading and transfer in all lanes (data not shown). The blot was subsequently hybridized with probes recognizing c-myc and mad1. The size of the indicated RNA transcripts is 2.4 kilobase pairs for c-myc and 6.5 kilobase pairs (upper band) and 3.8 kilobase pairs (lower band) for mad1. b, HL-60 were treated with TPA for 0, 2, 6, 12, and 24 h and lysed in F-buffer.Equal amounts of protein were separated on SDS-PAGE, blotted to nitrocellulose membrane, and probed for Sin3B (AK-12), c-Myc (N-262), and Max (C-17) as indicated. c, HL-60 cells were treated with TPA for 0, 6, and 20 h and labeled with [35S]methionine/cysteine for 30 min. Immunoprecipitations in AB-buffer lysates with anti-Myc serum IG13 (top) and in F-buffer lysates with mAbs anti-Mad1 5C9 and 5F4 (bottom) were carried out. The arrows indicate the c-Myc proteins of 64 and 67 kDa and Mad1 of 35 kDa. The mobilities of molecular weight markers run on the same gel are shown. d, down-regulation of endogenous c-Myc/Max DNA binding during TPA-induced differentiation of HL-60 cells. HL-60 cells were treated with TPA or G-CSF for 0, 2, 6, and 24 h; F-buffer lysates were prepared; and equal amounts of protein were used in EMSA experiments. For supershift experiments, the mAb 6A10 recognizing c-Myc was used. The assays were carried out in the presence of a minimal amount of anti-USF1 serum to minimize USF binding. This permitted clearer visualization of the c-Myc/Max complex. c-Myc/Max and Max/Max complexes are indicated. The circle denotes a nonspecific complex, and the asterisk shows the Mnt/Max complex.
Unlike Myc and Mad proteins, the newly identified Max partner Mnt (or Rox) is expressed constitutively (25, 26). Our findings suggest that the Mnt/Max heterodimer is the most prominent Myc/Max/Mad network complex that we can detect in COS7 and HL-60 whole cell extracts. The identification of the Mnt/Max complex is based on its sensitivity to several Myc-specific antisera (see e.g. Fig. 2) and its comigration with COS7 cell-derived overexpressed Mnt/Max. In addition, the Mnt/Max complex is also supershifted using a Mnt-specific antiserum. The apparent binding activity of this complex is not altered during differentiation (Fig. 8), and it has been detected in extracts of a wide variety of different murine tissues and cell lines. Since Mnt can negatively regulate Myc function, these findings suggest that other forms of regulation of Mnt function may exist. One possibility is that the interaction of Mnt with Sin3 could be modulated and thus the recruitment of regulators of gene transcription, including N-CoR and histone deacetylases.

Besides the Myc/Max/Mad network complexes, we have observed USF as a major Myc E-box-binding factor (Figs. 3 and 7). USF complexes contain two proteins of 43 kDa (USF1) and 44 kDa (USF2), which can form homo- and heterodimers (75). Similar to the Mnt/Max complex, the binding of USF to DNA appears to be rather constant during HL-60 differentiation. Since the USF complexes are very prominent, we have made attempts to use oligonucleotides that could distinguish between Myc/Max/Mad network complexes and USF. In binding site selection studies, evidence for such sequences has been obtained (36). Accordingly, we used an oligonucleotide (M36) that would have been expected to interact selectively with c-Myc and Max and Max but not USF. However, in this study (Fig. 4), M36 bound poorly to the c-Myc/Max complex and failed to interact with USF or Max/Max, suggesting that the proteins used in the two studies do not display comparable activities. A likely explanation is that denatured and renatured complexes have been used (36). We have not been able to compare the two protocols directly, since we were only able to recover Max but not c-Myc/Max DNA binding activity from denatured and renaturated immunoprecipitated Max complexes.

In conclusion, we have developed conditions for electrophoretic mobility shift assays that allow us to measure the specificity and the affinity of different Myc/Max/Mad network complexes. This has not been achieved previously, most likely due to the difficulties in handling c-Myc proteins as summarized above. Furthermore, using the established protocol, we have shown that some of the Myc/Max/Mad network complexes can be detected in HL-60 cells and that the DNA binding activities of the c-Myc/Max and Max/Max dimers are regulated during differentiation. In continuation of our findings, it will now be possible to analyze and determine the DNA binding activity of proteins isolated from cells subjected to various treatments as well as of mutant proteins. Thus, our findings provide a framework to further enhance our understanding of the regulation and functional role of the transcriptional regulators of the Myc/Max/Mad network.

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*References* are cited in the text.
53. Papoulas, O., Williams, N. G., and Kingston, R. E. (1992) J. Biol. Chem. 267, 10470–10480
54. Waters, C. M., Littlewood, T. D., Hancock, D. C., Moore, J. P., and Evan, G. I. (1991) Oncogene 6, 797–805
55. Gregor, P. D., Sawadogo, M., and Roeder, R. G. (1990) Genes Dev. 4, 1730–1740
56. Li, L.-H., Nerlov, C., Prendergast, G., MacGregor, D., and Ziff, E. B. (1994) EMBO J. 13, 4070–4079
57. Beckmann, H., Su, L.-K., and Kadesh, T. (1990) Genes Dev. 4, 167–179
58. Carr, C. S., and Sharp, P. A. (1990) Mol. Cell. Biol. 10, 4384–4388
59. Bousset, K., Oelgeschlager, M., Henriksson, M., Schreek, S., Burkhardt, H., Litcheff, D. W., Luscher-Firzlaff, J. M., and Luscher, B. (1994) Cell. Mol. Biol. Res. 40, 501–511
60. Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J. M., Varmus, H., and Lee, W. (1987) Mol. Cell. Biol. 7, 1697–1709
61. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
62. Halazonetis, T. D., and Kandil, A. (1992) Science 255, 464–466
63. Bousset, K., Henriksson, M., Luscher-Firzlaff, J. M., Litcheff, D. W., and Luscher, B. (1993) Oncogene 8, 3211–3220
64. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, M. (1985) Mol. Cell. Biol. 5, 3610–3616
65. Sommer, A., Hilfenhaus, S., Menkel, A., Kremmer, E., Loidl, P., and Luscher, B. (1997) Cell. Mol. Biol. Res. 40, 501–511
66. Timchenko, N., Wilsom, D. R., Taylor, L. R., Abdelsayed, S., Wilde, M., Sawadogo, M., and Darlington, G. J. (1995) Mol. Cell. Biol. 15, 1192–1202
67. Oelgeschlager, M., Krieg, J., Luscher-Firzlaff, J. M., and Luscher, B. (1995) Mol. Cell. Biol. 15, 5966–5974
68. Henriksson, M., Bakardjieva, A., Klein, G., and Luscher, B. (1993) Oncogene 8, 3199–3209
69. Luscher, B., and Eisenman, R. N. (1988) Mol. Cell. Biol. 8, 2504–2512
70. Campbell, A. M., Kessler, P. D., and Fambrough, D. M. (1992) J. Biol. Chem. 267, 9321–9325
71. Ceglarek, J. A., and Revzin, A. (1989) Electrophoresis 10, 360–365
72. Fried, M. G. (1989) Electrophoresis 10, 360–365
73. Collins, S. J. (1987) Blood 70, 1233–1244
74. Ayer, D. E., and Eisenman, R. N. (1993) Genes Dev. 7, 2110–2119
75. Sirio, M., Lin, Q., Maity, T., and Sawadogo, M. (1994) Nucleic Acids Res. 22, 427–433
76. Begley, C. G., Metcalf, D., and Niccola, N. A. (1987) Int. J. Cancer 39, 99–105
77. Nozaki, N., Naoe, T., and Okazaki, T. (1997) J. Biochem. 121, 550–559
78. Walbou, A. J. M., Gubbia, J. M., Bernard, B., van der Vliet, P. C., and Timmers, H. T. M. (1997) Nucleic Acids Res. 25, 1493–1501
79. Kato, G. J., Lee, W. M. F., Chen, L., and Dang, C. V. (1992) Genes Dev. 6, 81–92
80. Fabbri, C. O., and Sauer, R. T. (1992) Annu. Rev. Biochem. 61, 1053–1095
81. Evan, G. I., and Hancock, D. C. (1985) Cell 43, 253–261
82. Lutterbach, B., and Henn, S. R. (1997) J. Biol. Chem. 272, 967–975
83. Brownlie, P., Ceska, T. A., Lamers, M., Romier, C., Stier, G., Ten, H., and Suck, D. (1997) Cell 89, 509–520
84. Larsson, L.-G., Bahram, F., Burkhardt, H., and Luscher, B. (1997) Oncogene 15, 737–748
85. Larsson, L.-G., Bahram, F., Wu, S., Ober, F., Nilsson, K., and Luscher, B. (1997) Curr. Topics Microbiol. Immunol. 224, 191–200
86. Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997) Nature 387, 49–55
87. Hassig, C., Fleischer, T., Billin, A. N., Schreiber, S. L., and Ayer, D. E. (1997) Cell 89, 341–347
88. Heinzel, T., Lavinsky, R. M., Mullen, T.-M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W.-M., Brard, G., Ngo, D. D., Davie, J. R., Zets, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 45–48
89. Kadosh, D., and Struhl, K. (1997) Cell 89, 365–371
90. Laherty, C. D., Yang, W.-M., Sun, J.-M., Davie, J. R., Zets, E., and Eisenman, R. N. (1997) Cell 89, 394–399
Identification and Characterization of Specific DNA-binding Complexes Containing Members of the Myc/Max/Mad Network of Transcriptional Regulators
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