c-Myc/Max heterodimers bind cooperatively to the E-box sequences located in the first intron of the rat ornithine decarboxylase (ODC) gene

A. J. M. Walhout, J. M. Gubbels[+], R. Bernards[1], P. C. van der Vliet and H. Th. M. Timmers*

Laboratory for Physiological Chemistry, Utrecht University, PO Box 80042, 3508 TA Utrecht, The Netherlands and [1]Division of Molecular Carcinogenesis, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

ABSTRACT

The oncoprotein c-Myc plays an important role in cell proliferation, transformation, inhibition of differentiation and apoptosis. These functions most likely result from the transcription factor activity of c-Myc. As a hetero-dimer with Max, the c-Myc protein binds to the E-box sequence (CACGTG), which is also recognized by USF dimers. In order to test differences in target gene recognition of c-Myc/Max, Max and USF dimers, we compared the DNA binding characteristics of these proteins *in vitro* using vaccinia viruses expressing full-length c-Myc and Max proteins. As expected, purified c-Myc/Max binds specifically to a consensus E-box. The optimal conditions for DNA binding by either c-Myc/Max, Max or USF dimers differ with respect to ionic strength and Mg^{2+} ion concentration. Most interestingly, the c-Myc/Max complex binds with a high affinity to its natural target, the rat ODC gene, which contains two adjacent, consensus E-boxes. High affinity binding results from the ability of c-Myc/Max dimers to bind cooperatively to these E-boxes. We propose that differential cooperative binding by E-box binding transcription factors could contribute to target gene specificity.

INTRODUCTION

The oncoprotein c-Myc plays a pivotal role in important cellular processes such as proliferation, suppression of differentiation and apoptosis (reviewed in refs 1–4). The mechanism by which c-Myc exerts its functions is not completely understood. It has been shown that c-Myc has properties of a regulatory transcription factor *in vivo* (5–7) and integrity of this activity is required both for transformation and for apoptosis (8,9).

Like most regulatory transcription factors c-Myc contains two separable domains, a C-terminal dimerization and DNA binding domain of the basic region/helix–loop–helix/leucine zipper (BR/HLH/LZ) class, and an N-terminal transactivation domain. Via its BR/HLH/LZ domain c-Myc dimerizes with its partner

Max and the resulting heterodimer binds specifically to the E-box sequence CACGTG (10–13). The N-terminal domain of c-Myc mediates activation of transcription possibly via direct contacts with components of the basal transcription machinery, as has been shown for the basal transcription factor, the TATA binding protein TBP (14,15). Both the interaction with Max and transactivation are essential for the biological function of c-Myc (8,9,16,17).

In contrast to c-Myc, Max can form homodimers which are also capable of binding to E-box sequences. However, Max lacks a transcription activation domain and Max homodimers are transcriptionally inactive. Therefore Max homodimers are thought to repress c-Myc action in a passive manner (7,9,18). Other Max-dimerization partners include the Mad proteins, which are also members of the BR/HLH/LZ protein family (19–21). Mad/Max heterodimers antagonize the function of c-Myc/Max by active repression of transcription. For this the interaction with the co-repressor Sin3 is essential (22,23). Mad expression is induced upon differentiation and induction of Mad correlates with a decline in c-Myc expression and withdrawal from the cell cycle (24,25).

Besides members of the Max transcription network, other BR/HLH/LZ transcription factors also function through binding to E-boxes. One of these, the upstream stimulating factor USF (or MLTF) does not dimerize with c-Myc, Max or Mad and only forms dimers with other USF proteins (26,27). Co-crystal structures of both truncated Max and USF dimers with their target DNA sites have been determined (28,29). These structures showed that both dimers contact DNA in a similar manner. The dimers fold into a parallel, left-handed, four-helix bundle upon DNA binding. Amino acids located in their basic regions directly contact the E-box bases in the major groove and also make contact with the DNA backbone.

Although both c-Myc and USF dimers bind to the E-box CACGTG, they regulate the expression of different target genes. USF was first identified as a protein factor that activates transcription of the adenovirus major late gene via binding to the E-box located in the promoter of this gene (26,30,31). Despite its clear function in tumorigenesis and apoptosis only few target genes of c-Myc have been identified. These include the ornithine decarboxylase (ODC) and prothymosin α genes which contain

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*To whom correspondence should be addressed. Tel: +31 30 253 8981; Fax: +31 30 253 9035; Email: h.t.m.timmers@med.ruu.nl

[+]Present address: Department of Infectious Diseases and Immunology, Yalelaan 1, PO Box 80165, 3508 TD Utrecht, The Netherlands

two and one consensus E-boxes in their first intron, respectively (32–36). The ODC gene product is the first and rate limiting enzyme involved in polyamine synthesis and is required for cell proliferation. Furthermore, ODC is involved in c-Myc induced apoptosis (37). The function of prothymosin α is not known. Recently, the human cdc25A gene has been shown to be a target gene of c-Myc. In this gene the E-boxes through which activation by c-Myc is accomplished are also located downstream of the transcription start site (38). In contrast, the eukaryotic initiation factor 4E (eIF4E) gene, which is also responsive to c-Myc, contains E-boxes in a promoter-proximal position (39).

In addition to activation, repression of transcription by USF and c-Myc has also been reported. It is thought that repression is mediated via the initiator element (Inr) located at the transcription start site of many promoters (40–42).

It has been difficult to study the DNA binding and transactivation properties of c-Myc proteins in *in vitro* assays since full length c-Myc is a labile and extremely insoluble protein in various expression systems like bacteria, CHO cells and baculovirus-infected insect cells. Several approaches have been employed to circumvent the solubility problem. Either denatured and renatured full length c-Myc protein, *in vitro* translated proteins, chimaeric proteins or the isolated BR/HLH/LZ domain have been used (13,43–46). A disadvantage of these approaches is that the produced c-Myc proteins are either incomplete, may be misfolded or not properly modified. In order to examine the difference in target gene specificity between USF and c-Myc we constructed recombinant vaccinia viruses allowing expression of histidine-tagged full length c-Myc and Max proteins in mammalian cells and compared the DNA binding properties of purified c-Myc/Max heterodimers with USF and Max dimers on a natural c-Myc target, the rat ODC gene.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from ICN Biomedicals. Herring sperm DNA (HS-DNA), dithiothreitol (DTT), isopropyl β-D-thiogalactopyranoside (IPTG), BrdU and restriction enzymes were purchased from Boehringer Mannheim. Phenyl-methylsulfonylfluoride (PMSF), protease inhibitors, xanthine, hypoxanthine and mycophenolic acid (MPA) were obtained from Sigma. T4 polynucleotide kinase, Ficoll-400, oligonucleotides and chromatography media were obtained from Pharmacia. Protein markers were obtained from BRL. Nitrocellulose (BA45) filters were obtained from Schleicher & Schuell. Tissue culture media were from Imperial.

Plasmids

The 507 bp human short Max open reading frame (ORF) was obtained by PCR using pBSK-Max as template, a 5′-primer containing a *Nde*I site and the M13 reverse primer. The PCR product was digested with *Nde*I and *Bam*HI and cloned into pET15b (Novagen) digested with *Nde*I and *Bam*HI. The PCR fragment was verified by sequence analysis (T7 sequencing kit, Pharmacia). The resulting pET-His-Max plasmid encodes short Max with 20 additional N-terminal amino acids, MGSSHHHHH-HSSGLVPRGSH. pTM3-Max was obtained by cloning the Max ORF (obtained by PCR using a 5′ oligo and M13 reverse primer, creating a 5′ *Rca*I site) into pTM3 (47) digested with *Nco*I and

*Bam*HI. pTM3-His-c-Myc was obtained by cloning the hybridized oligos 5′-CATGGGGCACCACCATCACCACCATCATG-3′ and 5′-CCCGTGGTGGTAGTGGTGGTAGTACTTAA-3′ into pBSKS-ATG-Flag digested with *Nco*I and *Eco*RI, generating pBSK-ATG-His. The 1.3 kb c-Myc ORF was digested from pSP64-c-Myc using *Hinc*II and *Sac*I and cloned in pBSK-ATG-His digested with *Sma*I and *Sac*I. His-c-Myc was then cloned into pTM3 using *Nco*I and *Sac*I generating pTM3-His-Myc which encodes c-Myc with 17 additional amino acids on the N-terminus, MGHHHHHHHHEFLQPDSS.

pAlter-rODC was made by cloning the 478 bp *Eco*RI–*Hin*dIII rODC fragment from pUC13-rODC (48) into pAlter (Promega) digested with *Eco*RI and *Hin*dIII. pAlter-rODC box1m in which E-box 1 was mutated into CACGCG was made using the Altered sites II *in vitro* mutagenesis system (Promega) with the oligo 5′-GGGCCTCGCGACGCGTGGCCGCACA-3′ creating an *Mlu*I restriction site. pAlter-rODC∂box1 was constructed by digesting pAlter-rODCbox1m with *Mlu*I, treatment with S1 nuclease and religation. This resulted in a deletion of 20 bp including E-box 1. Both mutant constructs were verified by sequencing (T7 sequencing kit, Pharmacia).

Construction of recombinant vaccinia viruses

Both His-c-Myc and Max were cloned into pTM3 which carries the gene for the *Escherichia coli* enzyme guanine phosphorybosyl transferase (gpt), enabling positive selection for recombinants. Recombinant vaccinia viruses, vv-His-c-Myc and vv-Max, were made as described (49). First, recombinant viruses were selected by infection of HuTK⁻ cells in the presence of 25 μg/ml BrdU. Individual plaques were picked and positive plaques were identified by PCR using a pTM specific primer and either a c-Myc or Max specific primer. Subsequently, two rounds of gpt-selection were performed by culturing infected RK13 cells in the presence of 250 μg/ml xanthine, 15 μg/ml hypoxanthine and 25 μg/ml MPA. Positive plaques were amplified and the titer of the virus stocks was determined. Virus stocks were stored at –20°C. Prior to infection, virus stocks were sonicated for 25 s and then kept on ice.

Protein expression and purification

Max homodimers were purified from *E.coli* BL21(DE3)-pLysS containing pET-His-Max. Expression of Max was induced by addition of 0.4 mM IPTG for 2 h at 37°C. After centrifugation, bacteria were resuspended in lysis buffer (50 mM Tris–HCl pH 8.0, 20% sucrose, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 0.2 mM sodium-metabisulfite, 1 mM PMSF, 1 mg/l leupeptine, 2 mg/l aprotinin) containing 0.1% Triton X-100 and 50 mg/l lysozyme. One volume high salt buffer (50 mM Tris–HCl pH 8.0, 0.6 M KCl, 0.05 mM EDTA, 5 mM β-mercaptoethanol, 0.2 mM sodium-metabisulfite, 1 mM PMSF, 1 mg/l leupeptine, 2 mg/l aprotinin) was added and the extract was centrifuged for 15 min in a SS34 rotor at 12 000 r.p.m. The supernatant was sonicated and subsequently centrifuged for 45 min at 45 000 r.p.m. in a 50 Ti rotor at 4°C. Max was purified to apparent homogeneity as judged by Coomassie blue staining of protein gels. Briefly, the protein was bound to Ni-NTA agarose beads in batch, the matrix was washed and Max was eluted in buffer T (50 mM Tris–HCl pH 8.0, 10% glycerol, 100 mM KCl, 5 mM β-mercaptoethanol, 0.3 M imidazole).
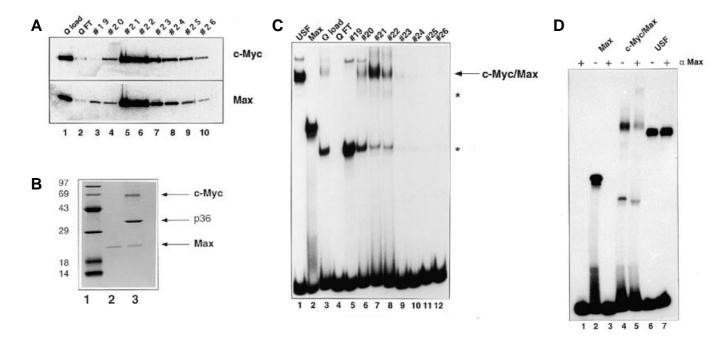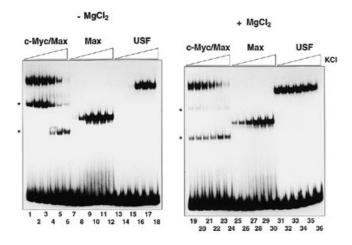
Figure 1. Purification of c-Myc/Max heterodimers. (**A**) Immunoblot analysis of final column protein fractions using a polyclonal Max antiserum and the c-Myc monoclonal antibody 9E10. Lanes 1–10 contain 0.7 µl of Mono Q fractions as indicated above the lanes. Comparison of the intensities of the bands with known amounts of bacterially expressed Max indicates that the concentration of the c-Myc/Max heterodimer in fractions #21 and #22 is ~750 µg/ml. (**B**) Coomassie blue staining of 2.5 µl of Mono Q fraction #21. Lane 1, molecular weight protein marker; lane 2, 250 ng purified Max. p36 indicates a protein of 36 kDa that copurifies with the c-Myc/Max complex. (**C**) Gelshift analysis using 0.5 µl Mono Q fractions as indicated above the lanes (3–12). Lane 1, 10 ng USF (20 ng/µl based on known affinity and compared to Max); lane 2, 50 ng purified Max. pCM1, which contains the optimal c-Myc/Max E-box cacCACGTGgtg, was used as a probe. The asterisks indicate non-specific complexes which do not contain either c-Myc or Max proteins. (**D**) Gelshift analysis in the presence of 0.8 µl Max antiserum as indicated. Lane 1, Max antiserum alone; lanes 2 and 3, 80 ng Max homodimer; lanes 4 and 5, 10 ng USF; lanes 6 and 7, 750 ng c-Myc/Max.

For purification of c-Myc/Max heterodimers, 12 l of Hela S3 cells was infected with vv-His-c-Myc at a multiplicity of infection (moi) of 2 plaque forming units (p.f.u.) per cell and vv-Max at an moi of 0.5 p.f.u./cell. Since both expression of c-Myc and Max are under control of the T7 promoter, cells were coinfected with a vaccinia virus expressing T7 RNA polymerase (vv-T7pol) (50) at an moi of 5 p.f.u./cell. Cells were incubated for 2 h at 37°C in 1/20 vol serum free medium, then the volume was adjusted to 12 l by adding medium containing 10% fetal calf serum and cells were maintained for another 21 h at 32°C. These conditions result in the highest amounts of soluble c-Myc/Max heterodimers. Infected cells were harvested, washed in ice-cold PBS, dounced six times in buffer T300 (50 mM Tris–HCl pH 8.0, 300 mM KCl, 10% glycerol, 0.1% NP-40, 0.05 mM EDTA, 0.5 mM PMSF, 0.2 mM sodium-metabisulfite, 1 mg/l leupeptine, 2 mg/l aprotinin and 5 mM β-mercaptoethanol) and sonicated three times for 25 s, while keeping on ice in between. After centrifugation for 30 min in a SW41 rotor at 30 000 r.p.m. at 4°C, the extract was loaded on a DEAE Sepharose Fast Flow column equilibrated in buffer T300. The flow-through was loaded on a Ni-NTA column equilibrated in buffer T300. After washing in buffer T100 (100 mM KCl) with 16 mM imidazole, proteins were eluted using a linear gradient in buffer T100 from 16 to 400 mM imidazole. Fractions containing c-Myc/Max (as judged by immunoblotting) were pooled and loaded on a Mono Q column (HR 5/5, Pharmacia) equilibrated in buffer B (20 mM Tris–HCl pH 8.0, 20% glycerol, 0.5 mM EDTA, 0.1% NP-40, 0.5 mM PMSF, 1 mM DTT, 1 mg/l leupeptin, 2 mg/l aprotinin, 0.2 mM sodium-metabisulfite) plus 0.1 M KCl. Bound proteins were eluted using a linear gradient in buffer B from 0.1 to 1 M KCl. The c-Myc/Max heterodimer elutes at around 0.3 M KCl.

USF (kindly provided by L. Chodosh) was purified from calf brain (51).

Gelshift analysis

The 70 bp E-box probe pCM1, which contains an optimal, artificial c-Myc/Max binding site cacCACGTGgtg, was labeled by digesting pCM1 with *Eco*RI and *Hin*dIII, and filling in the ends using Klenow DNA polymerase and [α-^{32}P]dCTP. The 74 bp MLP probe, containing the E-box located in the adenovirus major late promoter, was labeled as described for pCM1. The 151 bp rODC probe was labeled by digesting pAlter-rODC, which contains the +10 to +443 part of the rat ornithine decarboxylase (rODC) gene, with *Nco*I and *Pvu*II and end-filling the *Nco*I site with [α-^{32}P]dCTP. rODC mutant probes were labeled identically. Double stranded oligo probes were made by annealing the oligonucleotides 5′-CTCAGGCACCACGTGGTGGGGGAT-3′ and 5′-ATCCC-CCACCACGTGGTGCCTGAG-3′ to make the CM1 probe and 5′-GCCTCGCGACACGCGGCCGCACAATC-3′ and 5′-GATT-GTGCGGCCGCGTGTCGCGAGGC-3′ to make the CACGCG probe. Probes were labeled by phosphorylating 5′-ends using T4 polynucleotide kinase and [γ-^{32}P]ATP. Reactions were done in a 15 µl volume either in buffer T (12 mM Tris–HCl pH 8.0, 12% glycerol, 0.1 M KCl, 0.6 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 0.4 mg/ml BSA) (experiments of Figs 1C and D, 2 and 3) or in retardation buffer (20 mM HEPES pH 8.0, 4% Ficoll-400, 1 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 0.025% NP-40) (experiments of Figs 1D and 4) with 25 µg/ml HS-DNA and 0.3 pmol probe unless described differently in the legends. After incubation for
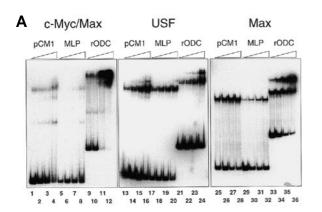
Figure 2. c-Myc/Max, Max and USF have different salt and $MgCl_2$ requirements for DNA binding. Gelshift analysis using pCM1 as a probe. The KCl concentration was varied in the absence or presence of 5 mM $MgCl_2$. KCl concentrations of the reactions are 10, 25, 50, 100, 200 and 400 mM in each panel. Lanes 1–6 and 19–24, 350 ng c-Myc/Max; lanes 7–12 and 25–30, 100 ng Max; lanes 13–18 and 31–36, 8 ng USF. The asterisks indicate non-specific DNA–protein complexes, which do not contain c-Myc or Max proteins.

30 min at 30°C, the samples were run on 6 or 7.5% (Fig. 3B) native polyacrylamide gels in TBE at 200 V for 4 h at 4°C.

DNAseI footprinting

The bottom strand of the rODC gene was labeled as described above for gelshift analysis. Binding reactions were done as described for gelshift analysis in 50 µl in buffer T. DNaseI (0.1 U) was added and after 40 s reactions were stopped by the addition of 1 vol stopmix (0.4% SDS, 40 mM EDTA and 20 µg/ml HS-DNA). After phenol extraction the DNA was precipitated and loaded in denaturing DNA loading buffer (50% formamide, 10 mM NaOH) on a 5.5% sequencing gel. Quantifications of both gelshifts and footprints were done by using PhosphorImager gel scanner (Molecular Dynamics, Sunnyvale, CA) and Imagequant 5.25 software.
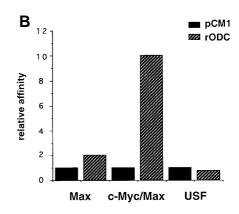
Antibodies and immunoblotting

The monoclonal anti-c-Myc antibody 9E10 was purified from 2 l hybridoma cells using protein A-sepharose column chromatography (Biorad) as described (52). The polyclonal anti-Max antiserum was obtained by immunizing rabbits with human Max (11) that was purified as a His-tagged protein from *E.coli*. Immunoblots were performed using nitrocellulose and filters were blocked in TBS-Tween (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% dry-milk. Filters were incubated with the antibodies described in the legends of the figures followed by incubation with goat-anti-mouse (9E10) or goat-anti-rabbit (anti-Max) coupled to horseradish peroxidase (HRP). After washing, bound HRP was visualised by enhanced chemiluminescence (ECL, Amersham).

RESULTS

Purified full-length native c-Myc/Max heterodimers are active in E-box binding

In order to obtain full-length native c-Myc/Max heterodimers, we constructed recombinant vaccinia viruses encoding histidine-tagged c-Myc and native Max. High levels of expression were achieved by coinfection with a vaccinia virus expressing T7 RNA polymerase. After optimization of extraction and purification procedures, ~1 mg of c-Myc/Max complex was purified from 12 l human HeLa S3 cells (6×10^9 cells). Figure 1A shows an immunoblot of final protein fractions (mono Q column chromatography) using a polyclonal Max antiserum and the c-Myc monoclonal antibody 9E10. Fractions #21 and #22 (Fig. 1A, lanes 5 and 6, Fig. 1B, lanes 6 and 7) contain the peak of c-Myc and Max proteins. In Figure 1B a Coomassie blue stained protein gel of peak fraction #21 is shown (lane 3). The heterodimer concentration is ~750 ng/µl. A protein of ~36 kDa (p36) copurifies with c-Myc/Max and we are presently investigating the function of this protein. The DNA binding activity of the purified c-Myc/Max heterodimers was tested using the optimal c-Myc/Max binding site cacCACGTGgtg (pCM1) as a probe. Although fractions #21 and #22 contain the same amount of c-Myc and Max protein, Figure 1C shows that the DNA binding activity of the

Figure 3. c-Myc/Max binds with high affinity to the rODC E-boxes. (**A**) Gelshift analysis using different E-boxes as probes. pCM1 is a synthetic, optimal E-box for c-Myc/Max, MLP is the E-box of the adenovirus MLP (from –60 to –55), rODC is the rODC intron which contains two consensus E-boxes (at positions +214 to +219 and +255 to +260). The three dimers were titrated in 3-fold dilutions as indicated. Highest amounts used were: 750 ng c-Myc/Max (lanes 4, 8 and 12), 20 ng USF fraction (lanes 16, 20 and 24) and 100 ng Max (lanes 28, 32 and 36). Equimolar amounts of probe were used as in Figure 2. (**B**) Relative affinity of the three dimers for pCM1, containing one E-box and rODC, containing two E-boxes. Large protein titrations were done using the two probes (data not shown) and the difference in amount of protein required to bind 50% of the DNA was used as relative affinity.
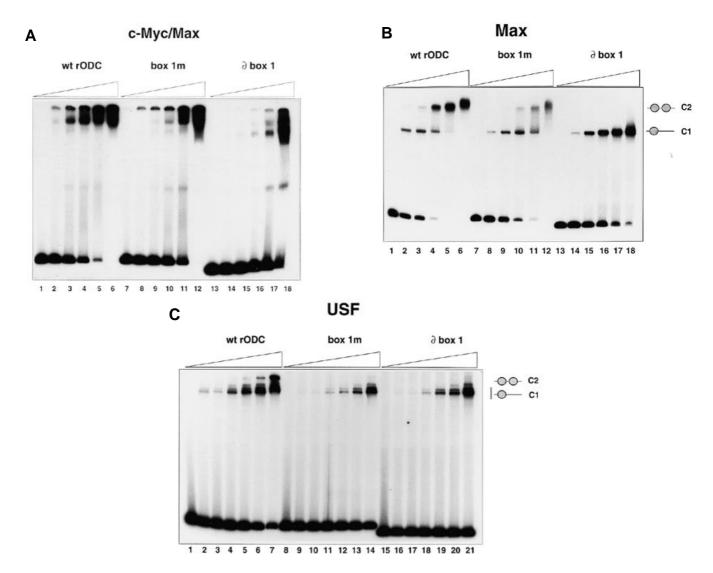
Figure 4. c-Myc/Max binds cooperatively to the rODC E-boxes. Gelshift analysis using probes derived from the first intron of the ODC gene. The wild type rat ODC fragment (wt rODC) contains the two E-boxes and flanking sequences, in the box 1m fragment the most proximal E-box (E-box 1) was mutated into CACGCG. In the ∂ box 1 fragment, 20 bp were deleted including E-box 1 (see also Fig. 6). (**A**) Lanes 6, 12 and 18, 900 ng c-Myc/Max, dilutions were 3-fold as indicated; lanes 1, 7 and 13, no added protein. (**B**) Lanes 6, 12 and 18, 300 ng Max (Max was titrated with 3-fold dilutions as indicated); lanes 1, 7 and 13, no added protein. (**C**) Eight ng of USF (lanes 7, 14 and 21) was titrated in 2-fold dilutions. Lanes 1, 8 and 15 show only free probe. C1 indicates the first and C2 the second DNA–protein complex formed.

heterodimer peaks in fraction #21 and therefore this fraction was used in the following experiments. To analyze the protein composition of the observed complexes, we added antibodies to the binding reactions. Figure 1D shows that the slowest migrating complex contains the c-Myc/Max heterodimer since this complex can be supershifted by the addition of Max antiserum (Fig. 1D, lanes 4 and 5). Binding of Max homodimers is completely abolished by the antibody whereas, as expected, binding of USF is not affected. (Fig. 1D, lanes 2, 3, 6 and 7). As already noted by others (39), the addition of different Myc antibodies, like 9E10, had no effect on the formation and migration of the protein–DNA complexes (data not shown).

c-Myc/Max has different DNA binding characteristics compared to Max homodimers and USF

In order to investigate target gene specificity of c-Myc/Max heterodimers and Max and USF dimers, the DNA binding

properties of the different complexes were compared. The DNA binding characteristics of native, purified USF have already been studied in more detail (26). Figure 2 shows a gelshift assay using pCM1 as a probe. The ionic strength was varied either in the absence or presence of 5 mM $MgCl_2$. Binding of c-Myc/Max and Max homodimers does not depend on the addition of $MgCl_2$ to the binding reaction (Fig. 2, lanes 1–12 and 19–30). However, one of the two non-specific complexes present in the c-Myc/Max fraction, disappears when $MgCl_2$ was included in the binding reaction (Fig. 2, compare lanes 1–6 with lanes 19–24). c-Myc/Max shows an optimum in DNA binding at 40 mM KCl (lanes 2 and 3) whereas Max homodimers have a binding optimum at ~150 mM KCl (lanes 10 and 11). Binding of USF is dramatically influenced both by salt and by $MgCl_2$ (Fig. 2, lanes 13–18 and 31–36). In the absence of $MgCl_2$, binding of USF requires relatively high salt concentrations, with an optimum at 200 mM KCl. In contrast, in the presence of 5 mM $MgCl_2$, binding of USF is largely
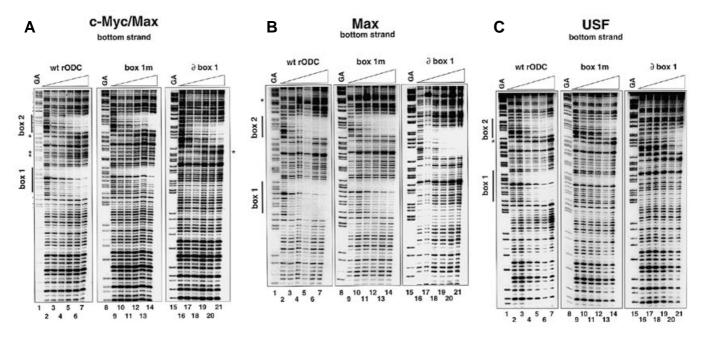
Figure 5. Mutation of one E-box in the ODC enhancer results in a lower affinity of c-Myc/Max for both sites. DNAseI footprinting analysis using the bottom strand of the rODC first intron and mutants box 1m and ∂ box 1 as probes. In lanes 2, 9 and 16 no protein was added. All proteins were titrated in 2-fold dilutions. Lanes 7, 14 and 21 contain respectively 750 ng c-Myc/Max (Mono Q fraction #21, **A**), 1000 ng bacterially expressed Max (**B**) or 160 ng USF (**C**). E-box 1 and 2 are indicated as box 1 and box 2 and protected regions are indicated by black bars. Asterisks indicate DNAseI hypersensitive sites. GA shows G+A tracts of the different probes.

independent of the addition of salt. This shows that for binding of USF the addition of 5 mM $MgCl_2$ can substitute for the addition of salt. (Fig. 2, lanes 1–6 and 19–24). Chodosh *et al.* (26) found that USF binding decreases at high salt concentrations in the presence of $MgCl_2$. This discrepancy could be the result of different reaction conditions, different DNA probes or the purity of the protein fraction used. However, our direct comparison between c-Myc/Max and USF using identical conditions and DNA fragments shows that the optimal binding conditions for each dimer are different. In the following experiments binding reactions contained 100 mM KCl and 5 mM $MgCl_2$.

c-Myc/Max binds with high affinity to the rODC element which contains two E-boxes

Since c-Myc and USF regulate transcription of different target genes and in the experiments described thus far a synthetic binding site was used, we analyzed the DNA binding characteristics of c-Myc/Max, Max and USF dimers to two natural target sites. The adenovirus major late promoter (MLP) contains one E-box required for activation of transcription by USF (26,30,31). The rODC gene contains two conserved consensus E-boxes in the first intron which are essential for activation of ODC gene expression by c-Myc *in vivo* (32,33). Using gelshift analysis we compared binding of c-Myc/Max heterodimers, Max homodimers and USF dimers with pCM1, MLP and rODC (Fig. 3). In contrast to USF and Max, c-Myc/Max binds with different affinities to these three elements (Fig. 3A, lanes 1–12). The affinity of c-Myc/Max for the rODC probe is significantly higher than for the single E-box containing pCM1 and MLP probes. In addition, as for the single E-box probes, only one specific DNA–protein complex is formed by c-Myc/Max on the double E-box containing rODC probe (Fig. 3A, lanes 9–12). This may indicate either that binding of one dimer excludes binding of a second dimer or that both E-boxes are

simultaneously bound by two dimers. USF binds with very similar affinities to both the pCM1, MLP and rODC elements (Fig. 3A, lanes 13–24). Max homodimers bind with a lower affinity to the MLP and pCM1 probes compared with the rODC element (Fig. 3A, lanes 25–36). Although this is not clear from this particular experiment, repetition of this experiment clearly indicates that Max binds with comparable affinities to pCM1 and MLP (data not shown). Both for USF and Max dimers two DNA–protein complexes are formed when the rODC element is used as a probe. Most likely, the faster migrating complex represents occupation of one E-box whereas the slower migrating complex consists of two dimers bound to DNA (Fig. 3A, lanes 21–24 and 33–36). With USF multiple bands can be seen in the faster migrating complex (Fig. 3A). This is most likely due to the presence of different forms of USF dimers (53). Quantifications of wide protein titrations showed that the affinity of Max is ~2-fold higher for rODC, whereas USF has a somewhat higher affinity for pCM1. However, c-Myc/Max binds with a 10-fold higher affinity to rODC compared to pCM1 (Fig. 3B). Taken together with the observation that one protein–DNA complex is formed by c-Myc/Max in this experiment, this suggests that c-Myc/Max binds to the two E-boxes of the rODC gene in a synergistic manner.

c-Myc/Max binds cooperatively to the E-boxes of the rODC gene

To investigate synergistic binding of c-Myc/Max to the E-boxes of the rODC gene, mutations were introduced in the proximal E-box (box 1, see also Fig. 6). In the first mutant the proximal E-box was mutated into CACGCG (box 1m) and in the second mutant box 1 was deleted (∂ box 1). Both for Max (Fig. 4B, lanes 13–18) and for USF dimers (Fig. 4C, lanes 15–21) deletion of box 1 results in the formation of one DNA–protein complex (C1), which runs at the same position as the faster complex with the
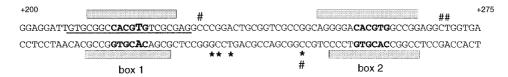
Figure 6. Schematic overview of DNAseI footprinting results on the wild type rODC intron. Bars indicate protection from cleavage by DNAseI of the three different dimers. Asterisks show DNAseI hypersensitive sites induced by c-Myc/Max binding, # hypersensitive sites induced by binding of USF. Positions relative to the transcription start site are indicated. E-boxes are depicted in bold. In mutant box 1m the T of the proximal E-box was mutated into a C and in∂ box 1 the underlined basepairs were deleted.

wild type probe. This indicates that the C1 complex represents binding of a single dimer. The point mutation in E-box 1 hardly affects formation of the C1 complex with Max (Fig. 4B, compare lanes 2 and 8). This shows that the affinity of Max for a single E-box, in this case box 2, remains unchanged. Adding higher amounts of protein results in the formation of the second complex C2 (Fig. 4B, lanes 7–12) although at a lower efficiency. This indicates that Max has a low affinity for the mutated E-box (see also below). Formation of C1 with USF is somewhat less efficient with the box 1m probe (Fig. 4C compare lanes 5 and 14) and binding of USF to the mutated E-box was no longer observed. In Figure 4A two protein–DNA complexes are observed with c-Myc/Max on the wild type rODC probe, one of which hardly enters the gel. The complex that enters the gel consists of two c-Myc/Max dimers bound, because deletion of box 1 results in the formation of a DNA–protein complex which migrates faster compared to the wild type DNA–protein complexes (Fig. 4A, lanes 13–18). The affinity of c-Myc/Max for this probe is ~10-fold lower than for the wild type probe and comparable to the affinity of c-Myc/Max to pCM1. Therefore, it is most likely that these complexes represent binding of c-Myc/Max to the single consensus E-box. Changing one base in E-box 1 results in a ~5-fold reduction in affinity of the c-Myc/Max heterodimer. The complexes formed run at the same position as for the wild type probe (Fig. 4A, lanes 7–12). This suggests that two dimers are bound and therefore that c-Myc/Max can bind with relatively high affinity to the mutant E-box 1 (CACGCG) when a wild type E-box is adjacent (see also below). To verify that both E-boxes are simultaneously bound, DNAseI footprinting experiments using the rODC wild type and mutant probes were performed.

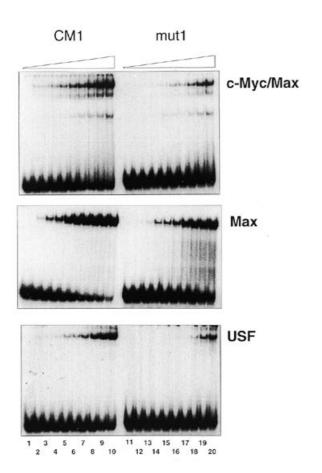**Mutation of one E-box in the rODC intron results in lower binding affinity of c-Myc/Max for both sites**

For a protein that binds cooperatively to two sites one expects that mutation of one binding site affects binding to the unaltered wild type site. In order to test this, DNAseI footprinting experiments were performed. In Figure 5 binding of c-Myc/Max, Max and USF to the rODC fragments was analyzed. For c-Myc/Max, mutation of one base in E-box 1 results in a ~5-fold lower affinity not only for this E-box, but also for E-box 2 (Fig. 5A, compare lanes 5–7 with lanes 12–14). This shows that c-Myc/Max binds in a cooperative fashion to both rODC E-boxes and agrees with the results obtained by gelshift analysis (Figs 3 and 4). Furthermore, it confirms that c-Myc/Max binding to a weak E-box can occur when one intact E-box is adjacent. As expected, for Max homodimers a point mutation in E-box 1 decreases the affinity of the protein for this site, but the affinity for the wild type E-box 2 remains unchanged (Fig. 5B, lanes 9–14). In the case of USF a point mutation of E-box 1 reproducibly results in a 2-fold

lower affinity for E-box 2 whereas binding to E-box 1m can no longer be detected. These results are in agreement with the gelshift assays shown in Figure 4. Binding of the three dimers to the wild type fragment results in similar DNAseI protection patterns (Fig. 5A, B and C lanes 1–7). However, binding of either c-Myc/Max, Max or USF results in differences in DNAseI cleavage outside the E-box sequences (Fig. 5A, B and C, lanes 1–7 and see below). The differences in DNAseI hypersensitive sites between c-Myc/Max, Max and USF (Figs 5 and 6) suggest that binding of these proteins induces different structural changes in the DNA. Figure 6 shows a scheme of the DNAseI footprinting results, also of the top strand (data not shown).

To ensure that c-Myc/Max binds with a very low affinity to a single, mutant E-box (CACGCG), binding to this site was compared with binding to a wild type CM1 E-box. Figure 7 shows a gelshift experiment in which the three different dimers were used. As expected, the affinity of the three dimers for the mutant E-box is at least 20-fold lower. For the rODC box1m probe, the affinity is only 5-fold lower, this shows that CACGCG is a very weak E-box for c-Myc/Max and that binding of this E-box by c-Myc/Max is enhanced when a consensus E-box is adjacent.

DISCUSSION

In addition to c-Myc and its dimerization partner Max, several other transcription factors bind to identical E-box sequences (reviewed in ref. 3). Since it has been established that the oncoprotein c-Myc functions as a transcription factor by binding to the E-box CACGTG, the question how it discriminates its target genes from other E-box binding factors, like USF, TFEB and TFE3, is important. It has been difficult to examine the DNA-binding characteristics of intact c-Myc *in vitro* since the protein is very labile and extremely insoluble in various systems tested. We have constructed vaccinia viruses expressing histidine-tagged, full-length c-Myc and native Max proteins and infection of mammalian cells with these viruses allowed us to purify the intact, soluble c-Myc/Max heterodimer. We show that the purified heterodimer binds to different E-boxes with different affinities (Fig. 3) and show that it is capable of cooperative binding to the two E-boxes located in the first intron of the rat ODC gene (Figs 4 and 5), a *bona fide* target of c-Myc (32,33). This cooperativity results in a 10-fold higher affinity of c-Myc/Max for the rODC gene compared to the adenovirus major late promoter which is activated by USF (Fig. 3). USF dimers do not discriminate in binding to the different E-boxes (Fig. 3). USF has a lower affinity for the rODC enhancer and probably does not bind in a cooperative manner to this element. This provides an explanation for the difference in target gene specificity between c-Myc/Max and other E-box binding transcription factors.

Figure 7. The mutant E-box CACGCG is a weak binding site for c-Myc/Max, Max and USF. c-Myc/Max, Max and USF were titrated using oligonucleotide probes containing a wild type E-box (CM1) or a mutant E-box CACGCG (mut 1). Proteins were titrated in 2-fold dilutions. Lanes 10 and 20 contain either 750 ng c-Myc/Max, 300 ng Max or 20 ng USF. In lanes 1 and 11 no protein was added.

Recently, it has been shown that not only the sequence of the E-box, and sequences surrounding the E-box determine which factor activates via this site, but also the distance of the element to the transcription start site is of importance. Analysis of the prothymosin α gene, another c-Myc target, showed that c-Myc activates transcription from a distal E-box whereas USF does not (36). In addition to the distance effect we propose that the synergistic effect in DNA binding by c-Myc/Max further discriminates c-Myc from USF function in regulation of transcription activation.

Mutation of one of the two E-boxes of the rODC intron still allows c-Myc/Max to bind cooperatively to both sites, although with somewhat lower affinity. This has an intriguing implication. Since binding of c-Myc/Max to a cryptic E-box still occurs when a wild type element is adjacent (Figs 4A and 5A) and since c-Myc/Max does not have a high affinity for such a site by itself (Fig. 7), additional targets for c-Myc may have been overlooked. The prothymosin α gene studied by Desbarats *et al*. (36) indeed contains a second E-box, CAAGTG, in close proximity to the wild type E-box. It will be interesting to see if c-Myc/Max heterodimers also bind the prothymosin α elements cooperatively, adding to its ability to act from a distance. It has been reported that binding of the transcription factor AP-2 to the prothymosin α gene impairs binding of truncated c-Myc/Max to the consensus E-box (54). However, it has not been tested whether full length

c-Myc/Max is also displaced by AP-2. Our results indicate that c-Myc/Max might bind both the consensus and the cryptic E-box whereas USF does not and therefore in this case c-Myc/Max binding might preclude binding of AP-2.

Previously, it has been shown that c-Myc/Max has some affinity for less well defined E-boxes CANNTG (10). It is likely that for a second cryptic E-box some sequence specificity is essential. Furthermore, it will be interesting to test whether the spacing between the two elements is of importance for cooperativity. In addition to both the rat and the mouse ODC gene, the human ODC gene also contains the two conserved E-boxes in its first intron. However, the spacing between these elements differs approximately half a helical turn (34 versus 28 bp). We are presently investigating whether the hODC is also bound by c-Myc/Max in a cooperative manner. The recently identified c-Myc target human cdc25A also contains multiple E-boxes one of which is the consensus CACGTG (38). Similarly, the eIF4E gene contains two consensus E-boxes separated by more than 100 bp.

Two possible mechanisms by which c-Myc/Max binds cooperatively can be envisaged. First, the c-Myc/Max heterodimer could transiently interact with itself and this interaction could stabilize binding to two, or more, E-boxes. Indeed, it has been reported that bacterially produced Myc proteins can form oligomers in solution (55). Secondly, another protein or protein complex may be responsible for the cooperativity. This protein could then act as a positive cofactor for transactivation by c-Myc. In addition, this putative protein may have transcription activating properties by itself when bound directly, or indirectly to DNA. We observe several proteins copurifying with the heterodimer the most prominent with a molecular mass of 36 kDa (Fig. 1B, p36). Microsequence analysis of p36 showed that this protein is encoded by vaccinia virus. Preliminary experiments indicate that p36 does not affect Myc binding to DNA nor transactivation by Myc in transient assays (manuscript in preparation).

Since Max is still able to bind the CACGCG E-box in the rODC box 1m probe and USF is not, this suggests that binding of Max to the rODC E-boxes shows a low degree of cooperativity. Therefore, it is likely that the potency of cooperative binding by c-Myc/Max resides in the N-terminal part of the c-Myc protein. We are currently testing which part of c-Myc is responsible for the cooperativity in DNA binding and it would be interesting to see if c-Myc mutants that cannot bind cooperatively also lose the preference for activation of the ODC gene. In addition it will be interesting to examine if Mad/Max heterodimers, which repress ODC transcription thereby antagonizing c-Myc function (56), also bind the ODC E-boxes in a cooperative manner.

Using transient transfection experiments it has been shown that mutation of one of the two E-boxes of the murine ODC gene results in a moderate decrease in transcriptional activation by c-Myc, whereas mutation of both E-boxes completely abolishes activation by c-Myc (32,33). These *in vivo* results show that the two E-boxes functionally cooperate which is in agreement with our *in vitro* binding studies. Since both sequence and spacing of the element are highly conserved between the rat and mouse ODC gene, high affinity binding of c-Myc/Max heterodimers to these E-boxes is most likely the result of cooperative binding.

In addition to the difference in cooperativity in DNA binding by either c-Myc/Max, Max or USF dimers, we observe a difference in optimal conditions for binding to a single E-box. Both ionic strength and magnesium ion requirements differ between the dimers. Together with the difference observed in DNAseI

hypersensitive sites induced by binding of the different dimers to the rODC E-boxes, this could reflect a difference in DNA structure induced by binding of these dimers.

Both USF and c-Myc have been proposed to bind to the initiator element located around the transcription start site of the adenovirus major late promoter (40,41). We have not been able to detect binding of c-Myc/Max nor USF to the adenovirus major late initiator sequence by gelshift and DNAseI footprinting analysis, even at high protein concentrations (data not shown). This suggests that both c-Myc and USF may require another protein, possibly TFII-I (40,41), which is not present in our protein fractions (data not shown).

Taken together, our data show that c-Myc/Max heterodimers, contrary to USF, are capable of cooperative binding to DNA when two E-boxes are present. This property is likely to contribute to the difference in target gene selection by different E-box binding transcription factors.

ACKNOWLEDGEMENTS

REFERENCES

1 Marcu,K.B., Bossone,S.A. and Patel,A.J. (1992) *Annu. Rev. Biochem.*, **61,** 809–860.
2 Meichle,A., Philipp,A. and Eilers,M. (1992) *Biochim. Biophys. Acta*, **1114,** 129–146.
3 Littlewood,T. and Evan,G. (1994) *Protein Profile*, **1,** 637–709.
4 Packham,G. and Cleveland,J.L. (1995) *Biochim. Biophys. Acta*, **1242,** 11–28.
5 Amati,B., Dalton,S., Brooks,M.W., Littlewood,T.D., Evan,G.I. and Land,H. (1992) *Nature*, **359,** 423–426.
6 Kretzner,L., Blackwood,E.M. and Eisenman,R.N. (1992) *Nature*, **359,** 426–429.
7 Amin,C., Wagner,A.J. and Hay,N. (1993) *Mol. Cell. Biol.*, **13,** 383–390.
8 Amati,B., Littlewood,T.D., Evan,G.I. and Land,H. (1993) *EMBO J.*, **12,** 5083–5087.
9 Amati,B., Brooks,M.W., Levy,N., Littlewood,T.D., Evan,G.I. and Land,H. (1993) *Cell*, **72,** 233–245.
10 Blackwell,T.K., Kretzner,L., Blackwood,E.M., Eisenman,R.N. and Weintraub,H. (1990) *Science*, **250,** 1149–1151.
11 Blackwood,E.M. and Eisenman,R.N. (1991) *Science*, **251,** 1211–1217.
12 Halazonetis,T.D. and Kandil,A.N. (1991) *Proc. Natl. Acad. Sci. USA*, **88,** 6162–6166.
13 Papoulas,O., Williams,N.G. and Kingston,R.E. (1992) *J. Biol. Chem.*, **267,** 10470–10480.
14 Hateboer,G., Timmers,H.T.M., Rustgi,A.K., Billaud,M., van't Veer,L.J. and Bernards,R. (1993) *Proc. Natl. Acad. Sci. USA*, **90,** 8489–8493.
15 Maheswaran,S., Lee,H. and Sonenshein,G.E. (1994) *Mol. Cell. Biol.*, **14,** 1147–1152.
16 Kato,G.J., Barrett,J., Villa-Garcia,M. and Dang,C.V. (1990) *Mol. Cell. Biol.*, **10,** 5914–5920.
17 Crouch,D.H., Fisher,F., Clark,W., Jayaraman,P.-S., Goding,C.R. and Gillespie,D.A.F. (1993) *Oncogene*, **8,** 1849–1855.
18 Gu,W., Cechova,K., Tassi,V. and Dalla-Favera,R. (1993) *Proc. Natl. Acad. Sci. USA*, **90,** 2935–2939.
19 Ayer,D.E., Kretzner,L. and Eisenman,R.N. (1993) *Cell*, **72,** 211–222.
20 Zervos,A.S., Gyuris,J. and Brent,R. (1993) *Cell*, **72,** 223–232.
21 Hurlin,P.J., Quevan,C., Koskinen,P.J., Steingrimsson,E., Ayer,D.E., Copeland,N.G., Jenkins,N.A. and Eisenman,R.N. (1995) *EMBO J.*, **14,** 5646–5659.
22 Schreiber-Agus,N., Chin,L., Chen,K., Torres,R., Rao,G., Guida,P., Skoultchi,A.I. and DePinho,R.A. (1995) *Cell*, **80,** 777–786.
23 Ayer,D.E., Lawrence,Q.A. and Eisenman,R.N. (1995) *Cell*, **80,** 767–776.
24 Ayer,D.E. and Eisenman,R.N. (1993) *Genes Dev.*, **7,** 2110–2119.
25 Chin,L., Schreiber-Agus,N., Pellicer,I., Chen,K., Lee,H.W., Dudast,M., Cordon-Cardo,C. and DePinho,R.A. (1995) *Proc. Natl. Acad. Sci. USA*, **92,** 8488–8492.
26 Chodosh,L.A., Carthew,R.W. and Sharp,P.A. (1986) *Mol. Cell. Biol.*, **6,** 4723–4733.
27 Gregor,P.D., Sawadogo,M. and Roeder,R.G. (1990) *Genes Dev.*, **4,** 1730–1740.
28 Ferre-D'Amare,A.R., Prendergast,G.C., Ziff,E.B. and Burley,S.K. (1993) *Nature*, **363,** 38–45.
29 Ferre-D'Amare,A.R., Pognonec,P., Roeder,R.G. and Burley,S.K. (1994) *EMBO J.*, **13,** 180–189.
30 Carthew,R.W., Chodosh,L.A. and Sharp,P.A. (1985) *Cell*, **43,** 439–448.
31 Sawadogo,M. and Roeder,R.G. (1985) *Proc. Natl. Acad. Sci. USA*, **82,** 4394–4398.
32 Bello-Fernandez,C., Packam,G. and Cleveland,J.L. (1993) *Proc. Natl. Acad. Sci. USA*, **90,** 7804–7808.
33 Tobias,K.E., Shor,J. and Kahana,C. (1995) *Oncogene*, **11,** 1721–1727.
34 Eilers,M., Schirm,S. and Bishop,J.M. (1991) *EMBO J.*, **10,** 133–141.
35 Gaubatz,S., Meichele,A. and Eilers,M. (1994) *Mol. Cell. Biol.*, **14,** 3853–3862.
36 Desbarats,L., Gaubatz,S. and Eilers,M. (1996) *Genes Dev.*, **10,** 447–460.
37 Packham,G. and Cleveland,J.L. (1994) *Mol. Cell. Biol.*, **14,** 5741–5747.
38 Galaktionov,K., Chen,X. and Beach,D. (1996) *Nature*, **382,** 511–517.
39 Jones,R.M., Branda,J., Johnston,K.A., Polymenis, M., Gadd, M., Rustgi, A., Callahan,L. and Schmidt,E.V. (1996) *Mol. Cell. Biol.*, **16,** 4754–4764.
40 Roy,A.L., Carruthers,C., Gutjahr,T. and Roeder,R.G. (1993) *Nature*, **365,** 359–361.
41 Roy,A.L., Meisterernst,M., Pognonec,P. and Roeder,R.G. (1991) *Nature*, **354,** 245–248.
42 Li,L.-H., Nerlov,C., Prendergast,G., MacGregor,D. and Ziff,E.B. (1994) *EMBO J.*, **13,** 4070–4079.
43 Evan,G.I. and Hancock,D.C. (1985) *Cell*, **43,** 253–261.
44 Wurm,F., Gwinn,K.A. and Kingston,R.E. (1986) *Proc. Natl. Acad. Sci. USA*, **83,** 5414–5418.
45 Kerkhoff,E., Bister,K. and Klempnauer,K.-H. (1991) *Proc. Natl. Acad. Sci. USA*, **88,** 4323–4327.
46 Lemaitre,J.-M., Bocquet,S., Thierry,N., Buckle,R. and Mechali,M. (1994) *Gene*, **150,** 325–330.
47 Moss,B., Elroy-Stein,O., Mizukami,T., Alexander,W.A. and Fuerst,T.R. (1990) *Nature*, **348,** 91–92.
48 van Steeg,H., van Oostrom,C.T.M., Hodemaekers,H.M. and van Kreyl,C.F. (1990) *Gene*, **93,** 249–256.
49 Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1992) *Short Protocols in Molecular Biology*. Second edition. Greene Publishing Associates and John Wiley & Sons, New York.
50 Nakano,R., Zhao,L.-J. and Padmanabhan,R. (1991) *Gene*, **105,** 173–178.
51 Chodosh,L.A. (1988) Ph.D. thesis, Massachusetts Institute of Technology, Cambridge.
52 Harlow,E. and Lane,D. (1988) *Antibodies, A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.
53 Sawadogo,M., van Dyke,M.W., Gregor,P.D. and Roeder,R.G. (1988) *J. Biol. Chem.*, **263,** 11985–11993.
54 Gaubatz,S., Imhof,A., Dosch,R., Werner,O., Mitchell,P., Buettner,R. and Eilers,M. (1995) *EMBO J.*, **14,** 1508–1519.
55 Dang,C.V., McGuire,M., Buckmire,M. and Lee,W.M.F. (1989) *Nature*, **337,** 664–666.
56 Wu,S., Pena,A., Korcz,A., Soprano,R.D. and Soprano,K.J. (1996) *Oncogene*, **12,** 621–629.