Analysis of E-box DNA binding during myeloid differentiation reveals complexes that contain Mad but not Max

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It has been shown that during myeloid differentiation the levels of *mad1* mRNA are induced before the loss of c-Myc protein. This suggests that inactivation of the differentiation-blocking activity of c-Myc might not occur primarily through the loss of Myc protein, but through an increase in the levels of its antagonist, Mad1. To investigate this question we have analysed the levels of *mad1* mRNA during differentiation of myeloid leukaemic HL60 cells. Although levels of *mad1* mRNA were moderately increased after induction with phorbol ester, we also found that differentiation could be achieved with other inducers without any concomitant up-regulation of *mad1* mRNA. In addition, analysis of E-box DNA binding revealed that, although

Myc–Max complexes were lost rapidly after differentiation induction, formation of Mad1-containing complexes only occurred during the later stages of the differentiation programme. Further analysis of these Mad-containing complexes revealed that they were also unlikely to have the capacity to antagonize c-Myc function, as they did not contain Max. Therefore these data suggest that an increase in the levels of *mad1* mRNA or the formation of a Mad–Max complex are unlikely to be essential or determining events for myeloid differentiation. In addition, the discovery of DNA-binding complexes that contain Mad1, but not Max, opens up this transcription factor network to include other Max-like proteins or proteins of an unrelated nature.

INTRODUCTION

The *myc* family of cellular proto-oncogenes encode nuclear phosphoproteins that are implicated in the establishment of numerous types of human malignancy [1–3]. Enforced expression of the most studied family member, *c-myc*, results in continuous cell-cycle progression [4], cellular immortalization [5] and blockages to differentiation in many lineages [6,7]. Although it is perceived that c-Myc functions as a transcription factor, it has been shown that it is only effective in transcriptional activation [8] and in inducing cell-cycle progression and immortalization [9,10] when dimerized with a second protein, termed Max. Unlike Myc, Max can also form a homodimeric complex which can bind to the same ‘E-box’ DNA-binding sites as the Myc–Max heterodimer (i.e. CACGTG, CATGTG) [11]. However, when assayed for transactivation potential, the Max–Max complex was considered relatively inert when compared with the activation seen for the Myc–Max heterodimer [12,13]. Max also forms complexes with four other proteins: Mad1 [14], Mxi1 [15], Mad3 and Mad4 [16]. These proteins are similar to Myc in that they do not bind DNA alone and do not form homodimers, but form heterodimeric complexes with Max and also bind to the CACGTG motif [14–16]. However, when their ability to trans-activate from this site was assessed, they were found to be more like the Max–Max homodimer and are therefore believed to act as transcriptional repressors [14–16]. As each of these proteins is thought to be in competition with c-Myc for both available Max protein and available E-box sites, any induction in their expression could result in antagonism of c-Myc function. Therefore the correct functioning of Myc is not only dependent on the presence of Max, but also on the levels of Mad1, Mxi1, Mad3 and Mad4. In this regard, it is noteworthy that expression of Mad1 and Mxi1 in a cell line expressing activated *c-myc* and *ras* genes resulted in a reduction in the number of cells exhibiting a transformed phenotype [17].

Studies that have analysed the expression of *c-myc*, *max* and

mad1 in myeloid leukaemia cells lines have revealed complex changes in the relative mRNA levels as the cells are induced to differentiate. While the levels of *c-myc* mRNA are down-regulated rapidly, the levels of *max* mRNA remain largely invariant and the levels of *mad1* mRNA are increased immediately after treatment with the differentiation inducer [18,19]. Since it is known that constitutive *c-myc* expression blocks differentiation within the myeloid lineage [20], it is not a surprise that the loss of c-Myc protein is an early event during this process. However, since the levels of *mad1* mRNA are increased before this down-regulation of *c-myc*, the possibility exists that abrogation of the differentiation-blocking activity of *c-myc* does not occur primarily through the loss of c-Myc protein, but as a result of the up-regulation of the Myc antagonist, Mad1.

In order to investigate further the role of *mad1* in processes of myeloid differentiation, we have utilized the HL60 leukaemic cell line, which can be released from its differentiation blockage by treatment with various inducers [21], including antagonists of c-Myc activity [22], to yield populations of terminally differentiated cells. Although we found, in agreement with previous studies [19], that induced differentiation of our clone of HL60 cells can also result in increased levels of *mad1* mRNA, we also found that differentiation to granulocytes can be achieved without any concomitant up-regulation of *mad1*. In addition, assessment of protein binding to the E-box motif during differentiation within this system revealed that, whereas loss of Myc–Max DNA binding was a rapid event after treatment with the differentiation inducers, Mad1 DNA binding was only detectable during the late stages of the differentiation programme. Furthermore, analysis of these Mad1-containing complexes revealed that they did not contain Max. These data therefore indicate that up-regulation of *mad1* expression is not an essential event for the differentiation of these cells and that Mad1 activity is unlikely to be the primary mechanism by which the differentiation-blocking activity of c-Myc is abrogated within this system.

Abbreviations used: EMSA, electrophoretic mobility-shift assay; NBT, NitroBlue Tetrazolium; NSE, non-specific esterase.

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MATERIALS AND METHODS

Culture of HL60 cells

Passage-25 HL60 cells were kindly provided by Dr. G. Brown (Department of Immunology, University of Birmingham, Birmingham, U.K.). They were grown in suspension culture in Special Liquid Medium (Gibco-BRL), supplemented with 10 % foetal calf serum (Gibco-BRL) and 2 mM glutamine (Gibco-BRL) in an atmosphere of 5 % CO₂ in air at 37 °C. Cultures were passaged every 48 h and were seeded at a density of 5×10^5 cells/ml.

Induction of differentiation

Cells were diluted with fresh medium to a concentration of 5×10^5 cells/ml, 16 h before induction. Differentiation to monocytes/macrophages was induced by addition of PMA (Sigma) to a final concentration of 1.6×10^{-7} M. Differentiation to granulocytes was achieved by the addition of DMSO to 1.5 % (v/v).

Markers of differentiation

Non-specific esterase (NSE)

Aliquots of 1×10^6 cells were collected on glass slides by centrifugation at 32 g for 5 min in a Cyto-tek cytocentrifuge. NSE activity was assessed using diagnostic kit 91-A (Sigma) and assays were performed as recommended by the manufacturer. Two slides were prepared for each time point and 100 cells were examined to determine the proportion staining positively for NSE.

NitroBlue Tetrazolium (NBT) reduction

Aliquots of 1×10^6 cells were harvested by centrifugation at 260 g for 5 min (MSE benchtop). The cell pellet was resuspended in 1 ml of PBS containing 10 μ M PMA (Sigma) and 0.2 % NBT (Sigma). Samples were then incubated at 37 °C for 25 min followed by centrifugation on to glass slides. Two slides were prepared for each time point and 100 cells were examined to determine the proportion staining positively for NBT reduction.

Northern-blot analysis

Total cellular RNA was isolated using RNAzol B (Biogenesis), as advised by the manufacturers. RNA was denatured by suspension in 50 % formamide/2.2 M formaldehyde in 40 mM Mops/10 mM sodium acetate/1 mM EDTA, pH 7.0, and heating at 65 °C for 15 min. A 15 μ g portion of RNA per lane was size-fractionated by electrophoresis through 1.25 % agarose gels containing 2.2 M formaldehyde. Gels were washed in distilled water (2 $\times$ 30 min) followed by transfer to Hybond N⁺ membranes (Amersham) via capillary blotting using 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7).

Preparation of hybridization probes

The *c-myc* probe was the 1.4 kbp *Cla*I–*Eco*RI fragment of the plasmid pMC41-3RC [23] comprising the third exon and 3' flanking sequence of the human *c-myc* gene. The *max* probe was a 500 bp fragment of the plasmid pSTMax7 (a gift from Dr. D. H. Crouch and Dr. D. A. F. Gillespie of the Beatson Institute) containing the entire coding region of the human *max* gene. The *mad1* probe was a 1 kb fragment of the plasmid pVZMad (a gift provided by Dr. D. Ayer, Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.) containing the entire coding region of the human *mad1* mRNA. The β_2 -microglobulin probe com-

prises 97 % of the coding region plus 3' untranslated sequence of the human cDNA which was previously cloned into the *Pst*I site of pBR322 [24]. All probes were radiolabelled with [³²P]dCTP by random priming using a kit supplied by Boehringer-Mannheim.

Hybridization

Nylon membranes were prehybridized in a solution containing 50 % formamide, 5 $\times$ SSPE [1 $\times$ SSPE is 0.15 M NaCl/0.01 M sodium phosphate (pH 7.4)/1 mM EDTA], 5 $\times$ Denhardt's reagent, 0.5 % (w/v) SDS and 200 μ g/ml sonicated salmon sperm DNA at 42 °C for 4 h. Radiolabelled probe was then added to this solution at a concentration of 1×10^6 c.p.m./ml and the incubation continued for a further 16–24 h. The blots were then washed (2 $\times$ 20 min in 2 $\times$ SSC/0.1 SDS at room temperature; then 45 min in 0.1 $\times$ SSC/0.1 % SDS at 65 °C), and exposed to Kodak X-OMAT film with intensifying screens at –70 °C. Autoradiographs were quantified by scanning laser densitometry using apparatus from Molecular Dynamics and software from PDI.

Western-blot analysis

Samples of cells were harvested by centrifugation or scraped from the tissue culture dish and subsequently washed twice in ice-cold PBS. They were then lysed in SDS/PAGE sample buffer [1 $\times$ sample buffer is 20 % (v/v) glycerol/2 % (w/v) SDS/100 mM Tris/HCl, pH 6.8 to which 5 % 2-mercaptoethanol is added immediately before lysis of the cells], boiled for 5 min then chilled on ice. Lysates were sonicated to shear high-molecular-mass DNA and then centrifuged at 15000 g for 2 min at 4 °C in a Microfuge. The resultant supernatants were divided into aliquots and stored at –70 °C until required.

Equal quantities of protein (measured using the Bradford assay) were separated by electrophoresis at 30 mA for 16 h through SDS/7 % polyacrylamide gels [30 % acrylamide stock solution: 29.6 % (w/v) acrylamide, 0.4 % (w/v) bisacrylamide]. The proteins from these gels were then transferred to Transblot nitrocellulose membrane (Bio-Rad) by semi-dry electroblotting at 180 mA for 1 h. c-Myc protein was detected on these blots with an antibody (237–6) raised against human c-Myc, which was a gift from Dr. D. Gillespie of the Beatson Institute. Blots were first blocked for 16 h in PBS containing 5 % (w/v) non-fat milk powder and 0.05 % Tween 20 (Sigma), and incubated in this solution with the c-Myc antiserum for 1 h, then with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Promega) for 1 h. The extent of reactivity against c-Myc was measured using the ECL Western-blotting system (Amersham) and exposure to Kodak X-OMAT film.

Electrophoretic mobility-shift assay (EMSA)

Typically 1×10^7 cells were used for the isolation of nuclear proteins for each sample and were prepared and stored as described by Andrews and Faller [25]. To analyse the DNA-binding activities during the differentiation programmes, 10 μ g of nuclear protein from each time point was incubated with 0.2 ng of ³²P-end-labelled 'CM1' double-stranded oligonucleotide (5'-CCCCACCACGTGGTGCCTGA-3', which has been selected as a consensus Myc-Max-binding site [11]), at room temperature for 20 min, in a buffer consisting of 20 mM Hepes (pH 7.2), 50 mM KCl, 1 mM EDTA, 3 mM MgCl₂ and 10 % (v/v) glycerol. A 200 ng sample of an unrelated double-stranded oligonucleotide (5'-TTCCGGCTGACTCATCAAGCG-3') was used as a competitor in each reaction to sequester non-specific DNA-binding proteins from the labelled probe.

Where indicated, the binding reaction mixtures were also incubated with either competitor DNA or antibody for 30 min, on ice, before the addition of the labelled probe. (Antisera added after incubation of protein with the labelled probe did not result in a clear-cut supershift of any of the complexes, possibly because the antibody disrupts the protein-oligomer complexes.) Subsequently, binding reactions were resolved by electrophoresis through a 6% polyacrylamide gel in 45 mM Tris/HCl/45 mM boric acid/1 mM EDTA, pH 8.0 for approx. 3 h at 25 mA. The gel was then dried and exposed to Kodak X-OMAT film with intensifying screens at -70°C . Antibodies used in these reactions were obtained as follows: Mad Ab and Max Ab1 were from Santa Cruz Biotechnology; Max Ab2 and Myc sera were a gift from David Gillespie of The Beatson Institute, and have been described previously [26,27]; Max Ab3 was a gift from Trevor Littlewood and Gerard Evan of the Imperial Cancer Research Fund, London, U.K. and has been described previously by Littlewood et al. [28] as MX.

During the course of this work, we found that the ability to detect the Myc-Max complexes in cellular extracts was highly dependent on the binding conditions used. In particular, as a result of a series of experiments designed to find the optimum conditions for binding (results not shown), it became apparent that the c-Myc-Max complex could be completely competed for by the double-stranded polynucleotide, poly(dI-dC)·poly(dI-dC). As this factor is a standard constituent of many EMSA protocols, this may be the reason why many workers have been unable to effectively detect Myc-Max DNA binding in nascent cellular extracts. Since we found that a number of other non-specific double-stranded DNA oligonucleotides did not affect the Myc-Max complex (results not shown), while still retaining the capacity to compete away non-specific proteins from radio-labelled probe, these findings should be a consideration of workers who are planning to conduct studies of a similar nature.

RESULTS

Induction of *mad1* expression is not essential for differentiation in HL60 cells

During this study, HL60 cells were differentiated with either PMA to yield mature populations of monocytes/macrophages

Figure 1 Percentage of cells staining positively for markers of terminal differentiation during HL60 cell differentiation

HL60 cells were analysed for their ability to reduce NBT during granulocytic differentiation induced by DMSO (○) and percentage staining positive for NSE during monocytic differentiation induced by PMA (●). Analysis of differentiation induced by PMA was only taken up to 3 days, as the cells die beyond this time. These findings were reproducibly seen in at least two individual experiments for each individual time point.

Figure 2 Northern-blot analysis of RNAs isolated from HL60 cells after differentiation induced by PMA and DMSO

HL60 cells were treated with PMA at a final concentration of 1.6×10^{-7} M or with DMSO at a final concentration of 1.5% (v/v). RNA was isolated at the times indicated after addition of the inducer, size-fractionated by electrophoresis through an agarose gel and transferred to a nylon membrane by capillary blotting. The blot was sequentially hybridized with probes for *mad1*, *c-myc* and β_2 -microglobulin (β_2m) and sections are labelled accordingly. Detection of *mad1* mRNAs usually required 72 h autoradiography whereas *c-myc* and β_2 -microglobulin mRNAs were detected in 16 h.

or DMSO to yield granulocytes. The proportion of cells displaying the characteristic phenotypes of differentiated cells was determined by specific staining as well as microscopic examination (Figure 1). Hybridization of HL60 cell RNA with a *mad1* cDNA revealed two RNA species that differ both in size and relative abundance (Figure 2). These two *mad1* RNAs have been described previously [18,19], but no reason is yet known for their existence, or for their relative abundances in cells. Analysis of these *mad1*-hybridizing RNAs after treatment of the cells with PMA revealed that, in agreement with previous studies [18,19], the levels of *mad1* mRNA reproducibly undergo a rapid, though small, increase after the addition of the phorbol ester (Figure 2).

Figure 3 Western-blot analysis of proteins isolated from HL60 cells after differentiation induced by PMA or DMSO

HL60 cells were incubated with PMA or DMSO as described for Figure 2, and protein was isolated at the times indicated. Equal quantities of protein were fractionated by SDS/PAGE, blotted and assayed for Myc protein with an antiserum against full-length human *c-Myc*.

Although this 2–3-fold increase was smaller than that observed by Larsson et al. [19], and may reflect a clonal variation between the HL60 cells used in the two studies, it occurred over a very similar time frame. Levels of *mad1* mRNA were found to increase over the first hour of the differentiation programme whereas down-regulation of the levels of *c-myc* mRNA (Figure 2) and protein (Figure 3) did not occur until after 2 h after PMA addition. In contrast, and in disagreement with the study by Larsson et al. [19], we found that, when we differentiated our HL60 cells to granulocytes by treatment with DMSO, we did not observe any concomitant increase in the levels of *mad1* mRNA (Figure 2). In spite of this, there was a rapid decrease in the levels of *c-myc* mRNA (Figure 2) and protein (Figure 3), and the cells subsequently developed a granulocytic morphology and showed activity for a marker of terminal differentiation (Figure 1). This indicated that increased levels of *mad1* mRNA were not essential for differentiation of our clone of HL60 cells to granulocytes by DMSO. Interestingly, the result was similar when this clone of cells was induced to differentiate to monocytes/macrophages by treatment with 1- α ,25-dihydroxyvitamin D₃ (results not shown).

Loss of Myc–Max DNA binding is an early event during induced differentiation of HL60

Although *mad1* up-regulation is not an essential event for differentiation of HL60 cells (Figure 2), uninduced cells express

a detectable basal level of *mad1* mRNA. This level may be sufficient for translation of effective levels of Mad1 protein and therefore our results cannot discount a role for Mad1 in any of the processes that occur after treatment with the differentiation inducers. As a more direct assay of the possible significance of Mad1 activity within this system, and with particular interest being given to its relevance to Myc and Myc target genes, we analysed the E-box DNA-binding activity of nuclear proteins isolated from the cells at various points during the differentiation programmes.

Our analysis of nuclear extracts from cells differentiated with PMA and DMSO (Figure 4) revealed that a large number of proteins were bound to the E-box sequence at all of the time points sampled. However, this was not unexpected as a number of non-Myc-related proteins have also been shown to bind this motif [29–31]. When a comparison was made between the changes in the abundances of the DNA-binding complexes in Figure 4 with the changes in mRNA levels that had been observed during differentiation of the cells with either PMA or DMSO (Figure 2), it was apparent that at least one of the complexes (complex I) had a very similar pattern of binding to the changes in the levels of *c-myc* mRNA that occurs during these processes. Subsequent analysis of complex I with affinity-purified antisera raised against Myc and Max confirmed that this complex was indeed Myc–Max (Figure 5).

Mad1-containing DNA-binding complexes are detectable in the late stages of differentiation to granulocytes, but are not seen during differentiation to monocytes/macrophages

During our analysis of the DNA-binding complexes that were detectable after treatment of the cells with PMA, we did not detect any complexes that reacted with our antiserum raised against Mad1. However, during granulocytic differentiation of the cells induced by DMSO, two additional complexes were observed which were not apparent during differentiation to monocytes/macrophages. These two complexes, designated II and IIa (Figure 4), had mobilities slower and faster respectively than that observed for the previously described complex I and were, particularly in the case of complex II, induced subsequent to the decrease in complex I. The ensuing analysis of complexes II and IIa using affinity-purified antisera (Figure 6) indicated that these complexes, as well as at least one other higher-molecular-mass complex, did indeed contain Mad1. Therefore Mad1-containing complexes are induced during the differentiation of HL60 cells to granulocytes, but are not induced to detectable levels during differentiation of the cells to monocytes/macrophages. In addition, as these profiles of DNA-binding activity do not mirror the observed differentiation-induced changes in *mad1* mRNA (Figure 2), this finding either indicates that Mad1 DNA binding is not solely determined by the levels of *mad1* mRNA or that other Mad1-containing complexes exist within the induced cell extracts.

Mad1-containing DNA-binding complexes induced during granulocytic differentiation do not contain Max

Further analysis of complexes II and IIa revealed that they reacted with the Mad1 antiserum, but not with the antiserum raised against Max (Figure 6). These data are in disagreement with previous results which showed that DNA binding by Mad1 was dependent on dimerization with Max [14]. To confirm these results, we used several different nuclear extracts (results not shown) and other Max antisera raised against different portions

Figure 4 EMSA of E-Box DNA-binding activity of nuclear proteins isolated from HL60 cells after treatment with PMA or DMSO

Nuclear protein samples were isolated at the times indicated and equal quantities (10 μ g) were added to binding reaction mixtures containing a 32 P-labelled 'CM1' double-stranded oligonucleotide, which contains the Myc–Max-binding CACGTG motif (see the Materials and methods section). Reaction mixtures were fractionated by electrophoresis through a 6% polyacrylamide gel, and autoradiographed for 16 h. A complex that has been shown to contain Myc and Max (see Figure 5) is indicated as I. Complexes that have been shown to contain Mad1 (see Figures 6 and 7) are indicated by II and IIa.

Figure 5 EMSA to investigate the nature of complex I that is lost during differentiation of HL60 cells induced by PMA

Equal quantities of nuclear proteins (10 μ g) from uninduced HL60 cells were incubated before addition of 32 P-labelled double-stranded oligonucleotide 'CM1' (Myc–Max binding) with, where shown, either a 100-fold excess of unlabelled double-stranded oligonucleotides or antibodies raised against the proteins indicated (see the Materials and methods section). Reaction mixtures were fractionated by electrophoresis through a 6% polyacrylamide gel and autoradiographed for 16 h. PI, preimmune serum.

Figure 6 EMSA to investigate the nature of complexes II and IIa that are induced during differentiation of HL60 cells

Equal quantities of nuclear proteins (10 μ g) from HL60 cells treated for 5 days (5 D) with DMSO were incubated before addition of 32 P-labelled double-stranded oligonucleotide 'CM1' (Myc–Max binding) with, where shown, either 100-fold excess of unlabelled double-stranded oligonucleotides or antibodies (Ab) raised against the proteins indicated (see the Materials and methods section). Reaction mixtures were fractionated by electrophoresis through a 6% polyacrylamide gel and autoradiographed for 16 h. PI, preimmune serum.

Figure 7 EMSA showing the reactivity of proteins in complexes I, II and IIa to antiserum raised against Mad1 and a series of antisera raised against Max

Nuclear proteins from uninduced cells or from cells treated for 5 days (5 D) with DMSO were incubated with or without the antisera indicated before the addition of 32 P-labelled 'CM1' double-stranded oligonucleotide. Sources of antisera are indicated in the Materials and methods section. PI, preimmune serum.

of the Max protein. As can be seen in Figure 7, whereas each of these antisera was equally effective in recognizing Max in a Myc–Max complex (complex I), they did not react with the Mad-containing complexes II and IIa. Therefore these complexes do indeed contain Mad, but not Max. Furthermore, in the light of the fact that two complexes were detected and that it has been shown that Mad is by itself ineffective in DNA binding, it must also be the case that complexes II and IIa contain factors, in addition to Mad, whose identities are yet to be determined.

DISCUSSION

It was the aim of this study to investigate the importance of the expression of *mad1* during processes of myeloid differentiation. It had previously been shown that increased *mad1* mRNA levels were an early event during the induced differentiation of myeloid leukaemia HL60 and U937 cells [18,19]. Coupled with the observation that *mad1* mRNA abundance could be increased during these processes without new protein synthesis [18,19], it was not unreasonable to assume that the up-regulation of *mad1* gene expression was a possible determining event during differentiation within these systems. In addition, as the induction of *mad1* expression occurred before the down-regulation of *c-myc* expression that occurs during these processes, this opened up the possibility that the induction of *mad1* expression was the initial and possibly primary event involved in the abrogation of Myc–Max function during the differentiation of these cells. However, when we analysed the expression patterns of *mad1*

during differentiation of our clone of HL60 cells, we found that, although the levels of the mRNA increased during differentiation to monocytes/macrophages after treatment with PMA, we were also able to differentiate the cells to granulocytes (using DMSO) without a concomitant increase in *mad1* mRNA levels. This therefore precludes the possibility that up-regulation of *mad1* gene expression is an essential event for differentiation within these cells. In addition, this would also indicate that increased *mad1* expression is not a necessary process for the inactivation of the differentiation-blocking activity of c-Myc. However, as our findings are in contrast with observations by Larsson et al. [19], which indicated that *mad1* mRNA levels were induced with all three inducers, it is most likely that this represents a clonal variation between the HL60 cells used in the previous study [19] and those used by ourselves. Therefore it must be pointed out that the findings presented here cannot be taken to suggest that induction of *mad1* expression is not an essential event for differentiation in the clone of HL60 cells used by Larsson et al. [19]. Furthermore, as the uninduced HL60 cells used by us contain a basal level of *mad1* mRNA, these data cannot exclude a role for Mad1 protein during differentiation of these cells induced by any of the three inducers used, whether in the regulation of Myc activity or in a non-Myc-related function.

We extended our studies to gain greater insight into the role Mad1 has within this system. As it is known that c-Myc is a key regulator of the growth and differentiation of these cells, it is reasonable to assume that the regulation of the target genes of c-Myc (e.g. ornithine decarboxylase [32] and α -prothymosin [4]) is critical to the way in which c-Myc controls these processes. Since Myc–Max and Mad1–Max have respectively been shown to activate and repress transcription through the same consensus DNA-binding site (CACGTG) [14], upon which expression of ornithine decarboxylase and α -prothymosin is dependent [33,34], we analysed the proteins that bind to this site during the induced differentiation of HL60 cells. These studies revealed that loss of Myc–Max DNA binding was an early event during differentiation via both monocytic and granulocytic pathways. Moreover, the changes in Myc–Max complex levels were entirely similar to the changes in the levels of *c-myc* mRNA. In this regard, it is easy therefore to perceive how a signal to up-regulate *c-myc* expression could be rapidly translated into target gene activation and ultimately phenotypic change. It is perfectly understandable therefore why the levels and activity of c-Myc have been found to be subjected to such intricate control [35].

In contrast with our findings for Myc–Max and despite extensive analysis using a variety of protein–DNA binding conditions (results not shown), we found that no Mad1-containing complexes were detectable during differentiation of the cells to monocytes/macrophages. In addition, those that were observed during the differentiation of the cells to granulocytes were only detected during the late stages of the differentiation programme. This therefore indicates that, since these profiles of DNA-binding activity are in contrast with the observed changes in the levels of *mad1* message, the regulation of Mad1 DNA binding during this process is not solely determined by the abundance of its mRNA. Moreover, as DNA binding by a Mad1-containing complex is only detectable subsequent to the loss of the Myc–Max complex, it would appear that involvement of Mad1 as a primary factor in the abrogation of c-Myc function is very unlikely. However, we cannot discount the possibility that these Mad1-containing complexes may well have a bearing on the differentiation process by a mechanism that is distinct from the ability of Mad1 to antagonize the function of c-Myc.

The most surprising finding from these analyses was the observation that the Mad1-containing complexes that were found

during the differentiation to granulocytes did not contain Max. From our analysis of these complexes with authenticated antisera raised against three different regions of the Max protein, it seems certain that there is no Max within these DNA-binding complexes. This finding is in contention with previous data that indicate that all Myc-related and Mad-related proteins are dependent on dimerization with Max in order to bind DNA effectively [36]. Analysis of the literature on Mad1-containing complexes in cellular extracts showed that they had only been detected in proteins that had previously been subjected to immunoprecipitation with antisera raised against Max [18]. Therefore the detection of any complex that contained Mad1 but not Max would not be possible under these conditions. Consequently, as our assay was conducted on unfractionated nuclear proteins, the observation of complexes that contain Mad1 but not Max is not in conflict with the current status of knowledge on these proteins. More interestingly, since it is known that Mad1 cannot bind the CACGTG motif alone [14], it must be bound as part of a heterodimeric complex that does not contain Max. In the light of this, the data presented here open up this transcription factor network to include proteins, which may or may not be related to Max, that can facilitate the binding of Mad1 in a sequence-specific manner.

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