Mad4 is regulated by a transcriptional repressor complex that contains Miz-1 and c-Myc

Louise KIME and Stephanie C. WRIGHT[1]

School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Myc and Mad family proteins are central regulators of cellular proliferation and differentiation. We show that various *Mad* family genes have distinct patterns of expression during the chemically induced differentiation of mouse erythroleukaemia (MEL) cells, suggesting that they each serve a different function. Mad4 RNA is highly induced and persists in terminally differentiated cells, in agreement with observations in other systems. Using reporter gene assays in stably transfected MEL cells, we show that induction of *Mad4* is mediated by a 49 nt core promoter region. We demonstrate that the initiator element is required for *Mad4* activation, and show that induction is associated with the loss from the initiator of a complex that contains Miz-1 and c-Myc. Miz-1 activates the *Mad4* promoter in transient transfection assays, and this effect is antagonized by c-Myc. We therefore identify *Mad4* as a novel target of transcriptional repression by c-Myc. These data suggest that the expression of *Mad4* in proliferating undifferentiated cells is suppressed by the binding of a c-Myc–Miz-1 repressor complex at the initiator, and that the activation of *Mad4* during differentiation results, at least in part, from a decrease in c-Myc-mediated repression.

Key words: cell cycle, differentiation, Mad, mouse erythroleukaemia cells, Myc, transcription.

INTRODUCTION

Fluctuations in the expression of genes of the c-*Myc* and *Mad* families (*Mad1*, *Mxi1*, *Mad3* and *Mad4*) play an important role in the control of cellular proliferation and differentiation (reviewed in [1,2]). Differentiation is usually associated with a reduction in c-Myc levels and a concomitant increase in Mad proteins; the differentiation of cultured cells can be blocked by the overexpression of transfected c-*Myc* genes (e.g. [3]), and is conversely accelerated by the overexpression of Mad1 [4]. Enforced expression of c-Myc leads to the transformation of certain cultured cells and to tumour formation in transgenic mice, and deregulation of c-Myc is associated with many human malignancies (reviewed in [5]). In contrast, overexpression of Mad1 inhibits the proliferation of several normal and malignant cell lines (e.g. [6,7]), and the targeted disruption of *Mad1* results in defects in cell-cycle exit during the late stages of granulocytic differentiation [8]. Previous reports have shown that different *Mad* family genes are expressed during specific stages of the cell cycle and differentiation. For example, *Mad3* is expressed in the S-phase of the cell cycle in proliferating cells [9,10], and is induced during the S-phase burst that precedes terminal differentiation in various systems [9–11]. In contrast, *in situ* hybridization studies have shown that the expression of *Mad1* and *Mad4* is restricted to the later stages of differentiation in the adult mouse and the developing embryo [11,12]. *Mad1* and *Mad4* are also induced during the later stages of differentiation in cultured 3T3-L1 adipoblasts [10] and P19 pheochromocytoma cells [11]. Since the induction of *Mad4* is associated with the differentiation of many cell lineages, it is likely to represent an integral feature of diverse differentiation programmes.

c-Myc and Mad are basic helix–loop–helix leucine zipper proteins which form heterodimers with the partner Max (reviewed in [2]) and recognize the E-box sequence CACGTG. c-Myc/Max heterodimers activate the transcription of reporter genes containing CACGTG in transfected cells, and CACGTG elements have been shown to mediate the activation of several c-Myc target genes *in vivo* (e.g. [13,14]). Transcriptional activation by c-Myc/Max is mediated by a variety of mechanisms that include the recruitment of histone acetyltransferase and of chromatin remodelling activities [15,16]. Mad/Max heterodimers also recognize CACGTG sequences, but act to repress transcription and to antagonize the activity of c-Myc [12,17,18]. Transcriptional repression by Mad/Max occurs via the tethering of an mSin3–histone deacetylase complex to the N-terminal domain of Mad proteins [19,20]. In addition to its role as a transcriptional activator, c-Myc can repress the transcription of certain physiological target genes (reviewed in [21]); this activity is thought to be important in the immortalization of primary cells by c-Myc (e.g. [22]). The transcriptional repression of certain target genes is mediated by the interaction of c-Myc with Miz-1 at the initiator element [22–24]; c-Myc thus opposes the transcriptional activation function of Miz-1 by interfering with the recruitment of p300 (e.g. [22,24]). c-Myc target genes have been identified by various complementary approaches, and are found to function in diverse physiological processes, such as the cell cycle, cell growth, metabolism, signalling and adhesion (reviewed in [25]). Few target genes of Mad family proteins have been identified (e.g. [26,27]) and the extent of target gene overlap among c-Myc and the various Mad family members is unknown.

The induction of tissue-specific genes during cellular differentiation has been well studied: transcriptional regulation is generally mediated via the binding of tissue-specific activators and repressors to defined *cis*-acting sequence elements of the target gene. In contrast, little is known of the mechanisms that regulate the expression of those genes that are not tissue-specific,

Abbreviations used: CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; DMS, dimethyl sulphate; EMSA, electrophoretic mobility shift assay; MEL, mouse erythroleukaemia.

[1] To whom correspondence should be addressed (e-mail s.c.wright@leeds.ac.uk).

but are instead activated during the differentiation of many different lineages. Such genes may be involved in more general processes, e.g. cell-cycle exit, that are common to the differentiation of diverse cell types. The induction of *Mad4* is not restricted to specific tissues, and may be considered an integral feature of diverse differentiation programmes. In the present study, we analysed the mechanisms that control the activation of *Mad4* during differentiation in cultured mouse erythroleukaemia (MEL) cells. Using reporter gene assays, we show that *Mad4* induction is mediated by a 49 nt sequence element that contains the core promoter, and demonstrate that the initiator element is required for appropriate regulation. We show that *Mad4* activation is associated with the loss from the initiator of a high-molecular-mass complex that contains Miz-1 and c-Myc, and demonstrate that the *Mad4* promoter is regulated by Miz-1 and c-Myc in transient transfection assays. We thus identify *Mad4* as a novel target of transcriptional repression by c-Myc, and suggest that a c-Myc–Miz-1 repressor complex regulates the expression of *Mad4* during the switch from cellular proliferation to differentiation.

EXPERIMENTAL

Cell culture

MEL and HeLa cells were grown in MEMα medium supplemented with 10% (v/v) calf serum. MEL cells were induced to differentiate by culturing in 2% (v/v) DMSO. Stable transfection of MEL cells was carried out by the calcium phosphate precipitation method [28] using 5 μg of pBabePuro [29] together with 20 μg of reporter construct per 10 cm-diam. plate (2×10^6 cells). Stably transfected clones were selected using 1 μg/ml puromycin. Transient transfection of HeLa cells was carried out using 5 μg of *Mad4*–chloramphenicol acetyltransferase (CAT) reporter together with 0–10 μg of cytomegalovirus (CMV)–Miz-1 and 0–10 μg of CMV–c-Myc; transfections contained 1 μg of human α-globin plasmid as a control, and the total amount of DNA was made up to 30 μg with pcDNA3 (InVitrogen). CMV–Miz-1 and CMV–c-Myc were constructed by inserting the coding regions of mouse *Miz-1* and c-*Myc* into pcDNA3.

RNA preparation and ribonuclease protection analysis

Total cellular RNA was isolated by the guanidinium isothiocyanate method [30]. Ribonuclease protection analysis was performed as described previously [31]. Hybridizations contained 10 μg of RNA and were carried out at 50 °C unless otherwise stated. Markers were end-labelled *Msp*I fragments of the plasmid pBR322. Plasmid constructs used for the generation of riboprobes comprised the following sequences cloned into pSP72 (Promega Biotech): mouse Mad4 cDNA, nt +1 to +630; mouse Mad3 cDNA, nt +366 to +621; mouse Mad1 cDNA, nt +246 to +684; mouse Mnt cDNA, nt +1384 to +1775; mouse Mxi1 cDNA, nt +1 to +204; mouse c-Myc genomic DNA, nt +17 to +238; CAT cDNA, nt +114 to +213; human α-globin cDNA, nt +1 to +132 (cDNA sequences are relative to a designation of +1 for the ATG translation initiation codon; mouse c-*Myc* genomic sequences are relative to a designation of +1 for the site of transcriptional initiation at the P1 promoter). Sizes of ribonuclease-protected fragments were: mouse *Mad4*, 630 nt; mouse *Mad3*, 256 nt; mouse *Mad1*, 439 nt; mouse *Mnt*, 392 nt; mouse *Mxi1-SR*, 204 nt; mouse *Mxi1-WR*, 136 nt; CAT, 100 nt; human α-globin, 132 nt; mouse c-*Myc*, 221 nt. Signals were quantified using a Fuji BAS1000 phosphorimager and AIDA software.

Isolation of a mouse *Mad4* genomic clone

A mouse *Mad4* genomic clone was isolated by screening a λ library (Stratagene; B6/CBA) with a Mad4 cDNA probe (nt +1 to +630) using protocols recommended by the manufacturer. A 5.2 kb *Bgl*II fragment containing the 5′ end of the gene was subcloned for further analysis.

Generation of *Mad4*–CAT reporter constructs

Restriction endonuclease fragments containing the *Mad4* 5′ flanking region were inserted upstream of the CAT gene in pCAT-Basic (Promega Biotech). Deletions within the *Mad4* core promoter region were made using the Erase-a-base system (Promega Biotech); the resulting fragments were inserted into pCAT-Basic to generate reporters containing *Mad4* sequences −26 to +23 and −26 to −1. The *Mad4* initiator within the *Mad4*–CAT (−26 to +23) construct was replaced with the TATA box of the adenovirus major late promoter using the Stratagene QuikChange site-directed mutagenesis system with the oligonucleotide CGACTTATGTGTATAAAAGAGGGCCTGAGG.

Preparation of nuclear protein extracts

The method was modified from the procedure of Andrews and Faller [32]. Cells (3×10^6) were rinsed with cold PBS, resuspended in 1.2 ml of PBS and centrifuged for 10 s at 10000 ***g***. Cells were resuspended in 400 μl of cold Buffer A (10 mM Hepes/KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF) and allowed to swell for 10 min on ice. The sample was vortexed for 10 s and then centrifuged for 10 s at 10000 ***g***. The pellet was resuspended in two packed cell volumes of cold Buffer C [20 mM Hepes/KOH, pH 7.9, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF] and incubated on ice for 20 min. Cellular debris was removed by centrifugation at 11000 ***g*** for 2 min at 4 °C. The supernatant was recovered and stored at −80 °C.

Electrophoretic mobility shift assay (EMSA)

Binding reactions were carried out in a volume of 25 μl and contained 4% (w/v) Ficoll 400, 16 mM Hepes/KOH, pH 7.9, 1 mM spermidine, 0.03% (v/v) Nonidet P40, 1 mM dithiothreitol, 1 μg of poly(dI-dC) and 5 μg of protein extract. Unlabelled competitor was used at a 100-fold molar excess, and was preincubated with the binding reaction for 30 min on ice. Radiolabelled DNA probe (1 ng) was then added, and the reaction was incubated for a further 20 min at 30 °C. Where appropriate, binding reactions were incubated with or without antibodies overnight at 4 °C. The binding reactions were electrophoresed on a 4% (w/v) polyacrylamide [37.5:1 (w/w) acrylamide/bisacrylamide] gel at 220 V for 2.5 h in 0.5 × TBE (1 × TBE = 45 mM Tris/borate/1 mM EDTA) and 0.03% (v/v) Nonidet P40. Antibodies were purchased from Santa Cruz (anti-c-Myc, N-262; anti-Miz-1, N-17).

Dimethyl sulphate (DMS) modification interference analysis

Double-stranded DNA probes that were end-labelled on one strand were dissolved in 10 μl of water. A 91 μl reaction mixture containing 10 μl of 0.5 M sodium cacodylate, pH 8.0, 1 μl of 1 M $MgCl_2$, 0.2 μl of 0.5 M EDTA and 0.5 μl of DMS was added.

The mixture was incubated at room temperature for 3.5 min and the reaction was quenched by the addition of 25 μl of DMS stop buffer [1.5 M sodium acetate, pH 7.0, 200 μg/ml *Escherichia coli* tRNA, 7 % (v/v) 2-mercaptoethanol]. The DNA was precipitated with ethanol and resuspended in water to a specific radioactivity of 15000–20000 c.p.m./μl. An EMSA was carried out in a 100 μl volume containing 4 μg of poly(dI-dC), 50 μg of protein extract and 120000 c.p.m. of radiolabelled DMS-modified probe. The reaction was incubated at 30 °C for 20 min and electrophoresed on a 4 % (w/v) polyacrylamide gel. Gel slices containing free probe and protein-bound probe (complex A) were excised, and the DNA was eluted in 500 μl of elution buffer (2 M NaCl, 50 mM Tris/HCl, pH 8.0, 1 mM EDTA) at 50 °C. The DNA was precipitated with ethanol and resuspended in 200 μl of 100 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 mM EDTA and 0.5 % (w/v) SDS. Samples were extracted twice with phenol/chloroform, precipitated with ethanol and resuspended in 90 μl of water. Piperidine (10 μl) was added and the samples were incubated at 90 °C for 30 min. A G-ladder was also generated by incubation of DMS-modified probe (60000 c.p.m.) with piperidine. DNA was precipitated with ethanol, resuspended in 50 % formamide, 20 mM EDTA, 0.05 % Bromophenol Blue and 0.05 % xylene cyanol, and electrophoresed on a 7 M urea/15 % (w/v) polyacrylamide [19:1 (w/w) acrylamide/bisacrylamide] gel in TBE.

RESULTS AND DISCUSSION

Different *Mad* family genes have strikingly distinct patterns of expression during differentiation in MEL cells

We initially sought to identify a cell line that would be suitable for analysing *Mad* gene regulation in transfection studies. MEL cells are virus-transformed erythroid precursor cells that may be induced to differentiate in culture by the addition of various chemicals such as DMSO. Differentiation is associated with well-characterized changes in gene expression, and culminates in the appearance of terminally differentiated cells that express globin [33–35]. MEL cells have proven amenable for the analysis of gene regulatory elements in stable transfections, and have been used for the characterization of sequence elements that mediate the tissue-specific activation of globin genes during erythroid differentiation (e.g. [36]). The patterns of expression of c-*Myc* and of *Mad* family genes during MEL cell differentiation have previously been partially characterized. Mad1 mRNA is induced during differentiation [37], whereas levels of c-Myc mRNA decline in a biphasic manner as differentiation proceeds [38]. In contrast, Mad3 mRNA is biphasically induced and is then down-regulated in terminally differentiated cells [9].

In order to evaluate MEL cells for studying *Mad* gene regulation, we used ribonuclease protection assays to analyse the pattern of expression of each *Mad* family member during DMSO-induced differentiation (Figure 1). The expression of mouse β-major globin mRNA was monitored as a control for the differentiation procedure (Figure 1E). c-*Myc* expression showed a characteristic biphasic decline: levels decreased immediately upon addition of inducer, and rose again at 8–12 h, before declining during the terminal differentiation stages (Figure 1A). The different *Mad* family genes showed distinct expression patterns. *Mad3* and *Mad4* were induced at the earliest time points (Figures 1A and 1D; 5–8 h), but thereafter showed different fluctuations: the induction of *Mad3* was biphasic, and expression was reduced in terminally differentiated cells, whereas the induction of *Mad4* was striking (> 30-fold) and persisted throughout differentiation. *Mxi1*, *Mad1* and *Mnt* were activated to a lesser extent (5-, 11- and 2-fold respectively) and at later time points during differentiation (Figures 1B–1D). The *Mxi1* probe simultaneously detected the Mxi1-SR (strong repressor) and Mxi1-WR (weak repressor) transcripts; the latter form of Mxi1 lacks the N-terminal Sin3 interaction domain and is therefore a less potent transcriptional repressor than full-length Mxi1-SR [39]. Individual *Mad* family genes thus have distinct patterns of expression during MEL cell differentiation, suggesting that they each serve a unique function and that they are each regulated by a different transcriptional mechanism. The induction of Mad4 mRNA during MEL cell differentiation was in agreement with observations in other systems (e.g. [10]). The analysis of *Mad4* activation in transfected MEL cells should thus provide information that is of fundamental importance to the understanding of differentiation.

Identification of regulatory elements that mediate *Mad4* activation during MEL cell differentiation

In order to identify regulatory elements that mediate the induction of *Mad4* during differentiation, we used reporter gene assays in stably transfected MEL cells. We initially isolated a mouse *Mad4* genomic clone from a λ library, and a 5.2 kb *Bgl*II restriction endonuclease fragment containing the 5′ end of the gene was subcloned for further analysis. The transcriptional start sites of *Mad4* were mapped by ribonuclease protection analysis using a probe that spanned the 5′ end of the gene (Figure 2A). This revealed multiple sites of transcriptional initiation within a region located 130–170 nt upstream from the ATG translation initiation codon. The transcriptional start sites within this region were confirmed by primer extension analysis (results not shown). Sequence analysis showed that the Mad4 promoter is TATA-less, GC-rich, and contains a putative initiator sequence (consensus YYANT/AYY) [40] that coincides with the start sites (Figure 2B).

A reporter gene was generated by inserting approx. 2 kb of *Mad4* 5′ flanking sequence upstream of the CAT gene (Figure 3A). This construct was introduced into MEL cells by stable transfection, and five independent clones were propagated for further analysis. The patterns of expression of endogenous *Mad4* and of transfected *Mad4*–CAT were measured during DMSO-induced differentiation using ribonuclease protection analysis. The expression of *Mad4*–CAT was induced during differentiation (5–20-fold) in all clones analysed (Figure 3A, lanes 1 and 2), thus validating the use of this system for analysing *Mad4* regulation. Serial deletions were generated within the *Mad4* 5′ flanking region of *Mad4*–CAT (Figure 3A) and expression of the resulting reporter constructs was analysed similarly in transfected MEL clones. The patterns of expression of *Max*, *Mnt* and c-*Myc* were monitored as controls for RNA loading and for differentiation (results not shown; examples in Figures 3 and 5). The expression of each *Mad4*–CAT reporter construct was induced in at least four out of the five clones analysed (Figure 3A). Induction levels ranged from 3- to > 30-fold; this variation probably reflected both clonal differences in endogenous *Mad4* induction and the different chromosomal integration sites of the transfected genes. This analysis thus revealed that the regulatory elements required for *Mad4* induction were located within a 161 nt fragment that spans the sites of transcription initiation. Further deletions within this 161 nt regulatory region were then created to generate reporter constructs containing *Mad4* sequences −26 to +23 and −26 to −1. The expression of *Mad4*–CAT (−26 to +23) was inducible during differentiation in all five stably transfected MEL clones analysed (Figure 3B, lanes 1–4). In contrast, a further

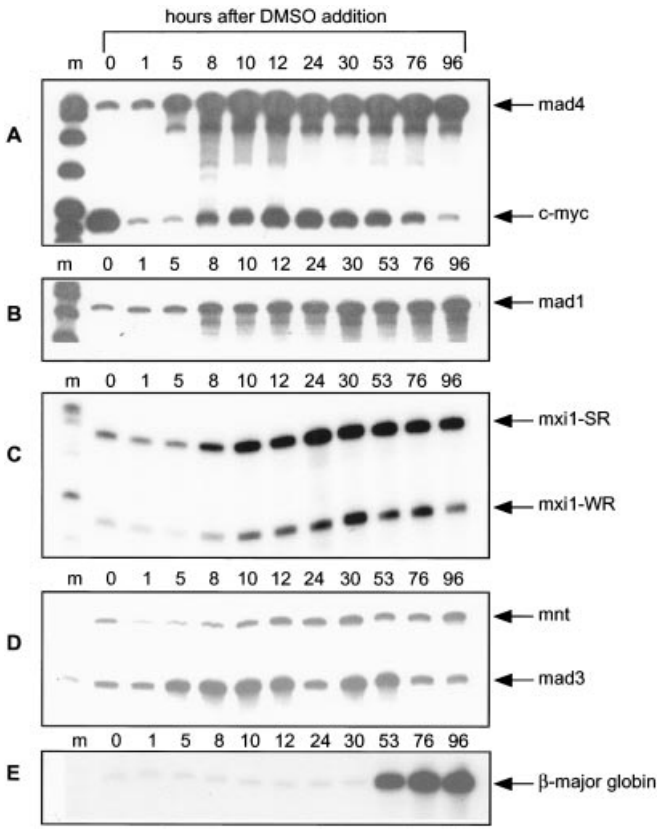

Figure 1 Expression of *Mad* family genes during differentiation in MEL cells

MEL cells were induced to differentiate by culturing in 2% (v/v) DMSO, and levels of *Mad* family transcripts were measured by ribonuclease protection mapping. Hybridizations contained probes that detected *Mad4* and c-*Myc* (**A**), *Mad1* (**B**), *Mxi1* (**C**), *Mnt* and *Mad3* (**D**) or mouse β-major globin (**E**). An α-tubulin probe was used as a loading control (not shown). SR and WR denote strong repressor and weak repressor respectively. Lane m contains size markers. These experiments were carried out three times and representative gels are shown.

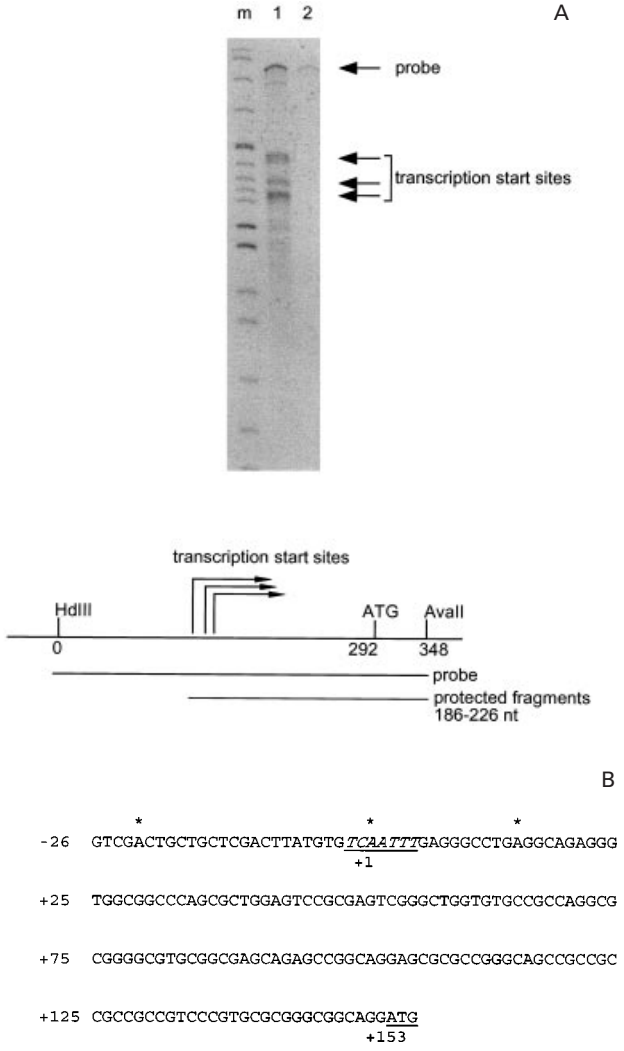

Figure 2 Identification of *Mad4* transcription start sites

(**A**) Ribonuclease protection mapping of *Mad4* transcription initiation sites. Samples of MEL cell RNA (lane 1) or tRNA (lane 2) were hybridized to a riboprobe derived from the 5′ end of the mouse *Mad4* gene. Bands representing the probe and the transcription start sites are indicated. Lane m contains size markers. (**B**) Schematic representation of the *Mad4* promoter. The nucleotide sequence of the *Mad4* 5′ flanking region is depicted. The ATG translation initiation codon is underlined, and sites of transcriptional initiation are indicated with asterisks. The initiator element is shown in italics and underlined. The transcription start site within the initiator is designated +1.

deletion [*Mad4*–CAT (−26 to −1); Figure 3B, lanes 5 and 6] resulted in a reduction in basal transcription and a loss of inducibility in all clones analysed. The DNA sequences required for *Mad4* induction are thus located within the 49 nt −26 to +23 region that contains the core promoter element and spans the sites of transcriptional initiation.

The timing of the transcriptional activation of individual genes is tightly regulated and takes place at distinct stages during MEL cell differentiation. For example, the induction of *Mad4* occurs 5–8 h after DMSO addition, whereas β-major globin mRNA is not increased until the later stages of differentiation (Figure 1). It was therefore important to verify that the transfected *Mad4*–CAT reporter constructs were induced at the same time as endogenous *Mad4* during the differentiation time course. The expression of endogenous *Mad4*, endogenous c-*Myc* and transfected *Mad4*–CAT (−26 to +23) was measured at different time points during DMSO-induced differentiation (Figure 3C). The expression of endogenous c-*Myc* and *Mad4* showed the characteristic fluctuations during differentiation. The induction of transfected *Mad4*–CAT (−26 to +23) occurred 5–8 h after the addition of DMSO, and was concomitant with the activation of endogenous *Mad4*. The *Mad4* (−26 to +23) sequence element thus mediates the correct timing of induction during MEL cell differentiation.

Protein interactions at the −26 to +23 *Mad4* regulatory element

In order to elucidate the mechanism whereby the −26 to +23 *Mad4* regulatory region mediates transcriptional activation, we used EMSAs to study the interaction of protein complexes with this element. The binding of proteins to the radiolabelled 49 nt double-stranded probe was analysed using nuclear extracts prepared from both undifferentiated (Figure 4A, lanes 2 and 4) and differentiated (Figure 4A, lanes 3 and 5) MEL cells. EMSAs

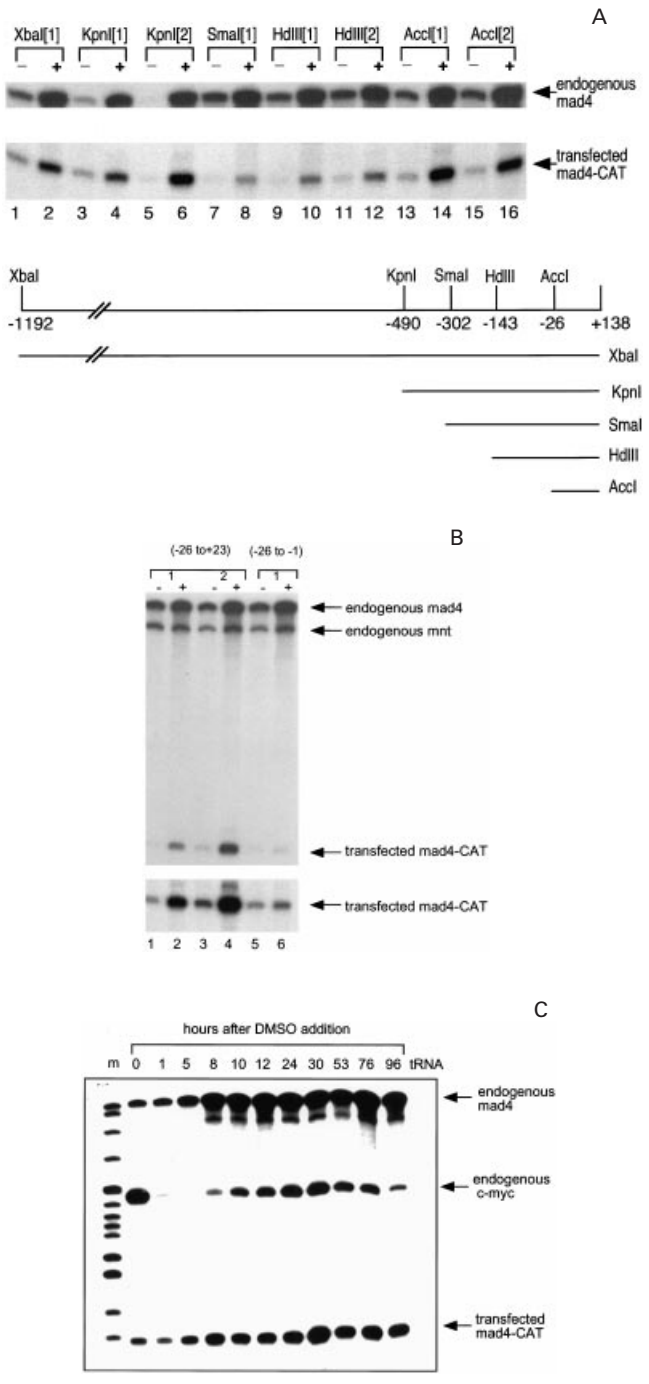

Figure 3 Activation of *Mad4* during MEL cell differentiation is mediated by a 49 nt core promoter element

(**A**) Expression of *Mad4*–CAT reporter genes in stably transfected MEL cells. Restriction fragments containing the indicated regions of *Mad4* were inserted upstream of the CAT gene in pCAT-Basic. Reporter constructs were stably transfected into MEL cells, and independent clones were induced to differentiate by culturing for 96 h in 2% (v/v) DMSO. RNA was harvested before (−) and after (+) differentiation and analysed by ribonuclease protection mapping using probes that detected endogenous *Mad4* or transfected *Mad4*–CAT. Representative clones (designated 1 or 2) are shown. (**B**) Expression of reporter genes containing the *Mad4* core promoter element. Reporter constructs containing the *Mad4* 5′ flanking region (nt −26 to +23 or nt −26 to −1) were introduced into MEL cells. The expression of endogenous *Mad4*, endogenous *Mnt* and transfected *Mad4*–CAT was measured by ribonuclease protection mapping using RNA samples harvested before (−) and after (+) differentiation. Representative clones are shown. The lower panel represents a longer exposure of the gel. (**C**) Time course of expression of the *Mad4*–CAT (−26 to +23) reporter during MEL cell differentiation. A MEL clone containing the *Mad4*–CAT (−26 to +23) reporter was induced to differentiate and RNA was harvested at the indicated time points. Levels of endogenous *Mad4*, endogenous c-*Myc* and transfected *Mad4*–CAT were measured by ribonuclease protection mapping. Lane m contains size markers.

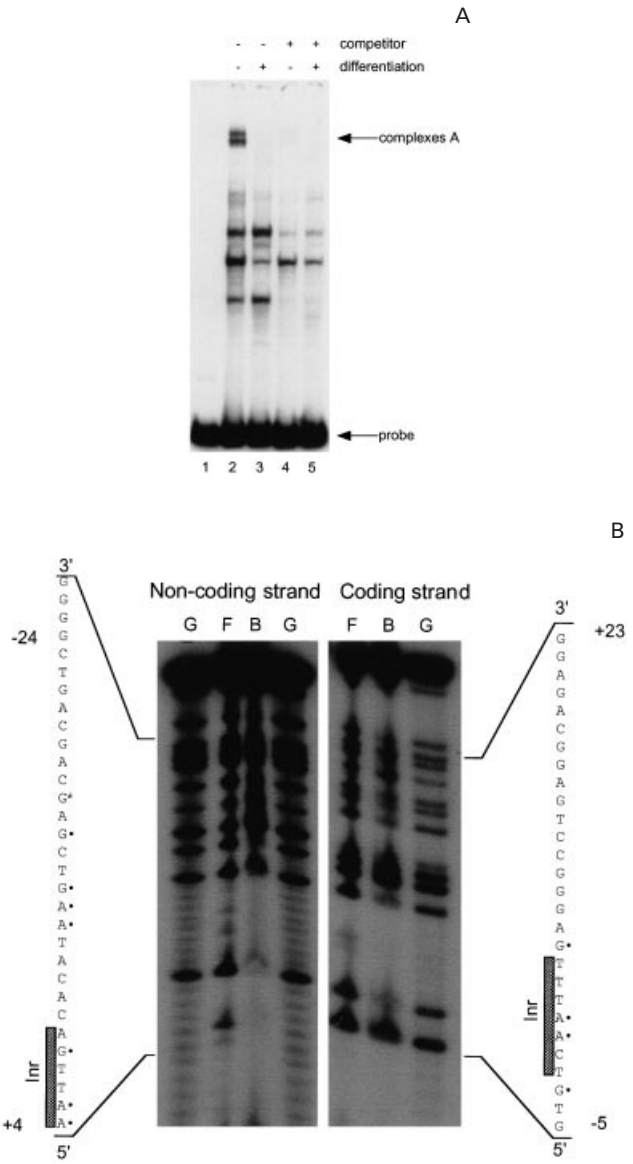

Figure 4 Analysis of protein binding to the *Mad4* 49 nt regulatory region

(**A**) EMSA of protein interactions with the *Mad4* −26 to +23 region. A DNA fragment comprising the *Mad4* −26 to +23 region was radiolabelled and EMSAs were carried out using extracts prepared from undifferentiated or differentiated MEL cells. Incubations were carried out in the presence or absence of excess unlabelled competitor −26 to +23 fragment. Lane 1 represents probe that was incubated in the absence of extract. The positions of free probe and of complexes A are indicated. (**B**) Methylation interference analysis. A DNA fragment comprising the −26 to +23 region was radiolabelled, treated with DMS and incubated in an EMSA using an extract derived from undifferentiated MEL cells. Bands corresponding to free probe (F) and to complex A (B) were excised, and the DNA was eluted and treated with piperidine. Filled circles and asterisks indicate residues that are under-represented or over-represented respectively in complex A as compared with free probe. Lane G represents a G sequence ladder corresponding to the −26 to +23 region. This experiment was carried out three times with similar results.

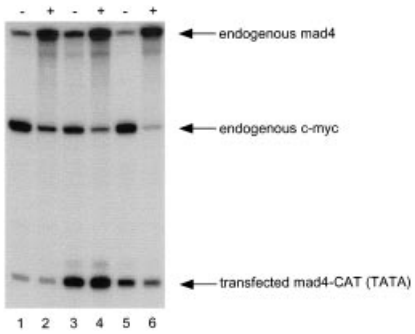

Figure 5 The initiator element is required for *Mad4* activation during MEL cell differentiation

The initiator element of the *Mad4*–CAT (−26 to +23) reporter was replaced with a TATA box. Expression of *Mad4*–CAT (TATA) was analysed by ribonuclease protection mapping in eight transfected MEL clones using RNA harvested before (−) and after (+) differentiation; three representative clones are shown.

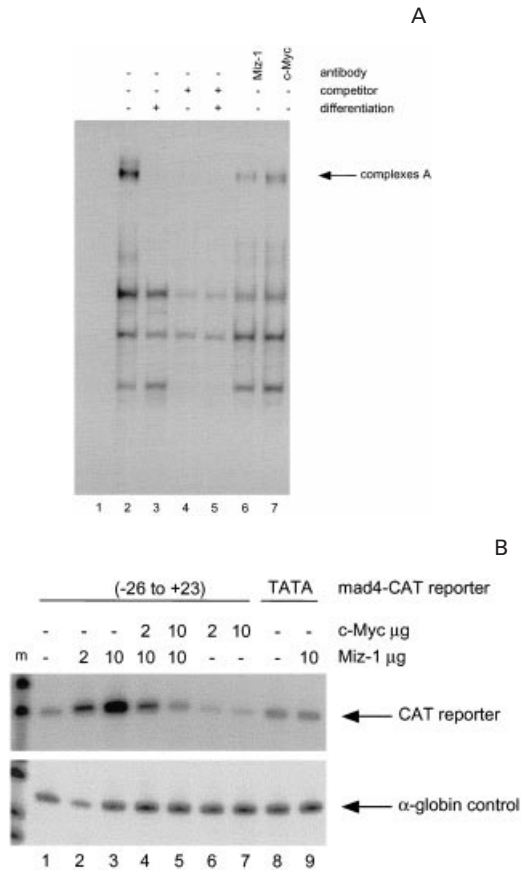

Figure 6 *Mad4* is regulated by Miz-1 and c-Myc

(**A**) Miz-1 and c-Myc bind to the *Mad4* initiator. EMSAs were carried out as in Figure 4 using extracts prepared from undifferentiated or differentiated MEL cells. Binding reactions were incubated with excess unlabelled competitor −26 to +23 fragment or with antibodies as indicated. (**B**) Regulation of *Mad4*–CAT (−26 to +23) by Miz-1 and c-Myc in transient transfection assays. *Mad4*–CAT (−26 to +23) or *Mad4*–CAT (TATA) was transiently transfected into HeLa cells together with the indicated amounts of plasmids expressing Miz-1 or c-Myc. Samples of 50 μg of RNA were analysed by ribonuclease protection mapping using a probe that detected the CAT reporter gene. Expression of the α-globin gene transfection control was monitored using 1 μg RNA samples.

containing excess unlabelled competitor 49 nt fragment revealed specific protein interactions at the −26 to +23 region (Figure 4A, lanes 4 and 5). Comparison of protein binding using extracts derived from undifferentiated and differentiated cells revealed that the interaction of two slow-migrating complexes (complexes A) was significantly decreased in differentiated cells (Figure 4A, lanes 2 and 3). DMS methylation interference analysis of these complexes revealed sites of protein interaction with the initiator element that is located centrally within the 49 nt region (Figure 4B); analysis of either the upper or the lower band within complex A yielded an identical result (not shown).

The initiator element is required for *Mad4* induction during MEL cell differentiation

Since the induction of *Mad4* is associated with changes in protein interactions at the initiator, we sought to determine if this sequence element is essential for transcriptional activation during differentiation. The initiator element of *Mad4*–CAT (−26 to +23) was replaced with a TATA box by *in vitro* site-directed mutagenesis, and expression of the resulting *Mad4*–CAT (TATA) construct was analysed in eight independent stably transfected MEL clones. Expression of *Mad4*–CAT (TATA) was not inducible during differentiation in any of these clones (Figure 5). In a simultaneous experiment, expression of the parent *Mad4*–CAT (−26 to +23) construct was inducible during differentiation in an additional seven out of eight MEL clones analysed (results not shown). EMSA analysis showed that replacement of the initiator element with a TATA box resulted in loss of binding of complex A to the 49 nt regulatory region (results not shown). These data thus strengthen the hypothesis that *Mad4* activation is mediated, at least in part, by changes in protein interactions at the initiator element.

Mad4 is regulated by the interaction of Miz-1 and c-Myc at the initiator

The DNA sequence of the *Mad4* initiator resembles the consensus initiator element that is found within genes that are transcriptionally repressed by c-Myc (TCA[+1]NYYY) [41]. An important mechanism whereby c-Myc represses transcription involves its interaction with Miz-1 at the initiator of target genes; c-Myc antagonizes the transcriptional activation function of Miz-1 by interfering with recruitment of the co-activator p300. The activation of *Mad4* during MEL cell differentiation is associated with a loss of complexes A from the initiator; since the loss of complexes A is correlated with a reduction in c-Myc levels during differentiation, we initially sought to determine whether these complexes contain Miz-1 and c-Myc. EMSAs were carried out in the presence of antibodies raised against either Miz-1 or c-Myc (Figure 6A); this resulted in a decrease in the level of complexes A, and the magnitude of this effect was consistent with results obtained for other initiator elements, such as p15INK4B and p21Cip1, that are regulated by c-Myc [22,24]. In order to determine whether the *Mad4* initiator is regulated by Miz-1 and c-Myc *in vivo*, we transiently transfected the *Mad4*–CAT (−26 to +23) reporter into HeLa cells together with various amounts of plasmids that express either Miz-1 or c-Myc. Miz-1 activated the transcription of *Mad4*–CAT (−26 to +23) (Figure 6B, lanes 1–3), and this effect was antagonized by the co-expression of c-Myc (Figure 6B, lanes 4 and 5). The expression of c-Myc in the absence of Miz-1 resulted in a modest 2-fold reduction in reporter gene expression (Figure 6B, lanes 6 and 7). Miz-1 was unable to

activate the *Mad4*–CAT (TATA) reporter, thus confirming that the *Mad4* initiator mediates transcriptional regulation by Miz-1 and c-Myc (Figure 6B, lanes 8 and 9).

Conclusion

Cellular differentiation is characterized by timely changes in gene expression, and culminates in the cessation of proliferation and the appearance of cell-type-specific differentiation markers. Fluctuations in the levels of c-Myc and Mad family proteins are central to the regulation of proliferation and differentiation, and the activation of *Mad4* is an integral feature of diverse differentiation programmes. Many studies have illustrated the role of tissue-specific transcriptional activators and repressors in gene control during the differentiation of specific lineages. In contrast, less is known about the mechanisms that regulate genes involved in more general processes, such as cell-cycle exit, that occur during the differentiation of diverse cell types.

In the present study, we show that the different *Mad* family genes have distinct patterns of expression during differentiation in MEL cells, suggesting that they each serve a unique function and that they are regulated by different mechanisms. The activation of *Mad4* in terminally differentiated MEL cells is in agreement with observations in other systems. Using reporter gene assays in stably transfected MEL cells, we show that the 49 nt core promoter region mediates *Mad4* induction. We demonstrate that the initiator element is required for *Mad4* regulation, and show that differentiation is associated with the loss from the initiator of a complex that contains Miz-1 and c-Myc. We also show that Miz-1 activates the *Mad4* promoter in transient transfection assays, and that c-Myc antagonizes this effect, thus identifying *Mad4* as a novel target of transcriptional repression by c-Myc. These data are consistent with a model in which a c-Myc–Miz-1 repressor complex suppresses the expression of *Mad4* in proliferating undifferentiated cells. The loss of c-Myc during differentiation thus leads to a relief of transcriptional repression and contributes to *Mad4* activation; high levels of *Mad4* expression in differentiated cells may then be directed by Miz-1 and/or other activators that bind to the 49 nt regulatory element. The present work and other studies suggest that c-Myc mediates the co-ordinate suppression of a variety of genes that are associated with cell-cycle arrest and differentiation; the aberrant expression of c-Myc may therefore contribute to tumorigenesis via the inappropriate suppression of such genes.

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REFERENCES

1 Grandori, C., Cowley, S. M., James, L. P. and Eisenman, R. N. (2000) The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu. Rev. Cell Dev. Biol. **16**, 653–699

2 Eisenman, R. N. (2001) Deconstructing myc. Genes Dev. **15**, 2023–2030

3 Coppola, J. A. and Cole, M. D. (1986) Constitutive c-myc oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. Nature (London) **320**, 760–763

4 Cultraro, C. M., Bino, T. and Segal, S. (1997) Function of the c-Myc antagonist Mad1 during a molecular switch from proliferation to differentiation. Mol. Cell. Biol. **17**, 2353–2359

5 Nesbit, C. E., Tersak, J. M. and Prochownik, E. V. (1999) MYC oncogenes and human neoplastic disease. Oncogene **18**, 3004–3016

6 Roussel, M. F., Ashmun, R. A., Sherr, C. J., Eisenman, R. N. and Ayer, D. E. (1996) Inhibition of cell proliferation by the Mad1 transcriptional repressor. Mol. Cell. Biol. **16**, 2796–2801

7 Chen, J., Willingham, T., Margraf, L. R., Schreiber-Agus, N., DePinho, R. A. and Nisen, P. D. (1995) Effects of the MYC oncogene antagonist, MAD, on proliferation, cell cycling and the malignant phenotype of human brain tumour cells. Nat. Med. (N.Y.) **1**, 638–643

8 Foley, K. P., McArthur, G. A., Queva, C., Hurlin, P. J., Soriano, P. and Eisenman, R. N. (1998) Targeted disruption of the MYC antagonist MAD1 inhibits cell cycle exit during granulocyte differentiation. EMBO J. **17**, 774–785

9 Fox, E. J. and Wright, S. C. (2001) S-phase-specific expression of the Mad3 gene in proliferating and differentiating cells. Biochem. J. **359**, 361–367

10 Pulverer, B., Sommer, A., McArthur, G. A., Eisenman, R. N. and Luscher, B. (2000) Analysis of Myc/Max/Mad network members in adipogenesis: inhibition of the proliferative burst and differentiation by ectopically expressed Mad1. J. Cell. Physiol. **183**, 399–410

11 Queva, C., Hurlin, P. J., Foley, K. P. and Eisenman, R. N. (1998) Sequential expression of the MAD family of transcriptional repressors during differentiation and development. Oncogene **16**, 967–977

12 Hurlin, P. J., Queva, C., Koskinen, P. J., Steingrimsson, E., Ayer, D. E., Copeland, N. G., Jenkins, N. A. and Eisenman, R. N. (1995) Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. EMBO J. **14**, 5646–5659

13 Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J. F., Obaya, A. J., O'Connell, B. C., Mateyak, M. K., Tam, W., Kohlhuber, F. et al. (2000) Identification of CDK4 as a target of c-MYC. Proc. Natl. Acad. Sci. U.S.A. **97**, 2229–2234

14 Miltenberger, R. J., Sukow, K. A. and Farnham, P. J. (1995) An E-box-mediated increase in cad transcription at the G1/S-phase boundary is suppressed by inhibitory c-Myc mutants. Mol. Cell. Biol. **15**, 2527–2535

15 McMahon, S. B., Wood, M. A. and Cole, M. D. (2000) The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. Mol. Cell. Biol. **20**, 556–562

16 Wood, M. A., McMahon, S. B. and Cole, M. D. (2000) An ATPase/helicase complex is an essential cofactor for oncogenic transformation by c-Myc. Mol. Cell **5**, 321–330

17 Ayer, D. E., Kretzner, L. and Eisenman, R. N. (1993) Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell **72**, 211–222

18 Zervos, A. S., Gyuris, J. and Brent, R. (1993) Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72**, 223–232

19 Ayer, D. E., Lawrence, Q. A. and Eisenman, R. N. (1995) Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell **80**, 767–776

20 Hassig, C. A., Fleischer, T. C., Billin, A. N., Schreiber, S. L. and Ayer, D. E. (1997) Histone deacetylase activity is required for full transcriptional repression by mSin3A. Cell **89**, 341–347

21 Claassen, G. F. and Hann, S. R. (1999) Myc-mediated transformation: the repression connection. Oncogene **18**, 2925–2933

22 Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massague, J., Hanel, F. and Eilers, M. (2001) Repression of p15INK4b expression by Myc through association with Miz-1. Nat. Cell Biol. **3**, 392–399

23 Peukert, K., Staller, P., Schneider, A., Carmichael, G., Hanel, F. and Eilers, M. (1997) An alternative pathway for gene regulation by Myc. EMBO J. **16**, 5672–5686

24 Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M. and Massague, J. (2001) TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. Nat. Cell Biol. **3**, 400–408

25 Dang, C. V. (1999) c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol. Cell. Biol. **19**, 1–11

26 O'Hagan, R. C., Schreiber-Agus, N., Chen, K., David, G., Engelman, J. A., Schwab, R., Alland, L., Thomson, C., Ronning, D. R., Sacchettini, J. C. et al. (2000) Gene-target recognition among members of the myc superfamily and implications for oncogenesis. Nat. Genet. **24**, 113–119

27 Oh, S., Song, Y. H., Yim, J. and Kim, T. K. (2000) Identification of Mad as a repressor of the human telomerase (hTERT) gene. Oncogene **19**, 1485–1490

28 Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. and Axel, R. (1979) Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell **16**, 777–785

29 Morgenstern, J. P. and Land, H. (1990) A series of mammalian expression vectors and characterization of their expression of a reporter gene in stably and transiently transfected cells. Nucleic Acids Res. **18**, 1068

30 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156–159

31 Wright, S. and Bishop, J. M. (1989) DNA sequences that mediate attenuation of transcription from the mouse protooncogene myc. Proc. Natl. Acad. Sci. U.S.A. **86**, 505–509

32 Andrews, N. C. and Faller, D. V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. **19**, 2499

33 Levy, J., Terada, M., Rifkind, R. A. and Marks, P. A. (1975) Induction of erythroid differentiation by dimethylsulfoxide in cells infected with Friend virus: relationship to the cell cycle. Proc. Natl. Acad. Sci. U.S.A. **72**, 28–32

34 Marks, P. A. and Rifkind, R. A. (1978) Erythroleukemic differentiation. Annu. Rev. Biochem. **47**, 419–448
35 Rifkind, R. A., Sheffery, M., Profous-Juchelka, H. R., Reuben, R. C. and Marks, P. A. (1983) Regulation of globin gene expression during induced erythroid cell differentiation. Prog. Clin. Biol. Res. **134**, 183–192
36 Wright, S., Rosenthal, A., Flavell, R. and Grosveld, F. (1984) DNA sequences required for regulated expression of beta-globin genes in murine erythroleukemia cells. Cell **38**, 265–273
37 Cultraro, C. M., Bino, T. and Segal, S. (1997) Regulated expression and function of the c-Myc antagonist, Mad1, during a molecular switch from proliferation to differentiation. Curr. Top. Microbiol. Immunol. **224**, 149–158
38 Lachman, H. M. and Skoultchi, A. I. (1984) Expression of c-myc changes during differentiation of mouse erythroleukaemia cells. Nature (London) **310**, 592–594
39 Schreiber-Agus, N., Chin, L., Chen, K., Torres, R., Rao, G., Guida, P., Skoultchi, A. I. and DePinho, R. A. (1995) An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. Cell **80**, 777–786
40 Smale, S. T. and Baltimore, D. (1989) The ''initiator'' as a transcription control element. Cell **57**, 103–113
41 Li, L. H., Nerlov, C., Prendergast, G., MacGregor, D. and Ziff, E. B. (1994) c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. EMBO J. **13**, 4070–4079

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