# S-phase-specific expression of the *Mad3* gene in proliferating and differentiating cells

Elizabeth J. FOX and Stephanie C. WRIGHT[1]

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

The Myc/Max/Mad transcription factor network plays a central role in the control of cellular proliferation, differentiation and apoptosis. In order to elucidate the biological function of Mad3, we have analysed the precise temporal patterns of *Mad3* mRNA expression during the cell cycle and differentiation in cultured cells. We show that *Mad3* is induced at the G1/S transition in proliferating cells; expression persists throughout S-phase, and then declines as cells pass through G2 and mitosis. The expression pattern of *Mad3* is coincident with that of *Cdc2* throughout the cell cycle. In contrast, the expression of *Mad3* during differentiation of cultured mouse erythroleukemia cells shows two transient peaks of induction. The first of these occurs at the onset of differentiation, and does not correlate with the S-phase of the cell cycle, whereas the second is coincident with the S-phase burst that precedes the terminal stages of differentiation. Our results therefore suggest that Mad3 serves a cell-cycle-related function in both proliferating and differentiating cells, and that it may also have a distinct role at various stages of differentiation.

Key words: cell cycle, differentiation, Mad, Myc, transcription.

## INTRODUCTION

The Myc/Max/Mad transcription factor network plays a central role in the control of cellular proliferation, differentiation and apoptosis (reviewed in [1,2]). Myc family proteins accelerate cell-cycle progression in diverse settings in cultured cells, and also play a role in initiating apoptosis under specific conditions (reviewed in [3]). Myc is generally expressed in proliferating cells and decreases during differentiation; enforced expression of Myc inhibits the differentiation of several cultured cell lines (for example, [4,5]; reviewed in [6]). Overexpression of Myc also leads to the transformation of various cultured cells, and aberrant Myc expression is associated with many human tumours (reviewed in [7,8]). The biological activities of Myc are thought to be antagonised by the Mad family proteins (Mad1, Mxi1, Mad3 and Mad4) *in vivo* [9–11]; Mad proteins generally inhibit cellular proliferation and oppose the transforming activity of Myc in cultured cells [12–21].

Myc and Mad are highly unstable basic helix-loop-helix-leucine zipper proteins that function via heterodimerization with the stable, ubiquitously expressed basic helix-loop-helix-leucine zipper partner, Max [22,23]. Myc–Max and Mad–Max heterodimers specifically bind CACGTG and related E-box sequences *in vitro* [24]; in addition, Myc–Max also recognises noncanonical sites [25]. Myc–Max heterodimers activate transcription from reporter genes containing the sequence CACGTG in yeast and in transiently transfected mammalian cells [26,27]. In contrast, Mad–Max represses transcription and opposes the transcriptional activation function of Myc in these assays [9–11]. Transcriptional activation by Myc is mediated by distinct regions in the N-terminal portion of the protein [26,28]. These sequences mediate contacts with components of the basal transcription apparatus, and importantly are also involved in the recruitment of histone acetyl transferase and chromatin remodelling activities [29–32]. The transcriptional repression function of Mad–Max is mediated via the N-terminal domain of Mad, which interacts with an mSin3 multi-protein complex that contains histone deacetylase activity [33–38]. It has therefore been suggested that the transcriptional regulation of target genes by Myc–Max and Mad–Max is mediated via alterations in the acetylation of histones, although this simplistic model has recently been partially challenged [39]. Target genes that are transcriptionally regulated by Myc have been identified by a variety of approaches; these genes function in diverse biological processes such as cell growth, the cell cycle, apoptosis, signalling and adhesion (reviewed in [40–42]; see also [43,44]). Few target genes for the Mad family proteins have so far been identified [44,45]; consequently, it is not known to what extent the physiological target genes for Myc–Max and Mad–Max overlap.

The precise physiological functions of the different Mad family proteins remain to be elucidated. Mad1 is generally induced during differentiation, and has been shown to inhibit both cellular proliferation and apoptosis (for example, [46–49]). Targeted deletion of *Mad1* in mice has indicated a role in cell-cycle exit during myeloid differentiation [50]. Mxi1 is also often induced during differentiation, although in certain conditions it is expressed in proliferating cells [11,47,49]. Targeted deletion of *Mxi1* generated a more severe phenotype, resulting in hyperplasia in several tissues [51]. In addition, *Mxi1* deficient mice showed an increased susceptibility to tumour formation when crossed with mice with a targeted deletion of *Ink4a*. In contrast to Mad1 and Mxi1, Mad3 is expressed in both the proliferating and differentiating compartments in developing mouse embryos and adult tissues [49]. The expression of *Mad3* correlated with regions of bromodeoxyuridine incorporation in the neural progenitors of developing mouse embryos, suggesting that *Mad3* is expressed during the S-phase in these cells; it was suggested that *Mad3* expression is closely linked to cell-cycle exit and is restricted to the last S-phase prior to terminal differentiation [52]. Targeted deletion of *Mad3* in mice resulted in an increased sensitivity of neural progenitors and thymocytes to cell death upon $\gamma$-irradiation [52], but no defects in cell-cycle exit were observed.

In order to gain insight into the biological function of Mad3, we have analysed the precise temporal patterns of *Mad3* ex-

Abbreviations used: MEL cells, mouse erythroleukemia cells.

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

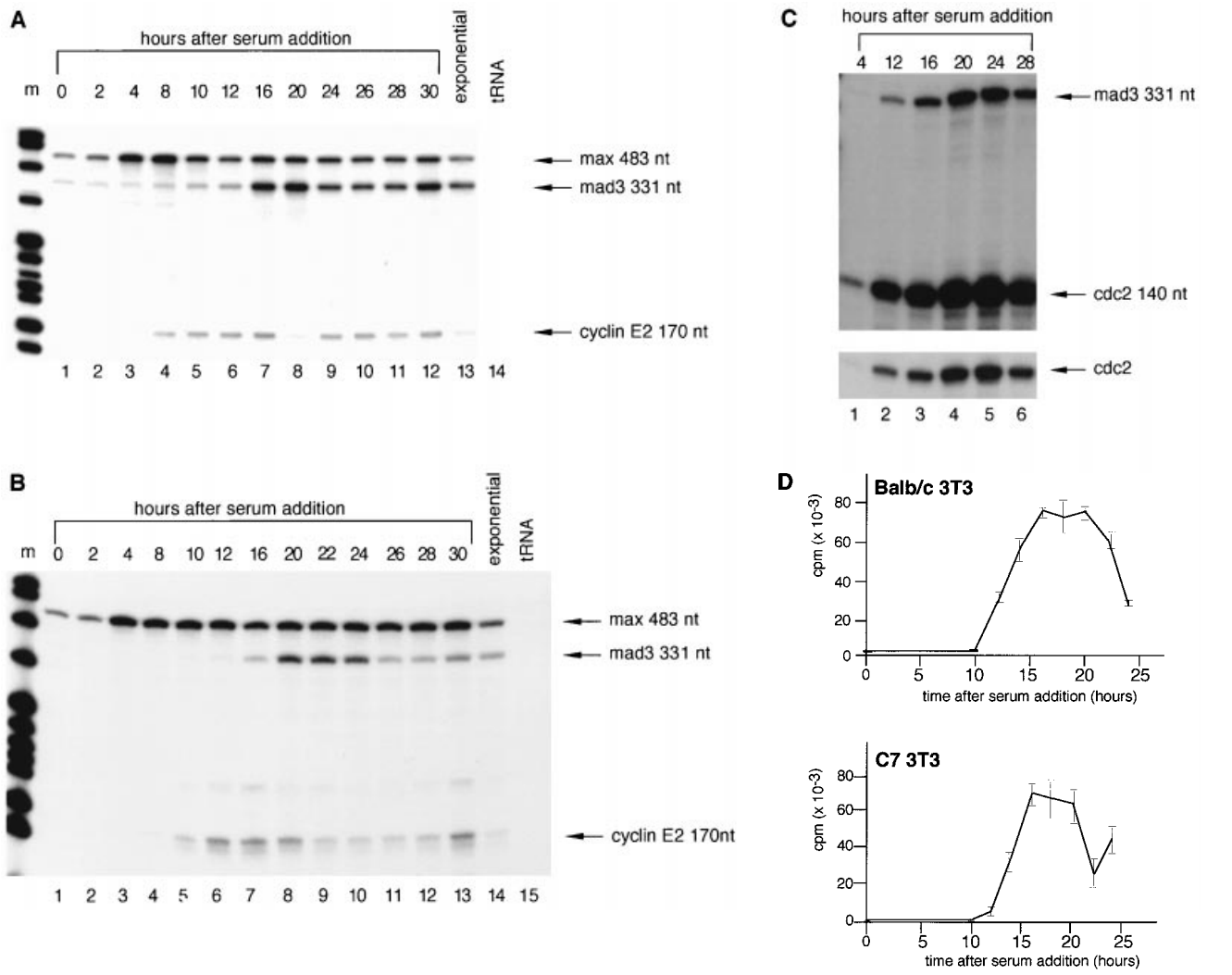


**Figure 1** ***Mad3*** **is expressed in the S-phase of the cell cycle following serum stimulation of quiescent cells**

(**A**) Expression of *Mad3*, *Max* and cyclin E2 during the cell cycle in Balb/c 3T3 fibroblasts. Quiescent Balb/c 3T3 fibroblasts were induced to re-enter the cell cycle by the addition of calf serum to 10%. Levels of *Mad3*, *Max* and cyclin E2 RNA were measured by ribonuclease protection analysis (lanes 1–12). Lane 13 represents the hybridization of the probes to an RNA sample derived from exponentially growing Balb/c 3T3 cells, and lane 14 represents the hybridization to *E. coli* tRNA. (**B**) Expression of *Mad3*, *Max* and cyclin E2 RNA during the cell cycle in C7 3T3 fibroblasts. C7 3T3 fibroblasts were analysed as described in (**A**). Lanes 1–13 represent RNA derived from the cell-cycle time course. Lane 14 represents the hybridization to RNA derived from exponentially growing cells, and lane 15 represents hybridization to *E. coli* tRNA. (**C**) *Mad3* and *Cdc2* show similar expression patterns during the cell cycle in C7 3T3 fibroblasts. Levels of *Mad3* and *Cdc2* RNA were measured by ribonuclease-protection analysis as described in (**A**). The lower panel shows a shorter exposure of the gel. (**D**) Measurement of DNA synthesis during the cell cycle in Balb/c 3T3 and C7 3T3 fibroblasts. Quiescent cells were induced to re-enter the cell cycle as described in (**A**). [$^3$H]Thymidine incorporation was measured at the times indicated. Results are expressed as the mean ± S.E.M. for three experiments.

pression during the cell cycle and differentiation in cultured cells. In conjunction with flow cytometric analysis and DNA synthesis assays, we show the *Mad3* is induced during the S-phase of the cell cycle in proliferating fibroblasts and epithelial cells in culture. By analysing the expression of several cell-cycle-regulated genes, we show that the induction of *Mad3* is coincident with that of *Cdc2*. In contrast, the expression of *Mad3* during differentiation of cultured mouse erythroleukemia (MEL) cells showed two transient peaks of induction. The first of these occurred at the onset of differentiation and did not correlate with the S-phase of the cell cycle, whereas the second was coincident with the S-phase burst that preceded the terminal stages of differentiation. Our results therefore suggest that the expression of *Mad3* is tightly linked to the S-phase of the cell cycle during proliferation and during the S-phase burst that immediately precedes terminal differentiation; the induction of *Mad3* that occurs at the onset of MEL differentiation is, however, clearly dissociated from cell-cycle regulation.

## EXPERIMENTAL

### Cell culture

Balb/c 3T3 fibroblasts, C7 3T3 fibroblasts, HeLa cells and MEL cells (line F412-B2) were cultured in MEM$\alpha$ medium supplemented with 10% calf serum. Balb/c 3T3 and C7 3T3 cells were made quiescent by culturing them for 28 h in serum-free medium at a density of $2 \times 10^6$ cells/10-cm-diameter dish. Cells were induced to re-enter the cell cycle by the addition of calf serum to 10%. Balb/c 3T3 and HeLa cells were arrested at metaphase by treatment with nocodazole. HeLa cells were treated with

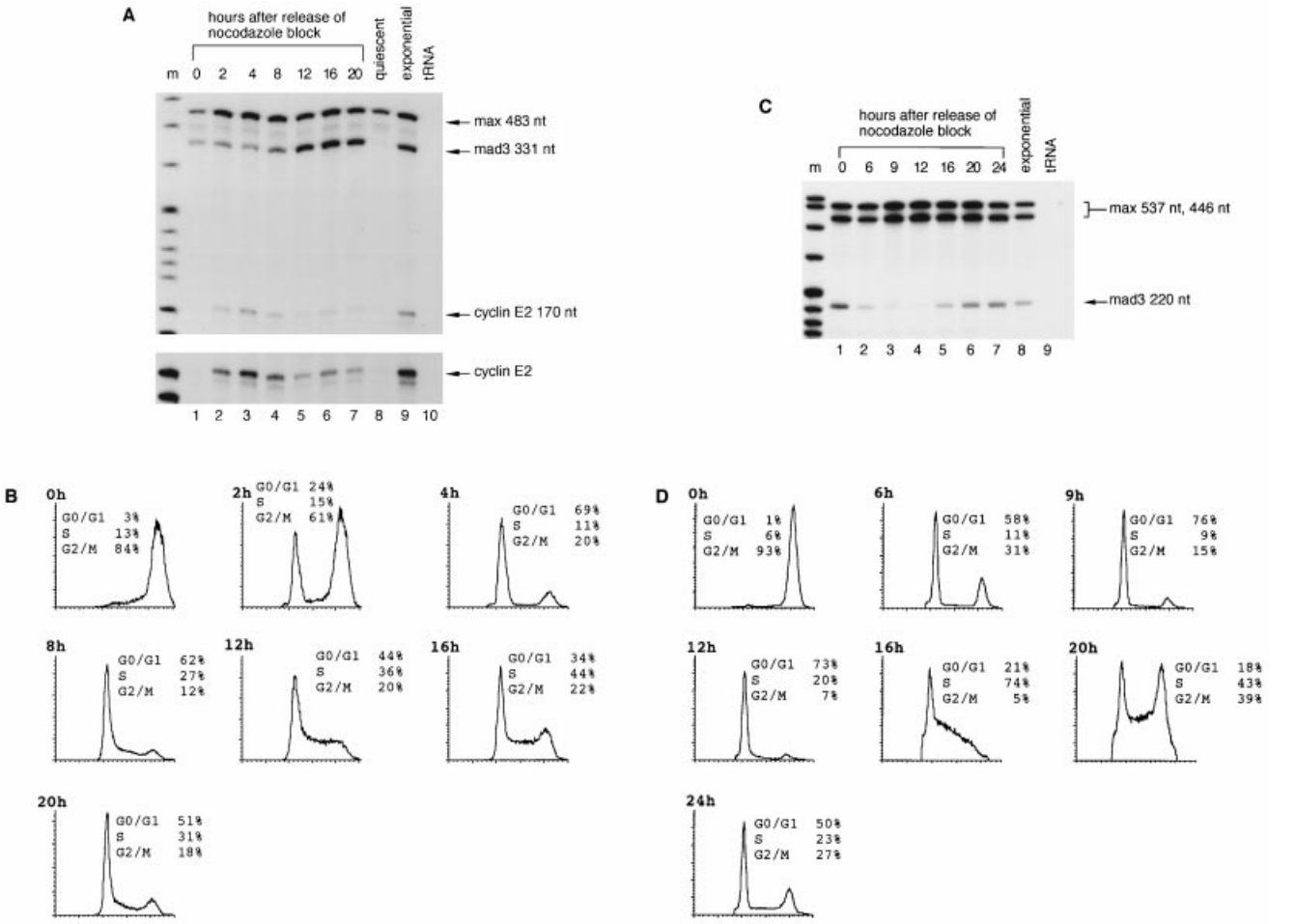


**Figure 2** ***Mad3*** **is expressed in the S-phase of the cell cycle following release from a nocodazole block**

(**A**) Expression of *Mad3*, *Max* and cyclin E2 during the cell cycle in Balb/c 3T3 fibroblasts. Balb/c 3T3 fibroblasts were arrested at metaphase by treatment with nocodazole. Cells were released from the block, and levels of *Mad3*, *Max* and cyclin E2 RNA measured by ribonuclease-protection analysis (lanes 1–7). Lane 8 represents the hybridization of the probes to RNA derived from serum-starved quiescent cells. Lane 9 represents the hybridization to RNA derived from exponentially growing cells. Lane 10 represents the hybridization to *E. coli* tRNA. The lower panel shows a longer exposure of the gel. (**B**) Flow cytometric analysis of Balb/c 3T3 cells following release from a nocodazole block. The cell-cycle distribution was measured by FACS at the times indicated. The *y*-axis represents cell number, and the *x*-axis represents propidium iodide (DNA) content. (**C**) Expression of *Mad3* and *Max* during the cell cycle in HeLa cells. HeLa cells were released from a nocodazole block as described in (**A**). Levels of *Mad3* and *Max* RNA were measured by ribonuclease protection analysis. (**D**) Flow cytometric analysis of HeLa cells following release from a nocodazole block. The cell-cycle distribution was measured by FACS at the times indicated. The *y*-axis represents cell number, and the *x*-axis represents propidium iodide (DNA) content.

0.2 $\mu$g/ml nocodazole for 20 h; mitotic cells were then 'shaken off' and re-plated in nocodazole-free medium in order to release the block. Balb/c 3T3 cells were firstly made quiescent and were stimulated to re-enter the cell cycle as above; 12 h after serum addition, they were treated with 0.2 $\mu$g/ml nocodazole for 12–14 h. MEL cells were induced to differentiate by the addition of DMSO to 2%.

### RNA preparation and ribonuclease-protection analysis

Total cellular RNA was isolated by the guanidinium isothiocyanate method [53]. RNA was analysed by hybridization to uniformly labelled probes as described previously [54]. Hybridization reactions contained 5 $\mu$g of RNA and were carried out at 50 °C. 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 *Mad3* cDNA, nt +290 to +621; mouse *Max* cDNA, nt +1 to +484 (see Figures 1 and 2) or nt +333 to +483 (see Figure 3); mouse cyclin E2 cDNA nt +1 to +171; mouse *Cdc2* cDNA, nt +775 to +915; human *MAD3* cDNA, nt +1 to +221; human *MAX* cDNA, nt −28 to +509; mouse $\alpha$5-tubulin cDNA, nt +63 to +187; mouse c-*Myc* genomic DNA, nt +17 to +238 (cDNA sequences are relative to a designation of +1 for the ATG translation initiation codon; mouse *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 *Mad3*, 331 nt; mouse *Max*, 483 nt or 150 nt; mouse cyclin E2, 170 nt; mouse *Cdc2*, 140 nt; mouse c-*Myc*, 221 nt; mouse $\alpha$5-tubulin, 124 nt; human *MAD3*, 220 nt; human *MAX*, 537 nt and 446 nt. The human *MAX* probe detected both *MAX* (537 nt) and a splice variant (446 nt).

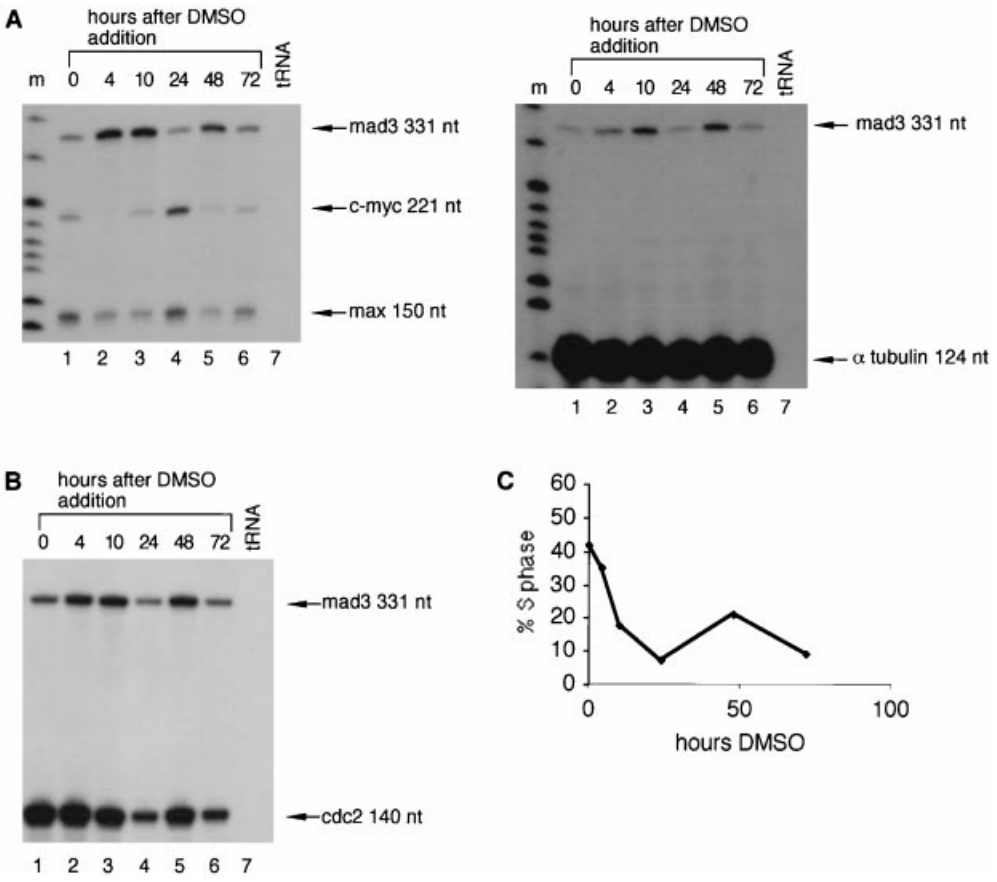


**Figure 3 Expression of *Mad3* during DMSO-induced differentiation of MEL cells**

(**A**) Expression of *Mad3*, c-*Myc* and *Max* during differentiation. MEL cells were induced to differentiate by the addition of DMSO to 2%. Levels of *Mad3*, c-*Myc*, *Max* and α5-tubulin RNA were measured by ribonuclease protection analysis (Lanes 1–6). Lane 7 represents the hybridization of the probes to *E. coli* tRNA. (**B**) Expression of *Mad3* and *Cdc2* is not co-ordinately regulated during differentiation. Lanes 1–6, levels of *Mad3* and *Cdc2* were measured as in (**A**). Lane 7 represents the hybridization of the probes to *E. coli* tRNA. (**C**) Flow cytometric analysis of MEL cells during DMSO-induced differentiation. The cell-cycle distribution of MEL cells during differentiation was measured by FACS; time points correspond to the samples shown in (**A**).

### [$^3$H]Thymidine-incorporation assays

$1.5 \times 10^5$ cells were seeded on to 35-mm-diameter dishes in 1 ml of medium. At the appropriate time point, 1 $\mu$Ci [$^3$H]thymidine was added for the duration of 1 h. Cells were rinsed twice with 1 ml of ice-cold 5% trichloroacetic acid, and twice with 1 ml of 95% ethanol. The precipitate was solubulized with 150 $\mu$l of 0.1 M NaOH, and [$^3$H]thymidine incorporation determined by liquid-scintillation counting.

### Flow-cytometric analysis

$3 \times 10^6$ cells were rinsed with PBS and fixed by incubation in 2 ml of ice-cold 70% ethanol at 4 °C for 30 min. Cells were pelleted, re-suspended in 400 $\mu$l of PBS, and treated with 1 mg/ml RNase A (heat-treated) for 10 min at room temperature (25 °C). Propidium iodide was added to a concentration of 50 $\mu$g/ml, and the sample was analysed using a FACSCalibur flow cytometer (Becton Dickinson). Cell-cycle profiles were calculated using the CELLQUEST and MODFIT software. For each plot shown, the *y*-axis represents cell number, and the *x*-axis represents propidium iodide (DNA) content.

## RESULTS AND DISCUSSION

### Expression of *Mad3* is induced during the S-phase of the cell cycle

During a preliminary analysis of *Mad3* expression patterns, we showed that *Mad3* mRNA is readily detectable in all the proliferating cultured cell lines analysed. Since the expression of *Mad* family genes has generally been associated with differentiating as opposed to proliferating cells in culture, we wished to determine whether *Mad3* expression was restricted to a specific phase of the cell cycle. We initially studied *Mad3* expression throughout the cell cycle in two mouse fibroblast cell lines that show good synchronization following re-entry into the cell cycle from G0. Balb/c 3T3 and C7 3T3 cells were synchronized in G0 by serum deprivation for 28 h, and then induced to re-enter the cell cycle by the addition of serum to 10%. Expression of *Mad3* and *Max* RNA throughout the cell cycle was measured by ribonuclease-protection analysis. Expression of a cell-cycle-regulated gene, cyclin E2, was also measured in order to monitor progress through the cell cycle. Expression of *Mad3* was very low in serum-starved quiescent cells, and was induced at approx. 16–20 h following serum addition. The expression of *Max* showed a modest induction within a few hours of serum addition, and thereafter remained constant throughout the cell cycle; this was

in agreement with previous results [55] and thus served as a control for fluctuations in *Mad3* at later stages in the cell cycle. The cyclin E2 gene was induced at approx. 8 h (Figures 1A and 1B). In order to precisely determine the stage of the cell cycle at which *Mad3* was induced, S-phase entry was monitored by assaying DNA synthesis throughout the time course (Figure 1D). The timing of *Mad3* induction correlated with entry into the S-phase of the cell cycle in both C7 and Balb/c 3T3 fibroblasts; the cyclin E2 gene was induced at mid-G1 as expected. Expression of *Mad3* persisted throughout the S-phase, and then declined as cells progressed through G2 and mitosis; thereafter, the cells tended to lose synchrony.

In order to pinpoint the timing of *Mad3* induction more precisely, we also measured the expression of *Cdc2* during the cell-cycle time course. The induction of *Cdc2* is known to occur at the G1/S transition, with expression then persisting throughout the S-phase of the cell cycle [56]. The temporal induction of *Mad3* closely coincided with that of *Cdc2* at the G1/S-phase transition, with the expression of both genes persisting throughout S-phase and then declining at the G2/M transition (Figure 1C).

The induction of *Mad3* in the S-phase of the cell cycle in serum-stimulated cells may represent genuine cell-cycle regulation; it was, however, possible that it reflected a serum effect or occurred only upon re-entry into the cell cycle from G0. We therefore analysed also *Mad3* expression in a sychronized population of cells that had been arrested in metaphase by treatment with nocodazole. Balb/c 3T3 cells were treated with nocodazole; cells arrested in mitosis were 'shaken off' and replated in the absence of drug. Following replating, the cells progressed synchronously through the cell cycle as monitored by flow-cytometric analysis and cyclin E2 gene expression (Figures 2A and 2B). As expected, cyclin E2 was induced at the mid-G1 phase of the cell cycle. *Mad3* expression correlated with the S-phase of the cell cycle; these results were therefore in agreement with the expression pattern observed in serum-stimulated cells. As a further control, we also measured *Mad3* expression throughout the cell cycle in the epithelial HeLa cell line following release from a nocodazole block (Figures 2C and 2D). In HeLa cells, there was some residual *Mad3* expression in the metaphase-arrested cells; this declined as the cells progressed through G1, and *Mad3* was then induced at the onset of S-phase.

### Expression of *Mad3* during differentiation of MEL cells

The expression of the *Mad* family genes during differentiation has been well documented in several systems. Different *Mad* family genes are generally expressed sequentially during the switch from proliferation to differentiation, and it has been suggested that *Mad3* is expressed in the proliferating compartment prior to differentiation. *Mad3* is the first member of the *Mad* family gene to be induced during the neuronal differentiation of P19 cells, with induction occurring about two days after inducer addition [49]. During the differentiation of 3T3-L1 cells into adipocytes, the induction of *Mad3* appeared to coincide with the S-phase burst that preceded the onset of the terminal differentiation stages [57]. The expression of *Mad3* at early time points during differentiation was not, however, analysed in these previous studies. In order to analyse precisely the relationship between *Mad3* expression and the cell cycle during differentiation, we determined the temporal pattern of *Mad3* expression during the well-defined programme of erythroid differentiation in cultured MEL cells. MEL cells are arrested at the pro-erythroblast stage of differentiation; when cultured with various chemicals, such as DMSO, they undergo a programme of erythroid differentiation that has many similarities to that observed *in vivo* (reviewed in [58–60]). The cell-cycle distribution of MEL cells during differentiation has been well characterized. Upon treatment with inducer, the G1-phase becomes prolonged and there is a transient block in the initiation of DNA synthesis; the S-phase population is lowest around 25 h after the addition of inducer [61]. This is followed by an S-phase burst that occurs around 48 h after addition of inducer; the S-phase population then declines as the cells proceed through the terminal stages of differentiation. Myc levels have been shown to undergo a biphasic expression pattern during DMSO-induced differentiation: Myc initially declines immediately upon addition of inducer, rises around 24 h, and then declines again during the terminal differentiation stages [62,63]. We therefore measured levels of *Mad3*, *Max* and c-*Myc* RNA during this well-defined differentiation programme. The cell-cycle distribution throughout the differentiation time course was monitored by flow cytometry. *Mad3* mRNA showed a 3–10-fold increase at 4 h following the induction of differentiation; levels then declined at 24 h and rose again at 48 h before decreasing to pre-induction levels (Figure 3A). The expression of $\alpha$5-tubulin RNA was unchanged during the differentiation time course and thus served as a control for fluctuations in *Mad3* (Figure 3A). The cell-cycle distribution and *Myc* expression pattern during differentiation (Figures 3A and 3C) were in agreement with previous results [61–63]. The second peak of *Mad3* expression (at 48 h) correlated with the S-phase burst that preceded terminal differentiation. In contrast, the induction of *Mad3* that occurred within a few hours of inducer addition took place at a time when the population of cells in S-phase was declining. Since we have shown that *Mad3* and *Cdc2* are co-ordinately expressed during the cell cycle, we also analysed *Cdc2* expression during MEL differentiation (Figure 3B). *Cdc2* expression declined at approx. 10 h after the addition of the inducer and reached a minimum at 24 h; levels then increased at 48 h before declining during terminal differentiation. *Cdc2* expression was thus closely correlated with the S-phase population throughout the differentiation time course. *Cdc2* and *Mad3* were therefore co-ordinately regulated during the S-phase burst that preceded terminal differentiation, but were clearly dissociated during the early stages of differentiation.

## CONCLUSION

We have shown that *Mad3* is specifically expressed in the S-phase of the cell cycle in both proliferating cells and in the S-phase burst that precedes the onset of terminal differentiation. This expression pattern suggests that Mad3 most likely has a function in normal cellular proliferation, in addition to its previously suggested role in cell-cycle exit during differentiation. The induction of *Mad3* that occurred at the onset of MEL cell differentiation did not correlate with the S-phase of the cell cycle, indicating that Mad3 may play a distinct role at this stage. No transcriptional targets of Mad3 have thus far been identified; given that the expression pattern of *Mad3* is strikingly distinct from that of the other *Mad* family members, it is likely that the different *Mad* genes regulate target genes that serve different biological functions. It will be of particular interest to determine whether those genes that are regulated by Mad3 during the S-phase in proliferating cells are identical with those that are regulated at various stages of differentiation.

We thank members of the laboratory for many useful discussions on the work and manuscript. This work was supported by a grant from Yorkshire Cancer Research and an MRC Research studentship to E. J. F.

## 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 behaviour. Annu. Rev. Cell Dev. Biol. **16**, 653–699
2 Baudino, T. A. and Cleveland, J. L. (2001) The Max network gone Mad. Mol. Cell. Biol. **21**, 691–702
3 Prendergast, G. C. (1999) Mechanisms of apoptosis by c-Myc. Oncogene **18**, 2967–2987
4 Freytag, S. O. (1988) Enforced expression of the c-myc oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G0/G1. Mol. Cell. Biol. **8**, 1614–1624
5 Coppola, J. A. and Cole, M. D. (1986) Constitutive c-myc oncogene expression blocks mouse erythroleukemia cell differentiation but not committment. Nature (London) **320**, 760–763
6 Henriksson, M. and Luscher, B. (1996) Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv. Cancer Res. **68**, 109–182
7 DePinho, R. A., Schreiber-Agus, N. and Alt, F. W. (1991) Myc family oncogenes in the development of normal and neoplastic cells. Adv. Cancer Res. **57**, 1–46
8 Nesbit, C. E., Tersak, J. M. and Prochownik, E. V. (1999) Myc oncogenes and human neoplastic disease. Oncogene **18**, 3004–3016
9 Ayer, D. E., Kretzner, L. and Eisenman, R. N. (1993) Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell (Cambridge, Mass.) **72**, 211–222
10 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
11 Zervos, A. S., Gyuris, J. and Brent, R. (1993) Mxi1, a protein that specifically interacts with Max to bind Myc–Max recognition sites. Cell (Cambridge, Mass.) **72**, 223–232
12 Lahoz, E. G., Xu, L., Schreiber-Agus, N. and DePinho, R. A. (1994) Suppression of Myc, but not E1a, transformation activity by Max-associated proteins, Mad and Mxi1. Proc. Natl. Acad. Sci. U.S.A. **91**, 5503–5507
13 Chen, J. T., Willingham, T., Margraf, L. R., Schreiber-Agus, N., DePinho, R. A. and Nisen, P. D. (1995) Effects of the myc ongogene antagonist, MAD, on proliferation, cell cycling and the malignant phenotype of human brain tumour cells. Nat. Med. **1**, 638–643
14 Cerni, C., Bousset, K., Seelos, C., Burkhardt, H., Henriksson, M. and Luscher, B. (1995) Differential effects by Mad and Max on transformation by cellular and viral oncogenes. Oncogene **11**, 587–596
15 Vastrik, I., Kaipainen, A., Penttila, T-L, Lybboussakis, A., Alitalo, R., Parvinen, M. and Alitalo, K. (1995) Expression of the mad gene during cell differentiation *in vivo* and its inhibition of cell growth *in vitro*. J. Cell Biol. **128**, 1197–1208
16 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
17 Koskinen, P. J., Ayer, D. E. and Eisenman, R. N. (1995) Repression of Myc-Ras cotransformation by Mad is mediated by multiple protein–protein interactions. Cell Growth Differ. **6**, 623–629
18 Queva, C., McArthur, G. A., Ramos, L. S. and Eisenman, R. N. (1999) Dwarfism and dysregulated proliferation in mice overexpressing the Myc antagonist Mad1. Cell Growth Differ. **10**, 785–796
19 Wechsler, D. S., Shelly, C. A., Petroff, C. A. and Dang, C. V. (1997) Mxi1, a putative tumor suppressor gene, suppresses growth of human glioblastoma cells. Cancer Res. **57**, 4905–4912
20 Gehring, S., Rottmann, S., Menkel, A. R., Mertshing, J., Krippner-Heidenreich, A. and Luscher, B. (2000) Inhibition of proliferation and apoptosis by the transcriptional repressor Mad1. J. Biol. Chem. **275**, 10413–10420
21 Bejarano, M. T., Albihn, A., Cornvik, T., Brijker, S. O., Asker, C., Osorio, L. M. and Henriksson, M. (2000) Inhibition of cell growth and apoptosis by inducible expression of the transcriptional repressor Mad1. Exp. Cell. Res. **260**, 61–72
22 Blackwood, E. M. and Eisenman, R. N. (1991) Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science (Washington, D. C.) **251**, 1211–1217
23 Blackwood, E. M., Luscher, B. and Eisenman, R. N. (1992) Myc and Max associate *in vivo*. Genes Dev. **6**, 71–80
24 Blackwell, T. K., Kretzner, L., Blackwood, E. M., Eisenman, R. N. and Weintraub, H. (1990) Sequence-specific DNA binding by the c-Myc protein. Science (Washington, D.C.) **250**, 1149–1151
25 Blackwell, T. K., Huang, J., Ma, A., Kretzner, L. and Alt, F. W. (1993) Binding of Myc proteins to canonical and noncanonical DNA sequences. Mol. Cell. Biol. **13**, 5216–5224
26 Kretzner, L., Blackwood, E. M. and Eisenman, R. N. (1992) Transcriptional activities of the Myc and Max proteins in mammalian cells. Curr. Top. Microbiol. Immunol. **182**, 435–443
27 Amati, B., Dalton, S., Brooks, M. W., Littlewood, T. D., Evan, G. I. and Land, H. (1992) Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature (London) **359**, 423–426
28 Kato, G. J., Barrett, J., Villa-Garcia, M. and Dang, C. V. (1990) An amino-terminal c-Myc domain required for neoplastic transformation activates transcription. Mol. Cell. Biol. **10**, 5914–5920
29 Sakamuro, D. and Prendergast, G. C. (1999) New Myc-interacting proteins: a second Myc network emerges. Oncogene **18**, 2942–2954
30 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
31 Brough, D. E., Hofmann, T. J., Ellwood, K. B., Townley, R. A. and Cole, M. D. (1995) An essential domain of the c-Myc protein interacts with a nuclear factor that is also required for E1A-mediated transformation. Mol. Cell. Biol. **15**, 1536–1544
32 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
33 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 (Cambridge, Mass.) **80**, 767–776
34 Laherty, C. D., Yang, W.-M., Sun, J.-M., Davie, J. R., Seto, E. and Eisenman, R. N. (1997) Histone deacetylases associated with the mSin3 corepressor mediate Mad transcriptional repression. Cell (Cambridge, Mass.) **89**, 349–356
35 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 (Cambridge, Mass.) **89**, 341–347
36 Ayer, D. E., Laherty, C. D., Lawrence, Q. A., Armstrong, A. P. and Eisenman, R. N. (1996) Mad proteins contain a dominant transcription repression domain. Mol. Cell. Biol. **16**, 5772–5781
37 Kasten, M. M., Ayer, D. E. and Stillman, D. J. (1996) SIN3-dependent transcriptional repression by interaction with the Mad1 DNA-binding protein. Mol. Cell. Biol. **16**, 4215–4221
38 Schreiber-Agus, N. and DePinho, R. A. (1998) Repression by the Mad(Mxi1)–Sin3 complex. Bioessays **20**, 808–818
39 Eberhardy, S. R., D'Cunha, C. A. and Farnham, P. J. (2000) Direct examination of histone acetylation on Myc target genes using chromatin immunoprecipitation. J. Biol. Chem. **275**, 33798–33805
40 Dang, C. V. (1999) c-Myc target genes involved in cell growth, apoptosis and metabolism. Oncogene **18**, 1–11
41 Cole, M. D. and McMahon, S. B. (1999) The myc oncoprotein: a critical evaluation of transactivation and target gene regulation. Oncogene **18**, 2916–2924
42 Grandori, C. and Eisenman, R. N. (1997) Myc target genes. Trends Biochem. Sci. **22**, 177–181
43 Coller, H. A., Grandori, C., Tamayo, P., Colbert, T. and Lander, E. S. (2000) Expression analysis with oligonucleotide microarrays reveals Myc regulates genes involved in growth, cell cycle, signaling, and adhesion. Proc. Natl. Acad. Sci. U.S.A. **97**, 3260–3265
44 O'Hagan, R. C., Schreiber-Agus, N., Chen, K., David, G. and Engelman, J. A. (2000) Gene-target recognition among members of the Myc superfamily and implications for oncogenesis. Nat. Genet. **24**, 113–119
45 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
46 Ayer, D. E. and Eisenman, R. N. (1993) A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. Genes Dev. **7**, 2110–2119
47 Larsson, L.-G., Pettersson, M., Oberg, F., Nilsson, K. and Luscher, B. (1994) Expression of mad, mxi1, max and c-myc during induced differentiation of hematopoietic cells: opposite regulation of mad and c-myc. Oncogene **9**, 1247–1252
48 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**, 2353–2357
49 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
50 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
51 Schreiber-Agus, N., Meng, Y., Hoang, T., Hou. Jr, H., Chen, K., Greenberg, R., Cordon-Cardo, C., Lee, H. W. and DePinho, R. A. (1998) Role of Mxi1 in aging organ systems and the regulation of normal and neoplastic growth. Nature (London) **393**, 483–487
52 Queva, C., McArthur, G. A., Iritani, B. M. and Eisenman, R. N. (2001) Targeted deletion of the S-phase-specific Myc antagonist Mad3 sensitizes neuronal and lymphoid cells to radiation-induced apoptosis. Mol. Cell. Biol. **21**, 703–712

53 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal. Biochem. **162**, 156–159
54 Wright, S. and Bishop, J. M. (1989) DNA sequences that mediate attenuation of transcription from the mouse proto-oncogene myc. Proc. Natl. Acad. Sci. U.S.A. **86**, 505–509
55 Berberich, S., Hyde-DeRuyscher, N., Espenshade, P. and Cole, M. (1992) Max encodes a sequence specific DNA binding protein and is not regulated by serum growth factors. Oncogene **7**, 775–779
56 Dalton, S. (1992) Cell cycle regulation of the human *cdc2* gene. EMBO J. **11**, 1797–1804
57 Pulverer, B., Sommer, A., McArthur, G. A., Eisenman, R. N. and Luscher, B. (2000) Analysis of the Myc/Max/Mad network members in adipogenesis: inhibition of the proliferative burst and differentiation by ectopicaly expressed Mad1. J. Cell. Physiol. **183**, 399–410

Received 10 April 2001/2 July 2001; accepted 13 August 2001

58 Marks, P. A. and Rifkind, R. A. (1978) Erythroleukemic differentiation. Annu. Rev. Biochem. **47**, 419–448
59 Gusella, J. F. and Housman, D. (1976) Induction of erythroid differentiation *in vitro* by purines and purine analogues. Cell (Cambridge, Mass.) **8**, 263–269
60 Levenson, R. and Housman, D. (1979) Developmental program of murine erythroleukemia cells. J. Cell Biol. **82**, 715–725
61 Terada, M., Fried, J., Nudel, U., Rifkind, R. A. and Marks, P. A. (1977) Transient inhibition of initiation of S-phase associated with dimethylsulfoxide induction of murine erythroleukemia cells to erythroid differentiation. Proc. Natl. Acad. Sci. U.S.A. **74**, 248–252
62 Lachman, H. M. and Skoultchi, A. I. (1984) Expression of c-myc changes during differentiation of mouse erytholeukemia cells. Nature (London) **310**, 592–594
63 Lachman, H. M., Hatton, K. S., Skoultchi, A. I. and Schildkraut, C. L. (1985) c-myc mRNA levels in the cell cycle change in mouse erythroleukemia cells following inducer treatment. Proc. Natl. Acad. Sci. U.S.A. **82**, 5323–5327