The transcriptional repressor gene *Mad3* is a novel target for regulation by E2F1

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Mad family proteins are transcriptional repressors that antagonize the activity of the c-*Myc* proto-oncogene product. *Mad3* is expressed specifically during the S-phase of the cell cycle in both proliferating and differentiating cells, suggesting that its biological function is probably linked to processes that occur during this period. To determine the mechanisms that regulate the cell-cycle-specific transcription of *Mad3*, we used reporter gene assays in stably transfected fibroblasts. We show that the activation of *Mad3* at the G_1–S boundary is mediated by a single E2F (E2 promoter binding factor)-binding site within the 5′-flanking region of the gene. Mutation of this element eliminated transcriptional activation at S-phase, suggesting that the positively acting E2F proteins play a role in *Mad3* regulation. Using electrophoretic mobility-shift assays and chromatin immunoprecipitation, we show that E2F1 binds to the *Mad3* 5′-flanking region both *in vitro* and *in vivo*. We thus identify *Mad3* as a novel transcriptional target of E2F1.

Key words: cell cycle, E2F, gene transcription, Mad, Myc.

INTRODUCTION

Mad family proteins (Mad1, Mxi1, Mad3 and Mad4) are transcriptional regulators that act as antagonists of the c-*Myc* proto-oncogene product (reviewed in [1]). Myc and Mad are basic helix–loop–helix leucine zipper proteins that function via heterodimerization with their partner Max. Myc:Max heterodimers recognize the E-box sequence CACGTG and activate transcription by recruiting activities that mediate histone acetylation, chromatin remodelling and transcriptional elongation [2,3]. Mad:Max heterodimers recognize the same E-box sequence, but in contrast function as transcriptional repressors via the recruitment of an mSin3–histone deacetylase complex [4,5]. Myc functions to promote cell-cycle progression and to enhance apoptosis in specific settings (reviewed in [6]), and levels are generally high in proliferating cells and decrease during growth arrest and differentiation. Enforced expression of Myc leads to the transformation of cells in culture, and de-regulation of Myc is a common feature of diverse tumour types (reviewed in [7]). The biological activities of Myc are thought to be antagonized by the Mad family proteins. The expression of Mad proteins has been associated generally with growth arrest and differentiation, and the overexpression of Mad1 has been shown to inhibit proliferation and oppose the transforming activity of Myc in cultured cells. The transition from proliferation to differentiation thus results in a switch from Myc:Max to Mad:Max heterodimers and in the consequent modulation of target gene expression. In agreement with the proposed biological role of the Mad proteins, targeted deletion of *Mad1* in mice resulted in defects in cell-cycle exit during myeloid differentiation [8], whereas disruption of *Mxi1* led to a more severe phenotype, with hyperplasia in several tissues [9].

In contrast with the other Mad family members, Mad3 is found in both proliferating and differentiating cells in developing mouse embryos and adult tissues [10,11], and a number of studies have shown that its expression is restricted to the S-phase of the cell cycle [11,12]. The biological function of Mad3 is unknown, although targeted disruption of *Mad3* resulted in an increased sensitivity to apoptosis in response to DNA damage [13].

The E2F (E2 promoter binding factor) transcription factors are central regulators of gene expression throughout the cell cycle (reviewed in [14,15]). The various E2F family members have distinct biological activities, and can function as either transcriptional activators or repressors; repression is mediated by interaction with the pocket proteins pRB, p107 and p130, which in turn act to recruit histone deacetylase, histone methyltransferase and chromatin remodelling activities. E2F1, E2F2 and E2F3 are generally referred to as the 'activating' E2Fs, and their main role involves the activation of genes that control cell proliferation and apoptosis (see e.g. [16]). The transcriptional activation function of E2F1, E2F2 and E2F3 is repressed by association with pRB in the G_1 phase of the cell cycle; the free 'activating' E2Fs are released in late G_1 phase after phosphorylation of the pocket protein. Mutant mouse models and overexpression studies have, however, indicated that the biological activities of E2F1, E2F2 and E2F3 are mediated by transcriptional activation rather than the pRB-associated repression of E2F-responsive genes. In contrast, E2F4 and E2F5 function as important transcriptional repressors during the G_1 phase of the cell cycle. E2F4 and E2F5 lack a nuclear localization signal, and the free proteins are thus cytoplasmic and are not able to function as transcriptional activators (see e.g. [17]). E2F4 and E2F5 are imported into the nucleus via interaction with the pocket proteins, and the resulting complexes then act as strong transcriptional repressors. These repressive E2Fs have been identified to play a role in the regulation of cell-cycle exit and terminal differentiation [18,19]. E2F6 is structurally distinct from the other E2F family members in that it lacks a transcriptional activation domain and is unable to bind pocket proteins. A recent study [20] has shown that E2F6 is involved

Abbreviations used: ChIP, chromatin immunoprecipitation; DHFR, dihydrofolate reductase; DOC, deoxycholate; E2F, E2 promoter binding factor; EMSA, electrophoretic mobility-shift assay; NP40, Nonidet P40; Sp1, specificity protein-1.

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in transcriptional silencing in quiescent G_0 cells; E2F6-mediated transcriptional repression occurs via the direct recruitment of chromatin modifiers such as histone methyltransferase, heterochromatin protein 1γ and polycomb group proteins.

The expression pattern of *Mad3* is strikingly different from that of the other *Mad* family members, suggesting that it is regulated by a distinct mechanism. Since the S-phase-specific expression of *Mad3* is probably intimately linked to its biological function, we sought to elucidate its transcriptional control. Using reporter gene assays in stably transfected cells, we show that the transcriptional activation of *Mad3* at the G_1–S transition requires an E2F-binding site within the 5′-flanking region of the gene. We show by electrophoretic mobility-shift assay (EMSA) and chromatin immunoprecipitation (ChIP) that the positively acting E2F family member, E2F1, binds to this regulatory element. This analysis thus identifies *Mad3* as a novel E2F target gene and may be relevant for our understanding of both E2F function and the biological role of Mad3.

EXPERIMENTAL

Cell culture

Mouse C7 3T3 fibroblasts were grown in minimum essential medium alpha supplemented with 10% calf serum. Cells were made quiescent by culturing for 28 h in serum-free medium and were then induced to re-enter the cell cycle by the addition of serum to a final concentration of 10%. Stable transfections were performed using the calcium phosphate precipitation method [21]. The precipitate contained 20 μg of reporter gene construct together with 5 μg of neo^r plasmid, and was applied to 10^6 cells on a 10-cm-diameter plate. Pools comprising approx. 500 stably transfected clones were generated after selection in 1 mg/ml G418.

RNA preparation and RNase protection mapping

RNA was prepared using the guanidinium isothiocyanate method [22] and RNase protection mapping was performed as described previously [23]. Hybridization was performed at 50 °C and contained 5 μg of RNA. Appropriate cDNA fragments were inserted into pSP72 (Promega, Chilworth, Southampton, U.K.) for the synthesis of riboprobes: mouse *Mad3* +264 to +621; mouse *Max* +1 to +484 nt (sequences are relative to a designation of +1 for the ATG translation-initiation codon). The probe used for the detection of *Mad3*-globin reporter gene transcripts comprised a 1175 nt fragment derived from the 3′-end of the rabbit β-globin gene; this yielded an RNase-protected fragment of 170 nt. Markers were end-labelled *Msp*I restriction fragments of the plasmid pBR322.

Isolation of a mouse *Mad3* genomic clone

A lambda library ('mouse strain' B6/CBA; Stratagene, Cambridge, U.K.) was screened with a *Mad3* cDNA probe (+1 to +114 nt) in accordance with the manufacturer's instructions. A 4.5 kb *Xba*I fragment containing the 5′-end of the gene was subcloned for further analysis.

Construction of *Mad3* reporter gene plasmids

A 4.5 kb *Xba*I fragment derived from the 5′-end of a mouse *Mad3* genomic clone was inserted into the polylinker of pSP72 (Promega); this region contains exons 1–4 of *Mad3*. A *Mad3*-globin hybrid gene was generated by the subsequent insertion downstream from *Mad3* of a 1.175 kb fragment derived from the 3′-end of the rabbit β-globin gene; this fragment comprises part of β-globin exon 2, intron 2, exon 3 and 450 nt 3′-flanking sequences. Deletions within the *Mad3* 5′-flanking region were generated using either the Erase-a-Base system (Promega; constructs −793 and −479) or PCR (constructs −429 and −365). The E2F-binding site within the *Mad3* 5′-flanking region was mutated using the Stratagene QuikChange site-directed mutagenesis system.

Preparation of nuclear protein extracts

The procedure for preparation of nuclear protein extracts was as described by Leone et al. [24]. Cells were harvested in ice-cold PBS, pelleted, re-suspended in 10 PCV (packed cell volumes) of hypo-osmotic lysis buffer [10 mM Hepes/KOH (pH 7.5), 10 mM KCl, 3 mM $MgCl_2$, 0.05% Nonidet P40 (NP40), 1 mM EDTA, 10 mM NaF, 0.1 mM $NaVO_4$, 1 mM dithiothreitol, 10 mM β-glycerophosphate and protease inhibitors (Complete; Roche, Lewes, East Sussex, U.K.)] and incubated on ice for 30 min. Nuclei were pelleted by centrifugation for 5 min at 500 ***g*** and rinsed with hypo-osmotic lysis buffer. They were re-pelleted and re-suspended in 10 PCV of nuclear extraction buffer [50 mM Hepes/KOH (pH 7.9), 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP40, 10% (v/v) glycerol, 10 mM NaF, 0.1 mM $NaVO_4$, 1 mM dithiothreitol, 10 mM β-glycerophosphate and protease inhibitors], and incubated on ice for 30 min. Cellular debris was removed by centrifugation for 10 min at 12000 ***g*** and the supernatant was snap-frozen in liquid nitrogen.

EMSA

Binding reactions were performed in 20 mM Hepes (pH 7.9), 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.1% NP40, 10% glycerol and contained 2–5 μg of nuclear extract, 500 ng of sonicated salmon testis DNA and 30 μg BSA; the total volume was 20 μl. Reactions were incubated for 30 min on ice with or without competitor oligonucleotides (200-fold excess) or antibodies as appropriate. End-labelled double-stranded probe (0.5 ng) was added, and the mixture was incubated for a further 20 min at room temperature (25 °C). Incubations with deoxycholate (DOC; 0.6% final concentration) were performed for 10 min on ice, followed by the addition of NP40 to a final concentration of 1%. Binding reactions were electrophoresed in Tris/borate/EDTA (0.5 × TBE) on a 5% acrylamide (37.5:1, acrylamide:bisacrylamide) gel containing 5% glycerol. Antibodies were purchased from Santa Cruz Biotechnology (anti-E2F1: sc-193X; anti-E2F3: sc-879X; normal rabbit IgG: sc-2027; Santa Cruz, CA, U.S.A.). Double-stranded oligonucleotides used as probes and competitors were the following: *Mad3* E2F site,CGTCCGGGTTTTCAAATTTGAAACCGGCAGCTTTTCCCGCCCGAGTCGG; dihydrofolate reductase (DHFR) E2F site, CTAGAGCAATTTCGCGCCAAACTTGGATC; specificity protein 1 (Sp1) binding site, ATTCGATCGGGGCGGGGCGAGC; *Mad3* E2F site mutant 1, GAAACCGGCAGCTTTTCCATCCCGAGTCGG; *Mad3* E2F site mutant 2, GAAACCGGCAGCCGGCCCCGCCCGAGTCGG.

Formaldehyde cross-linking and ChIP

ChIP was performed essentially as described by Boyd et al. [25]. Formaldehyde was added to the cell-culture medium to a final concentration of 1% and the cells were incubated for 15 min at room temperature (25 °C). The reaction was terminated by the addition of glycine (to a final concentration of 0.125 M) and incubated for a further 5 min at room temperature (25 °C). Cells

were rinsed with PBS, dissociated with trypsin, and lysed in 10 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 10 mM NaCl, 0.5 % NP40 and protease inhibitors (Complete, Roche). Nuclei were collected by centrifugation and lysed in 50 mM Tris/HCl (pH 8.1), 10 mM EDTA, 1 % SDS and protease inhibitors. The chromatin was sonicated and pre-cleared with blocked Staph A cells (Sigma–Aldrich, Poole, Dorset, U.K.). Aliquots of pre-cleared chromatin (from 1×10^7 cells/sample) were diluted (1:5) with 16.7 mM Tris/HCl (pH 8.1), 0.01 % SDS, 1.1 % (v/v) Triton X-100, 1.2 mM EDTA, 167 mM NaCl and protease inhibitors and incubated overnight with 2 μg of antibody at 4 °C. Antibodies were as described above. Immunocomplexes were harvested with blocked Staph A cells and washed extensively with 50 mM Tris/HCl (pH 8), 2 mM EDTA, 0.2 % Sarcosyl and then with 100 mM Tris/HCl (pH 9), 500 mM LiCl, 1 % NP40 and 1 % DOC. After elution with 50 mM $NaHCO_3$/1 % SDS, the cross-links were reversed and the extracted DNA was subjected to PCR analysis. PCR primers were the following: *Mad3* forward, CATTCGAAGACCAATGAAGGGCAGATACG; *Mad3* reverse, TACACGTGCGCGCGGGACCGGC; *Cdc2* forward, GTGGACTGTCACTTTGGTGGCTGGC; *Cdc2* reverse, GGTAAAGCTCCCGGGATCCGCCAAT; *Albumin* forward, GGACACAAGACTTCTGAAAGTCCTC; *Albumin* reverse, TTCCTACCCCATTACAAAATCATA.

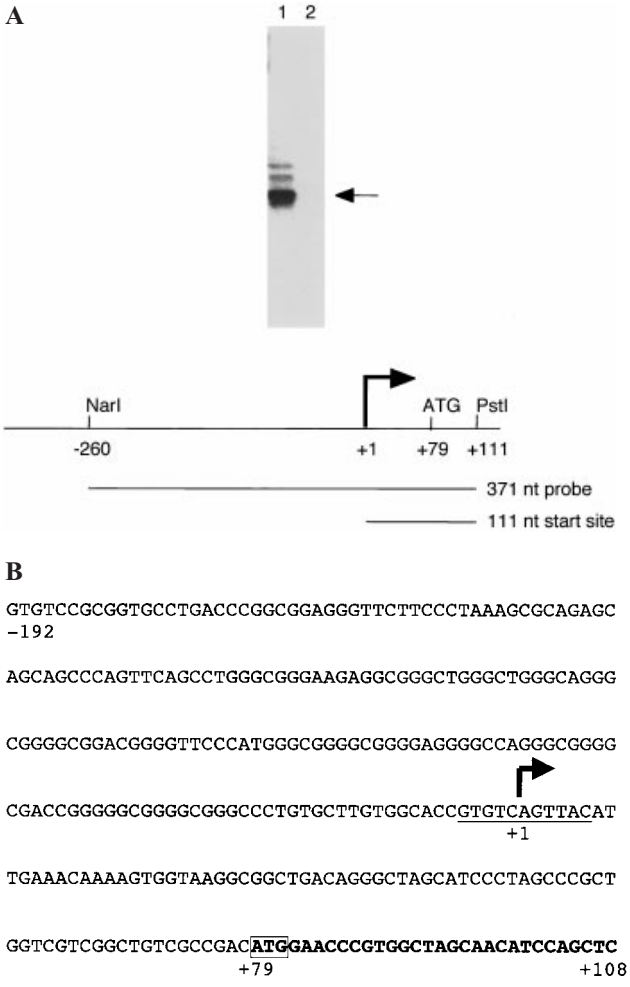

Figure 1 Identification of *Mad3* transcription start sites

(**A**) RNase protection mapping of *Mad3* transcription start sites. Samples of C7 3T3 RNA (lane 1) or tRNA (lane 2) were hybridized to a riboprobe corresponding to the 5′-end of the mouse *Mad3* gene. The major transcription start site is indicated by an arrow. (**B**) Schematic representation of the *Mad3* promoter. The DNA sequence of the *Mad3* 5′-flanking region is depicted. The initiator element is underlined and the major site of transcriptional initiation is indicated by an arrow. The coding region is shown in boldface, and the ATG translation-initiation codon is shown inside a box.

RESULTS

Regulation of *Mad3* reporter genes during the cell cycle in stably transfected C7 3T3 fibroblasts

To identify DNA sequence elements that mediate the S-phase-specific activation of *Mad3*, a reporter gene system was used in stably transfected cells. Initially, a clone containing the 5′-end of mouse *Mad3* was isolated from a lambda genomic library, and the site of transcriptional initiation was located by RNase protection mapping using a probe that spanned the 5′-end of the gene (Figure 1A). The major transcriptional start site was located 78 nt upstream from the ATG translation-initiation codon and coincided with a potential initiator element (Figure 1B). The location of this start site was confirmed using additional probes in RNase protection mapping and by primer extension analysis (results not shown), and is consistent with the sequence of the 5′-end of a clone present in public databases (National Center for Biotechnology Information accession no. AK014516).

A reporter gene was constructed that comprised the 5′-region of *Mad3* fused to the 3′-end of the rabbit β-globin gene. The hybrid gene contained approx. 800 nt of *Mad3* 5′-flanking sequences together with *Mad3* genomic DNA as far as exon 4 (Figure 2A; −793 reporter). The *Mad3*–globin hybrid gene was stably transfected into C7 3T3 fibroblasts and a pool of approx. 500 clones was generated. The expression of the reporter gene was monitored by RNase protection assays. The simultaneous detection of endogenous *Mad3* and *Mad3*–globin reporter was achieved by designing riboprobes that specifically detected the

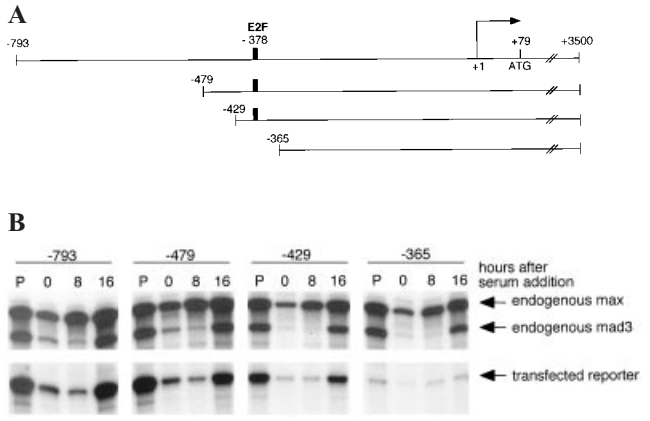

Figure 2 Cell-cycle regulation of *Mad3*-reporter constructs

(**A**) Schematic representation of *Mad3*-reporter gene constructs. The indicated regions of *Mad3* were inserted into the reporter construct. The transcriptional start site is depicted with an arrow, and the position of the E2F-binding site is shown. (**B**) Expression of *Mad3*-reporter constructs in stably transfected C7 3T3 cells. Reporter constructs were stably transfected into C7 3T3 fibroblasts and pools of approx. 500 clones were generated. Quiescent cells were induced to re-enter the cell cycle by the addition of serum to a final concentration of 10 %, and RNA harvested at the indicated time periods. Levels of endogenous *Mad3*, endogenous *Max*, and transfected reporter gene were measured by RNase protection mapping. Lanes depicted 'P' contain RNA samples prepared from asynchronously proliferating cells. These experiments were performed three times using two independent pools of transfected cells.

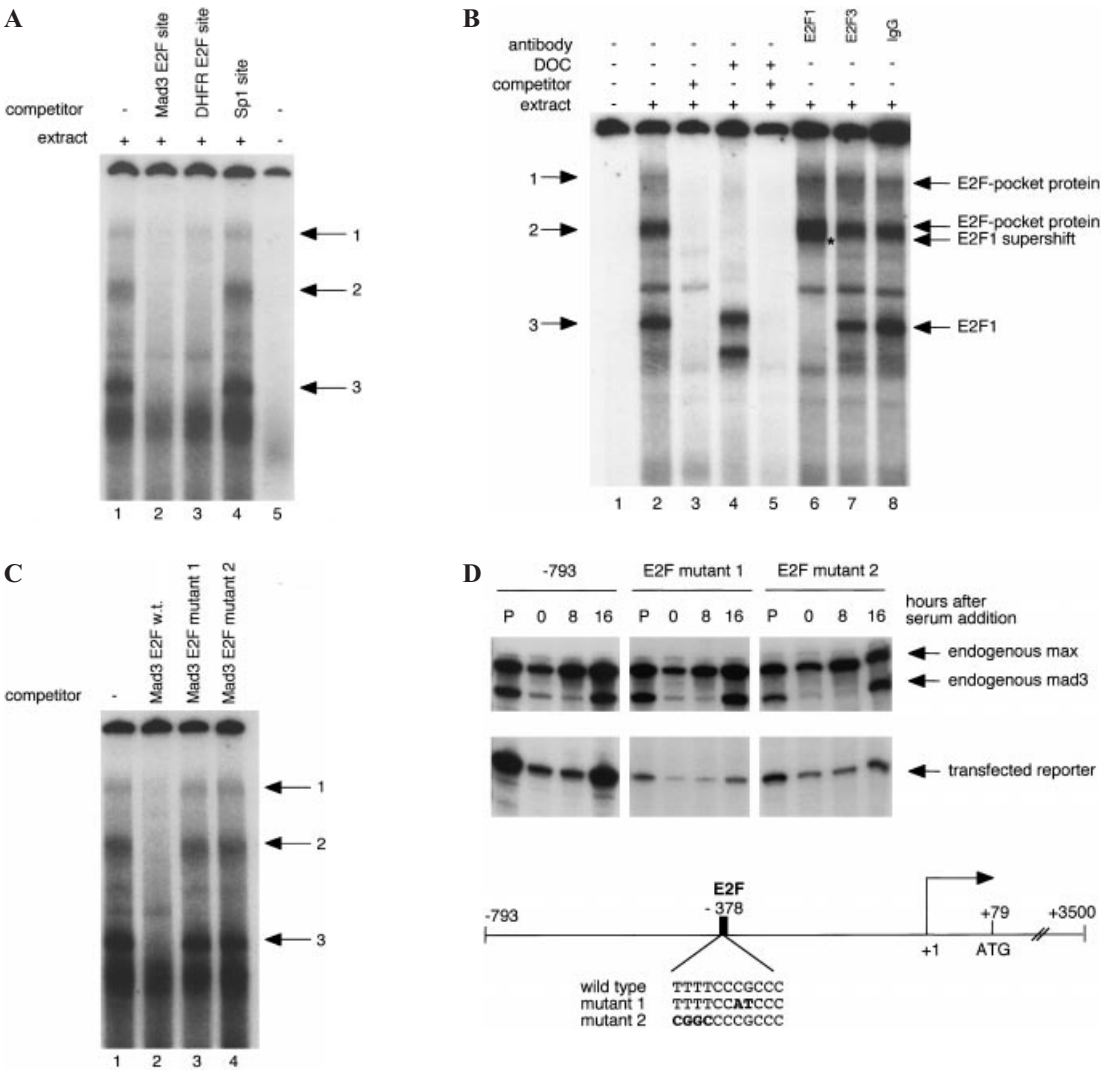

Figure 3 Cell-cycle regulation of *Mad3* is mediated by E2F

(**A**) EMSA of protein interactions with the *Mad3* E2F-binding site. An oligonucleotide corresponding to the *Mad3* E2F-binding site was radiolabelled and EMSA performed using nuclear extract prepared from asynchronously proliferating C7 3T3 fibroblasts (lanes 1–4). Lane 5 represents the probe that was incubated in the absence of extract. Incubations performed in the presence of excess unlabelled competitor oligonucleotides were *Mad3* E2F-binding site (lane 2), DHFR E2F-binding site (lane 3) and Sp1-binding site (lane 4). The positions of E2F-containing complexes (1–3) are indicated. (**B**) Identification of protein interactions with the *Mad3* E2F-binding site. EMSA was performed as in (**A**). Binding reactions were incubated with excess unlabelled *Mad3* E2F-binding site competitor, with DOC or with E2F-specific antibodies as indicated. The positions of complexes corresponding to E2F–pocket proteins and to E2F1 are shown. The complex corresponding to the E2F1 supershift is indicated by an asterisk. (**C**) E2F complexes do not recognize mutated *Mad3* E2F-binding sites. EMSA was performed as in (**A**). Binding reactions were incubated with competitor oligonucleotides corresponding to wild-type and mutant *Mad3* E2F-binding sites (mutant 1 or 2, see **D**). (**D**) Expression of transfected *Mad3*-reporter constructs containing mutated E2F-binding sites. *Mad3* reporters containing a wild-type or mutated E2F-binding site were stably transfected into C7 3T3 fibroblasts and pools of approx. 500 clones were generated. Expression of endogenous *Mad3*, endogenous *Max* and transfected reporter was measured throughout the cell cycle by RNase protection mapping as described in Figure 2(B). Lanes 'P' contain RNA samples derived from asynchronously proliferating cells. These experiments were performed three times using two independent pools of transfected cells.

3′-ends of the corresponding transcripts. The expression of endogenous *Mad3* and of the transfected reporter gene was measured in asynchronously proliferating cells and at various stages throughout the cell cycle; the expression of endogenous *Max* was also monitored as a loading control (Figure 2B; −793 reporter). The timing of each cell-cycle phase and the expression pattern of endogenous *Mad3* in the C7 3T3 cell line have been described previously [12]. The expression patterns of endogenous *Mad3* and of the transfected *Mad3*-reporter gene were identical: RNA levels were low in serum-starved quiescent cells and in mid-G_1 (Figure 2B; −793 reporter, 0 and 8 h), and were subsequently induced at the onset of S-phase (16 h) after re-entry into the cell cycle. *Max* mRNA was modestly induced (2–3-fold) at an earlier stage in the cell cycle as reported previously [26]. The expression of stably transfected *Mad3*–globin hybrid genes in C7 3T3 cells should thus provide a suitable system for identifying sequence elements that mediate the S-phase-specific induction of *Mad3*.

Serial deletions within the *Mad3* 5′-flanking region of the reporter construct were generated to localize approximately the DNA sequence elements that mediate S-phase-specific activation (Figure 2A). Expression of each reporter gene was measured during the cell cycle in a pool of stably transfected C7 3T3 cells. All pools comprised at least 500 independent clones, and all expressed equivalent amounts of co-transfected *neo*^{r}

transcript (results not shown). The *Mad3*-reporter gene that contained 479 nt of *Mad3* 5′-flanking sequences was expressed at a similar level to the −793 reporter and it was appropriately regulated during the cell cycle, i.e. the expression level was low in quiescent cells (Figure 2B; −479 reporter, 0 h) and was later induced at the onset of S-phase (16 h). The reporter that contained 429 nt of *Mad3* 5′-flanking region was expressed at a lower level than the −793 and −479 reporters, indicating that positively acting transcription factors bind between nt −793 and −429 within the 5′-flanking region of *Mad3*. Importantly, the expression of the −429 reporter was still strongly induced at the G_1–S transition, indicating that the cell-cycle-specific regulatory elements remained intact within this construct. Further deletions into the *Mad3* 5′-flanking region in the reporter resulted in a loss of cell-cycle-regulated expression in transfected cells (Figure 2B, −365 reporter; and results not shown). This reflected a loss of reporter gene induction at S-phase entry rather than an increased expression at earlier times in the cell cycle. Therefore these results are consistent with a model in which the S-phase-specific induction of *Mad3* is mediated by a positively acting transcriptional control element located between nt −429 and −365 within the 5′-flanking region.

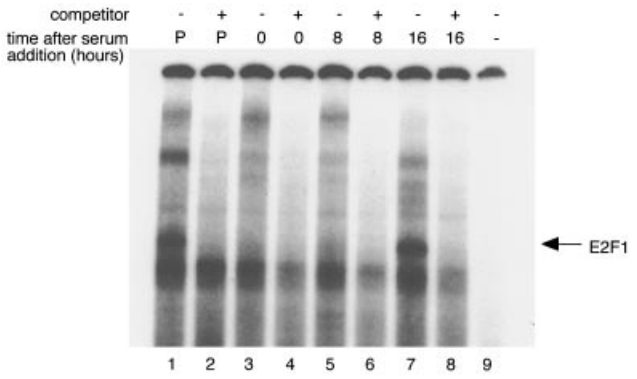

Figure 4 E2F1 binds to the *Mad3* promoter in a cell-cycle-regulated manner

Nuclear extracts were prepared from aysnchronously proliferating C7 3T3 fibroblasts (P) and from synchronized cells at different times throughout the cell cycle (G_0, 0 h; G_1, 8 h; S, 16 h). EMSA was performed using radiolabelled *Mad3* E2F-binding site probe in the presence or absence of the corresponding unlabelled competitor oligonucleotide as indicated. The position of the complex corresponding to E2F1 is indicated.

Cell-cycle regulation of *Mad3* is eliminated by mutation of an E2F-binding site in the 5′-flanking region

Deletion analysis of the *Mad3* 5′-flanking region indicated that a cell-cycle-specific regulatory element was present within the −429 to −365 region. This sequence contains a candidate-binding site for the E2F transcription factor family (Figure 2A, −378 reporter; TTTTCCCGCCC); this consensus element is notably conserved between the mouse and human genes with respect to both its sequence and its location relative to the transcriptional start site. Since the E2F transcription factors play a crucial role in the temporal control of gene expression during the cell cycle, we determined whether or not the S-phase-specific induction of *Mad3* is mediated by the candidate E2F-binding site. Using EMSA with nuclear extracts derived from asynchronously proliferating C7 3T3 fibroblasts, we initially determined if this sequence binds E2F complexes *in vitro* (Figure 3A). Three specific complexes interacted with a radiolabelled oligonucleotide probe corresponding to the *Mad3* candidate E2F-binding site; binding of these complexes was competed by incubation with an excess of the corresponding unlabelled oligonucleotide (Figure 3A, lane 2). Formation of these complexes was also reduced by incubation with a competitor oligonucleotide corresponding to a previously characterized E2F-binding site within the DHFR promoter, thus confirming that they contain E2F (Figure 3A, lane 3). The complexes were not competed by incubation with the unlabelled oligonucleotide corresponding to a binding site for the transcription factor Sp1 (Figure 3A, lane 4). To determine whether complexes 1–3 contain free E2F proteins or E2F in association with the pocket proteins pRB, p107 or p130, extracts were incubated with DOC to disrupt E2F–pocket protein interactions (Figure 3B). Incubation with DOC revealed that complexes 1 and 2 represent E2F–pocket protein interactions, whereas complex 3 contains a free E2F species (Figure 3B, lanes 4 and 5).

Multiple E2F proteins (E2F1–6) are involved in the activation and repression of gene expression at various stages throughout the cell cycle. E2F4 and E2F5 function in complexes with pocket proteins to mediate transcriptional repression during the G_1 phase of the cell cycle. In contrast, transcriptional activation of E2F-responsive genes is mediated by E2F1, E2F2 and E2F3. Since deletion analysis in reporter assays had indicated the importance of positively acting transcription factors in the cell-cycle regulation of *Mad3*, we determined if any of the activating E2Fs were present within the protein complexes bound to the *Mad3* E2F-binding site. EMSA was performed in the presence of antibodies that specifically recognize individual E2F family members, thus revealing that complex 3 contains E2F1 (Figure 3B, lanes 6–8).

To determine whether the E2F-binding site plays a role in the S-phase-specific activation of *Mad3*, this site was mutated within the context of the *Mad3* reporter that contained the longest 5′-flanking region (Figure 3D; −793 reporter, mutants 1 and 2). Mutations were made in residues of the consensus element that had been shown previously to be crucial for E2F binding within various target gene promoters. We initially confirmed that oligonucleotides containing mutated E2F sites were unable to act as competitors in EMSA, indicating that these mutations indeed eliminate the binding of E2F to the *Mad3* site (Figure 3C, lanes 3 and 4). Mutant reporter genes were then transfected into C7 3T3 fibroblasts, and their expression was analysed throughout the cell cycle in pools of approx. 500 stably transfected clones (Figure 3D). Mutation of the E2F-binding site resulted in an almost complete loss of regulation as compared with the parent reporter gene; the loss of regulation again reflected a striking reduction in transcriptional activation at the G_1–S phase boundary, rather than an increased expression during the earlier stages of the cell cycle. Thus it can be concluded that the deregulation results from loss of activator binding at G_1–S rather than from loss of a repressor during G_0 and G_1.

E2F1 binds the *Mad3* 5′-flanking region in a cell-cycle-regulated manner

To identify cell-cycle-specific fluctuations in the interactions of complexes with the *Mad3* E2F-binding site, EMSA was performed using nuclear extracts prepared from synchronously proliferating C7 3T3 fibroblasts at various stages of the cell cycle (Figure 4). The *Mad3* E2F site was mainly occupied by E2F–pocket protein complexes during the G_0 and early G_1 phases of the cell cycle (Figure 4, lanes 3 and 5). These species quite probably represent E2F in complex with p107 and p130, and may play a role in the repression of *Mad3* during the early stages

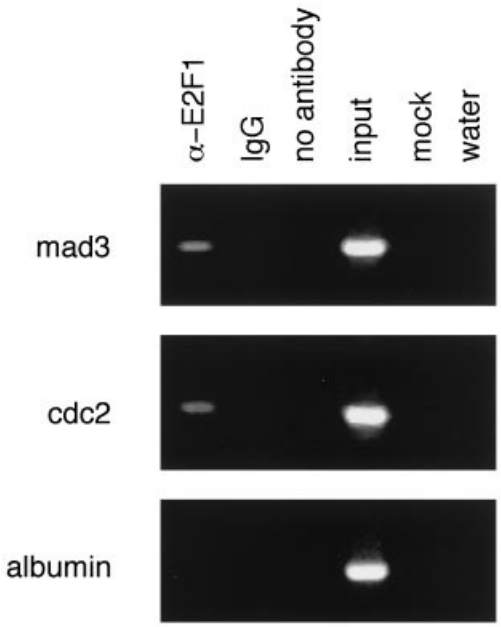

Figure 5 ChIP analysis of E2F1 binding to the *Mad3* 5′-flanking region *in vivo*

ChIPs were performed using asynchronously proliferating C7 3T3 fibroblasts with an antibody specific to E2F1 or with IgG. PCR was performed using primers designed to detect the E2F-binding site in the *Mad3* promoter, the E2F-binding site in the *Cdc2* promoter or an irrelevant region within the *Albumin* gene. Negative control reactions (no antibody, mock and water), and input material are indicated.

of the cell cycle. The EMSA also revealed a significant increase in E2F1-binding activity at the G_1–S transition (Figure 4, lanes 7 and 8). The transcriptional activation of *Mad3* at the G_1–S boundary thus correlates with an increased binding of E2F1 to the promoter as measured by EMSA.

E2F1 binds *in vivo* to the *Mad3* 5′-flanking region

Reporter gene assays and *in vitro* binding results have suggested that E2F1 plays a role in the S-phase-specific activation of *Mad3*. To confirm that E2F1 binds *in vivo* to the *Mad3* 5′-flanking region, ChIP was performed using antibodies specific to E2F1 (Figure 5). The binding of E2F1 to the *Cdc2* promoter was monitored as a positive control for the ChIP assay. E2F1 binding was detected on both the *Cdc2* and *Mad3* promoters, but not within the negative control albumin gene (Figure 5, α-E2F1). The E2F-binding sites within *Cdc2* and *Mad3* were not immunoprecipitated using the negative control antibody IgG, thus confirming the specificity of the ChIP assay. These results therefore confirm that E2F1 binds *in vivo* to the *Mad3* promoter.

DISCUSSION

The S-phase-specific transcription of *Mad3* is strikingly distinct from the expression patterns of the other *Mad* family members. In the present study, we show that the activation of *Mad3* at the G_1–S transition is mediated by a single E2F-binding site within the 5′-flanking region of the gene. Mutation of this element eliminated transcriptional activation at S-phase, implicating the positively acting E2F proteins in *Mad3* regulation. Using EMSA and ChIP, we show that E2F1 binds to the *Mad3* 5′-flanking region both *in vitro* and *in vivo*, thus identifying *Mad3* as a novel E2F target gene.

Transcriptional regulation by the E2F family members is mediated by both repressive and activating complexes that function at specific stages of the cell cycle. The mutagenesis studies described here clearly indicate that the activating E2Fs play a major role in the cell-cycle-dependent induction of *Mad3* at the G_1–S transition, as loss of binding results in a reduced activation rather than a relief of repression. Our results do not exclude a contribution of transcriptional repression in the regulation of *Mad3*; this may involve sequence elements that were not identified by the present study. However, our analysis strongly suggests that E2F–pocket protein complexes do not play a dominant role in the repression of *Mad3* during the G_1 phase of the cell cycle as mediated via the binding site studied here. We consider our analysis to be physiologically relevant, because we have used stably integrated reporter templates rather than transient transfections. Therefore the chromatin environment and transcriptional regulation of our transfected genes quite probably reflect that of endogenous *Mad3*.

A variety of approaches have been used to identify E2F target genes; more recently, these include ChIP with E2F-specific antibodies [27,28] and microarray analysis using E2F-overexpressing cell lines (see e.g. [29,30]). These analyses have revealed that E2F target genes are involved in diverse processes, which include cell-cycle progression, DNA repair, DNA replication, G_2–M checkpoints and apoptosis. Consistent with their distinct biological functions, the individual E2Fs were shown to have specific target genes. Only one recent analysis [30] has identified *Mad3* as a candidate E2F target; this may simply reflect the techniques employed and the fact that commercial microarrays represent a limited number of genes. Interestingly, Polager et al. [30] demonstrated the activation of rat *Mad3* after the induced overexpression of E2F1 but not of E2F3. This is in agreement with our results showing that *Mad3* is a transcriptional target of E2F1, and is consistent with our inability to detect E2F3 binding to the *Mad3* promoter either *in vitro* or *in vivo* (Figure 3B; results not shown).

The biological function of Mad3 is unknown, although targeted disruption of *Mad3* in mice has suggested a role in the control of apoptotic responses to DNA damage [13]. Whereas the E2F proteins are generally associated with apoptotic induction, target gene studies have indicated that they also activate anti-apoptotic genes such as *Bcl2* [31]. This implies that a balance of various signals is involved in apoptotic control by E2F. Deregulation of the E2F pathway is a common feature of most human cancers; it will therefore be of interest to determine the role of Mad3 both as an effector of E2F function and as a contributor to tumourigenesis.

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