Journal of Cellular Biochemistry 96:1071–1085 (2005)

c-Myc Creates an Activation Loop by Transcriptionally Repressing Its Own Functional Inhibitor, hMad4, in Young Fibroblasts, a Loop Lost in Replicatively Senescent Fibroblasts

Richard Marcotte,[1] Jen Mou Chen,[1] Sylvain Huard,[1] and Eugenia Wang[2]*

[1]The Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, The Sir Mortimer B. Davis-Jewish General Hospital, and Department of Medicine, McGill University, Montréal, Québec, Canada
[2]Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, Kentucky

Abstract c-Myc transcriptional activity in cells is dampened by the Mad family of transcriptional repressors. The expression of one member, hMad4, is increased in growth-arrested states such as quiescence or replicative senescence; hMad4 mRNA levels in replicatively senescent fibroblasts are about twice those seen in their young contact-inhibited quiescent counterparts. Moreover, the repression of hMad4 transcription following serum stimulation observed in quiescent young fibroblasts is lost in senescent cells. This loss results in persistent expression of hMad4, which leads to an inability to switch from an hMad4/Max complex to a c-Myc/Max complex on selected c-Myc target genes following serum stimulation. We have located an initiator element (Inr), a candidate for Miz-1 binding, in the hMad4 promoter. In reporter assays, Miz-1 enhances reporter GFP expression; this enhancement is inhibited by co-expressing c-Myc. Thus hMad4, as does its murine counterpart, contains the Inr element through which Miz-1 activates its expression; but this action is inhibited in the presence of c-Myc. This inhibition may explain the down-regulation of hMad4, corresponding to the up-regulation of c-Myc, in young serum-starved quiescent fibroblasts upon serum stimulation. However, this reciprocal change does not occur in replicatively senescent fibroblasts upon serum stimulation; instead, hMad4 persists in the presence of high levels of c-Myc activation. Our results suggest that: (1) replicative senescence-specific factors may block c-Myc inhibition of Miz-1 activation of hMad4 expression; and (2) the continual presence of hMad4 protein may transcriptionally repress selected c-Myc target genes, whose functions are key to the signaling pathways leading to apoptosis inhibition and permanent exit of cell cycle traverse in normal human fibroblasts. J. Cell. Biochem. 96: 1071–1085, 2005.

Key words: Miz-1; chromatin immunoprecipitation; promoter-reporter assays; primer extension; fluorescence protein

Upon serum stimulation, replicatively senescent cells enter G_1 phase by eliciting early and middle phase gene expressions, albeit slightly later than younger counterparts. Accordingly, the expressions of genes synthesized in early-G_1 (e.g., c-Myc) and mid-G_1 (e.g., ODC) are similar in young and replicatively senescent cells, while late G_1 genes, such as thymidine kinase (TK), cdc2, cyclin A, cyclin B, cdk2, and cdk4, are greatly downregulated in senescent cells [Chang and Chen, 1988; Richter et al., 1991; Lucibello et al., 1993]. The reduced expression of late G_1 genes explains why Rb is present only in its unphosphorylated form in senescent cells [Futreal and Barrett, 1991], which in turn explains the inability of senescent cells to enter S phase, since E2F is not released to synthesize genes involved in S phase progression [Dyson, 1998].

Grant sponsor: National Institute of Aging of the National Institutes of Health of the United States of America (to EW); Grant number: R37 AG07444.

*Correspondence to: Dr. Eugenia Wang, Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, Kentucky 40292.
E-mail: Eugenia.Wang@Louisville.edu

Received 31 January 2005; Accepted 10 March 2005
DOI 10.1002/jcb.20503

c-Myc is a major regulator of G_1/S transition; its function regulates expression of dozens of genes functionally needed at this checkpoint of cell cycle traverse [Dang, 1999]. c-Myc acts as a transcriptional activator by dimerizing with Max, an obligate partner, and then by binding the DNA consensus sequence CACGTG, located in the promoter region of many target genes, thus positively regulating several mid- to late-G_1 genes that are downregulated in senescent cells [Dang, 1999]. However, c-Myc expression itself is not altered in senescent cells upon serum stimulation, although its response is slightly delayed and repressed [Rittling et al., 1986]. Moreover, the mRNA levels of genes such as *ODC*, a well-characterized c-Myc target, follow the same pattern of expression in both young and senescent cells following serum stimulation [Chang and Chen, 1988].

In vivo c-Myc transcriptional activity is hampered by the family of Mad transcriptional repressors. These proteins also dimerize with Max and bind the same DNA consensus sequence CACGTG; but rather than activating transcription, Mad members lead to gene repression, because they lack a transactivating element which c-Myc has [Luscher, 2001]. Accordingly, expressions of c-Myc and Mad members are usually conversely regulated, in the sense that c-Myc is expressed in proliferating cells, but Mad members in growth-arrested and differentiated cells [Luscher, 2001].

Recently, c-Myc's ability to influence gene expression was found not to be limited to transactivation; microarray data indicate that c-Myc also represses the expression of several hundred genes involved in different cellular processes, although most of these genes are unlikely to be direct targets of c-Myc-mediated repression [Coller et al., 2000; Guo et al., 2000; Nesbit et al., 2000; O'Hagan et al., 2000; Boon et al., 2001; Schuhmacher et al., 2001]. This repression ability is not dependent upon the consensus sequence CACGTG ("E-box"), but rather, for the majority of the repressed genes, upon the Initiator element (Inr), with consensus sequence: Py Py A-1 N T/A Py Py [Lo and Smale, 1996]. While c-Myc does not by itself bind to this sequence, it interacts with several proteins that have this ability, including Miz-1, TFII-I, and YY-1 [Peukert et al., 1997]. The common characteristic of these Inr element-binding proteins is that they normally act as transcriptional activators in the absence of c-Myc. Upon c-Myc expression, they are sequestered away from this DNA consensus sequence, or prevented from interacting with transcriptional co-activators, resulting in gene repression [Gartel and Shchors, 2003].

In this study, we report that the expression of one Mad family member, hMad4, persists in senescent cells, unlike in their young quiescent counterparts, where upon serum stimulation its expression is down-regulated. We also show that this persistent expression of hMad4 expression leads to a defective switch from hMad4 to c-Myc on the promoter region of selected targeted genes, separating them into hMad-repressible and -irrepressible categories. This is based on the fact that upon serum stimulation, in the presence of activated c-Myc expression, selected examples of its target gene expression continue to be repressed, corresponding to hMad4 binding to E-box elements in their promoter regions. By cloning and sequencing the hMad4 promoter region, we have identified the specific site for the initiator element (Inr), and by reporter assays, we show that, as in its murine counterparts, overexpression of Miz-1 enhances hMad4 expression; this enhancement is annulled by co-expressing c-Myc. In all, our findings suggest that: (1) senescence-specific factor(s) may block c-Myc inhibition of Miz-1 activation of hMad4 expression, leaving the latter not down-regulated; and (2) the persistent presence of hMad4 may in turn exert transcriptional repression on selected c-Myc-targeted E-box-regulated genes, i.e., hMad-repressible ones. The cascade impact of c-Myc's failure to repress Miz-1 activation of hMad4, and the resulting continual presence of hMad4 in replicatively senescent fibroblasts, in turn causing downstream repression of the expression of selected E-box-regulated genes, may be the major block for these cells at the G_1/S checkpoint, thereby resulting in apoptosis inhibition and permanent exit from cell cycle traverse.

MATERIALS AND METHODS

Cell Culture

Human WI38 lung embryonic fibroblasts were passaged from 20 cumulative population doublings (CPDLs) until the cells underwent replicative senescence at 58 CPDLs. Cells were considered senescent if no growth was observed after 3 weeks, confirmed by tritiated thymidine labeling and >87% *s*-β-galactosidase staining.

In the case of confluent cultures, cells were left for three days at 100% confluency to ensure that all cells had stop dividing. For serum stimulation experiments, young cultures were plated at 30% confluency (4,000–5,000 cells/cm^2), and grown to 50% confluency over 72 h; cultures were then kept growth-arrested by leaving them at extremely low serum, 0.01%, for 72 h, following which cells were stimulated with 10% fetal bovine serum (FBS); senescent cultures were plated at 8,000 cells/cm^2, and kept in the same conditions for the same length of time before adding full-strength (10%) serum.

RT-PCR

Total RNA was isolated from normal young and senescent WI38 human fibroblasts using Trizol reagent, following the manufacturer's recommendations. Five micrograms of RNA were reverse transcribed using the THERMOSCRIPT RT-PCR system (Invitrogen, Burlington, ON) following the manufacturer's recommendations. PCR reactions included the following reagents: 1–2 μl of RT product, 125 ng of each primer, 1 mM dNTPs, and 2.5 U of *Taq* polymerase, in a total volume of 50 μl. After 30 cycles, 5 μl of the reaction were run on 2% agarose gels containing ethidium bromide, and visualized using a UV light photoimaging system. Bands were quantified using a Phosphoimager and normalized with regard to Max and β-actin controls.

Northern Blots

Total RNA was isolated from WI38 normal human fibroblasts using Trizol reagent, following the manufacturer's recommendations. Ten micrograms of total RNA were fractionated on a 1% agarose gel and blotted onto a nylon membrane; blots were hybridized with the ^{32}P-labeled cDNA SacI fragment, and hybridization was performed overnight at 42°C in a solution of 50% formamide, 10× Denhardt's solution, 0.1% sodium pyrophosphate, 50 mM Tris pH 7.5, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml of denatured salmon sperm DNA (Roche, Laval, QC). Membranes were washed once at room temperature in 2× sodium chloride/sodium citrate (SSC) buffer containing 1% SDS for 30 min, and three times at 65°C for 15 min. Membranes were then exposed to X-ray films at −70°C with intensifier screens. The same membranes were stripped and reprobed with human 18S ribosomal RNA probe, to verify the amount of total RNA loaded in each lane.

Chromatin Immunoprecipitation

A minimum of 4×10^7 cells were cross-linked using formaldehyde added directly to the culture media to a final concentration of 1%, and left for 20 min at room temperature. Formaldehyde was inactivated by adding 2.5M glycine, to a final concentration of 125 mM, for 5 min. Cells were washed twice with 10 ml ice-cold 1× phosphate-buffered saline (PBS), scraped, pelleted into 1× PBS, and resuspended in nuclear extraction buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and protease inhibitors (mini-complete from Roche)). Cells were left on ice for 20 min, vortexed for 30 s, and centrifuged at 2,000 rpm for 10 min to pellet down cell nuclei. Pellets were resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, mini-complete (Roche)) and left on ice for 10 min. The lysate was sonicated 12 times for 20 s at 50% constant maximal power, which resulted in DNA fragments averaging 500 bp, as determined by electrophoresis. The lysate was then centrifuged at 12,000 rpm for 2 min to remove cellular debris. The supernatant fraction was diluted fivefold in ChIP dilution buffer (1% Triton-X, 140 mM NaCl, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 7.8, and mini-complete) and incubated overnight at 4°C with constant rotation, with 2 μg of the following antibodies: anti-c-Myc (Santa Cruz # sc-764X, Santa Cruz, CA), anti-Max (Santa Cruz # sc-765X), anti-hMad4 [our own, Marcotte et al., 2003], anti-Miz-1 (Santa Cruz # sc-5987X), anti-Sp1 (Santa Cruz # sc-59X), anti-C/EBPα (Santa Cruz # sc-61X), anti-E2F1 (Upstate Biotechnology # 05-379, Charlotte, VA), anti-E2F4 (Santa Cruz # sc-866X), or anti-E2F6 (Santa Cruz # sc-8366).

For each experiment, an aliquot was used with no antibody to serve as a negative control. Protein-G agarose beads (Sigma, Oakville, ON) were then added to the mixture for 3 h under constant rotation at 4°C. Before the first wash, the supernatant from the negative control was saved as total input of chromatin, and later processed with the immunoprecipitates starting at the RNase treatment. Beads were washed ten times for 5 min with ice-cold washing buffer (1% Triton-X, 500 mM NaCl, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 7.8, and mini-complete), and chromatin was eluted by resuspending the

beads in 300 μl of TE buffer with 1% SDS and incubating at 65°C for 15 min, after which another 300 μl of TE were added, and beads were left at 65°C for at least 5 h to overnight, to reverse formaldehyde cross-linking. Beads were removed, and the lysates were treated for 30 min at 37°C with 3 μl of RNase A (10 mg/ml), following which they were treated for 2 h at 37°C with 10 μl of proteinase K (10 mg/ml). Following phenol–chloroform extraction, DNA was precipitated overnight at −30°C by the addition of 2 volumes of ethanol and 1 μl of glycogen (10 μg/μl) as a carrier. Precipitated DNA was resuspended in 30 μl of TE and stored at −20°C.

PCR reactions contained 4 μl of immunoprecipitated DNA or diluted total input (1:10), 125 ng of each primer (sequence and melting temperatures described in Table I), 1 mM dNTPs, 2.5 U of *Taq* polymerase, and radiolabelled ^{32}P-dCTPs in a total volume of 50 μl. After 35 cycles, 5–10 μl of the reaction were run on 2% agarose gel containing ethidium bromide, and visualized using a UV light, or on 5% acrylamide gel when radiolabelled dCTPs were used. In that case, gels were dried and exposed overnight at −80°C using an intensifier screen. Bands were quantified using a Phosphoimager.

Primer Extension Assay

Total cellular RNA was extracted using Trizol following the manufacturer's recommendations (Invitrogen). Two primers (5′ CCGGCGGAGGTCGTCCTAGTC 3′ and 5′ CTAGGGACGCGAGGTCCTAGA 3′) complementary to the region downstream of the hMad4 ATG were designed, and end-labeled with γ-^{32}P using T4 polynucleotide kinase. Primers were hybridized with 10 μg of total RNA from confluent WI38 fibroblasts at 95°C for 5 min, followed by 70°C for 30 min. After hybridization, the reverse transcription reaction was carried out at 37°C for 60 min, upon the addition of 1 mM dNTPs and 200 U of murine leukemia virus reverse transcriptase (Invitrogen). Product from the reaction was precipitated overnight using 2.5 volumes of 95% ethanol at −30°C. The pellet was resuspended in 5 μl of TE to which 3 μl of RNA loading dye was added, followed by heating at 95°C for 5 min, and finally loaded on an 8% acrylamide/8M urea sequencing gel, which was dried and exposed overnight at −80°C using an intensifier screen.

TABLE I. Sequences of the Primers Used for PCR Following ChIP

Promoter	Sequence	T_m (°C)
ODC	5′ CGGAGGAAGGGAGGGAGC 3′	62
	5′ GGTCTCCGGGTGGGTCTC 3′	62
Prothymosin α	5′ CCCACACAACCGATTTCTTAG 3′	62
	5′ GGGACTACCTGAAATTTGGG 3′	60
Cdk4	5′ GCGGGGGTTGTGGCAGCC 3′	64
	5′ GAAGCGCGGACGTTCTGGG 3′	64
hTert	5′ GGATTCGCGGGCACAGACG 3′	64
	5′ GCCGGGGCCAGGGCTTCC 3′	66
Cul1	5′ GTCGCCCAGGTAAGAGATG 3′	60
	5′ CTGTCTTGGATGAGCTTTC 3′	56
EIF4e	5′ CCAGCAAGCGAGTGTCCG 3′	60
	5′ GGCAATACTCACCGGTTCG 3′	60
hMad4	5′ CCTCTCGGCAACGCGTCC 3′	62
	5′ GATCAGCAGGGAGTTCAGC 3′	60

hMad4 Promoter-Reporter Assay

A 1,620 bp fragment of the human Mad4 promoter was cloned out by PCR, using genomic DNA from WI38 fibroblasts. The fragment was cloned into the EcoRI site of the pd2EGFP-1 promoter-less vector (Clontech, Mountain View, CA), and sequenced to verify that no mutations were introduced. Serial truncation of the promoter was done sequentially using *Hind III*, *Apa1*, *Nhe 1*, *Hinc II*, and *Sac II* directed at sites in the 5′ region, which yielded fragments of 762, 589, 391, 289, and 113 bp; all these constructs were sequenced. For a positive control, the CMV promoter was cloned 5′ in front of the EGFP. Five micrograms of each construct were transfected into 293 cells using lipofectamine, with 0.5 μg of a β-galactosidase plasmid to control transfection efficiency. Cells were collected by trypsin treatment, pelleted down, resuspended in 1× PBS, and subjected to flow cytometry 24 h after transfection. Units for promoter activity were normalized with β-galactosidase activity, to control transfection efficiency. In co-transfection experiments, 4 μg of the reporter construct were used along with 1 μg of either c-Myc, Miz-1, or Sp1 (the generous gift of Dr. Robert Tjan) expression vector, as well as the β-galactosidase vector. Mutations in the promoter were introduced using site-directed mutagenesis with the following primers: for Sp1, 5′ GCGGGGTGGAAGGCGAGCGGAGCC 3′ and 5′ CCCGGGAAGGACGGCGCGGA-AGGGAG 3′, where the regions underlined were previously GGGCGG; for the E-box, 5′ GGGGCCGGGCCACCACACGCTGTTGCTAAG 3′, where the region underlined was previously CACGTG; and for Miz-1, 5′ CTCCCTTATGTGTCGGTTTGAGGGCC 3′, where the region underlined was previously TCAATT.

RESULTS

hMad4 Is Not Down-Regulated in Senescent Cells Upon Serum Stimulation

The fact that hMad4 was originally cloned out of a cDNA library produced from senescent cells suggests that hMad4 may play a role in establishing replicative senescence. As demonstrated in Marcotte et al. [2003], the hMad4 mRNA transcript is upregulated in senescent cells compared to replicating cells of young and intermediate ages, but its expression is relatively similar in senescent cells and in confluent cultures of young- and intermediate-aged cells [Marcotte et al., 2003]; however, these Northern blots were performed with randomly selected WI38 clonal populations. Here we performed serial passaging of the same young fibroblast population of contact-inhibited quiescent cultures until it reached replicative senescence, at 58 cumulative population doubling levels (CPDL), to examine the trend of hMad4 expression. Total RNA was collected from cultures at 15, 24, 33, 42, 48, 51, and 58 CPDLs, and subjected to Northern blotting. Under these conditions, we observed a two-fold increase in hMad4 expression in confluent cells as they approached senescence (Fig. 1A, PD's 48–51). Therefore, hMad4 expression in senescent cells is increased significantly over that in their contact-inhibited quiescent counterparts.

Since c-Myc is upregulated following serum stimulation, and since Mad member expression is usually decreased upon c-Myc up-regulation, we examined their expression levels following serum stimulation and c-Myc activation in both young and senescent WI38 fibroblasts. Young replicating fibroblasts were plated at 30% confluency, and growth-arrested for 72 h using serum-free medium, at which point the cells reached 50% confluency; their senescent counterparts were prepared in the same way. This was followed by stimulation with 10% FBS. Total RNA was collected every 3 h for 15 h for Northern blot analysis, and for 18 h for RT-PCR assays, as an alternate method to determine expression levels. Expression of c-Myc mRNA is rapidly upregulated by fourfold at 3 h following serum stimulation of young WI38 cells, followed by a decline in expression at later time points (Fig. 1B,C), as reported previously [Rittling et al., 1986]. Results were similar when expression was measured by Northern blot or RT-PCR (compare Fig. 1B and C to D and E). c-Myc expression in senescent fibroblasts following serum stimulation also shows significant increase, although the maximum induction is only 2.4-fold, and peaks at 6 h (Fig. 1B–E). In contrast, hMad4 mRNA levels decrease by twofold following serum stimulation in young cells; this decrease, however, is not observed in senescent fibroblasts, where mRNA levels remain stable throughout stimulation (Fig. 1B,D). mRNA expression of hMxi (a human Mad family member) does not change following serum stimulation of either young or senescent cells (data not shown). Similarly, hMad1 mRNA expression remains unchanged following serum stimulation in both young and senescent fibroblasts (Fig. 1D). These observations suggest that hMad4 may be the functional c-Myc inhibitor in WI38 fibroblasts. Max expression is stable in all conditions, and was used as a loading control, along with β-actin (Fig. 1D).

Regulation of Selected c-Myc Target Genes Is Altered in Senescent Cells

To determine whether the differential regulation of hMad4 in senescent cells versus young replicating cells has a potential effect on the transcription of c-Myc target genes, we tested for the presence of c-Myc/Max and hMad4/Max dimers on the promoter regions of several known c-Myc target genes (Fig. 2). Both growth-arrested young and senescent cells at 30% confluency (after the actual deprivation time) were stimulated with 10% FBS for 6 h. Cells were processed for chromatin immunoprecipitation as described in "Materials and Methods." In young fibroblasts, all five genes tested (*ODC*, *pro α*, *cdk4*, *eIF4-E*, and *cul1*) show a two to fivefold increase in the presence of c-Myc on their promoters following serum stimulation (Fig. 2A, compare lanes 1 and 6). In contrast, hMad4's presence on all five promoters decreases following serum stimulation of young cells (Fig. 2A, compare lanes 3 and 8). These results constitute the first evidence that hMad4 actually binds the promoter regions of c-Myc target genes in vivo. Moreover, as shown by Northern blot and RT-PCR data, Max binding to promoters remains unchanged following serum stimulation (Fig. 2A, compare lanes 2 and 7). In senescent cells, c-Myc binding increases and hMad4 binding decreases on the promoters of two genes, *ODC* and *cul1*, following serum stimulation, similar to the case in young cells

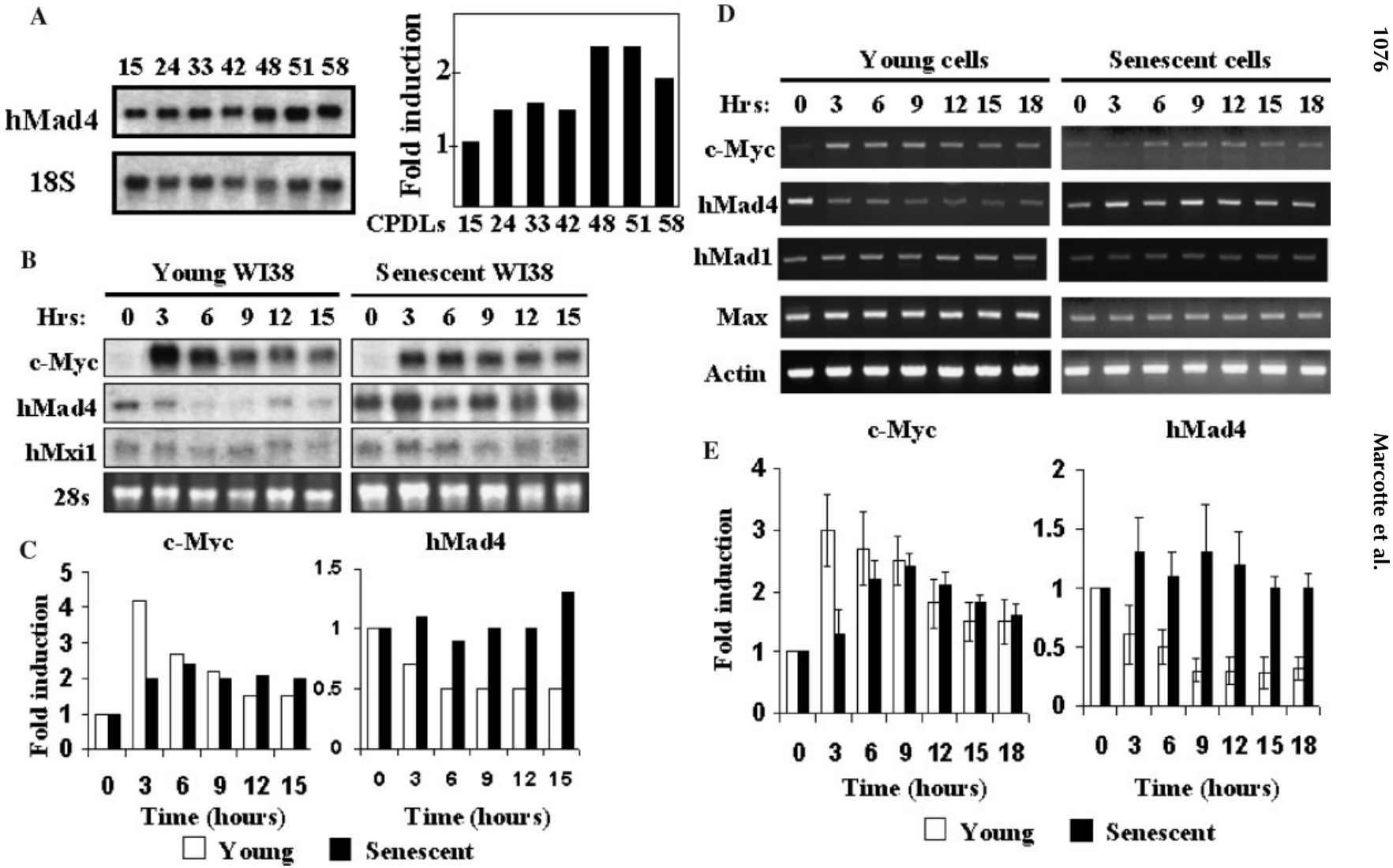

Fig. 1. Expression analysis of c-Myc and hMad members in young and senescent fibroblasts following serum stimulation. **A**: hMad4 mRNA levels in serially-passaged WI38 cells. Cells at CPDL 58 are senescent, as determined by SA-β-galactosidase activity. 18s RNA is shown as a loading control. The hMad4 signal relative to the 18s RNA signal was quantified for each CPDL, as depicted in the plot at the right. **B**: Young (CPDL 21) or senescent (CPDL 58) WI38 cells were kept in 0.01% serum condition for 72 h; extracted RNA was subjected to Northern blot analysis with probes for hMad members and c-Myc. The 28s RNA was detected as a loading control; quantification of c-Myc and hMad4 expression is depicted in (**C**). **D**: The same RNA from experiments described in (C) was subjected to RT-PCR using primers for c-Myc/Max/Mad members. Max and actin mRNAs were examined as controls. The experiments were performed in triplicate; quantification is shown in (**E**). c-Myc and hMad4 expression levels were normalized using the signals obtained for the Max loading control.

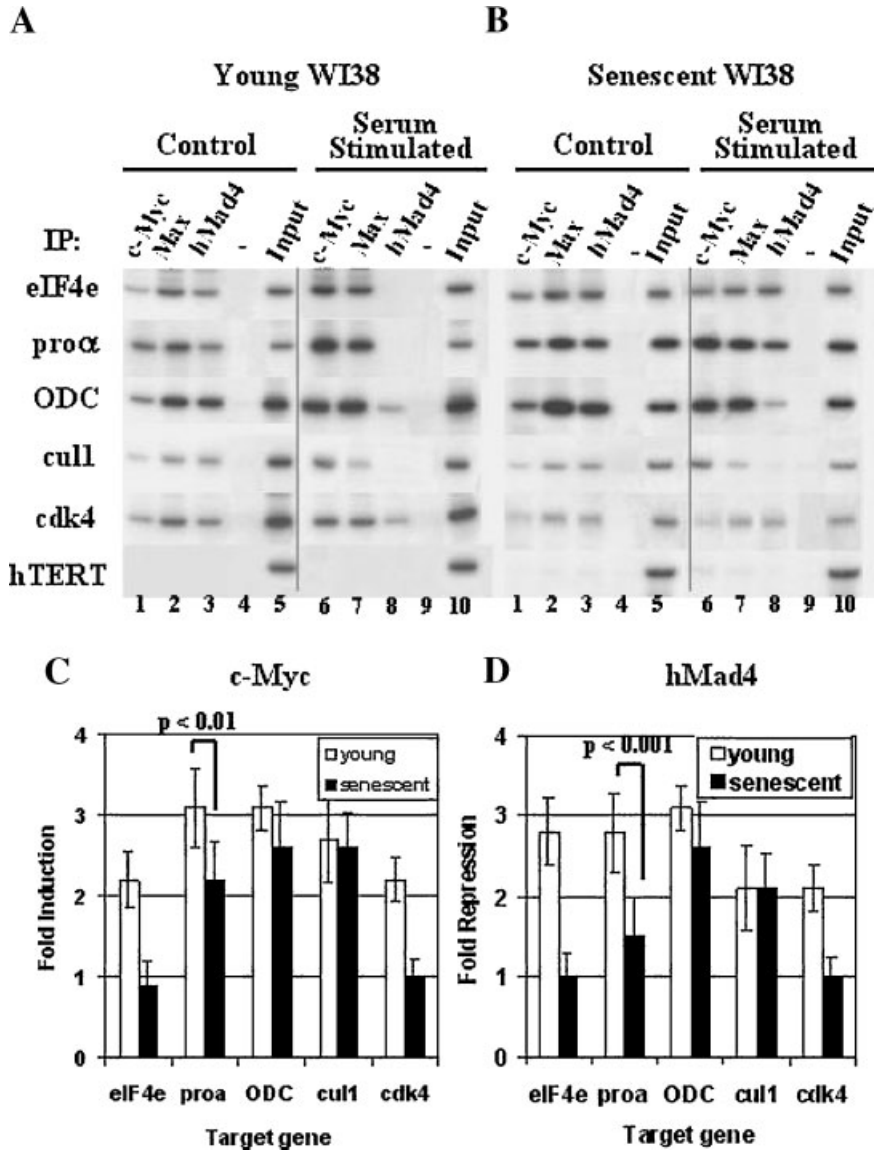

Fig. 2. ChIP analysis of c-Myc, Max, and hMad4 binding to the promoter of c-Myc target genes. Young and senescent cells were kept in 0.01% serum for 72 h, and then serum-stimulated for 6 hours. Cells were subjected to ChIP, as described in ''Materials and Methods.'' A representative result is shown for each gene tested; experiments were repeated at least three times for each gene. **A**: ''Young'' fibroblasts. **B**: Senescent fibroblasts. **C**, **D**: Quantification of c-Myc and hMad4 on the promoter region of the target genes of young and senescent fibroblasts, respectively. c-Myc and hMad4 induction is normalized for both Max and input signal. Quantifications include results from at least three experiments.

(Fig. 2B; for c-Myc compare lanes 1 and 6; for hMad4 compare lanes 3 and 8). However, a binding switch from hMad4 to c-Myc does not occur on the promoters of eIF4-E, pro α, or cdk4 following serum stimulation of senescent cells. These results correlate with previously published observations that ODC mRNA expression is the same in both senescent and young cells, whereas mRNA expression of cdk4, a late gene necessary for G_1/S traverse, is down-regulated in senescent cells [Rittling et al., 1986; Chang and Chen, 1988; Lucibello et al., 1993]. The failure to switch on eIF4-E, pro α, and cdk4 is statistically significant (Fig. 2C,D) by densitometric analysis, where c-Myc and hMad4 signals are expressed as fold induction and fold repression from serum-starved to serum-stimulated cells, respectively. The Max and input signals were used to normalize expression. Therefore, these data provide strong correlative evidence that hMad4 acts as a senescence-specific inhibitor of selected c-Myc target genes, such as eIF-4 and prothymosin α, following serum stimulation.

Expression of eIF4-E and Prothymosin α, but not cdk4, Is Altered in Senescent Cells

To test whether this lack of switching of hMad4 to c-Myc dimers on the promoter region of the target genes leads to an according decrease in mRNA expression, we performed RT-PCR analysis of c-Myc target genes in young and senescent fibroblasts following serum stimulation. In young cells ODC, prothymosin α, and eIF4-E mRNA levels are increased following serum stimulation, whereas no induction is seen for cdk4 or cul1 (Fig. 3A). In senescent cells, mRNA induction following serum stimulation is lost for eIF4-E and prothymosin α, whereas ODC peak mRNA induction is delayed (9 h vs. 12 h), similar to c-Myc mRNA induction following serum stimulation in senescent cells (Fig. 1B,D). As in young fibroblasts, no mRNA induction is seen for cdk4 and cul1 following serum stimulation in senescent cells, although overall levels seem lower, suggesting that c-Myc is not an important transcriptional regulator of these two genes in this particular cell type, although c-Myc binds their respective promoters (Fig. 2). In all, these results suggest that c-Myc transcriptional activity is probably impaired in senescent cells because of an increase in hMad4 expression.

Determination of the hMad4 Transcriptional Start Site

To determine the mechanism behind the altered regulation of hMad4 in senescent fibroblasts, the hMad4 promoter was isolated. *hMad4* was previously localized to chromosome 4p16.3, and known to be contained in two cosmid regions; the promoter DNA sequence was obtained from GenBank. Depicted in Figure 4A is the DNA sequence of the hMad4 promoter, including the translation start site and about 500 nucleotides upstream of it. Consensus sequences for binding of several transcription factors were found; interestingly, the hMad4 promoter contains several Inr elements and an E-box consensus sequence, two sequences to which c-Myc binds directly (E-box) or indirectly (Inr element). Also depicted in Figure 4A is the putative transcription start site (black arrow). Since the location of the hMad4 transcription start site had not previously been directly demonstrated, we performed a primer extension assay to determine its precise location. To this end, two primers were designed 3′ of the translation start site on the (−) DNA strand; the sequences and locations of these primers are depicted in Figure 4A. Total RNA from WI38 cells was used in a reverse transcription reaction, and the labeled DNA products were separated on an 8% sequencing gel. The two primers gave products with the expected sizes of 93 bp for primer #2, and 72 bp for primer #1 (lanes 2 and 3 of Fig. 4B). No product was obtained when RNA was omitted from the reaction, for either primer (lanes 2 and 4 of Fig. 4B). Therefore, the main transcription start site coincides with the predicted transcription start site, and is

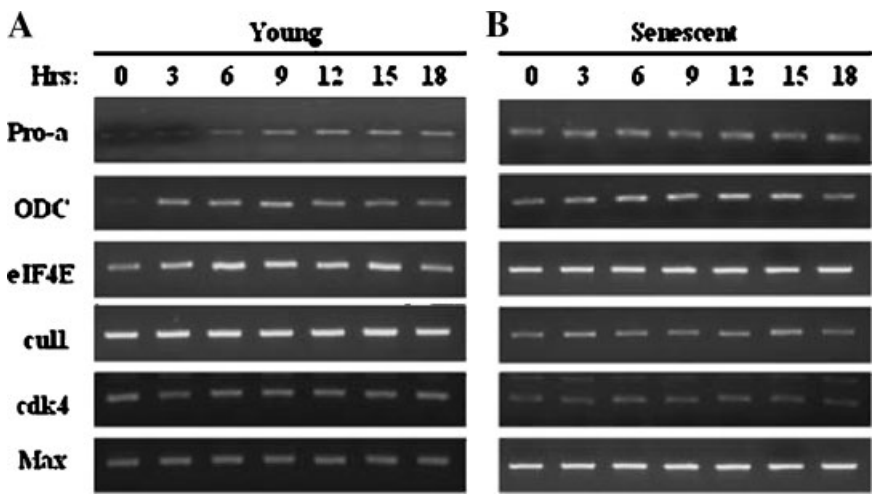

Fig. 3. Expression analysis of c-Myc target genes in young and senescent serum-stimulated fibroblasts. **A**: Young (CPDL 21) or senescent (CPDL 58) WI38 cells were kept in 0.01% serum for 72 h; extracted total RNA was subjected to RT-PCR analysis with primers specific for c-Myc target genes. Max and β-actin were used as loading controls.

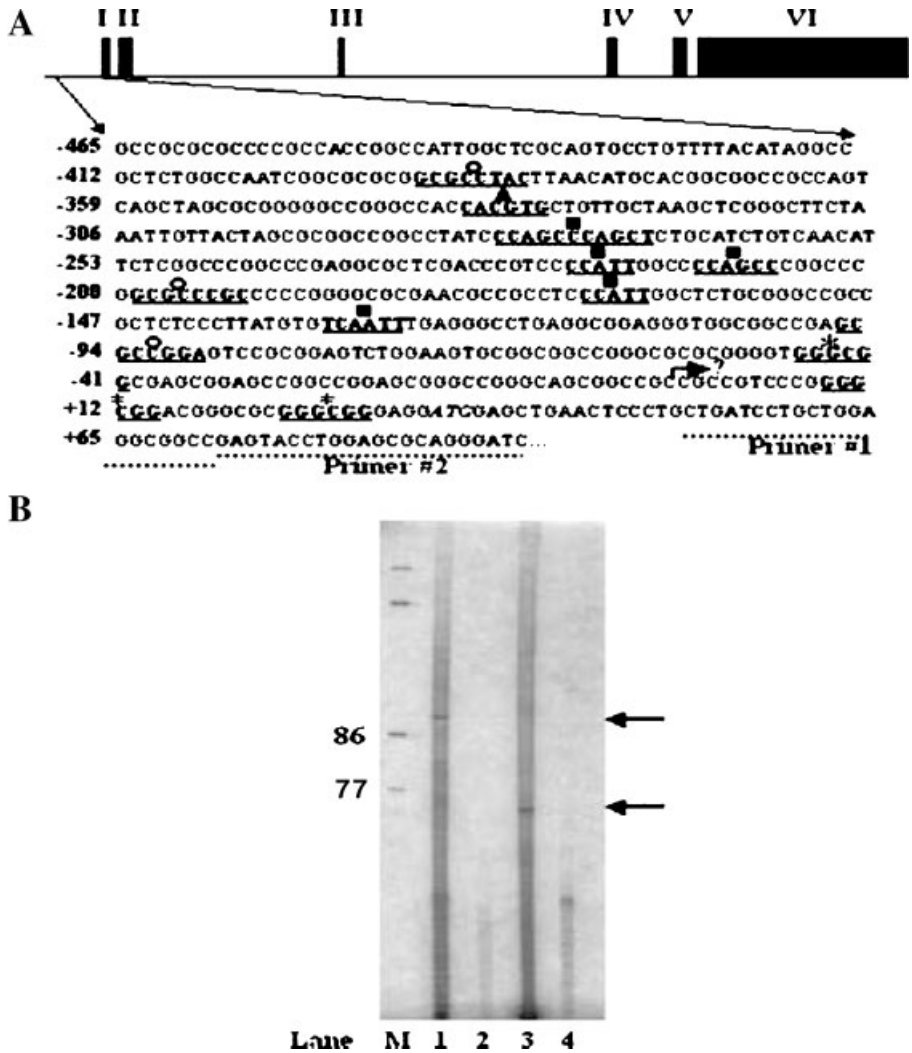

Fig. 4. Determination of the *hMad4* transcription start site. **A**: Nucleotide sequences of the 5′ upstream region of the *hMad4* gene, and following the translational start site. The annealing sites of primers used in primer extension assays are indicated by dotted lines. The consensus sequences for different transcription factors are indicated by bold underlined typeface: * (Sp1), ° (E2F), ■ (Inr), ▲ (E-box). The italicized ATG triplet represents the translational start site. An arrow indicates the potential transcriptional start site. **B**: Primer extension assays were performed using primers complimentary to nucleotides 39-19 (primer 1) and 61-40 (primer 2) on total RNA isolated from confluent cultures of WI38 fibroblasts. Negative control reactions, (−) in which no RNA was used, are shown in **lanes 2** and **4**. Arrows indicate the primer extension products. The ladder marker was produced by digestion of the hMad4 promoter with several restriction enzymes, and labeled as described for the primers in ''Materials and Methods.''

located 32 nucleotides (nt) upstream of the translation start site.

Identification of the Minimal Promoter of hMad4

To identify possible transcription factors regulating hMad4 promoter activity, we first used PCR to isolate a 1620 nt DNA fragment from the hMad4 promoter, including 5′ promoter sequences, the transcription start site, and the translation start site. This fragment was inserted 5′ of EGFP in the promoter-less vector pd2EGFP-1, and was serially digested to give rise to the different promoter fragments depicted in Figure 5A. After checking the integrity of the promoter as well as vector construction by sequencing, each construct was transfected into 293 cells, and EGFP expression was analyzed by flow cytometry. The full-length promoter induces a 68-fold increase in EGFP expression compared to the promoter-less vector (Fig. 5B). This is about one third of the level of induction obtained using the CMV promoter (data not shown), indicating that the *hMad4* promoter is

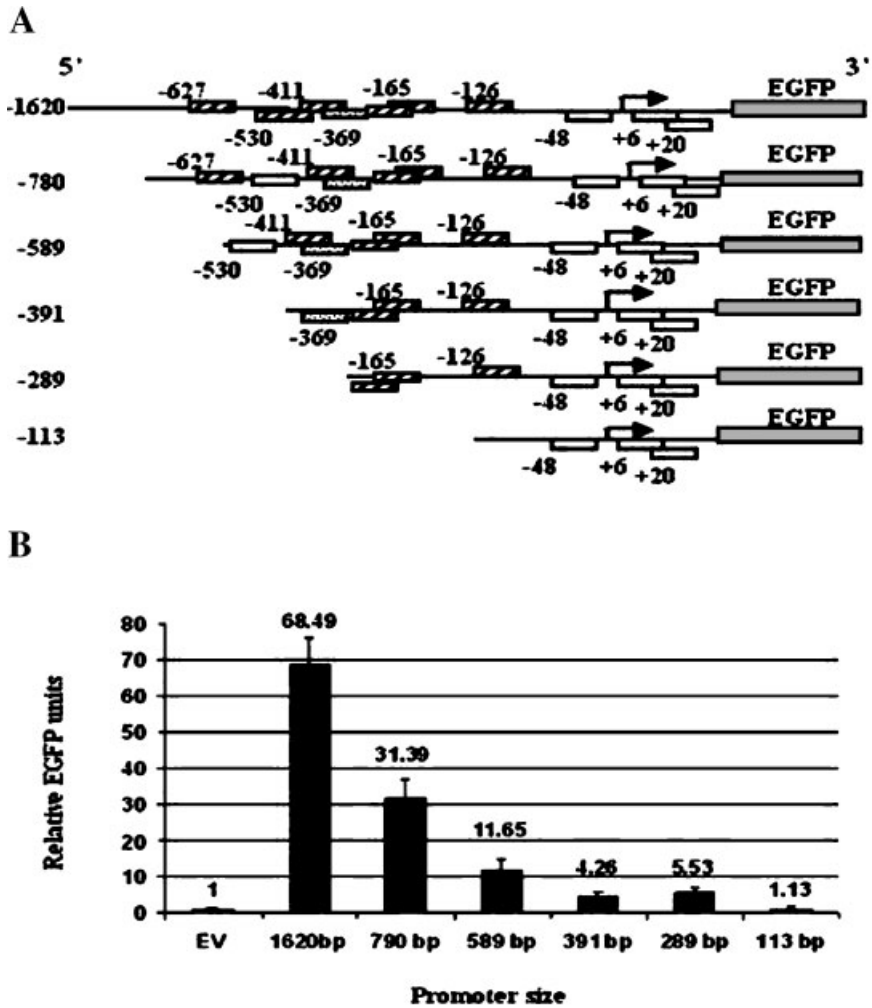

Fig. 5. *hMad4* promoter-reporter assay. **A**: Schematic of the constructs used in the reporter assay. Truncated versions of the hMad4 promoter were ligated 5′ of the EGFP-coding sequence. Black arrows indicate the hMad4 transcription start site. Sequences up to but not including the hMad4 translation start site are included. White boxes: Sp1 consensus binding sites; striped boxes: Inr elements; dotted box: E-box. Numbers indicate the starting nucleotide of the consensus sequence. **B**: Fold induction of EGFP reporter expression of different hMad4 promoter truncation constructs. Four micrograms of promoter constructs were used. EGFP expression was measured by FACS 24 h after transfection, and normalized to β-galactosidase (0.5 μg) expression to control for transfection efficiency. Results are expressed as fold increase over the empty vector, consisting of EGFP sequences with no promoter. Experiments were performed three to six times depending on the construct.

weaker than CMV. Serial truncation of the promoter to within 289 nt of the transcriptional start site reduces EGFP induction to fivefold, relative to promoter-less vector and the 113 nt promoter construct (Fig. 5B). The *hMad4* minimal promoter extends beyond the first 113 nt 5′ of the start site, since the 113 nt fragment does not induce the expression of more EGFP than the promoter-less vector. The minimal promoter region is probably located between 113 and 289 nt upstream of the transcription start site. In addition, sequences extending from 289 to 1620 nt upstream of the transcriptional start site enhance the activity of the *hMad4* promoter.

c-Myc, Sp1, and E2F4 Bind to the hMad4 Promoter

To identify transcription factors interacting with the minimal *hMad4* promoter, we performed ChIP assays with antibodies targeted towards transcription factors whose consensus binding sequences are found in the *hMad4* promoter (see Fig. 4A), and which are known to regulate genes involved in cell cycle progression. Young WI38 fibroblasts were plated at 30%

confluency, growth-arrested for 72 h (at which point the cells reached 50% confluency), and then stimulated for 5 h with 10% FBS. ChIP assays were used to identify changes in transcription factor binding to the *hMad4* promoter in serum-stimulated versus serum-starved cells (Fig. 6B). E2F-4 and Sp1 bind to the *hMad4* promoter more strongly than other candidates tested in serum-starved cells (Fig. 6B). Though other transcription factors appear to bind somewhat to the *hMad4* promoter, this binding is only slightly higher than the level of background signal in these assays (Fig. 6B). E2F4 interactions with the *hMad4* promoter do not change following serum stimulation, while there is a slight decrease in Sp1 binding (Fig. 6B). Moreover, there is a decrease in Miz-1 and E2F-6 binding to the *hMad4* promoter

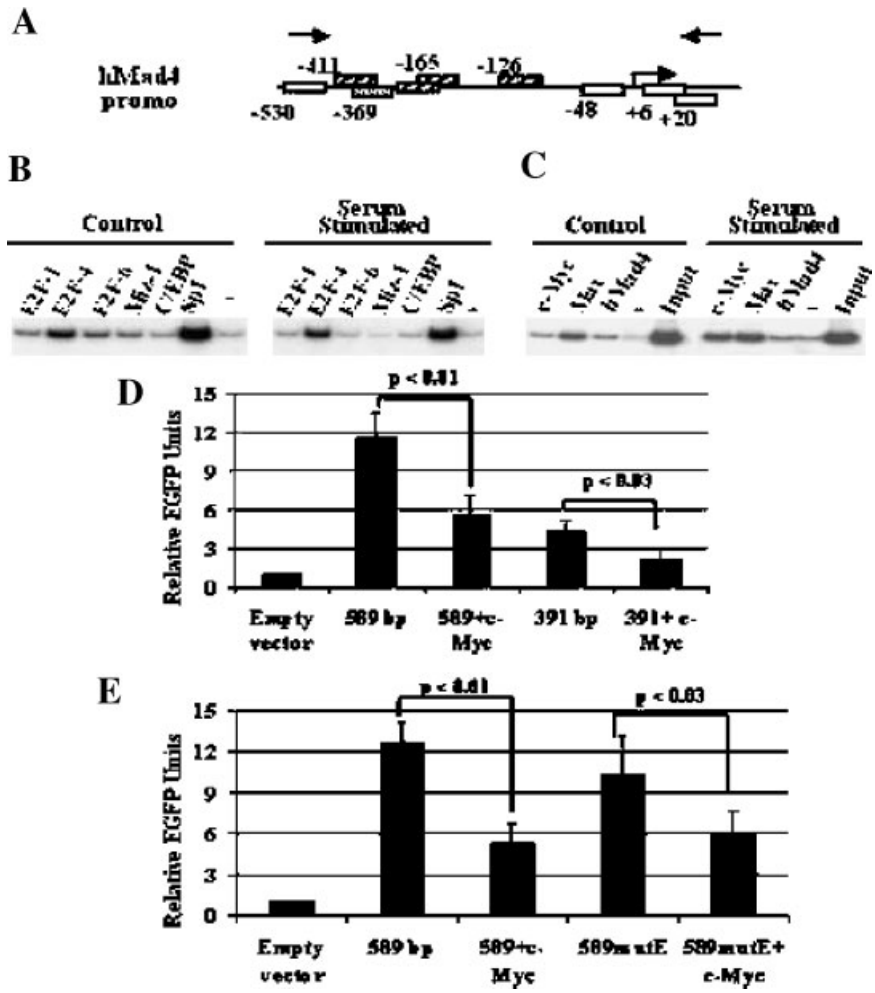

Fig. 6. ChIP assay detection of different transcription factors on the *hMad4* promoter. **A**: Schematic of the region amplified by PCR following ChIP. Black arrows represent the hMad4 transcription start site, and sequences up to but not including the translation start site. White boxes: Sp1 sites; striped boxes: Inr elements; dotted box: E-box. Numbers indicate the first nucleotide of each consensus sequence. **B**, **C**: PCR of the hMad4 promoter following ChIP, with antibodies targeted against several transcription factors. hMad4 primers are described in Table I in ''Materials and Methods.'' **D**: Changes in EGFP expression under the control of the *hMad4* promoter following transfection with c-Myc DNA. EGFP signals were measured by FACS, normalized to the β-galactosidase activity signal to control for transfection efficiency, and depicted graphically as fold induction relative to the EGFP signal induced by a promoter-less vector. Experiments were performed in triplicate. Four micrograms of *hMad4* promoter construct and 0.5 μg of each of the c-Myc- and β-galactosidase-expressing vectors were used in transfection. **E**: hMad4 promoter-dependent activation of EGFP expression in the presence of overexpressed c-Myc. EGFP signals were measured by FACS, normalized to the β-galactosidase activity signal to control for transfection efficiency, and depicted graphically as fold induction relative to the EGFP signal induced by a promoter-less vector. Experiments were performed in triplicate. Four micrograms of hMad4 promoter construct and 0.5 μg of each of the c-Myc- and β-galactosidase-expressing vectors were used in transfection.

following serum stimulation, consistent with published results which demonstrate that E2F-6 acts as a transcriptional repressor and inhibitor of other E2F family members [Gaubatz et al., 1998; Trimarchi et al., 1998]. Since there is an E-box consensus sequence located 369 nt upstream of the transcriptional start site, we also tested the *hMad4* promoter for the presence of c-Myc, Max, and hMad4 itself (Fig. 6C). In growth-arrested cells, Max is the only protein present in significant amounts; c-Myc and hMad4 signals are only slightly higher than background (Fig. 6C). In contrast, c-Myc binding to the *hMad4* promoter is greatly enhanced following serum stimulation (Fig. 6C), by at least three to fourfold.

There is no difference in Max binding to the *hMad4* promoter following serum stimulation, and as in serum-starved cells, hMad4 binding is barely greater than non-specific background signal. When compared to the known decreases in hMad4 expression following serum stimulation, these results suggest that whereas Miz-1 may act as a transcriptional activator of hMad4 expression, c-Myc may function as an endogenous repressor of its own inhibitor, hMad4.

Since c-Myc levels binding to the *hMad4* promoter change following serum stimulation, we tested its effect on hMad4 expression using the 589 and 391 nt promoter-EGFP reporter constructs. Human embryonic 293 kidney cells were transfected with a combination of *hMad4* promoter reporter construct, expression vectors for β-galactosidase, and c-Myc. After 72 h, cells were analyzed for EGFP expression using a flow cytometer. EGFP expression is induced almost 12-fold and 5-fold by the 589 and 391 nt reporter constructs alone, respectively (Fig. 6D); the EGFP expression induced by both reporter constructs is repressed by about twofold by c-Myc overexpression. In all, these results suggest that c-Myc acts as a transcriptional repressor of hMad4 expression.

To test whether E-box consensus sequences affect hMad4 expression, we mutated the E-box sequence from CACGTG to CACCAG in the 589 nt reporter construct, using site-directed mutagenesis. Again, 293 cells were transfected with the reporter construct, a β-galactosidase vector, and a c-Myc expression vector. After 72 h, cells were analyzed for EGFP expression using a flow cytometer. Transfection of the 589 nt reporter construct alone results in a 12.6-fold induction of EGFP expression relative to the promoterless reporter (Fig. 6E); this induction is slightly decreased to 10.4-fold upon mutation of the E-box, but the decrease is not significant statistically. Co-expression of c-Myc with the mutated E-box reporter construct nevertheless causes a 2-fold repression of EGFP expression, similar to the result obtained with the non-mutated reporter construct. Therefore, the E-box probably does not play a major role in c-Myc-mediated repression of hMad4 expression.

c-Myc Probably Represses hMad4 Expression by Interacting With Miz-1

Since c-Myc does not regulate hMad4 expression by binding to the E-box sequence, we tested the implication of Miz-1 on hMad4 expression, as well as the effect of Myc on that regulation. The 589 and 391 nt reporter EGFP constructs were transfected with a β-galactosidase vector and a Miz-1 expression vector into 293 cells. Upon adding Miz-1, there is a 2.2-fold increase in EGFP expression by the 589 reporter construct, and a 2.5-fold increase with the 391 reporter construct; both increases are statistically significant (Fig. 7A). These results suggest that Miz-1 acts as a transcriptional activator of hMad4.

To confirm that Miz-1 indeed regulates hMad4 through direct binding to the promoter, the Inr site located at −126 nucleotides from the transcriptional start site was mutated in both the 589 and 391 nt EGFP reporter constructs. These constructs were transfected along with Miz-1 expression vector; no induction of EGFP is seen with the mutated Inr reporter construct (Fig. 7B). Moreover, c-Myc no longer represses EGFP expression under the mutated Inr reporter construct, but inhibits the induction of EGFP driven by the non-mutated promoter following Miz-1 expression (Fig. 7C), in a dose-dependent manner. In all, this suggests that Miz-1 regulates hMad4 expression, and that c-Myc represses hMad4 expression by preventing Miz-1 from activating expression of the hMad4 promoter.

DISCUSSION

The c-Myc/Max/Mad protein network is a major regulator of both G_1/S progression and cell growth, both of which are defective in replicatively senescent fibroblasts. Here we demonstrate that expression of the endogenous inhibitor hMad4 is further up-regulated by

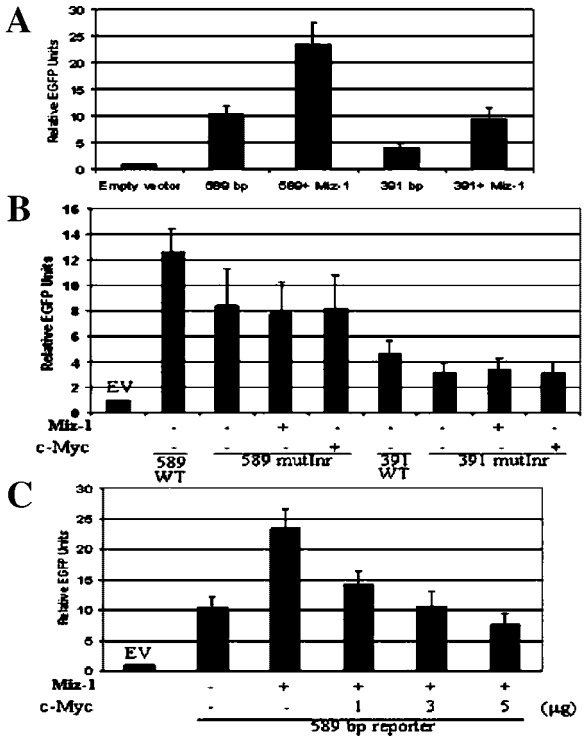

Fig. 7. **A**: Changes in EGFP expression under the control of the *hMad4* promoter following transfection with Miz-1 cDNA. EGFP signals were measured by FACS 24 h following transfection, normalized to the β-galactosidase activity signal to control for transfection efficiency, and depicted graphically as fold induction relative to the EGFP signal induced by a promoter-less vector. Experiments were performed in triplicate. Four micrograms of *hMad4* promoter construct and 0.5 μg of each of the Miz-1 and β-galactosidase-expressing vector were used in transfection. **B**: Same as (A), using an *hMad4* promoter mutated in the Inr element located at −126 nucleotides from the transcriptional start site. **C**: c-Myc was added in increasing amounts to the non-mutated *hMad4* promoter reporter construct and Miz-1.

twofold in replicatively senescent cells, compared with younger contact-inhibited quiescent ones (Fig. 1A). Moreover, hMad4 mRNA levels are no longer down-regulated following serum stimulation in senescent cells, as in their young quiescent counterparts following serum stimulation. Importantly, no other hMad family members seem to be regulated by serum stimulation in both young and senescent fibroblasts, suggesting that hMad4 is likely to be the only hMad family member regulating c-Myc targeted genes. Expression of c-Myc in serum-stimulated senescent cells, although delayed and slightly decreased (Fig. 1B–E), follows the same up-regulated pattern as in younger counterparts, as previously observed [Rittling et al., 1986]. Therefore, genes regulated by this network in senescent cells are not dependent on c-Myc expression levels, but probably affected by the failure of hMad4 to be downregulated following serum stimulation, as would be the case in younger counterparts.

Consistent with expression studies, there is a defective switch from the hMad4/Max complex to the c-Myc/Max complex on the promoters of three known c-Myc target genes following serum stimulation (Fig. 2). Specifically, a switch from hMad4/Max to c-Myc/Max complexes does not occur on the promoters of the *eIF4E* or *cdk4* genes, and only partially on *prothymosin* α, although this switch occurs on the *ODC* and *cul1* promoters (Fig. 2). This result is intriguing because it suggests that hMad4 promoter binding may mediate selective inhibition of a subset of genes in senescent cells normally activated by c-Myc. The mechanisms underlying differential gene expression in senescence, i.e., one subset of cMyc-target genes in replicative senescent fibroblasts is repressible by hMad4 while another subset is not, remain unknown. They may be dependent on changes in the types of modifying complexes that c-Myc recruits to promote transcriptional activation, and/or to overall chromatin structure near the affected gene.

Moreover, our results also demonstrate that hMad4, Max, and c-Myc bind in vivo to the promoters of five selected c-Myc target genes. Therefore, *ODC*, *prothymosin* α, *cul1*, *eIF4E*, and *cdk4* can be added to the growing list of true Myc/Max/Mad targets. Other studies demonstrate binding of Mad family members to the promoter region of target genes in vivo; in these reports, hMad1 is shown to bind to the *cyclin D2* and *hTERT* genes following differentiation of a human leukemic cell line [Bouchard et al., 2001; Xu et al., 2001]. In our case, no binding of any member of the Myc/Max/Mad network is detected on the promoter of *hTERT* gene. c-Myc might not be expected to bind to the *hTERT* promoter in the non-immortalized, non-transformed, telomerase-negative cells used in our experiments; nevertheless, our data suggest that a defective c-Myc response to serum due to persistent expression of hMad4 contributes in part to the inability of senescent cells to bypass the G_1/S restriction point.

Expression of Mad family members is usually enhanced in conditions of growth arrest, such as extremely low-serum growth conditions, and differentiation, but is reduced in cycling cells [Grandori et al., 2000]. hMad4 expression patterns are similar to those for other Mad family

members, since hMad4 expression is increased in both young confluent and senescent cells [Marcotte et al., 2003]. Moreover, hMad4 expression is also down-regulated following serum stimulation (Fig. 1B–E), and strikingly, this decrease correlates with an increase in c-Myc expression. These data suggest that c-Myc may regulate the expression of its own inhibitor. Indeed, c-Myc binds the *hMad4* promoter in vivo, an interaction that is enhanced following serum stimulation (Fig. 6C). In addition, overexpression of c-Myc represses *hMad4* promoter-driven EGFP expression in 293 cells (Fig. 6D), suggesting that c-Myc may indeed act as a transcriptional repressor of hMad4. However, this repression is not mediated by the E-box consensus sequence, located 369 nt from the transcriptional start site (Fig. 6E).

c-Myc represses the expression of several genes, for some of which the repression mechanism has been elucidated. In the case of $p15^{INK4b}$, c-Myc prevents the normal binding of Miz-1, a transcriptional activator, to an Inr element located in the promoter region [Seoane et al., 2001; Staller et al., 2001]. Interestingly, Miz-1 expression is increased in senescent cells [Staller et al., 2001], therefore probably limiting the repression potential of c-Myc. Moreover, overexpression of Miz-1 in fibroblasts induces replicative senescence, suggesting that the genes regulated by this transcriptional factor may be required for the senescent phenotype [Staller et al., 2001]. However, our results indicate that Miz-1 binding to the *hMad4* promoter in young fibroblasts in the presence of serum is nearly undetectable compared to the negative control (Fig. 6B). This may be due to low levels of Miz-1 expression in young fibroblasts, and/or a low affinity of the Miz-1 antibody for in vivo formaldehyde cross-linked Miz-1 protein.

Nevertheless, our reporter assay results suggest that Miz-1 can regulate hMad4 expression, through an Inr element located at −126 from the transcriptional start site (Fig. 7). Here, we suggest that c-Myc-mediated repression of hMad4 is dependent on Inr elements; this is supported by recent data pertaining to the transcriptional regulation of mouse *Mad4* [Kime and Wright, 2003]. This study reports that c-Myc regulates mMad4 expression by binding via Miz-1 to two Inr elements, located 130–170 nt upstream of the *mMad4* ATG translation initiation codon. However, the major transcriptional start site of *mMad4* is located immediately adjacent to the regulatory Inr elements, quite far from the translation start site. In contrast, the transcriptional start site of *hMad4* is located 32 nt, and the Inr elements are found 126–280 nt, upstream of the translational start site (Fig. 4A). Despite these differences in promoter organization, the fact that mMad4 is regulated through Inr elements lends support to our suggestion that Miz-1 regulates the human homologue of Mad4 expression, an event inhibited by c-Myc.

In conclusion, our results show that hMad4 expression is down-regulated in growth-arrested quiescent young fibroblasts upon serum stimulation, with a corresponding up-regulation of c-Myc. This reciprocal change in the two gene expressions is not lost in replicative senescent fibroblasts following serum stimulation, where significant expression of hMad4 persists even in the substantial presence of c-Myc. The persistent presence of hMad4 in replicatively senescent cells results in selected cMyc-target, E-box-regulated genes remaining repressed, such as prothymosin α, cdk4, and eIF4E. Our identification of the Initiator element (Inr) in the hMad4 promoter, and of Miz-1 activation and c-Myc inhibition of this activation, suggests that failure to down-regulate hMad4 may be due to the presence of senescence-specific factors which block the latter's inhibitory action. Future experiments will allow us to identify these factors, and investigate their functional impact on c-Myc action in inhibiting Miz-1 activation of hMad4 expression. Nevertheless, our results show that hMad4's transcriptional repression of selected cMyc-target, E-Box-regulated genes may be part of the key functionally responsible for blocking replicatively senescent fibroblasts at the G_1/S checkpoint of cell cycle traverse, rendering them both permanently growth-arrested and apoptosis-resistant.

ACKNOWLEDGMENTS

The authors wish to express their sincere thanks to Mr. Alan N. Bloch for editing the text, and Dr. Jacques Galipeau for insightful discussions and support during our study.

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