Transcriptional Control of the Human *High Mobility Group A1* Gene: Basal and Oncogenic Ras-Regulated Expression

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Abstract

Several studies have already shown that the high mobility group A1 (*HMGA1*) gene is up-regulated in most common types of cancer and immortalized tissue culture cell lines. *HMGA1* expression is also much higher during embryonic development than in adult life. The elevated expression of *HMGA1* in cancer thus likely occurs through oncofetal transcriptional mechanisms, which to date have not been well characterized. In the present study, we have cloned and functionally analyzed the TATA-less 5′-flanking regulatory region of human *HMGA1*. We identified two proximal regulatory regions that are important for basal transcription and in which specificity protein 1 (SP1) and activator protein 1 (AP1) transcription factors seem to be the regulating elements. In addition, we showed that the *HMGA1* promoter is strongly inducible by oncogenic Ras, via a distal regulatory region. An AP1 site and three SP1-like sites are responsible for this inducible activity. An even more convincing finding for a role of oncogenic Ras in the regulation of *HMGA1* in cancers is the discovery that *HMGA1* up-regulation in the HCT116 colon cancer cell line is abolished when the mutated *Ras* allele is removed from these cells. Our data constitute the first extensive study of the regulation of basal and Ras-induced human *HMGA1* gene expression and suggest that the elevated expression of *HMGA1* in cancer cells requires, among others, a complex cooperation between SP1 family members and AP1 factors by the activation of Ras GTPase signaling. [Cancer Res 2007;67(10):4620–9]

Introduction

The high mobility group A (HMGA) family of low molecular mass nonhistone chromatin proteins consists of three members: HMGA1a and HMGA1b, which are produced by alternative splicing from a single gene on chromosome 6p21 (1), and HMGA2, which is the product of a separate gene on chromosome 12q14-15 (2). HMGA proteins are called architectural transcription factors and have been implicated in both positive and negative regulation of gene transcription. They lack the intrinsic ability to transactivate a gene directly but are able to influence the regulation of gene expression by altering DNA conformation through binding to AT-rich regions and/or direct interaction with various transcription factors (reviewed in refs. 3, 4).

The level of expression of HMGA1 mRNAs and proteins is low or undetectable in most differentiated or nonproliferating cells (5) but is often exceptionally high in transformed cells, as well as in embryonic cells (6). Initially, *HMGA1* overexpression was described in rat thyroid transformed cells (7) and associated with aggressiveness of rat prostate cancer cells (8). Subsequently, elevated levels of *HMGA1* gene products and HMGA1 proteins have been observed in almost every cancer type investigated [thyroid (9), colon (10, 11), prostate (8, 12), cervix (13), ovarium (14), pancreas (15), gastrointestine (16), hepatocellular (17), breast (18, 19), and leukemia (20)]. Moreover, increased expression of HMGA1 correlates with invasiveness, metastatic potential, and stage progression in several human tumors of epithelial origin (8, 9, 12, 16). Additionally, it was shown that overexpression of HMGA1 proteins in 'normal' Rat1a cells and a human breast adenocarcinoma cell line (MCF-7) caused, respectively, neoplastic transformation and malignant metastatic progression of these cells (21, 22). Moreover, transgenic mice expressing full-length HMGA1a under control of the murine H-2K promoter and immunoglobulin μ enhancer, which drive expression of the transgene in B and T cells, have been shown to develop highly penetrant, aggressive lymphoid malignancy (23). HMGA1 was also shown to be induced by the tumor-promoting agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA; ref. 24). Although all these results together show that overexpression of full-length HMGA1 proteins in tumor cells is extremely widespread and biologically important, the basis for the elevated expression has mostly been unknown.

Growth factors initiate diverse intracellular signaling pathways that lead to the phosphorylation of transcription factors and ultimately to the regulation of target genes. Among the pathways often used to transduce signals are the mitogen-activated protein kinase (MAPK) cascades. The Ras/Raf/MAPK/extracellular signal-regulated kinase (ERK) kinase/ERK signaling cascade regulates cell proliferation and differentiation (25). Components of this pathway are often activated in human tumors. Mutated variants of Ras are found in 30% of all human cancers and are consequently rendered constitutively activated in their GTP-bound form (26, 27). In addition to mutational activation, Ras GTPase signaling can be up-regulated due to increased coupling to cell surface receptors. In particular, members of the epidermal growth factor (EGF) family of receptor tyrosine kinase (including EGF receptor/ErbB/HER1 and ErB2/HER2/Neu) or other tyrosine kinases (e.g., Bcr-Abl) are commonly overexpressed in many cancers, causing persistent activation of Ras in the absence of mutations in *Ras* genes (28). The aberrant activation of Ras proteins ultimately leads to the chronic stimulation of signaling cascades that promote activation of transcription factors involved in virtually all aspects of the malignant phenotype of the cancer cell (29).

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To better understand the potential role of HMGA1 in cell growth and tumorigenesis, we have been studying the transcriptional regulation of the human *HMGA1* gene in embryonic and cancer cells. In this article, we show that the *HMGA1* promoter consists of at least two proximal and a distal regulatory region. Basal *HMGA1* transcription is mainly controlled at proximal region 1 by specificity protein (SP) 1/SP3 factors and at proximal region 2 by activator protein 1 (AP1) factors. We also show that the higher expression of HMGA1 in tumors might be, in part, the result of activation by oncogenic Ras, which influences the transcription of *HMGA1* via the distal region (enhancer element).

Materials and Methods

Materials. Antibodies against SP1, SP3, c-fos (K-25), and c-jun (D) were from Santa Cruz Biotechnology. The actin antibody, mithramycin A, and TPA were from Sigma-Aldrich.

The plasmid pCMV-SP1, kindly provided by Dr. R. Tjian (University of California, Berkeley, CA), constitutively expresses SP1 under the control of the cytomegalovirus (CMV) long terminal repeat region. The plasmid pRSV-*Ha-Ras^{Leu61}*, kindly provided by R. Medema and B. Burgering (Utrecht University, the Netherlands), expresses constitutively active *Ha-Ras^{Leu61}* under the control of the RSV promoter.

Promoter constructs. *HMGA1*-luciferase reporter plasmids were constructed by PCR starting from a human BAC clone (513I15). A 2.0-kb fragment of the 5′-flanking region of the h*HMGA1* was amplified with an Expand High Fidelity PCR system (Roche Applied Science) using the oligonucleotides hHMGP1s 5′-CGGCTAGCCCACCACCACTCACGGCCTACTAA-3′ and hHMGP5 5′-CGAGATCTCGCGGGGTATTTATGTGTCACAGC-3′ and cloned into the polylinker (via *Nhe/Bgl*II) of the luciferase reporter vector pGL3-Basic (Promega). This construct was named pIC(−1745/+265). Sequential 5′-deletion constructs were generated by digesting the pIC(−1745/+265) construct with *Nhe*I and each of the following restriction enzymes: *Bsu*36I and *Asc*I (New England Biolabs) and *Sfi*I, *Bbr*PI, *Mlu*NI, *Eco*RI, *Xho*I, *Sma*I, and *Apa*I (Roche). The constructs containing additional 5′- or 3′-flanking sequences were made by insertion of a PCR fragment into pIC(−1745/+265) for extra 5′- and 3′-sequences and pIC(−78/+265) for extra 3′-sequences only. For PCR amplification, the following primers were used: prom5 5′-TTCCTCCTAAGTCTGTAAGCGCCTGAC-3′ (forward) and 5′-GAATGTGGCAGGAATGTTTCCGG-3′ (reverse) and start2 5′-AGAAGCTCCTTCGTGACTCCTCTGA-3′ (forward) and 5′-GGATCTGAAACCGGGAGAGAGCA-3′ (reverse). Additional deletions were made by digestion with *Nhe*I and *Pac*I or *Ssp*I. The internal deletion mutants were made by digestion with *Bbr*PI-*Bsu*36I (ΔRRE1), *Bbr*PI-*Asc*I (ΔRRE1-2), and *Bsu*36I-*Asc*I (ΔRRE2). Mutation of the SP1 and AP1 sites and of the E-box were generated by overhang PCR using the following primers (mutations are in bold): mSP1A 5′-ATGGCCCCG**AA**CCCTGAGTGAC-3′ (forward); mSP1B 5′-ACGGCTGGCGCG**TT**CGGGCC-3′ (forward); mSP1C 5′-CTTCGGG**TT**AGGGGGAATATTTTTG-3′ (forward); mSP1D 5′-GGCCTG**TT**CGGCGAGCACGCGG-3′ (forward); mAP1Re 5′-CGCCTGGCTGTGACA**TG**TAAATACCC-3′ (forward); mSP1overh 5′-CGGGACTGCGAGGAGTGGGCG-3′ (forward) and 5′-GCGAGGGGCAGGAAGCTGGGA-3′ (reverse); ST2overh 5′-GGTCGCTTTTTAAGCTCCCCTG-3′ (forward) and 5′-GGTGGCTTTACCAACAGTACCGGA-3′ (reverse); mEbox 5′-GGTCAAGCA**GC**TGCTGCCCGGGCCCCGA-3′ (forward); m3*SP1 5′-TGGGCCCA**AA**CCCCG**AA**CGCTCCCCA**AA**CCCACCGG-3′; and overhEbox 5′-TGCGCTCCAGTGACACACGGCTGTGAC-3′ (forward) and 5′-GGGACTTCTCAGGAACTGCGTTTCACCTGG-3′ (reverse). The AP1 mutation in the distal regulatory region (DRR) was generated by PCR using the primer set mAP1 5′-AGCCTGAGGAGGGGGCTGGGCCAGGGCCTCGGCTGACCGGGGAGGAAGAAGGGGAGCAGAGAAAAACA**C**GAGTC**G**CAGCC-3′ (forward) and 5′-TGCACCGAGCGAGAAGGAAGCC-3′ (reverse).

Cell culture, stable cell lines, and Western blot analysis. Cell lines included HEK293 (ATCC CRL-1573), HCT116 (ATCC CCL-247), SW480 (ATCC CCL-228), and Hke3 and e3MKRas#14 cells, both sublines of HCT116 cells (generous gift from Dr. T. Sasazuki, Kyushu University, Fukuoka, Japan). Cells were grown in DMEM (Invitrogen) supplemented with 10% FCS (Hyclone, Pierce Perbio Science) and penicillin/streptomycin (100 units/mL; Invitrogen) at 37°C in a humidified 5% CO_2 incubator.

Stable HEK293 cells were made expressing empty vector (Mock) or Ha-Ras^{Leu61}. Transfected cells were selected in medium containing 3 μg/μL puromycin (Becton Dickinson) and tested for the expression of the transfected gene by Western blot analysis as described (30). HMGA1 protein was detected with rabbit polyclonal antiserum directed against the NH_2-terminal peptide SSSKSSQPLASKQ (Eurogentec) of the human HMGA1 protein.

Cells were transfected using Fugene 6 transfection reagent (Roche).

Luciferase reporter assays. Twenty-four hours after seeding in 24-well plates, cells were cotransfected in duplicate with 400 ng of the different *HMGA1* promoter constructs and the indicated amount of a construct expressing *Ha-Ras^{Leu61}* (pRSV-*Ha-Ras^{Leu61}*) or *SP1* (pCMV-*SP1*). Total DNA concentration for each transfection was matched with empty vector. Routinely, 24 h after transfection, cells were washed and grown on serum-free medium (not absolutely necessary). Cell lysates were prepared 48 h after transfection and assayed for luciferase activity using the Luciferase Assay System (Promega) as described (31). Seventy-five nanograms of pEL1 (transcription elongation factor 1) β-galactosidase (β-gal)–expressing plasmid was cotransfected for normalizing the transfection efficiency. β-gal activity was assayed as described in Sambrook et al. (32). Luciferase activity and β-gal A_{420} were measured in a Wallac Victor2 1420 Multilabel Counter (Perkin-Elmer Life Sciences).

Preparation of nuclear extracts. Nuclear extracts were prepared from semiconfluent cells in 100-mm culture dishes according to the procedure described (33). Aliquots of the nuclear extracts were immediately frozen on dry ice and stored at −80°C. The protein concentration of the nuclear extracts was determined by the Bradford assay (Protein Assay Reagent Protein Assay kit, Pierce Perbio Science).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSA) were done using the LightShift Chemiluminescent EMSA kit (Pierce Perbio Science) with slight modifications. Briefly, 5 μg nuclear extracts were preincubated with 50 ng/μL poly(deoxyinosinic-deoxycytidylic acid), 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L KCl, 1 mmol/L DTT, 5 mmol/L $MgCl_2$, and 0.05% NP40 for 10 min at room temperature. After preincubation, the samples were either directly used in the binding assay, incubated for 10 min at room temperature with 6 pmol (200-fold molar excess) unlabeled competitor probe, or incubated for 60 min at 4°C with the appropriate antibody. Thirty femtomoles of biotin-labeled probe (Eurogentec) were added in a total volume of 20 μL, and the reaction mixture was incubated for 20 min at room temperature. Electrophoresis, electrophoretic transfer, and detection of the biotin-labeled DNA were done according to the manufacturer's instructions.

The sequences of the upper strand of the oligonucleotides used were as follows: WTSP1A 5′-ATTTGCATGGCCCCGCCCCCTGAGT-Biotin-3′ (forward); MuSP1A 5′-ATTGCATGGCCCCG**AA**CCCTGAGT-Biotin-3′ (forward); CoSP1 5′-GCTCGCCCCGCCCCGATCGAAT-3′ (forward); WTAP1 5′-AGCAGAGAAAAACTTGAGTCACAGCCGTGT-Biotin-3′ (forward); MuAP1 5′-AGCAGAGAAAAACT**C**GAGTC**G**CAGCCGTGT-Biotin-3′ (forward); WTAP1 5′-AGCAGAGAAAAACTTGAGTCACAGCCGTGT-3′ (forward); and MuAP1 5′-AGCAGAGAAAAACT**C**GAGTC**G**CAGCCGTGT-3′ (forward).

Quantitative reverse transcription-PCR. Total cellular RNA was isolated using the NucleoSpin RNA II kit (Filter Service, S.A). cDNA was prepared from 5 μg DNaseI-treated total RNA, using the SuperScript First-Strand system for reverse transcription-PCR (RT-PCR; Invitrogen) with a random primer, according to the manufacturer's instructions. Quantitative real-time PCR was done using the qPCR Master Mix Plus for SYBR Green I with fluoresceine (Eurogentec) in a 15 μL reaction. Target cDNA was amplified in triplicate with the MyIQ Single-Color Real-time PCR detection system (Bio-Rad). The following PCR conditions were used: enzyme activation at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. A nontemplate reaction was included as negative control. After completion of the amplification, the samples were subjected to dissociation to evaluate the presence of a single amplification product and the absence of nonspecific PCR products and primer dimer formation. Primer sets were

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designed with the Primer Express software (version 2.0; Applied Biosystems). Primers included hHMGA1qPCR 5′-CCCGGAAAACCACCACAAC-3′ (forward) and 5′-TTCTCCAGTTTTTTGGGTCTGC-3′ (reverse) and hSP1qPCR 5′-CAAATGCCCCAGGTGATCAT-3′ (forward) and 5′-CACCAGCCCCATGGAGAC-3′ (reverse). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as reference gene to normalize for variations in the amount of input cDNA [hGAPDHqPCR 5′-TGGAGTCCACTGGCGTCTTC-3′ (forward) and 5′-CTCCCCCCTGCAAATGAG-3′ (reverse)]. The results obtained from quantitative RT-PCR (qRT-PCR) were analyzed using the Data Analysis software provided by Bio-Rad.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were done by following the ChIP assay protocol from Upstate Biotechnology (Millipore). Lysates were sonicated using a Branson 250 sonicator applying 70% power, duty cycle of 25% during 5 min on ice. After elution of the histone complex and reversing of histone-DNA cross-links, DNA was recovered using the QIAquick PCR purification protocol (Qiagen). PCR amplification was done using the following primer set: SP1MP 5′-CGGGACTGCGAGGAGTGGGCG-3′ (forward) and 5′-GCGAGGGGCAGGAAGCTGGGC-3′ (reverse).

Statistical analysis. The results of the luciferase experiments are a representative set of experiments done in duplicate that were repeated at least thrice. Significant effects of the different deletion and mutation constructs were assessed using an unpaired Student's t test. The degree of significance is indicated by asterisks, where asterisk indicates $P < 0.05$.

Results

Functional analysis of the *HMGA1* gene promoter. To define key regulatory regions in the human *HMGA1* gene, luciferase reporter constructs bearing various portions of a fragment of 2010 bp of the *HMGA1* 5′-flanking region were generated (Fig. 1*A*) and transiently transfected into HEK293 cells and two colon cancer cell lines, SW480 and HCT116. Luciferase analysis of these deletion mutants revealed that the proximal regions −78/−24 and +222/+335 bp were necessary for basal expression (Fig. 1*B*). Inclusion of additional 5′-flanking sequences to −1745 bp resulted in an increase of promoter activity and suggests the presence of additional positive regulatory elements in the region between −1745 and −1353 bp for the HEK293 cells and between −1745 and −1161 bp for the colon cancer cell lines investigated. No further increase of promoter activity was observed after addition of another 1-kb fragment to −2706 bp (data not shown). Inclusion of the second transcription start site at the 3′-end of the promoter sequence caused a strong increase of luciferase activity (Fig. 1*B*), which confirms an important role for both transcription start sites (24). From these data, it is evident that the *HMGA1* promoter contains at least three positive regulatory regions: two proximal regions at the level of transcription start site 1 (PRR1; −78/−24 bp) and transcription start site 2 (PRR2; +222/+335 bp), respectively, and a distal region (DRR; −1745/−1161 bp). The importance of these regulatory regions is reflected in the high degree of homology between human and mouse *HMGA1* nucleotide sequences at the level of these regions (Fig. 1*C*).

Analysis of putative transcription factor binding sites in the proximal regulatory region. Base pair alignment of the human proximal promoter region and the homologous mouse region (Fig. 2*A*) shows a very high sequence identity for PRR1 and PRR2 (also see ref. 34). The *HMGA1* promoter sequence is very GC rich (65–70%) and lacks an obvious TATA box. SP1 is known to play a role in the regulation of genes lacking a TATA box, by binding to GC-boxes to activate transcription (reviewed in ref. 35). Analysis of PRR1-2 by the MatInspector program[3] (36) revealed four putative binding sites for SP1 (Fig. 2*A*). Therefore, we introduced mutations in these SP1-binding sites in pIC(−78/+265) and pIC(−78/+335) and determined the luciferase activity. Mutation of SP1-A caused a decrease in luciferase activity of ∼65% compared with cells transfected with the wild-type (WT) plasmid, whereas mutation of SP1-B, SP1-C, and SP1-D had little or no effect (Fig. 2*B*). These results indicate that the SP1-A-binding site at nucleotide positions −62/−52 bp of the *HMGA1* gene plays an essential role in basal promoter activity.

In addition, we analyzed whether the AP1-binding site located immediately before start site 2, and earlier identified as a TPA-responsive element in human K562 erythroleukemia cells (24), is important for the regulation of the human *HMGA1* promoter in the cell lines used for this study. Therefore, we mutated the AP1 site in pIC(−78/+265) and pIC(−78/+335) and transiently transfected HEK293, HCT116, and SW480 cell lines and investigated the promoter activity. As can be seen on Fig. 2*B*, the AP1 mutation had a considerable effect on promoter activity in all cell lines investigated.

SP1 binds to the *HMGA1* minimal promoter. To investigate whether the SP1 protein is able to bind to the SP1-A-binding site, we did EMSA analysis. Nuclear extracts from HEK293, HCT116, and SW480 cells were incubated with an oligonucleotide probe covering the SP1-A site. DNA-protein complexes were formed with extracts from each of these cell lines, whereas no complexes were formed in the absence of nuclear extract (Fig. 2*C*). The formation of the DNA-protein complexes was abolished or markedly reduced by incubation with an oligonucleotide probe with mutated SP1-A or excess amounts of unlabeled SP1 consensus oligonucleotides (Fig. 2*C*). Moreover, the major DNA-protein complex was detected as a supershifted band by incubation with anti-SP1 antibody. Two lighter bands were supershifted by incubation with anti-SP3 antibody (Fig. 2*C*).

Activation of the human *HMGA1* gene by SP1. To show the biological importance of SP1 in regulating *HMGA1* expression, we cotransfected the pIC(−78/+265) construct and increasing amounts of SP1-expressing construct (pCMV-*SP1*) into HEK293 cells. We found a dose-dependent increase in reporter activity (Fig. 3*A*). In addition, we analyzed the effect of mithramycin A, a drug that is used to block activity of SP1 family members by binding to GC-rich regions (37). Treatment of the different cell lines induced a decrease of *HMGA1* mRNA levels as detected by qRT-PCR (Fig. 3*B*; data not shown). To verify whether the SP1 protein binds to the SP1-binding site *in vivo*, we did ChIP experiments. HEK293 cells were cross-linked followed by immunoprecipitation with SP1 antibody. As shown in Fig. 3*C*, compared with the no-antibody control, a band resulting from amplification of the SP1-binding region in PRR1 is clearly present.

Activation of the human *HMGA1* gene by oncogenic Ha-Ras$^{\mathrm{Leu61}}$. Because the proximal regulatory regions seem to be the most important for basal promoter activity and in an attempt to find a role for the distal regulatory region, we speculated whether the DRR could be functioning as an inducible element in the *HMGA1* promoter. An important pathway that is activated by growth factors and, moreover, is constitutively active in many tumor cells is the Ras pathway. To examine the possible effect of oncogenic Ras on *HMGA1* expression, we stably transfected HEK293 cells with an oncogenic *Ha-Ras^{Leu61}*–expressing construct. Expression of Ha-Ras$^{\mathrm{Leu61}}$ correlated with elevated *HMGA1* mRNA and corresponding protein levels (Fig. 4*A* and *B*). In addition, transient cotransfection of the pIC(−1745/+265) construct and an

[3] http://www.genomatix.de/

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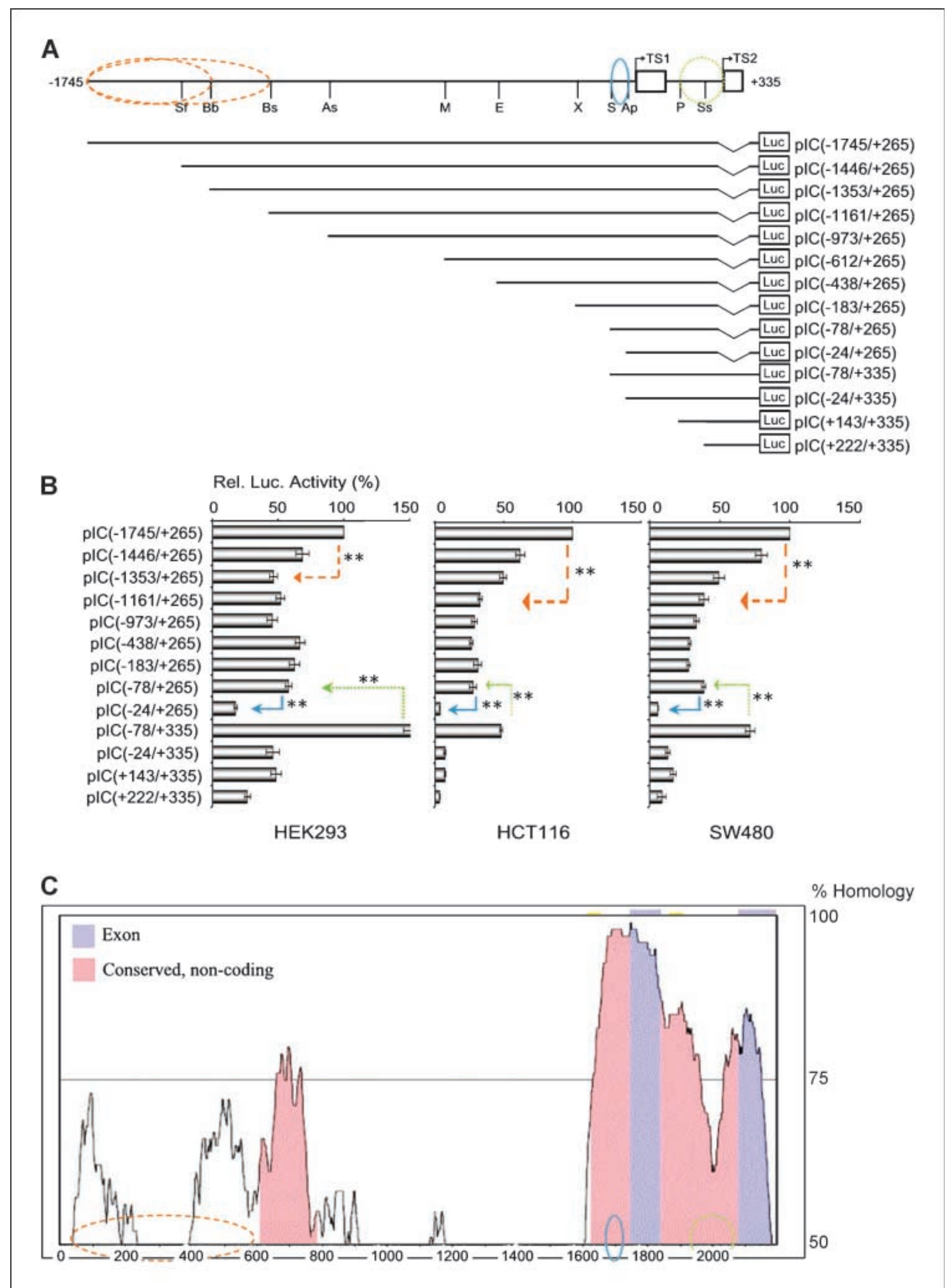

Figure 1. Deletion studies of the human *HMGA1* promoter. *A,* schematic overview of the progressive 5′-deletion mutations of the *HMGA1* promoter. *B,* HEK293, HCT116, and SW480 cells were transiently transfected with *HMGA1* promoter constructs containing the indicated deletions as described in Materials and Methods. The ratio of luciferase (*Luc*)/β-gal activity was determined in each case and normalized to the respective value of the empty pGL3 basic vector. The relative activity is presented as a percentage of the activity of the pIC(−1745/+265) vector, which is set to 100% for each individual experiment. *Columns,* mean of at least three independent experiments done in duplicate; *bars,* SE. *, $P < 0.05$, two-tailed *t* test. *Red,* DRR; *green,* PRR1; *blue,* PRR2. *C,* comparison between human and mouse *HMGA1* promoter sequences, using the VISTA program (http://genome.lbl.gov/vista/index.shtml; ref. 50). *X-axis,* percentage identity; *Y-axis,* position of the region.

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oncogenic *Ha-Ras^{Leu61}*–expressing construct into HEK293 cells showed 15- to 20-fold increase in *HMGA1* promoter activity (Fig. 5*A*).

Identification of enhancer elements required for Ha-Ras^{Leu61}–induced *HMGA1* expression. To define the promoter regions important for induction by Ha-Ras^{Leu61}, deletion studies were undertaken. HEK293 cells were transiently cotransfected with a series of 5′-truncations of the original *HMGA1* promoter fragment pIC(−1745/+265 bp) along with pRSV-*Ha-Ras^{Leu61}*. Removal of the most 5′ located 392 bp decreased basal induction of the *HMGA1* promoter but did not affect Ha-Ras^{Leu61}–mediated induction (Figs. 1*B* and 5*A*). Further shortening down to −973 bp abolished Ha-Ras^{Leu61}–induced promoter activity from ∼16-fold to only 3-fold (Fig. 5*A*). Internal deletion of region −1353/−1161 bp (RRE1) and −1161/−973 bp (RRE2) showed a significant role for both domains in oncogenic Ha-Ras stimulation (Fig. 5*B*). In HCT116 and SW480 cells, which both harbor Ras mutations, we could identify these same domains as important for *HMGA1* promoter activity (Fig. 5*C*).

Further investigation of the two Ras-responsive elements shows that RRE2 contains a putative binding site for the AP1 transcription factor at position −1097 bp (also see ref. 38). Mutation of this site resulted in an impaired induction by oncogenic Ha-Ras^{Leu61}, comparable with the removal of the whole RRE2 (Fig. 5*B*), whereas basal activity was retained. EMSA analysis suggested that both Jun and Fos family members are able to bind to this AP1 site (Fig. 5*D*; also see ref. 38). For RRE1, the MatInspector software program predicted three SP1-like sites (−1301, −1294, and

−1283 bp) in close proximity of each other and an E-box at position −1353 bp. Induction by oncogenic Ha-Ras^{Leu61} was not affected by mutating the E-box (Fig. 5*B*). In contrast, mutation of the three SP1-like sites showed a significant decrease in activation of the *HMGA1* promoter caused by Ha-Ras^{Leu61}, comparable with the removal of the whole RRE1 (Fig. 5*B*). The same results were observed in HCT116 and SW480 cells (Fig. 5*C*). In addition, nuclear extracts taken from HCT116 and SW480 cells formed DNA-protein interactions with the WT AP1 oligonucleotide-bio, whereas no complexes were formed when the mutated AP1 oligonucleotide was used (Fig. 5*D*). These results support the finding that RRE2, and more particular the AP1-binding site at position −1097 bp, is important for *HMGA1* regulation in these cell lines.

Role of oncogenic Ras in *HMGA1* induction in colon cancer cell lines. To see whether there could be a correlation between oncogenic activation of Ras and induction of *HMGA1* gene expression in colon cancer cell lines, we analyzed protein extracts taken from different colon cancer cell lines with WT or mutant Ras. As shown in Fig. 6*A*, HMGA1 was readily detectable in cell lines harboring mutant Ras (indicated by asterisk; ref. 39) but barely observable in cell lines with WT Ras. In addition, we looked at *HMGA1* expression in HCT116 cells and in clone cells derived from the HCT116 cell line, in which activated *Ki-Ras* was disrupted (Hke3; ref. 40). qRT-PCR analysis showed that *HMGA1* mRNA levels were strongly reduced in Hke3 cells compared with the parental HCT116 cells (Fig. 6*B*). Furthermore, Hke3-stable transfectants

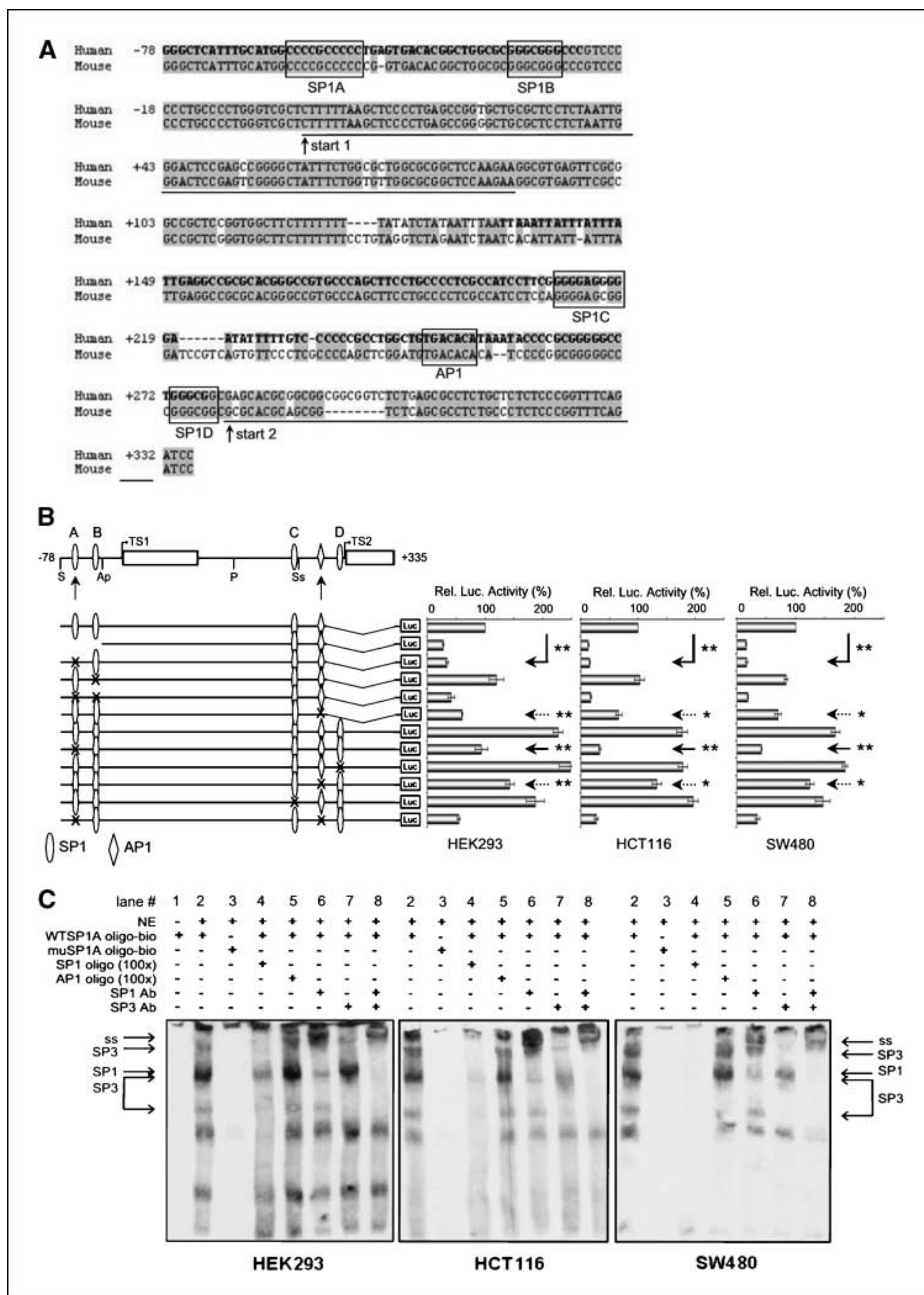

Figure 2. Analysis of putative transcription binding sites in the PRR. *A,* nucleotide sequence and alignment of the 5′-flanking region of the human and mouse *HMGA1* gene, using AlignX, a component of Vector NTI Advance 10 software (Invitrogen). *Underlined,* exons 1 and 2; *bold,* PRR1 and PRR2. The predicted binding sites for SP1 and AP1 are marked on the sequence. *B,* mutation analysis of the *HMGA1* promoter. HEK293, HCT116, and SW480 cells were transiently transfected with *HMGA1* promoter constructs containing the indicated mutations as described in Materials and Methods. The ratio of luciferase/β-gal activity was determined in each case and normalized to the respective value of the empty pGL3 basic vector. The relative activity is presented as a percentage of the activity of the pIC(−78/+265) vector, which is set to 100% for each individual experiment. *Columns,* mean of at least three independent experiments done in duplicate; *bars,* SE. *, $P < 0.05$, two-tailed *t* test. *C,* SP1 binding to the SP1-A binding site. Biotin-labeled *HMGA1* probes containing the WT (*lanes 1* and *2*) or mutant SP1-A-binding site (*lane 3*) were used in EMSA. For competition experiments, unlabeled oligonucleotides (*oligo*) representing a consensus SP1-binding site (*lane 4*) or a consensus AP1-binding site (*lane 5*) were included in the binding reaction. Supershift experiments were done by adding 2 μg SP1 (*lanes 6* and *8*) or Sp3 antibody (*lanes 7* and *8*) to the reaction. Data are representative of two independent experiments.

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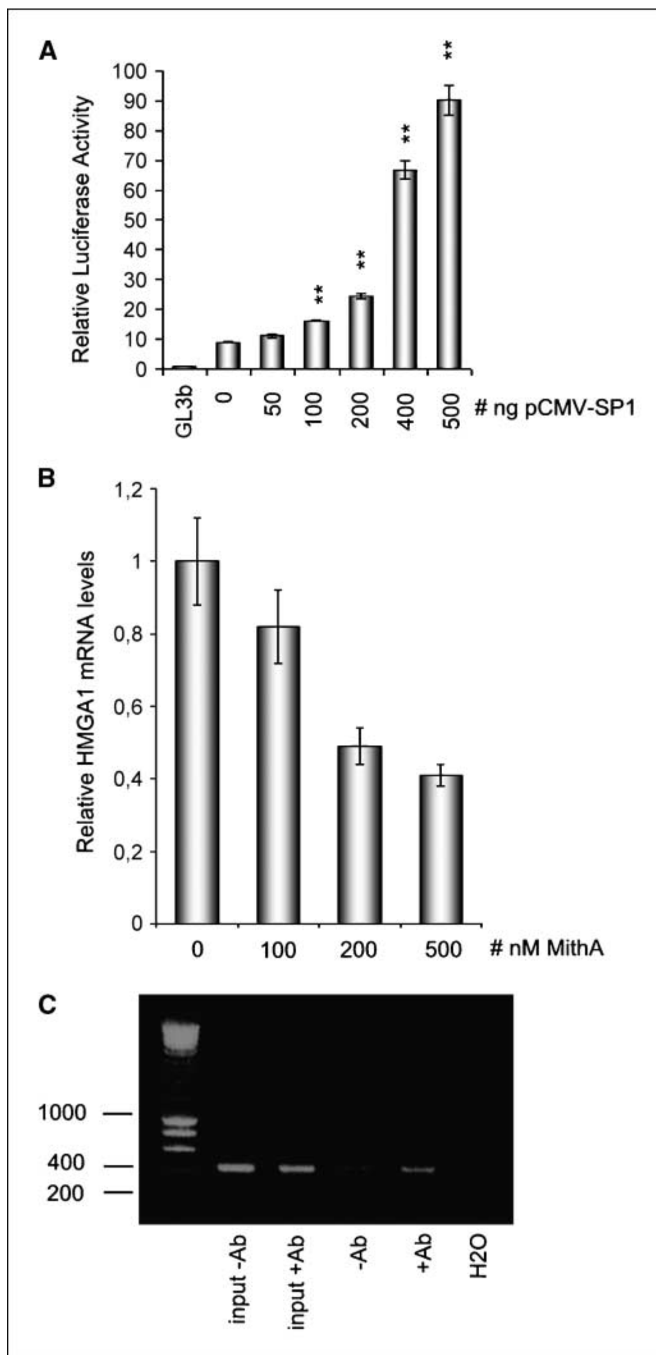

Figure 3. Biological importance of SP1 in regulating *HMGA1* promoter activity. *A,* SP1 up-regulates expression of *HMGA1*. The pIC(−78/+265) vector was cotransfected with the indicated amount of pCMV-*SP1* into HEK293 cells. Empty vectors were added to match total DNA concentration for each transfection. Luciferase activity was determined 48 h after transfection and is expressed as fold induction over the empty pGL3 basic vector. *Columns,* mean of at least three independent experiments done in duplicate; *bars,* SE. *, $P < 0.05$, two-tailed *t* test. *B,* mithramycin A (*MithA*) down-regulated *HMGA1* endogenous expression. HEK293 cells were treated with the indicated concentration of mithramycin A 24 h before RNA extraction. cDNA was prepared from 5 μg total RNA followed by qRT-PCR (40 cycles). Relative *HMGA1* mRNA expression was normalized to the levels of *GAPDH* that served as an internal control. *Columns,* mean of two independent experiments; *bars,* SE. *C, in vivo* association between SP1 and the SP1-binding element in PRR1. Immunoprecipitated DNA (+Ab) was amplified using a primer set specific for the SP1-binding region (see Materials and Methods). DNA prepared from the input chromatin, taken before immunoprecipitation, was used as a positive control for PCR amplification (input −Ab and input +Ab).

expressing the activated Ki-Ras (e3-MKRAS#14; ref. 41) evidently showed an increased expression of *HMGA1* compared with the Hke3 cells (Fig. 6*B*). These findings were confirmed at protein level as seen on Fig. 6*C*. These data suggest an important role for oncogenic Ras in the elevated expression of HMGA1 during tumor development.

Discussion

Several studies have shown that HMGA1 is up-regulated in a wide variety of carcinomas (42), including colon carcinomas (10, 11). Transcriptional deregulation is probably a major mechanism involved in the aberrant expression of HMGA1 in cancers. However, little is known about transcriptional regulation of *HMGA1* in both normal cells and cancer cells. A partial explanation for this comes from the extremely complex structure and transcriptional regulation of both the mouse and human *HMGA1* gene. It comprises four different transcription start sites, of which the first two are considered to be the major ones (24).

The present work represents the first extensive characterization of the human *HMGA1* promoter and evaluation of transcription factors that could be involved in its regulation. The cloning and sequence analysis of a 2-kb region upstream from the major transcription start sites revealed several potential regulatory elements, which are conserved in the mouse *hmga1* promoter (Fig. 1*C*). The high degree of homology between the human and mouse *HMGA1* nucleotide sequences reflects the importance of these regulatory regions. The combined study of progressive deletions of the 2-kb promoter (Fig. 1) and of individual site mutations in the pIC(−78/+265) construct (Fig. 2*B*) pointed to a major role of a SP1-binding element, located at position −57 bp from transcription start 1. Indeed, deletion or mutation of this site led to a loss of ∼65% of the promoter activity. Based on complementary EMSA (Fig. 2*C, lanes 3* and *4*), supershift (Fig. 2*C, lanes 5–7*), expression experiments (Fig. 3*A*), mithramycin A treatment (Fig. 3*B*), and ChIP (Fig. 3*C*), we can argue that SP1

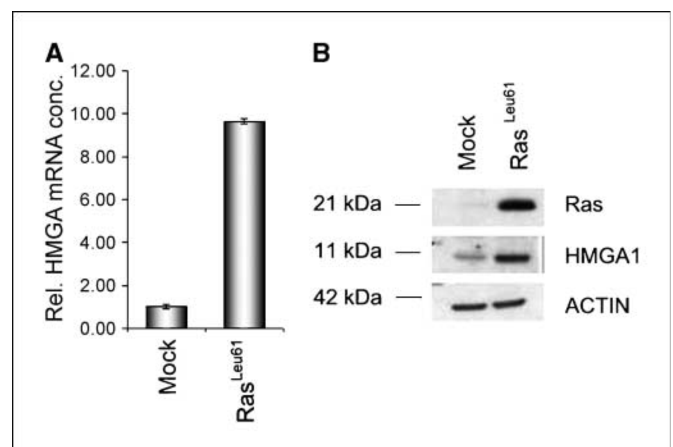

Figure 4. Correlation between oncogenic Ha-Ras and *HMGA1* expression. *A,* stable transfection of HEK293 cells with *Ha-Ras^{Leu61}* correlated with elevated HMGA1 mRNA levels. cDNA prepared from RNA extracted from HEK293-Mock and HEK293-*Ha-Ras^{Leu61}* cells was subjected to qRT-PCR analysis. Relative *HMGA1* mRNA expression was normalized to the level of *GAPDH* that served as an internal control. *B,* stable transfection of HEK293 cells with *Ha-Ras^{Leu61}* correlated with elevated HMGA1 protein levels. Western blotting was done on whole-cell protein extracts of HEK293-Mock and HEK293-*Ha-Ras^{Leu61}* cells. On blotting proteins to nitrocellulose membranes, these were incubated with antibodies specifically detecting Ha-Ras or HMGA1. Equal loading was verified by incubation with the actin antibody.

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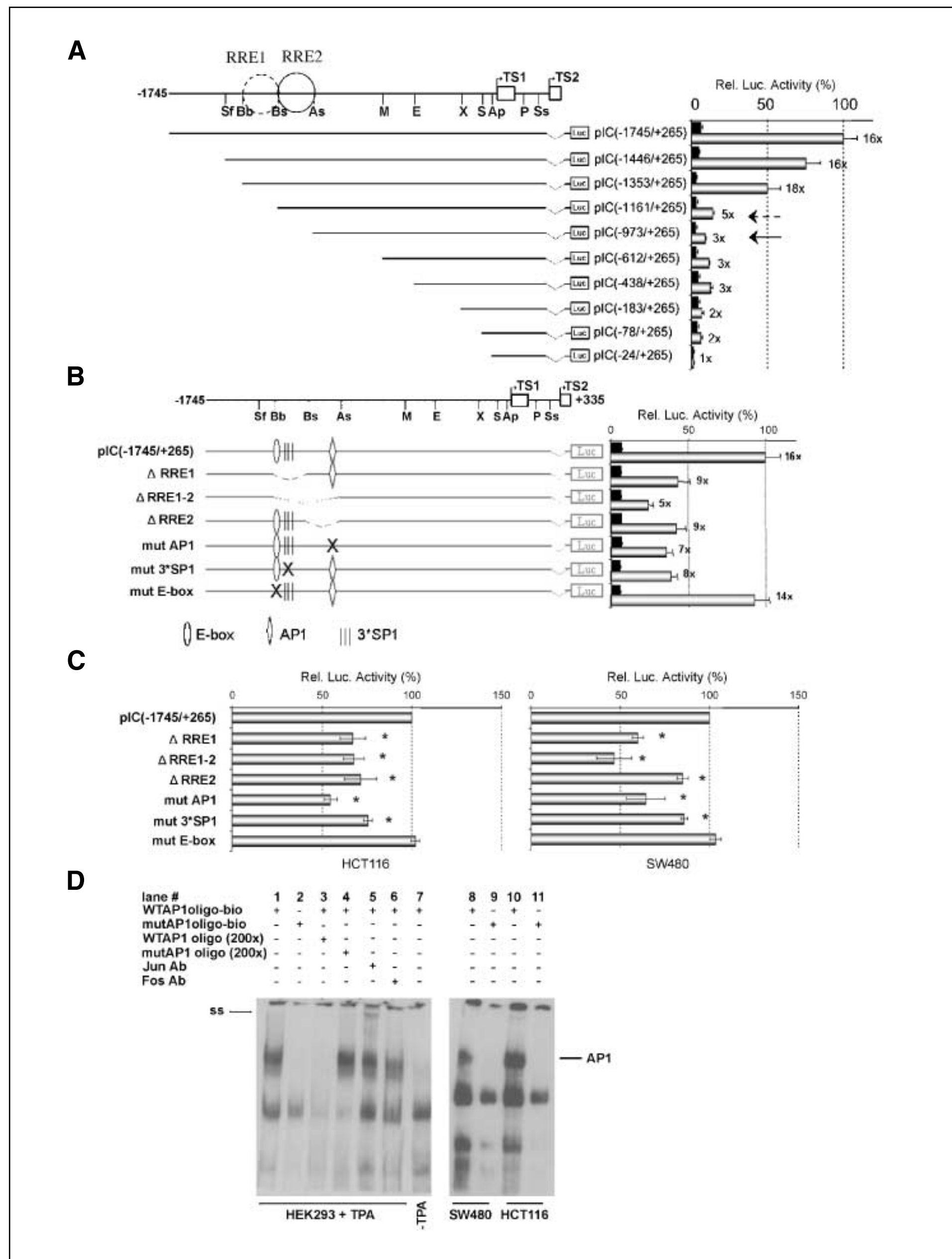

Figure 5. Identification and analysis of enhancer elements required for oncogenic Ha-Ras–induced *HMGA1* gene expression. *A,* HEK293 cells were transiently transfected with *HMGA1* promoter constructs containing the indicated deletions or mutations, along with either a *Ha-Ras*^{Leu61} expression plasmid or empty vector as a control. Promoter activity normalized to β-gal was determined after 48 h. The relative activity is presented as a percentage of the activity of the pIC(−1745/+265) vector, cotransfected with *Ha-Ras*^{Leu61}, which is set to 100% for each individual experiment. The numbers followed by "x" on the side of each column stand for the fold induction caused by Ha-Ras^{Leu61} (luciferase activity when *Ha-Ras*^{Leu61} is cotransfected/luciferase activity when *Ha-Ras*^{Leu61} is not cotransfected). *Columns,* mean of at least three independent experiments done in duplicate; *bars,* SE. *B,* effect of internal deletions and mutations in *HMGA1* promoter sequences on *Ha-Ras*^{Leu61}–induced *HMGA1* promoter activity in HEK293 cells. HEK293 cells were transiently transfected with *HMGA1* promoter constructs containing the indicated deletions or mutations, along with either a *Ha-Ras*^{Leu61} expression plasmid or empty vector as a control. Promoter activity normalized to β-gal was determined after 48 h. The relative activity is presented as a percentage of the activity of the pIC(−1745/+265) vector, cotransfected with Ha-Ras^{Leu61}, which is set to 100% for each individual experiment. The numbers followed by "x" on the side of each column stand for the fold induction caused by Ha-Ras^{Leu61} (see above). *Columns,* mean of at least three independent experiments done in duplicate; *bars,* SE. *C,* effect of internal deletions and mutations in *HMGA1* promoter sequences on promoter activity in HCT116 and SW480 cells. HCT116 and SW480 cells were transiently transfected with *HMGA1* promoter constructs containing the indicated deletions or mutations. Promoter activity normalized to β-gal was determined after 48 h. The relative activity is presented as a percentage of the activity of the pIC(−1745/+265) vector, which is set to 100% for each individual experiment. *Columns,* mean of at least three independent experiments done in duplicate; *bars,* SE. *, $P < 0.05$, two-tailed *t* test. *D,* EMSA analysis of the AP1-binding site in DRR2. Biotin-labeled *HMGA1* probes containing the WT (*lanes 1, 3–8*, and *10*) or mutant AP1-binding site (*lanes 2, 9*, and *11*) were incubated with nuclear extracts taken from TPA-treated HEK293 cells. Cells treated with TPA, from which it is known to induce *HMGA1* expression (24), were chosen because HEK293 cells transiently transfected with *Ha-Ras*^{Leu61} did not seem to meet with the demands needed to do EMSA analysis. Luciferase experiments showed that the AP1-binding site at position −1097 bp was also important for TPA-mediated induction of HMGA1, which agrees with the results obtained for Ha-Ras^{Leu61}. For competition experiments, unlabeled WT (*lane 3*) and mutant (*lane 4*) oligonucleotides were included in the binding reaction. Supershift EMSA was done by adding an antibody that recognizes all Jun family members (*lane 5*) or all Fos family members (*lane 6*) to the reaction. No DNA-protein interactions were formed with extracts from non-TPA–treated cells (*lane 7*). When nuclear extracts taken from SW480 (*lanes 8* and *9*) and HCT116 (*lanes 10* and *11*) cells were taken, DNA-protein interactions were formed with the WT AP1 oligonucleotide but not with the mutated AP1 oligonucleotide. Data are representative of two independent experiments.

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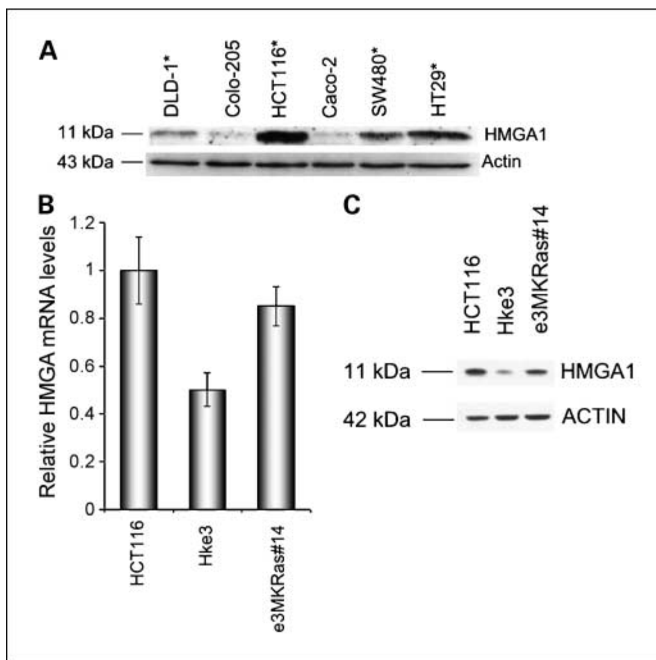

Figure 6. Biological importance of oncogenic Ras. *A,* levels of HMGA1 in different colon cancer cell lines. Whole-cell protein extracts (20 μg) were size fractionated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and probed with anti-HMGA1 as described in Materials and Methods. Cell lines that harbor mutations in Ras are marked with an asterisk. *B,* disruption of activated Ras in colon cancer cells leads to decreased *HMGA1* mRNA levels. cDNA prepared from RNA extracted from HCT116, Hke3, and e3MKRas#14 cells was subjected to qRT-PCR analysis. Relative *HMGA1* mRNA expression was normalized to the level of *GAPDH* that served as an internal control. *C,* disruption of activated Ras in colon cancer cells leads to decreased HMGA1 protein levels. Western blotting was done on whole-cell protein extracts of HCT116, Hke3, and e3MKRAS#14 cells. On blotting proteins to nitrocellulose membranes, these were incubated with HMGA1-antiserum and anti-actin antibody.

proteins are important for basal transcription of *HMGA1*. Another option to show the importance of SP1 in regulating *HMGA1* expression would be an experimental approach using the RNA interference (RNAi) technology. However, HEK293 cells contain relatively low levels of HMGA1; therefore, immunologic detection of the HMGA1 protein is rather difficult even in this embryonic cell line. Ideally, RNAi should be carried out in cells that contain very high levels of HMGA1, such as the colon cancer cell lines used in this article. However, the transfection efficiency in these cell lines is rather low; in addition, the more distally situated inducible promoter element becomes very important whereby the SP1-binding site in PRR1 becomes relatively less important, making it difficult to assess this approach in these colon cancer cell lines. Another possibility is to set an artificial environment by cotransfecting HEK293 cells with a HMGA1-expressing construct and shSP1. This way, HMGA1 expression is clearly detectable and down-regulated by the shSP1 approach (data not shown), which is in support with the data presented in the article. The data obtained in this article are in good agreement with the fact that these zinc finger transcription factors are responsible for recruiting TATA-binding proteins and fixing the transcriptional start site at TATA-less promoters, such as the *HMGA1* promoter (35).

In addition to the SP1-binding element, an AP1-binding site located −31 bp from the second transcriptional start site, and earlier identified as a TPA-responsive element (24), seemed to be of importance for basal transcriptional activity of the human *HMGA1* promoter. The basal activity of the human *HMGA1* promoter therefore seems to depend on the cooperation between SP1 and AP1 transcription factors.

Because of the role that HMGA1 plays in tumor development and progression (42), it is of considerable interest to clarify how HMGA1 expression is regulated in tumor cells. Receptor-mediated Ras activation and Ras activation by oncogenic mutation is common in human tumors and contributes to the development and maintenance of the malignant phenotype. Because *HMGA1* up-regulation is found in the majority of malignant epithelial tumors, we hypothesized that oncogenic activation of the Ras signal transduction pathway could be, at least in part, responsible for the increased *HMGA1* gene expression seen in tumors. We tested this hypothesis by doing some analyses in the HEK293 cell line. HEK293 cells, stably expressing oncogenic Ha-Ras, contained increased *HMGA1* mRNA and corresponding protein levels (Fig. 4*A* and *B*) compared with Mock HEK293 cells. Oncogenic Ha-Ras also induced an increase in *HMGA1* promoter activity in this embryonic cell line (Fig. 5*A*). Through truncational analysis, we were able to locate two distal promoter regions essential for induction of *HMGA1* by oncogenic Ha-Ras (Fig. 5*A* and *B*). These same regions are important in SW480 and HCT116 colon cancer cells (Fig. 5*C*). In RRE2, we pinpointed the AP1 site, located at position −1097 bp, as a key player for inducible promoter activity (Fig. 5*B*). EMSA experiments indicated that both Fos and Jun family members are able to bind to this site (Fig. 5*D*). Furthermore, it was shown by Hommura et al. (38) that Hmga1 is a direct target of AP1 in Rat1a cells via binding of c-Jun to the homologous AP1 site of the mouse *Hmga1* promoter.

RRE1 contains an E-box at position −1353 bp, which is not important for Ha-Ras–induced expression of *HMGA1* in HEK293 cells (Fig. 5*B*). In contrast, this site is shown to be essential for c-*myc*–dependent induction of both the mouse and human *HMGA1* promoter in Burkitt's lymphoma cell lines and HEK293 cells, respectively (43, 44). RRE1 also contains three juxtaposed SP1 sites important for Ha-Ras–dependent induction of *HMGA1*. EMSA analysis showed that neither SP1 nor SP3 was able to bind to these sites (data not shown). Preliminary results suggest that other members of the SP/KLF family might bind to these sites, but this is currently under investigation (to be published elsewhere). Mutational analysis (Fig. 5*C*) and EMSA experiments (Fig. 5*D*) done in HCT116 and SW480 cells showed the same results, suggesting that oncogenic Ras might be the cause of HMGA1 up-regulation seen in these cell lines.

We further focused on colon cancer cell lines and found a close correlation between the presence of oncogenic Ras mutations and increased HMGA1 protein levels in different colon cancer cell lines (Fig. 6*A*). Moreover, disruption of activated Ki-Ras in HCT116 cells led to a down-regulation of endogenous *HMGA1* mRNA and corresponding protein levels (Fig. 6*B* and *C*) compared with the parental HCT116 cells. Reintroduction of activated Ki-Ras in the Hke3 cells (e3MKRas#14 cells) resulted in an up-regulation of *HMGA1* expression, comparable with the levels seen in the parental HCT116 cell line (Fig. 6*B* and *C*). It is known that colon cancer is caused by a defined set of molecular events (45). During the progression of small adenomas to larger, more dysplastic forms, mutation in the Ki-Ras oncogene frequently occurs. It is shown that HMGA1 is expressed in all colorectal carcinoma tissues, whereas there is no expression in normal colorectal mucosa. Interestingly, early-stage adenomas contain only very low levels of HMGA1, whereas HMGA1 expression is found to be especially high in

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adenomas with clear dysplasia (10, 11). Given that this is the stage at which Ras is found to be mutated in colorectal cancers, and together with the data presented in this article, it could be assumed that Ras mutation serves as a gateway for booming expression of HMGA1 in these cancers. Because oncogenic Ras induces the constitutive activation of the Raf-MEK-ERK pathway (46), we postulated that this pathway might play a role in our system. Preliminary results show that inhibition of the Ras-Raf-MEK-ERK pathway leads to a down-regulation of endogenous *HMGA1* mRNA and corresponding protein levels (data not shown). The significance of these results and possible involvement of other MAPK pathways is currently under investigation. Taken together, these data suggest a role for oncogenic Ras in HMGA1 up-regulation in different cell lines. Interestingly, a vice versa mechanism is published, in which Ras/ERK signaling is regulated by HMGA1 proteins. Treff et al. (22) showed that HMGA1a proteins significantly increase the sensitivity to the activation of the Ras/ERK signaling pathway in MCF-7 cells overexpressing HMGA1a. They believe that this might be one of the key ways by which overexpression of HMGA1 proteins promotes tumor progression and increased metastatic potential in human MCF-7 mammary breast cancer cells.

In conclusion, we believe that the definition of the promoter region able to confer basal activity to the *HMGA1* gene and the identification of an enhancer region, reported in this article, represents an important step in understanding the molecular events that regulate the expression of the *HMGA1* gene during the processes of cell transformation and development. Indeed, one can speculate that the elevated expression of *HMGA1* in embryos and cancers is, in part, the result of a complex interplay between Ras, SP family members, and AP1 factors. SP1, which we identified as essential for basal expression of *HMGA1*, is a well-characterized, sequence-specific, DNA-binding protein that is important in the transcription of many genes that contain GC-boxes in their promoters (47). Its levels and function may change with differentiation, transformation, and cell growth, suggesting that these changes have important biological consequences (48). In agreement with this is the recent observation that SP1 sites have been found to mediate transcription in response to diverse stimuli, including growth factors, cytokines, and oncogenes, such as Ras (49).

Acknowledgments

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