HMGA2 Regulates Transcription of the Imp2 Gene via an Intronic Regulatory Element in Cooperation with Nuclear Factor-κB

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Introduction

The Imp2 (insulin-like growth factor-II mRNA binding protein 2) gene encodes a member of the VICKZ (Vg1 RBP/Vera, IMP1, IMP2, IMP3, CRD-BP, KOC, and ZBP-1) family of highly related mRNA-binding proteins that are implicated in posttranscriptional processes such as mRNA localization, turnover, and translational control. In this way, they are involved in the temporal and spatial control of gene expression at the level of mRNA rather than at the level of gene transcription. The three mammalian VICKZ proteins (IMP1/CRD-BP, IMP2, and IMP3/KOC) are primarily expressed during embryonic development, and their expression is generally not detected in adult tissues, with the exception of placenta and testes (1–4). In contrast, they are overexpressed in various cancers (reviewed in refs. 5, 6), classifying them as oncofetal proteins. Concerning IMP2, Lu et al. (7) detected overexpression of a splice variant of IMP2 (p62) in liver cancer (hepatocellular carcinoma and cholangiocarcinoma), and Zhang et al. (8) found autoantibodies in sera of more than 10% of patients with esophageal, hepatocellular, lung, lymphoma, pharyngeal, or uterine cancer. Testing for the presence of such autoantibodies against a panel of autoantigens, including IMP proteins, has been proposed as a valuable tool for cancer detection and diagnosis (9). Until now, the mechanisms by which the oncofetal VICKZ proteins are overexpressed in certain cancers remain elusive. IMP1, IMP2, and IMP3 are each encoded by a separate gene, and previous work from our group clearly indicates that the mRNA level of Imp2, but not of Imp1 and Imp3, is developmentally regulated by the architectural transcription factor HMGA2 (high mobility group A2; ref. 10). Like IMP2, HMGA2 is an oncofetal protein: it is expressed at high levels during embryonic development (11, 12), whereas it is hardly detectable in adult tissues (13). In many tumor types, the HMGA2 gene is disrupted or aberrantly rearranged in human neoplasias (reviewed in refs. 14, 15). The HMGA2 protein consists of three short basic domains (AT-hooks), enabling its binding to the minor groove of AT-rich DNA, followed by an acidic COOH-terminal tail (16). Disruption of the gene in tumors, as a result of specific chromosomal rearrangements, results in the expression of a truncated protein (HMGA2Tr) only containing the DNA-binding AT-hooks (17, 18). Previous work from our group identified Imp2 as a target gene of both wild-type and tumor-specific truncated HMGA2 proteins, suggesting an HMGA2-dependent Imp2 expression

Abstract

IMP2 (insulin-like growth factor-II mRNA binding protein 2) is an oncofetal protein that is aberrantly expressed in several types of cancer. We recently identified the Imp2 gene as a target gene of the architectural transcription factor HMGA2 (high mobility group A2) and its tumor-specific truncated form HMGA2Tr. In this study, we investigated the mechanism via which HMGA2 regulates Imp2 gene expression. We show that HMGA2 and HMGA2Tr directly regulate transcription of the Imp2 gene by binding to an AT-rich regulatory region located in the first intron. In reporter experiments, we show that this AT-rich regulatory region mimics the response of the endogenous Imp2 gene to HMGA2 and HMGA2Tr. Furthermore, we show that a consensus nuclear factor-κB (NF-κB) binding site located immediately adjacent to the AT-rich regulatory region binds NF-κB and that NF-κB and HMGA2 cooperate to regulate Imp2 gene expression. Finally, we provide evidence that there is a strong and statistically significant correlation between HMGA2 and Imp2 gene expression in human liposarcomas.

regulation that plays an important role during embryonic development and in tumorigenesis (10). In this study, we investigated the mechanism via which HMGA2 regulates Imp2 gene expression. We show that HMGA2 and HMGA2Tr directly regulate transcription of the Imp2 gene via an AT-rich regulatory region located in the first intron. We show that HMGA2 binds to this region both in vitro and in vivo. In reporter experiments, we show that the AT-rich regulatory region mimics the response of endogenous Imp2 to HMGA2 and HMGA2Tr. We also identified a consensus nuclear factor-κB (NF-κB) binding site located immediately adjacent to the AT-rich regulatory region that binds NF-κB and show that NF-κB and HMGA2 cooperate to regulate Imp2 gene expression. Finally, we investigated whether there is a relationship between HMGA2 and IMP2 expression in a collection of human well-differentiated and myxoid liposarcomas and found a positive correlation. Collectively, our data show that Imp2 gene transcription is directly regulated by HMGA2 in cooperation with NF-κB.

**Results**

**The Mouse Imp2 Gene Contains Two Putative Gene-Regulatory Regions**

We determined the mouse Imp2 genomic organization and searched for possible gene-regulatory regions. The genomic organization of the Imp2 gene was obtained by performing a BLAT search against the University of California at Santa Cruz Genome Browser database using the Imp2 cDNA sequence (19). As shown in Fig. 1A, the Imp2 gene contains 16 exons spanning a region of about 100 kb. The transcription start site (TSS) region was identified by searching the Database of Transcriptional Start Sites (DBTSS) using RefSeq sequence NM_183029 as query. This search revealed a region containing 58 potential TSSs corresponding to 58 oligo-capped cDNA clones. The most upstream TSS, corresponding to the clone with accession no. BY310082, was located 113 bp 5-prime to the translational start site of Imp2. 5′-RNA ligase-mediated rapid amplification of cDNA ends (5′-RLM-RACE) analysis, done on RNA isolated from NIH/3T3 cells, confirmed that the TSS of the Imp2 gene was indeed located in this region, the most 5′ TSS obtained being 29 bp downstream of the most 5′ TSS in the DBTSS. In what follows, position 113 bp upstream of the ATG translation initiation site was chosen as +1 (Fig. 1).

To have a first indication of regions that are important in the regulation of the mouse Imp2 gene, we analyzed part of the Imp2 genomic sequence using different *in silico* promoter prediction programs. The genomic sequence that was analyzed spanned 7 kb and included 5 kb upstream of the assumed transcription initiation site, the first and the second coding exons, and the intron in between (intron 1; Fig. 1B). Online promoter prediction programs used included PromoterInspector and Gene2Promoter from GenomatixSuite, grailEXP (Grail Experimental Gene Discovery Suite), and PROSCAN. Combination of the obtained predictions resulted in the identification of two putative gene-regulatory regions for the mouse Imp2 gene (Fig. 1B, light gray bars). The first predicted regulatory region (A′, 604 bp) encompasses both the transcription initiation site and the ATG translation initiation codon, whereas the second region (B′, 276 bp) is located in the first intron. The first region has 64.6% identity, whereas the second region has 73.2% identity, to the homologous regions of the *hIMP2* genomic sequence, pointing towards evolutionary conserved noncoding regions (Fig. 2A).

**The Intronic Gene-Regulatory Region Mimics the Response of Endogenous Imp2 to HMGA2 and HMGA2Tr**

Previous work from our group identified Imp2 as a target gene of both wild-type and tumor-specific truncated HMGA2 proteins (10). HMGA2 and HMGA2Tr contain three DNA-binding domains, called AT-hooks, enabling their binding to AT-rich DNA. Interestingly, the *in silico* predicted regulatory region B′ was preceded by three AT-rich stretches, the latter being possible HMGA2-binding sites (Fig. 2A). We cloned regions A and B covering the *in silico* predicted regions A′ and B′, respectively, along with flanking sequences (Fig. 1B) in front of the firefly luciferase reporter gene. To measure the influence of HMGA2 and HMGA2Tr proteins on reporter gene expression, we transfected equal amounts of retrovirally transduced NIH/3T3 fibroblasts stably expressing wild-type HMGA2, HMGA2Tr proteins, or Mock cells (10) with reporter plasmid A or B. Twenty-four hours after transfection, luciferase and β-galactosidase activities were measured. Figure 2B shows normalized luciferase values plotted relative to their values in Mock cells. Both HMGA2 and HMGA2Tr proteins had a slight repressing effect on reporter plasmid A. More important, however, HMGA2 overexpression enhanced luciferase expression from reporter plasmid B almost 2-fold, whereas HMGA2Tr strongly repressed the activity of the same reporter. This latter result reflects the endogenous Imp2 mRNA expression changes induced by HMGA2 and HMGA2Tr, as seen previously in Northern blot analyses (10). Furthermore, measuring the effect of HMGA2 and HMGA2Tr on reporter gene expression via combination of region A and B, where A is functioning as a promoter and B as an enhancer, gave a similar result. Therefore, we can conclude that region B is the major regulatory region via which the HMGA2 proteins exert their effects on Imp2 gene expression. This region encompasses 419-bp intronic sequence of the mouse Imp2 gene (Fig. 1B) and contains three AT-rich repeats (Fig. 2A). Deletion of the AT-rich repeats completely abolished the effect of the HMGA2 proteins on luciferase reporter gene expression (Fig. 2B), suggesting that this conserved AT-rich stretch represents HMGA2-binding sites.

**HMGA2 and HMGA2Tr Bind in vitro to the AT-Rich Fragment of the Mouse Imp2 Intronic Gene-Regulatory Region**

To investigate whether HMGA2 proteins are directly involved in Imp2 transcriptional regulation, we evaluated the HMGA2 DNA-binding activity to the Imp2 promoter. In particular, we analyzed an 84-bp region spanning nucleotides 935 to 1018 in intron 1 of the murine Imp2 gene (boxed sequence in Fig. 2A), containing three AT-rich putative
HMGA2-binding sites (TATTTTT, AATTTTTT, and ATTATT). As shown in Fig. 3A, a biotin-labeled double-strand Imp2 oligonucleotide probe covering the three AT-stretches formed DNA-protein complexes with nuclear extracts of the NIH/3T3 cell lines, stably expressing wild-type HMGA2 or HMGA2Tr proteins (lanes 2). No retarded protein-DNA complexes were formed when a mutant biotin-labeled probe was used (lanes 4). Specificity of these HMGA2-DNA and HMGA2Tr-DNA complexes was confirmed by supershifts of the bands upon incubation with HMGA2-specific antiserum (lanes 5). Both HMGA2 full length and HMGA2Tr proteins bound to the Imp2 oligonucleotide probe.

In what follows, we limited our studies to full-length HMGA2 because HMGA2 and HMGA2Tr, both having identical AT-hooks, bind the same DNA structures (20, 21). Direct binding of the HMGA2 protein was confirmed by using recombinant HMGA2-His full-length protein (Fig. 3B, lanes 2-4). The formation of this DNA-protein complex was markedly reduced by incubation with 200× molar excess of unlabeled Imp2 oligonucleotide (lane 5) and supershifted when incubating with HMGA2-specific antiserum (lane 7). In contrast 200× excess of mutant Imp2 probe (mutImp2), in which the AT-rich sequences are mutated, did not affect retardation of the HMGA2-DNA complex (lane 6). Furthermore, no complex was formed when incubating the purified protein with the mutant biotin probe (Fig. 3B, lane 9).

To verify the binding of Hmg2 to the Imp2 promoter in primary cells, we assayed the DNA-binding activity of nuclear extracts prepared from mouse embryonic fibroblasts derived from E12.5 wild-type, heterozygous, or knockout Hmg2 mouse embryos. As shown in Fig. 3C, specific DNA-protein complexes were present in extracts from both wild-type and heterozygous mouse embryonic fibroblasts (lanes 1 and 4), whereas they were absent in extracts derived from homozygous Hmg2 null mutant fibroblasts (lane 7). These complexes were specifically displaced by the incubation with antisera directed against the HMGA2 protein (lanes 2 and 5). No complex was formed when the nuclear extracts were incubated with the mutImp2 oligo (lanes 3 and 6). Together, these results show that the intronic AT-rich regulatory region of the Imp2 gene binds HMGA2 proteins in vitro.

Hmg2 Binds In vivo to the Intronic Regulatory Region of the Mouse Imp2 Gene

To verify whether the HMGA2 protein binds to the intronic regulatory region in vivo, we did chromatin immunoprecipitation experiments. Therefore, the mouse NIH/3T3 fibroblasts that stably express the wild-type HMGA2 protein were cross-linked (see Materials and Methods) followed by immunoprecipitation of the cross-linked protein-DNA complexes with HMGA2-specific antiserum. As a negative control, we included a no-antibody reaction. The precipitated double-stranded DNA was amplified by PCR using the primer set depicted in Fig. 2B. As shown in Fig. 4, compared with the no-antibody control, a band resulting from amplification of the Imp2 region was clearly present. To rule out the possibility of nonspecific precipitation by the HMGA2 antibody, amplification of region −1606 bp relative to the TSS (Fig. 4B) was assayed as negative control. We did not observe any association of HMGA2 with this upstream region (Fig. 4A) establishing a specific, in vivo binding of the HMGA2 protein to the AT-rich intronic region of the Imp2 gene, indisputably confirming our in vitro studies.

NF-κB Binds to a Consensus Binding Site Immediately Adjacent to the AT-Rich Regulatory Region

Detailed analysis of region B revealed a consensus binding site for NF-κB immediately adjacent to the AT-rich regulatory region (Fig. 2A). To investigate whether NF-κB is able to bind to this site, we did an electrophoretic mobility shift assay using...
nuclear extracts from NIH/3T3 cells transiently cotransfected with equal amounts of p50 and p65 expressing constructs (NIH/3T3+). As shown in Fig. 5 (lane 2), incubation of these extracts with the 85-bp biotin-labeled double-stranded DNA probe (Fig. 2A) results in the formation of a protein-DNA complex, which is not present when nuclear extract from nontransfected NIH/3T3 cells is used (lane 1). Specificity of these protein-DNA complexes was confirmed by the detection of a supershifted band when the mix was incubated with anti-p50 or anti-p65 antibody (lanes 3 and 4). Interestingly, the intensity of the complex diminishes when the mix was incubated with HMGA2-specific antiserum (compare lane 2 and lane 5) and when a mutant biotin-labeled oligo was used (mutImp2-bio, mutated AT-hooks; compare lane 2 and lane 6). Together, these results indicate that NF-κB binds to the NF-κB consensus binding site adjacent to the AT-rich regulatory region, and that HMGA2 enhances this binding.

FIGURE 2. Identification of the Hmga2-regulated region. A. Nucleotide sequence and alignment of part of the intronic sequence containing reporter plasmid B (bold) of the human and mouse IMP2 gene. The 84-bp probe used in electrophoretic mobility shift assay experiments is boxed. Upper and lower primers used to amplify a 180-bp fragment of this genomic region in chromatin immunoprecipitation analysis (dashed arrows). AT-rich regions and the NF-κB binding site (underlined). B. HMGA2 and HMGA2Tr have opposite effects on the second regulatory region of the mouse Imp2 gene. NIH/3T3 cells retrovirally transduced with the empty retroviral vector (Mock), HMGA2, or HMGA2Tr were seeded in 24-well plates. Twenty-four hours after seeding, cells were cotransfected with 400 ng reporter construct or empty pGL3basic plasmid and 100 ng pEL1 h-galactosidase – expressing plasmid to normalize for transfection efficiency. Cells were harvested 24 h after transfection and assayed for luciferase and h-galactosidase activity. The ratio of luciferase/h-galactosidase activity was determined in each case and normalized to the respective luciferase value of the empty pGL3basic vector. Normalized luciferase values were calculated relative to their respective luciferase values in control (Mock) cells. Columns, mean of at least three independent experiments done in duplicate; bars, SE.

FIGURE 3. Hmga2 binding to the AT-rich stretch contained in reporter B. A. Biotin-labeled wild-type (WTimp2-bio, lanes 2) or mutant (mutimp2-bio, lanes 4) Imp2 promoter oligonucleotides were used in electrophoretic mobility shift assay. Binding assays were done using 5 μg nuclear extract of Mock, HMGA2, or HMGA2Tr NIH/3T3 cells with or without HMGA2-specific antiserum (lanes 5). Representative data of two independent experiments. B. Electrophoretic mobility shift assay was done by incubating biotin-labeled wild-type (lanes 1-7) or mutant (lanes 8-9) Imp2 promoter oligonucleotide with 5 ng (lane 2), 20 ng (lanes 3, 5-7, and 9), or 50 ng (lane 4) of the recombinant full-length HMGA2-HIS protein. Where indicated, a 200-fold molar excess of unlabeled wild-type (lane 5) or mutant (lane 6) oligonucleotides was added, or HMGA2-specific antiserum (lane 7). Representative data of two independent experiments. C. Electrophoretic mobility shift assays were done by incubating 5 μg nuclear extracts from mouse embryonic fibroblast derived from wild-type (WT), Hmga2+/- and Hmga2-/- mice with biotin-labeled wild-type (lanes 1, 4, and 7) or mutant (lanes 3, 6, and 9) probes. Where indicated, the samples were preincubated with HMGA2-specific antiserum. Representative data of two independent experiments.
HMGA2 Enhances NF-κB–Mediated Transcriptional Activation of the Intronic Gene-Regulatory Region

To verify whether HMGA2 is able to enhance transcriptional activation by NF-κB, we did luciferase experiments in NIH/3T3 cells. First, we looked at the transactivation capacity of NF-κB. Cotransfection of a fixed amount of reporter B construct with increasing amounts of both p50- and p65-expressing constructs resulted in a dose-dependent increase of luciferase activity (data not shown). When we cotransfected the reporter B construct with a fixed amount of both p50- and p65-expressing constructs and an HMGA2-expressing construct, a further increase of luciferase activity was seen (Fig. 6A). Deletion of the AT-rich repeats clearly inhibited the enhancing effect of HMGA2 (Fig. 6B). When the NF-κB site was mutated, no induction was seen when p50/p65 was cotransfected. However, HMGA2 cotransfection still resulted in a 2-fold increase in luciferase activity (Fig. 6C). These results indicate that HMGA2 is able to enhance the activity of NF-κB by binding to the AT-rich repeats immediately adjacent to the NF-κB consensus site.

A Positive Correlation between HMGA2 and IMP2 Gene Expression in Liposarcomas

Having shown that Imp2 gene expression is directly regulated by HMGA2 in cultured cells (this work) and during embryonic development (10), we next investigated whether there is a relationship between HMGA2 and IMP2 gene expression in tumors. Therefore, we compared expression levels of HMGA2 and IMP2 in human well-differentiated and myxoid liposarcomas. The most important cytogenetic feature of well-differentiated liposarcoma is the presence of supernumerary ring and giant marker chromosomes (22). These aberrant chromosomal rings and giant markers often contain an amplification of the 12q13-q15 region, which harbors the HMGA2 gene. Several studies have shown immunopositivity of

FIGURE 4. In vivo association between HMGA2 and the intronic Imp2 gene-regulatory region. The binding of HMGA2 to region B of the mouse Imp2 gene was evaluated with a chromatin immunoprecipitation (ChIP) assay in NIH/3T3 cells stably expressing the wild-type HMGA2 protein. A, Immunoprecipitated DNA was amplified by PCR using a primer set specific for the Imp2 region (+891 to +1070 bp: right). As a negative control, immunoprecipitated DNA was amplified by a primer set specific to an off-target region of the Imp2 gene (−1789 to −1606 bp: left). DNA prepared from the input chromatin, taken before immunoprecipitation, was used as a positive control for PCR amplification. B, Schematic representation of the position of the regions amplified by PCR after chromatin immunoprecipitation. All sequences are drawn to scale, and nucleotide positions indicated are relative to the most 5′ position of mouse expressed sequence tag with Genbank accession no. BY310082 (+1). Introns and genomic sequences (lines). Coding sequences (dark gray boxes); 5′ and 3′ untranslated regions (open boxes). Amplified PCR regions (light gray boxes; see A).

FIGURE 5. NF-κB binds to the NF-κB binding site immediately adjacent to the AT-rich regulatory region. Electrophoretic mobility shift assay was done by incubating 5 μg nuclear extract of nontransfected (lane 1) or p50/p65–transfected (NIH/3T3+, lanes 2-6) NIH/3T3 cells with biotin-labeled wild-type (lanes 1-5) or mutant (lane 6) Imp2 promoter oligonucleotides. Where indicated, the samples were preincubated with antibody directed against p50 (lane 3) or p65 (lane 4) or with HMGA2-specific antiserum (lane 5). Representative data of two independent experiments.
HMGA2 in these tumors and/or amplification of the HMGA2 gene by Southern blotting or fluorescence in situ hybridization (23-26). Furthermore, Pentimalli et al. (27) have shown that HMGA2 protein suppression results in growth inhibition of HMGA2-overexpressing primary cell cultures of well-differentiated liposarcoma, indicating that HMGA2 plays an important role in the growth of these tumors. The cytogenetic hallmark of myxoid liposarcoma is the presence of t(12;16)(q13;p11) that leads to the formation of the FUS-DDIT3 fusion protein (28-31). In contrast to well-differentiated liposarcoma, the HMGA2 gene is not amplified in myxoid liposarcoma.

We analyzed the expression levels of HMGA2 and IMP2 by quantitative real-time reverse transcription-PCR in 11 samples of human well-differentiated liposarcoma and 3 specimens of myxoid liposarcoma, which were obtained from 14 different patients. The karyotypes of the tumors are depicted in Table 1. As can be seen in Table 1, the 11 well-differentiated liposarcoma (S4-S14) were characterized by supernumerary ring chromosomes and/or giant marker chromosomes, whereas the 3 myxoid liposarcoma (S1*-S3*) showed the presence of t(12;16)(q13;p11) translocation, specific for this entity. The obtained DCt values for HMGA2 and IMP2, which are inversely related to their expression level, are plotted in Fig. 7. A strong and statistically significant relationship was observed between HMGA2 and IMP2 gene expression (r = 0.8066, P = 0.000244, Spearman rank test). From Fig. 7, it is also clear that the three myxoid liposarcoma samples that did not have ring chromosomes and/or giant marker chromosomes (S1*-S3*) and thus were not expected to have a high expression level of HMGA2 indeed had low expression levels (high DCt) of HMGA2 and also of IMP2.

Discussion

Here, we present the first data on the direct transcriptional regulation of the Imp2 gene. A first major finding is the identification of an AT-rich regulatory region in the first intron of the Imp2 gene. In reporter experiments, this AT-rich region mimics the response of the endogenous Imp2 gene to the architectural transcription factor HMGA2 and its truncated tumor-specific counterpart HMGA2Tr, as shown by Brants et al. (10). In addition, we show that the HMGA2 protein binds directly to the AT-rich region and is also associated with this site in the complex.

![Figure 6](image_url)

**FIGURE 6.** Ability of HMGA2 to enhance transcriptional activation by NF-κB. NIH/3T3 cells, seeded in 24-well plates, were transiently cotransfected with 400 ng reporter B (A), Bdel (B), or BmutNF-κB (C) with or without an HMGA2-expressing construct and p50/p65 expression vectors as indicated. pEL1 β-galactosidase-expressing plasmid (100 ng) was added to normalize for transfection efficiency. Cells were harvested 48 h after transfection and assayed for luciferase and β-galactosidase activity. The ratio of luciferase/β-galactosidase activity was determined in each case and normalized to the respective luciferase value of the empty pGL3basic vector. Normalized luciferase values were calculated relative to their respective luciferase values in control (no HMGA2 or p50/p65 cotransfected) cells. Columns, mean of at least three independent experiments; bars, SE.

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Abbreviations: F, female; M, male.
region in vivo. HMGA2 is a member of the HMGA family of proteins that comprises four known members: HMGA1a, HMGA1b, and HMGA1c, which are encoded by alternative splice products of the HMGA1 gene, and HMGA2, which is encoded by a different gene (32). HMGA proteins do not have intrinsic transcriptional activation capacity but instead play a role in the formation and stabilization of higher-order nucleoprotein complexes (enhanceosomes) in promoter/enhancer regions of their target genes, as such acting as “architectural” transcription factors. The best characterized example of how HMGA proteins modulate gene expression is the transcriptional regulation of the IFN-β gene, where HMGA1 orchestrates the assembly of different transcription factors into an enhanceosome (33). In contrast to HMGA1, the role of HMGA2 as an architectural transcription factor is less well studied. To our knowledge, to date, besides Imp2, only five other genes have been identified as target genes of HMGA2, being the CDC2, TK1, and cyclin E genes (34); the DNA repair gene ERCCI (21); and the cyclin A gene, which is a key factor in cell cycle control (35). In the two latter cases, it has been shown that HMGA2 binds to AT-rich regions in the promoter of these target genes. Concerning the Imp2 gene, we show here that the intronic AT-rich regulatory region is mandatory for the regulation of the gene. This AT-rich regulatory region is located in the first intron of the gene, about 1 kb downstream of the promoter, suggesting that this region has a function as a cis-acting transcriptional enhancer, and that HMGA2, like HMGA1, also regulates transcription from an enhancer (36, 37). However, by screening the DBTSS, a mouse Imp2 expressed sequence tag (accession no. BB643209) was found that has its TSS at –27 bp relative to the beginning of exon 2. By performing a BLAST (Basic Local Alignment Search Tool) search, two additional mouse expressed sequence tags (accession nos. CJ171095 and CJ169368) were identified both containing 144 bp of intron 1 sequence located immediately upstream of exon 2. These three mouse expressed sequence tags suggest the presence of a second TSS, which is located downstream of the AT-rich regulatory region. This implies that the AT-rich regulatory region could be part of an alternative promoter directing the transcription of a shorter alternative Imp2 transcript lacking exon 1. Additional evidence for such an alternative transcript was found in human: two human IMP2 expressed sequence tags were identified (accession nos. BX491079 and CN398355), which have a similar composition as the three mouse expressed sequence tags described above. Both in human and mouse, an alternative in frame ATG start codon is present in exon 2, which is preceded by an in-frame stop codon. This suggests that the protein that is translated from the shorter alternative transcript lacks the first 67 amino acids, which correspond to the RNA recognition motif-1 domain, one of the six RNA-binding domains in the Imp2 protein (4). At present, to the best of our knowledge, the function of this domain in the Imp2 protein is not known. Further experiments are needed to investigate whether the intronic AT-rich regulatory region regulates expression of the ordinary longer transcript as an enhancer, or of the alternative shorter transcript as a promoter, or both. As shown in Fig. 2, the AT-rich regulatory region is functioning both as promoter element (reporter B) and as enhancer element (reporter A + B) in luciferase reporter assays, thereby each time mimicking the response of the endogenous Imp2 gene to HMGA2 and HMGA2Tr.

A second major finding is that transcription of the Imp2 gene is regulated by NF-κB, and that this regulation takes place in cooperation with HMGA2. The fact that NF-κB and HMGA proteins work together in regulating transcription is not novel: it was first discovered by Thanos and Maniatis (38) who showed that HMGA1 is required for NF-κB–dependent virus induction of the human IFN-β gene. Later, Mantovani et al. (39) found that NF-κB–mediated transcriptional activation is also enhanced by HMGA2, and in a follow-up study by Noro et al. (20), a physical interaction between HMGA2 and the p50/p65 subunits of NF-κB was shown. The sequence-specific transcription factor NF-κB portrays various dimeric complexes of members of the Rel protein family. Of the various dimeric combinations, the p50/p65 dimer, which was used in this study, is most common (40).

The third and last major finding is a relationship between HMGA2 and IMP2 gene expression in well-differentiated versus myxoid liposarcomas. Well-differentiated liposarcomas seemed to be especially suited to study the question about a possible relationship between HMGA2 and IMP2 expression in tumors because they displayed different levels of HMGA2 expression as can be seen in Fig. 7. We found a strong positive, statistically significant correlation between HMGA2 and IMP2 expression levels in these tumors. Together with the other results presented in this study, this suggests that IMP2 overexpression in tumors is accomplished via HMGA2 in association with NF-κB.
Materials and Methods

Cloning of mHmp2 Regulatory Regions

BAC clone RP23-50H13 covering the mouse genomic region containing the Imp2 gene was selected by using the University of California at Santa Cruz Genome Browser (19) and purchased from Invitrogen/Life Technologies (Carlsbad, CA). Promoter regions A (901 bp) and B (419 bp; Fig. 1B) were amplified from this BAC clone using Invitrogen TaqPCRx DNA Polymerase in combination with PCRx Enhancer Solution according to the manufacturer’s protocol for GC-rich templates. Primer sets used were 5’-CTTGTGCACTCCTCGGCACATC-3’/5’-GGGCTAGGGGACCTTAAGGAGAAGTGCCTGTC-3’ and 5’-CCCTCGGCTTCTTTCCCCGAAGCTCTGTGCGC-3’, respectively. The obtained fragments were purified and TA-cloned in the pGEMTeasy vector (Promega, Madison, WI). A DNA gel containing pGL3Basic vector. 53-mer template. Primer sets used were 5’-GTCTCGAGAGGCCGAGGACAGCTCGGCT-3’/5’-AGGCTTCCAATGTACAGGAGAAGTGCCTGTC-3’, respectively. The obtained fragments were purified and TA-cloned in the pGEMTeasy vector (Promega, Madison, WI). A DNA gel containing pGL3Basic vector. 53-mer template. Primer sets used were 5’-GTCTCGAGAGGCCGAGGACAGCTCGGCT-3’/5’-AGGCTTCCAATGTACAGGAGAAGTGCCTGTC-3’, respectively. The obtained fragments were purified and TA-cloned in the pGEMTeasy vector (Promega, Madison, WI). A DNA gel containing pGL3Basic vector.

Cell Culture and Transfection

Mouse embryonic fibroblasts were derived from E12.5 embryos (Hmg2a+/+, Hmg2a+/−, and Hmg2a−/−; ref. 17) as described previously (41). In brief, mouse embryonic fibroblasts were explanted, maintained on a 3T9 protocol, and propagated in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 2 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 55 μmol/L 2-mercaptoethanol, and 10 μg/mL gentamicin (Invitrogen).

Cell Culture and Transfection

Cell lines used included NIH/3T3 (mouse embry fibroblast cells; ATCC CRL-1658) and NIH/3T3 cells stably expressing HMG2A or HMG2AT, or stably transfected with empty vector (mock) as described previously (10). Cells were incubated at 37°C and 5% CO2 in a humidified incubator. Plasmid DNA transfections were done using FuGene 6 Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer’s protocol.

Luciferase Reporter Assays

Twenty-four to 48 hours after transfection of semiconfluent cells, cells were lysed and assayed for luciferase activity using the luciferase assay system (Promega) as described in Crombez et al. (42). A β-galactosidase expressing plasmid was cotransfected for normalizing the transfection efficiency. β-Galactosidase activity was assayed as described in Sambrook et al. (43). Luciferase activity and β-galactosidase A420 were measured in a Wallac Victor 1420 Multilabel Counter (Perkin-Elmer Life Sciences, Wellesley, MA). For each experiment, luciferase and β-galactosidase activities were determined in duplicate wells. The results are the mean of at least three individual experiments.

Preparation of Nuclear Extracts for Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from NIH/3T3 cells according to the procedure described (44). 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 (BCA Protein Assay kit, Pierce Perbio Science, Rockford, IL).

Protein Purification

The coding sequence of human HMGA2, which was adapted for enhanced bacterial expression, was cloned in the NdeI/XhoI sites of the pET-31b(+) bacterial expression vector (Novagen, Darmstadt, Germany), resulting in a construct expressing COOH-terminally HIS-tagged HMGA2 protein (kindly provided by Torik Ayoubi, University of Maastricht, the Netherlands).

The HMGA2 protein was expressed in the Escherichia coli BL21 strain in the following way: BL21 was grown in 500 mL Luria-Bertani medium containing ampicillin at 37°C. When the A600 reached a value of about 0.5, 1 mmol/L isopropyl β-D-thiogalactopyranoside (Roche) was added, and the culture was incubated overnight at 30°C. The cells were collected by centrifugation and dissolved in buffer A [50 mmol/L Tris/HCl (pH 8), 500 mmol/L NaCl] containing 10 μg/mL DNase, 10 μg/mL RNase, and 1 mg/mL lysozyme. After incubating 30 min on ice followed by sonication, the sonicate was centrifuged for 15 min at maximum speed. Purification of the HMGA2-HIS protein from the supernatant fraction was carried out as follows: after washing in buffer A, 100 μL Ni-Beads were added to the clear sonicate and rotated for 1 h at 4°C. Then the beads were washed twice for 1 min and twice for 5 min in buffer A and retained in a glass wool column by 1-min low-speed centrifugation. The column was washed once in buffer B [50 mmol/L Tris/HCl (pH 8), 100 mmol/L NaCl] before elution of the HIS-protein with 200 μL buffer B containing 250 mmol/L imidazole. All buffers used contained Complete.
protease inhibitors (Roche). Elution was repeated, and the purity of both elutes was checked by SDS-PAGE. The concentration of the purified proteins was determined by the Bradford assay (BCA Protein Assay kit, Pierce).

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assays were done using the LightShift Chemiluminescent EMSA kit (Pierce). Briefly, 5 µg of NIH/3T3 nuclear extracts or different amounts (5, 20, and 50 ng) of purified proteins were preincubated in the binding buffer containing 5 mM MgCl₂, 50 ng/µL poly(dexoyinosinic-dexoycytidylic acid), 0.05% NP40, and 0.2 µg/µL bovine serum albumin 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 unlabelled competitor probe, or incubated for 1 h at 4°C with 5 µL HMGA2fix antiserum (10), 2 µg p50, or 2 µg p65 antibody (Santa Cruz, Santa Cruz, CA). Biotin-labeled probe (30 fmol) was added in a total volume of 20 µL, and the reaction mixture was incubated for 10 min at room temperature. The protein–DNA complexes were separated by electrophoresis on a 6% polyacrylamide gel in 0.5× Tris-borate EDTA buffer at 4°C. After size fractionation, protein–DNA complexes were electrophoretically transferred to Hybond N+ membrane (GE Healthcare, Uppsala, Sweden) for 40 min at 380 mA in Tris-borate EDTA buffer.

**Chromatin Immunoprecipitation Assay**

Chromatin immunoprecipitation assays were done by following the chromatin immunoprecipitation assay protocol from Upstate Biotechnology (Millipore, Billerica, MA) with minor modifications. Briefly, NIH/3T3 cells stably overexpressing the wild-type HMGA2 protein were seeded in 10-cm cell culture dishes, grown to nearly confluence, and overexpressing the wild-type HMGA2 protein were seeded in 10-cm cell culture dishes, grown to nearly confluence, and rinsing twice with ice-cold PBS containing Complete protease inhibitors (Roche). Cells were collected and pelleted by centrifugation at 350 × g for 4 min at 4°C. Cell pellets were resuspended in 200 µL chromatin immunoprecipitation SDS lysis buffer containing Complete protease inhibitors (Roche) and incubated for 10 min on ice. Pooled lysate from two culture dishes was sonicated using a Branson 250 sonicator, applying 70% power, duty cycle of 25% during 6 min on ice. Immunoprecipitation was done using 10 µL HMGA2fix antiserum. Salmon sperm DNA/Protein A-Sepharose (50%) slurry used to preclar the lysates and to collect the antibody/histone complex was prepared as follows: 2.5 mL packed protein A-Sepharose CL-4B beads (GE Healthcare) were incubated by overnight rotation at 4°C in a final volume of 5 mL TE buffer (pH 8) containing 1 mg sonicated salmon sperm DNA, 2.5 mg bovine serum albumin, and 0.05% sodium azide. After elution of the histone complex and reversing of histone-DNA cross-links, DNA was recovered using the Qiaquick PCR purification protocol (Qiagen) followed by elution in 50 µL water before PCR amplification. The amplification was done using 3 µL DNA template with the following primer sets. Promoter region B, 5’-TCGTTCTTTCCCCAGCTC-3’/5’-CCGATG- TGAGCACAGGGTACC-3’; the upstream negative control –1789/–1606 bp region (Fig. 4B), 5’-CTCAGAGGCAGTGAAAGAGGGCTCC-3’/5’-GTTGTGTTGTTCTCCCTGGCATGAAAAAG-3’. The obtained PCR product (20 µL) was analyzed by electrophoresis on a 2% agarose gel. DNA was visualized by ethidium bromide staining.

**Tumor Samples and RNA Isolation**

Eleven well-differentiated liposarcomas and three myxoid liposarcomas were resected from 14 patients. Single-cell suspensions of the tumors were obtained by overnight collagenase treatment, whereafter suspensions were frozen in liquid nitrogen. All tumors were karyotyped according to standard procedures (45).

To isolate total RNA, collagenase–treated cells were thawed, washed twice in cold PBS, and pelleted. Total RNA was isolated using the NucleoSpin RNA II total RNA Isolation kit (Macherey-Nagel, Düren, Germany), according to conditions described by the manufacturer. DNase digestion was done directly on the purification column before elution of total RNA, as described in the manual.

**Quantitative Reverse Transcription-PCR**

cDNA was prepared from 5 µg Dnase I–treated total RNA, using the Superscript II First-Strand system for reverse transcription-PCR (Invitrogen) according to the conditions described by the manufacturer. Real-time PCR was done using the qPCR Mastermix for SYBR green I (Eurogentec) and was carried out using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Target cDNA was amplified in duplicate using the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of amplification (15 s at 95°C and 1 min at 60°C). A non-template reaction was included as negative control. After amplification, samples were subjected to a dissociation protocol to verify the presence of a single amplification product and the absence of nonspecific PCR products. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a reference gene to normalize for variations in the amount of input cDNA. Primer sets were chosen in such a way that they anneal in exons separated by large intronic sequences to avoid amplification of contaminating genomic DNA and were designed with Primer Express 2.0 (Applied Biosystems). Primers included qHMG2ap, 5’-TCCCTCTAAAGCAGCTCAAAAGA-3’;
Statistical Analysis

The Spearman rank correlation test was used to test a possible correlation between HMG A2 and IMP2 expression. The P value reported is one-tailed.

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References

HMGA2 Regulates Transcription of the Imp2 Gene via an Intronic Regulatory Element in Cooperation with Nuclear Factor-κB

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