Molecular Dissection of the Architectural Transcription Factor HMGA2†

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ABSTRACT: HMGA2 protein belongs to the High Mobility Group A (HMGA) family of architectural transcription factors. These proteins establish a network of protein−protein and protein−DNA interactions resulting in the formation of enhanceosomes at promoters and enhancers regulating the expression of several genes. HMGA2 dysregulation, as a result of specific chromosomal rearrangements, has been identified in a variety of common benign mesenchymal tumors, and transgenic mice expressing a truncated form of HMGA2 protein demonstrated a causal relationship between the expression of the HMGA2 protein and tumorigenesis. In this paper, using several recombinant mutant proteins, we have investigated the role played by the different domains of HMGA2 in protein−protein and protein−DNA interaction. Using the IFN-β gene as a model, we have shown that a short region of HMGA2, comprising the second DNA-binding domain, is critical for enhancing the NF-κB complex formation, for binding to the PRDII element, and also for protein−protein interaction with the NF-κB p50 subunit. Moreover, we have analyzed the interaction of HMGA2 mutant proteins with different DNA targets demonstrating that the absence of the C-terminal tail alters HMGA2/DNA complexes in a subset of DNA sequences. Our results suggest possible implications for the role of HMGA2 in tumorigenesis.

The nuclear factor HMGA2[1] together with the highly homologous HMGA1a and HMGA1b proteins constitute the HMGA family of proteins (*1*, *2*). HMGA1a and HMGA1b result from alternative splicing of the same gene (*1*). All HMGA proteins contain about 100 amino acid residues and have three DNA-binding domains that interact with the narrow minor groove of AT-rich DNA sequences that are therefore called AT-hooks (*3*). Although these nuclear proteins possess a highly acidic region at the C-terminus tail, similar to the activation domain of several transcription factors, they are not able to activate transcription per se. Rather by binding to DNA and/or to transcription factors, the HMGA proteins can organize the assembly of nucleoprotein transcriptional complexes at the level of enhancers or promoters of several genes, enhancing or repressing transcription (recently reviewed in ref *4*). HMGA1 proteins are in fact essential for inducible expression of several genes such as cytokines, cell adhesion molecules, growth, and transcription factors (*4*). The interferon-β (IFN-β) gene is the best-characterized example of how HMGA1 proteins can modulate gene transcription (*5*). Using the PRDII element of the IFN-β gene, we have previously demonstrated that HMGA2 also behaves as an architectural factor enhancing NF-κB binding to DNA and transcriptional activation (*6*).

In addition to their role in gene-specific regulation, HMGA proteins have a role in chromatin structure. They have in fact been localized to AT-rich G/Q and C bands of mammalian metaphase chromosomes (*7*, *8*) and to AT-rich scaffold-associated regions (SARs) where they have been shown to compete in vitro with histone H1 for DNA binding (*9*).

HMGA proteins are preferentially expressed during embryonic and fetal stages of development (*10*−*12*). In adults HMGA2 is not expressed, and HMGA1 expressed at very low levels. High levels of HMGA1 expression is limited to adult retina and in tissues that have undergone neoplastic transformation (*13*−*15*). A role for the HMGA2 protein in embryonic cell growth and differentiation is provided by the finding that the *pygmy* phenotype in mice (small size and drastic reduction of body fat content) is caused by the disruption of both *Hmga2* alleles (*10*). HMGA2 modulation in vivo also reduces obesity (*16*).

Cytogenetic studies have provided extensive evidence implicating the *HMGA2* gene in the development of a variety

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[1] Abbreviations: HMGA, high mobility group A; bp, base pairs; EMSA, electrophoretic mobility shift assay; TBE, Tris-borate-EDTA buffer; EDTA, ethylenediaminetetraacetic acid; GST, glutathione *S*-transferase; ORF, open reading frame; LPP, lipoma preferred partner; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TRX, thioredoxin; IFN-β, interferon-β.

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of benign tumors, mostly of mesenchymal origin, including lipomas, uterine leiomyomas, pleomorphic adenomas, fibroadenomas, aggressive angiomyxomas, endometrial polyps, and pulmonary hamartomas. Indeed, the gene is probably the most commonly rearranged gene in human neoplasms (*17*−*20*). The majority of the breakpoints are clustered within the third intron of the gene and result in chimeric transcripts containing exons 1−3, which encode the three DNA-binding domains, and ectopic sequences from other genes. A variety of different fusion partners have been identified, sometimes providing only few amino acid residues, thus suggesting that the removal of the last two exons encoding the acidic C-terminus tail, rather than the presence of ectopic sequences from other genes, is the key event in the genesis of these tumors. Indeed, both the truncated and a chimeric form of HMGA2 with the LIM domain of LPP were able to transform NIH-3T3 cells without any relevant difference (*21*).

The final demonstration that HMGA2 is sufficient for causing tumors comes from the generation of transgenic mice expressing a truncated form of HMGA2. Despite the ubiquitous expression of the truncated form, the transgenic mice developed a selective abundance of fat tissue and have an abnormal high incidence of lipomas (*22*−*24*). These findings demonstrate that the DNA binding domains of HMGA2, in the absence of C-terminal fusion partners, are sufficient to perturb adipogenesis and predispose to lipomas in vivo. Many differentially expressed genes have been identified in adipocytes expressing the truncated HMGA2 protein (*23*), but the molecular mechanisms through which the truncated HMGA2 acts are completely unknown. We therefore decided to investigate this aspect by producing several recombinant mutant forms of HMGA2 and studying their interaction with different DNA sequences by EMSA and footprinting. Interestingly, all HMGA2 forms retaining the three AT-hooks but missing the acidic C-terminus are able to bind DNA with the same specificity as the wild-type protein. However, the DNA−protein complex is altered due, probably, to enhanced protein−protein interaction. We therefore suggest that the truncated form is able to recognize the same target genes as the wild-type protein but could affect transcription by altering protein−protein interactions at promoters or enhancers.

EXPERIMENTAL PROCEDURES

Construction of the Prokaryotic Expression Vectors. The HMGA2 expression vectors were generated by PCR using the human cDNA for HMGA2 (*25*) as template. The primers used for expression vector construction were as follows:

primer C5: 5′-GAGCTCATATGAGCGCACGCGCACGCGGTGAGGGCG-3′;

primer C3-109: 5′-AAGCTTGGATCCTTATTAGTCCTCTTCGGC-3′;

primer C3-94: 5′-AAGCTTGGATCCTTATTACTGAGCAGGCTT-3′;

primer C3-83: 5′-AAGCTTGGATCCTTATTACCATTTCCTAGG-3′;

primer C3-MAD: 5′-AAGCTTGGATCCTTATTATGAGTTCTTCTG-3′;

primer C3-54: 5′-AAGCTTGGATCCTTATTAGCCTTTGGGTCT-3′;

primer C3-43: 5′-AAGCTTGGATCCTTATTAGGGCTCACCGGT-3′.

Primer C5-e95: 5′-CCTAGGAAATGGGAGGAAACTGAA-3′ was used to generate the internal deletion mutant. The PCR products were obtained using primer C5 in combination with the different C3 primers and cloned between the *Nde*I and the *Bam*HI sites of the bacterial expression vector pAR3038 under the bacteriophage T7 promoter (*26*). The resulting clones pAR HMGA2$_{109}$, pAR HMGA2$_{94}$, pAR HMGA2$_{83}$, pAR HMGA2$_{MAD}$, pAR HMGA2$_{54}$, and pAR HMGA2$_{43}$ were verified by sequencing. The internal deletion in pAR HMGA2$_{\Delta 83-94}$ plasmid was obtained by overlapping PCR, first amplifying the 1−83 fragment by primer C5 and C3−83 and the bridging 95−109 fragment using primer C5-e95 and C3-109, then the two fragments were used as template and amplified by primer C5 and C3-109.

Protein Expression and Purification. Recombinant HMGA2 proteins were expressed and purified essentially as previously described (*6*). Briefly, proteins were expressed using the BL21 (DE3) *Escherichia coli* strain that contains the T7 RNA polymerase under lacUV5 promoter control. Cultures were grown to $OD_{600} = 0.6$, induced with 0.5 mM isopropyl-β-D-thiogalactopiranoside, grown overnight, and harvested. HMGA2 proteins were selectively extracted from bacterial cells with 5% (v/v) perchloric acid and precipitated by acetone-HCl. The proteins were purified by reverse-phase HPLC on a BioRad RP304 column using a Waters apparatus as described elsewhere (*27*). The purified recombinant HMGA2 proteins were analyzed by SDS−PAGE and by mass spectrometry (Perkin-Elmer API 1 spectrometer) to confirm their molecular masses. To confirm the identity of HMGA2$_{73}$, a partial sequence from the N terminus was also obtained. The concentration of the HMGA2 proteins was obtained by measuring the tryptophan absorbance at 280 nm using an absorption coefficient for tryptophan of 5500 M^{-1} cm^{-1}. Since HMGA2$_{73}$, HMGA2$_{54}$, and HMGA2$_{43}$ have removed the tryptophan residue, their concentration was estimated according to Waddel measuring the absorbance at 225 and 215 nm using the following equation: protein concentration (μg/mL) = $(A_{215} - A_{225}) \times 154.2$. The coefficient of 154.2 was determined empirically using the other HMGA2 proteins.

Electrophoretic Mobility Shift Assays (EMSAs). EMSAs with NF-κB and HMGA2 mutants were performed with the oligonucleotide PRDII: 5′-GATCGTGGGAAATTCCGATC-3′, using 2, 1, 0.5, and 0.25 fmol of NF-κB (a generous gift of D. Thanos) and 2 pmol of HMGA2, as previously described (*6*). The fold-stimulation was calculated as the ratio between the intensity of the NF-κB/DNA complex, measured with the Multi-Analyst software (Biorad), obtained in the presence and in the absence of HMGA2.

EMSA experiments with the mutated HMGA2 proteins were performed with the following double-stranded oligonucleotides (only the upper strand sequence is shown):

CAnt1: 5′-CACTGCCCAGTTAATTGTTCTTGA-3′;

HCRII: 5′-GACACATTAATCTATAATCAAATAC-3′;

OCT-DRA: 5′-AGAGTAATTGATTTGCATTTTAAT-3′.

EMSAs were carried out as previously described (*6*). Briefly, purified proteins (1−16 pmol as indicated) were incubated with 50 fmol of labeled double-stranded DNA in 20 μL reactions containing 20 mM Tris-HCl pH 7.5, 75 mM

KCl, 100 mM NaCl, 5 μg/μL BSA, 1 mM EDTA, 13% glycerol for 20 min at room temperature. After incubation, protein-bound DNA and free DNA were separated on a native 7% polyacrylamide gel run in 0.5x TBE at 15 V/cm at 4 °C. The equilibrium dissociation constant (K_d) was determined by EMSA, considering three different experiments. The amount of free DNA was measured with Multi-Analyst software on a Biorad GS-525 Molecular Imager. K_d was defined according to Carey (*28*) by the following equation: K_d = [free DNA] × [free protein]/[complexes]. For protein−DNA cross-linking experiments, formaldehyde was added at the end of the incubation at 1% final concentration for 20 min at 4 °C. Cross-linking was stopped by addition of H_2SO_4 0.2 M and NH_4HCO_3 2 M final concentrations. Protein−DNA complexes were separated on a 15% SDS−PAGE.

DNase I Footprinting. DNase I footprinting followed standard protocols (*29*). 0.5−12 pmol of HMGA2 proteins were bound to the labeled HCR DNA fragment *Bam*HI−*Hind*III (10.000 cpm) from the HOXD9 promoter (*30*) in a 50 μL binding reaction containing 25 mM HEPES pH 7.6, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 μg of BSA, 0.01% NP-40, and 1 μg of poly dG-dC as a nonspecific competitor DNA. Products of Maxam−Gilbert chemical cleavage reactions of HCR DNA served as reference standards in the sequencing gels used in the footprinting experiments.

GST Pull-Down Assays. Human HMGA2 ORF was cloned in the *Bam*HI and *Xho*I restriction sites of the pGEX-4T2 plasmid in frame with the glutathione *S*-transferase (GST). pGEX-TRX44/63 and pGEX-TRX53/72 were obtained cloning the DNA sequence coding for the peptides from amino acid 44−63 and from 53−72 of the human HMGA2 protein in the *Rsr*II restriction site of the pGEX-4T1 TRX plasmid. The pGEX-4T1 TRX vector was obtained cloning the *E. coli* thioredoxin gene into the *Eco*RI and *Xho*I restriction site of the pGEX-4T1 plasmid in frame with the GST (*31*). Expression and purification of recombinant GST fusion proteins were carried out using standard protocols. To produce p50 and p65 proteins in vitro, p50 and p65 cDNAs (*32*) were digested with *Hind*III and *Bgl*II and with *Hind*III and *Bam*HI, respectively, and subcloned in the *Hind*III and *Bam*HI sites in the pcDNA3 vector. p50 and p65 polypeptides of NF-κB were then generated in vitro using the commercial in vitro transcription/translation kit (TNT, Promega), with ^{35}S-methionine (NEN Life Science) following the manufacturer's instructions. GST pull-down assays were carried out essentially as described (*33*). Essentially, 5 μg of recombinant GST proteins bound to beads were incubated with 10 μL of the in vitro translated proteins in 200 μL of binding buffer (25 mM HEPES pH 7.9, 50 mM NaCl, 1 mM DTT, 0.01% NP-40). After 2 h incubation of proteins with beads at room temperature, the protein-bound beads were washed twice in complete binding buffer (binding buffer plus 0.25% BSA) and twice in binding buffer without BSA. The bound proteins were then eluted and analyzed by SDS−PAGE and autoradiography.

Farwestern Blot Analysis. One microgram of each of the HMGA2 recombinant proteins were separated by SDS−PAGE and transferred to PVDF membrane. Farwesterns were then carried out as described (*33*). Briefly, after blotting the membrane was air-dried, and after incubation with methanol, the proteins were denatured for 10 min in 6 M guanidine hydrochloride (GuHCl) in a buffer containing 10 mM Hepes pH 7.5, 60 mM KCl, 1 mM EDTA, and 1 mM DTT (HBB). The proteins were renatured in the same HBB buffer with progressively decreasing GuHCl concentration. The membrane was then rinsed in HBB and blocked with HBB supplemented with 5% nonfat milk and 0.5% NP-40. The in vitro-translated p50 protein was incubated with the membrane in a buffer containing 15 mM HEPES pH 7.5, 13 mM NaCl, 50 mM KCl, 1 mM DTT, 1% milk, and 0.5% NP-40 for 4 h at 4 °C. The unbound protein was removed with extensive washes for 30 min in the same buffer. The membrane was air-dried, and the bound proteins were visualized by autoradiography.

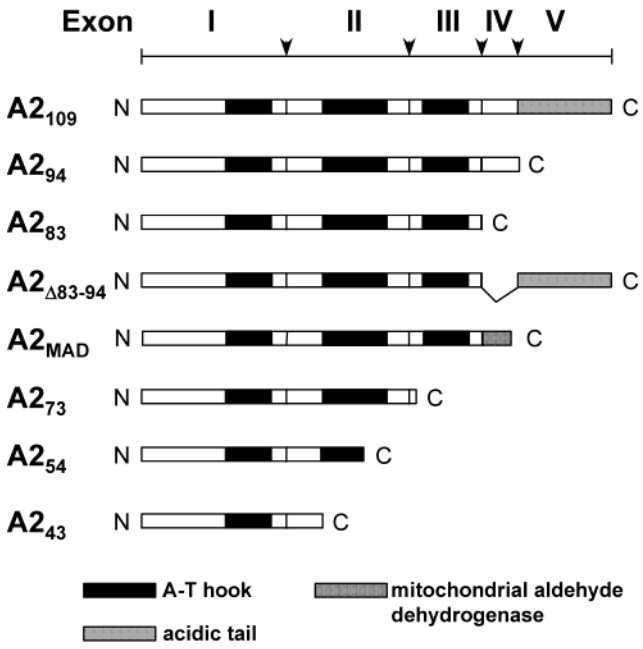

FIGURE 1: HMGA2 deletion mutants used in the study. Schematic representation of the *HMGA2* gene exon organization (top) and the different recombinant proteins used in the study (bottom).

RESULTS

Expression and Analysis of Recombinant Wild Type and Deletion Mutants of HMGA2. The architectural factor HMGA2 is composed of different functional elements each encoded by a different exon (*34*, *35*). The first three exons in fact encode the three DNA-binding domains (AT-hooks). A spacer region is encoded by exon IV. This is absent in the highly homologous *HMGA1* gene. An acidic C-terminus is encoded by exon V (Figure 1). To understand the modality of interaction of HMGA2 with DNA and to investigate the contribution of the single regions of the protein to its architectural function, we generated a series of deletion mutants where single protein elements where progressively removed. Figure 1 shows the panel of recombinant proteins used in the present study. $HMGA2_{109}$ is the human wild-type protein. In $HMGA2_{94}$, the last exon encoding the acidic C-terminus tail has been removed. $HMGA2_{83}$ represents a further deletion where the short region between the acidic C-terminus tail and the last DNA binding domain has been deleted. This mutant is particularly interesting since it corresponds to the truncated form of HMGA2 generated

following translocations involving the third intron of the *HMGA2* gene. This truncated protein is expressed in a large number of mesenchymal tumors, and its expression in transgenic mice is sufficient to induce lipoma formation (*22*, *23*). $HMGA2_{73}$ was obtained as a degradation product of $HMGA2_{83}$ and was included in the study since it has the third AT-hook deleted and almost corresponds to the product of the first two exons. In $HMGA2_{\Delta 83-94}$, the spacer region from amino acid 83−94 was removed from $HMGA2_{109}$ to evaluate its contribution in the wild-type protein. $HMGA2_{MAD}$ corresponds to a chimeric protein expressed as a consequence of a rearrangement involving the HMGA2 and the mitochondrial aldehyde dehydrogenase gene found in a uterine leiomyoma (*36*). The resulting fusion transcript encodes for the three DNA binding domains of HMGA2 fused to the last 10 amino acids of the mitochondrial aldehyde dehydrogenase protein. $HMGA2_{54}$ and $HMGA2_{43}$ are further deletions where the central AT-hook has been partially or completely removed, respectively.

Particular care has been taken to choose the expression system since the use of GST or HIS tags could have altered the binding properties of the mutants. Therefore, the T7 expression system (*26*) that allows the production of recombinant proteins without any additional amino acid was used.

All the recombinant proteins were expressed and purified to homogeneity as described in the Experimental Procedures, and their integrity was assessed by mass spectrometry, which indicates that all proteins have the initial methionine removed. The purified recombinant proteins were also checked on SDS−PAGE to assess that purification was achieved (data not shown).

Region from Amino Acid 54−73 in HMGA2 Is Critical for Enhancing the Binding of NF-κB to the PRDII Element. HMGA1 proteins have been shown to regulate the expression of a large number of genes by enhancing or repressing the binding of transcription factors to promoters and enhancers, the best-characterized example being the IFN-β gene. For HMGA2, functioning as an architectural transcription factor, there are instead only few reports in the literature (*6*, *37*). We have previously demonstrated that HMGA2, in analogy to HMGA1, is able to enhance the binding of the p50/p65 NF-κB heterodimer to the PRDII element of the IFN-β promoter (*6*). We have now used the same model to test the ability of different HMGA2 deletion products, in particular $HMGA2_{83}$ and $HMGA2_{MAD}$, which were described in many mesenchymal tumors, to enhance NF-κB binding to the PRDII element. The PRDII oligonucleotide was therefore incubated with decreasing amounts of recombinant NF-κB heterodimer in the presence of a constant amount of wild-type HMGA2 or two mutants, $HMGA2_{83}$ and $HMGA2_{MAD}$. Figure 2A shows that, as previously described (*6*), at low concentrations of NF-κB, $HMGA2_{109}$ enhances the binding of NF-κB to DNA (compare lanes 9−11 with lanes 4−6) and that both HMGA2 mutants stimulate the formation of the band-shift complex in a similar manner (compare lanes 14−16 and 19−21 with 9−11).

To map on HMGA2 the region responsible for this effect, a similar experiment was therefore performed with $HMGA2_{73}$, $HMGA2_{54}$, and $HMGA2_{43}$. Figure 2B shows that $HMGA2_{73}$ is still able to enhance the band-shift complex formation (compare lanes 14−16 with lanes 9−11), while

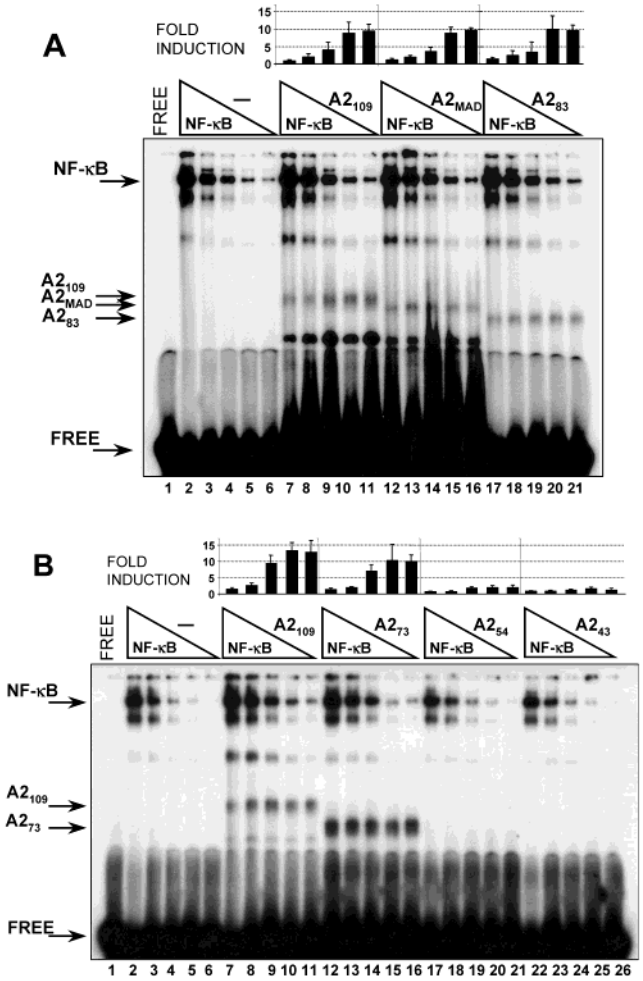

FIGURE 2: Role of the different HMGA2 deletion mutants in enhancing the binding of NF-κB to the PRDII element. (A) An EMSA analysis is shown where a range of NF-κB concentrations were incubated with the PRDII oligonucleotide in the presence or absence of $HMGA2_{109}$, HMGA2 $_{MAD}$, and $HMGA2_{83}$. Lane 1 free probe. Decreasing amounts (2, 1, 0.5, 0.25 fmol) of NF-κB without HMGA2 (lanes 2−6); with 2 pmol of $HMGA2_{109}$ (lanes 7−11); with 2 pmol of HMGA2 $_{MAD}$ (lanes 12−16); and 2 pmol of $HMGA2_{83}$ (lanes 17−21). The fold-stimulation, reported above the lanes, was calculated as the ratio between the intensity of the NF-κB/DNA complex obtained in the presence and in the absence of HMGA2 proteins. Columns and bars represent the mean and standard deviation, respectively, of three different experiments. (B) An EMSA analysis is shown where a range of NF-κB concentrations were incubated with the PRDII oligonucleotide in the presence or absence of $HMGA2_{109}$, $HMGA2_{73}$, $HMGA2_{54}$, and $HMGA2_{43}$. Lane 1 free probe. Decreasing amounts (2, 1, 0.5, 0.25 fmol) of NF-κB without HMGA2 (lanes 2−6); with 2 pmol of $HMGA2_{109}$ (lanes 7−11); with 2 pmol of $HMGA2_{73}$ (lanes 12−16); with 2 pmol of $HMGA2_{54}$ (lanes 17−21); and 2 pmol of $HMGA2_{43}$ (lanes 22−26). The fold-stimulation, reported above the lanes, was calculated as the ratio between the intensity of the NF-κB/DNA complex obtained in the presence and in the absence of HMGA2 proteins. Columns and bars represent the mean and standard deviation, respectively, of three different experiments.

both $HMGA2_{54}$ and $HMGA2_{43}$ do not enhance the binding of NF-κB to DNA (compare lanes 19−21 and 24−26 with lanes 9−11). Moreover, it is possible to see in the lower part of the band-shift that while $HMGA2_{109}$ and $HMGA2_{73}$ are able to bind to DNA forming a complex, mutants $HMGA2_{54}$ and $HMGA2_{43}$ (lacking part or the entire second AT-hook) cannot form complexes with DNA. It appears therefore that a region between residues 54 and 73 in

HMGA2 is critical for both binding to DNA and for stimulating the binding of NF-κB to DNA.

HMGA Proteins Share a Common Protein−Protein Interaction Region. Several reports indicate that HMGA1 proteins can interact with transcription factors in the absence of DNA. The data also indicate that this is relevant for higher-order nucleoprotein complex formation at promoters and enhancers (*38*). In particular, a protein−protein interaction has been evidenced with the NF-κB subunits p50 and p65 (*39*). To explore this interaction, a GST pull-down experiment was performed using recombinant HMGA2 fused to GST and in vitro ^{35}S-labeled p50 and p65. Figure 3A shows that the interaction of HMGA2 with p50 is strong (compare the input in lane 3 with the bound protein in lane 1) and instead with p65 is very weak (compare the input in lane 6 with the bound protein in lane 4).

Since HMGA2 interacts mainly with p50, a farwestern experiment was performed using pure recombinant HMGA2 proteins and in vitro ^{35}S-labeled p50 to map the region in HMGA2 responsible for this interaction. Figure 3B shows that HMGA2$_{109}$, HMGA2$_{94}$, HMGA2$_{MAD}$, HMGA2$_{83}$, and HMGA2$_{73}$ mutants all bind p50, while this interaction is severely impaired when HMGA2$_{54}$ is present and completely absent with HMGA2$_{43}$. Two peptides spanning the sequence presumably involved in protein−protein interaction with p50 were then expressed in the active site of *E. coli* thioredoxin (TrxA) to asses which region was sufficient for binding with p50. The TrxA was chosen since its scaffold provides conformational constraint and stability to short peptide sequences and was successfully used to select peptides from combinatorial libraries of aptamers (*31*). Figure 3C shows a GST pull-down experiment where peptides 44−63 and 53−72 expressed within the TrxA protein fused to GST were able to bind in vitro translated p50 (lanes 3 and 4), whereas the control GST-TrxA was not (lane 5). The interaction with p50 is much stronger with peptide 44−63, demonstrating therefore that this region within HMGA2 is necessary and sufficient for p50 binding. Therefore, the region that is crucial for enhancing the NF-κB complex formation and for binding to DNA is also responsible for protein−protein interaction with the NF-κB p50 subunit. Interestingly, the corresponding region in HMGA1 has been shown to be necessary for binding to p50 and to other transcription factors as well (*29*, *39*-*43*). Therefore, a region critical for the interaction with different transcription factors is conserved between HMGA1 and HMGA2.

Absence of the Acidic C-Terminus Alters the HMGA2/DNA Complex. HMGA2 proteins that have either lost the acidic tail (HMGA2$_{83}$) or had it replaced with ectopic sequences (HMGA2$_{MAD}$) are able to enhance the binding of NF-κB to the PRDII element similarly to wild-type HMGA2. We therefore tested the panel of mutants for their ability to interact with low and high affinity DNA target sites, exploring a wide range of protein concentrations, to see whether it was possible to find any difference among them. The oligo CAnt1 was used since it has been shown that both HMGA1 and HMGA2 are able to interact with it recognizing the core sequence 5′-TTAATT-3′ (*44*). As previously shown, HMGA2 is able to interact with CAnt1 forming a stable complex (Figure 4A, lanes 2−6), while a completely different result is observed for the HMGA2 mutants that retain all three DNA binding domains but have lost the acidic tail

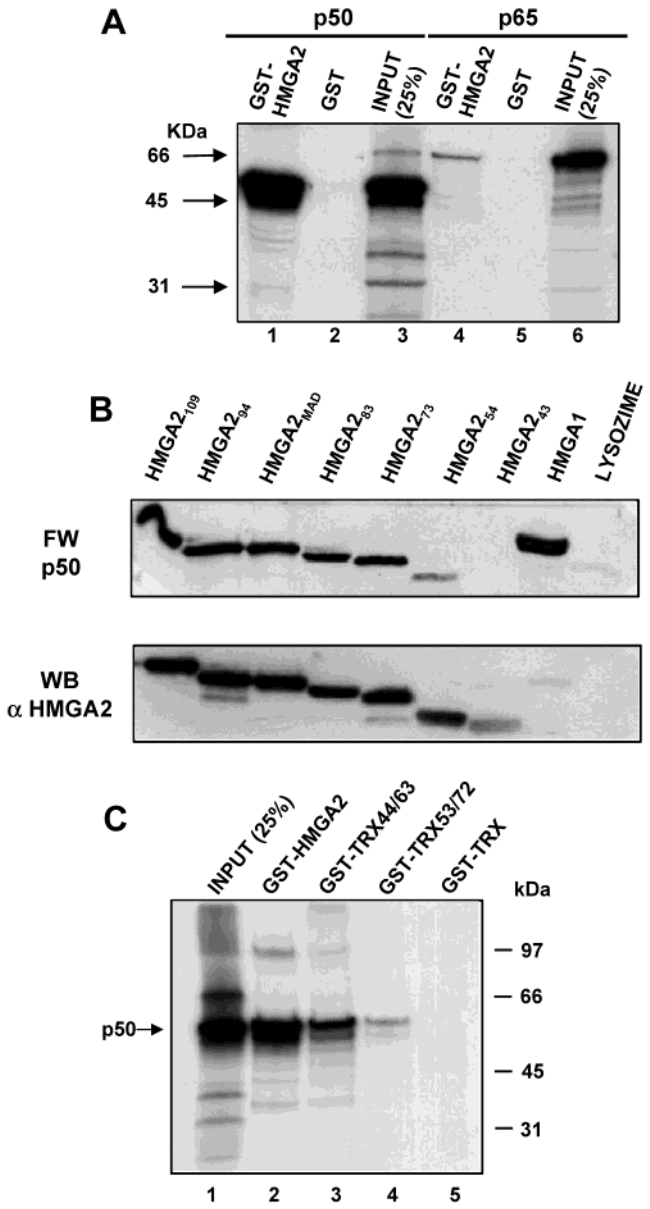

FIGURE 3: Protein−protein interaction between p50/p65 of NF-κB and HMGA2. (A) In vitro translated ^{35}S-labeled p50 and p65 subunits of NF-κB were incubated with GST or GST-HMGA2 immobilized to sepharose beads, and the bound protein was loaded in lanes 2 and 5, and 1 and 4, respectively, on a SDS−PAGE. Lanes 3 and 6 contain 25% of the in vitro translated reaction used in the GST pull-down experiment. (B) Farwestern blot analysis of HMGA2 deletion mutants probed with radiolabeled p50 (upper panel). HMGA1 and the lysozime are positive and negative control, respectively. Western blot analysis of the membrane with α-HMGA2 antibodies (lower panel). (C) In vitro translated ^{35}S-labeled p50 subunit of NF-κB was incubated with GST-HMGA2, GST-TRX44/63, GST-TRX53/72, and GST-TRX immobilized to sepharose beads, and the bound protein was loaded in lanes 2, 3, 4, and 5, respectively, on a SDS−PAGE. Lane 1 contains 25% of the in vitro translated reaction used in the GST pull-down experiment.

(HMGA2$_{94}$ and HMGA2$_{83}$) or had it replaced with an ectopic sequence (HMGA2$_{MAD}$). A smear is generated instead of a discrete complex, which is particularly evident at high protein concentrations (compare lanes 7 and 8 with lanes 2 and 3). HMGA1 protein has been shown to interact with itself (*39*), and this is true also for HMGA2 (data not shown); in our opinion therefore this result could be due to protein−protein interactions. To exclude that this result is due to the reduction

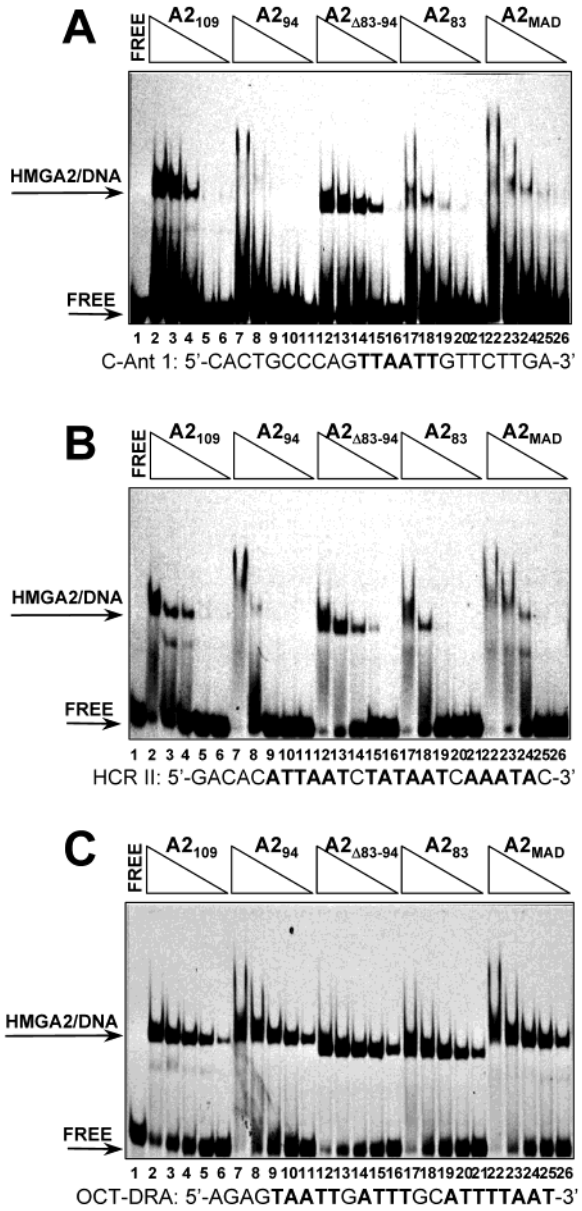

FIGURE 4: EMSA experiments with the HMGA2 deletion mutants. EMSA analyses were performed incubating decreasing amounts (16, 8, 4, 2, 1 pmol) of the different recombinant HMGA2 deletion mutant proteins as indicated in the figure with ^{32}P-labeled Cant1 (A), HCRII (B), and OCT-DRA (C) oligonucleotides. Lane 1, free probe.

of the size of the protein, the construct $HMGA2_{\Delta 83-94}$, where the internal spacer region was removed, was used. The complex formed with $HMGA2_{\Delta 83-94}$ is similar to that obtained with the wild-type protein (compare lanes 12−16 to 2−6), thus pointing out to a role played by the acidic tail itself.

Similar results were obtained using an other oligo, HCRII, derived from the HOXD9 gene promoter that was used in a previous study (*44*) (Figure 4B), thus confirming the different behavior of mutants lacking the acidic C-terminus tail.

Even though CAnt1 and HCRII contain AT-tracts, they are not high affinity binding sites for HMGA2 protein, with the estimated K_d =170 and 60 × 10^{-9} M for these sites, respectively. High affinity binding sites for HMGA proteins typically have multiple AT-tracts permitting multiple interactions between a single HMGA molecule and the target DNA.

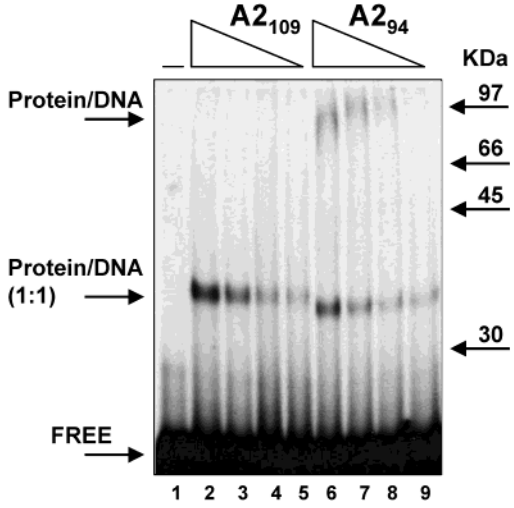

FIGURE 5: Formaldehyde cross-linking experiments. Decreasing amounts (16, 8, 4, 2 pmol) of recombinant $HMGA2_{109}$ (lanes 2−5) and $HMGA2_{94}$ (lanes 6−9) were incubated with labeled oligo CAnt1, cross-linked with formaldehyde, resolved in SDS gel electrophoresis, and exposed to autoradiography. Lane 1 is free probe. Bands corresponding to DNA, protein/DNA complexes (1:1), and higher order protein/DNA complexes are indicated by arrows. Protein molecular weights are indicated on the right.

We thus also studied the interactions of our panel of proteins with the OCT-DRA oligonucleotide, containing a high affinity binding site for HMGA2 (K_d = 10 × 10^{-9} M, see Experimental Procedures). This oligo is derived from the promoter of the human class II MHC gene HLA-DRA and contains several AT-tracts (*29*). Figure 4C shows that differences between the wild type, and the different mutants lacking the acidic tail ($HMGA2_{94}$, $HMGA2_{83}$, and $HMGA2_{MAD}$) are much less pronounced than those observed with the previous oligos.

Formaldehyde cross-linking experiments were carried out to analyze the protein−DNA complexes formed. $HMGA2_{94}$ was used as representative of the truncated forms of HMGA2 with the CAnt1 probe. As shown in Figure 5, a band corresponding to a protein−DNA complex (1:1 ratio) is detected in all lanes where HMGA2 proteins were added (lanes 2−9); instead, high molecular weight protein−DNA complexes are detected only where $HMGA2_{94}$ but not $HMGA2_{109}$ was used. In particular, these complexes are detectable with the highest concentration of proteins (lanes 6−8), which is consistent with what was observed in the EMSA experiment reported above (Figure 4A). In conclusion, HMGA2 mutants that have either removed the acidic tail or had it replaced with ectopic sequences are not able to properly bind to a subset of DNA sequences.

Acidic C-Terminus Tail Is Not Involved in Determining the HMGA2 DNA-Binding Specificity. The specificity of the interaction of HMGA2 mutants with the minor groove structure associated with stretches of AT-rich DNA was also tested. Figure 6 shows the results of the experiments in which a DNaseI footprinting experiment was performed on the HOXD9 promoter region that contains the sequence of the oligo HCRII, comparing equimolar amounts of $HMGA2_{109}$ and $HMGA2_{MAD}$ proteins. $HMGA2_{109}$ protects the AT-tracts that are present in the HCRII oligo, as expected, but no differences in the protected regions were detected when comparing the two proteins. Similar results were obtained

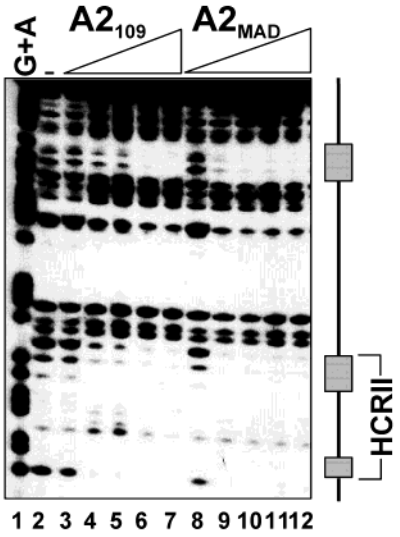

FIGURE 6: DNA binding specificity of HMGA2 deletion mutants. A DNaseI footprinting experiment is shown using the ^{32}P-labeled antisense HCR region of HOXD9 promoter. Increasing concentrations (0.2, 2, 6, 12 pmol) of $HMGA2_{109}$ and $HMGA2_{MAD}$ were used in lanes 3−7 and 8−12, respectively. In lane 2, the − indicates that the DNaseI digestion was performed with naked DNA. In lane 1, the HCR DNA chemically cleaved by Maxam−Gilbert G+A reaction has been included as a reference. The hatched boxes on the right indicate the protected regions. The region corresponding to the HCRII oligo used in EMSA experiments is shown.

using two other truncated proteins $HMGA2_{94}$ and $HMGA2_{83}$ (data not shown).

In addition, EMSA experiments were performed with $HMGA2_{83}$ and $HMGA2_{109}$ and the radiolabeled OCT-DRA oligo in the presence of increasing concentration of two different nonlabeled DNA competitors: dG-dC, a nonspecific competitor DNA, or dI-dC, a specific competitor for AT-rich DNA sequences (*45*). Again no relevant differences are observed between $HMGA2_{109}$ and $HMGA2_{94}$ suggesting therefore no difference in binding specificity between them (data not shown).

DISCUSSION

In the last years, cytogenetic studies revealed that the locus 12q13−15 is commonly rearranged in a variety of mesenchymal tumors identifying *HMGA2* as the gene participating in all these translocations (*17*-*20*). The other highly related gene HMGA1 at 6p21 has also been shown to be rearranged in these tumors although at much lower frequency (*36*). The pivotal role of HMGA2 in the genesis of these tumors has been demonstrated with the generation of transgenic mice that, although expressing the truncated protein in all tissues, show high tissue specificity developing lipomas (*22*, *23*) and natural killer cell lymphomas (*46*). Another important consequence of these studies is that the fusion partners of HMGA2 are not necessary for the tissue specificity nor for the transforming ability, thus pointing to the removal of the acidic C-terminal tail as the key molecular event responsible for tumor formation. Very recently, transgenic mice were obtained expressing the wild-type HMGA2 protein (*47*). These mice develop a similar phenotype, although other pathologies were described that were not observed in the transgenic mice expressing the truncated HMGA2. Therefore, the truncated HMGA2 form could confer a different activity in comparison to the wild-type protein, a role which was also evidenced by a previous finding where truncated HMGA2 protein, but not wild type, was shown to transform NIH-3T3 cells (*21*).

The acidic C-terminus tail is a characteristic feature not only of the HMGA proteins but of all the HMG family members including HMGB1, HMGB2, HMGN1, and HMGN2, but its function is still not understood. The removal of the acidic tail has been shown to modestly increase the affinity of HMGA1 to AT-rich DNA and to consistently enhance the ability of introducing negative supercoils (*48*), while it does not affect the binding of HMGA1 to nucleosomes (*49*). More controversial is the involvement of the C-terminus tail of HMGA1 in enhancing transcription factor activity. In fact, it has been shown that its removal does not affect the enhancement of the binding of Serum-response Factor (SRF) to DNA nor the transcription of SRF-target genes in transfection assays (*43*); on the contrary, it affects transcriptional activation of the interferon-β gene (*39*). Interestingly, the C-terminus tail of both HMGA1 and HMGA2 has no activating or repressing activity when fused to the DNA-binding domain of GAL4 and assayed for transcriptional effect (*45*, *50*); moreover, although many protein partners have been identified for HMGA1 and the regions of HMGA1 involved in the interactions mapped, the C-terminus acidic tail has never been directly involved in protein−protein interaction. It is therefore difficult to understand the mechanism through which the removal of the C-terminal acidic tail can affect HMGA function and in particular cell growth in tumors. In this paper, using different mutants of HMGA2, we start to dissect the role played by the different domains of HMGA2 involved in DNA binding and in protein−protein interaction.

Since we have previously demonstrated that HMGA2 enhances the binding of NF-κB to the PRDII element of the IFN-β gene (implying therefore that also HMGA2 can participate in the formation of enhanceosomes), we used this system as a model to test whether removal of domains from HMGA2 protein could alter its ability to enhance the binding of NF-κB to DNA. Results clearly indicate that removal of the C-terminal tail does not alter the ability to facilitate the binding of NF-κB to DNA and that a region from amino acid 44−63 of HMGA2 is critical for protein−protein interaction. The corresponding region in HMGA1 is required for the interaction with DNA, with p50/p65 and for the stimulatory effect on the binding of NF-κB to DNA, as demonstrated by alanine-scanning mutagenesis (*51*). Moreover, this region is involved in the interaction with other transcription factors as well (*29*, *40*−*43*). The interesting point is that residues K65 and K71 in HMGA1a, shown to be acetylated by CBP/p300 and P/CAF, respectively, and to be critical for the disassembly/assembly of the enhanceosome (*5*), map to this region and are conserved (Figure 7). This suggests that enhanceosomes containing HMGA2 protein could be modulated by acetyl transferases similarly to HMGA1. However, there are also important differences in this region. At S59, between the two acetylation sites, there is a consensus for the cell cycle dependent p34/cdc2 kinase in HMGA2 that has been shown to be phosphorylated in vitro by p34/cdc2 (*52*), and which is absent in HMGA1. This modification, in this critical location, could alter the stability

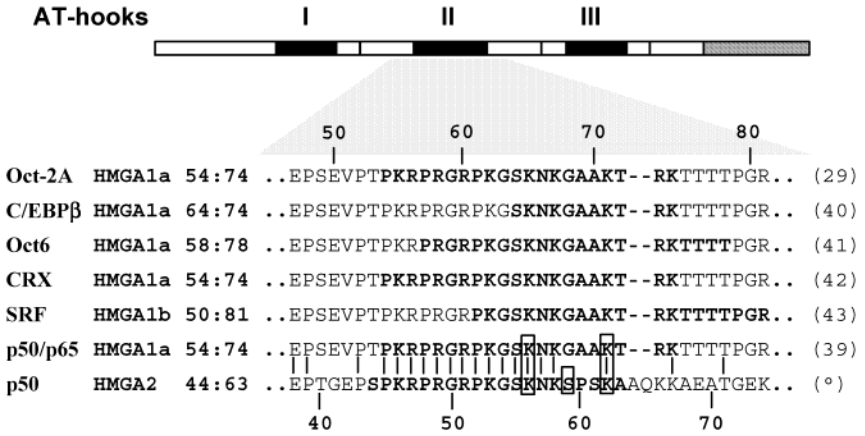

FIGURE 7: Several transcription factors interact with the same region in HMGA proteins. The upper line of the diagram illustrates the organization of the HMGA2 protein. The black boxes correspond to the AT-hooks and the shaded box to the acidic C-terminus tail. In the lower part, the aligned sequences of HMGA1a, HMGA1b, and HMGA2 are shown. Numbers refer to HMGA1a (upper part) and HMGA2 (lower part) considering the first residue the initial methionine. In bold is evidenced the amino acid sequence involved in the interaction with the different transcription factors (indicated on the left). On the right, the corresponding bibliographic references are indicated, (°) this paper. Amino acid residues that can be subjected to posttranslational modifications are boxed.

of enhanceosomes containing HMGA2 proteins that, as a consequence, could be regulated by a different posttranslational modification than those regulating HMGA1.

We show that the removal of the last exon of the *HMGA2* gene, resulting in a HMGA2 protein missing the acidic C-terminus, could alter the resulting protein/DNA complex. This effect is evident at high protein concentration and for low affinity HMGA2 binding sites such as HCRII and Cant1, while it is less evident for the high affinity binding sites in OCT-DRA. This suggests therefore that when the truncated HMGA2 protein is engaged with DNA in high affinity binding sites it is less available for protein−protein interaction with itself. In other words, if the K_d value of the protein−DNA complex increases, the ability to interact with itself increases as well, resulting in the formation of protein−protein complexes that are responsible for the smear detected in EMSA and evidenced in the cross-linking experiments. The further removal of exon IV, encoding the spacer region, from the *HMGA2* gene or the substitution of the sequences encoded by the last two exons with an ectopic sequence from the mitochondrial aldehyde dehydrogenase ($HMGA2_{MAD}$) does not introduce any further difference to the effect of $HMGA2_{94}$. It is possible to explain this effect considering that HMGA2, as well as HMGA1 proteins, possesses three highly conserved AT-hooks that are not equivalent in terms of DNA binding. It has in fact been demonstrated that the second AT-hook has the highest affinity for DNA (*53*) and that, depending on the DNA binding sites, the protein can use in addition to this also the other two AT-hooks in different combinations. For example, with poly(dA-dT) all three AT-hooks of HMGA2 are employed in DNA binding, while in the IFN-β promoter only the first and second AT-hooks bind the NRDI and the PRDII, respectively, while the third does not contribute to the protein−DNA interaction (*53*). In this view therefore the third AT-hook, in the context of the entire protein, could be free to interact with DNA or with proteins depending on the sites present on the target DNA, and the C-terminus tail could modulate this activity. This hypothesis has been proposed for HMGA1 by Thanos (*39*) and is supported by the observation that the region from residue 50 to 81, comprising the region between the second and the third AT-hook in HMGA1b, interacts with SRF (*43*).

The specificity of DNA interaction remains always the same for all the mutants tested as it has been demonstrated by footprinting and competition experiments in EMSA with different DNA sequences. This result is in agreement with those of Reeves and co-workers that, using a chimeric protein with the second AT-hook of HMGA1 fused to the B-box of HMGB1, demonstrated that the AT-hook is dominant over the HMG box, in fact, the chimeric protein recognizes specifically only AT-rich sequences (*54*).

An important conclusion of this work is therefore that truncated HMGA2, as that expressed in many tumors, binds to the same sequences as the wild type but has impaired the ability to interact with itself and probably with other transcription factors, an event which could account for its transforming ability. The identification of protein partners for HMGA2, which is ongoing in our laboratories, will provide the tools to test this hypothesis.

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