Transcriptional Regulation of the *HMGA1* Gene by Octamer-Binding Proteins Oct-1 and Oct-2

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Abstract

The High-Mobility Group AT-Hook 1 (*HMGA1*) protein is an architectural transcription factor that binds to AT-rich sequences in the promoter region of DNA and functions as a specific cofactor for gene activation. Previously, we demonstrated that *HMGA1* is a key regulator of the insulin receptor (INSR) gene and an important downstream target of the INSR signaling cascade. Moreover, despite of this biological and pathological relevance, the mechanisms that control *HMGA1* gene expression remain unknown. In this study, to define the molecular mechanism(s) that regulate *HMGA1* gene expression, the *HMGA1* gene promoter was investigated by transient transfection of different cell lines, either before or after DNA and siRNA cotransfections. An octamer motif was identified as an important element of transcriptional regulation of this gene, the interaction of which with the octamer transcription factors Oct-1 and Oct-2 is crucial in modulating *HMGA1* gene and protein expression. Additionally, we demonstrate that *HMGA1* binds its own promoter and contributes to its transactivation by Oct-2 (but not Oct-1), supporting its role in an auto-regulatory circuit. Overall, our results provide insight into the transcriptional regulation of the *HMGA1* gene, unveiling a differential control exerted by both Oct-1 and Oct-2. Furthermore, they consistently support the hypothesis that a putative defect in Oct-1 and/or Oct-2, by affecting *HMGA1* expression, may cause INSR dysfunction, leading to defects of the INSR signaling pathway.

Introduction

*HMGA1* is an architectural transcription factor that specifically interacts with the narrow minor groove of AT-rich regions of DNA and regulate gene expression [1,2,3]. By modifying DNA conformation and by recruiting transcription factors to the transcription start site, it facilitates the assembly and stability of steroespecific DNA-protein complexes (so-called enhanceosomes), thus driving gene transcription in response to multiple extracellular and intracellular signals [4,5,6]. In addition to its role in gene transcription, *HMGA1* is also involved in numerous other processes, including embryogenesis, differentiation, and neoplastic transformation [7,8,9]. Recent evidence assigns to *HMGA1* an important role in the transcriptional regulation of glucose homeostasis [10,11,12,13,14]. Consistent with these latter findings, we have shown that defects in *HMGA1* expression and/or function, by negatively affecting insulin receptor (INSR) signaling in insulin target tissues, may cause insulin resistance and increase susceptibility to type 2 diabetes mellitus [11,15]. Also, evidence has been provided implicating the *HMGA1* locus as one conferring a high cross-race risk of development of type 2 diabetes and metabolic syndrome [16,17,18,19]. However, despite this variety of studies, the mechanisms that regulate *HMGA1* gene expression are mostly unknown.

Cloning and characterization of the *HMGA1* gene reveals a very complex structure [2,20], with a regulatory region comprising the untranslated exons 1–3, lacking TATA and CAAT, but rich in G and C. Its complexity includes at least two transcription start sites and an extensive alternative mRNA splicing, particularly in the 5′- untranslated region [2,20], resembling the genes that are regulated without the preferential utilization of any specific start site [21]. Instead, a preferential utilization of start site 2 has been demonstrated under certain experimental conditions, in which tight gene regulation is achieved, following the production of specific mRNA in response to different stimuli [20]. A mechanism of transcriptional regulation of the human *HMGA1* gene has been postulated before both in embryonic and cancer cells highly expressing *HMGA1* [22]. It has been proposed that elevated expression of *HMGA1* in these cell models is mainly controlled by specificity protein 1 (Sp1) and activator protein-1 (AP-1) transcription factors, both of which seem to act as positive
regulating elements, after binding to the transcription start site 1 and the transcription start site 2, respectively [22]. In the current study, we sought to further investigate the mechanisms that underlie HMGA1 gene expression in different human and rat cell lines widely used in biochemical and molecular genetic studies of gene function and regulation. We report herein the identification of a new mechanism of regulation of the HMGA1 gene, which involves the octamer DNA motif “ATGCCAAT” originally described in immunoglobulin (Ig) gene regulation [23]. As a cis-acting transcriptional regulatory element found in both promoters and enhancers of a wide variety of ubiquitously expressed and cell-type-specific genes, this octamer motif is specifically bound by a family of at least 11 distinct nuclear proteins, named octamer-binding (Oct) transcription factors, designated from Oct-1 to Oct-11 [24]. All these factors are characterized by two Pit-Oct-Unc (POU) domains, in which the N-terminal subunit is known as the POU-specific (POUS) domain, while the C-terminal subunit is a homeo domain (POUH). Both domains are required for high affinity, site-specific binding to the octamer motif, and mediate specific protein-protein interactions with other transcription factors or co-factors, thus activating or inhibiting gene expression [24]. Here, we focused our attention on the regulation of HMGA1 gene transcription by the octamer-binding proteins Oct-1 and Oct-2, whose ubiquitous expression differs from other Oct proteins that exhibit, instead, a developmental and tissue-specific distribution [24]. Our data indicate a role for Oct-1 and Oct-2 in regulating HMGA1 expression through direct interaction with the upstream HMGA1 promoter region.

Materials and Methods

Cells, protein extracts and Western Blot (WB)

HepG2 human hepatoma cells, and human cervix epithelioid carcinoma cells (HeLa) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), IM-9 cultured human lymphocytes (ATCC) were maintained at subconfluent densities in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified 5% CO2 atmosphere at 37°C. L6 muscle rat cells were differentiated in DMEM containing 2% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine in a humidified 5% CO2 atmosphere at 37°C. The medium was replaced every 3 days until myotube formation was reached. Total cellular lysates or nuclear extracts were prepared as previously [25] and final protein concentrations in the extracts reached. Total cellular lysates or nuclear extracts were prepared as previously [25] and final protein concentrations in the extracts reached. Total cellular lysates or nuclear extracts were prepared as previously [25] and final protein concentrations in the extracts reached. Total cellular lysates or nuclear extracts were prepared as previously [25] and final protein concentrations in the extracts reached. Total cellular lysates or nuclear extracts were prepared as previously [25] and final protein concentrations in the extracts reached. Total cellular lysates or nuclear extracts were prepared as previously [25] and final protein concentrations in the extracts reached.

Probes and electrophoresis mobility shift assay (EMSA)

To obtain probe DNA for EMSA, the regulatory region of the HMGA1 gene was amplified by PCR reaction, using the following specific oligonucleotide primers for the HMGA1 gene (GenBank AL354740): HMGA1 forward 1 5’TGTGATCTACACATT-CAGGC-3’ (sense primer) and HMGA1 reverse 1 5’TGCAC-GAAGGAGCTTCTGCGG-3’ (antisense primer); HMGA1 forward 2 5’TGTGATCTACACATT-CAGGC-3’ (sense primer) and HMGA1 reverse 2 5’TGCAC-GAAGGAGCTTCTGCGG-3’ (antisense primer). The obtained fragments (311 and 1242 bp, respectively) were purified using the Qiagen Gel Extraction Kit (Qiagen), and an aliquot of recovered DNA was sequenced by automatic genetic analyzer and compared to the published sequence (Genbank n. NC_000006.11). Then, both genomic sequences for HMGA1 were digested into eight smaller fragments (P1 to P8, see Figure 1) with EcoRI and DdeI restriction endonucleases (Promega), end-labeled with (^32P)ATP (GE Healthcare) using DNA Polymerase I (Promega), and electrophoresed on 8% non-denaturing polyacrylamide gel. Double-stranded probes were excised from the gel, purified according to a standard protocol [27] and used in EMSA, as previously described [25,26]. Briefly, cell nuclear extract or either Oct-2 enriched nuclear extract (Roche) or pure HMGA1 [26] were incubated with 2 ng of radiolabeled probe, in the presence of poly(dI-dC), which was used as competitor DNA, in a buffer containing 15 mM Hepes pH 7.9, 1 mM EDTA, 5% glycerol, 40 mM KCl and 0.5 mM DTT. After 30 min of incubation at 20°C, the reaction products were separated by electrophoresis through a 6% non-denaturing polyacrylamide gel in 0.5× TBE (1× TBE = 50 mM Tris, 50 mM boric acid and 1 mM EDTA), and free and bound DNA were detected by autoradiography [25,26]. Unlabeled 22-mer double stranded oligonucleotides containing consensus binding site for the transcription factors AP-1, AP-2, AP-3, Sp1, Oct-1, Oct-2 (Promega) and N-Myc (Santa Cruz Biotech) were used in competition studies, together with a 27-mer double-stranded oligonucleotide containing the PRDII element of the β-interferon promoter which specifically binds HMGA1 [4,26,28].

Chromatin immunoprecipitation (ChiP)

ChiP was performed in HepG2 cells, either untreated or pretreated with Oct-1 or Oct-2 small interfering RNA (siRNA), as described previously [13]. Formaldehyde-fixed DNA-protein complex was immunoprecipitated with either anti-Oct-1 or Oct-2 antibody. Sequence-specific primers for the HMGA1 gene promoter [human HMGA1 (NC_000006.11): HMGA1 forward 5’TGTGACCGGAGGGAAGTAC-3’ and HMGA1 reverse 5’ATGCGAGGGCGACGTAAGG-3’] were used for PCR amplification of immunoprecipitated DNA (30 cycles), using PCR ready-to-go beads (GE Healthcare). PCR products were electrophoretically resolved on 1.5% agarose gel and visualized by ethidium bromide staining. Specificity of PCR product was confirmed by direct sequencing. Primers for quantitative reverse transcriptase PCR (qRT-PCR) of ChiP-ed samples are available upon request.

Transfection studies

The HMGA1-luciferase (HMGA1-luc) reporter plasmid was obtained by cloning the HIF1α promoter which specifically transactivation of the human HMGA1 gene promoter into pGL3-basic vector (Promega). Recombinant HMGA1-luc reporter construct, in the presence or absence of expression vectors for Oct-1 or Oct-2 [29], were transiently transfected into cultured cells using LipoFectA-MINE 2000 reagent (Invitrogen), and luc activity was assayed 48 hrs later, using the dual-luciferase reporter assay system (Promega) [30]. Renilla control vector served as an internal control of transfection efficiency, together with measurements of protein expression levels. siRNA targeted to human HMGA1, Oct-1 or Oct-2, plus non-specific siRNA controls with a similar GC content (Invitrogen) were transfected into cells at 50% to 60% confluency and cells were analyzed 48 to 96 hrs later, as described previously [31].

Real-time PCR

For qRT-PCR, total cellular RNA was extracted from cells using the RNAqueous-4PCR kit and subjected to DNase treatment (Ambion). RNA levels were normalized against 18s
ribosomal RNA in each sample, and cDNAs were synthesized from 2 μg of total RNA using the RETROscript first strand synthesis kit (Ambion). Primers for human and rat HMGA1, Oct-1, Oct-2, INSR, Sp1 and RPS9 were designed according to sequences from the GenBank database. A real-time thermocycler (Eppendorf Mastercycler ep realplex ES) was used to perform quantitative PCR. SYBR Green fluorescence was measured, and relative quantification was made against the RPS9 cDNA used as an internal standard [12]. All PCR reactions were done in triplicate.

Statistical analysis
All calculations were performed with the SPSS 20.0 software (SPSS Inc.). Mean values were compared with t-tests. A p-value <0.05 (two-tailed) was considered significant.

Results
Putative binding sites on the HMGA1 gene promoter
In an attempt to identify the mechanism(s) that regulate HMGA1 gene expression, we initially performed functional experiments aimed at identifying protein-DNA interactions within the P1 to P8 sequence elements of the HMGA1 gene promoter (Figure 1). Consensus transcription factor-binding sites within these eight regions were explored by using the TRANSFAC database (containing a large library of predefined matrix descriptions for known transcription factor recognition sequences) searched with MatInspector professional software [32]. Putative binding sites for the octamer-binding proteins Oct-1 and Oct-2, and HMGA1 were identified and characterized by EMSA using homologous radiolabeled fragments. As shown in Table 1, the octamer motif “ATTGCAAT” and the binding sequence for HMGA1 within fragment P5 are underlined. Mutagenized bases in each consensus site are in lowercase letters.

Nuclear protein-DNA interaction within the HMGA1 promoter region
As shown in Figure 2A, DNA-protein binding activity was observed following incubation of either probe P3 or P5 with nuclear extracts from IM-9 cells, a human B lymphocyte cell line

| Species        | Genbank     | Sequences          | Position   |
|----------------|-------------|--------------------|------------|
| Mus musculus   | NC_000083   | cccggggtc atttgcatg | 3020 from ATG |
| Bos taurus     | NC_007324   | cccggagctc atttgcatg | 3668 from ATG |
| Pan troglodytes| NC_006473   | cccgggctc atttgcatg | 3979 from ATG |
| Homo sapiens   | NC_000066.11| cccggggtc atttgcatg | 3980 from ATG |

1 The octamer sequence is underlined.

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expressing both Oct-1 and Oct-2 transcription factors, in addition to HMGA1. No binding activity was observed with any of the other six \(P1, P2, P4, P6, P7, P8\) DNA probes (not shown). To determine specificity of DNA-protein binding, competition assays were performed. A 10-30-fold molar excess of either unlabeled fragment \(P3\) or unlabeled fragment \(P5\) considerably reduced the binding of labeled \(P3\) and labeled \(P5\) to the DNA binding proteins, respectively (Figure 2B). Moreover, mutations within the consensus sequences abolished DNA-protein binding activity of either probe \(P3\) or \(P5\) (Figure 2B). As shown in Figure 3A, a 30-fold molar excess of 22-mer double stranded oligonucleotides representing the consensus binding sites for the nuclear factors AP-1, AP-2, AP-3, Sp1 and N-Myc did not compete for binding of nuclear proteins to \(P3\) and \(P5\) probes. Instead, unlabeled competitor DNA for the octamer motif “ATGCAAAT”, completely abolished protein-DNA complex formation with \(P3\) probe (Figure 3A), indicating that the POU domain is essential for the formation of a DNA-protein complex at this site. On the other hand, binding of HMGA1 to \(P5\) probe was displaced by excess unlabeled double-stranded oligonucleotides containing the PRDII element motif (GGGAAATTCCGTGGGAAATTCCGAGCT) (Figure 3A), which, as reported previously by us and others, specifically binds HMGA1 in the minor groove of the central AT-rich region \(4,26,28\), thus suggesting a potential autoregulatory role of HMGA1 on its own promoter. Next, we showed that the protein-DNA complexes formed upon incubation of nuclear extracts from IM-9 cells with labeled \(P3\) were recognized and supershifted to a slower-migrating form by the anti-Oct-1 or anti-Oct-2 antibody (Figure 3B); instead, binding of HMGA1 to labeled \(P5\) produced a protein-DNA complex that was recognized

![Figure 2. Nuclear protein-DNA interaction within \(P3\) and \(P5\) fragments of the HMGA1 promoter.](image)

(A) Nuclear extracts (NE) from IM-9 cells were incubated with \(P3\) (left) or \(P5\) (right) probe (0.2 ng each), in the presence of 0.2 \(\mu\)g of poly(dI-dC), which was used as the competitor DNA for nonspecific DNA-binding proteins in the nuclear extracts. DNA-protein complexes were resolved on a 6% non-denaturing polyacrylamide gel. (B) Labeled \(P3\) and \(P5\), and a mutant version of either \(P3\) (P3m) or \(P5\) (P5m) probe were used in EMSAs with 4 \(\mu\)g of NE from IM-9 cells under the same conditions as in (A). Sequence specificity of HMGA1 DNA binding proteins was determined by using 10–30 fold molar excess of unlabeled fragment \(P3\) (left) or \(P5\) (right) as competitors. Free (DNA) and bound (DNA-P) probe is indicated. A representative of three separate assays is shown for each condition.

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and supershifted by the anti-HMGA1 antibody (Figure 3B). Binding of Oct-1 and Oct-2 to P3 sequence was substantiated further by ChIP coupled with qRT-PCR of ChIP-ed samples, showing that binding of both Oct-1 and Oct-2 to the endogenous HMGA1 chromosomal locus was present in living IM-9 cells naturally expressing Oct-1 and Oct-2, and was considerably decreased in cells exposed to siRNA against either Oct-1 or Oct-2 (Figure 3C).

HMGA1 is necessary for recruitment and binding of Oct-2 to the HMGA1 promoter

Physical association between HMGA1 and Oct-1 and Oct-2 has been reported previously in pull-down assays indicating that direct contact of Oct-1 and Oct-2 with HMGA1 occurs through their conserved POU homeodomains [33]. In the light of the abovementioned data indicating that a relationship may exist between these factors, to see whether HMGA1 had any influence on the binding of Oct-1 and Oct-2 to the endogenous HMGA1 gene promoter, we carried out ChIP experiments in living IM-9 cells, either untreated or pretreated with siRNA against HMGA1. As detected by qRT-PCR of ChIP-ed samples, whereas binding of Oct-1 to the HMGA1 gene promoter was not affected in IM-9 cells expressing reduced levels of endogenous HMGA1 (Figure 4A), the binding of Oct-2 was significantly decreased in cells exposed to siRNA against HMGA1 (Figure 4B), thus indicating that HMGA1 is required for binding of Oct-2, but not Oct-1, to the HMGA1 gene promoter in vivo, in intact cells.

Oct-1 and Oct-2 differentially regulate HMGA1 gene promoter

To test whether Oct-1 and Oct-2 had a functional role in regulating the HMGA1 gene at the transcriptional level, human HeLa and HepG2 cells and rat skeletal muscle cells (differentiated from L6 myoblasts), three cell lines naturally expressing HMGA1, were cotransfected transiently with the HMGA1-luc reporter plasmid, plus increasing amounts of Oct-1 or Oct-2 expression vector. As shown in Figure 5A, overexpression of Oct-1 significantly reduced HMGA1-luc activity in all cell lines and this effect occurred in a dose-dependent manner. In contrast, overexpression of Oct-2 caused a significant increase of HMGA1-luc activity in all these cells (Figure 5B), supporting the notion that, by binding the same consensus site in the HMGA1 promoter, Oct-1 and Oct-2 exert an opposite effect on the HMGA1 gene; while the former decreases the transcriptional activity and protein level of HMGA1, the latter increases them.

Oct-1 and Oct-2 may affect INSR expression

Based on the above data indicating that interaction of Oct-1 and Oct-2 with the HMGA1 gene promoter may play a role in HMGA1 expression, it was important to ask whether this interaction could have an effect on the expression of the INSR gene, a well-characterized molecular target for HMGA1 which...
causes an increase in intracellular INSR in cells and tissues [10,11,26]. For this purpose, the ability of Oct-1 and Oct-2 to drive INSR gene expression was measured in HepG2 cells, in which protein levels of Oct-1 or Oct-2 were increased by transfecting cells with plasmids for expression of Oct-1 or Oct-2. As shown in Figure 6A, overexpression of Oct-1 in HepG2 cells caused a decrease in endogenous HMGA1 mRNA and protein levels and this was followed by a decline in INSR mRNA and protein expression. The opposite was observed when HepG2 cells were transfected with an expression vector coding for Oct-2: in this case, a significant increase was observed in the levels of HMGA1 mRNA and protein abundances, which were paralleled by increased levels of mRNA and protein for the INSR (Figure 6A). These effects were specific for HMGA1, as both mRNA and protein expression for the ubiquitously expressed transcription factor Sp1 were unaffected under both conditions. Similar results were obtained in differentiated L6 skeletal muscle cells naturally expressing INSR (Figure 6B). Thus, these data indicate that Oct-1 and Oct-2, by differentially modulating HMGA1 gene transcription, may affect INSR gene and protein expression and perhaps the expression of other HMGA1-target genes.

**Discussion**

For the first time in this work we identified and characterized a novel molecular mechanism that contribute to the regulation of HMGA1 gene expression. Using DNA-protein interaction studies combined with ChIP coupled with qRT-PCR, we demonstrated a differential role for Oct-1 and Oct-2 transcription factors in the transcriptional regulation of the HMGA1 gene which, in addition to the regulation of HMGA1 mRNA and protein expression, may contribute to the modulation of HMGA1-target genes. The observation that Oct-1 and Oct-2 differentially affect HMGA1 gene expression by binding to the same octamer motif “ATG-CAAAAT” is consistent with previous reports, in which it has been demonstrated that both Oct-1 and Oct-2 bind to the same DNA sequence but may differ in their activation potential [29,34,35,36]. Furthermore, here we show that, by facilitating the interaction of Oct-2 (but not Oct-1) with the HMGA1 gene, HMGA1 enhances...
transactivation of its own promoter by this octamer transcription factor, providing evidence for the existence of an auto-regulatory circuit in which HMGA1 activates its own transcription. In relation to Oct-1, it needs to be considered that several isoforms of this transcription factor were described previously, each of which is active in a tissue-specific manner, being able to mediate different and even opposing effects [24]. Thus, we cannot exclude that in other cells the effect of Oct-1 on HMGA1 may be different from that reported here in our work. In this regard, results have been provided indicating that Oct-1, NF-kappa B and HMGA1 cooperate for transactivation of the iNOS promoter in pancreatic β-cells [37].

As we previously reported, HMGA1 is a main activator of the INSR gene [10,26], and individuals with defects in HMGA1 have decreased INSR expression and increased susceptibility to type 2 diabetes mellitus [11,15,16,18]. Also, we showed that, as a novel downstream nuclear target of the INSR signaling pathway [13], HMGA1 represents an important novel mediator of glucose disposal and glucose homeostasis [12,14]. Therefore, based on these observations and the results from the present study indicating that a functional relation can be established between HMGA1, Oct-1 and Oct-2 in the context of the INSR gene, it is tempting to hypothesize that a putative defect in these nuclear proteins may cause INSR dysfunction with subsequent impairment of insulin signaling and action. In this regard, an association between Oct-1 gene variants and type 2 diabetes mellitus, which may support this intriguing hypothesis, has been reported [38]. Moreover, recent evidences suggest that Oct-1 may play a role in metabolic homeostasis by acting as a sensor for cAMP (cyclic AMP). In particular, elevation of cAMP in pancreatic and intestinal endocrine cells reduces nuclear Oct-1 content [39,40], and this is consistent with previous observations indicating that expression of HMGA1 increases in response to cAMP [12,41], thus

Figure 6. Role of Oct-1 and Oct-2 in HMGA1 expression and the consequent effects on INSR. (A,B) HepG2 and differentiated L6 cells were transfected with 2.0 μg of either Oct-1 or Oct-2 expression vector, and HMGA1 and INSR mRNA (qRT-PCR) and protein (IP/WB) levels were measured. mRNA is expressed as percent of the value in untransfected (basal) HepG2 and L6 cells (100%). Results are the mean ± SEM of three independent experiments. *p<0.05 versus basal cells. Immunoprecipitation (IP) of the INSR and Western Blot (WB) were performed using an anti-INSR β-subunit antibody. Sp1 and β-actin, controls.
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suggesting that increased HMGA1 expression could be due, at least in part, to the reduction of Oct-1 content.

In conclusion, our findings from this study reveal new mechanistic insights into the transcriptional mechanisms governing HMGA1 gene expression. While additional investigations will be needed to further define the roles of Oct-1 and Oct-2 in HMGA1 expression in other cell systems and models, these results provide a paradigm to suggest other types of molecule, Oct-1 and Oct-2, in addition to HMGA1, which should be searched for in investigations designed to elucidate the causes of impairment of INSR signaling in humans with INSR dysfunction and insulin resistance.

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Author Contributions

Conceived and designed the experiments: EC BA AB. Performed the experiments: EC BA KP MG SI VV RP. Analyzed the data: EC AB. Contributed reagents/materials/analysis tools: AB. Wrote the paper: EC AB. Critically reviewed the manuscript and carried out bioinformatic analysis of the HMGA1 regulatory region: DF FSB.

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