HMGA Proteins Up-regulate CCNB2 Gene in Mouse and Human Pituitary Adenomas

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Abstract
The high mobility group As (HMGA) belong to a family of nonhistone nuclear proteins that orchestrate the assembly of nucleoprotein complexes. Through a complex network of protein-DNA and protein-protein interaction, they play important roles in gene transcription, recombination, and chromatin structure. This protein family is involved, through different mechanisms, in both benign and malignant neoplasias. We have recently reported that transgenic carrying the Hmga1 or Hmga2 genes under transcripational control of the cytomegalovirus promoter develop pituitary adenomas secreting prolactin and growth hormone. We have shown that the mechanism of the HMGA2-induced pituitary adenoma is based on the increased E2F1 activity. The expression profile of mouse normal pituitary glands and adenomas induced in HMGA transgenic mice revealed an increased expression of the cccnb2 gene, coding for the cyclin B2 protein, in the neoplastic tissues compared with the normal pituitary gland. Here, we show, by electrophoretic mobility shift assay and chromatin immunoprecipitation, a direct binding of HMGA proteins to the promoter of cccnb2 gene, whereas luciferase assays showed that HMGA are able to up-regulate cccnb2 promoter activity. Finally, we report an increased CCNB2 expression in human pituitary adenomas of different histotypes that is directly correllated with HMGA1 and HMGA2 expression. Because cyclin B2 is involved in the regulation of the cell cycle, these results taken together indicate that HMGA-induced cyclin B2 overexpression gives an important contribution to experimental and human pituitary tumorigenesis. [Cancer Res 2009;69(5):1844–50]

Introduction
The high mobility group A (HMGA) protein family includes HMGA1a and HMGA1b, which are encoded by the same gene through alternative splicing (1), as well as the closely related HMGA2 protein (2). These proteins are nonhistone architectural nuclear factors, which bind the minor groove of AT-rich DNA sequences through three short basic repeats, called “AT-hooks”, located at the NH2-terminal region of the proteins. The involvement of HMGA proteins in embryogenesis, cell proliferation, differentiation, apoptosis and, above all, cancer development has been extensively shown (3). In particular, HMGA proteins seem to play their major physiologic role during embryonic development. In fact, their expression is very high during embryogenesis, whereas it is very low or negligible in normal adult tissues (4, 5). Conversely, HMGA protein expression has been found abundant in several malignant neoplasias, including pancreas, thyroid, colon, breast, lung, ovary, prostate carcinomas, squamous carcinomas of the oral cavity, and head and neck tumors (6).

Both HMGA1 and HMGA2 show oncogenic activity in vitro and in vivo: they are able to transform mouse and rat fibroblasts in culture (7, 8), and both the HMGA1 and HMGA2 transgenic mice develop GH/PRL-secreting pituitary adenomas and T/NK lymphomas (9–11). Recently, we have reported that HMGA2-mediated E2F1 activation is a crucial event in the onset of these tumors in transgenic mice (12), and likely also in human prolactinomas where HMGA2 gene amplification and overexpression have been shown (13). To identify other genes involved in the process of pituitary tumorigenesis induced by the HMGA genes, we have analyzed the gene expression profile of two HMGA1- and three HMGA2-pituitary adenomas in comparison with a pool of 10 normal pituitary glands from control mice using the Affymetrix MGU11K oligonucleotide array representing ~13,000 unique genes. Among these genes, we previously focused our attention on the Mia/Cd-rap gene, whose expression was essentially suppressed in all pituitary adenomas tested by cDNA microarray. We showed that the HMGA proteins directly bind to the promoter of the Mia/Cd-rap gene and are able to down-regulate its expression (14).

Here, we focus our attention on the cccnb2 gene, coding for the cyclin B2 protein, which showed an 8-fold change increase in the pituitary adenomas in comparison with normal pituitary glands from control mice (14). Cyclin B2 is a member of the B-type cyclin family, including B1 and B2. The B-type cyclins associate with p34cdc2 kinase and are essential components of the cell cycle regulatory machinery, being directly involved in the G2-M transition (15). Consistent with this finding, cccnb2-knockout mice, although develop normally, are smaller than normal mice and have reduced litter sizes, which suggests that cyclin B2 expression gives some growth advantage (16). Moreover, several studies have reported the accumulation of Cyclins B1 and B2 in human malignant tumors, such as colorectal and lung cancer (17–20). Also, in human pituitary adenomas cyclins amplification and...
overexpression have been observed (21–23). More specifically, in human prolactinomas, an increase of CCNB1 mRNA expression, related to aggressiveness, has been recently reported (24).

Here, we report that HMGA proteins are able to bind in vitro and in vivo the promoter of cyclin B2 gene, and to up-regulate its activity. Furthermore, an increased expression of Cyclin B2, correlated with HMGA1 and HMGA2 expression, has been shown in human pituitary adenomas of different histotypes, suggesting its contribution to the process of human pituitary tumorigenesis where HMGA proteins are involved.

**Materials and Methods**

**Cell cultures.** NIH3T3 cells were grown in DMEM supplemented with 10% FCS. A1T20 and A1T3-1 are murine pituitary adenoma cell lines secreting adrenocorticotropin hormone (ACTH) and gonadotroph hormones, respectively. RC-4B/C are rat pituitary adenoma cells secreting GH, follicle-stimulating hormone (FSH), luteinizing hormone, GnRH, ACTH, and TSHβ, whereas GH1, GH3, and GH4c1 are rat pituitary adenoma cells secreting prolactin and growth hormone. All these cell lines, purchased from American Type Culture Collection, were cultured in DMEM containing 10% fetal bovine serum (HyClone) and 50 μg/mL gentamicin (Life Technologies, Inc.) in a humidified atmosphere of 95% air and 5% CO2.

**Cyclin B2 promoter construct, transient transfection, and luciferase assay.** The wild-type cyclin B2-luciferase vector (B2-luci) was generously provided by Giulia Piaggio (25). Transfections for luciferase assays were performed with complete protease inhibitors mixture (Roche Diagnostic Corp.). Total proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk and incubated with antibody against Cyclin B2 (N-20 Santa Cruz, sc-2325). Bound antibody was detected by the appropriate secondary antibody and revealed with an enhanced chemiluminescence system (Amersham-Pharmacia Biotech).

**Tissue samples.** The human samples were obtained from 45 patients as surgical excision biopsies from patients of “Federico II” University (Naples) and “Faculte de Médecine Lyon-RTH Laennec” (Lyon). One part of pituitary adenoma was saved for routine histopathology evaluation and the other one taken from each patient was immediately frozen at −80°C until the extraction of nucleic acids. All tissue samples were fixed immediately after surgical removal in a solution of 4% paraformaldehyde in PBS w/v. The criteria for inclusion in the study were that the routinely processed paraffin blocks were suitable for immunohistochemistry and adequate clinical information.

**Immunohistochemical analysis.** Paraffin sections (5–6 μm) were deparaffinized, placed in a solution of absolute methanol and 0.3% hydrogen peroxide v/v for 30 min, and then washed in PBS before immunoperoxidase staining. The slides were subsequently incubated in diaminobenzidine (DAB) and with streptavidinhorseradishperoxidase for 20 more min. For immunostaining, the slides were incubated in diaminobenzidine (DAB-Dako) solution containing 0.06 mmol/L DAB and 2 mmol/L hydrogen peroxide in 0.05% PBS v/v (pH 7.6) for 5 min. After chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent mounting medium (Permount). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system. For Cyclin B2 immunohistochemistry, antibodies (2 μg/mL) raised against the recombinant Cyclin B2 protein were used. The specificity of the reaction was validated by the absence of staining when carcinoma samples were stained with antibodies preincubated with the peptide against which the antibodies were raised. Similarly, no positivity was observed when tumor samples were stained with a preimmune serum.

**RNA extraction, cDNA preparation, semiquantitative and quantitative reverse transcription-PCR.** Total RNA isolation from human tissues was performed with Trizol (Invitrogen) according to the manufacturer’s instructions. RNA was extracted from fresh specimens after pulverizing the
tumors with a stainless steel mortar and pestle that were chilled on dry ice. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis. One microgram of total RNA of each sample was reverse-transcribed with the QuantiTect Reverse Transcription (QIAGEN group) using an optimized blend of oligo-dT and random primers according to the manufacturer's instructions. One microgram of total RNA of each sample was reverse-transcribed with the QuantiTect Reverse Transcription (QIAGEN group) using an optimized blend of oligo-dT and random primers according to the manufacturer's instructions. To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing the PCR on samples that were not reverse-transcribed but identified positively. For semiquantitative PCR, reactions were optimized for the number of cycles to ensure product intensity within the linear phase of amplification. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and scanned using a Typhoon 9200 scanner.

Electrophoretic mobility-shift assay. Protein/DNA-binding was determined by electrophoretic mobility shift assay (EMSA), as previously described (28). Briefly, 5 to 20 ng of recombinant protein were incubated in the presence of radiolabeled oligonucleotide (specific activity, 8,000–20,000 cpm/nmol). A 200-fold excess of specific unlabeled competitor oligonucleotide was used. The double-strand oligonucleotides used was a region spanning from base –1189 to –69 of the mouse ccnb2 promoter region, and incubated with 5 ng (lane 1) and 20 ng (lanes 2, 5, 8, and 9) of the recombinant HMGA1-His protein or with 5 ng (lanes 3, 6, 10, and 11) and 20 ng (lane 4) of the recombinant HMGA2-His protein. To assess the specificity of the binding, a 100-fold excess of unlabeled oligonucleotides (lanes 5 and 6) or anti-HMGA1 (lane 9) and anti-HMGA2 (lane 11) antibodies were incubated as specific competitors. B, ChIP assay performed on NIH3T3 cells transfected with pCEFLHA (HA), pCEFLHA-HMGA1 (HA-A1), or pCEFLHA-HMGA2 (HA-A2). C, ChIP performed on pituitary adenomas from HMGA1 and HMGA2-transgenic mice to detect the endogenous in vivo binding of HMGA proteins to ccnb2 promoter gene. Input, PCR products with chromosomal DNA without immunoprecipitation. As an immunoprecipitation control, IgG was used.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was carried out with an acetyl-histone H3 immunoprecipitation assay kit (Upstate Biotechnology) according to the manufacturer's instruction. Approximately 3 × 10^7 cells of the NIH3T3 cell line or –1 ng of chopped HMGA-transgenic pituitary adenomas were cross-linked by the addition of formaldehyde (1% final concentration). Cross-linking was allowed to proceed at room temperature for 5 min and was terminated with glycine (final concentration, 0.125 mol/L). Cells and tissues were lysed in buffer containing 5 mmol/L Pipes (pH 8.0), 85 mmol/L KCl, 0.5% NP-40, and protease inhibitors (1 mmol/L; phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), on ice for 10 min. Nuclei were pelleted by centrifugation at 5,000 rpm for 5 min at 4°C and resuspended in buffer containing 50 mmol/L Tris–Cl (pH 8.1), 100 mmol/L EDTA, 1% SDS, the same protease inhibitors (1 mmol/L); phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), on ice for 10 min. Nuclei were pelleted by centrifugation at 5,000 rpm for 5 min at 4°C and resuspended in buffer containing 50 mmol/L Tris–Cl (pH 8.1), 100 mmol/L EDTA, 1% SDS, the same protease inhibitors, and incubated on ice for 10 min. Chromatin was sonicated on ice to an average length of ~400 bp with a Branson sonicator model 250. Samples were centrifuged at 14,000 rpm for 10 min at 4°C. Chromatin was precleared with protein A-Sepharose (blocked previously with 1 mg/ml bovine serum albumin) at 4°C for 2 h. Precleared chromatin of each sample was incubated at 4°C overnight with 2 μg of antibody anti-HA (sc-7392; Santa Cruz Biotechnology), for transfected cells, anti-HMGA1 (26), for HMGA1-transgenic pituitary adenomas, and anti-HMGA2 (12), for HMGA2-transgenic pituitary adenomas. An aliquot of wild-type samples was incubated also against IgG antibody. Next, 60 μl of a 50% slurry of blocked protein G Sepharose were added, and immune complexes were recovered. The supernatants were saved as “input.” Immunoprecipitates were washed twice with 2 mmol/L EDTA, 50 mmol/L Tris–Cl (pH 8.0) buffer, and 4 times with 100 mmol/L Tris–Cl (pH 8.0), 500 mmol/L LiCl, 1% NP-40, and 1% deoxycholic acid buffer. The antibody-bound chromatin was eluted from the beads with 200 μl of elution buffer (50 mmol/L NaHCO3, 1% SDS). Samples were incubated at 67°C for 5 h in the presence of 10 μg RNase and NaCl to a final concentration of 0.3 mol/L to reverse formaldehyde cross-links. Samples were then precipitated with ethanol at –20°C overnight. Pellets were resuspended in 10 mmol/L Tris (pH 8.0) buffer and treated with proteinase K to a final concentration of 0.5 mg/ml at 45°C for 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol, ethanol-precipitated, and resuspended in water. Input DNA and immunoprecipitated DNAs were analyzed by PCR for the presence of cyclin B2 promoter sequence. PCR reactions were performed with AmpliTaq gold DNA polymerase (Perkin-Elmer). The primers used to amplify the sequence of the ccnb2 promoter were 5′-TAAAGATGATGGAACAAAG-3′ (forward) and 5′-CCCTGACCTAAAATCACA-3′ (reverse). PCR products were...
resolved on a 2% agarose gel, stained with ethidium bromide, and scanned using a Typhoon 9200 scanner.

**Statistical analysis.** For the comparison between two groups of experiments, Student’s *t* test was used. The statistical significant difference was considered when *P* value was <0.05. The Pearson correlation was performed to determine the association of CCNB2 with HMGA1 or HMGA2 expression levels in human pituitary adenomas. A correlation coefficient (*r*) close to 1 was considered indicative of a significant direct correlation.

**Results**

Cyclin B2 is overexpressed in pituitary adenoma developing in HMGA-transgenic mice. The analysis of mRNA expression profile of HMGA1- and HMGA2-induced pituitary adenomas showed an 8-fold change increase expression of the *ccnb2* gene in the neoplastic tissues in comparison with the normal pituitary from control mice (14). This result has been validated by semiquantitative (Fig. 1A) and quantitative reverse transcription-PCR (qRT-PCR; Fig. 1B) in two HMGA1- and three HMGA2 pituitary adenomas. In fact, the quantitative PCR showed a higher than 20-fold increase in specific *ccnb2* mRNA expression. Western blot analysis showed the accumulation of cyclin B2 protein in pituitary adenomas of the HMGA1 and HMGA2 transgenic mice but not its presence in normal pituitary gland (Fig. 1C). However, in the case of the pituitary adenoma sample PA2, no correlation has been found between RNA and protein level. This could be due to the possible action of some still unidentified microRNA/s in this adenoma. Subsequently, we analyzed by RT-PCR the expression of the *ccnb2* gene also in murine and rat cell lines derived from pituitary adenomas of different histotype. As shown in Fig. 1D, the expression of *ccnb2* is undetectable in normal pituitary (NG) gland, both from mouse and rat, whereas it is expressed in all analyzed cell lines. These results suggest cyclin B2 as a direct target of HMGA proteins in pituitary tumorigenesis.

HMGA proteins bind to the *ccnb2* promoter and positively regulate its activity. To investigate whether the HMGA proteins are directly involved in *ccnb2* transcriptional regulation, we evaluated the HMGA ability to bind the *ccnb2*-promoter *in vitro* using oligonucleotides spanning from base −1189 to −69 of the mouse *ccnb2* promoter region (29) including AT-rich putative HMGA-binding sites. As shown in Fig. 2A (lanes 1–2) increasing amounts (5 and 20 ng) of the recombinant HMGA1 protein were capable of binding the 32P-end–labeled double-strand oligonucleotide in EMSA. The binding specificity was shown by competition experiments showing loss of binding with the addition of 100-fold molar excess of the specific unlabeled oligonucleotide (lane 5). In addition, competition experiments with specific anti-HMGA1 and anti-HMGA2 antibodies further confirmed the specificity of the HMGA/DNA complexes (Fig. 2A, lanes 8–9). Analogous results were obtained when the same experiments were performed using the recombinant HMGA2 protein (Fig. 2A, lanes 3–4, 6, and 10–11).

To verify that HMGA proteins are able to bind to *ccnb2* promoter also *in vivo*, we performed experiments of ChiP in the NIH3T3 cell line transiently transfected with either the HA-HMGA1 or HA-HMGA2 expression plasmids. Chromatin prepared as described under Materials and Methods was immunoprecipitated with anti-HA or rabbit IgG antibodies, used as internal control. The results, shown in Fig. 2B, confirmed that both HMGA1 and HMGA2 proteins bind to the promoter of the *ccnb2* gene. In fact, the *ccnb2* promoter region was amplified from the DNA recovered with anti-HA antibody in HA-HMGA1– and HA-HMGA2–transfected cells but not in the cells transfected with the backbone vector. Moreover, no amplification was observed in the same samples immunoprecipitated with rabbit IgG. To confirm this result in our *in vivo* model system of pituitary adenoma, the ChiP of both HMGA1 and HMGA2 was also analyzed in pituitary adenomas coming from HMGA1 and HMGA2 transgenic mice, respectively. As shown in

![Figure 3](image-url)
Fig. 2C, both the HMGA proteins do bind the ccnb2 promoter in pituitary adenoma tissues.

To investigate the functional effect of the binding of HMGA proteins to the ccnb2 promoter, we transiently transfected the NIH3T3 cell line, which expresses low levels of HMGA proteins, with a construct expressing the luciferase gene under the control of the mouse ccnb2 promoter region (B2-luci). As shown in Fig. 3A, cotransfection with the HMGA1, or HMGA2 expression vectors, led to a significant increase of the luciferase activity in a dose-dependent manner. Neither synergistic nor additive effect on the activity of the ccnb2 promoter was observed when both the HMGA1 and HMGA2 expression vectors were transfected together. To confirm these results in a pituitary cell context, we also performed analogous luciferase assays in the GH3 cell line. The results, shown in Fig. 3B, are quite similar to those observed in the NIH3T3 cells.

To further validate that cyclin B2 expression is dependent on HMGA proteins, we analyzed cyclin B2 mRNA and protein levels in murine embryonic fibroblasts (MEFs) derived from knockout mice for Hmga1, Hmga2, or both genes. As shown in Fig. 3C, the cyclin B2 transcript is significantly lower in Hmga1 and Hmga2 minus MEFs compared with the wild-type ones, and almost undetectable in the MEFs where both members of the HMGA family have been disrupted. Analogous results were obtained for the protein levels (Fig. 3D).

**Cyclin B2 is overexpressed in human pituitary adenomas.**

The high expression of cyclin B2 in HMGA-induced mouse pituitary adenomas and in murine and rat cell lines derived from pituitary adenomas prompted us to verify whether cyclin B2 overexpression could be a feature also of human pituitary adenomas. Therefore, we analyzed the expression of CCNB2 in a panel of 45 human pituitary adenomas (including 15 nonfunctioning adenomas, 6 somatotrophic adenomas GH-secreting, 16 prolactinomas, 2 mixed GH/PRL-cell adenomas, 6 gonadotrophic adenomas FSH-secreting) by qRT-PCR. As shown in Fig. 4A, CCNB2 was expressed in all the adenomas analyzed at a much higher level in comparison with the normal pituitary gland. Interestingly, HMGA1 and HMGA2 expression was increased in all the tumors analyzed when compared with normal tissue and a direct correlation between HMGA1, HMGA2, and CCNB2 mRNA levels was observed. In fact, as shown in Fig. 4B and C, the correlation coefficients for the fold changes between adenomas and normal gland, calculated in both CCNB2 and HMGA1, as well as CCNB2 and HMGA2 expression levels, were $r = 0.80$ ($P < 0.001$) and $r = 0.90$ ($P < 0.001$), respectively.

To verify whether the increase in Cyclin B2–specific mRNA levels corresponded to an increase also in protein levels, we evaluated Cyclin B2 protein expression in pituitary adenomas by Western blot and immunohistochemistry. As shown in Fig. 5A, a band of 51 kDa

![Figure 4. qRT-PCR analysis of CCNB2, HMGA1 and HMGA2 expression in human pituitary adenomas.](image_url)
Figure 5. Analysis of Cyclin B2 protein expression in human pituitary adenomas. A, cyclin B2 protein expression in extracts coming from pituitary adenomas of different histotypes and normal pituitary gland. Blot against vinculin has been performed as control for equal protein loading. B, paraffin sections from a representative prolactinoma and normal pituitary tissue were analyzed by immunohistochemistry using antibodies raised against Cyclin B2. a, immunostaining of a normal prolactinoma (×200) showing a cytoplasmic positivity; b, immunostaining of a representative prolactinoma (×200) showing a cytoplasmic positivity; c, immunostaining of the same samples as in b, in the absence of the primary antibody; no immunoreactivity was observed; d, immunostaining of the same prolactinoma as in b at a higher magnification (×400). NP, normal pituitary gland; NF, nonfunctioning adenoma; GH, GH-secreting adenoma; GH/PRL, GH/prolactin-secreting adenoma; PRL, prolactin-secreting adenoma.

Our above reported and previously published data suggested that the increase in cyclin B2 expression might have a role in the generation of pituitary adenomas in Hmga1- and Hmga2-transgenic mice. Therefore, we considered interesting to evaluate whether the induction of cyclin B2 by HMGA2 expression. Moreover, luciferase assays showed that both the HMGA proteins were able to up-regulate ccnb2 promoter activity. Finally, we showed an increased expression of cyclin B2 in human pituitary adenomas of different histotypes that is associated to HMGA1 and HMGA2 expression.

A clear validation of the induction of the ccnb2 gene expression comes from our analysis of ccnb2 mRNA levels in Hmga1- or Hmga2-null MEFs. In fact, ccnb2 expression was reduced in MEFs carrying a disrupted Hmga1 or Hmga2 gene, and was almost undetectable in MEFs where neither Hmga gene was expressed.

It is reasonable to retain that the decreased cyclin B2 expression in Hmga2-null and Hmga1/Hmga2-double-null mice may also account for the pygmy phenotype of the mice knockout for Hmga2 and the very small size (less than half of that of the pygmy mice) of the mice double-knockout for Hmga1 and Hmga2.7

We have previously shown that a critical mechanism in the induction of pituitary adenomas by HMGA2 is the ability of HMGA2 to interact with pRB and induce E2F1 activity in mouse pituitary adenomas by displacing HDAC1 from the pRB/E2F1 complex—a process that results in E2F1 acetylation. Subsequently, we showed that Mia/Cd-rap gene expression was suppressed in HMGA-induced pituitary adenomas. Therefore, the induction of cyclin B2 by HMGA2 could represent a further mechanism by which HMGA2 would contribute to the development of pituitary adenomas in mice and humans because HMGA2 gene amplification and overexpression have been reported by our group in human prolactinomas.13. It is reasonable to hypothesize that cyclin B2 induction by HMGA proteins may contribute to high-rate cell proliferation of the malignant neoplasias where HMGA proteins are abundantly expressed.

In conclusion, our data show that HMGA proteins positively regulate cyclin B2 expression, indicating a new possible mechanism by which they may be correlated to mouse and human pituitary tumorigenesis.

7 I. De Martino et al. manuscript in preparation.
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