

Derepression of *HMGA2* Gene Expression in Retinoblastoma Is Associated with Cell Proliferation

KAI-YIN CHAU,¹ GUIDALBERTO MANFIOLETTI,² KAM-WA CHEUNG-CHAU,¹ ALFREDO FUSCO,³
NATHALIE DHOMEN,⁴ JANE C SOWDEN,⁴ TETSUO SASABE,⁵ SHIZUO MUKAI,⁶ AND SANTA JEREMY ONO¹

To assess whether retinoblastoma formation is associated with the expression of high mobility group (HMG) A2 protein, a transcription factor that is highly expressed during embryogenesis and completely repressed in normal adult tissues, we performed Northern and Western blots and RT-PCR analyses, and immunohistochemistry to test for *HMGA2* expression. We used established retinoblastoma cell lines in tumors grown in nude mice and clinical retinoblastoma specimens, and contrasted these tumors with normal embryonic and adult retina. Adenoviral-mediated antisense experiments were conducted on the retinoblastoma cell lines to suppress *HMGA2* expression and determine if cell proliferation is *HMGA2*-dependent. We also transfected a retinoblastoma cell line to identify *cis*-regulatory elements and transcription initiation sites on the *HMGA2* gene promoter. *HMGA2* gene expression was silenced in terminally differentiated retina of 6-wk-old mice, but it was detected in retina of a 13.5-d postcoitum embryo. Reactivation of *HMGA2* gene expression was observed in the retinoblastoma cell lines Y79, WERI-Rb1, and TOTL-1, in tumors derived from some of these cells propagated in nude mice, and in a high frequency of retinoblastomas excised from human patients. This suggests that expression of *HMGA2* gene in retinoblastoma cells involves a derepression process. By using an antisense approach to block *HMGA2* expression, we observed a decrease in the number of proliferating retinoblastoma cells. As a 1st step toward understanding *HMGA2* gene reactivation in retinoblastoma, we mapped the 2 transcription initiation sites and associated positive regulatory elements within the WERI-Rb1 cells. Our discovery of derepression of *HMGA2* gene expression in retinoblastoma provides the 1st evidence that this protein might contribute to neoplastic transformation of retina cells.

INTRODUCTION

Retinoblastoma is a malignant neoplasm composed of embryonic tumor cells arising from retinoblasts of neuroepithelial origin (1). Retinoblastoma development is associated with *Rb* gene inactivation. The molecular cloning of the *Rb* gene was accomplished by 3 independent groups—a collaboration between laboratories of Thaddeus Dryja and Robert Weinberg, the lab of Wen-Hwa Lee, and the lab of Yuen-Kai Fung and William Benedict (2–4). Despite this landmark discovery, retinoblastoma formation is not understood completely. Additional “pathogenic” factors, especially those associated with dedifferentiated and/or undifferentiated retinal cells, are thought to exist. The high mobility group (HMG) A2 protein is one of the candidates we have examined, because it appears to coordinate cell proliferation and differentiation during mammalian development (5–9).

HMGA2 is a nuclear protein of 109 amino acid residues. Both *HMGA* family members, *HMGA1* and *HMGA2*, contain 3 basic DNA-binding domains termed “AT-hooks.” The interaction of *HMGA* transcription factors with AT-rich DNA sequences results in conformational changes in the DNA helix, which facilitate

loading of other transcription factors onto their *cis*-elements. Because of this feature, *HMGA* proteins are known as architectural transcription factors (10–12).

The *HMGA2* gene is strongly expressed during early stages of fetal development in all cells. In later stages, expression is restricted to particular cell types and ceases before birth (13–17). Aberrant expression of the *HMGA2* protein is observed in thyroid cells, transformed by selected oncogenic retroviruses (18–20), and in other kinds of tumor (21–25). The general correlation of *HMGA2* expression with malignancy suggests a general role for this factor in the genesis or maintenance of the transformed phenotypes (26). Indeed, overexpression of *HMGA2* results in neoplastic transformation of Rat-1 fibroblast cells (27); in addition, pituitary adenomas develop in *HMGA2* transgenic mice (28). Reactivation of *HMGA2* gene expression also is observed in mesenchymal tumors, where there are frequent rearrangements of *HMGA2* gene (29–34). Interestingly, chimeric transcripts (either as an *HMGA2* fusion or truncation) were observed in several such tumors. Aberrant expression of truncated forms of *HMGA2* protein is thought to contribute to neoplastic transformation through their roles in inducible gene expression. Experimentally, ectopic

¹Department of Immunology, Institutes of Ophthalmology and Child Health, University College London, University of London, UK;

²Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, Italy; ³Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università degli Studi di Napoli “Federico II,” Italy; ⁴Developmental Biology Unit, Institute of Child Health, University College London, UK; ⁵Department of Ophthalmology, Osaka Habikino Hospital, Osaka, Japan;

⁶Massachusetts Eye & Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, MA.

expression of the truncated HMGA2 protein, deprived of its acidic tail and carrying only the 3 DNA-binding domains, could transform NIH3T3 cells (35). In transgenic mice, ubiquitous expression of the same truncation leads to an abnormally high prevalence of lipomas (36,37). Additional evidence that the HMGA2 product contributes to cell growth stems from the discovery that somatic inactivation of the HMGA2 gene results in growth retardation and a pygmy phenotype in mice (13). This is characterized by a marked inhibition of adipose tissue development. In vitro culturing of embryonic fibroblasts from these mice also exhibits a reduced proliferative capacity.

We report here that HMGA2 expression is derepressed in a high frequency of retinoblastomas (6 of 7 retinoblastoma cell and tumor samples examined). Our finding represents the 1st example of derepression of HMGA2 gene expression in an ocular neoplasm. We provide evidence that derepression of HMGA2 expression in retinoblastoma likely is relevant to retinoblast transformation, and that reduction of HMGA2 expression significantly affects cell proliferation. Finally, we have found 2 regions on the HMGA2 gene promoter responsible for positive transcription activity in a representative retinoblastoma cell line.

MATERIALS AND METHODS

Cell Culture

Human retinoblastoma cells Y79, WERI-Rb1, and TOTL-1 were maintained as described (38,39). Human hepatoma cells PLC/PRF/5 (22), mammary epithelial cells HBL-100 (40), and osteosarcoma cells OSH50T (4) were cultured in Dulbecco's Modification of Eagle's Medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL), 50 µg per mL penicillin/50 units per mL streptomycin (Sigma, St. Louis, MO, USA), and 2 mM glutaMaxI (Gibco BRL).

Northern Blot Analysis

Total RNA was prepared from cells and tumors as described (38). RNA samples were electrophoresed in 1.5% denaturing gels and transferred to Hybond-N membrane (Amersham Pharmacia, Arlington Heights, IL, USA). The blots were hybridized separately by radiolabeled human HMGA2 (22) and HMGA1 (41) coding regions in a stringent manner as described (38); then autoradiography was performed. Comparable amount of RNA loaded between samples was monitored by the probing of 28S rRNA. For reprobing, the membranes were boiled in 0.1% SDS for 30 min to remove the previous probe.

Western Blot Analysis

Perchloric acid extract (5%) of the cells and tumor samples were resolved and transferred to Hybond-P membrane (Amersham Pharmacia) as described (22). The intensity of the histone H1 bands after Coomassie staining was used as a control for uniform loading of the protein level in each extract. HMGA proteins were detected using Western-Light Chemiluminescent Detection System (Tropix, Bedford, MA, USA), with the anti-HMGA (22) and A1-specific polyclonal antibodies (in 1:1000 dilution) (42).

Nude Mouse Injection and Immunohistochemistry

All animal procedures used in this study were conducted in accordance with The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Six-week-old Swiss *nu/nu* (nude) mice (Taconic Farms, Germantown, NY, USA) were injected subcutaneously with 10 million retinoblastoma cells or intramuscularly with 1 million osteosarcoma cells in a volume of 0.1 mL. Mice were sacrificed when tumors reached 1 cm (1 to 2 mo after inoculation), and the tumors were excised and embedded in Tissue-Tek OCT medium (Sakura, Torrance, CA, USA). Adult nude mouse thigh muscle, 6-wk-old Balb/c eyeballs, and 129/sv embryos 13.5 d postcoitum were embedded in the same manner. Frozen sections (6 microns) of OCT-embedded samples were incubated in a solution of methanol and 1% hydrogen peroxide for 30 min before immunoperoxidase staining. For HMGA protein detection, the slides were blocked overnight at 4 °C with 2% normal goat serum (NGS; Vector, Burlingame, CA, USA), then incubated with the polyclonal antibodies specifically recognizing HMGA1 (42) and HMGA2 protein (raised against a peptide specific to HMGA2: RGEAGQPSTSA), diluted 1:100 in phosphate-buffered saline (PBS) and 2% NGS, for 1 h at room temperature. Afterward, the slides were washed in PBS 3 times for 10 min and subsequently incubated with biotinylated goat anti-rabbit IgG for 1 h (Vectastain Elite ABC kits; Vector). The slides were washed for 10 min in PBS containing 0.05% Tween20 followed by 2 washes for 10 min in PBS and then incubated with the secondary antibodies as above. Staining was performed using DAB substrate (Vector).

RT-PCR Analysis

Total RNA was prepared from each surgically removed retinoblastoma samples (RBT952, RBT962, RBT965, and RBT942). First-strand cDNAs were synthesized separately from 2 µg each of the RNA samples using an oligonucleotide with sequence specific to the human HMGA2 exon 4 (5'-CTGAGCAGGCTTCTCTGAA CAAC-3') and Random Primers (Gibco BRL), by SuperScript II RNase H⁻ Reverse Transcriptase (Gibco BRL) according to the instructions of the manufacturer, with RNasin ribonuclease inhibitor (Promega, Madison, WI, USA) added. PCR was then performed from the cDNA product using 2.5 units of Promega's Taq DNA Polymerase, 2.5 units of Taq Extender PCR Additive (Stratagene), 0.2 mM dNTPs, and 1 µM of each primer in a final volume of 50 µL. Amplification involved denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, in the Programmable Thermal Blok II thermal cycler (Lab-line Instruments, Melrose Park, IL, USA). For the HMGA2 gene amplification, the oligonucleotides used have the sequence 5'-ATGAGCGCACGCGGTGAGGG-3' and 5'-CCATTCCTAG GTCTGCCTCTTGG-3', which annealed to exons 1 and 3 of the HMGA2 coding region respectively. To specifically amplify the GAPDH gene, a pair of oligonucleotides with sequence 5'-TGAT GACATCAAGAAGGTGGT-3' and 5'-CAGTGAGGGTCTCTCTC TTCC-3' was used. The PCR products were resolved onto 1.5% agarose gels. To detect amplified products of the HMGA2 gene in a specific and sensitive manner, DNA was blotted onto nylon membrane from the agarose gel and the membrane was hybridized

with the radiolabeled *HMGA2* coding region, and then subjected to autoradiography. GAPDH amplification products were visualized on agarose gels stained with ethidium bromide. Oligonucleotides used were obtained from GenoSys (Woodlands, TX, USA).

Adenoviral-mediated Antisense Expression Analysis

Replication-defective recombinant adenoviruses carrying the *HMGA2* coding sequence in an antisense orientation (Ad-A2as), and its parental vector (Ad-GFP), were prepared by Alfredo Fusco using the described method (43). Retinoblastoma cells WERI-Rb1 were exposed to Ad-A2as or Ad-GFP, in the same multiplicity of infection (MOI) of 1000 pfu per cells for 12 h, and then grown in fresh medium for 7 d. Uninfected cells were included as control. The number of viable cells was determined by Trypan blue exclusion and hemacytometry. Proliferation of cells was determined using the ViaLight HS kit (Lumitech, Nottingham, UK), in which measurements for comparison were based on the same number of cells used. Briefly, cells were lysed by the Nucleotide Releasing Reagent, ATP Monitoring Reagent was added to the lysate, and luciferase activity was measured using the Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA, USA). Light output was integrated over a 15-s period and displayed as relative light units. Background activity was found to be < 0.1% of all readings. The effectiveness of antisense *HMGA2* RNA expression in suppressing the *HMGA2* protein level in transduced retinoblastoma cells was confirmed by Western blot analysis as described above.

Transient Transfection Analysis

In each transfection (as described in 44), 3×10^6 WERI-Rb1 cells were electroporated with 10 μ g of pGL-2-Basic construct (Promega) harboring the human *HMGA2* gene 5' UTR fragment and 10 μ g of pCAT-Control Vector (Promega; served as an internal control to determine transfection efficiency by monitoring CAT expression) that were prepared by Qiagen Plasmid kit (Chatsworth, CA, USA). Transfected cells were maintained in complete RPMI medium for 44 h, before luciferase (Promega) and CAT (Roche, Indianapolis, IN, USA) assays were conducted as suggested by the manufacturers. The pGL2-Promoter Vector (Promega) containing the luciferase gene driven by the SV40 promoter was used as a positive control for comparison of promoter activities. Mock transfection and transfection with the empty reporter were included to determine the background noise. Transfections were carried out in duplicate and repeated 3 times. For OSH50T cell transfection, 2×10^5 cells were seeded onto a 35-mm plate and exposed to LipofectAMINE (Gibco BRL) mixed with 3 μ g pGL-2-*HMGA2* promoter constructs and 7 μ g pCAT-Control Vector.

Primer Extension Analysis

Oligonucleotides of sequence 5'-GGAGATGAGGTGATAGGCC TGGGG-3' and 5'-TGTTGGTGTGTGTCGTCACGCGCTTG-3' (Genosys) were end-labeled with 32 P and hybridized with total RNA, as described (45). Extension products obtained by reverse-transcription were resolved through 8% sequencing gels, along with sequencing ladders that were generated using the same oligonucleotides as markers.

RESULTS

High Level of *HMGA2* and *HMGA1* Gene Expression in Retinoblastoma Cell Lines

Figure 1A shows a Northern blot assessing *HMGA2* and *HMGA1* transcript levels in the Y79 and WERI-Rb1 retinoblastoma cells. The levels are compared with those of the hepatoma cell line PLC/PRF/5 (previously reported to express high levels of both transcripts) (22), a nonmalignant human mammary epithelial cell line HBL-100 (40), and an osteosarcoma cell line, OSH50T, lacking the Rb protein (4) as also occurs in retinoblastoma cells. The top panel shows that the level of *HMGA2* transcripts exceeds that expressed in the human hepatoma cell line PLC/PRF/5. Interestingly, the size of *HMGA2* transcripts (that is, approximately 3.9/4.6 kb) appears different from that of PLC/PRF/5 cells, suggesting different transcription initiation/termination site usage. HBL-100 and OSH50T cells do not express *HMGA2*. As expected, the 2-kb *HMGA1* mRNAs are seen in the middle panel of the 5 cell lines examined (as well as many other cultured cell lines), and are abundant in the 2 retinoblastoma cell lines (several fold higher than that in PLC/PRF/5). The same blot was reprobed with 28S rRNA to control for equal RNA loading, as shown in the bottom panel. The experiment was repeated and the same results were obtained.

In Figure 1B, Western blot shows the level of *HMGA2* (upper panel) and *HMGA1* (middle panel) protein expression in the above 5 cell lines, and an additional retinoblastoma cell line TOTL-1 (39). *HMGA2* protein (12 kD) is expressed in all 3 lines of retinoblastoma cells Y79, WERI-Rb1, and TOTL-1, and in PLC/PRF/5 hepatoma cells as previously shown (22), but not in HBL-100 or OSH50T. Results in the middle panel indicate that *HMGA1a* and *HMGA1b* proteins (isoforms of each other, 11.8 and 10.6 kD respectively) are expressed at similar levels between the 6 cell lines. In short, there is a good correlation of *HMGA2* and *HMGA1* transcript levels and protein expression. The polyclonal antibodies recognize predominantly the *HMGA2* protein (22), whereas *HMGA1* protein was specifically detected with the polyclonal antibodies raised against a N-terminal region of *HMGA1* (42). Equal total protein loading of cell extracts was assessed, by monitoring levels of histone H1 on a separate gel stained with Coomassie blue (lower panel). Similar results were obtained in the repeated experiment.

Overexpression of *HMGA2* Protein Found in Only Embryonic Retinoblastoma Cells in Tumors Grown in Nude Mice

To determine if *HMGA2* and *HMGA1* expression in cultured retinoblastoma cells is more than an in vitro phenomenon in tissue culture, 3 cell lines (Y79, WERI-Rb1, and OSH50T) were subsequently injected into and grown in nude mice (Each cell line was implanted in 5 mice). All of the mice developed tumors, and these tumors were excised and analyzed for *HMGA2* and *HMGA1* expression. Immunohistochemical analysis was performed on cryosections of tumors derived from Y79, WERI-Rb1, and OSH50T in adult nude mice thigh muscle, a retina of 129/sv mouse 13.5 d postcoitum, and a 6-wk-old Balb/c retina. *HMGA2* was detected with new and specific polyclonal antibodies;

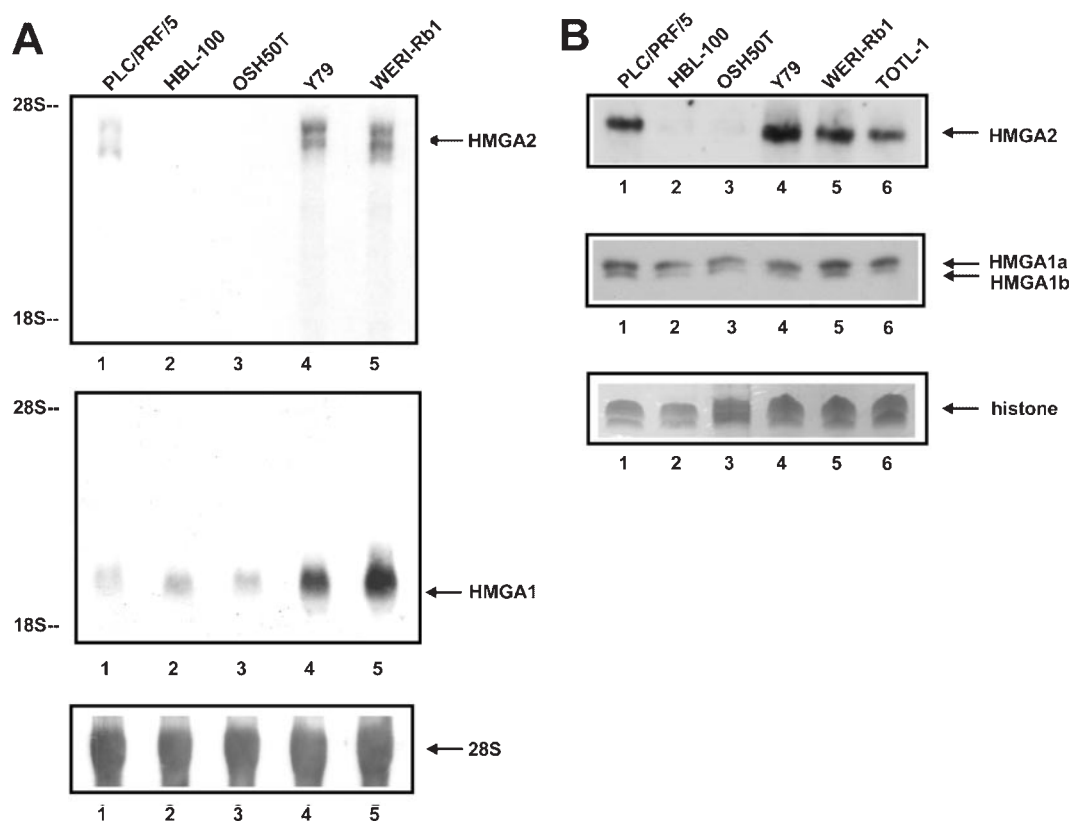


Figure 1. High-level expression of *HMGA2* and *HMGA1* mRNA and protein in cultured retinoblastoma cells. A: Northern blot of total RNA isolated from the Y79 (lane 4) and WERI-Rb1 (lane 5) retinoblastoma cells, PLC/PRF/5 hepatoma cells (lane 1), HBL-100 mammary epithelial cells (lane 2), and OSH50T osteosarcoma cells (lane 3), probed, respectively, with the radiolabeled *HMGA2* and *HMGA1* coding region DNA (upper and middle panels). 28S and 18S rRNA provide size markers of 4.8 and 1.8 kb. Uniform RNA loading in individual sample was shown by hybridizing the same Northern blot with the 28S rRNA probe (lower panel). B: Western blot of perchloric acid extracts from cells of PLC/PRF/5, HBL-100, OSH50T, Y79, and WERI-Rb1 (lanes 1 to 5), and an additional retinoblastoma cell line TOTL-1 (lane 6); cells were prepared and equal protein loading between lanes confirmed by the histone H1 band stained by Coomassie (lower panel). Anti-*HMGA2* and A1 polyclonal antibodies were used to detect *HMGA2* (upper panel) and *HMGA1a/b* (middle panel) proteins.

although they were moderate in affinity. *HMGA1* was specifically recognized by the polyclonal antibodies described above. In Figure 2A, nuclear staining with *HMGA2* and *HMGA1* antibodies was observed in the 2 retinoblastoma-induced tumors, derived from Y79 and WERI-Rb1 cells. Not every nucleus was immunostained for *HMGA2*, indicating that some cells of the tumor mass, such as the stromal cells, do not express the protein. *HMGA2* and *HMGA1* proteins were distinctively stained in the nuclei of embryonic cells, including the retina at 13.5 d postcoitum. This is consistent with previous reports (13,42). It is known that *HMGA2* is not expressed in normal adult tissues (13–17). The lack of *HMGA2* immunostaining observed in nude mouse thigh muscle and adult mouse retina confirmed this and served as background controls for our staining. Tumors derived from the osteosarcoma cell line OSH50T expressed *HMGA1* but not *HMGA2* protein. As noted earlier, *HMGA2* has a highly restricted expression pattern and the protein has been detected in only a subset of tumors (18–25). The top row of Figure 2A shows the results of the hematoxylin & eosin staining. The bottom row depicts the results of immunostaining without primary antibodies, indicating no endogenous peroxidase enzyme activity was detected. Further-

more, no immunostaining was observed when the tissues were analyzed after the antibodies had been preincubated with the corresponding immunogens (data not shown), demonstrating the specificity of the antibodies in recognizing *HMGA2* and *HMGA1* proteins. Overall, the immunohistochemical data indicates that *HMGA2* and *HMGA1* are expressed in experimental retinoblastoma and embryonic retina, and *HMGA2* is absent in adult nude mouse thigh muscle and retina. These results were highly reproducible. A high level of *HMGA1* expression in adult mouse retina was reported previously by us (38).

Because the *HMGA2* antibodies, although specific, do not provide strong immunostaining signals, Northern blot analysis for *HMGA2* (and *HMGA1*) expression of these experimental tumors was performed. The results shown in Figure 2B demonstrate the high level *HMGA2* and *HMGA1* expression in nude mouse tumors derived from 2 retinoblastoma cell lines, Y79 and WERI-Rb1.

***HMGA2* and *HMGA1* Gene Expression in Surgically Removed Retinoblastoma Samples**

To extend the analysis of *HMGA2* and *HMGA1* gene expression to clinical retinoblastoma, Northern blot and RT-PCR analysis was

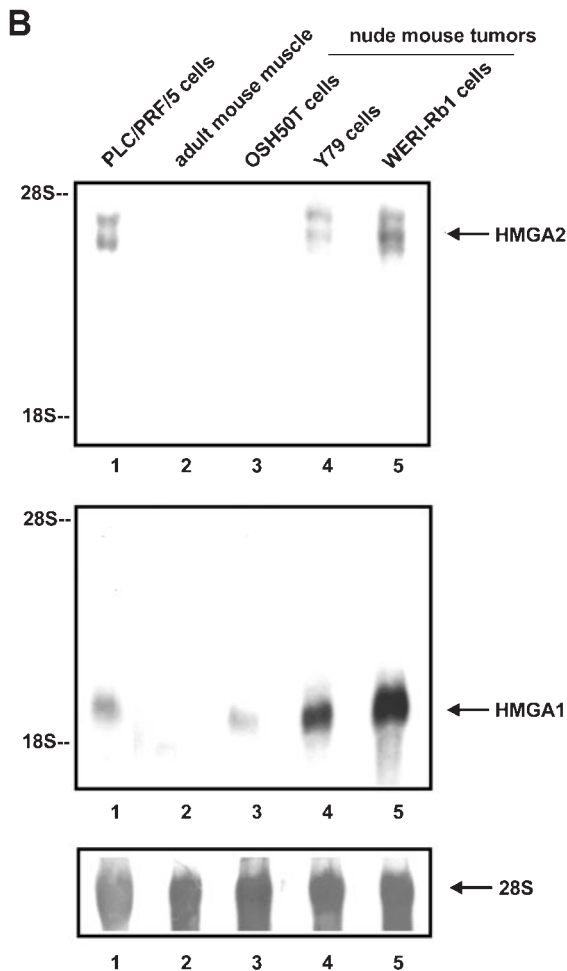
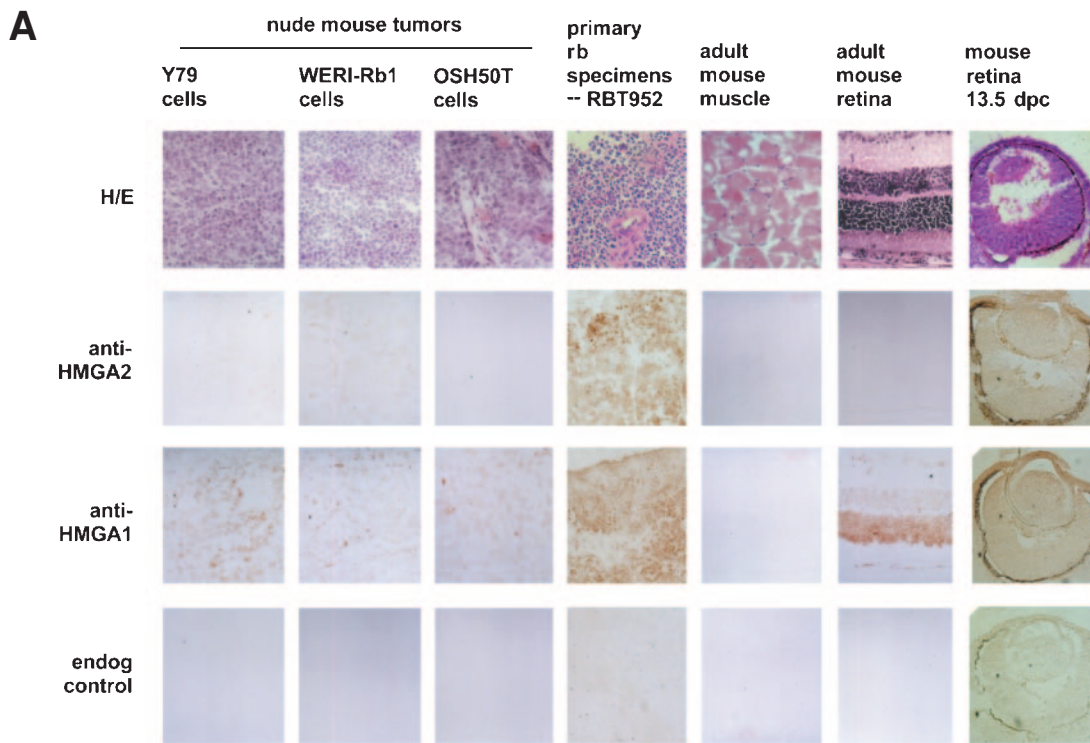


Figure 2. HMGA2 expression is in established lines of retinoblastoma cells propagated in nude mice, surgically removed retinoblastoma samples, and mouse embryonic retina, but absent in adult mouse tissues. A: Immunohistochemistry of cryosections of nude mouse tumors derived from the retinoblastoma cells Y79 and WERI-Rb1, and that from osteosarcoma cells OSH50T were hematoxylin & eosin stained, immunoperoxidase stained with the polyclonal antibodies specifically recognizing only HMGA2 protein (anti-HMGA2 panel), immunostained with the polyclonal antibodies which recognize only HMGA1 protein (anti-HMGA1 panel), and endogenous peroxidase controlled (endog control panel). HMGA proteins were detected in the primary retinoblastoma sample RBT952 (see Figure 3) as well. Retina of 129/sv mouse embryo 13.5-d postcoitum showed positive HMGA2 and HMGA1 immunostaining, whereas 6-wk-old Balb/c retina demonstrated undetectable HMGA2 protein but abundant HMGA1 protein. Thigh muscle from adult nude mouse did not stain with HMGA2 or HMGA1. Photomicrographs are magnified 400x. B: Northern blot of total RNA prepared from PLC/PRF/5 hepatoma cells (lane 1), adult mouse thigh muscle (lane 2), and nude mouse tumors derived respectively from OSH50T (lane 3), Y79 (lane 4), and WERI-Rb1 (lane 5), probed separately with radiolabeled HMGA2 (upper panel) and HMGA1 (middle panel) coding region DNA, and 28S rRNA (lower panel).

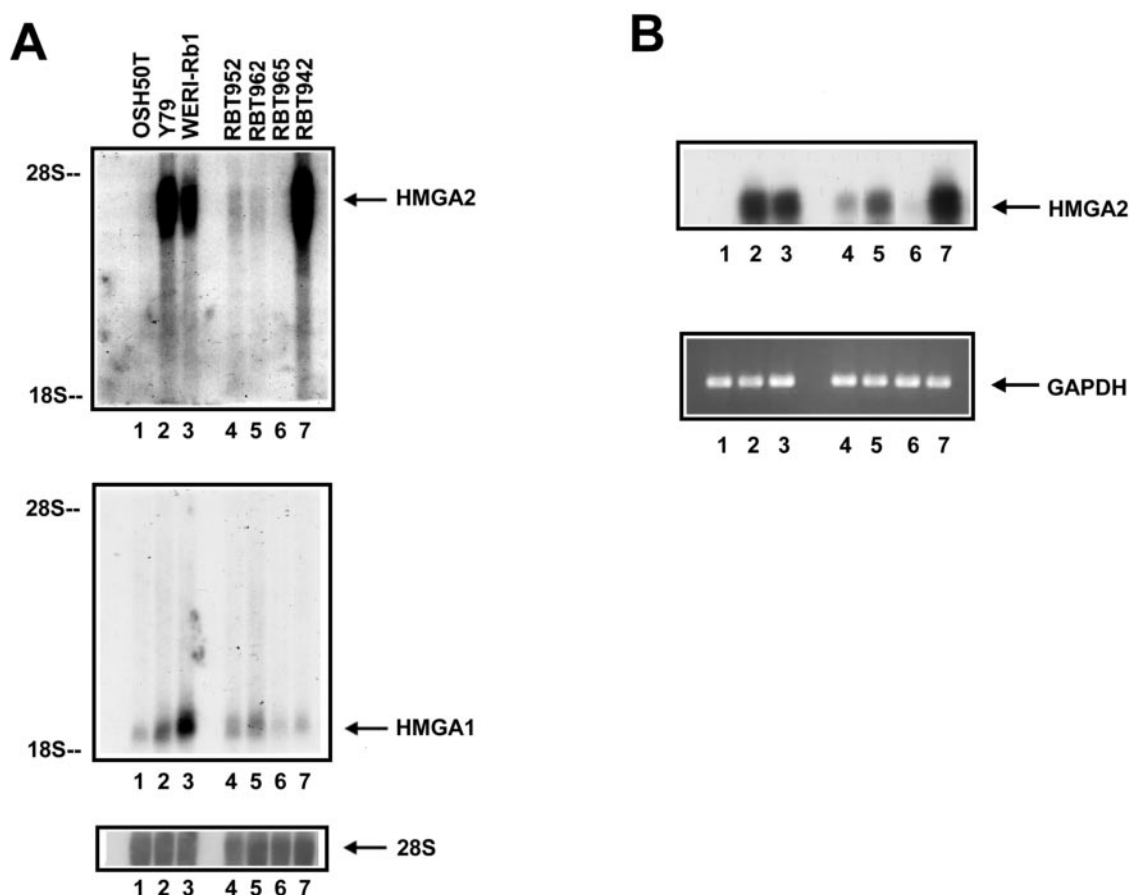


Figure 3. High prevalence of *HMGA2* gene expression in surgically removed retinoblastoma samples. Intact RNA was prepared from the cells: OSH50T (lane 1), Y79 (lane 2) and WERI-Rb1 (lane 3), and the surgically removed retinoblastoma specimens: RBT952, RBT962, RBT965, and RBT942 (lanes 4 to 7). A: Northern blot of the *HMGA2* (upper panel) and *HMGA1* (middle panel) mRNA, in which equal RNA loading was controlled by 28S probing (lower panel). Autoradiograph of the upper panel was overexposed to reveal weak signals. B: RT-PCR analysis of the same RNA samples, in which reverse transcription was performed separately using a gene-specific primer for *HMGA2* and random primers. cDNA synthesized were used in PCR amplification with pairs of *HMGA2* and *GAPDH*-specific primers. To ensure amplification is specific, PCR products from *HMGA2* gene amplification were size fractionated onto an agarose gel, blotted onto a nylon membrane and hybridized with *HMGA2* probe (upper panel). PCR products from *GAPDH* amplification, electrophoresed through an agarose gel and stained with ethidium bromide (lower panel), showed the equal quantities of cDNA used.

performed on intact RNA that was prepared from surgically removed primary retinoblastoma specimens. RNA extracted from WERI-Rb1 and Y79 cells served as positive controls and that from OSH50T as a negative control for the detection. Four specimens (RBT952, RBT962, RBT965, RBT942) were examined. Northern blot analysis detected abundant *HMGA2* transcripts in the 2 retinoblastoma cell lines and RBT942, and significant expression in RBT952 and RBT962 (Figure 3A, top panel). *HMGA1* mRNA, as expected, was present in all RNA samples (see Figure 3A, middle panel). *HMGA2* expression was evaluated also by RT-PCR in a semi-quantitative fashion (that is, linear amplification conditions) followed by blotting the amplification products for radioactive detection. (This more sensitive approach of detection also confirmed specific amplification and has been employed elsewhere [34].) RBT942 gave a strong signal of *HMGA2* expression that was comparable to that of cultured retinoblastoma cells (see Figure 3B, top panel). A weaker signal was detected in RBT952 and an even weaker one in RBT962. No reaction products were observed after PCR amplification and hybridization detection of *HMGA2* from

the RNA of OH50T cells and RBT965. Thus, 3 of 4 samples of retinoblastoma primary tumor were reproducibly shown to express the *HMGA2* gene. Taken together, of 7 retinoblastoma cell and tumor samples examined, 6 expressed the *HMGA2* gene. These results indicate that *HMGA2* and *HMGA1* gene expression is a frequent event in retinoblastoma.

Cryosections of specimen RBT952 were available for *HMGA2* and *HMGA1* immunohistochemistry. Their positive staining results, shown in Figure 2A, confirm the reactivation of *HMGA2* and *HMGA1* expression in clinical retinoblastoma.

Adenoviral-mediated Expression of Antisense *HMGA2* RNA Blocks A2 Protein Expression and Results in Growth Inhibition of Retinoblastoma Cells

Having established that *HMGA2* and *HMGA1* proteins are expressed in retinoblastoma, we investigated whether retinoblastoma cells grown in vitro are *HMGA2*-dependent. We chose to ectopically express antisense *HMGA2* RNA in WERI-Rb1 as a means to inhibit *HMGA2* expression. This approach has been

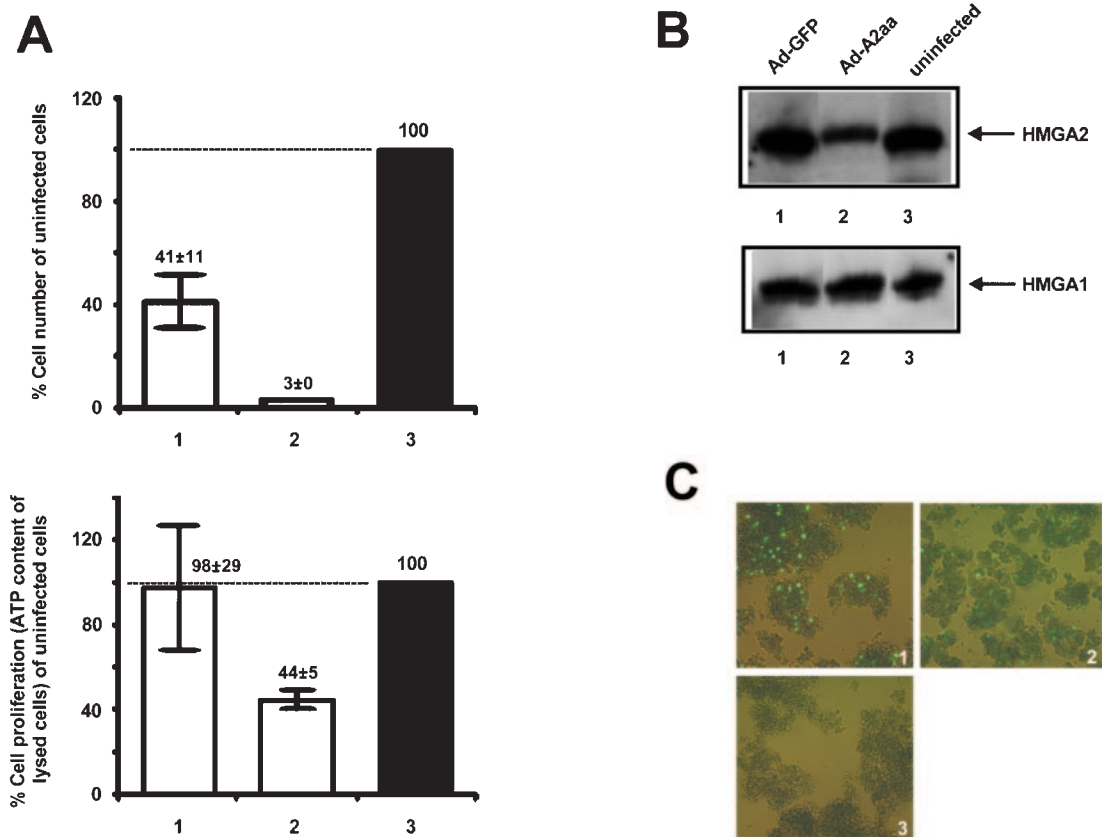


Figure 4. Antisense reduction of HMGA2 protein level affects proliferation of retinoblastoma cells. Retinoblastoma cells WERI-Rb1 were harvested after 7 d of infection (at 1000 pfu/cell) with the replication-defective recombinant adenovirus expressing GFP only (sample 1), or GFP and the *HMGA2* gene in an antisense orientation (sample 2). Same number of cells was used initially in samples 1 and 2 and in the uninfected control sample 3. A: Cell number was determined in individual infection, and the results of 3 experiments are summarized in the upper panel as mean percentage of uninfected cells, with standard error of mean values (error bars) shown. Cell proliferation was measured from the same number of cells in samples 1 to 3, and the result (mean percentage of uninfected cells and standard error) is shown in the lower panel. B: Western blot showing the level of HMGA2 and HMGA1 protein in samples 1 to 3, in which perchloric acid extracts of the samples were prepared and the amount of HMGA proteins equalized by histone H1. Anti-HMGA2 and A1 polyclonal antibodies were used to detect HMGA2 (upper panel) and A1 protein (lower panel). WERI-Rb1 cells infected with the recombinant adenovirus carrying an antisense of HMGA2 demonstrate a reduced HMGA2 protein but HMGA1 level remained unchanged. C: Fluorescent micrographs reveal the comparable green fluorescence between cells infected with recombinant GFP (panel 1) and GFP/antisense HMGA2 (panel 2) adenovirus, for 3 to 4 d, indicating similar degree of infection. No GFP fluorescence is observed in uninfected cells (panel 3). Identical sensitivity and exposure time were used.

used previously to successfully block HMGA2 expression (46). Adenoviral-mediated expression was used, in which an MOI of 300 is optimal for Y79 cells transduction (47); in our study, an MOI of 1000 appeared to be the most efficient to infect WERI-Rb1 by recombinant adenovirus. This resulted in some cytotoxicity, but a specific antisense effect was achieved (with respect to both HMGA2 expression and cell numbers). WERI-Rb1 cells were infected separately with the replication-deficient recombinant adenovirus expressing green fluorescent protein (GFP) as control, and the same recombinant carrying the *HMGA2* gene in an antisense orientation (created by Fusco's group). A comparable MOI of infections was confirmed by fluorescent microscopy on both infected cells 3 to 4 d after adenovirus exposure (Figure 4C). The WERI-Rb1 cells were harvested 7 d after exposure to the recombinant viruses; cell counts were determined and expressed as a percentage of the number of the uninfected cells (see Figure 4A, upper panel). Proliferation of the same number of infected cells

were assayed by measuring the ATP content of lysed cells (that are metabolically active) (see Figure 4A, lower panel). We note that ATP levels drop to 44%, indicative of a drop in cell proliferation. A significant reduction in the mean cell number and proliferation of WERI-Rb1 cells transduced with GFP/antisense HMGA2 adenovirus was observed when compared with uninfected cells or cells transduced with adenovirus-expressing GFP. The fact that a high MOI results in cell death could explain the baseline decrease in cell number when WERI-Rb1 was infected by adenovirus-expressing GFP. Our results suggest that change of cell number is not linearly related to change of cell proliferation, which could be explained by the ATP measurement rather than tritiated thymidine uptake as readouts for proliferation assays.

To confirm that expression of antisense *HMGA2* RNA resulted in the suppression of HMGA2 protein in WERI-Rb1 cells, perchloric acid extracts were prepared from infected and uninfected cells; the protein content of the individual cell lysate was

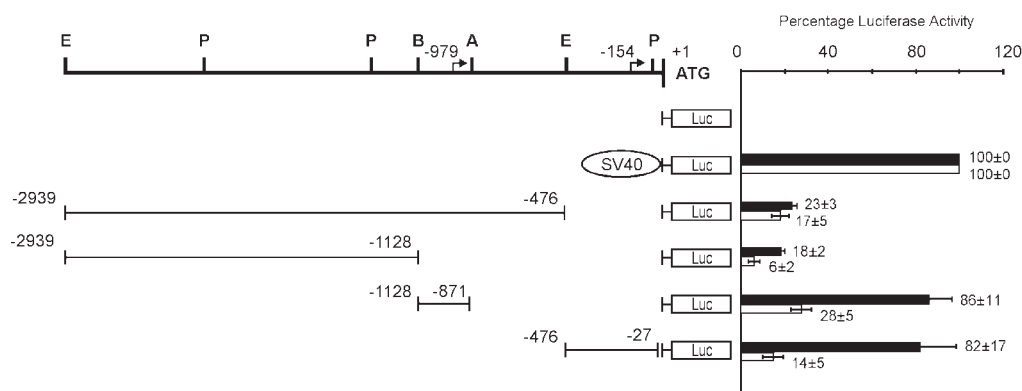


Figure 5. Identification of 2 *HMGA2* gene promoter regions active in retinoblastoma cells. Analysis was conducted in *HMGA2*-positive WERI-Rb1 and *HMGA2*-negative OSH50T cells. The schematic diagrams on the left show the organization of various plasmids constructed by subcloning different segments of the human *HMGA2* 5' UTR genomic fragments in forward orientation into the upstream region of the firefly luciferase gene (in pGL2-Basic vector) used in the transient transfection experiments. +1 corresponds to the A of the initiator methionine codon, and residues preceding it are represented by negative numbers. The top bold line shows the restriction sites of the 5' UTR (A, *ApaI*; B, *BamHI*; E, *EcoRI*; P, *PstI*) and the location of the 2 transcription start sites are represented by bent arrows (at nt -154 and -979; Figure 6 and 7). On the right is the relative luciferase activity (expressed as percent of pGL2-SV40 Promoter expression) upon cotransfection of different luciferase reporter plasmids and pCAT-Control Vector (used as internal control). Luciferase activity measured was normalized to the corresponding CAT activity for each sample. Values represent the average of 3 double transfections, and bars of standard error of mean are shown, of which black and white bars show that of WERI-Rb1 and OSH50T cells accordingly.

normalized by histone H1 on a separate gel stained with Coomassie blue (data not shown), for Western blot analysis of level of *HMGA2* and *HMGA1* proteins. The results of a representative blot, shown in the upper panel of Figure 4B, indicate a strong suppression of *HMGA2* protein expression in WERI-Rb1 cells infected with the GFP/antisense *HMGA2* adenovirus, and the *HMGA2* protein level appears unchanged by infecting with adenovirus expression GFP only. Unaltered *HMGA1* protein level was seen in the lower panel, suggesting the event leading to a reduction in cell number is independent to *HMGA1*.

Similar results were obtained from using Y79 cells for this experiment (data not shown).

Expression of *HMGA2* Gene Driven by 2 Promoters Coupled with Distinct Transcription Start Sites

After determining the expression pattern of *HMGA* proteins, in particular *HMGA2*, in retinoblastoma, the next goal was to understand the mechanism of derepression of *HMGA2* gene expression. The 1st step we took was to identify regulatory regions necessary for *HMGA2* gene expression in retinoblastoma. To accomplish this, luciferase reporter constructs harboring various regions of the *HMGA2* gene 5' UTR (48) in the forward orientation were generated and then transiently transfected into WERI-Rb1 and OSH50T cells. The results are summarized in Figure 5. The nucleotide immediately upstream from the methionine containing ATG was numbered as -1. Sequences flanking nt -27 to -476 and nt -871 to -1128 each possess strong promoter activity in WERI-Rb1 cells (82% and 86% compared with the SV40 promoter), whereas such activity was substantially less in OSH50T cells (26% and 37%, respectively). Therefore, these 2 regions function as proximal and distal promoters of the *HMGA2* gene in retinoblastoma cells. Their similar positive promoter strength matched the comparative level of the 2 *HMGA2* transcripts detected in these cells. Regions covering nt -476 to -2939 and nt -1128 to -2939 contribute minimal promoter activity in

WERI-Rb1 cells (23% and 18% of that of the SV40 promoter), suggesting the presence of negative regulatory activity between region nt -1128 to -2939 and/or region nt -476 to -871. These results indicate that expression of the *HMGA2* gene involves utilization of alternative transcription start sites. We are investigating whether the 2 transcript species utilize identical polyadenylation signals.

Subsequently, the transcription start sites associated with the 2 promoters of *HMGA2* gene were mapped, by performing primer extension on the RNA prepared from WERI-Rb1 and Y79 cells. Because *HMGA2* transcripts were not detected in OSH50T cells, RNA from this cell type served as *HMGA2* negative control in the experiment. Several oligonucleotides were made within the promoter regions to test for the presence of extended products only from retinoblastoma RNA. As shown in Figures 6A and 7A, 1 primer each from the extension reactions produced retinoblastoma-specific bands; their size predicts the proximal and distal *HMGA2* transcription start sites to be located at nt -154 and nt -979 5' to the translational start, respectively. The results also support our promoter mapping data generated via transfections.

The genomic DNA sequences of the proximal and distal promoter regions of the human *HMGA2* are shown in Figures 6B and 7B accordingly. Comparative analysis of the sequences with a database containing known binding sites for transcription factors and regulatory regions of other genes revealed several homologies (49). They are the Sp1 (CCCGCC or GGGCGG), AP-2 (CCMNSSS or YCSCCMNSSS), GCF (SCGSSSC) and S1_HS (TCCTCCTCC) sites. GCF and S1_HS both represent regulatory motifs of the epidermal growth factor receptor gene (50). Neither promoter harbors a TATA box, as also occurs in the human *HMGA1* gene 5' UTR (51).

DISCUSSION

The results of this study suggest that expression of the *HMGA2* gene is derepressed in human retinoblastoma, and that this con-

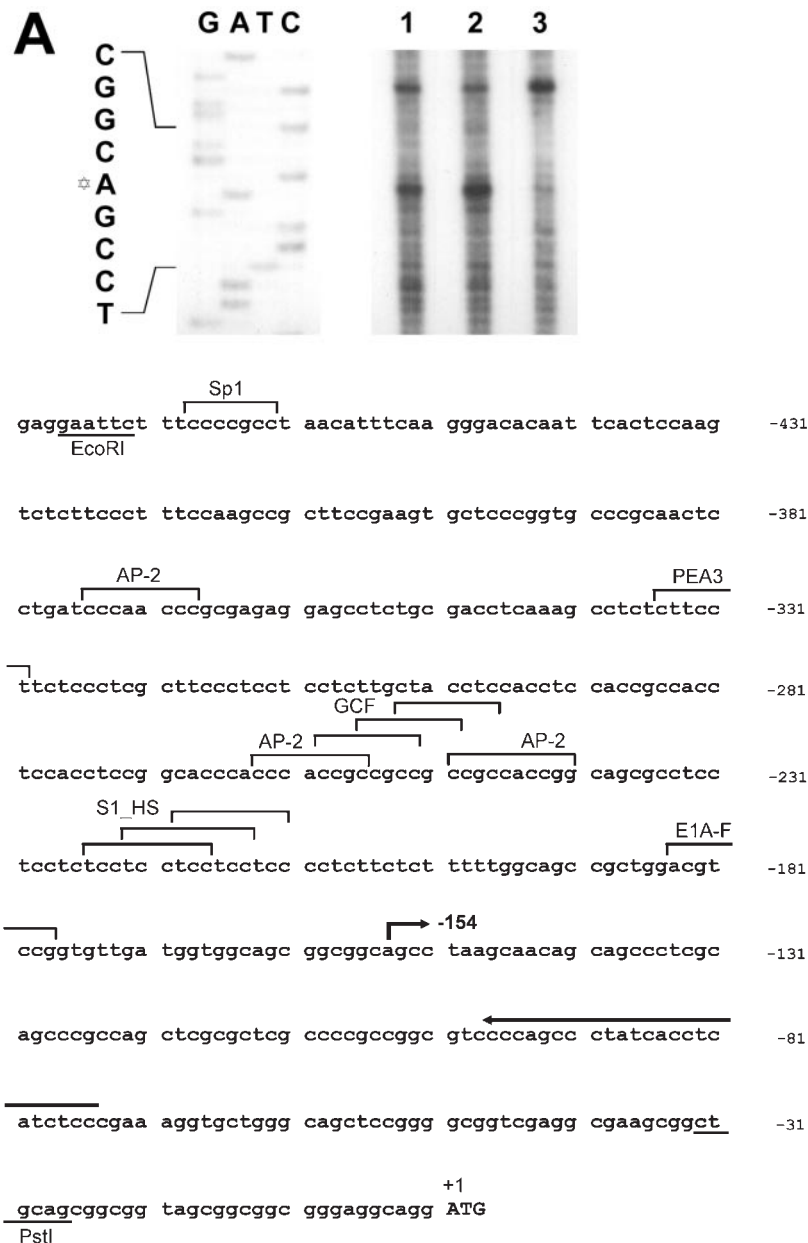


Figure 6. Analysis of the proximal *HMGA2* gene promoter region (flanking nt -27 to -476 of the genomic sequence with respect to the translational start). A: Primer extension of the *HMGA2* RNAs prepared from WERI-Rb1 (lane 1) and Y79 (lane 2) cells using an oligonucleotide complementary to nt -75 to -98 in the proximal promoter region of *HMGA2* gene. RNA extracted from OSH50T cells (lane 3) was used as negative control template. Extension products were analyzed in a 7M urea/8% polyacrylamide gel. Sequencing ladder generated from the same primer was electrophoresed simultaneously as a marker. The Davidic star to the left of the sequence indicates the transcription start site at nt -154. B: Nucleotide sequence of the proximal *HMGA2* gene promoter region. Consensus transcription factor recognition sites are shown. The arrow depicts the location of primer annealed. The bent arrow indicates the mapped transcriptional start site. Restriction sites are underlined. The upper-case ATG is the methionine start codon.

tributes to the proliferation of retinoblastoma cells. *HMGA2* is abundantly expressed in embryonic tissue, including the retina, but its expression is repressed in virtually all adult tissues, also including the retina (13–17). Our evidence of derepression of *HMGA2* expression stems from the analysis of 3 established retinoblastoma cell lines, namely Y79, WERI-Rb1, and TOTL-1 (grown in culture or in nude mice), and in 3 of a panel of 4 primary tumor specimens. In contrast to *HMGA1*, whose expression is generally induced in neoplastic cells as well as in many acti-

vated and proliferating cells (5–8), *HMGA2* gene expression is more tightly regulated. *HMGA2* expression is observed in cells of ectodermal origin such as in thyroid, breast, and lung cancers (18–25). Chimeric transcripts are also detected in several tumors of mesenchymal origin (29–34). Full-length *HMGA2* proteins were observed in the 3 retinoblastoma cell lines examined, and in 2 of them, genetic translocation of the *HMGA2* locus was not detected (data not shown), suggesting genetic alteration is not the cause of inducing *HMGA2* expression. Our characterization on

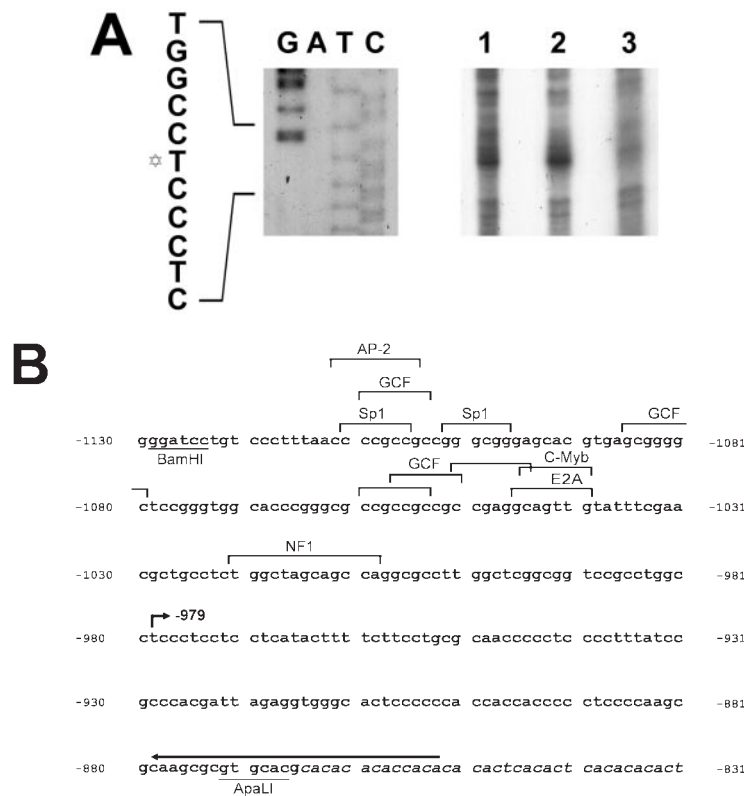


Figure 7. Analysis of the distal *HMGA2* gene promoter region (flanking nt -871 to -1128 of the genomic sequence with respect to the translational start). A: Primer extension of the *HMGA2* RNA prepared from WERI-Rb1 (lane 1) and Y79 (lane 2) cells using an oligonucleotide complementary to nt -853 to -879 in the distal promoter region of *HMGA2* gene. RNA extracted from OSH50T cells (lane 3) was used as negative control template. Extension products were analyzed in a 7M urea/8% polyacrylamide gel. Sequencing ladder generated from the same primer was electrophoresed simultaneously as a marker. The Davidic star to the left of the sequence indicates the transcription start site at nt -979. B: Nucleotide sequence of the distal *HMGA2* gene promoter region. Consensus transcription factor recognition sites are shown. The arrow depicts the location of primer annealed. The bent arrow indicates the mapped transcriptional start site. Restriction sites are underlined. Sequence of the CA repeat is shown in *italic*.

HMGA2 (and *HMGA1*) expression pattern provides the 1st evidence that aberrant *HMGA2* expression correlates with and perhaps contributes to the pathogenesis of retinal tumor. In addition, specific blockade of *HMGA2* (in which *HMGA1* remained unchanged) gene expression, with an antisense adenoviral-mediated approach, resulted in a decrease in cell number/proliferation of retinoblastoma cells. Finally, we isolated 2 regions on the *HMGA2* promoter in retinoblastoma cells responsible for such gene reactivation and specifically mapped their corresponding transcription initiation sites. This is the 1st step to assess the mechanisms controlling *HMGA2* gene derepression.

The 1st evidence implicating *HMGA2* in the pathogenesis of thyroid tumors came from antisense experiments (46). However, *HMGA2* protein did not appear to behave as a classical oncogene because ectopic expression did not by itself transform thyroid cells and NIH3T3 fibroblasts (46,35). Instead, it behaves as an oncogene in a subset of tumors, in particular those composed of undifferentiated or dedifferentiated cells and some other highly transformed cells (5-8). Therefore it appears that *HMGA2* is necessary but not sufficient for cell transformation in selected cell types.

Although the results of our antisense experiments support a role of *HMGA2* in the growth of cultured retinoblastoma cells, we

have yet to demonstrate a general requirement *in vivo* of *HMGA2* in retinoblastoma development. The 3 classical retinoblastoma cell lines used in this study, namely WERI-Rb1, Y79, and TOTL-1, are spontaneously transformed cells lacking an *Rb* signal. *Rb* inactivation, however, does not seem to directly activate *HMGA2* gene expression. The OSH50T cell line established from a patient with bilateral retinoblastoma and a homozygous deletion in the *Rb* gene (4) did not express *HMGA2*. Rather, the undifferentiated/dedifferentiated character of retinoblastoma cells appears to permit *HMGA2* gene expression. The possibility that *HMGA2* expression may be required to maintain the undifferentiated/dedifferentiated nature of this "embryonic" tumor is ruled out by the general observation that *HMGA2* is expressed in "differentiated" transformed cells as well, such as malignant PLC/PRF/5 hepatoma cells (22). Indeed, retinoblastoma cells transduced with the recombinant adenovirus to suppress *HMGA2* expression did not become differentiated, indicating that *HMGA2* level does not control such differentiation.

To investigate the mechanism of *HMGA2* gene expression in retinoblastoma, which may allow us in the future to inhibit the derepression process of *HMGA2* gene expression, we demonstrated the presence of 2 distinct promoter regions in WERI-Rb1 cells used for transcription, each with its own start sites. This was

shown by transfection studies using luciferase reporter constructs carrying various regions of the 5' flanking sequence of the *HMGA2* gene, and by primer extension analysis performed on RNA prepared from the 2 retinoblastoma cells compared with that from the osteosarcoma cells OSH50T that do not express *HMGA2*. Together, our results demonstrate that the 2 transcriptional start sites are located at nt 154 and 979 5' upstream from the translational start codon, and they are associated with putative transcriptional elements like AP-2, Sp1, and selected regulatory elements in the epidermal growth factor receptor gene promoter (50). Because 2 different sized transcripts were identified from Northern blot analysis (3.9 and 4.6 kb), these results suggest that expression of *HMGA2* gene in retinoblastoma utilizes alternative transcription starts and may have a common terminus.

Understanding the role that *HMGA2* plays in pathobiology and molecular mechanisms of retinoblastoma occurrence is biologically and clinically significant, and *HMGA2* may become a therapeutic target. One approach would be to block *HMGA2* protein expression in retinoblastoma cells, hopefully leading to inhibition of cell proliferation. Another approach is to inhibit *HMGA2* gene expression by targeting its promoters.

Our future experiments will determine (1) whether *HMGA2* expression is required for, and collaborates with other factors in, retinoblastoma formation by modifying expression of *HMGA2* protein and relevant factors (such as p107, Rb) in retinal cells by mouse modeling; and (2) to further identify and characterize functional *cis*- and *trans*-factors associated with the *HMGA2* promoter, leading to the derepression of *HMGA2* gene expression in retinoblastoma, as was performed in PLC/PRF/5 cells (45).

ACKNOWLEDGMENTS

We thank Drs Gilbert Jay, C Kathleen Dorey, Patricia Pearson, and other members of our laboratory, in particular: Drs Andrea Keane-Myers, Dai Miyazaki, Paola Arlotta, Takao Nakamura, and Jason Kreisberg who provided advice and help with the manuscript. We also thank Dr Robert Weinberg, Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, for the gifts of Y79, WERI-Rb1, and OSH50T cells; and the Tissue Culture Core Facility of the Lombardi Cancer Center for HBL-100 cells. This work was supported by National Institute of General Medical Science grant RO1 GM49661, the Lucille P Markey Charitable Trust, the Research to Prevent Blindness Foundation, and the "Associazione Italiana per la Ricerca sul Cancro." GM was supported by "Fondazione Italiana per la Ricerca sul Cancro" while he was a Visiting Scientist of the Ono Lab. SJO was a JSPS Visiting Professor at Kyoto University during the preparation of this manuscript. K-YC was a Fellow of the Research to Prevent Blindness, America Fund (PD97054). SM was supported by the Massachusetts Lions Eye Research Fund and Gifts to the Retina Genetics Fund, Massachusetts Eye & Ear Infirmary.

Address correspondence and reprint requests to Santa Jeremy Ono, Department of Immunology, Institutes of Ophthalmology and Child Health, University College London, University of London, UK. Phone: +44-20-7608-4069; Fax: +44-20-7608-4044; e-mail: santa.ono@ucl.ac.uk.

Submitted June 2, 2003; accepted for publication June 11, 2003.

REFERENCES

- Albert DA, Jakobiec FA (eds). (1994) *Principles and practice of ophthalmology*. Saunders, Philadelphia.
- Friend SH et al. (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 323:643-6.
- Lee WH et al. (1987) Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* 235:1394-9.
- Fung YK et al. (1987) Structural evidence for the authenticity of the human retinoblastoma gene. *Science* 236:1657-61.
- Goodwin G. (1998) Molecules in focus: the high mobility group protein, HMGI-C. *Int J Biochem. Cell Biol.* 30:761-6.
- Zhou X, Chada K. (1998) HMGI family proteins: architectural transcription factors in mammalian development and cancer. *Keio J. Med.* 47:73-7.
- Liu F, Chau KY, Arlotta P, Ono SJ. (2001) The HMGI proteins: dynamic roles in gene activation, development, and tumorigenesis. *Immunol. Res.* 24:13-29.
- Reeves R. (2001) Molecular biology of HMGA proteins: hubs of nuclear function. *Gene* 277:63-81.
- Fedeles M et al. (2001) Role of the high mobility group A proteins in human lipomas. *Carcinogenesis* 22:1583-91.
- Wolffe A. (1994) Architectural transcription factors. *Science* 264:1100-1.
- Mantovani F et al. (1998) NF- κ B mediated transcriptional activation is enhanced by the architectural factor HMGI-C. *Nucleic Acids Res.* 26:1433-9.
- Noro B et al. (2003) Molecular dissection of the architectural transcription factor HMGA2. *Biochemistry* 42:4569-77.
- Zhou X et al. (1995) Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. *Nature* 376:771-4.
- Zhou X et al. (1996) Genomic structure and expression of the murine HMGI-C gene. *Nucleic Acids Res.* 24:4071-7.
- Rogalla P et al. (1996) HMGI-C expression patterns in human tissues, implications for the genesis of frequent mesenchymal tumors. *Am. J. Pathol.* 149:775-9.
- Hirning-Folz U et al. (1998) The expression pattern of the HMGI-C gene during development. *Gene Chrom. Cancer* 23:771-4.
- Chieffi P et al. (2002) HMGA1 and HMGA2 protein expression in mouse spermatogenesis. *Oncogene* 21:3644-50.
- Giancotti V et al. (1985) Changes in nuclear proteins following transformation of rat thyroid epithelial cells by a murine sarcoma retrovirus. *Cancer Res.* 45:6051-7.
- Giancotti V et al. (1987) Elevated levels of a specific class of nuclear phosphoproteins in cells transformed with v-ras and v-mos oncogenes and by co-transfection with c-myc and polyoma middle T genes. *EMBO J.* 6:1981-7.
- Giancotti V et al. (1991) Comparison of multiple forms of the high mobility group I proteins in rodent and human cells. Identification of the human high mobility group I-C protein. *Eur. J. Biochem.* 198:211-6.
- Manfioletti G et al. (1991) cDNA cloning of the HMGI-C phosphoprotein, a nuclear protein associated with neoplastic and undifferentiated phenotypes. *Nucleic Acids Res.* 19:6793-7.
- Patel UA et al. (1994) Expression and cDNA cloning of human HMGI-C phosphoprotein. *Biochem. Biophys. Res. Commun.* 201:63-70.
- Rogalla P et al. (1997) Expression of HMGI-C, a member of the high mobility group protein family, in a subset of breast cancers: relationship to histologic grade. *Mol. Carcinog.* 19:153-6.
- Rogalla P et al. (1998) HMGI-C expression patterns in non-small lung cancer and surrounding tissue. *Anticancer Res.* 18:3327-30.
- Finelli P et al. (2002) The High Mobility Group A2 gene is amplified and overexpressed in human prolactinomas. *Cancer Res.* 62:2398-405.
- Wunderlich V, Bottger M. (1997) High-mobility-group proteins and cancer: an emerging link. *J. Cancer Res. Clin. Oncol.* 123:133-40.
- Wood LJ, Maher JF, Bunton TE, Resar LM. (2000) The oncogenic properties of the HMGI-C gene family. *Cancer Res.* 60:4256-61.
- Fedeles M et al. (2002) Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. *Oncogene* 21:3190-8.
- Schoenmakers EFP et al. (1995) Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours. *Nature Genet.* 10:436-44.
- Ashar HR et al. (1995) Disruption of the architectural factor HMGI-C: DNA-binding AT hook motifs fused in lipomas to distinct transcriptional regulatory domains. *Cell.* 82:57-65.
- Kazmierczak B et al. (1996) HMGI-C rearrangements as the molecular basis for the majority of pulmonary chondroid hamartomas: a survey of 30 tumors. *Oncogene* 12:515-21.
- Berner JM et al. (1997) HMGI-C, the gene for an architectural transcription factor, is amplified and rearranged in a subset of human sarcomas. *Oncogene* 14:2935-41.
- Pedeutour F et al. (2000) Dysregulation of HMGI-C in a uterine leiomyoma with a complex rearrangement including chromosomes 7, 12, and 14. *Genes Chrom. Cancer* 27:209-15.
- Finelli P et al. (2002) The High Mobility Group A2 gene is amplified and overexpressed in human prolactinomas. *Cancer Res.* 62:2398-405.

35. Fedele M et al. (1998) Truncated and chimeric HMGI-C genes induce neoplastic transformation of NIH3T3 murine fibroblasts. *Oncogene* 17:413-8.
36. Battista S et al. (1999) The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. *Cancer Res.* 59:4793-7.
37. Arlotta P et al. (2000) Transgenic mice expressing a truncated form of the high mobility group i-c protein develop adiposity and an abnormally high prevalence of lipomas. *J. Biol. Chem.* 275:14394-400.
38. Chau KY et al. (2000) The architectural transcription factor high mobility group I(Y) participates in photoreceptor-specific gene expression. *J Neurosci.* 20:7317-24.
39. Sasabe T et al. (1991) Cyclic nucleotides and differentiation of retinoblastoma. *Folia Ophthalmol.* 42:1200-6.
40. Vanhamme L, Szpirer C. (1988) Transforming activity of the human mammary line HBL100 DNA is associated with SV40 T antigen genetic information integrated in its genome. *Carcinogenesis* 9:653-5.
41. Johnson KR et al. (1988) Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG-I(Y). *J. Biol. Chem.* 263:18338-42.
42. Chiappetta G et al. (1996) High level expression of the HMGI(Y) gene during embryonic development. *Oncogene* 13:2439-46.
43. Scala S et al. (2000) Adenovirus-mediated suppression of HMGI(Y) protein synthesis as potential therapy of human malignant neoplasias. *Proc. Natl. Acad. Sci. U.S.A.* 97:4256-61.
44. Chau KY, Ono SJ. (1999) Gene transfer into retinoblastoma cells. *Biotechniques* 26:444-6.
45. Chau K et al. (1999) A novel downstream positive regulatory element mediating transcription of the human high mobility group (HMG) I-C gene. *FEBS Lett.* 457:429-36.
46. Berlingieri MT et al. (1995) Inhibition of HMGI-C protein synthesis suppresses retrovirally induced neoplastic transformation of rat thyroid cells. *Mol. Cell Biol.* 15:1545-53.
47. White JB, Taylor RE, Pittler SJ. (2001) Reproducible high efficiency gene transfer into Y79 retinoblastoma cells using adenofection. *J. Neurosci. Methods* 106:1-7.
48. Chau K-Y et al. (1995) The gene for the human architectural transcription factor HMGI-C consists of five exons each coding for a distinct functional element. *Nucleic Acids Res.* 23:4262-6.
49. Prestridge DS. (1991) Signal scan: a computer program that scan DNA sequences for eukaryotic transcriptional elements. *CABIOS* 7:203-6.
50. Ishii S et al. (1985) Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. *Proc. Natl. Acad. Sci. U.S.A.* 82:4920-4.
51. Friedmann M et al. (1993) Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene. *Nucleic Acids Res.* 21:4259-67.