MOLECULAR AND CELLULAR BIOLOGY, Dec. 2003, p. 9104–9116 Vol. 23, No. 24
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.24.9104–9116.2003

Transcriptional Activation of the Cyclin A Gene by the Architectural Transcription Factor HMGA2

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Received 5 June 2003/Returned for modification 15 July 2003/Accepted 19 September 2003

The HMGA2 protein belongs to the HMGA family of architectural transcription factors, which play an important role in chromatin organization. HMGA proteins are overexpressed in several experimental and human tumors and have been implicated in the process of neoplastic transformation. *Hmga2* knockout results in the *pygmy* phenotype in mice and in a decreased growth rate of embryonic fibroblasts, thus indicating a role for HMGA2 in cell proliferation. Here we show that HMGA2 associates with the E1A-regulated transcriptional repressor p120^{E4F}, interfering with p120^{E4F} binding to the cyclin A promoter. Ectopic expression of HMGA2 results in the activation of the cyclin A promoter and induction of the endogenous cyclin A gene. In addition, chromatin immunoprecipitation experiments show that HMGA2 associates with the cyclin A promoter only when the gene is transcriptionally activated. These data identify the cyclin A gene as a cellular target for HMGA2 and, for the first time, suggest a mechanism for HMGA2-dependent cell cycle regulation.

HMGA2 belongs, together with HMGA1a and HMGA1b, to the HMGA family of nuclear proteins, whose expression has functional implications in the pathogenesis of several human tumors (2, 20, 47, 50). A strong association between the expression of these proteins and the transformed phenotype has been observed since they were first described, in transformed rat thyroid cells (24, 25), and was later found in several human neoplasias (e.g., colon, prostate, cervical, and thyroid carcinomas) (4, 10, 17, 51). Their expression is very low, if not absent, in adult tissues and is restricted to embryogenesis (11, 28, 32, 55).

The first evidence of a direct role played by these factors in tumorigenesis came from transfection of an antisense construct for HMGA2 in normal rat thyroid cells that prevented the neoplastic transformation induced by myeloproliferative sarcoma virus and kirsten murine sarcoma virus (7). More recently, the increased expression of all three HMGA family members was shown to lead to transformation with anchorage-independent cell growth (53, 54), and the overexpression of HMGA1 was also shown to promote tumor progression in human breast epithelial cells (41). Of additional interest, rearrangements of the *HMGA2* gene, resulting in the loss of the acidic C-terminal tail, have been frequently detected in benign human tumors of mesenchimal origin (50). Indeed, transgenic mice expressing the truncated HMGA2 protein develop tumors such as lipomas and natural killer lymphomas (1, 3, 6, 18), while mice expressing wild-type HMGA2 develop pituitary adenomas (19).

Despite this evidence, the molecular events and the precise role played by HMGA2 in cell proliferation and tumorigenesis still need to be defined.

HMGA proteins contain about 100 amino acid residues and have three DNA-binding domains which have been named AT hooks because of their ability to interact with the narrow minor groove of AT-rich DNA sequences (42). These nuclear proteins, by binding to DNA and/or to transcription factors, can organize the assembly of nucleoprotein-DNA transcriptional complexes (called enhanceosomes) at the level of enhancers or promoters, resulting in enhancement or repression of transcription. For this reason, they are referred to as architectural transcription factors (42).

Since a critical step in the assembly of enhanceosomes is the ability of HMGA to establish protein-protein interactions with other nuclear factors, we used different approaches to search for new HMGA2-interacting proteins and identified the transcription factor p120^{E4F}. p120^{E4F} is a ubiquitously expressed GLI-Krüppel-related mammalian transcription factor which was first identified as a cellular factor binding to regulatory regions of the adenovirus E4 promoter and which is responsible for E4 regulation of expression in the course of adenoviral infection (21). p120^{E4F} is likely to play a key role in mammalian cell cycle control. In fact, ectopic expression of p120^{E4F} leads to growth suppression, an effect which is mediated by the interaction with the tumor suppressors pRB (15), p14ARF (43), and p53 (46). Moreover, overexpression of p120^{E4F} in NIH 3T3 cells inhibits progression from G_1 to S phase by a mechanism that involves the repression of cyclin A (16). This effect is mediated by p120^{E4F} binding to a cyclic AMP-responsive element (CRE) which is required for full transcriptional

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activation of cyclin A gene (5, 13). The CRE site is also recognized by CREB and ATF family members, raising the question of how the access of these factors to DNA is modulated.

Here we report that HMGA2 associates in vitro and in vivo with $p120^{E4F}$. Ectopic expression of HMGA2 activates the cyclin A promoter, counteracting the repressing activity of $p120^{E4F}$ exerted through the CRE element. Moreover, we show that HMGA2 is able to induce the expression of the endogenous cyclin A gene. Finally, chromatin immunoprecipitation experiments indicate that association of HMGA2 with the promoter is regulated during the cell cycle, positively correlating with transcriptional activation. The identification of cyclin A, a key factor in cell cycle control, as a functional cellular target for HMGA2 provides for the first time a mechanism that might reveal the oncogenic functions of this factor.

MATERIALS AND METHODS

Cell lines. All of the cell lines used were grown at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). CHO is a Chinese hamster ovary cell line, 293 is a human embryonic kidney cell line, and SaOS-2 is a human osteosarcoma cell line.

Plasmids. Plasmids pGEX HMGA2, pGEX TRX44/63, and pGEX TRX53/72 expressing the human HMGA2 and two HMGA2 peptides within the *Escherichia coli* thioredoxin in fusion with the glutathione *S*-transferase (GST) were already described (36). Plasmids pcDNA3HA-E4F 2.5K, -E4F 694, -E4F 551, -E4F 427, -E4F Δ60-358, -E4F 262, -E4F 162, -E4F Δ60-783, and -E4F Δ350-783, expressing wild-type $p120^{E4F}$ and deletion mutants in frame with a hemagglutinin (HA) epitope used for in vitro translation and transfections, were described previously (46). pcDNA3HA-E4F Δ153-299 was generated by PCR with pcDNA3HA-E4F 2.5K as a template and the primers 5′-CTGCTGAATTCGAGCTGGGAGAC GGTGAGA-3′ and 5′-CCAATAGTCTAGATCCAGCTCCAGAACCTG-3′. pGEX E4F Δ60-358 was obtained by cloning the sequence coding for the deletion mutant Δ60-358 into the *Eco*RI restriction site of the pGEX-4T1 vector. Plasmids pAR $HMGA2_{wt}$, pAR $HMGA2_{94}$, pAR $HMGA2_{83}$, pAR $HMGA2_{MAD}$, pAR $HMGA2_{73}$, pAR $HMGA2_{54}$, and pAR $HMGA2_{43}$, used for the expression of recombinant proteins used in far-Western experiments, were described previously (36). The pcDNA3HA-HMGA2 and pcDNA3-HMGA2 plasmids for the expression of wild-type HMGA2 in transfection experiments were obtained by cloning the HMGA2 open reading frame into the *Bam*HI and *Xho*I restriction sites of the pcDNA3HA vector and the *Bam*HI site of the pcDNA3 vector, respectively.

The $CycA_{wt}$ reporter plasmid was obtained by cloning into the *Kpn*I and *Hin*dIII restriction sites of the pGL2Basic vector a 213-bp fragment of the human cyclin A promoter (from bp −165 to +48 relative to the most 3′ transcription initiation site), which was generated by PCR with oligonucleotides 5′-CTCCG GTACCAGCCAGTTTGTTTCTC-3′ and 5′-TGGCAAGCTTAAGACGCCC AGAGATG-3′. The $CycA_{mut}$ reporter plasmid was obtained by using a splice overlap extension PCR technique with the internal oligonucleotides 5′-TTGAC TCGAGTCAAGGCCGCGAGCGCTTT-3′ and 5′-CCTTGACTCGAGTCAA GGCGACAGGGTCA-3′, in order to mutate the CRE site with the insertion of an *Xho*I restriction site (underlined).

GST pull-down assays and far-Western blot analysis. Expression and purification of GST fusion proteins for GST pull-down assays were carried out by standard protocols. Proteins were translated in vitro by using a commercial in vitro transcription-translation kit (TNT; Promega), with [^{35}S]methionine (NEN Life Science) according to the manufacturer's instructions. GST pull-down assays were carried out essentially as described previously (36). For far-Western experiments, recombinant HMGA2 proteins were expressed and purified as previously described (33). One microgram of each of the HMGA2 recombinant proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. Far-Western blotting was then carried out as described previously (36).

Immunoprecipitation and Western blot analysis. Subconfluent 293 cells seeded on 100-mm-diameter petri dishes were transfected with the indicated expression vectors by the conventional calcium phosphate procedure. Thirty-six hours later, cells were washed with ice-cold phosphate-buffered saline and then harvested in 1 ml of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris HCl [pH 7.5], 0.5% NP-40, 10% glycerol) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitors. Lysis was performed at 4°C for 20 min. The lysates were then clarified by centrifugation and incubated for 3 h at 4°C with anti-HMGA2 antibody (36) prebound to 20 μl of protein A–Sepharose CL-4B (Amersham Biosciences). The beads were then washed three times in 1 ml of ice-cold lysis buffer, and the bound proteins were solubilized by addition of 20 μl of SDS-containing sample buffer. Western blot analysis was performed by standard procedures with anti-HA primary antibody (Roche). Bound primary antibodies were visualized by enhanced chemiluminescence.

Transfections, luciferase assays, and immunocytochemical analysis. Transfections were performed by the standard calcium phosphate precipitation method. CHO cells were plated at a density of 0.7×10^6 cells per 60-mm-diameter culture dish and processed 32 h after removal of the precipitates. For luciferase assay, cells were transfected with 1 μg of the reporter construct, the indicated amount of the expression vectors, and 0.1 μg of pRL-CMV *Renilla* luciferase expression vector (Promega) to normalize for transfection efficiencies. The assay was performed with the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

For immunocytochemistry, SaOS-2 cells were plated 24 h before transfection at a density of 0.5×10^6 cells per 60-mm-diameter culture dish with a glass coverslip and transfected with 5 μg of pcDNA3HA-HMGA2 expression vector. At 32 h after removal of the precipitates, cells were fixed with 3% paraformaldehyde, permeabilized with 0.3% Triton X-100, and incubated with an anti HA-specific mouse monoclonal antibody (Roche) and an anti-cyclin A rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.). Immunofluorescence was monitored by incubation with an anti-mouse Cy3-conjugated secondary antibody (Sigma) and an anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Sigma). Nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole).

RT-PCR. SaOS-2 cells were plated 24 h before transfection in 100-mm-diameter culture dishes and transfected with 5 μg of pcDNA3HA-HMGA2 or pcDNA3HA expression vector. At 36 h after transfection, RNA was extracted by the guanidinium thiocyanate method, and reverse transcription (RT) was performed in a final volume of 50 μl with 2 μg of total RNA, 50 nmol of random nonamer primers (Sigma), 1× reaction buffer, and 200 U of Moloney murine leukemia virus reverse transcriptase (RNase H Minus; Promega). Twenty-five cycles of PCR were performed in a 50-μl volume with 10 μl of the RT mixture, 0.05 μl of [α-^{32}P]dCTP (Perkin-Elmer Life Science), 1× PCR buffer, and 50 pmol of each cyclin A primer or 5 pmol of each GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer. cDNA templates were incubated for 4 min at 94°C and amplified with cycles of 92°C for 1 min, 45°C for 1 min, and 75°C for 90 s, with a final extension for 5 min at 75°C. In order to compare the PCR products semiquantitatively, 20 to 30 PCR cycles were performed to determine the linearity of the PCR amplification, and the amplified GAPDH cDNA served as an internal control for cDNA quantity and quality. The primers for cyclin A were 5′-GCCATTAGTTTACCTGGACCCAGA-3′ and 5′-CACTGACATGGA AGACAGGAACCT-3′, and those for GAPDH were 5′-CAGCGACACCCAC TCCTCCACCTT-3′ and 5′-CATGAGGTCCACCACCCTGTTGCT-3′. Amplified PCR products were separated on an 8% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer and visualized by autoradiography. Amplification yielded amplified fragments of the predicted sizes (cyclin A, 354 bp; GAPDH, 124 bp).

EMSAs. The indicated amounts of purified recombinant GST-E4F Δ60-358 were incubated with ^{32}P-labeled DNA in 20 μl of binding buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 0.4 mM $ZnCl_2$, 0.25 mg of bovine serum albumin per ml, and 4% glycerol. For competition binding reactions, the unlabeled competitors were included in the reaction mixtures at the indicated molar excess. When assayed, high-pressure liquid chromatography-purified recombinant $HMGA2_{wt}$ and BSA proteins were included in the indicated amounts. After incubation for 20 min at room temperature, samples were loaded onto a native 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer, electrophoresed at 4°C at 15 V/cm, dried, and exposed to X-ray film (Hyperfilm MP; Amersham Biosciences). The following oligonucleotides (only the upper strand is shown) were used in electrophoretic mobility shift assay (EMSA) experiments: $CycA_{wt}$, 5′-TGTCGCCTTGAATGA CGTCAAGGCCGCGA-3′; $CycA_{mut}$, 5′-TGTCGCCTTGACTCGAGTCAAG GCCGCGA-3′.

Chromatin immunoprecipitation. Chromatin immunoprecipitations were performed essentially as described previously (52). NIH 3T3 cells, either serum starved for 60 h or restimulated for the indicated time with 10% FCS, were incubated for 10 min with 1% formaldehyde. After addition of 0.1 M glycine, the cross-linked material was broken with a Dounce homogenizer and sonicated until the DNA was fragmented to 500- to 800-bp fragments. Immunoprecipitation was performed with ProtG-Sepharose (KPL), using 3 to 5 μg of the follow-

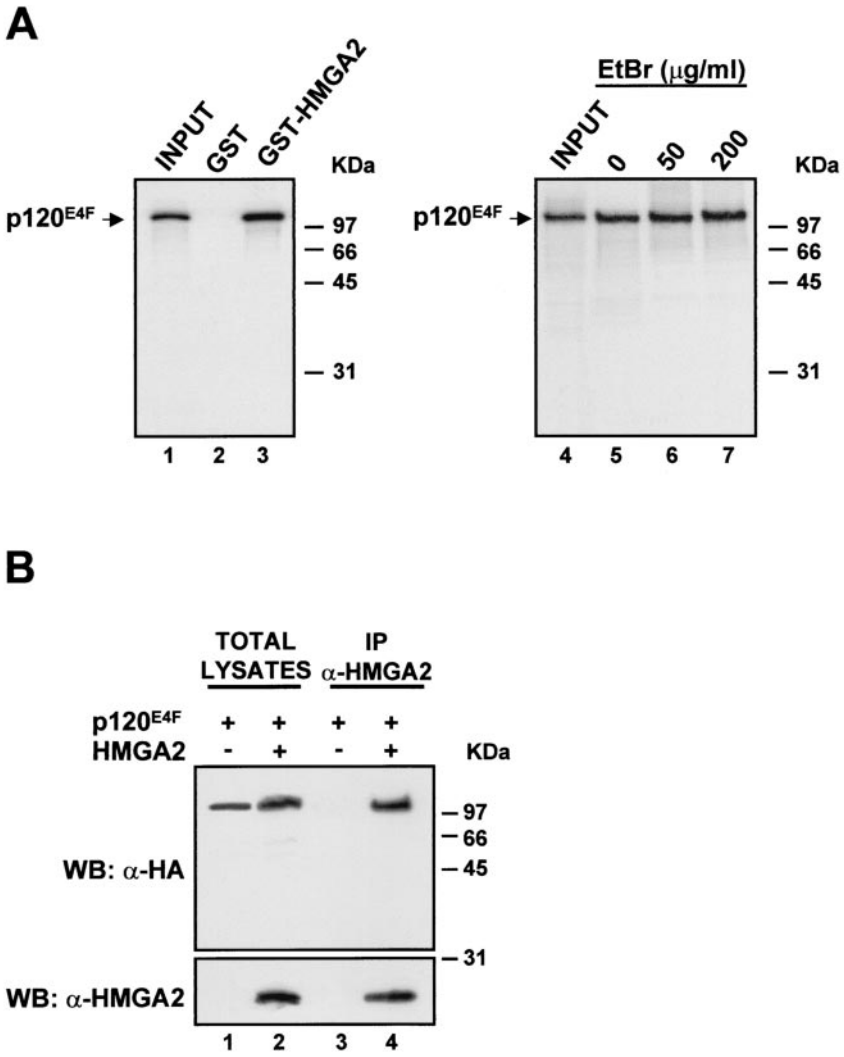

FIG. 1. p120^{E4F} interacts with HMGA2. (A) ^{35}S-labeled, in vitro-translated p120^{E4F} was incubated with either Sepharose-bound GST (negative control) (lane 2) or GST-HMGA2 (lane 3). Incubation of ^{35}S-labeled p120^{E4F} with GST-HMGA2 was also performed in the presence of increasing amounts of ethidium bromide (EtBr) (lanes 5 to 7). The bound proteins were resolved by SDS-PAGE and visualized by autoradiography. Lanes 1 and 4 contain 25% of the in vitro-translated reaction products used in the pull-down experiments. (B) Lysates from 293 cells cotransfected with 5 μg of pcDNA3HA-E4F 2.5K and 2 μg of pcDNA3-HMGA2 expression vectors, as indicated, were immunoprecipitated (IP) with an anti-HMGA2 antibody. Total lysates and immunoprecipitates were then analyzed by Western blotting (WB) with an anti-HA (α-HA) antibody to reveal p120^{E4F} (upper panel) or with an anti-HMGA2 antibody to detect HMGA2 protein (lower panel).

ing antibodies: anti-HMGA2 (36), anti-NF-YB (9), anti-human p120^{E4F} (46), anti-mouse p120^{E4F} (23), and anti-leader binding protein 1 (anti-LBP1) (9). The chromatin solution was precleared by treatment with ProtG-Sepharose for 2 h at 4°C, aliquoted, and incubated with the antibodies overnight at 4°C. Before use, ProtG-Sepharose was blocked twice at 4°C with 1 μg of salmon sperm DNA (sheared at 500 bp) per μl and 1 μg of BSA per μl for 2 h and overnight. PCRs were performed with the following primers: cyclin A, 5′-CTGTAAGATTCCC GTCGGGCCTTCG-3′ and 5′-GTAGAGCCCAGGAGCCGCGAG-3′; α-globin, 5′-GGGCAACTGATAAGGATTCC-3′ and 5′-AGCACCATGGCCACCA ATCT-3′.

RESULTS

Identification of p120^{E4F} as an HMGA2-interacting protein. To identify nuclear proteins interacting with HMGA2, a pull-down assay using a GST-HMGA2 fusion protein with a number of different transcription factors known to be involved in growth control and apoptosis was employed. One of the HMGA2-interacting factors was the E1A-regulated transcription factor p120^{E4F} (21). p120^{E4F} is a GLI-Krüppel-related transcription factor containing six C_2H_2 zinc finger motifs which are clustered in two separate regions; two motifs are contained within an N-terminal domain and mediate the DNA-binding activity, while the remaining four are grouped within a central region. As shown in Fig. 1A, in vitro-translated ^{35}S-labeled p120^{E4F} was retained by GST-HMGA2 but not by GST alone. Moreover, a bona fide interaction between HMGA2 and p120^{E4F} was observed in the presence of high concentrations of ethidium bromide (Fig. 1A, lanes 5 to 7), which has been shown to disrupt DNA-dependent protein-protein contact (30).

To demonstrate that HMGA2 and p120^{E4F} can also associate in vivo, vectors expressing HMGA2 and an HA-tagged p120^{E4F} were cotransfected in 293 cells. Cell lysates were immunoprecipitated with anti-HMGA2 antibody, and subsequently the immunocomplexes were analyzed by Western blotting with anti-HA antibody. As shown in Fig. 1B, p120^{E4F} associates with HMGA2 (lane 4). Complex formation was not detected in the absence of HMGA2 overexpression in the cells (lane 3). As control, cell lysates used in the experiment were tested in Western blots for the expression of p120^{E4F} and HMGA2 proteins (Fig. 1B, lanes 1 and 2). Taken together, these results clearly demonstrate that HMGA2 and p120^{E4F} physically associate both in vitro and in vivo.

HMGA2 activates the cyclin A promoter by relieving the p120^{E4F}-mediated repression. Next we assessed the functional consequences of the p120^{E4F}-HMGA2 interaction. p120^{E4F} behaves as a transcriptional repressor, and it recently has been demonstrated that inhibition of cyclin A is an event required for p120^{E4F}-dependent cell cycle arrest in G_1 and that the cyclin A promoter activity can be repressed by the binding of p120^{E4F} to the CRE site (16). Therefore, we asked whether HMGA2, by associating with p120^{E4F}, could be involved in controlling the cyclin A promoter. The activity of a reporter construct containing the promoter region of the human cyclin A gene cloned upstream of the luciferase gene (CycA$_{wt}$) (Fig. 2A) was therefore tested in transient-transfection assays. Cotransfections of CHO cells with the reporter construct and the p120^{E4F} expression vector result, in agreement with previous data (16), in a twofold repression of the luciferase activity (Fig. 2B, bar 2). Strikingly, this repression could be completely relieved by cotransfecting increasing amounts of the HMGA2-expressing vector (bars 3 and 4). Moreover, the transfection of the HMGA2 expression vector alone, in the absence of p120^{E4F} expression vector, induced the activity of the reporter vector (bars 5 and 6). To confirm that the observed promoter activity was linked to an increase in the amount of the expressed proteins, aliquots of the lysates were subjected to Western blot analysis (Fig. 2B, lower panels).

The effect on the cyclin A promoter is specific, since the activity of a reporter construct containing a cyclin A promoter mutated within the CRE (CycA$_{mut}$) (Fig. 2A) was not modified upon cotransfection of expression vectors for p120^{E4F}, HMGA2, or a combination of both (Fig. 2C). All of these results suggest that HMGA2 plays an important role in the regulation of the cyclin A promoter through the CRE.

HMGA2 activates the expression of the endogenous cyclin A gene. To further strengthen these observations, we carried out transfection experiments with SaOS-2 cells, which express very low levels of HMGA proteins (our unpublished data), and an HMGA2-expressing vector and analyzed the expression of endogenous cyclin A by a semiquantitative RT-PCR. RNA extracted from cells transfected with the HMGA2-expressing vector or with the empty vector was amplified by using primers for cyclin A and for GAPDH as a control. As can be seen in Fig. 3A, the expression of cyclin A in HMGA2-transfected cells (lane 3) was higher than that in control cells (lane 2), while the expression of GAPDH was constant.

Finally, SaOS-2 cells were transfected with the HMGA2-expressing vector and immunostained for overexpressed HMGA2 and endogenous cyclin A. Figure 3B (left panel)

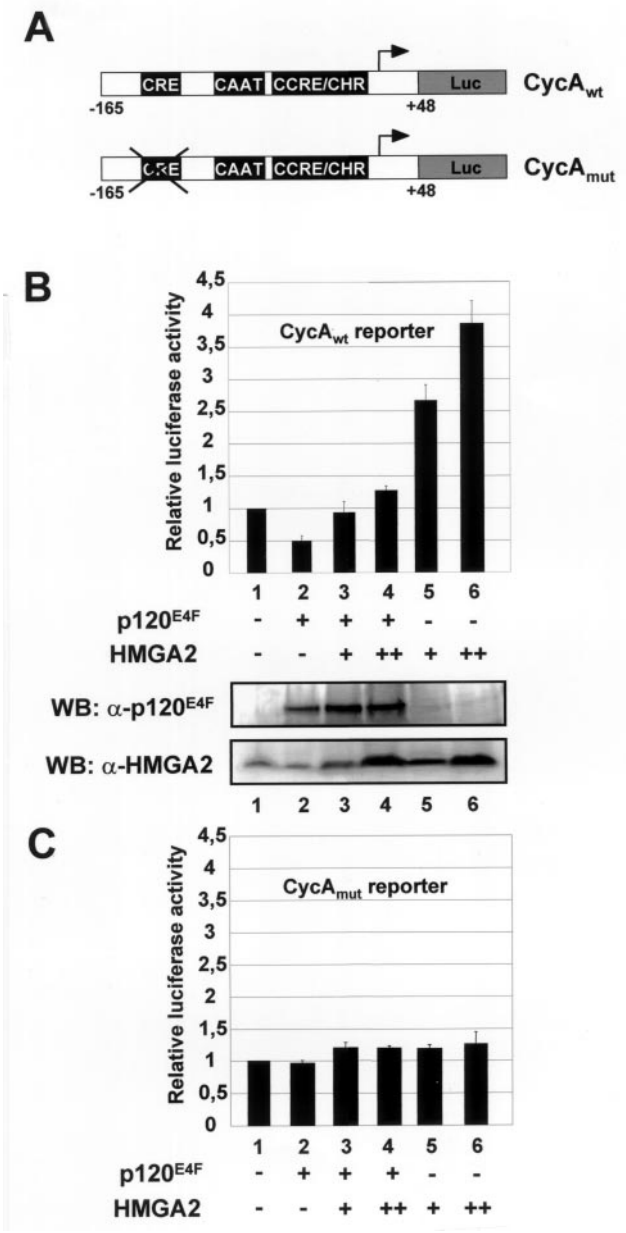

FIG. 2. HMGA2 overexpression relieves the inhibitory effect of p120^{E4F} on cyclin A transcription. (A) Diagrams of luciferase reporter genes under the transcriptional regulation of the wild-type human cyclin A promoter (CycA$_{wt}$) as well as the promoter mutated at the CRE (CycA$_{mut}$). The CRE, CAAT box, and CCRE/CHR elements are indicated. (B) CHO cells were transiently cotransfected with 1 μg of the luciferase reporter plasmid CycA$_{wt}$ (bars 1 to 6), 1 μg of the expression plasmid pcDNA3HA-E4F 2.5K (bars 2 to 4), and 1 or 2 μg of the expression plasmid pcDNA3HA-HMGA2 (bars 3 and 5 and bars 4 and 6, respectively). One hundred nanograms of pRL-CMV *Renilla* luciferase expression vector was included to normalize for transfection efficiencies. Values are reported as relative luciferase activity. Standard deviations are indicated for experiments repeated three times. The amounts of transfected p120^{E4F} and HMGA2 in each sample were controlled by Western blot (WB) analysis. p120^{E4F} protein was revealed with an anti-p120^{E4F} (α-p120^{E4F}) antibody, while the amount of HMGA2 was controlled by using an anti-HMGA2 antibody. (C) An identical transfection experiment was carried out with the luciferase reporter plasmid CycA$_{mut}$, which contains a cyclin A promoter mutated in the CRE.

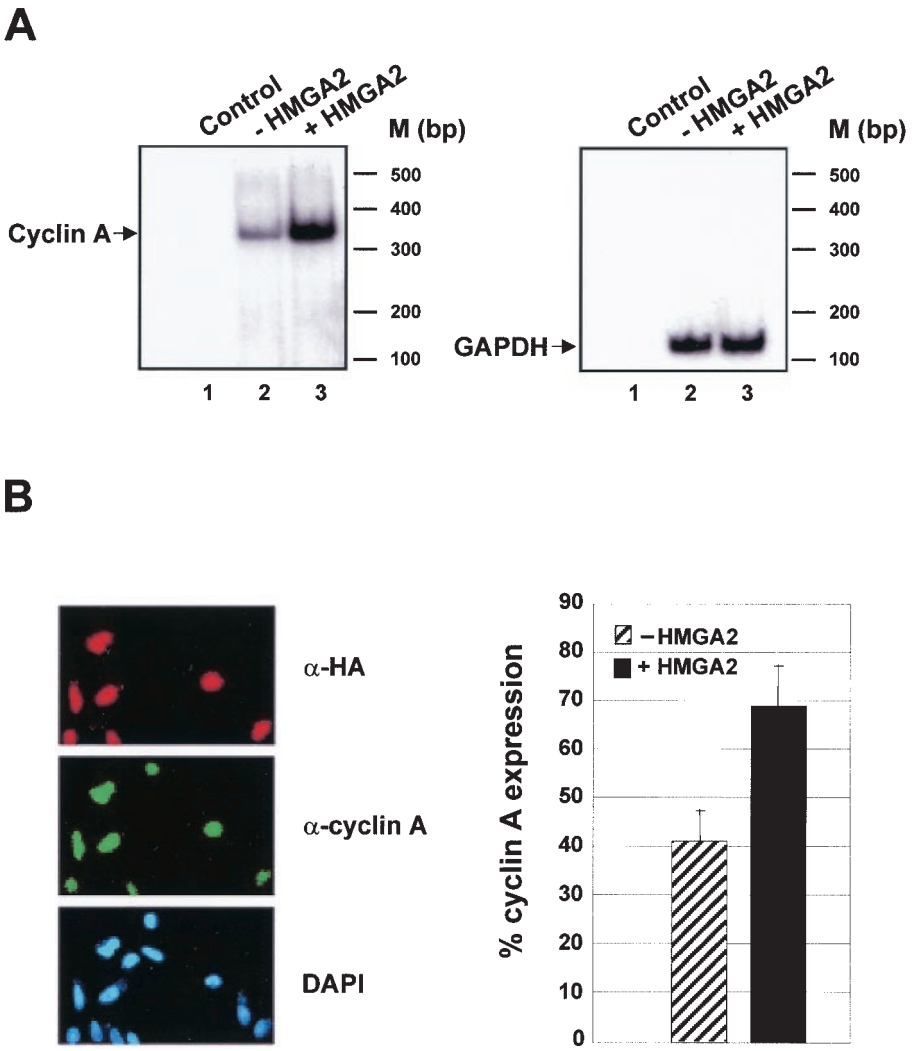

FIG. 3. HMGA2 induces the expression of the endogenous cyclin A. (A) SaOS-2 cells were transfected with 5 μg of pcDNA3HA-HMGA2 expression plasmid (lane 3) or with the same amount of pcDNA3HA empty vector (lane 2). At 48 h after transfection, cells were harvested and the extracted mRNA was amplified by RT-PCR with primers for cyclin A (left panel) and for GAPDH as a control for cDNA quantity and quality (right panel). In lane 1, the reverse transcriptase was omitted from the RT-PCR. (B) SaOS-2 cells were transfected with 5 μg of HMGA2 expression plasmid and immunostained for overexpressed HMGA2 and endogenous cyclin A. Left panel, typical result of this experiment. HMGA2-expressing cells were identified by using an anti-HA (α-HA) antibody directed to the tagged protein (red). Cells expressing endogenous cyclin A were monitored by using an anti-cyclin A antibody (green). Nuclei were stained with DAPI (blue). Right panel, percentages of cyclin A-expressing cells among HMGA2-positive or -negative cells. Standard deviations are indicated for experiments repeated three times.

shows a typical result of this experiment. As can be seen in Fig. 3B (right panel), the percentage of cyclin A-expressing cells which stained negative for HMGA2 was about 40%, while it increased to about 70% in HMGA2-positive cells. Statistical analysis with Student's *t* distribution revealed that the increase in the percentage of cyclin A-expressing cells is significant to a confidence level of greater than 99%. No significant difference was detected in the percentage of S-phase cells, as detected by bromodeoxyuridine incorporation, in HMGA2-positive and -negative cells under these experimental conditions (data not shown).

Together, these results demonstrate an HMGA2-dependent induction of the endogenous cyclin A gene.

Binding to HMGA2 requires the two N-terminal zinc-fingers of p120^{E4F}. As a first step to gain insight into the molecular mechanism responsible for this effect, we mapped the regions of p120^{E4F} that are involved in the interaction with HMGA2. To this end, various deletion mutants of p120^{E4F} cloned in the pcDNA3HA vector were in vitro translated and tested for their ability to bind recombinant GST-HMGA2 in pull-down experiments (Fig. 4A). As summarized in Fig. 4B, progressive deletions from the p120^{E4F} C-terminal end (constructs E4F 694, E4F 551, E4F 427, E4F Δ60-358, and E4F 262; lanes 1 to 13) removing residues 262 to 783 had no effect on HMGA2 binding even though the four-zinc-finger cluster, which is involved in the binding to p53 (46) and p14ARF (43), was completely

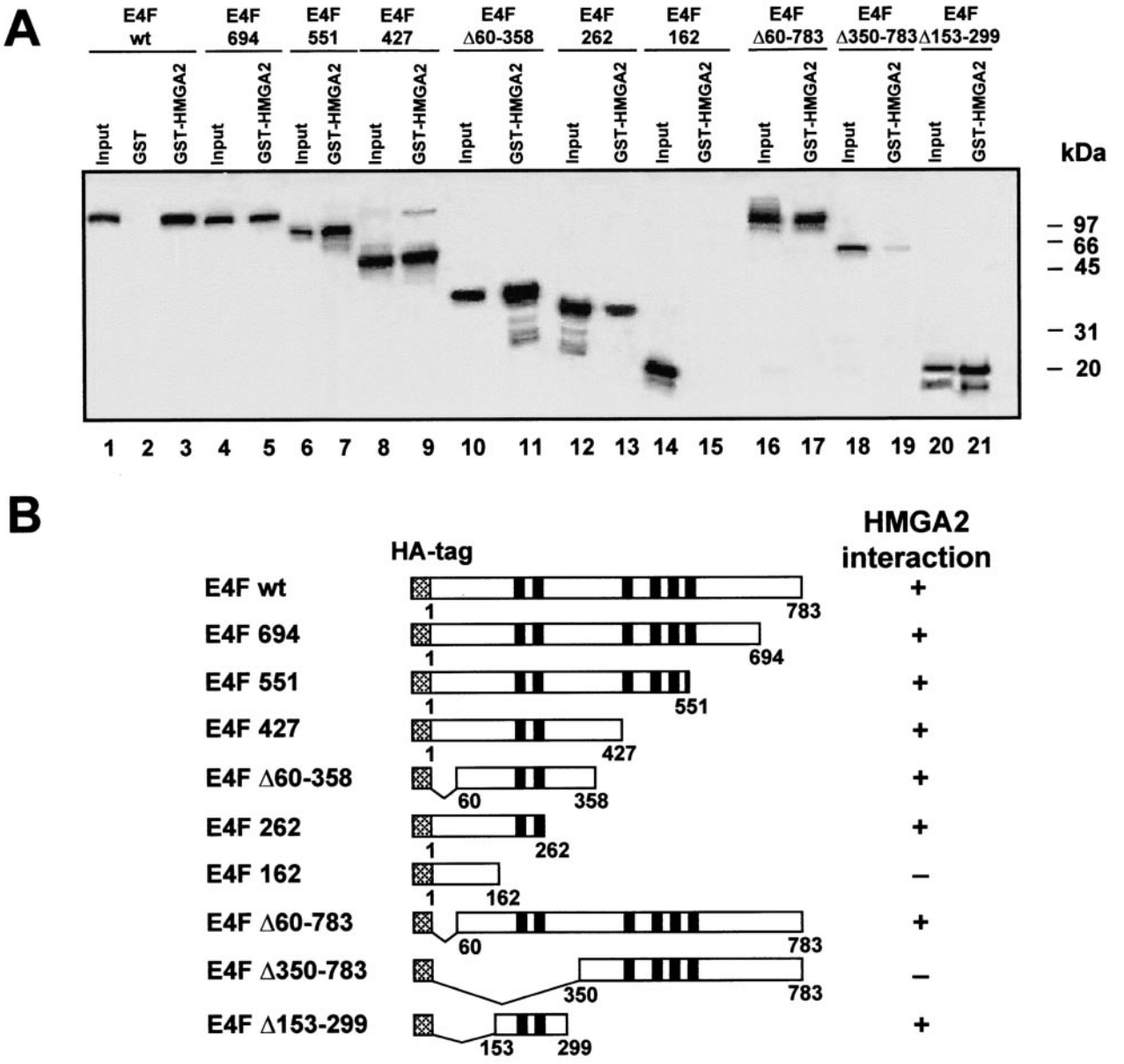

FIG. 4. Binding to HMGA2 requires amino acids 162 to 262 of $p120^{E4F}$. (A) ^{35}S-radiolabeled, in vitro-translated wild-type (wt) $p120^{E4F}$ and various E4F N-terminal and C-terminal deletion mutants were incubated with GST-HMGA2 or GST immobilized to Sepharose beads in GST pull-down assays. The bound proteins were resolved by SDS-PAGE and visualized by autoradiography. Twenty-five percent of the in vitro-translated reaction products used in the pull-down experiments were included (input). The pull-downs of the E4F deletion mutants with GST gave negative results and are not shown. (B) Schematic representation of the various E4F constructs used in the pull-down assays and summary of their abilities to bind HMGA2. The zinc finger domains are shown as black boxes. Numbers refer to amino acids.

deleted. A more extended deletion completely abolished the interaction (construct E4F 162, lanes 14 and 15), suggesting that the region containing the first two zinc fingers is involved in the binding with HMGA2. This result was further confirmed by using the construct E4F Δ153-299, which contains the two N-terminal zinc fingers and shows a strong and specific interaction with HMGA2 (lanes 20 and 21). Deletions from the N-terminal end, E4F Δ60-783 and E4F Δ350-783, confirm this result. The weak binding detected with E4F Δ350-783 is very likely due to the presence of four zinc fingers that, although they have a function different from those at the N-terminal region, share structural similarities.

To map the interaction domains also in vivo, vectors expressing wild-type $p120^{E4F}$ and different $p120^{E4F}$ deletions in fusion with an HA tag were cotransfected with wild-type HMGA2 in 293 cells. As shown in Fig. 5 (upper panel), complex formation was detected with E4F 262 (lane 3) and E4F Δ60-358 (lane 5) but not with E4F 162 (lane 4). As a control, the cell lysates used in the experiment were tested by Western blotting for the expression of $p120^{E4F}$ and HMGA2 proteins (Fig. 5, middle

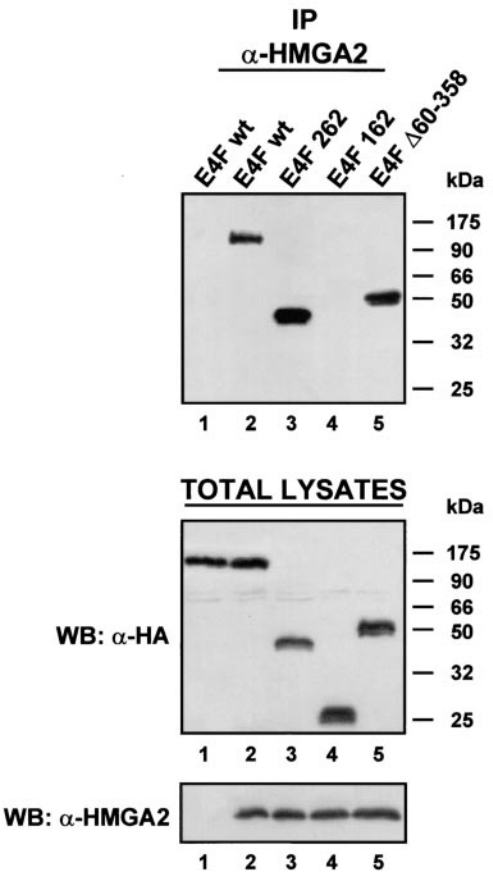

FIG. 5. Mapping of the interaction domains of $p120^{E4F}$ in vivo. Lysates from 293 cells cotransfected with 5 μg of pcDNA3HA vectors, expressing wild-type (wt) $p120^{E4F}$ and different $p120^{E4F}$ deletion mutants, and with 2 μg of pcDNA3-HMGA2 expression vector (lanes 2 to 5) or empty vector (lane 1) were immunoprecipitated (IP) with an anti-HMGA2 (α-HMGA2) antibody. The immunoprecipitates were then analyzed by Western blotting (WB) with an anti-HA antibody (upper panel, lanes 1 to 5). Total lysates used in the immunoprecipitation experiment were controlled for the amount of transfected E4F constructs with an anti-HA antibody (middle panel). Transfected HMGA2 protein was revealed with an anti-HMGA2 antibody (lower panel).

and lower panels, respectively). These results are consistent with those obtained in vitro and clearly demonstrate that binding in vivo requires the region containing the two N-terminal zinc fingers of $p120^{E4F}$.

Binding to $p120^{E4F}$ requires amino acids 44 to 63 of HMGA2. To identify the domains in HMGA2 that interact with $p120^{E4F}$, we generated deletions of the HMGA2 protein and tested for their ability to interact with in vitro-translated $p120^{E4F}$ in a far-Western assay. Data obtained from the experiment shown in Fig. 6A (upper panel) demonstrate that the interaction with $p120^{E4F}$ is maintained when the acidic C-terminal tail is removed ($HMGA2_{94}$, $HMGA2_{83}$, and $HMGA2_{MAD}$). Similarly, the deletion of the region containing the third AT hook ($HMGA2_{73}$) is not effective, while further deletions ($HMGA2_{54}$ and $HMGA2_{43}$) completely abolish the binding. As a control, a Western blot analysis was performed in parallel with an antibody raised against a peptide, derived from the N-terminal sequence of HMGA2, common to all the recombinant proteins (Fig. 6A, lower panel).

These data, summarized in Fig. 6B, indicate that the region between the second and third AT hooks, from amino acid 54 to 73 of HMGA2, is critical for the interaction with $p120^{E4F}$. Two peptide sequences spanning the region presumably involved in the interaction were expressed within the active site of the *E. coli* thioredoxin (TrxA) to assess which region was sufficient for binding with $p120^{E4F}$. TrxA was chosen since its scaffold provides conformational constraint and stability to short peptide sequences (12). Figure 6C shows a GST pull-down experiment in which peptides from amino acid 44 to 63 and from amino acid 53 to 72 of the HMGA2 sequence were expressed within the TrxA protein fused to GST and incubated with in vitro-translated $p120^{E4F}$. GST-TRX44/63 (lane 4) was able to bind $p120^{E4F}$, whereas GST-TRX53/72 (lane 5) behaved as the negative control GST-TRXA (lane 3), demonstrating that the region from amino acid 44 to 63 within HMGA2 is necessary and sufficient for $p120^{E4F}$ binding. These data indicate that, in the context of HMGA2, the minimal region required for specific interaction with $p120^{E4F}$ corresponds to the second AT hook flanked by the spacer region between the second and third AT hooks, revealing, in this respect, a behavior similar to that shown for HMGA2 and NF-κB (36).

HMGA2 interferes with E4F binding to the CRE element. We then investigated at the molecular level how the effect of HMGA2 on the cyclin A promoter could be explained. To this end, we tested by EMSA the effect of a purified $HMGA2_{wt}$ recombinant protein on E4F binding to the cyclin A CRE site (Fig. 7). An affinity-purified GST-E4F Δ60-358 fusion protein gave rise to a protein-DNA complex in a dose-dependent fashion (lanes 2 to 5). This complex is specific, since it was competed by an excess of the self cold probe $CycA_{wt}$ (lane 6) but not by the mutant $CycA_{mut}$ probe (lane 7). The E4F-DNA complex was then tested with increasing amounts of $HMGA2_{wt}$ recombinant protein (lanes 8 to 11). As can be seen, increasing amounts of $HMGA2_{wt}$ progressively inhibited the formation of the E4F-DNA complex, which disappeared with the highest concentration of $HMGA2_{wt}$ added (lane 11) while in parallel a fast-migrating complex appeared. This complex is due to the association of $HMGA2_{wt}$ with the DNA, since it also appears in the absence of GST-E4F Δ60-358 fusion protein (lanes 17 to 19), and is specific, since mutations that replace the AT bases present within and 5′ to the CRE in GC abolish the binding of $HMGA2_{wt}$ to the DNA (data not shown). It is interesting that there is a supershifted complex (lane 10), derived from the association of $HMGA2_{wt}$ with the E4F-DNA complex, as an intermediate before the complete disappearance of the E4F-DNA complex. The $HMGA2_{wt}$-mediated effect is specific, since the addition of BSA had no effect on E4F-DNA complex formation (lanes 12 to 15).

HMGA2 binds in vivo to the cyclin A promoter. To further demonstrate that HMGA2 could bind to the cyclin A promoter, we performed chromatin immunoprecipitation experiments with anti-HMGA2 affinity purified antibodies (Fig. 8A). We used mouse NIH 3T3 fibroblasts, which can be arrested in G_0 by incubation with 0.5% FCS and restimulated upon serum addition to reenter in the cell cycle. We took samples at different time points (6, 12, 18, and 24 h) poststimulation, corresponding to the G_1, G_1/S, S, and G_2/M phases of the cell cycle,

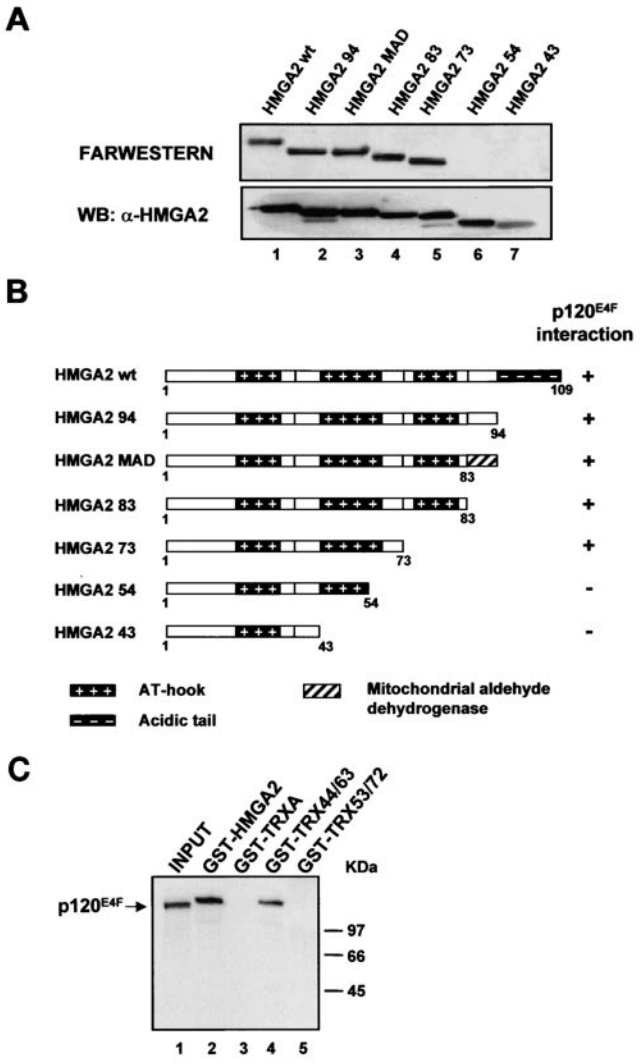

FIG. 6. Binding to $p120^{E4F}$ requires amino acids 44 to 63 of HMGA2. (A) Upper panel, purified recombinant wild-type (wt) HMGA2 and several HMGA2 deletion mutants were probed in a far-Western blot analysis with ^{35}S-radiolabeled in vitro-translated $p120^{E4F}$. Lower panel, Western blot analysis of the membrane with an anti-HMGA2 antibody. (B) Schematic representation of the various HMGA2 constructs used in the far-Western experiment and summary of their abilities to bind $p120^{E4F}$. The functional domains of HMGA2 and the ectopic sequence fused to HMGA2 described for a uterine leiomyoma (29) are indicated. Numbers refer to amino acids. (C) ^{35}S-radiolabeled, in vitro-translated $p120^{E4F}$ was incubated with GST-HMGA2 (positive control) (lane 2), GST-TRXA (negative control) (lane 3), GST-TRX44/63 (lane 4), and GST-TRX53/72 (lane 5) in a GST pull-down assay. All of the GST proteins used were bound to Sepharose beads. The bound proteins were resolved by SDS-PAGE and visualized by autoradiography. Lane 1 contains 25% of the in vitro-translated reaction products used in the pull-down assay.

as previously shown and checked by fluorescence-activated cell sorter analysis (reference 9 and data not shown). The immunoprecipitated DNA was used to amplify the cyclin A promoter, as well as an α-globin promoter that is not expressed in these cells. As shown in Fig. 8A, compared to the control antibody, binding of HMGA2 to cyclin A is negligible in G_0 and G_1 cells but becomes apparent at 18 h, corresponding to S-phase cells, while at 24 h the binding is no longer visible. We could not observe any association of HMGA2 with the control α-globin promoter at the different time points.

If HMGA2 competes with $p120^{E4F}$ for the binding to the cyclin A promoter, as suggested by the EMSA experiments, then the association of these two proteins with the promoter could be mutually exclusive. To test this hypothesis, antibodies against human and mouse $p120^{E4F}$ were used to immunoprecipitate chromatin from growth-arrested and serum-stimulated NIH 3T3 fibroblasts, and the immunoprecipitated DNA was used to amplify the cyclin A promoter. As shown in Fig. 8B, both human and mouse antibodies failed to detect $p120^{E4F}$ associated with the promoter before 24 h, when essentially all cells have passed S phase and are in G_2/M or have reentered G_1 (reference 9 and data not shown). In particular, $p120^{E4F}$ is not bound at 18 h, when the promoter is active and HMGA2 is bound. As a control for cell cycle progression, we used anti-NF-YB antibodies; the protein was found associated in G_1/S, S, and G_2/M as previously described (9). These data document the specific, phase-dependent, and mutually exclusive association of HMGA2 and $p120^{E4F}$ with the cyclin A promoter and

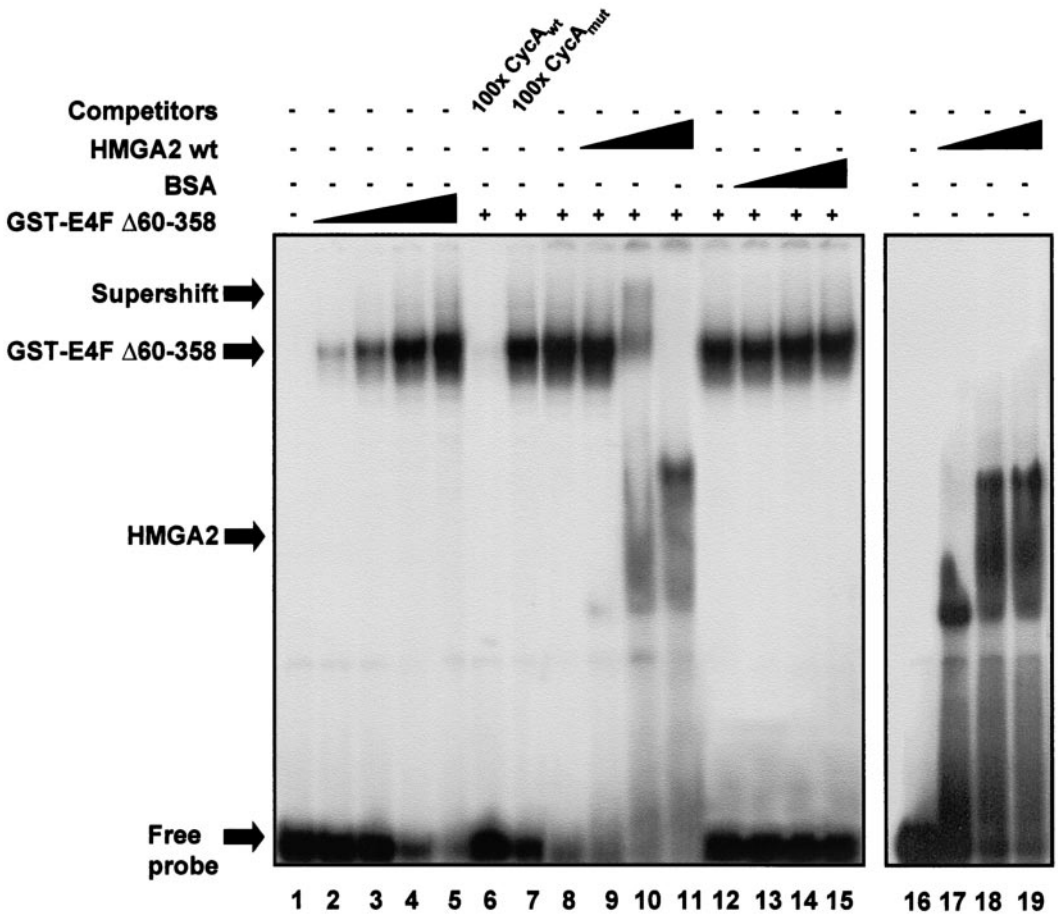

FIG. 7. HMGA2 interferes with E4F binding to the CRE element. Increasing amounts of purified GST-E4F Δ60-358 fusion protein (0.3, 0.6, 1.2, and 2 pmol [lanes 2 to 5, respectively]) were incubated with the ^{32}P-radiolabeled CycA$_{wt}$ oligonucleotide (from bp −140 to −112 relative to the most 3′ transcription initiation site) in an EMSA experiment. Lane 1, free probe. Competitions were performed on the maximal amount of GST-E4F Δ60-358 tested by using a 100-fold molar excess of unlabeled wild-type CycA$_{wt}$ (lane 6) or CRE-mutated competitor CycA$_{mut}$ (lane 7). Increasing amounts of purified recombinant wild-type (wt) HMGA2 (0, 4, 8, and 12 pmol [lanes 8 to 11, respectively]) or BSA (0, 4, 8, and 12 pmol [lanes 12 to 15, respectively]) were incubated with a fixed amount of GST-E4F Δ60-358 (2 pmol). Increasing amounts of purified recombinant wild-type HMGA2 (0, 4, 8, and 12 pmol [lanes 16 to 19, respectively]) were also incubated with the CycA$_{wt}$ probe in the absence of GST-E4F Δ60-358. Lane 16, free probe.

show the binding of HMGA2 under conditions in which the promoter is transcriptionally active (26).

DISCUSSION

Growth is one of the fundamental aspects of the development of an organism. This process is tightly controlled by the coordination of proliferation, differentiation, and apoptosis, with disruption of these processes commonly resulting in tumor formation.

In this study we report that HMGA2, an architectural transcription factor which is directly involved in cell proliferation and neoplastic transformation, associates with the transcriptional repressor p120^{E4F}, resulting in the induction of the cyclin A gene. Cyclin A is a key factor controlling cell cycle progression both at the S phase entry and at the G_2/M transition by binding to cdk2 and cdc2 kinases, respectively (37, 45).

HMGA2-mediated cyclin A induction is due to interference with p120^{E4F} repressing activity. Although several reports indicate that HMGA proteins are architectural factors, able to enhance the DNA binding of DNA-interacting proteins promoting the formation of enhanceosomes, data have been published showing an inhibitory effect of HMGA on DNA binding of other factors, usually by sterically blocking the functional binding of transcription factors to their recognition sites in gene promoters (42). Would it thus be possible to extend the mechanism that we found for the cyclin A gene to other p120^{E4F} target genes? Although the cyclin A gene is the only cellular target thus far demonstrated for p120^{E4F}, it is likely that other genes involved in the control of cell cycle progression are regulated by p120^{E4F}, as ectopic expression of p120^{E4F} can induce cell cycle arrest at the G_1/S and G_2/M transitions even when cyclin A mRNA and protein levels are not reduced (22, 44). The association of HMGA2 with p120^{E4F} through the region containing the DNA-binding domain could therefore constitute a switch that displaces p120^{E4F}-containing complexes from cyclin A and other, yet-to-be-defined, p120^{E4F} target genes. According to this hypothesis, the expression of

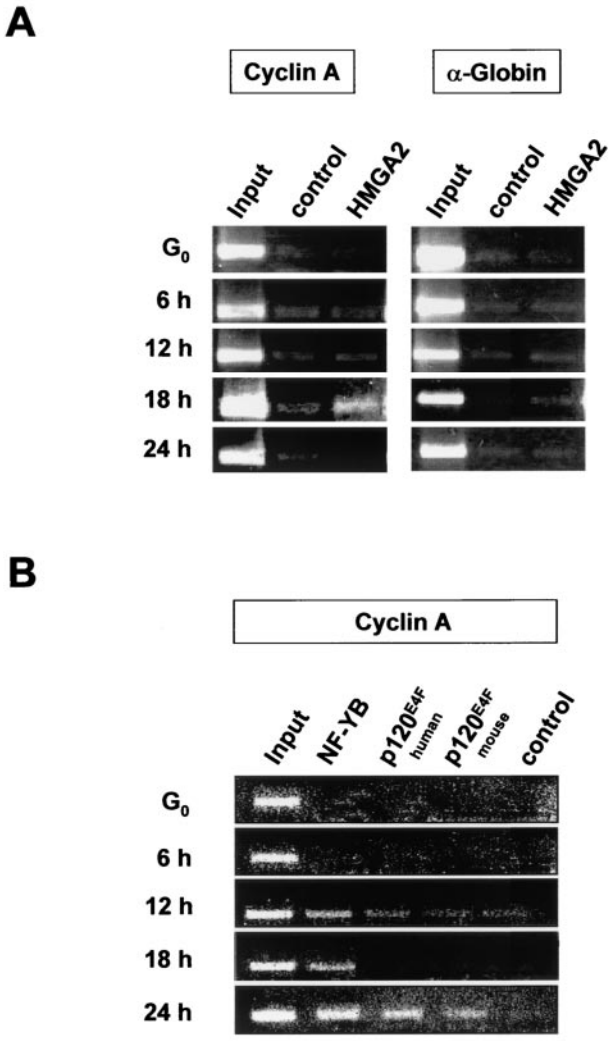

FIG. 8. Association of HMGA2 and $p120^{E4F}$ with the cyclin A promoter is regulated during the cell cycle. (A) Chromatin immunoprecipitation analysis of HMGA2 binding to the cyclin A promoter in vivo was performed with NIH 3T3 cells. Immunoprecipitations of sonicated, cross-linked chromatin samples, taken at G_0 and at different time points (6, 12, 18, and 24 h) poststimulation, corresponding to the G_1, G_1/S, S, and G_2/M phases of the cell cycle, were performed with antibodies raised against HMGA2 and LBP1, a cellular DNA-binding protein used as a negative control. DNAs corresponding to cyclin A (left panel) and to α-globin used as negative control (right panel) were PCR amplified with the indicated oligonucleotides (see Materials and Methods). The input chromatin taken prior to immunoprecipitation was used as a template for PCR amplification (input). (B) Chromatin immunoprecipitation analysis of $p120^{E4F}$ binding to the cyclin A promoter was performed as described for panel A with antibodies against NF-YB, human $p120^{E4F}$, mouse $p120^{E4F}$, and LBP1 as a negative control.

HMGA2 in human tumors could inactivate the repressing effect of $p120^{E4F}$ on cell growth.

Another important consideration refers to the region of HMGA2 involved in $p120^{E4F}$ protein-protein interaction. This is a very short region comprising and flanking the second AT hook, which we have previously shown to be involved in the interaction with NF-κB (36). Moreover, the corresponding region in HMGA1 is involved in the interaction with other transcription factors as well. Two lysine residues within this region were shown to be acetylated by CBP/p300 and P/CAF and to be critical for the disassembly and assembly of the enhanceosome at the beta interferon gene (34). This short domain, which can be targeted by posttranslational modifications, plays an important role in HMGA proteins in establishing connections with nuclear factors and is therefore crucial for the architectural role played by these factors.

HMGA2-mediated cyclin A gene induction occurs through the CRE element. The cyclin A promoter is regulated during G_0 and G_1 by contiguous *cis*-acting elements, the CDE-CHR bipartite element (8, 27, 38). Different transcription factors, among which are the pocket proteins (pRb and p107), have been shown to be involved in the binding to these elements, but the mechanism remains to be characterized and is still a matter of controversial discussion (27, 38). The cyclin A promoter region also contains a CRE site. Several reports show that the CRE site plays an important role in transcriptional activation of the cyclin A gene (5, 13, 35, 48, 49). Most studies indicate that CRE is constitutively occupied in G_1-phase cells, and it has been proposed that the CDE-CHR could act in concert with CRE to confer proper cell cycle regulation to cyclin A gene expression (49). The CRE is recognized by CREB and ATF family members that belong to the family of basic leucine zipper proteins, which are able to form homo- and heterodimers and cross-family heterodimers with members of the AP1 family. As a consequence, different factors binding to CRE have been identified in different cell types. It has been shown that the different compositions of the subunits, as well as their posttranslational modifications, are involved in the response to different stimuli (e.g., serum induction or cyclic AMP) acting through the CRE to regulate the cyclin A gene (5, 13, 35, 48, 49). The CRE site is also recognized by $p120^{E4F}$, raising the question of how the access of $p120^{E4F}$ to this site and subsequent transcriptional regulation are controlled (16). Interestingly, it has been hypothesized that auxiliary proteins, such as HMGA, could be possible regulators (16). Indeed, HMGA proteins can modulate the access of transcription factors to DNA and are regarded as highly connected nodes of protein-DNA and protein-protein interactions that influence a diverse array of normal and pathological processes, participating in a wide variety of nuclear processes, ranging from chromosome and chromatinic mechanics to architectural transcription factors (40). In this report we show that HMGA2 activates the cyclin A gene through the CRE and that this effect also occurs in the absence of ectopic expression of $p120^{E4F}$. This could be explained by assuming a competition with the endogenous $p120^{E4F}$, but it could also be due to an effect of HMGA2 on other transcription factors binding to CRE. Indeed, it has been demonstrated that HMGA1a could enhance the binding of basic leucine zipper-containing factors, increasing the stability of dimers (14). In addition, we demonstrated by EMSA experiments that HMGA2 can enhance the binding of ATF-2 to the CRE (data not shown). Therefore, we propose that HMGA2 regulation of the cyclin A gene occurs by modulating the access of transcription factors binding to the CRE. This occurs through protein-protein interactions, but since we demonstrated that HMGA2 interacts with DNA in a region containing the CRE, it is also possible that structural modifications of DNA induced by HMGA could play a role in this effect. In

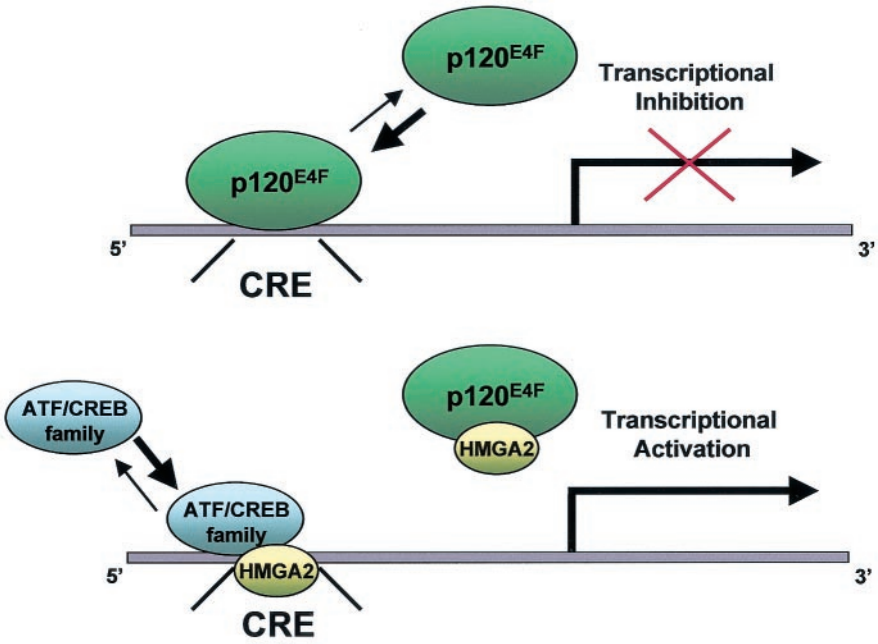

FIG. 9. HMGA2-dependent activation of the cyclin A gene. A model explaining the effect mediated by HMGA2 at the CRE of the cyclin A gene is proposed. HMGA2 interferes with $p120^{E4F}$ binding to the CRE and facilitates the binding of ATF/CREB family members, resulting in the activation of transcription.

this scenario, HMGA2 could therefore function by shifting the balance between $p120^{E4F}$-mediated repression and CREB/ATF activators binding to the CRE, favoring the latter (Fig. 9). Importantly, in this view HMGA2 is not constitutively bound to the cyclin A promoter throughout the cell cycle. Chromatin immunoprecipitation experiments in fact show HMGA2 bound to the promoter only at 18 h after serum induction, which corresponds to the time when the cyclin A gene is activated. In fibroblasts stimulated by serum, HMGA2 is a delayed-early-responsive gene with peak expression at 7.5 to 20 h (31). The expression of HMGA2 is therefore modulated during the progression through the cell cycle, and its kinetic is compatible with the activation of the cyclin A gene. Moreover, the timely regulated association of $p120^{E4F}$ with the promoter is consistent with the proposed model, since $p120^{E4F}$ binds in G_2/M but not during S phase, when the promoter is occupied by HMGA2 and the gene is active. Interestingly, $p120^{E4F}$ is not detected in G_0, suggesting that is not implicated in cyclin A repression in growth-arrested cells.

Repression of the cyclin A promoter in quiescent cells was found to be associated with recruitment of the E2F-4 transcriptional repressor (reference 39 and references therein). Our finding that another repressor, $p120^{E4F}$, associates with the cyclin A promoter defines a second level of repressive regulation for this gene.

Cyclin A is the first identified HMGA2 target gene involved in neoplastic transformation. Our finding that cyclin A is a direct target gene of HMGA2 is consistent with the role proposed for HMGA2 in cell transformation and cell growth during embryogenesis. The HMGA2 protein has been implicated in the pathogenesis of a variety of benign solid human tumors (2, 20, 47, 50), and its role has been demonstrated by using transgenic mice overexpressing wild-type HMGA2 or HMGA2 mutants (1, 3, 6, 18, 19). Moreover, it was reported that the *pygmy* phenotype in mice was caused by the disruption of both *Hmga2* alleles (55). These mice show a small size and a drastic reduction of body fat content, and, interestingly, $Hmga2^{-/-}$ embryonic fibroblasts have a decreased rate of cell proliferation. This suggested an involvement of HMGA2 in the control of critical steps of the progression through the cell cycle, and our results involve cyclin A as a possible effector of HMGA2 in this phenotype.

In conclusion, this work establishes cyclin A as a mediator of HMGA2-dependent cell cycle regulation. The characterization of other molecular partners of HMGA2 will allow definition of the role played by this factor in cell proliferation and tumorigenesis.

ACKNOWLEDGMENTS

We thank A. Skabar for his contribution in the initial part of this work and P. Sandy for critical reading of the manuscript.

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), Milan, Italy, to R.M., G.D.S, and V.G.; by ASI grant I/R/129/00 to V.G.; by Università degli Studi di Trieste, Italy, to G.D.S., G.M., and V.G.; and by a MIUR Cofin grant to R.M. and V.G. M.G., R.S., C.I., and G.C. are supported by a FIRC fellowship.

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