HMG-I/Y Is a c-Jun/Activator Protein-1 Target Gene and Is Necessary for c-Jun–Induced Anchorage-Independent Growth in Rat1a Cells

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Abstract

The transcription complex activator protein-1 (AP-1) plays a role in a diverse number of cellular processes including proliferation, differentiation, and apoptosis. To identify AP-1–responsive target genes, we used a doxycycline-inducible c-Jun system in Rat1a cells. The HMG-I/Y chromatin binding protein was found to be up-regulated by c-Jun. Following induction of c-Jun expression, Rat1a cells under nonadherent growth conditions have sustained *HMG-I/Y* mRNA expression and 2-fold higher protein than uninduced cells. *HMG-I/Y* promoter reporter assays show that *HMG-I/Y* promoter activity increases in the presence of c-Jun expression, and gel mobility shift assays demonstrate that induced c-Jun binds to an AP-1 consensus site at position −1,091 in the *HMG-I/Y* promoter. Suppression of HMG-I/Y expression by its antisense sequence significantly reduces the ability of c-Jun–overexpressing Rat1a cells to grow in an anchorage-independent fashion. HMG-I/Y transforms Rat1a cells (although the colonies are smaller than that observed for the cells overexpressing c-Jun). Taken together, these results suggest that *HMG-I/Y* is a direct transcriptional target of c-Jun necessary for c-Jun–induced anchorage-independent growth in Rat1a cells. (Mol Cancer Res 2004;2(5): 305–14)

Introduction

c-Jun is a major component of the activator protein-1 (AP-1) transcription factor. It binds to numerous sites, including AP-1 (TGAG[C]TCA) and activating transcription factor (TGACGTCA) sites found in the promoters of a large number of genes. Interaction at these sites occurs as either a homodimer or a heterodimer with members of the Fos family (c-Fos, FosB, Fra1, and Fra2) or activating transcription factor family, thus regulating the expression of genes containing AP-1 binding sites in their promoters. c-Jun plays a critical role in several cellular processes including transformation (1, 2), cell cycle progression (3), differentiation (4), and apoptosis (5). We have previously shown that c-Jun transforms the immortalized rat fibroblast cell line Rat1a as a single gene (6), and overexpression of c-Jun allows the cells to grow in an anchorage-independent manner. Although we and others have identified several c-Jun target genes (7-12), the precise molecular mechanisms by which c-Jun/AP-1 mediates biological processes are yet to be determined.

The *HMG-I/Y* gene encodes the HMG-I and HMG-Y protein isoforms, which result from alternatively spliced mRNA (13, 14). HMG-I and HMG-Y proteins differ by an internal 11 amino acids present only in the HMG-I isoform (13, 14). Both HMG-I and HMG-Y proteins contain AT hook DNA binding domains that mediate binding to AT-rich sequences in the minor groove of chromosomal DNA (15-19). These proteins appear to play an important role in regulating gene expression (15-25) and have been called architectural transcription factors because they alter the conformation of DNA by modulating nuclear protein-DNA complexes. HMG-I/Y proteins also physically interact with transcription factors such as AP-1, nuclear factor-κB (20), Sp1, and CAAT/enhancer binding protein-β (21) to modulate their ability to activate gene expression.

HMG-I/Y is localized to the short arm of chromosome 6 in a region commonly involved in chromosomal abnormalities associated with human cancers (13, 14, 26). *HMG-I/Y* expression is increased in neoplastic transformation and proliferation (27-33). Increased expression of *HMG-I/Y* mRNA and/or protein has also been associated with numerous human malignancies (34-37) as well as mouse models of skin (38) and prostate carcinoma (39) and is also associated with metastasis (40, 41). In addition, HMG-I/Y may play a direct role in tumorigenesis by interfering with the ability of p53 to recognize damaged DNA (42). It also cooperates with AP-1 and CBP/p300 in transcription of HPV18 sequences (43).

A previous study has shown that *HMG-I/Y* is a c-Myc target gene important in transformation in Burkitt's lymphoma (33). HMG-I/Y also has several oncogenic properties (33, 36, 44, 45). Increased expression of HMG-I/Y in Rat1a cells, human lymphoid cells (33), or human breast cells (36, 44) results in a transformed phenotype. In addition, rat cells overexpressing HMG-I/Y form tumors in nude mice (33, 45). *HMGI-I/Y* was also shown to be induced by phorbol esters, although this induction was relatively modest (46). We used our previously

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reported Rat1a cell system in which c-Jun expression is regulated by doxycycline (9) to examine whether *HMG-I/Y* is a target gene involved in c-Jun–induced transformation and anchorage-independent growth. We report that c-Jun binds to the AP-1 site at position −1,091 in the *HMG-I/Y* promoter and enhances HMG-I/Y expression under nonadherent growth conditions. Inhibition of HMG-I/Y expression by an antisense construct inhibits transformation by c-Jun in soft agarose, and overexpression of HMG-I/Y alone allows cells to grow in an anchorage-independent manner. Taken together, these results indicate that *HMG-I/Y* is a c-Jun target gene that is necessary, and partially sufficient, for c-Jun–induced transformation and anchorage-independent growth of Rat1a cells.

Results

HMG-I/Y Transcription Is Increased in the Presence of c-Jun Overexpression

We have previously shown that overexpression of c-Jun in Rat1a cells results in the activation of target genes, many of which may have roles in proliferation and transformation (9, 10). *HMG-I/Y* is a likely candidate for regulation by c-Jun/AP-1 because several potential AP-1 binding sites have been identified in its promoter (33, 46, 47). To determine the effect of c-Jun on *HMG-I/Y* expression, we used Rat1a cells with doxycycline-inducible c-Jun expression (9). Northern blot analysis showed that *HMG-I/Y* transcription was considerably up-regulated following c-Jun induction in cells grown in nonadherent conditions for 48 hours, while a GFP-expressing control cell line showed no change (Fig. 1A). This regulation was observed in two independent c-Jun–expressing clones, Rat1a-J2 (data not shown) and Rat1a-J4. These results were confirmed by Western blot analysis showing a concomitant increase in HMG-I/Y protein following c-Jun induction (Fig. 1B). There was no change in the relative ratios of HMG-I and HMG-Y isoforms under c-*jun* induction (data not shown). The increase in basal HMG-I/Y expression in Rat1a-J4 cells (−dox) compared with GFP control cells is likely due to leakiness of c-Jun expression in the absence of doxycycline.

c-Jun/AP-1 Binds to a Consensus AP-1 Binding Site in the HMG-I/Y Promoter

In addition to the E-box at −1,337 and AP-2 site at −1,321 in the mouse *HMG-I/Y* promoter (33, 47), three potential AP-1 binding sites are located at −1,457, −1,423, and −1,091 (Fig. 2A; refs. 46, 47). To determine if *HMG-I/Y* is a target for regulation by c-Jun/AP-1, we analyzed these potential AP-1 binding sites by in vitro gel mobility shift assays. All three oligomers showed DNA-protein interactions with nuclear extracts isolated from Rat1a-J4 cells grown in the absence and presence of doxycycline (Fig. 2B). The oligomers corresponding to the "AP-1 sites" at positions −1,457 and −1,091 had increased protein complex binding in the presence of doxycycline, suggesting that c-Jun/AP-1 may interact at these locations. The −1,091 oligomer, in particular, showed increased binding and migration of two complexes in the doxycycline-treated cell extracts. To determine if c-Jun is contained in the DNA-protein complexes at −1,457 and −1,091, we performed supershift analysis with an antibody against c-Jun. The complex binding at −1,457 was not supershifted, suggesting that c-Jun is not contained in the complex (Fig. 2C). Similarly, no supershifted complexes were detected at −1,423 (data not shown). Supershifted complexes were detected, however, at −1,091, and both complexes detected in doxycycline-induced extracts were supershifted (Fig. 2D). The complex binding at −1,091 was also supershifted by an antibody to JunD, suggesting that JunD, in addition to c-Jun, was a component of the complex (Fig. 2E). In the presence of doxycycline, the amount of the complex supershifted by the c-Jun antibody increased and that by the JunD antibody decreased; in addition, a supershifted band was detected with an antibody against Fra1 (Fig. 2E). An isotype control antibody did not result in any reproducibly supershifted complexes. These results suggest that c-Jun is a component of the complex interacting at −1,091 in the *HMG-I/Y* promoter, and in the presence of induced c-Jun, this complex is altered.

c-Jun/AP-1 Activates the HMG-I/Y Promoter Through the AP-1 Site at −1,091

To further determine if AP-1 could activate *HMG-I/Y* transcription in cells, we performed transfection experiments and promoter analysis in Rat1a, Rat1a-GFP, and Rat1a-J4 cells. Transient transfection of Rat1a cells with a −1,895 to +75 HMG-I/Y promoter luciferase construct in combination with the doxycycline-inducible pLRT-c-Jun plasmid resulted in a significant increase in promoter activity (Fig. 3A). In contrast to *HMG-I/Y* promoter activation by c-Jun, the AP-1 dominant negative mutant Tam67 had no effect (Fig. 3A). Transfection of the −1,895 to +75 HMG-I/Y promoter construct into Rat1a-J4 cells resulted in a significant increase in promoter activity in the presence of doxycycline (Fig. 3B). This increase in promoter activity was prevented by mutation of the AP-1 site at −1,091. In addition, deletion of the region −1,895 to −974 containing the E-box, AP-1 and AP-2 sites, also prohibited promoter activation by c-Jun/AP-1 (Fig. 3B). The slight increase in induced promoter activity seen in the truncated promoter construct may reflect the loss of an inhibitory element for which we have preliminary evidence.[3] No increase in promoter activity in the presence of doxycycline was observed for any of the constructs when transfected into the control Rat1a-GFP cells (Fig. 3B). Taken together, these results suggest that the AP-1 site at −1,091 and sequences upstream of −974 are important for c-Jun/AP-1 regulation of the *HMG-I/Y* promoter.

HMG-I/Y Expression Is Required for c-Jun/AP-1–Induced Anchorage-Independent Growth

To determine if HMG-I/Y is necessary for c-Jun–regulated anchorage-independent growth, we isolated pools of independent isolated clones of Rat1a-J4 cells with constitutive antisense *HMG-I/Y* expression. The antisense expression suppressed the

[3] L.M.S. Resar, unpublished results.

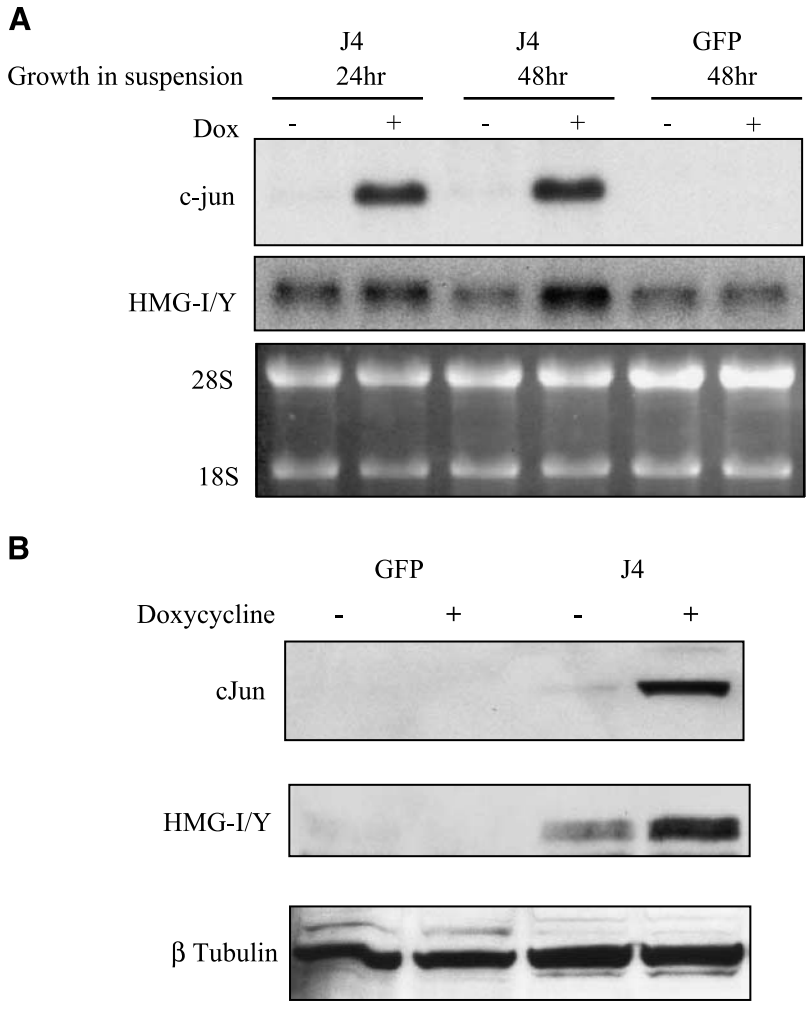

FIGURE 1. *HMG-I/Y* mRNA and protein levels in c-Jun–expressing Rat1a cells. **A.** Time course showing increased *HMG-I/Y* mRNA expression in Rat1a-J4 cells at ~48 hours of doxycycline incubation. **B.** Western blot analysis of c-Jun and HMG-I/Y proteins in control Rat1a-GFP and Rat1a-J4 cells after 3 days of incubation with doxycycline. The HMG-I/Y proteins are detected at ~19 kDa with a chicken anti-HMG-I/Y antibody (see Materials and Methods). Rat1a-J4 cells have increased HMG-I/Y protein compared with uninduced cells and control Rat1a-GFP cells.

c-Jun–induced nonadherent cell growth of J4 cells (Fig. 4A). Each pool of antisense clones was suppressed ~80% compared with the cloning of the parental cell line. The colonies were smaller, and the number of colonies was significantly reduced compared with those of c-Jun–expressing Rat1a-J4 cells. No suppression of cloning was observed for a pool of Rat1a-J4 cells transfected with the pSG5 vector alone (Fig. 4A). In addition, the pools expressing antisense *HMG-I/Y* demonstrated ~50% suppression of HMG-I/Y when determined by Western blot (Fig. 4B). Western blot analysis of HMG-C demonstrated no change in expression (data not shown). Constitutive expression of antisense *HMG-I/Y* did not affect the growth rate of the cells. The pools of Rat1a-J4 cells overexpressing antisense *HMG-I/Y* had a comparable doubling time to parental Rat1a-J4 cells both in the presence and in the absence of doxycycline (data not shown). Further, we isolated two independent clones of Rat1a-J4-asHMG-I/Y and tested them for their ability to clone in soft agarose. J4HMGas-9 and J4HMGas-23 were suppressed in their ability to grow in soft agarose (>90%; Fig. 4C), whereas two independent Rat1a-J4 clones transfected with the pSG5 vector alone were not. Western blot analysis demonstrated suppression of HMG-I/Y expression (Fig. 4D). The discrepancy in colony formation between the antisense *HMG-I/Y*-expressing pools and clones (compare Fig. 4A and D) was due to leakiness of HMG-I/Y expression in the pool cells. HMG-I/Y expression in the antisense clones was reduced by 98% (Fig. 4D). These results indicate that HMG-I/Y expression in Rat1a-J4 cells is necessary for anchorage-independent growth by c-Jun.

Because our antisense experiments indicated that HMG-I/Y is required for transformation by c-Jun in this system, we next sought to determine if HMG-I/Y was sufficient for transformation in our Rat1a cells. The overexpression of HMG-I/Y in Rat1a cells induced colony formation (Fig. 5B), although the colonies were slightly smaller, less compact, and fewer than those induced by c-Jun overexpression. This is similar to that reported previously (33, 45).

Discussion

In this article, we show that c-Jun binds to the *HMG-I/Y* promoter and induces expression of the HMG-I/Y chromatin binding protein. Decreasing HMG-I/Y proteins using an antisense approach inhibits c-Jun/AP-1–induced transformation, while

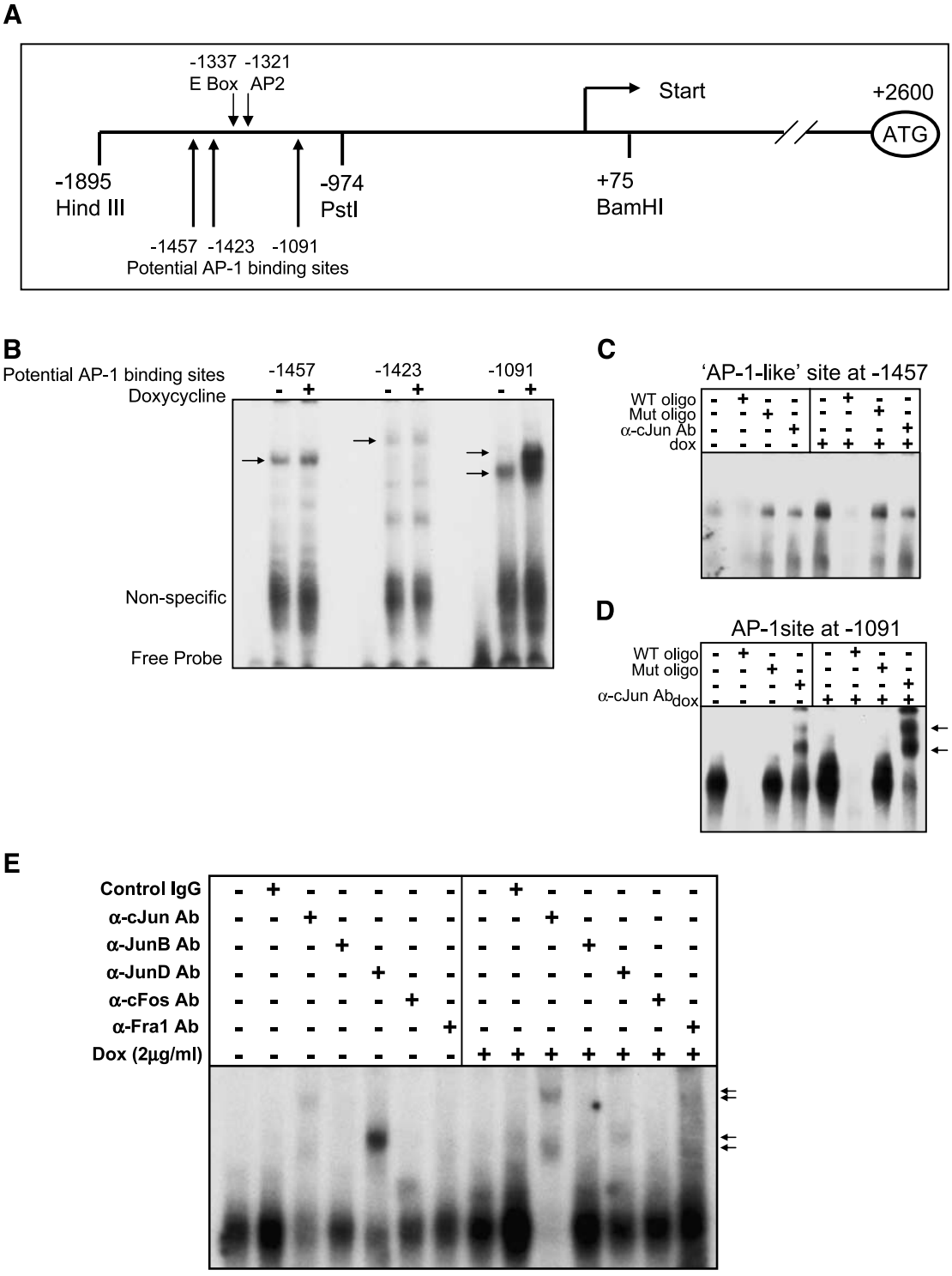

FIGURE 2. AP-1 binding at the *HMG-I/Y* promoter. **A.** The mouse *HMG-I/Y* promoter contains three potential AP-1 binding sites located at positions −1,457, −1,423, and −1,091. The E-box is located at −1,337, and an AP-2 binding site is located at −1,321. The translational initiation site is located at +2,600, producing a 2.6-kb 5′ untranslated region. **B.** Gel mobility shift assay using the potential AP-1 binding sites as probes with Rat1a-J4 nuclear proteins extracted from cells grown in the absence (−) and presence (+) of doxycycline to induce c-Jun expression. *Arrows,* DNA-protein complex formations. **C.** Gel shifts showing competition for complex formation with the oligomer containing −1,457 with cold wild-type oligomer and a mutated oligomer. Incubation with antibodies against c-Jun, however, does not result in a supershifted complex. **D.** Gel shifts showing competition for the DNA-protein complex formation at the AP-1 site located at −1,091 with wild-type oligomer and not the mutated oligomer. The complexes detected in doxycycline-treated nuclear extracts (*arrows*) were supershifted with the antibody against c-Jun. **E.** AP-1 complex composition at the −1,091 site in the absence (−) and presence (+) of doxycycline (2 μg/mL). Nuclear extracts were incubated with an end-labeled AP-1 probe and specific antibodies to various Jun and Fos family members as well as an isotype control antibody (*lanes 2* and *9*). *Arrows,* supershifted complexes.

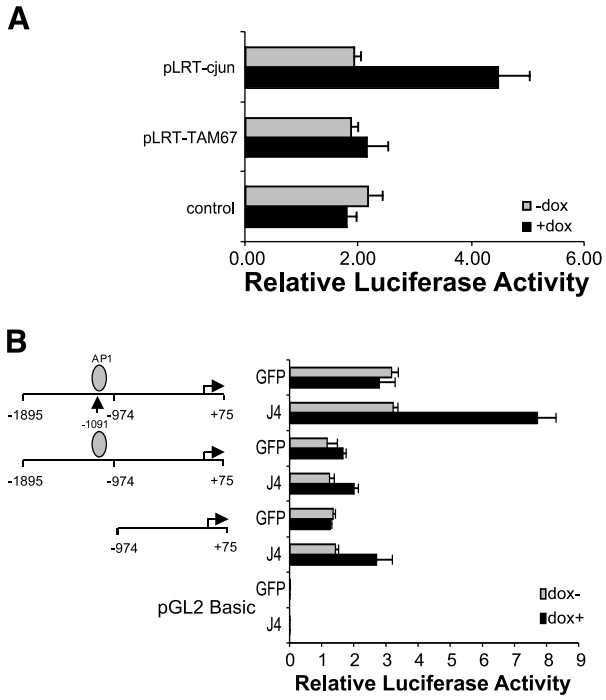

FIGURE 3. c-Jun/AP-1 regulation of the *HMG-I/Y* promoter. **A.** Rat1a cells were transiently cotransfected with pLRT-c-Jun or the AP-1 dominant negative mutant construct, pLRT-Tam67, and the −1,895 to +75 HMG-I/Y promoter fused to the luciferase reporter gene. *Control,* luciferase activities in cells transfected with one reporter alone. *Columns,* mean of triplicate experiments; *bars,* SD. **B.** Rat1a-J4 and Rat1a-GFP cells were transfected with *HMG-I/Y* promoter luciferase constructs representing −1,895 to +75, −1,895 to +75 with a mutated AP-1 site at −1,091, and a deletion construct representing −974 to +75. Luciferase activities are expressed relative to *Renilla* luciferase activity as a control for transfection efficiency. *Columns,* mean of triplicate experiments; *bars,* SD.

overexpression of HMG-I/Y confers anchorage-independent cell growth. The colonies in Rat1a cells overexpressing HMG-I/Y were smaller and fewer, suggesting that HMG-I/Y expression only partially recapitulates the c-Jun/AP-1 phenotype. HMG-I/Y is a chromatin remodeling protein with DNA binding domains called AT hooks, which bind to the minor groove of stretches of AT-rich DNA (13-21, 26). Several studies suggest an important role for *HMG-I/Y* in regulating gene transcription (13-26, 42-44). The *HMG-I/Y* gene is highly conserved among species; the transcribed regions of the human and mouse *HMG-I/Y* genes are ~80% identical at the nucleotide sequence level and >90% identical when only the protein-coding exons are considered (48). Both mouse and human *HMG-I/Y* promoters have a consensus AP-1 site at position −1,091 (47). The conservation of this site reflects the importance of the transcription factor binding site in the regulation of this gene. Ogram and Reeves (46) demonstrated that the AP-1 complex regulates HMG-I/Y expression during differentiation of human K562 erythroleukemic cells after treatment with 12-*O*-tetradecanoylphorbol-13-acetate. However, promoter analysis in this case revealed that a variant AP-1 site with one-base mismatch (TGACACA) within the human *HMG-I/Y* promoter was responsible for 12-*O*-tetradecanoylphorbol-13-acetate induction. This site (which is conserved within the mouse promoter) was downstream to the promoter sequence used for our studies. The variant AP-1 site was not included in our studies because it is in a region of the *HMG-I/Y* promoter that is not responsive to serum or growth factor stimulation in murine fibroblasts. Thus, it may not contribute to the induction of *HMG-I/Y* in response to growth-regulatory signals. Our gel mobility shift and reporter assays demonstrated that the AP-1 site at position −1,091 is a novel and critical element for c-Jun/AP-1 up-regulation of *HMG-I/Y.* Likely, the usage of different AP-1 sites depends in part on the transcriptional milieu, which in turn reflects the biological system being tested.

There is substantial evidence supporting the role of HMG-I/Y in cellular transformation and human malignancy. HMG-I/Y is expressed at low levels in normal or differentiated cells while higher in response to a wider range of growth factors (30, 49-52). *HMG-I/Y* is a direct target of the c-*myc* oncogene. Moreover, HMG-I/Y is necessary for c-*myc*–mediated transformation in Burkitt's lymphoma cells, a c-*myc*–driven human malignancy. Further, expression of HMG-I/Y in nontumorigenic breast cells converts them to a tumorigenic state (44). Previous work has demonstrated that several malignant tumors have aberrant expression of HMG-I/Y proteins (26-41, 48, 53, 54). In addition, use of an adenovirus carrying the mouse *HMG-I/Y* gene in an antisense orientation suppressed HMG-I/Y protein synthesis and induced programmed cell death of a human thyroid anaplastic carcinoma cell line ARO (54). Interestingly, in the ARO cell line, c-Jun, JunD, Fra1, and AP-1 binding activities are all increased (55).

Critical to our understanding of how c-Jun/AP-1 mediates such diverse biological actions is to precisely identify its mechanisms of transcriptional activity and its relevant "downstream" targets. c-Jun/AP-1 regulates the expression of a myriad of genes (9, 10, 56). Many of these genes are direct transcriptional targets for c-Jun/AP-1 with AP-1 consensus sequences within their promoters. Determining the relevance of these genes for a given biological activity has proven more difficult. We have previously characterized the c-Jun–responsive genes in Rat1a cells using microarrays (9, 10) and demonstrated that several of these genes are necessary for nonadherent cell growth. *HMG-I/Y* is clearly another one and perhaps the most intriguing. Not only is *HMG-I/Y* expression necessary for c-Jun/AP-1–induced nonadherent cell growth, forced expression of the gene produces transformation of Rat1a cells. Thus, *HMG-I/Y* appears to partially recapitulate the biological system. The precise role of c-Jun/HMG-I/Y in the nonadherent growth of Rat1a cells remains to be defined. Because there is no effect on cellular proliferation of antisense *HMG-I/Y,* c-Jun/HMG-I/Y acts by a different mechanism(s). Rat1a cells undergo anoikis when in the nonadherent state, and c-Jun/HMG-I/Y may be inhibiting this process. Further, the role of HMG-I/Y in chromatin remodeling (15-19, 22-26) suggests a new and novel way in which c-Jun/AP-1 can regulate gene expression. c-Jun/AP-1–induced expression of HMG-I/Y may result in alterations in chromatin structure and in turn availability of c-Jun/AP-1–regulated targets. Of interest, genes that are induced by HMG-I/Y expression include many genes involved in the extracellular matrix including the matrix metalloproteinases

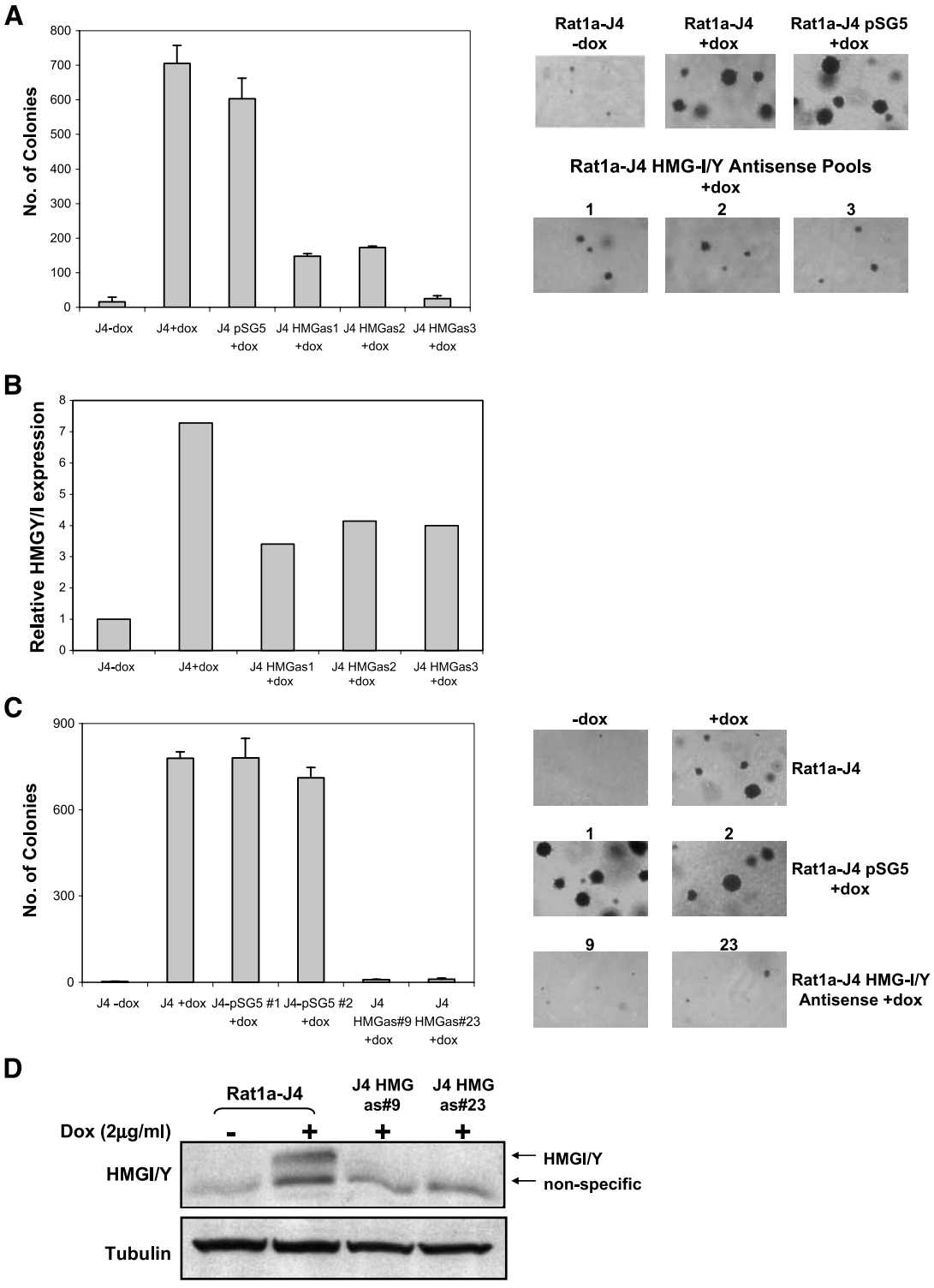

FIGURE 4. Antisense *HMG-I/Y* inhibits c-Jun–regulated anchorage-independent growth. **A.** Colony formation in Rat1a-J4 control (*–dox*), c-Jun–expressing clones (*+dox*), and pools of Rat1a-J4 pSG5 and Rat1a-J4-asHMG-I/Y clones after growth for 14 days in soft agarose. *Columns,* mean of triplicate experiments; *bars,* SD. *Right,* colony size. **B.** HMG-I/Y protein levels in Rat1a-J4 and pools of Rat1a-J4-asHMG-I/Y after normalization using tubulin levels to control for loading differences. Antisense *HMG-I/Y* decreases the HMG-I/Y protein levels by ~50%. **C.** Colony formation in Rat1a-J4 control (*–dox*), c-Jun–expressing clones (*+dox*), and two clones of Rat1a-J4 pSG5 and Rat1a-J4-asHMG-I/Y (*J4HMGas#9* and *J4HMGas#23*) after growth for 14 days in soft agarose. *Right,* colony size. **D.** Western blot analysis of HMG-I/Y expression in Rat1a-J4 cells (*–dox* or *+dox*) and Rat1a-J4-asHMG-I/Y clones after treatment for 7 days with doxycycline. The *nonspecific band* below HMG-I/Y was consistent between experiments, and its level was not altered in the presence of doxycycline.

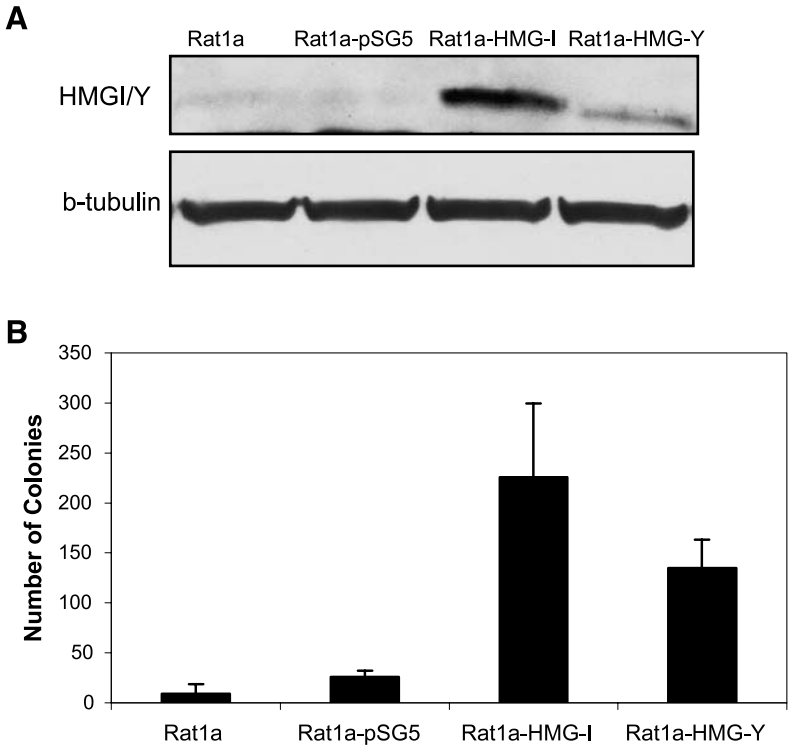

FIGURE 5. HMG-I/Y overexpression in Rat1a cells induces soft agarose colony formation. **A.** Western blots showing expression of HMG-I/Y in Rat1a-HMG-I/Y cells compared with parental Rat1a and vector control Rat1a-pSG5 cells. **B.** Number of colonies observed in Rat1a, Rat1a-pSG5, Rat1a-HMG-I, and Rat1a-HMG-Y after 14 days of growth in soft agarose.

and collagen genes (44, 57, 58), which are also AP-1–regulated genes (59-62). This suggests that HMG-I/Y may mediate some of the downstream AP-1 effects. In fact, direct interactions between HMG-I/Y and AP-1 components have been described (17, 20, 63-65). A systematic comparison of genes up-regulated by HMG-I/Y and AP-1 might identify genes with transcription dependent on c-Jun induction of HMG-I/Y.

In conclusion, the results in this article establish that *HMG-I/Y* is a direct target of c-Jun/AP-1. In Rat1a cells, up-regulation of *HMG-I/Y* is necessary and partially sufficient for c-Jun–induced nonadherent cell growth.

Materials and Methods

Cells and Cell Culture

Rat1a cells expressing either c-Jun (Rat1a-J2 and Rat1a-J4) or green fluorescent protein (Rat1a-GFP) in a doxycycline-controlled manner have been described previously (9). All cells were maintained in DMEM (Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) and 1% L-glutamine (Invitrogen Life Technologies). For nonadherent growth, cells were plated in PolyHeme-coated dishes in the presence or absence of doxycycline (2 μg/mL) to induce c-Jun expression.

Reporter Constructs and Expression Plasmids

A 2-kb *Hin*dIII/*Bam*HI fragment from the 4.6-kb HMG-I/Y-GH plasmid (33) was subcloned into pBluescript II KS. The resulting plasmid was digested with *Kpn*I and *Bam*HI, and the released fragment was cloned into pGL2-basic (Promega, Madison, WI). This plasmid was designated pGL2 HMG-I/Y −1,895/+75. A 5′ deletion fragment of the HMG-I/Y promoter from −974 to +75 was cloned into pBluescript II KS using *Hin*dIII and *Bam*HI. A 1.1-kb *Kpn*I/*Bam*HI fragment from HMG-I/Y−974/+75 pBluescript II KS was subcloned into pGL2-basic and designated pGL2 HMG-I/Y−974/+75. The plasmid pGL2 HMG-I/Y−1,895/+75 AP-1 mutant was created by mutating the AP-1 consensus site in the HMG promoter in plasmid pGL2 HMG-I/Y−1,895/+75. The site was mutated using the GeneEditor in vitro site-directed mutagenesis system (Promega) according to the manufacturer's recommendations with the following oligonucleotide with the mutated bases italicized: GGGGAACAGAGTTAT*CTCGAG*CAGTCGTG-TGTCACT. The plasmid expressing murine HMG-I in the antisense orientation (pSG5-AS-HMG-I) was made by cloning an HMG-I *Bam*HI fragment (a gift from J. Maher, Jackson, MS) in the antisense orientation into pSG5 at the same site. pLRT-c-Jun, pLRT-Tam67, pSG5-HMG-I, and pSG5-HMG-Y have been described previously (9, 16).

Northern Blot Analysis

Total cellular RNA from Rat1a-J2, Rat1a-J4, and Rat1a-GFP cells was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (1 μg) was separated on 1% agarose gels and transferred to nitrocellulose membranes. The membranes were

probed with [α-^{32}P]dCTP-labeled cDNA probes, and the signal was detected by autoradiography. The cDNA probe for *HMG-I* was obtained from pSG5-HMG-I (33), and that for c-*jun* was obtained from pBlue-c-*jun* (10).

Western Blot Analysis

Cell lysates from Rat1a-J4 and Rat1a-GFP cells grown in the absence or presence of doxycycline (2 μg/mL) were prepared by lysing the cells in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mmol/L Tris (pH 7.4)] supplemented with 0.1 mmol/L phenylmethylsulfonyl fluoride, 100 μg/mL aprotinin, and 100 μg/mL leupeptin. The cell lysates were sonicated and centrifuged to remove debris, and protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of protein were separated on 12% or 15% SDS gels, transferred to nitrocellulose membranes (PROTRAN, Schleicher & Schuell, Keene, NH), and incubated with the following antibodies: anti-HMG-I/Y antibody (29), anti-c-Jun (sc45x), and anti-β-tubulin (sc9104, Santa Cruz Biotechnology, Santa Cruz, CA). The signal was detected by enhanced chemiluminescence.

Transient Transfections and Luciferase Reporter Assays

Exponentially growing Rat1a-J4 or Rat1a cells were seeded at a density of 3×10^5 cells per 60 mm dish. The cells were transfected with 1 μg HMG-I/Y promoter reporter constructs using FuGene6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN). Forty nanograms of *Renilla* luciferase (Promega) were cotransfected to control for transfection efficiency. After overnight incubation, cells were trypsinized and plated under nonadherent growth conditions in PolyHeme-coated 12-well dishes in the presence or absence of doxycycline (2 μg/mL). Luciferase activity was measured 2 days after induction with doxycycline using the Dual-Luciferase Reporter Assay System (Promega).

Gel Mobility Shift and Supershift Assays

Nuclear proteins were prepared from Rat1a-J4 cells grown for 3 days under nonadherent conditions in the presence or absence of doxycycline (2 μg/mL) as described previously (66). Briefly, cells were pelleted and washed once with cold PBS. The cell pellet was resuspended in five packed volumes of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, and 0.5 mmol/L DTT and allowed to swell on ice for 10 minutes. The cells were lysed by slow uptake and rapid ejection (5×) through a 25 gauge needle. Nuclei were pelleted and resuspended in one-third volume of 20 mmol/L HEPES (pH 7.9), 25% glycerol, 0.45 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 100 μg/mL aprotinin, and 1 μg/mL leupeptin and rotated for 30 minutes at 4°C. The resulting nuclear proteins were dialyzed against 50 volumes of 20 mmol/L HEPES (pH 7.9), 25% glycerol, 0.1 mol/L KCl, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL each of aprotinin and leupeptin for 2 hours at 4°C. Supernatants were stored at −70°C, and protein concentrations were determined as described above. Five micrograms of nuclear extract were incubated with 2 μg of poly-deoxyinosinic-deoxycytidylic acid and 4 μL of incubation buffer [100 mmol/L HEPES (pH 7.9), 250 mmol/L KCl, 2.5 mmol/L EDTA, 5 mmol/L $MgCl_2$, and 20% Ficoll 400] in a final volume of 20 μL for 10 minutes on ice prior to the addition of double-stranded oligonucleotides labeled with [γ-^{32}P]ATP. The protein-DNA mixtures were incubated on ice for a further 20 minutes and separated on nondenaturing 5% polyacrylamide gels at 180 volts for 2 to 3 hours at 4°C in 0.5× Tris-borate EDTA. Unlabeled (100 ng) wild-type or mutated oligonucleotides were used as competitors to confirm the specificity of the complexes formed. The following oligonucleotides corresponding to the murine *HMG-I/Y* promoter were used as probes: AP-1–like (1), wild-type GCCCCTCCATGACTTCCTCCTTCTCCA and mutated GCCCCTCCATGGCTTGCTCCTTCTCCA; AP-1–like (2), wild-type CCCTGGAACTGAGGGTCACCTGGACC and mutated CCCTGGAACTGGGTGTCACCTGGACC; and AP-1, wild-type GGAACAGAGTTATGAGTCACAGTCGTGTGT and mutated GGAACAGAGTTATGGGTTGCAGTCGTGTGT.

For supershift assays, antibodies (sc45x, sc46x, sc74x, sc52x, and sc605x, Santa Cruz Biotechnology) were added to the DNA-protein mixture and incubated on ice for 20 minutes prior to the addition of the probe.

Colony-Forming Assays

Cells (1×10^4) were plated in triplicate in 6 mL of 0.35% agarose (Sea Plaque, FMC Bioproducts, Rockland, ME) in complete growth medium in the presence or absence of doxycycline (2 μg/mL) overlaid on a 0.7% agarose base, also in complete growth medium, and incubated for 2 to 3 weeks at 37°C. Colonies were stained with *p*-iodonitrotetrazolium violet (1 mg/mL) and counted using a GS710 Calibrated Imaging Densitometer and Quantity One software (Bio-Rad).

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