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). HMG-I/Y was also shown to be induced by phorbol esters, although this induction was relatively modest (46). We used our previously
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 expression 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. 2A; refs. 46, 47). To determine if HMG-I/Y is a target gene that is necessary, and partially sufficient, for c-Jun–induced transformation and anchorage-independent growth of Rat1a cells.

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.

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

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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 Rat1α-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 induc-
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.
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 HindIII/BamHI fragment from the 4.6-kb HMG-I-Y-
GH plasmid (33) was subcloned into pBluescript II KS. The
resulting plasmid was digested with KpnI and BamHI, 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
HindIII and BamHI. A 1.1-kb KpnI/BamHI 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: GGGGAACAGAGTTATCTCGAGCAGTCGTG-
TGTCACT. The plasmid expressing murine HMG-I in the
antisense orientation (pSG5-AS-HMG-I) was made by cloning
an HMG-I BamHI 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

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.

Mol Cancer Res 2004;2(5). May 2004
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probed with [α-32P]dCTP-labeled cDNA probes, and the signal was detected by autoradiography. The cDNA probe for HMG-I was obtained from pBSG-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.

**Transgenic 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 MgCl2, 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 MgCl2, 0.2 mol/mL 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 mol/mL 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-deoxyuridylic acid and 4 μL of incubation buffer [100 mmol/L HEPES (pH 7.9), 250 mmol/mL KCl, 2.5 mmol/mL EDTA, 5 mmol/L MgCl2, 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-32P]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 GCCCTTCCCATGACTTCTCCTCTTCCA and mutated GCCCTTCCCATGCTTGTTCCTCTTCCA; AP-1–like (2), wild-type CCCTGGAACCTGGTACACTGACGACC and mutated CCGTGGAACCTGGTACACGTGACC; and AP-1, wild-type GGAACAGTTATATAGCTTAGTGTGTTGGTTTGTGTTGTTGTGT.

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.

**Colonization-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-iomodinitrotetrazolium violet (1 mg/mL) and counted using a GS710 Calibrated Imaging Densitometer and Quantity One software (Bio-Rad).

**References**


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