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Role for BRG1 in Cell Cycle Control and Tumor Suppression

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Human BRG1, a subunit of the Swi/Snf chromatin remodeling apparatus, has been implicated in regulation of cellular proliferation and is a candidate tumor suppressor. Reintroduction of BRG1 into a breast tumor cell line, ALAB, carrying a defined mutation in the *BRG1* gene, induced growth arrest. Gene expression data revealed that the arrest may in part be accounted for by down-regulation of select E2F target genes such as cyclin E, but more dramatically, by up-regulation of mRNAs for the cyclin-dependent kinase inhibitors p21 and p15. Protein levels of both p15 and p21 were induced, and p21 protein was recruited to a complex with cyclin-dependent kinase, CDK2, to inhibit its activity. BRG1 can associate with the p21 promoter in a p53-independent manner, suggesting that the induction of p21 by BRG1 may be direct. Further, using microarray and real-time PCR analysis we identified several novel BRG1-regulated genes. Our work provides further evidence for a role for BRG1 in the regulation of several genes involved in key steps in tumorigenesis and has revealed a potential mechanism for BRG1-induced growth arrest.

The evolutionarily conserved Swi/Snf chromatin remodeling apparatus has functions in both transcriptional activation and repression (29, 36, 55). The Swi/Snf complex has been shown to alter the arrangement of nucleosomes along DNA in an ATP-dependent manner in vitro, thus allowing access for transcription factors to bind their target DNA sequences (29). The mammalian Swi/Snf complex is a 1.5- to 2.0-MDa multisubunit complex, the ATPase activity being generated by BRG1, a 190-kDa component, or a related protein, hBRM (58).

In mammalian cells BRG1 is known to play a role in the regulation of cellular proliferation (8, 49). This activity of BRG1 has been shown to be dependent on its association with the retinoblastoma tumor suppressor gene product (pRB) and, consequently, via the repression of E2F target gene expression (29). Specifically, it was demonstrated that BRG1/pRB complexes repress certain E2F target genes such as *cyclin E*, *cyclin A*, and *CDC2* (48, 61). The temporally ordered association of BRG1 with histone deacetylase and pRB complexes ensures sequential activation of *cyclin E* and then *cyclin A* and maintains the order of G_1 and S phases of the cell cycle (61).

Studies have now revealed that *BRG1* is frequently deleted or mutated in a variety of tumor cell lines (59), implicating *BRG1* as a potential tumor suppressor gene in its own right. The tumor suppressor function of BRG1 is supported by the generation of mice harboring a *BRG1* null mutation. Heterozygotes for *BRG1* are susceptible to neoplasia and display large subcutaneous tumors, suggesting that a partial reduction in cellular BRG1 level leads to critical loss of proliferation control (5). More recent work has shown loss of expression of BRG1/BRM expression in a subset of primary lung tumors associated with poor prognosis (39). Several studies have also revealed that the *INI1*/*SNF5* conserved component of the SWI/SNF complex is also mutated in human tumors, providing further evidence of a role for the SWI/SNF complex in growth regulation and tumor suppression (3, 21, 57).

It has been demonstrated that BRG1 regulates the expression of other non-E2F-regulated genes that are important in cancer. Murphy et al. demonstrated that BRG1 repressed transcription of the oncogene *c-FOS* in an E2F-independent manner (32), and recent work from Strobeck et al. suggests that BRG1 regulates *CD44* expression (47). Misregulation of *CD44*, encoding a transmembrane glycoprotein involved in cell-cell interactions and cell-matrix adhesion, has been implicated in tumor growth and metastasis. The recent identification of distinct classes of transcription factors that associate with BRG1 and BRM may provide clues as to which of and how these factors are recruited to specific promoters (19). Although the total number of genes regulated by mammalian Swi/Snf is unknown, the yeast Swi/Snf complex is estimated to control expression of 5 to 6% of genes (18, 50).

The majority of studies of BRG1 function in mammalian cells have relied on two cell lines, C33A and SW13, that lack BRG1 expression for unknown reasons. Our recent work has identified a collection of cell lines with defined mutations in the BRG1 gene, providing a broader spectrum of cell lines to dissect out BRG1's role in gene regulation (59). Using the breast tumor cell line ALAB, which contains a mutation (C→T) at nucleotide 1630 in exon 10 of *BRG*, resulting in a STOP codon and consequently no detectable protein expression, we have studied global gene induction and repression using microarray and real-time PCR analysis by reintroduction of BRG1. By comparison with earlier studies, we show here that BRG1 induces a number of novel genes in this cell type that are involved in cell adhesion, motility, and proliferation. Gene expression profiles in different cellular contexts will help provide a more comprehensive understanding of the mechanism of action of BRG1 in the control of tumorigenesis and metastasis.

MATERIALS AND METHODS

Recombinant *BRG1* adenovirus. Recombinant adenoviruses expressing full-length wild-type *BRG1* cDNA or an ATPase mutant (K785R) BRG1 cDNA were constructed using the method described by He et al. (16). Human *BRG1* cDNA was subcloned into the pShuttle-CMV vector. *Escherichia coli* BJ5183 cells were

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transformed with pShuttle-CMV*BRG1* and the adenoviral genome plasmid, pAdEasy, and transformants were selected on Luria-Bertani agar plates containing 50 μg of kanamycin/ml. The recombinant adenovirus was produced and amplified in 293A cells and purified according to the method of He et al.

Cell culture and adenovirus infection. ALAB, SW13, and SAOS2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum, nonessential amino acids, and penicillin-streptomycin. 293A cells (QBIOGENE) were cultured in DMEM supplemented with 5% serum, nonessential amino acids, and penicillin-streptomycin. In experiments to optimize infection conditions, BRG1 was visualized by immunofluorescence with a monoclonal antibody (MAb) to BRG1 (5D11) (59). For experiments presented here, cells were infected with 1,000 particles per cell (multiplicity of infection = 10) using cationic lipids for enhanced infection (10). The cells were incubated with adenovirus at 37°C for 3 h. Following the incubation, the virus cocktail was replaced with fresh medium and cells were harvested at the indicated hour postinfection (p.i.). Time zero corresponds to virus addition to cells.

Proliferation assay. Cells were plated in Cytostar-T scintillating microtiter plates (Amersham), incubated for 24 h at 37°C, and then incubated with the indicated viruses for 3 h at 37°C. The virus cocktails were removed at 3 h p.i., and medium was replaced on all samples with medium containing [^{14}C]thymidine. Counts per minute were measured using a Packard TopCount plate reader at 0, 24, 48, and 72 h after addition of medium containing [^{14}C]thymidine. Staurosporine (Calbiochem) was used at 500 nM.

Cell cycle analysis. Cells were plated in 10-cm dishes, incubated for 24 h at 37°C, and then infected with the adenoviruses as described above. AdVector was used as control virus because of AdGFP interference with the fluorescence of the DNA-binding dye, 7-amino-actinomycin D (7-AAD; BD Biosciences). At the indicated times, cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdU) for 30 min, harvested, fixed in 70% ethanol, and stained with anti-BrdU fluorescein isothiocyanate (Becton Dickinson) for flow cytometric analysis following the manufacturer's recommendations.

mRNA analysis. Total RNA was extracted using an RNeasy mini kit (Qiagen) with an on-column DNase treatment (Qiagen). Five micrograms of RNA in a 100-μl reaction volume was used for first-strand cDNA synthesis. The reaction mixture included 2.5 μg of oligo(dT)$_{12-18}$ (Gibco-BRL), 250 ng of random hexamers (Promega), 10 mM dithiothreitol (Gibco-BRL), 1× First-Strand buffer (Gibco-BRL), 500 μM deoxynucleoside triphosphate mix (Pharmacia), 80 U of RNasin (Roche), and 500 U of Superscript II reverse transcriptase (Gibco-BRL) and was incubated for 50 min at 42°C. The cDNA was diluted and used as template in a quantitative real-time PCR (Taqman) using a GeneAmp 5700 sequence detection system (Perkin-Elmer). Reactions were done in a final volume of 20 μl in SYBR Green PCR mix (Perkin-Elmer) and included 10 ng of cDNA and primers, each at a concentration of 400 nM. Primer sequences are listed in the Appendix. The mRNA expression level for all samples was normalized to the housekeeping gene S9. Data from triplicate samples of a representative experiment are shown.

Immunoblot analysis and immunoprecipitations. Cell lysates were prepared as described previously (45), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted to Immobilon (Millipore) or nitrocellulose (Schleicher & Schuell). Protein concentration in each clarified lysate was determined by the dye-binding method using bovine serum albumin as the standard. For CDK2 immunoprecipitations, 1 mg of protein was diluted to a final volume of 400 μl in lysis buffer and mixed with 2 μl of normal rabbit serum and protein A-agarose beads to preclear the lysate. After incubation at 4°C on a rotating shaker for 45 min, the precleared supernatant was collected and mixed with either 2 μg of protein A-purified anti-CDK2 antibody or 2 μg of protein A-purified normal rabbit antibody and incubated as before for 45 min. Protein A-agarose beads were added to the mixture, and incubation was continued for another 45 min. Beads were collected by brief centrifugation, washed three times with 1 ml of lysis buffer, and recollected. Bead-bound immune complexes were solubilized in SDS-PAGE sample buffer, immediately boiled for 5 min, resolved by SDS-PAGE, and analyzed by immunoblotting. Immunoblotting (50 to 100 μg of protein for each sample) was performed as described previously (59). Antibodies to BRG1, cyclin E (HE12), CDK2, and p15 have been described elsewhere (35, 45, 59). Other antibodies were purchased from the following sources: anti-RB polyclonal antibody (C-15; Santa Cruz); anti-p21 Ab-11 MAb (clone CP74; Neomarkers); anti-Waf1 Ab-1 MAb (clone EA10; Oncogene Research Products); anti-p21 MAb (clone 70; Transduction Labs); anti-pSTAIR (P-7962) MAb (Sigma); anti-p53 MAb (Ab-6 clone DO-1; Oncogene Research Products); anti-rabbit HRP (Amersham); anti-mouse HRP (Amersham).

Immobilized template assay. Template assays were performed by the method of Holloway et al. (17). Fragments of the human p21 promoter (−2328 to −2224 and −306 to −5) or the p21 open reading frame (+314 to + 631) were amplified by PCR with either pWWP-Luc (9) or CMV-p21 as template and biotinylated primers (−2328, −5, +314). PCR products were resolved by gel electrophoresis and purified with a Qiagen QIAquick gel extraction kit. For each reaction, 150 ng of template was bound to Dynabeads M280 streptavidin (Dynal) and blocked as described previously (38). Nuclear extracts were prepared (100 μg per reaction mixture) and supplemented with sheared salmon sperm DNA and poly(dI-dC) and allowed to bind the templates as described elsewhere (17). Precipitated proteins were eluted from the beads with SDS-PAGE sample buffer and subjected to SDS-PAGE and immunoblotting with anti-BRG1 or anti-p53 antibodies.

cDNA microarray analysis. ALAB cells were seeded in 500-cm^2 plates and incubated for 24 h at 37°C. Cells were infected with either Ad*BRG1* or AdGFP by the cationic lipid method at a multiplicity of infection of 10 (see above). For one experiment cells were harvested at 18 h p.i., and for the other experiment cells were harvested at 24 h p.i. Cells were washed in DMEM without additives, and cell pellets were frozen at −80°C. RNA preparations, probe synthesis, and microarray hybridizations were performed as described previously (62). The probe pairs were used for hybridization to six microarrays that contained more than 40,000 human cDNA elements (Incyte Genomics).

RESULTS

To examine the expression profile of genes regulated by BRG1, we constructed a recombinant adenovirus that expresses full-length *BRG1* cDNA to allow BRG1 expression in a variety of cell lines bearing mutant forms of BRG1. ALAB, a breast tumor cell line that contains a mutation (C→T) at nucleotide 1630 in exon 10 of *BRG1*, resulting in a STOP codon and consequently no detectable protein expression (59), was selected for our studies. SW13 cells were used as a means of correlating our data with those published by other investigators. Using the *BRG1* adenovirus, we were able to achieve *BRG1* expression in all cells of an infected population as determined by anti-BRG1 immunofluorescence (data not shown). In a time-course immunoblot analysis of *BRG1* expression, BRG1 could be detected as early as 6 h p.i., and expression increased dramatically at 11 and 24 h p.i. and was maintained for at least 72 h p.i. (Fig. 1A).

Earlier studies have demonstrated that reintroducing *BRG1* expression by stable transfection into several different BRG1 mutant cell lines causes growth arrest (8, 45, 49, 59). As shown in Fig. 1B, Ad*BRG1* blocked proliferation of ALAB cells, inhibiting the [^{14}C]thymidine uptake by 30% at 24 h p.i., a margin that increased to 50% by 48 h p.i. compared to untreated cells and cells expressing AdGFP. As expected, cells treated with staurosporine incorporated very little [^{14}C]thymidine. As a final analysis, we compared BrdU incorporation rates of cells infected with either Ad*BRG1* or AdVector by using anti-BrdU staining and flow cytometric analysis (Fig. 1C). At 48 h p.i., 9% of Ad*BRG1*-infected cells were in S phase compared to 26% of AdVector-infected cells in S phase. Thus, it appears that BRG1 expression in ALAB cells reduces the population of cells in S phase.

Differential gene induction profile in ALAB cells. To validate our system for the study of gene regulation by BRG1, we tested the expression of CD44, mCSF-1, and osteonectin, three genes which have previously been shown to be regulated by BRG1 in SW13 cells (25, 47). ALAB and SW13 cells were infected with either AdGFP or Ad*BRG1*, and gene expression was examined by real-time PCR. As shown in Fig. 2A, we observed a 42-fold induction over AdGFP of CD44 by Ad*BRG1* in SW13 cells and a similar albeit smaller induction (8- to 9-fold) in ALAB cells at 18 h p.i. Osteonectin was

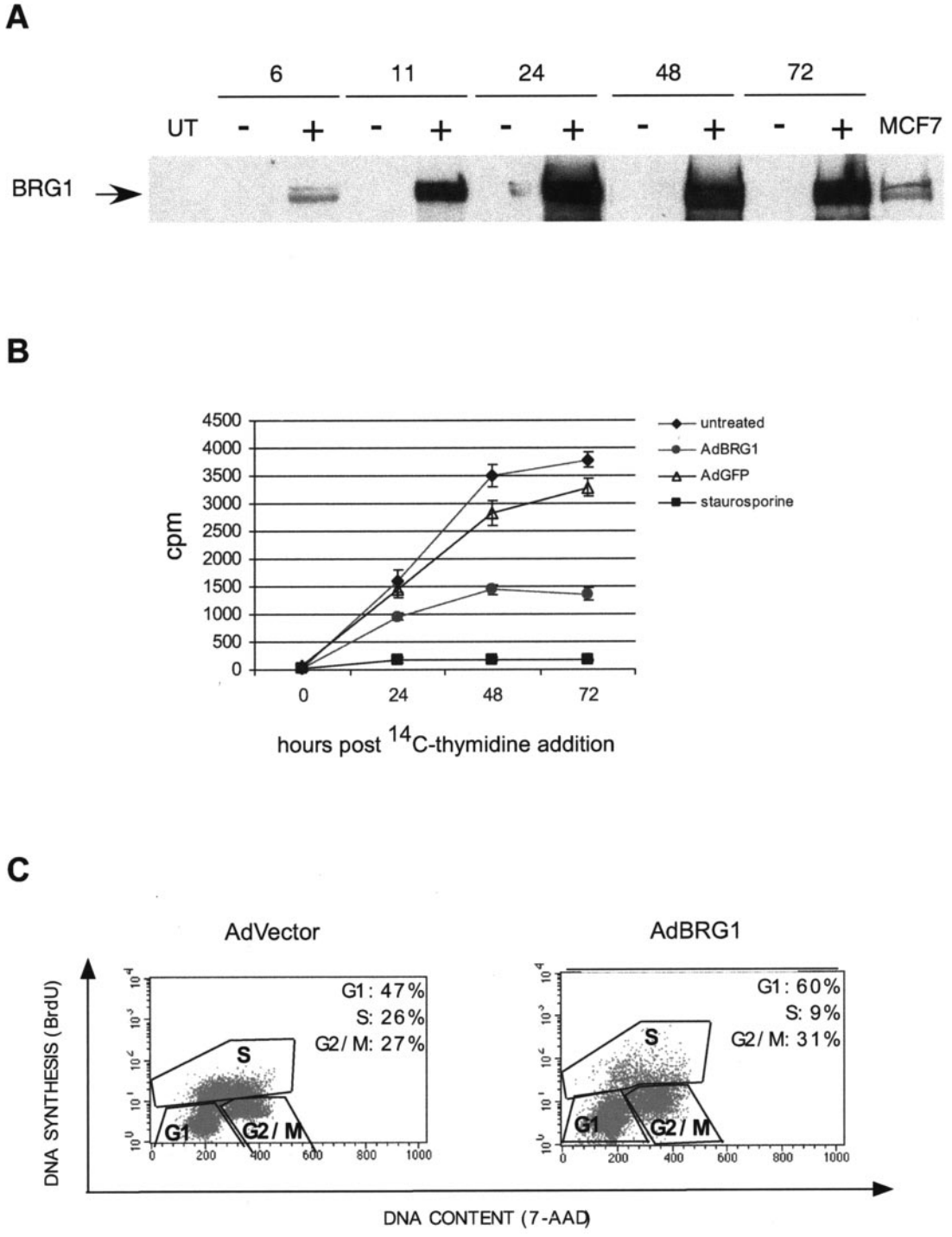

FIG. 1. Expression of *BRG1* from Ad*BRG1* causes cell cycle arrest. (A) Lysates were prepared from asynchronous ALAB cells infected with either Ad*BRG1* (+) or AdGFP (−) at the indicated times p.i., subjected to SDS-PAGE, and analyzed by immunoblotting using polyclonal anti-BRG1 sera. Lysates from untreated (UT) ALAB cells and from MCF7 were loaded as negative and positive controls, respectively. (B) Asynchronous ALAB cells seeded in a Cytostar-T scintillating microplate were untreated (♦), treated with staurosporine (■), or infected with adenoviruses expressing *BRG1* (●) or GFP (△). Three hours after virus addition, medium was replaced on all samples with medium containing [^{14}C]thymidine, and incorporated ^{14}C was measured at the indicated times (after [^{14}C]thymidine addition) to assay proliferation. (C) Asynchronous ALAB cells infected with the indicated adenoviruses for the indicated times were pulse-labeled with BrdU for 30 min prior to harvest for anti-BrdU flow cytometry cell cycle analysis. The *x* axis represents DNA content as assayed by 7-AAD (BD Biosciences). The *y* axis represents DNA synthesis as assayed by BrdU content.

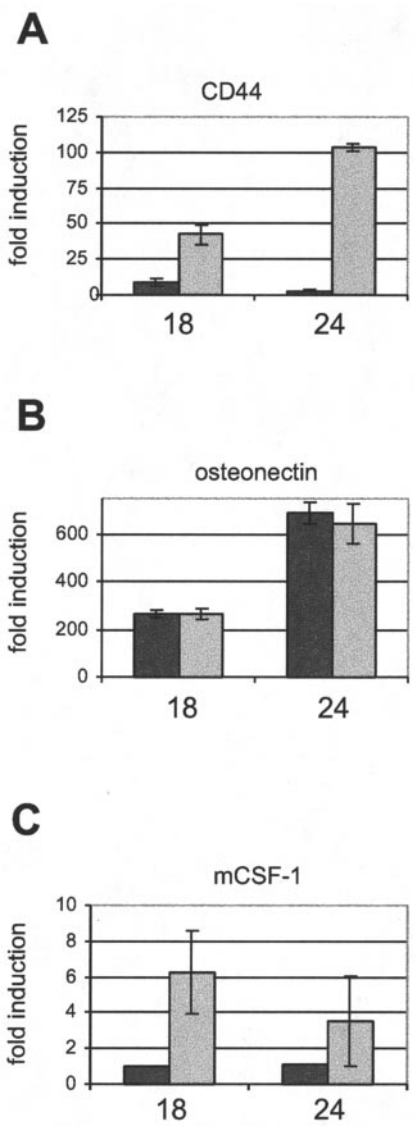

FIG. 2. mRNA levels of known BRG1-regulated genes in ALAB cells (black bars) and SW13 cells (gray bars) infected with Ad*BRG1* or control adenovirus for 18 or 24 h. Data for each gene are normalized to S9 ribosomal protein gene expression and expressed as Ad*BRG1* induction over control adenovirus.

robustly induced in both SW13 cells and ALAB cells, exceeding 600-fold induction at the 24-h time point in both cell types (Fig. 2B). In contrast, the regulation of mCSF-1 expression was not conserved between cell lines. In SW13 cells, mCSF-1 was induced sixfold over AdGFP at 18 h p.i., in agreement with the published microarray data (25); however, no induction was seen in ALAB cells (Fig. 2C). BRG1 was expressed at similar levels in both cell lines (data not shown).

cDNA microarray analysis revealed novel genes regulated by BRG1. Given the significant differences seen in the effects of BRG1 on selected gene expression in ALAB compared to SW13 cells, we decided to perform cDNA microarray hybridization experiments to look at broader effects of BRG1 on gene expression in ALAB cells. cDNA microarrays containing 40,000 human cDNA elements were probed in a competitive hybridization with cDNA probes from ALAB cells infected with either Ad*BRG1* or AdGFP. The hybridization was performed from cells harvested at 18 h p.i. and from cells harvested at 24 h p.i. Included in Table 1 are some selected genes for which the balanced differential expression (induction and repression) among the two probes was twofold or greater for both of the hybridizations. A full list of regulated genes is provided in the Appendix. The total number of genes induced at least twofold in both hybridizations was 70. In addition, 65 genes were repressed at least twofold in both hybridizations among 40,000 genes screened. In comparison, studies in SW13 cells by Liu et al. observed 80 genes activated greater than threefold and 2 genes repressed greater than threefold in at least two of three microarray hybridizations of 22,000 genes (25).

Aside from BRG1, which was induced 18- to 44-fold, the most significantly induced gene in the microarray experiments was *hBRM* (9.2- to 10.2-fold induction). This was surprising, given earlier work from Reisman et al. (40), who reported no such modulation. To verify these findings, we confirmed our data by real-time PCR. We observed an eightfold induction of hBRM at 24 h p.i. in ALAB cells (Fig. 3A, left panel) using three independent sets of hBRM-specific primers. This induction was seen with wild-type BRG1 but not with an ATPase-dead mutant of BRG (K→R) as shown in Fig. 3B. Immunoblot analysis with an antibody specific for hBRM (40) verified the induction of hBRM in ALAB cells at the protein level (data not shown). Confirming published data, hBRM mRNA levels were unchanged by BRG1 reintroduction into SW13 cells, demonstrating a cell type selectivity to this response.

We also observed changes in the expression of the proto-oncogenes c-*JUN* and c-*MYC* by BRG1. c-*JUN* is a proto-oncogene encoding a member of the AP-1 family of transcription factors (15). Using real-time PCR, we confirmed the microarray result that BRG1 promoted expression of c-*JUN* in ALAB cells (Fig. 3B). The proto-oncogene c-*MYC*, which encodes a transcription factor with important roles in growth and proliferation (26, 44), was repressed in the microarrays (2.4-fold at 18 h p.i. and 3.3-fold at 24 h p.i.). Real-time PCR analysis of c-*MYC* expression confirmed a slight repression (threefold at 24 h p.i.) by BRG1 in ALAB cells (Fig. 3C) and in SW13 cells (two- to threefold at 24 h p.i. [data not shown]). Immortalized rat fibroblasts with homozygous deletion of c-*MYC* have a much longer cell doubling time and accumulate in the G_1 and G_2/M phases of the cell cycle, suggesting that myc promotes proliferation (26). Therefore, repression of c-*MYC* is consistent with BRG1-regulated growth arrest.

Consistent with an antiproliferative phenotype, we also detected up-regulation in expression of p21. $p21^{Cip1,Waf1}$ was induced in both hybridizations: 2.9-fold in the 18-h sample and 5.1-fold in the 24-h sample. p21 is one of several cyclin-dependent kinase inhibitors (CKIs), which are key negative regulators of the cell cycle by inhibition of cyclin-dependent kinases (CDKs) (14, 60). We were able to confirm the effect of Ad*BRG1* on expression of p21 in ALAB cells by real-time PCR, with p21 mRNA up-regulated 3-fold by 6 h p.i. and nearly 50-fold at 24 h p.i. (Fig. 4A). We also examined the expression of the related CKI p27 (37) but saw no change in mRNA levels by BRG1 (Fig. 4A). Interestingly, the ATPase mutant BRG(K→R) was able to induce p21, but to a lower

TABLE 1. Genes exhibiting differential expression upon reintroduction of BRG1[a]

Accession no.	Description	Induction or repression[b] (fold) at:	
		18 h	24 h
Cell cycle			
U03106	p21 (WAF1, Cip1)	2.9 ↑	5.1 ↑
M60974	GADD45	4.0 ↑	3.9 ↑
Transcriptional regulation			
X72889	hBRM	10.2 ↑	9.2 ↑
J04111	c-JUN	2.6 ↑	2.2 ↑
L42176	DraL	3.3 ↑	2.5 ↑
V00568	c-MYC	2.4 ↓	3.3 ↓
Signal transduction and regulation			
D29767	Tec protein-tyrosine kinase	3.0 ↑	2.3 ↑
D11327	HEPTP phosphatase	2.5 ↑	2.6 ↑
AF106858	GPR56	3.5 ↑	2.1 ↑
M24545	MCP-1	2.0 ↑	3.8 ↑
L20688	GDP-dissocation inhibitor (Ly-GDI)	2.8 ↓	2.7 ↓
U45984	CCR6	5.3 ↓	3.4 ↓
L09230	CCR1	4.7 ↓	4.4 ↓
U57940	CCR2	3.3 ↓	3.4 ↓
HNFGF45R	GPR43	3.7 ↓	2.4 ↓
L29219	CLK1	3.8 ↓	2.3 ↓
Ca^{2+} regulation and signaling			
M80899	AHNAK	3.9 ↑	5.2 ↑
Y07755	S100A2	3.3 ↑	4.1 ↑
M25874	PMCA4	2.5 ↑	2.4 ↑
Cell-cell or cell-matrix interactions			
U58516	Breast epithelial antigen BA46	7.1 ↑	9.2 ↑
M59911	Integrin α-3	3.2 ↑	2.1 ↑
BC008786	Integrin α-5	4.8 ↑	2.6 ↑
AK024818	MMP-1	3.4 ↑	2.9 ↑
U20982	IGFBP4	3.4 ↑	2.8 ↑
U89942	LOXL2	3.5 ↑	3.6 ↑
M17783	Glial-derived nexin	4.5 ↑	5.5 ↑
M11749	Thy-1 glycoprotein	3.3 ↑	2.3 ↑
NM_003872	Neuropilin 2	3.5 ↓	2.7 ↓
X07979	Integrin β-1	3.9 ↓	2.3 ↓
NM_004429	Ephrin B1	3.7 ↓	2.9 ↓
Cytoskeleton or cell architecture			
AL050179	Tropomyosin	3.3 ↑	4.5 ↑
L06237	Microtubule-associated protein 1B	2.9 ↑	2.4 ↑
M25384	Pregnancy-specific β-1-glycoprotein	7.4 ↑	4.0 ↑
U48734	α-Actinin, nonmuscle	2.1 ↑	2.2 ↑

[a] Listed are selected known genes whose expression was altered at least twofold in both microarray experiments.
[b] Arrows indicate induction (↑) or repression (↓).

level than the wild type (Fig. 4C). This suggests that BRG1 may have a function, in addition to chromatin remodeling, in the activation of p21. To complete our analysis of cell cycle inhibitors, we also examined the effect of Ad*BRG1* on the expression of the INK family of CKIs (13) (Fig. 4B). Ad*BRG1* induced a fivefold up-regulation of p15INK4b mRNA as early as 11 h p.i. Induction of p15 increased to 20-fold at 20 h p.i. In contrast, mRNA expression of the three other INK family members, p16, p18, and p19, remained unchanged. Thus, BRG1 up-regulates mRNA levels of both p15 and p21, with kinetics that precede the eventual cell cycle arrest induced by BRG1 reintroduction.

BRG1 also induces cell cycle arrest in SW13 cells, so we tested whether the induction of CKIs also occurred in SW13 by real-time PCR. In fact, both p21 and p15 were induced in SW13, albeit to a lower level than in ALAB cells (Fig. 4C). p21 was induced 3-fold at 18 h p.i. and 24 h p.i. in SW13, compared to 30- to 35-fold induction in ALAB in that experiment. The trend was similar for p15. At 24 h p.i., p15 was induced nearly 12-fold in ALAB and 4-fold in SW13.

Mechanism of p21 induction by BRG1. p21 is a known p53-responsive gene (9). Three other p53-regulated genes, GADD45, S100A2, and DRAL, were also induced by BRG1 (Table 1) (20, 43, 53). GADD45 induction (40-fold at 24 h p.i.) was confirmed by real-time PCR (data not shown), supporting a potential p53 link. As an alternative connection, both p21 and p15INK4b have also been identified as transforming growth factor β (TGF-β)-induced genes (13, 42). It was therefore of note that several other genes induced by BRG1 in the microarray experiments have been reported to be TGF-β re-

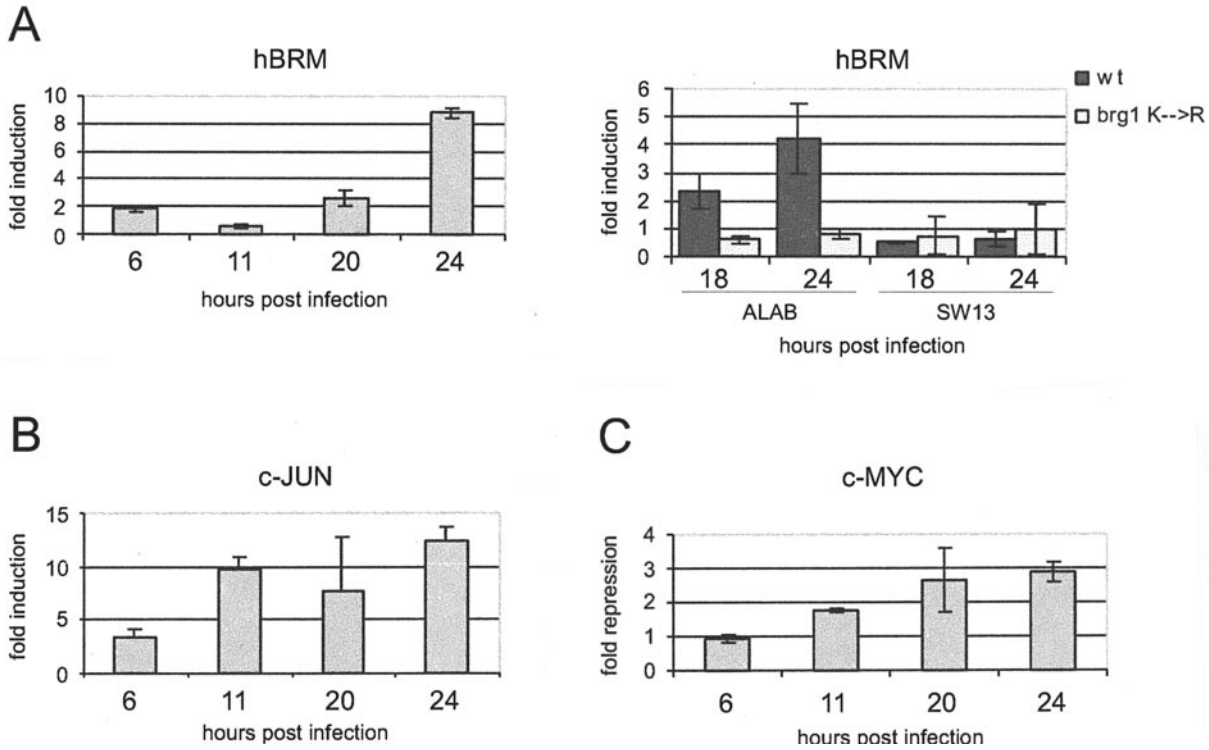

FIG. 3. Regulation of gene expression by BRG1. Results of mRNA expression analysis of *hBRM* (A), c-*JUN* (B), and c-*MYC* (C) in asynchronous ALAB cells or SW13 cells infected with either Ad*BRG1* or control adenovirus for the indicated times are shown. Data for each gene are normalized to S9 ribosomal protein gene expression and expressed as Ad*BRG1* (or Adbrg K→R) induction (or repression for c-*MYC*) over control adenovirus.

sponsive (Table 1): insulin-like growth factor binding protein-4 (2.5- to 3.4-fold), integrin alpha-5 (2.6- to 4.8-fold), integrin alpha-3 (2.1- to 3.2-fold), matrix metalloproteinase-1 (2.3- to 3.4-fold), c-JUN (2.2- to 2.6-fold), and tropomyosin α (3.3- to 4.5-fold) (2, 4, 6, 7, 12, 27, 56).

To further investigate the mechanism of p21 induction by BRG1, we first examined the status of p53 in the ALAB cell line. The p53 protein was undetectable by immunoblot analysis, even after treatment of cells with ionizing radiation or camptothecin, agents known to induce stabilization of p53, suggesting that these cells are functionally null for p53 (Fig. 5A). As further confirmation of the absence of functional p53, p21 was not induced either (Fig. 5A). Normal fibroblasts containing functional p53 (WS1) and a cell line containing mutant p53 (DU145) were used as controls. Reintroduction of p53 was able to induce expression of p21 in ALAB cells (Fig. 5B), suggesting that the p53 element in the p21 promoter in ALAB cells is functional. We also tested whether expression of p53 itself was regulated by BRG1, and we saw no induction of p53 mRNA (Fig. 5C). Taken together, the results in Fig. 5 demonstrate that it is unlikely that BRG1 exerts its effect on p21 via p53 in this system.

To determine whether BRG1 modulates gene expression directly via binding to particular activation sequences in the p21 promoter, we performed immobilized template assays (17) using PCR products corresponding to fragments of the human p21 promoter (Fig. 6). Probe a corresponds to a 105-bp fragment that includes the p53 element 2.3-kb upstream of the transcription start site. Probe b is a 302-bp fragment immediately upstream of the start site, containing the binding sites for several transcription factors, including TGF-β mediators Smad2/3. We tested the ability of these fragments of the p21 promoter to precipitate BRG1 from a nuclear extract of ALAB cells expressing Ad*BRG1* (Fig. 6). Surprisingly, BRG1 was precipitated by the 105-bp fragment (a) containing the p53 element and did not associate with the proximal activation sequences (b) of the p21 promoter. As a positive control, p53 expressed from Adp53 in ALAB cells associated with the 105-bp fragment (a) containing the p53 element (Fig. 6). A 318-bp fragment within the p21 open reading frame (c), used as a negative control, did not precipitate BRG1. These experiments demonstrated that BRG1 can bind directly to the p21 promoter in a p53-independent manner.

BRG1-induced p21 associates with CDK2. To establish the consequence of CKI induction by BRG1, we next tested whether the p21 and p15 protein levels also increased upon *BRG1* expression in ALAB cells. Immunoblot analysis using anti-p21 and anti-p15 antibodies was performed on whole-cell lysates prepared from ALAB cells infected with either Ad*BRG1* or AdGFP. The levels of both p21 and p15 increased significantly in ALAB cells expressing *BRG1* (Fig. 7A, + lanes) compared to green fluorescent protein (GFP) (Fig. 7A, − lanes). p21 is visible at 11 h p.i. and p15 is detectable at 24 h p.i. in Ad*BRG1*-infected cells.

We next performed coimmunoprecipitation experiments from whole-cell lysates of ALAB cells infected with either

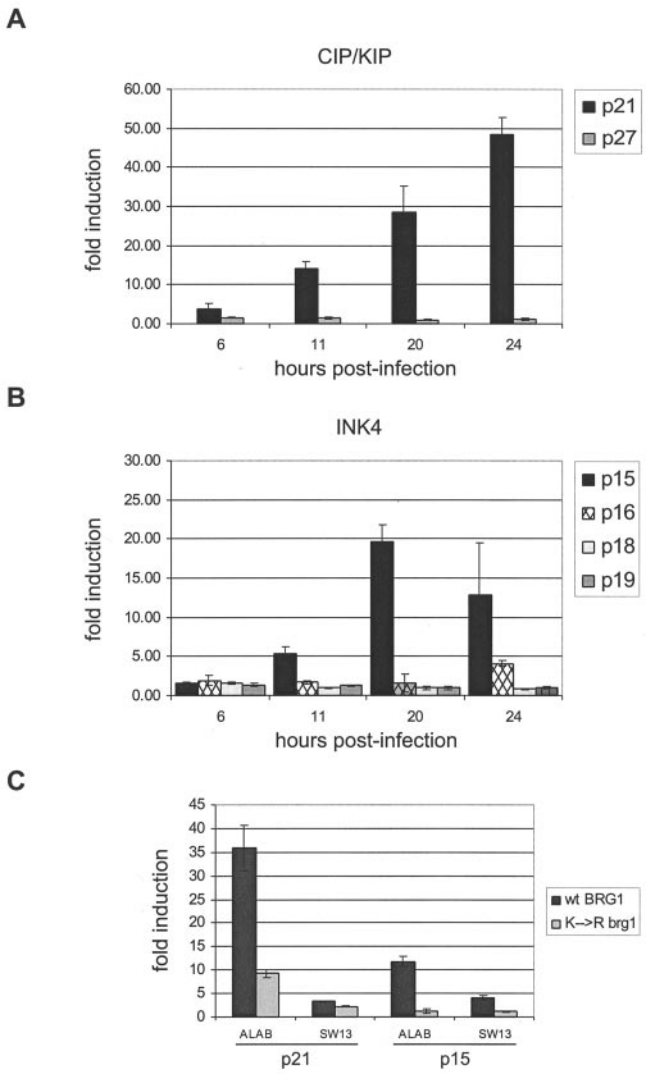

FIG. 4. BRG1 induces the expression of select CKIs, as shown by mRNA expression analysis of CKI expression in asynchronous ALAB cells infected with either Ad*BRG1* or control adenovirus for the indicated times. (A) Analysis of the Cip/Kip family of CKIs. (B) Analysis of the INK4 family of CKIs. (C) Analysis of p21 and p15 expression in ALAB and SW13 cells infected with either Ad*BRG1*, AdbrgK→R, or control adenovirus. Data for each gene are normalized to S9 ribosomal protein gene expression and expressed as Ad*BRG1* (or AdbrgK→R) induction over control adenovirus.

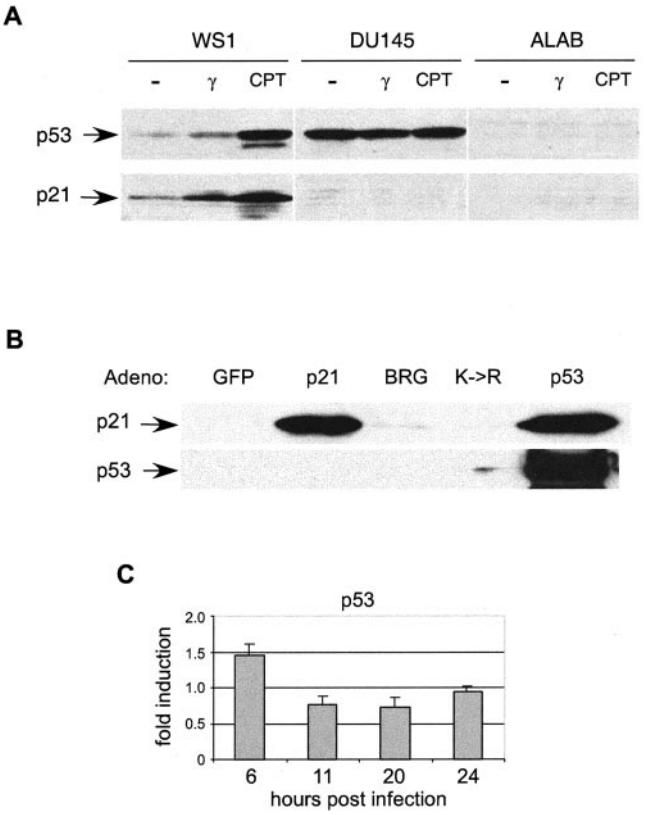

FIG. 5. BRG1 induction of p21 occurs in a p53-independent manner. (A) Lysates from cells harboring either wild-type p53 (WS1) or mutant p53 (DU145) and ALAB cells, untreated (−) or treated with ionizing radiation (γ) or camptothecin (CPT), were subjected to SDS-PAGE and analyzed by immunoblotting with anti-p53 or anti-p21 MAbs. (B) Lysates from asynchronous ALAB cells infected for 24 h with either AdGFP, Adp21, Ad*BRG1*, Ad*brg1*K→R (ATPase dead), or Adp53 were subjected to SDS-PAGE and immunoblot analysis with anti-p53 and anti-p21 MAbs. (C) mRNA expression analysis of p53 expression in asynchronous ALAB cells infected with either Ad*BRG1* or control adenovirus for the indicated times. Data were normalized to S9 ribosomal protein gene expression and expressed as Ad*BRG1* induction over control adenovirus.

Ad*BRG1* or AdGFP, using purified anti-CDK2 antibodies. Immunoblot analysis of precipitated proteins revealed that p21 coprecipitated with CDK2 from cells expressing *BRG1* as early as 11 h p.i. (Fig. 7B, + lanes). p21 did not coprecipitate with CDK2 from control cells (Fig. 7B, − lanes), nor was it present in immune precipitates from normal rabbit sera (Fig. 7B, NR lanes). Anti-CDK2 immune precipitates were analyzed for the presence of cyclin E, which was coprecipitated with CDK2 in all samples. These data suggest that BRG1 induces transcription of p21 mRNA, which results in an increase in cellular p21 protein that associates with CDK2.

pRB hyperphosphorylation is inhibited in Ad*BRG1*-infected cells. The anticipated result of p21 and p15 induction would be the inhibition of CDK2, resulting in cell cycle arrest prior to S phase. To determine if CDK activity was affected by BRG1 expression, we examined the phosphorylation status of pRB, which is a substrate of cyclin D-CDK4/6 and cyclin E-CDK2 in vivo and is hyperphosphorylated at the G_1-to-S transition. Immunoblot analysis (Fig. 7C) using a polyclonal pRB antibody that recognizes all forms of pRB demonstrated that in cells expressing *BRG1*, very little hyperphosphorylated pRB was detected by 20 h p.i., and almost none was detected at 24 h p.i. while hyperphosphorylated pRB was still present in cells expressing AdGFP. The reduction in hyperphosphorylated pRB follows the appearance of p21 in Ad*BRG1*-infected cell lysates and the association of p21 with CDK2, which is a consistent timeframe of molecular events. The hypophosphorylated, growth-suppressive form of pRB persisted in the Ad*BRG1*-infected samples. These data suggest that CDK activity is inhibited in cells expressing *BRG1*.

BRG1 effect on E2F target genes. Our data strongly support a major role for p21 in the growth arrest seen upon BRG1

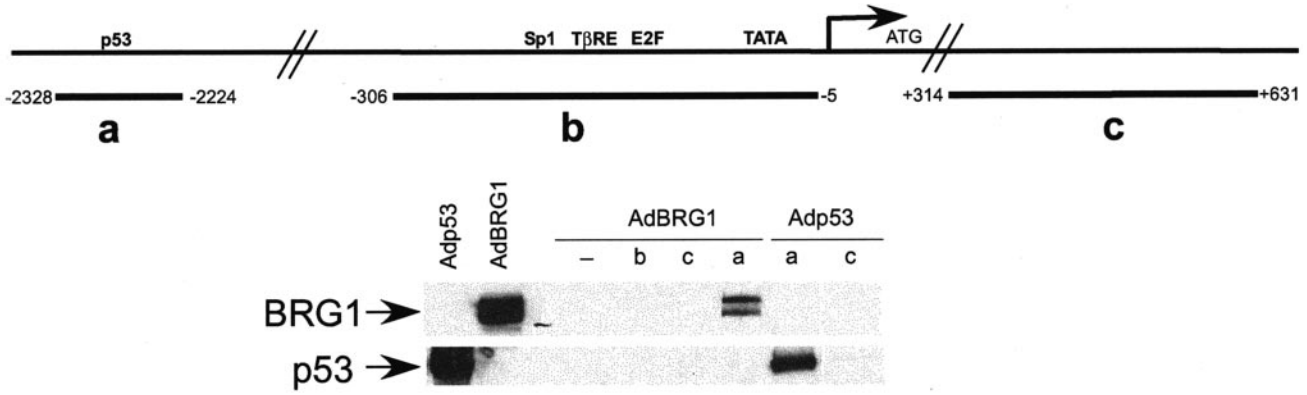

FIG. 6. BRG1 binds a region of the p21 promoter containing the p53 element. Biotinylated PCR products corresponding to the p53 element 2.3 kb upstream of the transcription start site (a), the proximal p21 promoter containing several transcription factor regulatory elements (b), and a fragment of the p21 open reading frame as a negative control (c) were immobilized on magnetic streptavidin-conjugated beads and used to precipitate proteins from nuclear extracts of ALAB cells infected with either Ad*BRG1* or Adp53. Streptavidin-conjugated beads without template were an additional negative control (−). Material precipitated by each template was eluted in SDS sample buffer and subjected to SDS-PAGE and immunoblotting with polyclonal anti-BRG1 or monoclonal anti-p53 antibodies. Samples of the nuclear extracts (Adp53 and Ad*BRG1*) were loaded as controls.

reintroduction into ALAB cells. Existing models of BRG1 function also predict a role for E2F gene regulation in this activity (48, 61). We did not see E2F target genes substantially modulated by microarray, so we took a number of classic E2F-regulated genes (30) and analyzed them more thoroughly by real-time PCR in ALAB cells infected with either Ad*BRG1* or AdGFP. Data from the 24-h time point are presented in Fig. 8A. Consistent with the microarray data, the expression of most E2F target genes examined was reduced only modestly by BRG1. We observed the same modest repression of some E2F target genes in SW13 cells (Fig. 8B).

To assess the relative repressive effects of BRG1 and pRB, we examined the effect of *RB* expression in SAOS2 cells ($RB^{-/-}$ cell line) on expression of the same group of E2F target genes (Fig. 8C). pRB had a much greater repressive effect on expression of other E2F targets than did BRG1. As an example, *E2F1* and *ORC1* were repressed 11-fold and 10-fold, respectively, by *RB* expression in SAOS2 cells, whereas in ALAB cells expressing *BRG1*, *E2F1* and *ORC1* were repressed approximately 2-fold and in SW13 cells *E2F1* and *ORC1* were not repressed by BRG1. In conclusion, pRB appears to be a much broader and stronger repressor of E2F activity than BRG1 under these experimental conditions.

DISCUSSION

Research from several laboratories has implicated BRG1 in the regulation of cellular proliferation and tumor suppression. We have undertaken a gene expression analysis approach to identify the targets of BRG1 that may be important for its growth regulation and tumor suppressor functions. Several genes have already been identified that may participate in the regulation, including *cyclin E* and *cyclin A*, which are E2F-regulated genes repressed by BRG1 in collaboration with pRB (48, 61). We found a very slight repressive effect by BRG1 in ALAB and SW13 cells on E2F targets.

We demonstrated that the CKIs p21 and p15 were induced upon reintroduction of *BRG1* into $BRG1^{-/-}$ ALAB cells. Both p21 and p15 protein levels increased dramatically, and p21 protein was recruited to CDK2 complexes. Concomitantly, a marked reduction in hyperphosphorylated pRB was observed, consistent with CDK inhibition. Given the kinetics of CKI induction and subsequent cell cycle arrest, it appears that growth arrest by BRG1 in ALAB cells is likely to occur through direct induction of CKIs and inhibition of CDK activity. We also saw significant induction of the plasminogen activator inhibitor-1 (*PAI-1*) by 24 h (data not shown). PAI-1 has been shown to play a role in arrest of cellular proliferation during senescence (31). Subsequent down-regulation of E2F-mediated transcriptional activation and other genes may contribute to the maintenance of the arrest phenotype. Our results do not rule out an involvement from direct E2F-mediated events, such as lowering of cyclin E levels, but they contrast significantly from what is observed for pRB. Interestingly, arrest induced by pRB in SAOS2 cells was quite different, with a more dramatic down-regulation of E2F targets and no induction of CKIs (Fig. 8C and data not shown). This supports the notion that BRG1 and pRB functions may overlap but are distinct.

The observation that *hBRM* is induced upon BRG1 expression is interesting, considering the phenotype of mice harboring homozygous null mutations in either *BRG1* or *hBRM* (5, 41, 51). $hBRM^{-/-}$ mice are viable, whereas $BRG1^{-/-}$ mice are not, suggesting that BRG1 has an important and essential role during development whereas hBRM is dispensable. The difference between the knockout mice can be explained by a model wherein BRG1 regulates the expression of *hBRM*. This model is further supported by a study of mRNA and protein levels of BRG1 and hBRM in mice. *mBRG1* mRNA and protein are present throughout preimplantation development, whereas *mbrm* mRNA and protein are detectable only when differentiation occurs at the blastocyst stage (23). Interestingly, in other studies we and others have found that in several BRG1 mutant cell lines and in primary tumors, hBRM levels were generally much lower, or undetectable, suggesting that hBRM

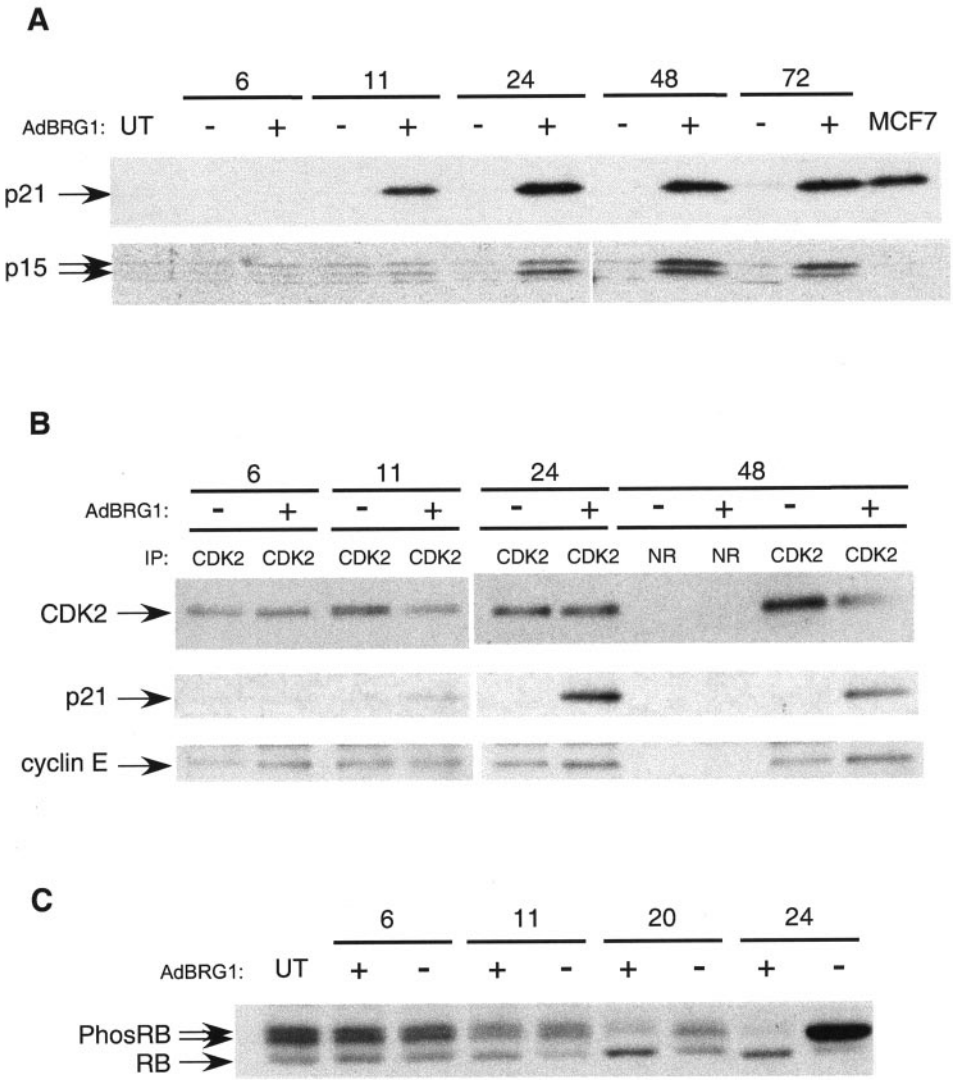

FIG. 7. BRG1-induced p21 associates with CDK2 and inhibits pRB phosphorylation. Lysates were prepared from asynchronous ALAB cells infected with either Ad*BRG1* (+) or AdGFP (−) at the indicated times p.i. and subjected to immunoblot analysis with anti-p21 MAbs and anti-p15 polyclonal antibodies (A), or immunoprecipitation with purified polyclonal anti-CDK2 antibodies (CDK2) or normal rabbit sera (NR), followed by immunoblot analysis with the MAbs anti-PSTAIR, anti-cyclin E (HE12), or anti-p21 (B), or immunoblot analysis using polyclonal anti-pRB antisera (C). Lysate from untreated (UT) ALAB cells was loaded as a control.

expression may be lost when BRG1 is mutated (39, 40, 59). Our conclusions are in disagreement with a report by Reisman et al., who failed to see hBRM regulation by BRG1 in SW13 and H522 cells (40). Our data in ALAB cells are to the contrary and illustrate that there is a cell type difference in BRM induction and that BRG1 can indeed regulate BRM expression under certain circumstances. It is of interest that the defect in BRG1 in SW13 and H522 cells that leads to loss of BRG1 expression remains undefined and may be a reason for this difference in response. The fact that we see both BRG1 and hBRM induction in our system may provide some explanation into the gene expression profile differences that we saw, compared to those in the earlier studies with SW13 cells. While earlier studies showed that BRM and BRG1 are essentially interchangeable, at least in restoring cell cycle arrest (40), there is growing evidence to suggest that BRG1 and BRM may have transcriptional specificity by virtue of unique protein-protein interactions with distinct classes of transcription factors (19).

We tested the effect of the ATPase mutant (K→R) BRG on gene expression and observed that for the majority of genes, hBRM and CD44 for example, the ATPase mutant did not affect gene expression, suggesting that ATPase activity (and by inference, chromatin remodeling) is necessary for the activation by BRG1. In contrast, p21 was modestly induced by the ATPase mutant (Fig. 4C). This suggests that chromatin remodeling may not be the only contribution that BRG1 makes in the activation of p21. This is in agreement with earlier observations that the ATPase mutant causes growth arrest in BRG1 mutant cells, though not as well as wild-type BRG1 (8, 45).

Several of the genes induced by BRG1 in the microarray and real-time PCR experiments encode proteins with functions in

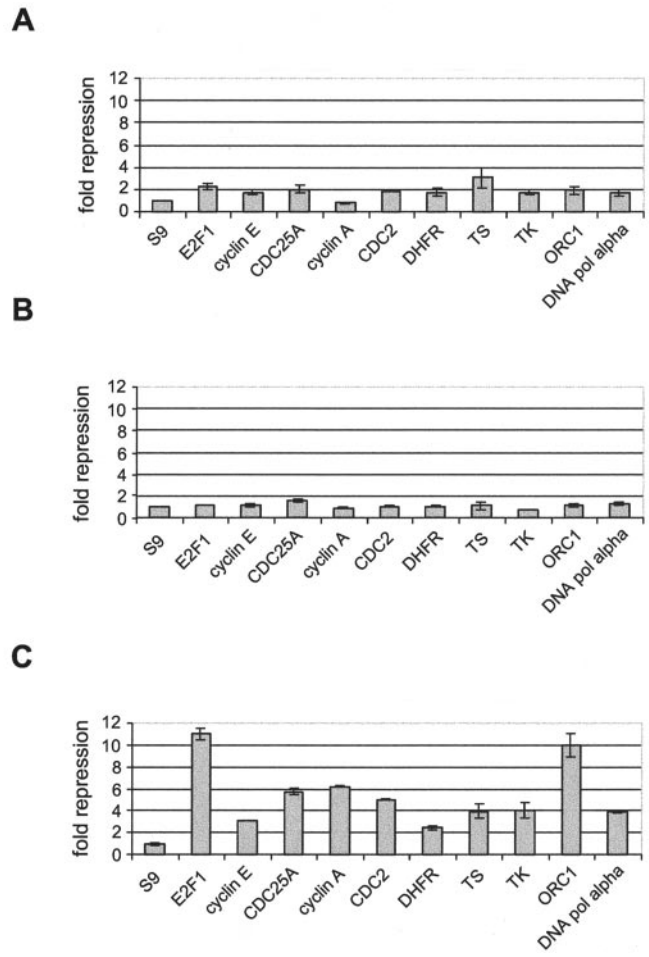

FIG. 8. Effect of *BRG1* overexpression on E2F target genes. mRNA levels of E2F target genes were measured by quantitative PCR analysis of RNA isolated from asynchronous ALAB (A) and SW13 (B) cells infected with either Ad*BRG1* or control adenovirus for 24 h or from asynchronous SAOS2 cells infected with Ad*RB* or control adenovirus (C). Data for each gene are normalized to S9 ribosomal protein gene expression and are expressed as repression over control adenovirus.

cell adhesion and differentiation that may play a role in tumor suppression. These were of interest considering the dramatic effect that BRG1 reintroduction has on cellular morphology. Typically, cells become flattened and exhibit altered cytoskeletal organization (1, 45). *CD44* encodes a hyaluronic acid cell surface receptor with important implications for growth and metastasis of tumors and is a known BRG1-regulated gene (47). CD44 associates with moesin (54), an ERM (ezrin, radixin, moesin) protein family member which links the plasma membrane with the actin cytoskeleton. Reduction of ERM protein expression by antisense oligonucleotides in cultured cells destroys cell adhesion sites (52). Thus, maintaining adequate levels of ERMs may be critical for cell adhesion. The expression of both CD44 and moesin was induced by BRG1 in ALAB cells (Fig. 2A and data not shown). These observations, taken together, raise important questions regarding a role for BRG1 in maintaining cell integrity and preventing cell spreading and metastasis. In a study using subtractive hybridization to survey genes which may be repressed during the development of neuroblastoma, Shtivelman and Bishop identified *CD44* as well as AHNAK/PM227 (46). We found that AHNAK was also induced by BRG1 in the microarray experiments (Table 1). S100A2, a putative tumor suppressor that is down-regulated in tumor cells and appears to inhibit cell motility (33), was induced by BRG1 (Table 1). In addition, the breast epithelial antigen BA46, integrin alpha-5 (fibronectin receptor subunit), and integrin alpha-3 were also induced, indicating that BRG1 overexpression affected genes involved in cell adhesion, also.

It is of interest that several cell surface receptors were down-regulated by BRG1, including the chemokine receptors CCR1, CCR2, and CCR6. Chemokines and their receptors are involved in directed migration of immune cells by chemoattraction. Two other G protein-coupled receptors, GPR56 and GPR43, were also repressed by BRG1 expression.

We have not yet elucidated the precise mechanism by which BRG1 promotes induction of p21 and p15. BRG1 has been implicated in the induction of p21 by p53 in another report (22). Those authors demonstrated a physical association of BRG1 with the p21 promoter in a p53-dependent manner in SAOS2 cells and observed stimulation of p53 transactivation by BRG1 overexpression. While we have clearly shown that BRG1 can bind to elements within the p21 promoter in the absence of p53, it is plausible that in normal cells there is cooperativity in the activities of BRG1 and p53 on p21 induction, given that we observed BRG1 binding to the p21 promoter in the vicinity of the p53 element.

Both p15INK4b and p21 have also been described as TGF-β-responsive genes. In addition, a number of other TGF-β-regulated genes were detected in the microarray analysis, including insulin-like growth factor binding protein-4, integrin alpha-5, integrin alpha-3, matrix metalloproteinase-1, c-*JUN*, c-*myc*, and tropomyosin α. If the effect of *BRG1*-mediated gene induction in *BRG1*$^{-/-}$ cells is related to restoration of a TGF-β response, it may occur at any of several levels, including direct association with the promoter. We did not observe BRG1 on the proximal region of the p21 promoter that contains a TGF-β-responsive element. Transcriptional induction of p15 and p21 by TGF-β is in part Smad dependent (11, 24, 28, 34). Interactions between Smad and BRG1 have not been reported, but it would be interesting to test that hypothesis.

In conclusion, our studies elucidate a complex gene regulation profile for BRG1. Some of the effects on cell cycle regulation can be accounted for by induction of CKIs, which would be potentiated by indirectly affecting E2F transactivation of S-phase genes after the inhibition of CDK2. An array of other BRG1-regulated genes involved in cell adhesion, migration, and possible TGF-β responses suggest overlapping but nonredundant pathways of tumor suppression for BRG1 and pRB.

APPENDIX

Microarray revealed novel BRG1-regulated genes. Table A1 includes all known genes altered at least twofold (induced or repressed) in both microarray hybridizations. Incyte expressed sequence tags were not included. There are 70 genes that were induced by BRG1 and 65 genes that were repressed by BRG1.

The primers listed in Table A2 were used in the quantitative real-time PCR (Taqman) with a GeneAmp 5700 sequence detection system (Perkin-Elmer). Each PCR generated only the expected specific amplicon, as shown by the melting temperature profiles of the final prod-

TABLE A1. Genes induced or repressed at least twofold in both microarray hybridizations

Gene name	Accession no.	Fold at: 18 h	Fold at: 24 h	BLAST results
Induced				
Clone FLJ22920, highly similar to HSU29175 human BRG1	AK026573	18.2	46.2	
Human hbrm	X72889	10.2	6.2	
Human, clone MGC:14528 IMAGE:4043484	BC008405	8	3.7	Pregnancy-specific beta-1 glycoprotein
Human fetal liver nonspecific cross-reactive antigen-3	M25384	7.4	4	Pregnancy-specific beta-1 glycoprotein
Human breast epithelial antigen BA46	U58516	7.1	9.2	
Human mRNA for pregnancy-specific beta-1 glycoprotein	X17610	6.7	3.8	
Human, fatty acid desaturase 3	BC004901	5.4	3.2	
Human, clone IMAGE:4299322	BC007910	5.4	2.2	Similar to PhD finger protein 3
Human, integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	BC008786	4.8	2.6	
Human, clone IMAGE:4299322	BC007910	4.7	2.5	
Human glia-derived nexin	M17783	4.5	5.5	
Human growth arrest and DNA damage-inducible protein (gadd45)	M60974	4	3.9	
Human pHS1-2 mRNA with ORF homologous to membrane receptor proteins	X12433	4	2.8	Lung a/b hydrolase domain containing protein 2
Human novel protein AHNAK	M80899	3.9	5.2	
Human, angiotensinogen (serine [or cysteine]) protease inhibitor	BC011519	3.9	3.3	
Human lysyl oxidase-related protein (WS9-14)	U89942	3.5	3.6	
Human cDNA, FLJ22761 fis, clone KAIA0893	AK026414	3.5	3.6	Similar to hexokinase 1, brain form (82% identity)
Human G-protein-coupled receptor (GPR56)	AF106858	3.5	2.1	
Human non-lens beta gamma-crystallin-like protein (AIM1)	U83115	3.4	3.2	
Human, FLJ21165 highly similar to HSCOLL1, type I interstitial collagenase	AK024818	3.4	2.9	MMP-1
Human insulin-like growth factor binding protein-4	U20982	3.4	2.8	
Human pyruvate dehydrogenase	AF155661	3.4	2.7	
Human growth arrest and DNA damage-inducible protein (gadd45)	M60974	3.4	2.3	
Human mRNA, cDNA DKFZp586K2222	AL050179	3.3	4.5	Tropomyosin a chain, fibroblast isoform 2
Human S100A2 gene, exon 1 to 3	Y07755	3.3	4.1	
Human (clone 35.3) DRAL	L42176	3.3	2.5	
Human Thy-1 glycoprotein gene	M11749	3.3	2.3	
Human mRNA, cDNA DKFZp434G171	AL133035	3.3	2.3	Homology with LIM domains
Human MT1H gene for metallothionein Ih	X64834	3.2	2.9	
Human integrin alpha-3 chain	M59911	3.2	2.1	
Human insulin-like growth factor binding protein-4	U20982	3.1	2.7	
Human mRNA for Tec protein-tyrosine kinase	D29767	3	2.3	
Human mRNA for ADP ribosylation factor-like protein	AB016811	3	2.1	
Human wild-type p53 activated fragment-1 (WAF1)	U03106	2.9	5.1	
Human cDNA, FLJ20866 fis, clone ADKA02201	AK024519	2.9	2.8	
Human mRNA for metallothionein isoform 1R	X97261	2.9	2.4	
Human microtubule-associated protein 1B	L06237	2.9	2.4	
Human monocyte chemotactic protein gene	M37719	2.8	4.3	
Human plasma membrane calcium ATPase4	M25874	2.8	2.6	
Human, FLJ21165, highly similar to HSCOLL1, type I interstitial collagenase	AK024818	2.8	2.3	MMP-1
Human actin-binding protein homolog ABP-278	AF043045	2.8	2	
AHNAK nucleoprotein	AAA69899	2.7	3.8	
Human keratin 18	M26326	2.7	2.4	
Human CLP	L54057	2.6	2.2	
Human c-*jun* proto-oncogene (JUN)	J04111	2.6	2.2	
Human, cathepsin H	BC002479	2.6	2.1	
Human, lectin, galactoside-binding, soluble, 1 (galectin 1)	BC001693	2.6	2	
Human insulin-like growth factor binding protein-4	U20982	2.5	2.6	
hHEPTP phosphatase	D11327	2.5	2.6	
Human plasma membrane calcium ATPase4	M25874	2.5	2.4	
Human mRNA for KIAA0315	AB002313	2.5	2.3	Homology with plexin-B1/SEP receptor, plexin B3
	AI041332	2.5	2.3	
Human cDNA clone similar to cathepsin B precursor				
Human keratin 18	AF179904	2.4	2.6	
Human (clone FBK III 11c) protein-tyrosine kinase (DRT)	L41939	2.4	2.2	
Human hematopoietic neural membrane protein 1	U87947	2.3	2	
Proteoglycan link protein	AAK676397	2.2	3.1	
Human cDNA, FLJ23306 fis, clone HEP11541	AK026959	2.2	2.3	
Human cDNA, FLJ10500 fis, clone NT2RP2000369	AK001362	2.2	2.3	
Cathepsin B mRNA	L16510	2.2	2.3	
Human, keratin 15	BC002641	2.2	2.1	
Human mRNA for transmembrane protein (THW gene)	AJ251830	2.1	2.7	
Human nonmuscle α-actinin	U48734	2.1	2.2	
Human cathepsin B mRNA	L22569	2.1	2.2	
	AA559314	2.1	2.1	Probable cathepsin B-like cysteine protease precursor
MCP-1/CCL2/SCYA2/MCAF	M24545	2	3.8	
Human AHNAK nucleoprotein	M80902	2	2.9	
Human mRNA for KIAA0638 protein	AB014538	2	2.6	Similar to LL5 protein with PH domain
Human, clone MGC:16121 IMAGE:3627113	BC007360	2	2.3	

Continued on following page

TABLE A1—*Continued*

Gene name	Accession no.	Fold at: 18 h	Fold at: 24 h	BLAST results
Human mRNA for nuclear envelope protein lamin A precursor	X03444	2	2.1	
Human gamma interferon-inducible protein (IP-30)	J03909	2	2	
Galectin-1 (beta-galactoside-binding lectin L-14-I)	P48538	2	2	
Repressed				
STRL33	U73531	16.2	5.8	
Human lumican	U21128	6.3	2.3	
Human cDNA clone similar to Ig kappa chain precursor v-iv region	AA581006	6	5.2	
EBI2	L08177	5.3	3.4	
CCR6	U45984	5.2	5.2	
Human NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase	L76465	5.2	4.1	
Melanocortin receptor subtype 5	L27080	5	4.2	
Human lung cytochrome P450 (IV subfamily) BI protein	J02871	5	2.6	
Human I-plastin	L20826	5	2.2	
Melanocortin receptor subtype 4	L08603	4.9	4.9	
Human fibronectin 3′ coding region	HUMFN2	4.8	2.2	
CCR1	L09230	4.7	4.4	
Human gastrointestinal tumor-associated Ag GA733-1 protein	J04152	4.5	2.8	
GIR	H7MAA112R	4.4	2.4	
Human HLA-DR a-chain	K01171	4.4	2.2	
Homo sapiens Sprouty 2	AF039843	4.3	2.7	
Human latent TGF-β binding protein	Z37976	4.2	3	
Human α-2 macroglobulin	M36501	4.2	2.2	
Human selenoprotein P	Z11793	4.1	2.6	
Human mf galactose-specific lectin (hMAC-2)	M35368	4.1	2	
Human DNA-binding protein (NF-E1)	M76541	4	2.1	
Macaca mulatta surfactant protein C	U06694	4	2.1	
Human fibronectin receptor beta subunit	X07979	3.9	2.3	
Homo sapiens clk1	L29219	3.8	2.3	
Human gastrointestinal tumor-associated antigen GA733-1 protein gene	J04152	3.7	2.9	
Ephrin B1	NM_004429	3.7	2.9	
GPR43	HNFGF45R	3.7	2.4	
Human DNA for p58 NK receptor gene	AJ000542	3.6	3.2	
Human pulmonary-associated surfactant protein B (SP-B) and SP-B (SFTP3)	J02761	3.6	2.2	
Neuropilin 2	NM_003872	3.5	2.7	
Human HLA-DR antigen-associated invariant chain	X00497	3.5	2.2	
Human beta-hexosaminidase beta chain	M19735	3.5	2.1	
Homo sapiens JM5 protein	AJ005897	3.5	2.1	
CD28-like	S78540	3.4	2.7	
Human MHC gene HLA-DR beta-I	V00522	3.4	2.1	
CCR2	U57940	3.3	3.4	
Neuropilin 1	NM_003873	3.3	2.6	
Human secreted cement gland protein XAG-2 homolog (hAG-2/R)	AF0384	3.3	2.4	
Hylobates lar epithelial mucin [MUC1]	L41589	3.1	2.1	
GPR6	L36150	3	2.5	
FIL1eta	NM_014438	3	2.5	
Human stimulatory G protein	JO3198	2.9	3.1	
Macaca mulatta flavin-containing monooxygenase	U59453	2.9	2.2	
Human rearranged immunoglobulin lambda light chain	X57822	2.8	2.9	
Human GDP-dissociation inhibitor protein (Ly-GDI)	L20688	2.8	2.7	
Human asparagine synthetase	M27396	2.8	2.4	
huPTPCAAX1 phosphatase	U48296	2.6	3.7	
DSP-7	AI301873	2.6	2.3	
DAP10	AA516481	2.6	2.1	
Human mRNA encoding the c-*myc* oncogene	V00568	2.4	3.4	
Human inwardly rectifying potassium channel Kir2.1 (KCNJ2)	AF153820	2.4	2.4	
Human, similar to KIAA0769 gene product	BC010394	2.4	2	
Human immunoglobulin heavy chain variable region	Z98693	2.2	2.3	
Human HLH 1R21 mRNA for helix-loop-helix protein	X69111	2.2	2.2	
Melanocortin receptor subtype 3	L06155	2.2	2.1	
Human protein kinase related to rat ERK2	Z11695	2.2	2	
Human mRNA, cDNA DKFZp761C121	AL136560	2.1	2.7	
Human G-protein-coupled receptor (NPGPR)	AF119815	2.1	2.2	
Human immunoglobulin kappa heavy chain	Y14735	2.1	2.1	
Human mRNA for period (*Drosophila*) homolog 3 hPER3	AB047686	2	2.7	
hPTPCAAX2 phosphatase	U48297	2	2.6	
Human, clone MGC:4408 IMAGE:2906200	BC012198	2	2.3	
Human mRNA for KIAA1057 protein	AB028980	2	2.1	Ubiquitin carboxy-terminal hydrolase 24
Human mRNA for FLJ00018 protein	AK024429	2	2.1	Lymphoma/leukemia guanine nucleotide exchange factor

TABLE A2. Primers used for cDNA amplification

Gene	Primer direction	Sequence 5′ to 3′
CDC25A	Forward	AGTAAGACCTGTATCTCGTGGCTGC
CDC25A	Reverse	CACGAAGCCATCATCCTCATCAGACAA
CyclinD1	Forward	CGATGCCAACCTCCTCAACGAC
CyclinD1	Reverse	CCAGCATCCAGGTGGCGACG
Cyclin A	Forward	TTCATTTAGCACTCTACACAGTCACGG
Cyclin A	Reverse	TTGAGGTAGGTCTGGTGAAGGTCC
DHFR	Forward	CAGAGAACTCAAGGAACCTCCACAAG
DHFR	Reverse	GAACTGCCACCAACTATCCAGACCAT
Cdc2	Forward	CAGGTCAAGTGGTAGCCATGAAA
Cdc2	Reverse	GCATAAGCACATCCTGAAGACTGA
Thymidine kinase	Forward	CATCCTGAACCTGGTGCCGCT
Thymidine kinase	Reverse	CTTGAAGTAGCAGAGCCGACACAC
E2F1	Forward	GGTTTCCAGAGATGCTCACCTTGTC
E2F1	Reverse	CACACAGACTCCTTCCCTTCCTG
DNA pol alpha	Forward	GGTTACCTCCTTCGGCTTAGCAC
DNA pol alpha	Reverse	CTTGTAATGTTCCTTGAGTTCGCTGC
S9	Forward	GGGATGTTCACCACCTG
S9	Reverse	GCAAGATGAAGCTGGATTAC
Cyclin E	Forward	GGAAGAGGAAGGCAAACG
Cyclin E	Reverse	GCAATAATCCGAGGCTTG
Thymidine synthetase	Forward	TCCAACACATCCTCCGCTGC
Thymidine synthetase	Reverse	ATGCCGAATACCGACAGGGTG
ORC1	Forward	CACTTTCCTCAGAACTATTTGCGGA
ORC1	Reverse	CTTACTCTTGGCTCTCACGGGTTTC
p21 Cip1	Forward	TGGAGACTCTCAGGGTCGAAA
p21 Cip1	Reverse	GGCGTTTGGAGTGGTAGAAATC
p27	Forward	CAGGAGAGCCAGGATGTC
p27	Reverse	AGTAGAAGAATCGTCGGTTG
p15 INK4b	Forward	CGGAGTCAACCGTTTCGG
p15 INK4b	Reverse	GGTGAGAGTGGCAGGGTCTG
p16	Forward	AGCAGCATGGAGCCTTC
p16	Reverse	CCTCCGACCGTAACTATTC
p18	Forward	GGAACCTGCCCTTGCACTT
p18	Reverse	GTGTGCTTCACCAGGAACTCC
p19	Forward	GGCACTTCCAATCCATCT
p19	Reverse	CAGGATGTCCACGAGGTC
CD44	Forward	CGTGGAGAAAAATGGTCGCTACAG
CD44	Reverse	GTGGGCAAGGTGCTATTGAAAGC
c-MYC	Forward	CTGGATCACCTTCTGCTG
c-MYC	Reverse	ACATTCTCCTCGGTGTCC
c-JUN	Forward	CATCGACATGGAGTCCC
c-JUN	Reverse	GCCAGCTCCGAGTTCTG
hBRM	Forward	GACGGCTCTCAACTCCAAAGCATAC
hBRM	Reverse	GACGGCGTTTCCTCTCCTGC
Osteonectin	Forward	AGACAGAGGTGGTGGAAGAAACTGTG
Osteonectin	Reverse	CCTCCTCTTCGGTTTCCTCTGC
p53	Forward	GCTGCTCAGATAGCGATGGTCT
p53	Reverse	CATCCAAATACTCCACACGCAA
BRG	Forward	CCTCTCTCAACGCTGTCCAACTG
BRG	Reverse	ATCTTGGCGAGGATGTGCTTGTCTT

ucts (dissociation curve, automatically measured by the Taqman 5700 apparatus).

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