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Transcription Regulation of the rRNA Gene by a Multifunctional Nucleolar Protein, B23/Nucleophosmin, through Its Histone Chaperone Activity[▽]

Kensaku Murano,[1] Mitsuru Okuwaki,[1,2] Miharu Hisaoka,[1] and Kyosuke Nagata[1]*

Department of Infection Biology, Graduate School of Comprehensive Human Sciences and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575,[1] and PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, 322-0012,[2] Japan

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It is well established that the transcription rate of the rRNA gene is closely associated with profound alterations in the cell growth rate. Regulation of rRNA gene transcription is likely to be dependent on the dynamic conversion of the chromatin structure. Previously, we identified B23/nucleophosmin, a multifunctional nucleolar phosphoprotein, as a component of template activating factor III that remodels the chromatin-like structure of the adenovirus genome complexed with viral basic proteins. It has also been shown that B23 has histone chaperone activity. Here, we examined the effect of B23 on rRNA gene transcription. B23 was found to be associated with the rRNA gene chromatin. Small-interfering-RNA-mediated down-regulation of the B23 expression level resulted in reduction of the transcription rate of the rRNA gene. We constructed a B23 mutant termed B23ΔC, which lacks the domain essential for the histone chaperone activity and inhibited the histone binding activity of B23 in a dominant-negative manner. Expression of B23ΔC decreased rRNA gene transcription and the rate of cell proliferation. These results suggest that B23 is involved in the transcription regulation of the rRNA gene as a nucleolar histone chaperone.

To ensure an optimal protein synthesis level, growing cells require continuous ribosome supply by synthesis of ribosomal proteins and rRNA. The rate of transcription of the rRNA gene by RNA polymerase I (Pol I) is closely correlated with the cell growth rate (19). In eukaryotic cells, rRNA genes are found in multiple tandem-arrayed copies in the nucleolus, known as the nucleolar organizer region. The tandem rRNA gene repeats exist in two distinct types of chromatin, an open one, which is permissive for transcription, and a closed one, which is found in the transcriptionally repressive state (6). Several epigenetic characteristics, including differential DNA methylation and specific histone modification, also differ between potentially active and inactive rRNA genes (18), so the epigenetic control mechanism is important for transcription regulation of the rRNA gene. For instance, it has been reported that the nucleolar remodeling complex (NoRC) is a member of ATP-dependent chromatin remodeling factors and consists of ATPase Snf2h and TIP5 localized in nucleoli (54). It has been suggested that NoRC plays an active role in nucleosome dynamics at the rRNA gene promoter in an ATP-dependent manner, and specific nucleosome positions determine the transcriptional readout of the rRNA gene (33). NoRC interacts with histone deacetylases, histone methyltransferases, and DNA methyltransferases, and it directs these enzymatic activities to the rRNA gene promoter to form heterochromatin (18). It has been shown that in addition to histone modification enzymes and ATP-dependent chromatin remodeling factors, nucleolin, a histone chaperone localized in the nucleolus, enhances the activity of chromatin remodeling machineries, SWI/SNF and ACF, and promotes the transcription from nucleosomal template in vitro (1). Experiments using small interfering RNA (siRNA) for nucleolin showed that nucleolin is involved in the transcription of the rRNA gene in vivo. Moreover, chromatin immunoprecipitation (ChIP) experiments showed that nucleolin is associated with the chromatin around the 18S rRNA gene (48).

To identify a factor involved in structural change of chromatin, we have taken advantage of the adenovirus (Ad) DNA complexed with viral basic core proteins, called Ad core (28, 36, 43). The viral genome is packed around core protein VII hexamer, and a unit of the viral nucleosome is bridged by core protein V (8, 55). Because of the chromatin-like structure of Ad core, access to the viral genome of the factors required for the replication and transcription is restricted. Using a cell-free DNA replication system with Ad core as a template, we have identified three host factors, termed template activating factor I (TAF-I), TAF-II, and TAF-III. TAF-I and TAF-II are identical to the leukemia-associated gene product SET (58) and nucleosome assembly protein 1 (NAP-1) (28), respectively. The major component of TAF-III is B23/nucleophosmin, a nucleolar phosphoprotein (43). Recombinant B23 induces the structural alteration of Ad core, thereby stimulating the Ad core DNA replication (43, 49). TAF-I and NAP-1 bind to core histones directly and transfer them to naked DNA (25, 35). TAF-I not only is involved in the replication and transcription of Ad core (20, 21, 37) but also has been suggested to be involved in regulation of chromatin structure in eukaryotic cells (14, 27). In addition, TAF-I promotes vitamin D_3 receptor- and Gal4-VP16-dependent transcription from the chroma-

* Corresponding author. Mailing address: Department of Infection Biology, Graduate School of Comprehensive Human Sciences and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan. Phone and fax: 81-29-853-3233. E-mail: knagata@md.tsukuba.ac.jp.

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tin template in vitro (14) and stimulates the transcription of a subset of genes in HeLa cells in a histone acetylation-independent manner (27). TAF-I also plays an important role in transcription regulated by transcription factors such as MLL (mixed lineage leukemia), Sp1, and KLF5 (39, 53, 56).

We have demonstrated that B23 binds to core histones directly and transfers them to naked DNA (44), as do TAF-I (35) and NAP-1 (25), and the acidic regions of B23 are important for its activity (44). The amino-terminal domain of B23 shows significant similarity to nucleoplasmin, which is the first identified histone chaperone (32). A recent report indicated that B23 is acetylated and that acetylated B23 has enhanced histone chaperone activity and stimulates in vitro transcription from chromatin templates by RNA Pol II in the presence of p300 (57). The crystal structure of the N-terminal domain of NO38, a *Xenopus* homolog of human B23, indicates that NO38 forms a pentamer and that the pentamers further assemble into decamers, as does nucleoplasmin (10, 41). These studies suggest that oligomer formation of nucleoplasmin family proteins may be important for their histone chaperone activity. Based on its nucleolar localization and biochemical properties, it can reasonably be assumed that B23 is involved in regulation of the chromatin structure around the rRNA gene through its histone chaperone activity.

B23 is also termed nucleophosmin, numatrin, or NPM1 and was initially identified as an abundant nucleolar phosphoprotein (46). These properties are similar to those of nucleolin, alternatively termed C23 (46). The expression level of B23 is up-regulated in association with growth factor-induced mitogenesis (12). A positive correlation among the quantity of B23 and nucleolin, the rate of Pol I transcription, and the cell proliferation rate has been reported (7). There are two subtypes of B23, B23.1 and B23.2, the latter of which lacks the carboxyl-terminal region present in B23.1 (4). B23 is a multifunctional protein: it has been suggested that B23 is involved in rRNA maturation through its nucleic acid binding and RNase activities (9, 22). B23 has been shown to play a critical role in centrosome duplication (42). Inactivation of B23 in mouse embryonic fibroblasts (MEFs) leads to unrestricted centrosome duplication and severe genomic instability in addition to reduction of the cell proliferation rate (17). B23 is also involved in regulation of the cell cycle progression. In normal cells, B23 targets ARF in the nucleolus by binding to ARF through the Mdm2 binding domain of ARF, thus allowing Mdm2 to interact with p53 for degradation of p53. Upon exposure to genotoxic stresses, the interaction between B23 and ARF is abrogated, so that the stability of p53 is increased (2, 5, 29). These indicate that B23 controls the cell cycle through the p53 pathway.

Here, we examined the effect of the histone chaperone activity of B23 on rRNA gene transcription. We showed that down-regulation of B23 by siRNA reduces the transcription rate of the rRNA gene. Nucleolar ChIP analyses demonstrated that B23 binds to the chromatin around the rRNA gene promoter. In order to characterize the function of B23 as a histone chaperone in the nucleolus, we constructed deletion mutants lacking the histone binding activity. B23ΔC, devoid of the functional acidic regions, formed a hetero-oligomer with B23 and inhibited the histone binding activity in a dominant-negative manner. Overexpression of B23ΔC reduced the transcription level from the rRNA gene. Taken together, these results suggest that B23 regulates the transcription from the rRNA gene chromatin as a nucleolar histone chaperone.

MATERIALS AND METHODS

Cell culture and transfection. 293T and $p53^{-/-}$ MEFs were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells were cultured at 37°C in MEM supplemented with 10% FBS. Transient transfection was performed using Transit-293 (Mirus) for 293T cells, Transit-LT1 (Mirus) for $p53^{-/-}$ MEFs, and GeneJuice (Novagen) for HeLa cells. HeLa cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen).

Preparation of plasmids. pCHA-B23.1 was prepared as previously described (43). To generate pCHA-B23ΔC, a fragment of B23ΔC3 cDNA was prepared by digestion of pET14b-hB23ΔC3 (43) with NdeI and BamHI. The fragment was then cloned in-frame to BstEII- and BglII-digested pCHA (43). To generate pUC119-HurDNA, the human rRNA gene (around −192 to +703 relative to the transcription start site [+1]) was amplified by PCR using genomic DNA purified from HeLa cells as the template. To amplify the human rRNA gene, two oligonucleotides, 5′-CGCGGATCCTGTCCTTGGGTTGACCAGAG-3′ and 5′-CCGGAATTCGCAAGTCGACAACCACTGGA-3′, were used as primers. The amplified human rRNA gene fragment was cloned into the BamHI- and EcoRI-digested pUC119. For expression of Flag-tagged B23.1, B23.1 cDNA attached to a Flag tag was excised from pBS-Flag-B23.1 (45) by BamHI and subcloned into BamHI-digested pcDNA3 vector (Clontech). To create pCAGGS-Flag-B23.1, a DNA fragment encoding Flag-tagged B23.1 was excised from pBS-Flag-B23.1 by BamHI, and subcloned into BglII-digested pCAGGS vector. To generate pET-14b-B23ΔA1, a DNA fragment of B23ΔA1 was amplified by two-step PCR using four oligonucleotides (5′-GCTAGTTATTGCTCAGCGG-3′ plus 5′-AGTAGCTGTGGTGAAACTCTTAAGTATA-3′ and 5′-TAATACGACTCACTATAGG-3′ plus 5′-AGAGTTTCACCACAGCTACTAAGTGCTG-3′) and pET-14b-B23.1 as a template and cloned into NdeI- and BamHI-digested pET-14b. To generate pET-14b-B23ΔA, a DNA fragment of B23ΔA was amplified by two-step PCR using four oligonucleotides (5′-GCTAGTTATTGCTCAGCGG-3′ plus 5′-ACTTGCTGCTAAAGCGCCAGTGAAGAAA-3′ and 5′-TAATACGACTCACTATAGG-3′ plus 5′-CTGGCGCTTTAGCAGCAAGTTTTACTTT-3′) and pET-14b-B23ΔA1 as a template and cloned into NdeI- and BamHI-digested pET-14b. To generate pBS-Flag-B23ΔA, a DNA fragment of B23ΔA was prepared by digestion of pET-14b-B23ΔA with NdeI and HindIII and cloned into NdeI- and HindIII-digested pBS-Flag. To generate pCAGGS-Flag-B23ΔA, a DNA fragment of Flag-B23ΔA was prepared by digestion of pBS-Flag-B23ΔA with BamHI and cloned into BglII-digested pCAGGS.

Antibodies. Antibodies used in this study were as follows. Mouse monoclonal antibody for B23 (Zymed) recognizes both endogenous B23.1 and B23ΔA proteins but not B23ΔC protein. For immunofluorescence assays of B23, goat polyclonal anti-B23 antibody (Santa Cruz Biotechnology) was used; for histone H3, rabbit polyclonal antibody (Abcam); for acetylated histone H3, rabbit polyclonal antibody (Upstate); for TATA-binding protein (TBP), rabbit polyclonal antibody (Santa Cruz Biotechnology); for upstream binding factor (UBF), rabbit polyclonal antibody (F-9; Santa Cruz Biotechnology); for nucleolin, mouse monoclonal antibody (Santa Cruz Biotechnology); for fibrillarin, rabbit polyclonal antibody (Santa Cruz Biotechnology); for the hemagglutinin (HA) tag, rabbit polyclonal antibody (MBL) or rat monoclonal antibody (3F10; Roche); for the Flag tag, mouse monoclonal antibody (M2; Sigma); for the His tag, mouse monoclonal antibody (Sigma); and for β-actin, mouse monoclonal antibody (Sigma).

Immunoprecipitation. Cells were lysed and sonicated in buffer A (50 mM Tris-HCl [pH 7.4], 0.1% NP-40) containing 100 mM NaCl. Antibodies, bovine serum albumin (1 mg/ml, final concentration), and protein A-Sepharose beads (GE Healthcare) were added to the lysate, and the mixture was incubated at 4°C for 2 to 4 h. The beads were washed extensively with buffer A containing 300 mM NaCl, and proteins bound to the beads were eluted with a sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and detected by immunoblotting using anti-HA, anti-B23, anti-Flag, or anti-histone H3 antibodies.

Pulse-labeling of B23. 293T cells (1×10^5) were suspended in 200 μl per assay of methionine-free MEM (Invitrogen). Met ^{35}S label (American Radiolabeled Chemicals) containing [^{35}S]methionine (20 μCi per assay) was added, and cells were incubated at 37°C for 1 h. Cell lysates were prepared, and B23 was immunopurified using anti-B23 antibody. Labeled proteins were separated by 10% SDS-PAGE and visualized by autoradiography.

TABLE 1. Primer pairs for the rRNA gene[a]

Region (bp)	Forward	Reverse
42960–11	TTTCGCTCCGAGTCGGC	AGCGTGTCAGCATATAACCCG
4104–4154	AACGGCTACCACATCCAAGG	GGGAGTGGGTAATTTGCGC
5309–5359	AGTGCGGGTCATAAGCTTGC	GGTGTGTACAAAGGGCAGGG
6626–6676	CTCTTAGCGGTGGATCACTCG	GCTAGCTGCGTTCTTCATCGA
9483–9533	GAAACTCTGGTGGAGGTCCG	CGGACGACCGATTTGCAC
12412–12442	AGCGTTGGATTGTTCACCCA	CGGTCTAAACCCAGCTCACG
15567–15617	GGTTCAAGCAAACAGCAAGTTTT	GGTGAAATTACGTCTTTACTCGAAATAGA
18370–18420	TTGTGGAATCCTCAGTCATCGA	TGTCCCTGCCTAGTCACCTGT
21422–21472	TGTGGCCCTTACGCTCAGA	GGAGTCAACCTACGGCAGAGA
23810–23860	CCTGACCACAAATGATCCACC	TCGTTTCCAGCACTTTGGG
27857–27907	CAGGCAGGCATCGGTTGT	TCAATAACAGTGGCCGCTAGGT
30857–30907	GGCCTAAGCCTGCTGAGAACT	AAGCATGGTCCCGAGGATC
33024–33074	CGCAATAAATGTCAACGGTGA	TGTGGTCCTACTTCTGCCTCAAC
36109–36160	ATCTCTCCTCTACGCGCGG	ACGTGTGTCCCGAGCTCC
39354–39404	TTCGTGTCTTTAACCCGCG	TGCCATCTGTCAAACCCGA
41087–41137	ACGTTTCTGTACGCTTATATGCAAA	AATGCAGAGATACACGTTGTCG

[a] Sequences derived from GenBank accession no. U13369.

siRNA for B23. B23 Stealth RNAi Select containing siB23-A (NPM1-HSS143152), siB23-B (NPM1-HSS143153), and siB23-C (NPM1-HSS143154) and Stealth RNAi (negative control; catalog no. 12935-200; Invitrogen) was introduced into HeLa cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 24 h posttransfection, the medium was replaced, and cells were harvested at 72 h after siRNA introduction. Proteins in HeLa cell lysates were separated by 10% SDS-PAGE and detected by immunoblotting.

Total RNA was prepared with an RNeasy minikit (Qiagen) and RNase-free DNase I (Qiagen). For analysis of 45S pre-rRNA, total RNA was subjected to reverse transcription with a primer, 5′-CCATAACGGAGGCAGAGACA-3′, corresponding to the 5′ external transcribed spacer (ETS) of the rRNA gene, and the synthesized cDNA was used for quantitative PCR using FullVelocity SYBR Green Q-PCR Master Mix (Stratagene) in the presence of a primer set, 5′-GC CTTCTCTAGCGATCTGAGAG-3′ and 5′-CCATAACGGAGGCAGAGAC A-3′, corresponding to the 5′ ETS of rRNA as described elsewhere (48). For analysis of β-actin, total RNA was subjected to reverse transcription with oligo(dT) as a primer, and the synthesized cDNA was used as a template for PCR amplification for β-actin in the presence of the primer set ATGGGTCA GAAGGATTCCTATGT-3′ and 5′-GGTCATCTTCTCGCGGTT-3′.

For immunofluorescence, HeLa cells transfected with siRNA on glass slides were permeabilized with 0.1% Triton X-100 and then subjected to cross-linking with 4% formaldehyde. Then, B23 was detected with goat anti-B23 polyclonal antibody. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI).

Nucleolar ChIP. Preparation of nucleoli and nucleolar ChIP were carried out according to the protocol described previously (47). Nucleoli were prepared from 6×10^6 cells prefixed by formaldehyde (0.25% for 10 min at 37°C). Nucleolar ChIP assays were carried out according to the manual for the ChIP assay kit (Upstate). Quantitative PCR was carried out using FullVelocity SYBR Green QPCR Master Mix (Stratagene). PCRs were carried out using primer sets corresponding to the rRNA gene (Table 1).

Run-on assay. Transcriptionally active nuclei were prepared from 293T cells (1×10^6) by treatment with NP-40 buffer (10 mM Tris-HCl [pH 7.9], 10 mM NaCl, 3 mM $MgCl_2$, and 0.1% NP-40), resuspended in 30 μl of glycerol stock buffer (10 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 3 mM $MgCl_2$, and 40% glycerol), and stored at −80°C until use. The isolated nuclei (30 μl) were mixed with 30 μl of a reaction buffer containing 10 mM Tris-HCl (pH 7.9), 300 mM KCl, 5 mM $MgCl_2$, 0.5 mM each ATP, CTP, and UTP, 50 μCi [α-^{32}P]GTP (~400 Ci/mmol) (Amersham), and 40 U of RNase inhibitor (Toyobo) and incubated at 30°C for 30 min. Genomic DNA was digested with 200 U of DNase I at 30°C for 10 min, followed by incubation with 100 μg proteinase K at 42°C for 30 min. RNA was extracted with phenol-chloroform, and the newly transcribed RNA was monitored by hybridization with 1 μg of denatured pUC119-HurDNA or pUC119 immobilized on Hybond-N^+ membranes. The intensity of hybridization dots was measured by using the Image Gauge program (Fuji-film).

In vitro histone binding assay. His-tagged B23.1, B23ΔA, and B23ΔC proteins were prepared as described previously (43). His-B23.1 and His-B23ΔC proteins were mixed at appropriate ratios and precipitated by cold acetone. The precipitated proteins were denatured in a guanidine buffer (50 mM HEPES-NaOH [pH 7.9], 150 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.1% NP-40, and 6 M guanidine-HCl) at room temperature for 15 min and renatured by dialysis against buffer containing 20 mM HEPES-NaOH (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Renatured B23 proteins were mixed and incubated at 4°C for 30 min with core histones purified from HeLa cells in 50 μl of 50 mM Tris-HCl (pH 7.9), 270 mM NaCl, 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml BSA, and 0.1% NP-40. Immunoprecipitation was carried out with anti-His or anti-B23 antibodies. Precipitated proteins were analyzed by 12.5% SDS-PAGE and visualized by silver staining and immunoblotting with anti-His and anti-histone H3 antibodies.

Nucleosome assembly assay. Supercoiled plasmid DNA (pCAGGS) was relaxed by incubation with topoisomerase I (TaKaRa) at 37°C for 30 min. The supercoiling assay was performed essentially as described previously (24).

RESULTS

Relationship between the cell growth rate and the rRNA gene transcription level. The relationship among the cell growth rate, the transcription rate of the rRNA gene, and the expression level of B23 was examined using 293T cells maintained in the presence of either 1% or 10% serum. The expression level of B23 was examined by pulse-labeling with [^{35}S]methionine. The pulse-labeled B23 was immunoprecipitated with an anti-B23 antibody and analyzed by 10% SDS-PAGE followed by autoradiography. The expression level of B23 was approximately fourfold higher in the presence of 10% serum than in the presence of 1% serum at 48 h (Fig. 1A, lanes 3 and 4). This result is consistent with the report that the expression of B23 is associated with growth factor-induced mitogenesis in Swiss 3T3 cells (12). The expression level of nucleolus-related proteins was examined in cells maintained in the presence of 1% or 10% serum by immunoblotting using anti-UBF, antinucleolin, antifibrillarin, and anti-β-actin antibodies. The expression level of UBF and nucleolin, both of which are known to be involved in the transcription of the rRNA gene, was reduced in the presence of 1% serum compared with that in 10% serum at 48 h (Fig. 1B), as was the case for B23. On the other hand, the expression level of fibrillarin involved in the processing of pre-rRNA and β-actin was almost equal between cells maintained in 1% and 10% serum (Fig. 1B). The reductions in levels of mRNAs of UBF, nucleolin, TIF-IA, fibrillarin, and β-actin by low serum levels were virtu-

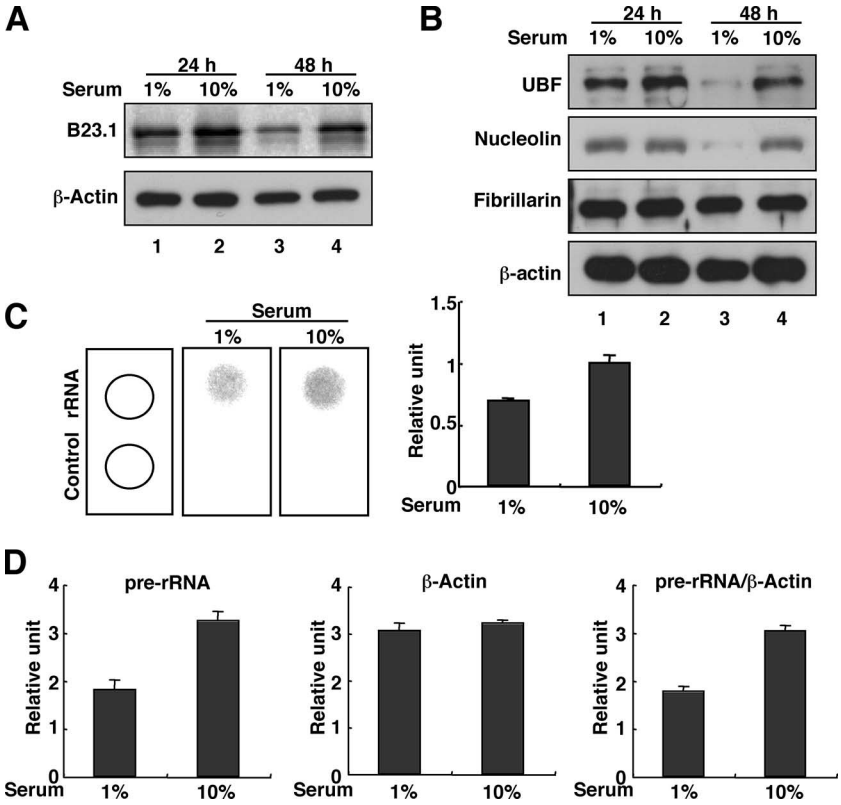

FIG. 1. Relationship between cell growth and the level of rRNA gene transcription. (A) Expression level of B23. 293T cells were maintained in DMEM in the presence of FBS for 24 or 48 h and then pulse-labeled with [^{35}S]methionine for 1 h. ^{35}S-labeled B23 proteins were immunopurified using anti-B23 antibody and analyzed by 10% SDS-PAGE followed by autoradiography. As a loading control, 10% of input lysates was separated by 10% SDS-PAGE, and β-actin was detected by immunoblotting. (B) Expression level of nucleolus-related genes. 293T cells were maintained in DMEM in the presence of FBS for 24 or 48 h. Protein expression levels were analyzed by immunoblotting. (C) Transcription rate of the rRNA gene. 293T cells were maintained in DMEM in the presence of FBS for 72 h. The expression level of the rRNA gene was detected by run-on assays using [α-^{32}P]GTP. Labeled rRNA was used as a probe for hybridization to membrane-blotted plasmid DNAs containing the rRNA gene (pUC-119-HurDNA) and pUC119 as a control. Results are means and standard deviations (SD) from three independent experiments. (D) Accumulation of pre-rRNA. 293T cells were maintained in DMEM in the presence of FBS for 72 h. The expression levels of pre-rRNA and β-actin mRNA as a loading control were determined by quantitative RT-PCR, and the amount of pre-rRNA normalized to that of β-actin mRNA is presented on the right. Results are means and SD from three independent experiments.

ally equal (available on request). These results suggest that the transcription by RNA Pol II is evenly affected by serum concentrations and that the expression level of proteins involved in the rRNA gene transcription is coordinately regulated at the level of translation and/or protein degradation. In parallel, the transcription rate of the rRNA gene was monitored by run-on assays. The transcription level of the rRNA gene in cells maintained in the presence of 1% serum was 0.7-fold lower than that in the presence of 10% serum (Fig. 1C). The amount of pre-rRNA was also monitored by quantitative reverse transcriptase PCR (RT-PCR) using a primer set corresponding to the 5′ ETS of pre-rRNA (48) as described in Materials and Methods. The results were normalized to that of β-actin. The amount of pre-rRNA in cells maintained in the presence of 1% serum was 0.6-fold lower than that in the presence of 10% serum (Fig. 1D). The number of rRNA transcripts in cells under the growing conditions is about 80% of the total cellular RNA quantity (40). Thus, this difference in the rRNA gene transcription rate between cells in 1% and 10% serum affects the cellular metabolism significantly. These results indicate that the rate of rRNA gene transcription and cell growth are positively correlated with the expression level of genes involved in rRNA gene transcription, including B23. From this point on, we focused functional studies on B23.

KD of B23 decreases the transcription rate of the rRNA gene. To investigate the role of B23.1 in transcription of the rRNA gene, knock-down (KD) of B23.1 expression was performed using siRNA specific for B23.1, siB23-A, -B, and -C. Introduction of the siRNA decreased the protein level of B23.1, but not β-actin, whereas a control siRNA had no effect (Fig. 2A). The expression level of other nucleolus-related genes was also examined in cells treated with siB23-C, and siB23-C was found to have no effect on the expression of these genes (Fig. 2B). Therefore, we could examine the function of B23.1 without a possible indirect effect of siB23-C. KD of B23.1 was also confirmed in nucleoli of HeLa cells by immunofluorescence using anti-B23 antibody (Fig. 2C). The amount of pre-rRNA in HeLa cells transfected with siB23 or control siRNA was examined by quantitative RT-PCR using 5′ ETS-specific primers for the rRNA gene. The amount of pre-rRNA was normalized as a ratio to that of β-actin mRNA. KD of the B23 expression level resulted in decreased transcription of the

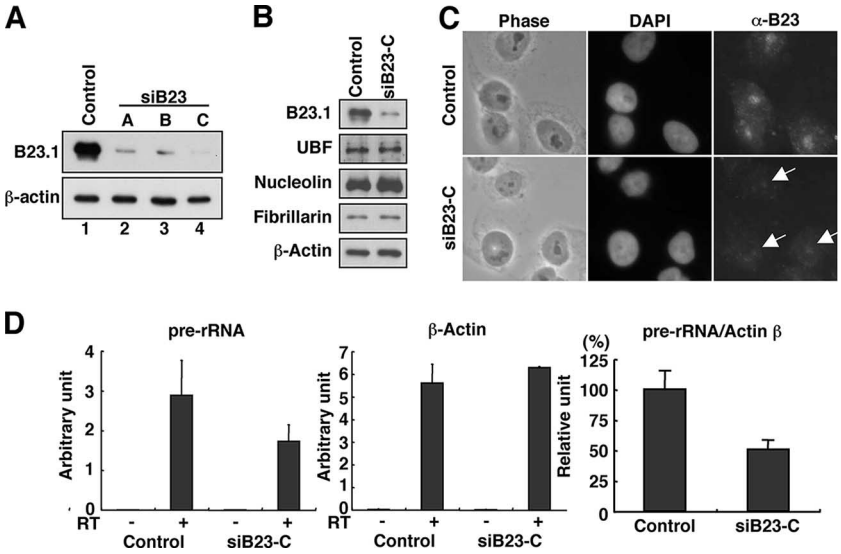

FIG. 2. Repression of the rRNA gene transcription by siB23. (A) Expression level of B23.1. Immunoblotting was performed with lysates from HeLa cells treated with 40 pmol of control siRNA (lane 1) and siB23-A (lane 2), -B (lane 3), and -C (lane 4) (siRNA for B23.1). The mRNA level of B23.1 was also decreased by transfection of siB23-C compared with control siRNA (available on request). (B) Expression level of nucleolus-related proteins. Protein expression levels were determined by immunoblotting using HeLa cells treated with control siRNA and siB23-C. (C) Expression of B23 in nucleoli. Cellular B23 was stained by an indirect immunofluorescence method using anti-B23 antibody in HeLa cells treated with control siRNA or siB23-C. Nuclei were visualized with DAPI. Arrows indicate the positions of nucleoli. (D) Quantitative determination of pre-rRNA by RT-PCR. RNA isolated from HeLa cells treated with control siRNA or siB23-C was examined by RT-PCR using 5′ ETS-specific primers for pre-rRNA and primers for β-actin. The amount of pre-rRNA normalized to that of β-actin mRNA is shown on the right. Results are means and SD from three independent experiments.

rRNA gene (Fig. 2D). The transcription level of the rRNA gene in cells treated by siB23-C was 0.5-fold lower than that of control siRNA. These results indicate that B23 plays an important role in transcription of the rRNA gene by Pol I.

B23 is associated with the chromatin around the rRNA gene. The amino-terminal domain of B23 shows significant similarity to nucleoplasmin, which is the first identified histone chaperone (32). B23 has been shown to bind with histones (44) and is localized in nucleoli. Thus, it is reasonable to hypothesize that B23 binds to chromatin around the rRNA gene. To examine this, we performed nucleolar ChIP as described in Materials and Methods. Nucleoli isolated from HeLa cells cross-linked with formaldehyde were sonicated to release chromatin fragments. DNA purified from chromatin fragments was less than 1 kbp (available on request). Sonicated chromatin was subjected to immunoprecipitation with anti-Flag antibody as a negative control and anti-B23 antibody, followed by quantitative PCR using a primer set specific for the rRNA gene promoter. The antibody against TBP was also used, since TBP is one of the features of the active promoter. Figure 3A indicates that B23 as well as TBP is associated with the chromatin around the rRNA gene promoter. Next, quantitative PCR was performed using primer sets corresponding to the entire rRNA gene (Table 1) and immunoprecipitated chromatin DNA as a template. The results showed that B23 binds the overall rRNA gene unit but is concentrated at the transcription initiation site and the region encoding 5.8S rRNA (Fig. 3B). Binding of B23 at the region encoding 5.8S rRNA could correspond to the function of B23 in processing internal transcribed spacer 2 (ITS2) of pre-rRNA (22, 26, 51). The binding activity of B23 at the transcription initiation site was examined in cells maintained in the presence of 1% or 10% of serum. The binding activity of B23 to the rRNA gene in cells maintained in the presence of 10% serum is higher than that in the presence of 1% serum (Fig. 3C). Moreover, we examined the chromatin structure of the rRNA gene promoter region in cells treated with siB23-C. The amount of B23 associated with the rRNA gene promoter region was decreased in cells treated with siB23-C, while that of fibrillarin or transcription factors of the rRNA gene (TBP and UBF) was not influenced upon treatment with siB23-C (Fig. 3D). In contrast, the amount of histone H3 associated with the rRNA gene increased upon treatment with siB23-C (Fig. 3D). The amount of histone H3 associated with the rRNA gene chromatin was twofold higher in cells treated with siB23-C than that of control siRNA. Histone distribution over the rRNA gene locus was examined (Fig. 3E). The amount of histone H3 associated with the rRNA gene in the cells treated with siB23-C was higher than that of control cells over the rRNA gene locus. The ratio of the histone density on the rRNA gene in cells treated with siB23-C relative to that in control cells was plotted (Fig. 3E). This plot shows that the histone density around the transcription start site and noncoding region between +15 kbp and +20 kbp relative to the transcription start site is increased more than that in other regions. It is possible that the increase of the histone density in the promoter region may decrease the transcription efficiency of the rRNA gene, although it is unclear how the high histone density in the noncoding region affects rRNA gene transcription. These results suggest that B23 is involved in control of the histone density over the rRNA gene.

B23ΔC inhibits the histone binding activity of B23.1 in vitro. To understand the function of B23 as a histone chaperone, we

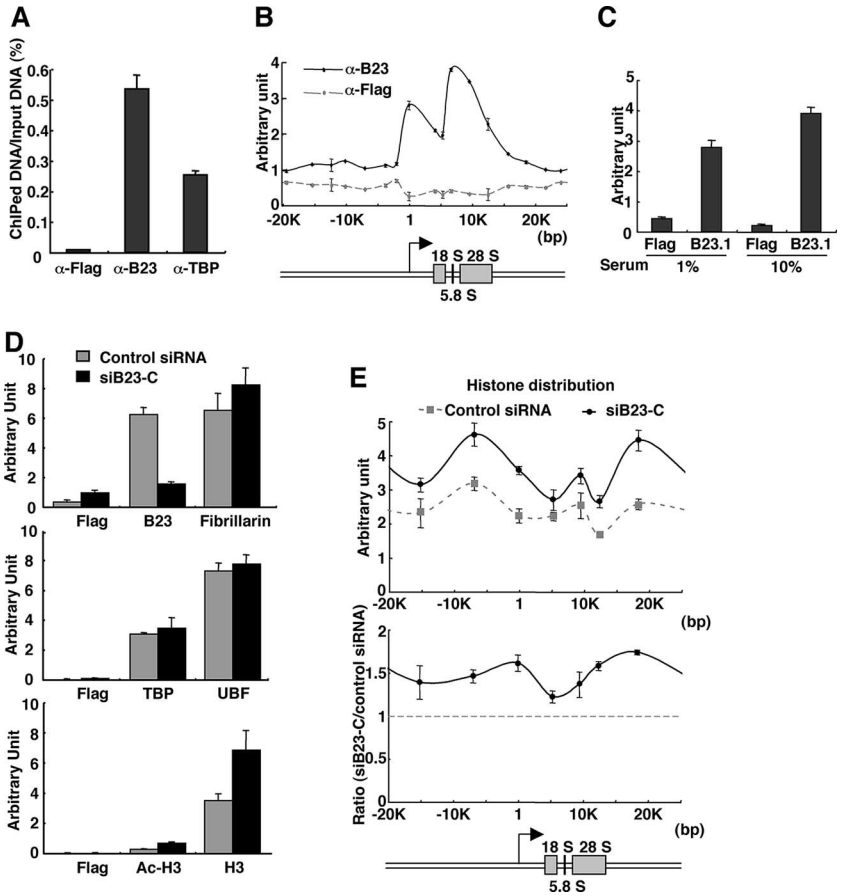

FIG. 3. Association of B23 with the chromatin around the rRNA gene. (A) Nucleolar ChIP analysis using quantitative PCR (Q-PCR). DNA was extracted from soluble nucleolar chromatin immunoprecipitated using anti-Flag, anti-B23, and anti-TBP antibodies. Q-PCR was performed using promoter-specific primers for the rRNA gene and chromatin-immunoprecipitated DNA as templates. Results are means and SD from three independent experiments. (B) ChIP analysis using Q-PCR through the whole rRNA gene unit. The structure of the human rRNA gene unit is shown under the graph. Boxes indicate rRNA-encoding sequences. The arrow indicates the transcription initiation site (+1) of the rRNA gene transcribed by Pol I. DNA was extracted from soluble chromatin immunoprecipitated with anti-Flag and anti-B23 antibodies. Q-PCR was performed using chromatin-immunoprecipitated DNA as the template and the primer sets in Table 1. Results are means ± SD from three independent experiments. (C) ChIP analysis using Q-PCR. 293T cells were maintained in DMEM in the presence of FBS for 72 h. DNA was extracted from chromatin that had been immunoprecipitated with anti-Flag and anti-B23 antibodies. Q-PCR was performed using promoter-specific primers for the rRNA gene and chromatin-immunoprecipitated DNA as templates. Results are means and SD from three independent experiments. (D) Histone density on the rRNA gene promoter. The soluble chromatin fraction from HeLa cells treated with control siRNA or siB23-C was examined by ChIP analyses with anti-Flag, anti-B23, anti-UBF, anti-TBP, anti-acetylated-histone H3 (Ac-H3), and anti-histone H3 antibodies. Q-PCR was performed with promoter-specific primers for the rRNA gene and chromatin-immunoprecipitated DNA as templates. Results are means and SD from three independent experiments. (E) Histone distribution over the rRNA gene. At the same time as the analysis for panel D, Q-PCR of the rRNA gene was carried out (top). The ratio of the histone density over the rRNA gene in cells treated with siB23-C relative to that in cells treated with control siRNA was plotted (middle). Results are means and SD from three independent experiments.

tried to prepare deletion mutants of B23.1 with the goal of constructing a dominant-negative mutant to block the histone chaperone function of B23 specifically. The acidic regions of B23 are crucial for both histone binding and histone chaperone activities (43, 44). The crystal structure of the N-terminal domain of NO38, a *Xenopus* homolog of human B23, indicates that NO38 forms a pentamer and the pentamers further assemble into decamers, as does nucleoplasmin (10, 41). These studies suggest that oligomer formation of nucleoplasmin family proteins is important for their histone chaperone activity. Thus, we hypothesized that a B23 protein containing the oligomeric domain but lacking domains responsible for histone chaperone activity would be a dominant-negative protein. To test this, B23 mutants B23ΔA and B23ΔC, lacking the acidic region and both the acidic and the carboxyl-terminal regions, respectively, were prepared (Fig. 4A). Recombinant hexahistidine-tagged B23.1, B23ΔA, and B23ΔC proteins were expressed in *Escherichia coli* and purified (Fig. 4B).

To examine the histone binding activity of B23 mutants, histone binding assays were carried out in vitro using recombinant B23 mutant proteins and core histones purified from HeLa cells. B23 proteins and core histones were mixed and incubated, followed by immunoprecipitation with anti-His antibody and immunoblotting with anti-His and anti-histone H3 antibodies. His-B23ΔC did not bind to core histones, whereas the histone binding activity of His-B23ΔA was essentially the same as that of wild-type His-B23.1 (Fig. 4C). The fact that B23ΔA binds to histone H3 indicates that other domains, ex-

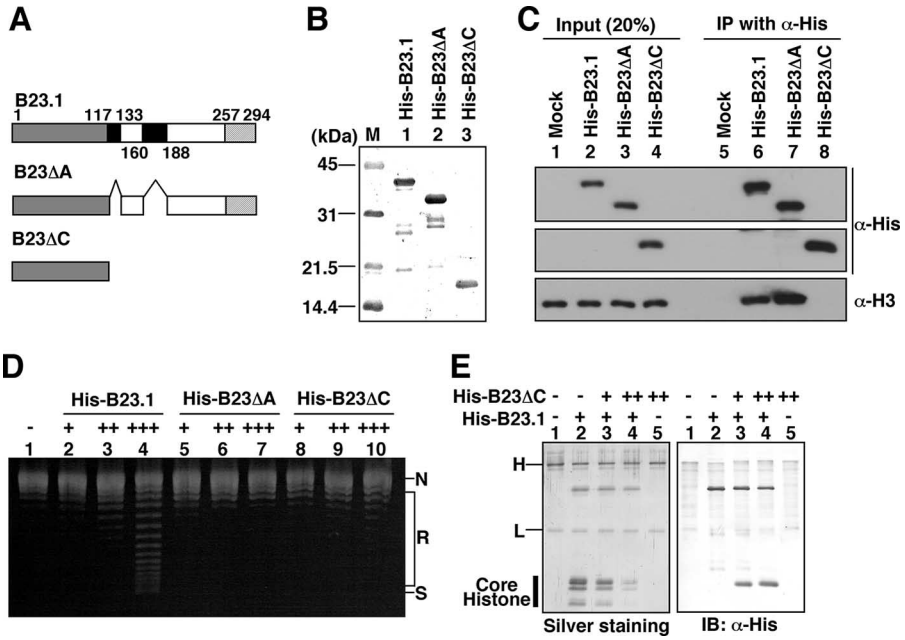

FIG. 4. Inhibition of the histone binding activity of B23.1 by B23ΔC in vitro. (A) Schematic representation of the structure of B23.1, B23ΔA, and B23ΔC. Gray, black, and striped boxes indicate the oligomeric domain, acidic domain, and RNA binding domain, respectively. (B) Purification of recombinant proteins. Recombinant His-B23.1, His-B23ΔA, and His-B23ΔC were purified and separated (400 ng) by 12.5% SDS-PAGE followed by Coomassie brilliant blue staining. (C) Histone binding activity of B23 mutants. Recombinant B23 proteins (500 ng) were mixed with 300 ng core histones purified from HeLa cells. Immunoprecipitation was carried out with an anti-His antibody. Precipitated proteins were analyzed by 12.5% SDS-PAGE followed by immunoblotting with anti-His and anti-histone H3 antibodies. (D) Nucleosome assembly activity of B23 mutants. The nucleosome assembly activity of B23 mutants was examined by a supercoiling assay. Core histones (200 ng) preincubated without His-B23 (−) or with His-B23.1 (+, 167 ng; ++, 500 ng; +++, 1,500 ng), His-B23ΔA (142, 425, and 1,275 ng), and His-B23ΔC (66.8, 200, and 600 ng), were mixed with closed circular DNA relaxed by topoisomerase I and further incubated. The amounts of B23 mutant proteins were adjusted according to their sizes so as to be added at the same molecular numbers. The DNA was purified and separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. Positions of relaxed (R), supercoiled (S), and nicked (N) circular plasmid DNA are indicated. (E) Inhibition of the histone binding activity of B23.1 by B23ΔC. His-B23.1 (200 ng) was mixed without (lane 2) or with 200 ng (lane 3) or 400 ng (lane 4) of B23ΔC and subjected to the denature-renature protocol. Renatured B23 complexes were mixed with 300 ng core histones purified from HeLa cells. His-B23.1 was immunoprecipitated with an anti-B23 antibody that cannot recognize B23ΔC protein. Precipitated proteins were analyzed by 12.5% SDS-PAGE followed by silver staining and immunoblotting (IB) with an anti-His antibody. H and L indicate heavy and light chains of immunoglobulin, respectively.

cept for acidic domains, are important for histone binding. The nucleosome assembly activity of B23ΔA was examined using a supercoiling assay (Fig. 4D). His-B23.1 assembled nucleosomes in a dose-dependent manner and His-B23ΔC did not, as previously described (44). Although His-B23ΔA bound with core histones, it could not transfer core histones to plasmid DNA (Fig. 4D). This result indicates that acidic domains of B23.1 are crucial for histone chaperone activity.

We confirmed that B23ΔC does not have histone binding and nucleosome assembly activities (Fig. 4C and D). Then, we examined whether B23ΔC is a dominant-negative protein for B23.1. When mixed directly, His-B23ΔC did not form a hetero-oligomer with His-B23.1 in vitro, suggesting that B23 forms a stable oligomer (data not shown). However, when a mixture of His-B23.1 and His-B23ΔC was subjected to the denature-renature protocol described in Materials and Methods, two proteins were found to form a hetero-oligomer successfully. The interaction between B23 oligomers and core histones was examined by immunoprecipitation using an anti-B23 antibody that cannot recognize B23ΔC (data not shown). Immunoprecipitated proteins were separated by 12.5% SDS-PAGE, followed by silver staining (Fig. 4E) and immunoblotting (Fig. 4E) using anti-His antibody. B23.1 formed a hetero-oligomer with His-B23ΔC (Fig. 4E, right, lanes 3 and 4). B23.1 interacted efficiently with core histones (Fig. 4E, left, lane 2), as shown in Fig. 4C and a previous report (44). However, the histone binding activity of B23.1 was decreased by oligomer formation with increasing amounts of His-B23ΔC (Fig. 4E, left, lanes 3 and 4; the molar ratios of His-B23.1 and His-B23ΔC were 1:2.5 and 1:5, respectively). Since B23 was shown to form a pentamer, it is likely that a His-B23.1 pentamer including four His-B23ΔC molecules lost the histone binding activity completely. We concluded that oligomer formation of B23 is critical for its histone binding activity and that B23ΔC inhibits the histone binding activity of B23.1 in a dominant-negative manner.

B23ΔC inhibits the histone binding activity of B23.1 in vivo. To use the B23 mutant proteins as tools for functional analyses of B23 in vivo, we examined whether B23 mutants inhibit the histone binding activity of B23.1. First, we confirmed the expression of each B23 protein by immunoblotting using lysates of 293T cells transfected with plasmids encoding HA-B23.1, HA-B23ΔC, Flag-B23.1, and Flag-B23ΔA proteins (Fig. 5A). To examine the histone binding activity of B23 mutants, im-

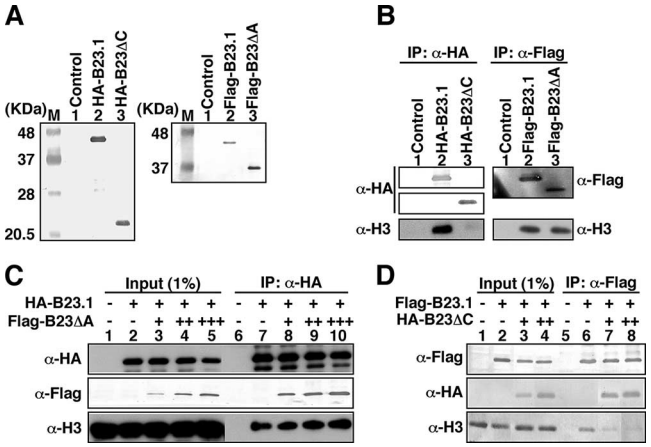

FIG. 5. Inhibition of the histone binding activity of B23.1 by B23ΔC in vivo. (A) Expression of B23 proteins in 293T cells. Immunoblotting was carried out using lysate prepared from 293T cells transfected with the indicated plasmids. (B) Histone binding activity of B23 mutants. Immunoprecipitation (IP) with anti-HA and anti-Flag antibodies was performed using lysates of 293T cells transfected with plasmids expressing B23 proteins. Immunoprecipitated proteins were analyzed by immunoblotting. (C) Lack of effect of B23ΔA on the histone binding activity of B23.1. Lysates of 293T cells (24 h posttransfection) transfected with pCHA-B23.1 (160 ng) and pCAGGS-Flag-B23ΔA (+, 160 ng; ++, 480 ng; +++, 1,440 ng) were subjected to immunoprecipitation with an anti-HA antibody. Immunoprecipitated proteins were analyzed by immunoblotting. (D) Inhibition of the histone binding activity of B23.1 by B23ΔC in vivo. Lysates of 293T cells (24 h posttransfection) transfected with pcDNA3-Flag-B23.1 (100 ng) and pCHA-B23ΔC (+, 300 ng; ++, 1,400 ng) were subjected to immunoprecipitation with anti-Flag antibody. Immunoprecipitated proteins were analyzed by immunoblotting.

munoprecipitation with anti-HA and anti-Flag antibodies was performed with lysates prepared from 293T cells expressing HA-B23.1, HA-B23ΔC, Flag-B23.1, or Flag-B23ΔA, followed by immunoblotting with anti-histone H3 antibody (Fig. 5B). B23ΔC did not bind to histone H3, whereas B23ΔA had histone binding activity, as did wild-type B23.1. This result is consistent with the result of histone binding assays in vitro (Fig. 4C).

We investigated whether B23ΔA and B23ΔC inhibit the histone binding activity of wild-type B23.1 in vivo (Fig. 5C and D). HA-B23.1 was expressed in 293T cells with or without Flag-B23ΔA, and immunoprecipitation was carried out using anti-HA antibody, followed by immunoblotting with anti-HA, anti-Flag, and anti-histone H3 antibodies. As expected, Flag-B23ΔA did not influence the histone binding activity of HA-B23.1, but the amount of coprecipitated histone H3 with B23ΔA was slightly increased (Fig. 5C, lanes 8, 9, and 10). Then, Flag-B23.1 was expressed in 293T cells with or without HA-B23ΔC, and immunoprecipitation was carried out with anti-Flag antibody, followed by immunoblotting with anti-Flag, anti-HA, and anti-histone H3 antibodies (Fig. 5D). In contrast to Flag-B23ΔA, HA-B23ΔC inhibited the histone binding activity of Flag-B23.1 in a dose-dependent manner through interaction with Flag-B23.1 (Fig. 5D, lanes 7 and 8). This result indicates that B23ΔC forms a hetero-oligomer with B23.1 and inhibits the histone binding activity of B23.1 in vivo.

Inhibition of the rRNA gene transcription by B23ΔC. We have shown that B23ΔC inhibits the histone binding activity of B23.1 in a dominant-negative manner. Therefore, B23ΔC could be a powerful tool to clarify the cellular function of B23 as a histone chaperone. Next, we examined the effect of B23ΔC on rRNA gene transcription. 293T cells expressing HA-B23ΔC were lysed, and HA-B23ΔC was immunoprecipitated with anti-HA antibody (Fig. 6A). Approximately 50% of endogenous B23.1 was coprecipitated with HA-B23ΔC under the conditions employed here. Therefore, it is quite likely that HA-B23ΔC can quench the function of endogenous B23 in cells. In order to examine the effect of HA-B23ΔC on the chromatin binding activity of B23, nucleolar ChIP with anti-B23 antibody was carried out using nucleoli purified from 293T cells transfected with plasmids encoding HA-B23.1 and HA-B23ΔC. The association of B23 with the rRNA gene promoter region was examined by quantitative PCR using immunoprecipitated DNA and a specific primer set (Fig. 6B). We found that B23 binds to the promoter region of the rRNA gene, and overexpression of HA-B23ΔC decreased this association by 25%. Next, we examined the effect of HA-B23ΔC on the chromatin structure of the rRNA gene promoter region. Binding of transcription factors of the rRNA gene, UBF and TBP, was not affected or was slightly up-regulated by expression of HA-B23ΔC (Fig. 6C), while the amount of histone H3 or acetylated histone H3 associated with the rRNA gene increased upon expression of HA-B23ΔC (Fig. 6D). Histone H3 distribution over the rRNA gene locus was examined (Fig. 6E). The amount of histone in cells expressing B23ΔC was higher than that in mock-treated cells over the rRNA gene locus. Overexpression of B23ΔC increased the histone density of the coding region of 18S rRNA gene (Fig. 6E, bottom). The histone density on the rRNA gene, except in the region encoding 18S rRNA, is approximately 1.3- to 1.8-fold higher in cells expressing B23ΔC than that in mock-treated cells, consistent with the result obtained with siB23 (Fig. 3E), whereas the histone density on the region coding 18S rRNA was 2.9-fold higher in cells expressing B23ΔC than in mock-treated cells. The reason for this discrepancy between the results in Fig. 3E and Fig. 6E is presently unclear, but it is assumed to be due to the different cell types used. The results shown in Fig. 3D and Fig. 6D indicate that the ratio of acetylated histone H3 to histone H3 on the rRNA gene in HeLa cells was lower than that in 293T cells. In other words, this could be interpreted as indicating that the ratio of the number of fully active rRNA genes to the total number of the rRNA genes is higher in 293T cells than that in HeLa cells. Nevertheless, it is probable that B23 controls the histone density on the rRNA gene.

Next, we analyzed the transcription activity of Pol I in cells expressing HA-B23ΔC. Nuclei were isolated from 293T cells expressing HA-B23ΔC, and run-on assays were carried out (Fig. 6F). Run-on assays demonstrated that the transcription level of the rRNA gene in 293T cells expressing HA-B23ΔC was 0.6-fold lower than that of control cells, suggesting that overexpression of HA-B23ΔC down-regulates the activity of Pol I, possibly by blocking the function of endogenous B23. Moreover, we examined the effect of B23ΔA on the transcription of the rRNA gene, which binds to histones but lacks histone chaperone activity (Fig. 4D). Quantitative RT-PCR for pre-rRNA demonstrated that the amount of pre-rRNA in cells

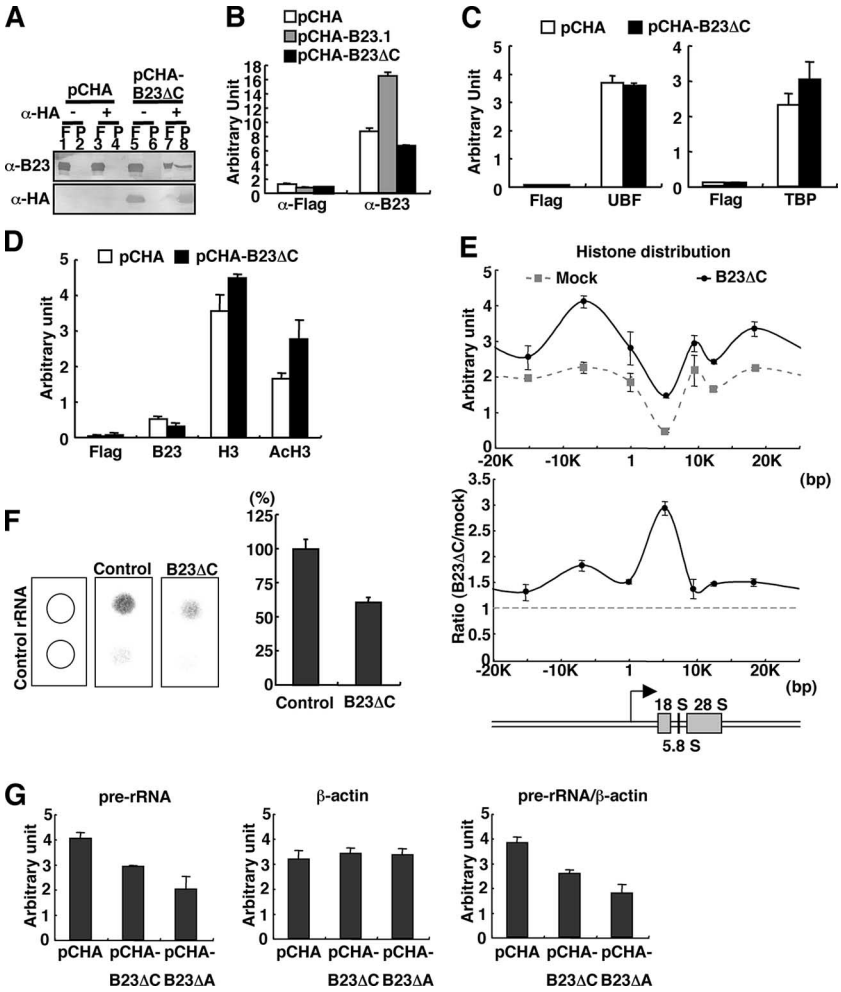

FIG. 6. Inhibition of rRNA gene transcription by B23ΔC. (A) Interaction of B23ΔC with endogenous B23. 293T cells were transiently transfected with pCHA (control) and pCHA-B23ΔC and cultured for 72 h posttransfection. Lysates prepared from 293T cells were used for immunoprecipitation with anti-HA antibody. Immunoprecipitates (P) and flowthrough fractions (F) were resolved by 12.5% SDS-PAGE, transferred to a membrane, and probed with anti-B23 and anti-HA antibodies. (B) Inhibition of association of B23 with the rRNA gene by B23ΔC. 293T cells were cotransfected with pBabe-puro and the indicated plasmids, and 0.5 μg/ml puromycin was added 24 h posttransfection. After puromycin selection for 30 h, 293T cells were maintained in fresh medium for 18 h. Nucleolar ChIP was carried out with anti-B23 and anti-Flag antibodies. Quantitative PCR was performed with primers around the promoter region of the rRNA gene. Results are means and SD from three independent experiments. A paired Student's *t* test gave a *P* value of 0.02 between pCHA- and pCHA-B23ΔC-transfected cells. (C) Association of transcription factors on the rRNA gene promoter. ChIP was carried out with anti-UBF and anti-TBP antibodies, and lysates were prepared for mock-treated cells or cells expressing B23ΔC. Q-PCR was performed with primers around the promoter region of the rRNA gene. Results are means and SD from three independent experiments. (D) Histone density on the rRNA gene promoter. ChIP was carried out using anti-B23, anti-histone H3, and anti-acetylated-histone H3 (AcH3) antibodies in the presence or absence of B23ΔC. Q-PCR was performed with primers around the promoter region of the rRNA gene. Results are means and SD from three independent experiments. (E) Histone distribution over the rRNA gene. ChIP was performed with anti-histone H3 antibody using HA-B23ΔC-expressing or mock-treated cells. The amount of chromatin-immunoprecipitated DNA was determined by Q-PCR using primer sets for the rRNA gene (top). The ratio of the histone density over the rRNA gene in cells expressing B23ΔC relative to that in mock-treated cells was plotted (middle). Results are means and SD from three independent experiments. (F) Inhibition of the rRNA gene transcription by B23ΔC. 293T cells were transiently transfected with pCHA (control) and pCHA-B23ΔC and cultured for 72 h posttransfection. Nuclei isolated from these 293T cells were used for run-on transcription assays. Radiolabeled nuclear RNA was isolated and hybridized with 1 μg of pUC119 (control) and pUC119-HurDNA (rRNA) on a membrane. This assay was done in triplicate, and a typical pattern is shown. The right panel shows a densitometry quantitation of the hybridization signals. Results are means and SD from three independent experiments. (G) Inhibition of the rRNA gene transcription by B23ΔA. 293T cells were cotransfected with pBabe-puro and one of the indicated plasmids, and 0.5 μg/ml puromycin was added 24 h posttransfection. After puromycin selection for 30 h, 293T cells were maintained in fresh medium for 18 h. RNA isolated from 293T cells was examined by RT-PCR using 5′ ETS-specific primers for pre-rRNA and primers for β-actin mRNA. Results are means and SD from three independent experiments.

expressing B23ΔA was lower than that of control cells (Fig. 6G). This result may be interpreted to mean that B23ΔA forms oligomers with endogenous B23.1 and inhibits rRNA transcription, as does B23ΔC. Thus, it is quite possible that B23 controls the histone density on the rRNA gene and thereby regulates transcription of the rRNA gene as a nucleolar histone chaperone.

We tried to confirm the above notion by complementation

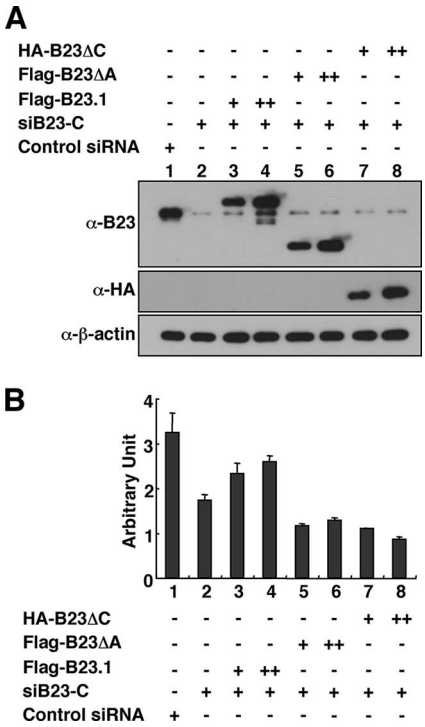

FIG. 7. Involvement of acidic domains of B23 in rRNA gene transcription. (A) Expression of B23 proteins in HeLa cells transfected with siB23. HeLa cells were transfected with 40 pmol of control siRNA and 40 pmol of siB23-C. At 12 h after siRNAs transfection, HeLa cells were supertransfected with pCAGGS-Flag-B23.1 (+, 180 ng; ++, 360 ng), pCAGGS-Flag B23ΔA (180 and 360 ng), pCHA-B23ΔC (180 and 360 ng), and pEF321-T (40 ng) encoding SV40 T antigen for amplification of transfected plasmid. At 72 h after siRNA transfection, lysates of 2.5×10^3 cells were analyzed by immunoblotting using anti-B23, anti-HA, and anti-β-actin antibodies. (B) Quantitative determination of pre-rRNA by Q-PCR. At the same time as the analysis for panel A, total RNA extracted from HeLa cells were examined by RT-PCR using 5′ ETS-specific primers for the rRNA gene. The amount of pre-rRNA was normalized to that of β-actin mRNA. Results are means and SD from three independent experiments.

experiments. By monitoring the transcription of the rRNA gene, we examined the rescue ability of B23 mutant proteins for HeLa cells treated with siRNA specific for B23.1. After treatment of siB23-C, HeLa cells were supertransfected with plasmids encoding various B23 proteins and with pEF321-T, encoding simian virus 40 (SV40) T antigen, in order to amplify the expression level of B23 proteins supplied by pCAGGS and/or pCHA, which contain the replication origin of SV40. siB23-C recognizes the 3′ UTR of endogenous mRNA of B23.1, and we constructed pCAGGS-based plasmids encoding B23 mutant proteins without the siB23-C target sequence. Therefore, siB23-C does not affect the expression of exogenous B23 mutant proteins. The expression level of B23 proteins was detected by immunoblotting using anti-B23 or anti-HA antibodies (Fig. 7A). The amount of 45S pre-rRNA in HeLa cells was examined by quantitative RT-PCR using the 5′ ETS-specific primer set for the rRNA gene. The reduction in synthesis of 45S pre-rRNA by siB23-C was recovered by the expression of Flag-B23.1 in a dose-dependent manner (Fig. 7B, lanes 2 to 4). In contrast, Flag-B23ΔA and HA-B23ΔC did not rescue the synthesis of 45S pre-rRNA reduced by siB23-C, but rather B23ΔC decreased the synthesis of 45S pre-rRNA (Fig. 7B, lane 2 and lanes 5 to 8). These results indicate that Flag-B23.1 promotes the transcription of the rRNA gene but Flag-B23ΔA and HA-B23ΔC do not. B23ΔA was shown to be capable of binding to histones (Fig. 4C), but it does not have histone chaperone activity (Fig. 4D), indicating that the acidic domain of B23.1 is critical for the histone chaperone activity and rRNA gene transcription-promoting activity. Thus, it is concluded that the histone binding activity of B23 is not sufficient for the rRNA gene transcription promotion, and not only the histone binding activity but also the histone chaperone activity is required (27).

Inhibition of cell growth by overexpression of B23ΔC. We have shown the positive correlation between the B23 histone chaperone activity and the rRNA gene transcription. Next, we investigated the function of B23 in cell growth. To this end, we took advantage of B23ΔC, a dominant-negative mutant for the histone chaperone activity of B23. 293T cells were cotransfected with plasmids encoding B23ΔC and enhanced green fluorescent protein (EGFP). The number of EGFP-positive cells, and thus quite possibly B23ΔC-positive cells, was counted every 24 h posttransfection (Fig. 8A). The growth of 293T cells expressing B23ΔC seemed to be stopped. The cell growth rate was decreased in proportion to the expression level of B23ΔC (Fig. 8B). In cells expressing B23ΔC3, the rRNA synthesis was inhibited (Fig. 6). Moreover, KD of the expression of B23.1 by siB23-C decreased the cell growth rate (data not shown). Thus, it is possible that B23ΔC suppresses the synthesis of the rRNA gene and thereby affects the cell growth rate.

Alternatively, B23ΔC may affect the cell growth through another pathway(s), since B23 is a multifunctional protein. We examined the cell cycle profile of cells expressing B23ΔC. It is suspected that the growth inhibition by expression of B23ΔC is due to apoptosis. However, the population of apoptotic cells was not increased by expression of B23ΔC (Fig. 8C, inset). As shown in Fig. 8C, the G_2/M population was slightly increased by expression of B23ΔC. This result may be consistent with the observation with $B23^{-/-}$ MEFs (17, 31). Tetraploid cells contain 4n DNA, as do cells in G_2/M phase. Along this line, the p53 pathway involved in centrosome duplication that is essential for ploidy maintenance is one of the candidates for the cell growth defect caused by expression of B23ΔC. It was reported that B23 regulates the stability and the transcriptional activity of p53 directly or indirectly (5, 29). To exclude the possibility that B23ΔC inhibited cell growth by affecting the p53 pathway, we examined the effect of overexpression of B23ΔC on the growth rate of $p53^{-/-}$ MEFs. As shown in Fig. 8D, the growth rate of $p53^{-/-}$ MEFs was similarly decreased by overexpression of B23ΔC. The effect of B23ΔC on the growth of $p53^{-/-}$ MEFs seems weaker than that on growth of 293T cells. This could be due to the fact that a pCAGGS-based expression vector is amplified in 293T cells expressing SV40 T antigen but not in $p53^{-/-}$ MEFs, so that the expression level of B23ΔC is higher in 293T cells than $p53^{-/-}$ MEFs. Thus, the cell growth inhibition activity of B23ΔC is independent of the p53 pathway. From these results, we concluded that overexpression of B23ΔC leads to the growth inhibition, most possibly through down-regulation of the rRNA gene transcription.

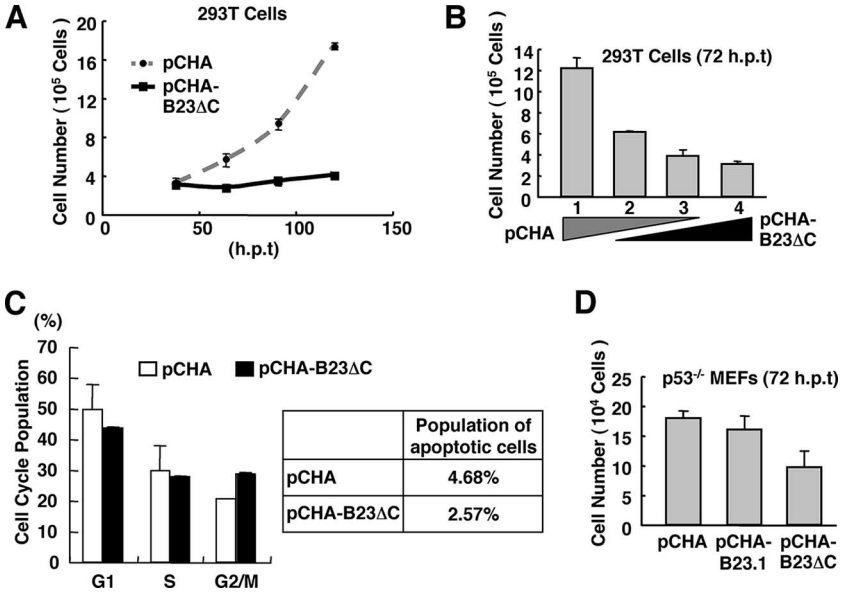

FIG. 8. Inhibition of cell proliferation by B23ΔC. (A) Proliferation inhibition of 293T cells by expression of B23ΔC. 293T cells were cotransfected with 1,400 ng of pCHA and 200 ng of pEGFP-N1 or with 1,400 ng of pCHA-B23ΔC and 200 ng of pEGFP-N1. The number of EGFP-positive cells was counted every 24 h as a function of culture time. Results are means and SD from three independent experiments. (B) B23 dose-dependent inhibition of the cell proliferation rate. The number of EGFP-positive cells was counted at 72 h after transfection of 200 ng of pEGFP-N1 with pCHA-B23ΔC (350 ng [lane 2], 700 ng [lane 3], and 1,400 ng [lane 4]), and pCHA (1,400 ng [lane 1], 700 ng [lane 2], and 350 ng [lane 3]). Results are means and SD from three independent experiments. (C) Cell cycle population of cells expressing B23ΔC. 293T cells were cotransfected with 1,400 ng of pCHA and 200 ng of pEGFP-N1 or with 1,400 ng of pCHA-B23ΔC and 200 ng of pEGFP-N1 and then incubated for 72 h. Cell population of 293T cells expressing EGFP was determined by fluorescence-activated cell sorting analysis. The sub-G_1 fraction was quantitatively determined as a population of apoptotic cells (inset). (D) p53-independent effect of B23ΔC on cell proliferation. EGFP-positive $p53^{-/-}$ MEFs were counted at 72 h after transfection of pEGFP-N1 (125 ng) with pCHA-B23.1 (875 ng) or pCHA-B23ΔC (875 ng). Results are means and SD from three independent experiments.

DISCUSSION

In this study, we examined the function of B23 as a nucleolar histone chaperone. Here, we have confirmed that the expression level of B23 is correlated with the rate of the rRNA gene transcription (Fig. 1). KD of B23 by siRNA decreased the transcription rate of the rRNA gene (Fig. 2). It was shown by using nucleolar ChIP assays that B23 is associated with the rRNA gene in cells (Fig. 3). To test the histone chaperone activity of B23 in cells, we constructed B23ΔC, a dominant-negative mutant, which inhibited the histone binding activity of B23.1 (Fig. 4 and 5). B23ΔC overexpressed in 293T cells bound to endogenous B23.1 and decreased the transcription rate of the rRNA gene in vivo (Fig. 6). Expression of exogenous B23.1 but not that of B23ΔA or B23ΔC could rescue the rRNA synthesis reduced by siRNA (Fig. 7). Finally, overexpression of B23ΔC results in inhibition of the cell growth (Fig. 8). Taken together, these results suggest that B23 regulates the transcription of rRNA gene as a nucleolar histone chaperone and thus affects the cell growth rate.

The inhibitory effect of siB23-C (Fig. 2D) and B23ΔC (Fig. 6F and G) on the rRNA transcription seemed incomplete. However, these changes in the transcription rate of the rRNA genes are in almost the same range as the difference between the rRNA synthesis levels of cells maintained in 10% and 1% serum (Fig. 1C and D). The tandem rRNA gene repeats exist in two distinct types of chromatin, an open one, permissive for transcription, and a closed one, in the transcriptionally repressive state. It has been reported that the number of the rRNA genes existing as open chromatin is independent of the cell growth rate (6). Based on these, it is possible that B23 may not be involved in remodeling of chromatin around the rRNA gene between open and closed states. In that case, we would consider it possible that B23 is required for maintenance of an open chromatin. The results of this study show that the level of rRNA gene transcription is proportional to the amount of B23. B23 remodels the chromatin structure and stimulates the transcription from chromatin template (reference 57 and our unpublished results; also, see below). We assume that the amount of B23 is a switch for the rRNA transcription fluctuation in response to a variety of continuous stresses and/or stimuli from outside and within cells.

In eukaryotic cells, the chromatin structure plays an important role in regulation of gene expression. It has been shown that in the nucleolus, NoRC, a nucleolar ATP-dependent chromatin remodeling complex, recruits histone deacetylase and DNA methyltransferase activities on the rRNA genes to induce formation of heterochromatin on the rRNA genes (38, 50). B23 has been shown to be acetylated by p300 and stimulate transcription preferentially from a chromatin template containing acetylated histones, although the stimulation by acetylated B23 occurs in transcription assays using RNA Pol II in the presence of the transactivator Gal4-VP16 in vitro (57). This observation suggests that B23 facilitates disassembly of histones over the rRNA gene in combination with p300 to stimulate the transcription of the rRNA gene. Nonacetylatable B23 mutants would be powerful tools for further analysis of the

coordination mechanism between B23 and p300 on rRNA gene transcription. In addition, B23 was shown to interact with nucleolin, which is a nucleolar histone chaperone and enhances the activity of the chromatin remodeling machineries, SWI/SNF and ACF (1, 34, 48). B23 and nucleolin as nucleolar histone chaperones may regulate the transcription of the rRNA gene through the structural change of chromatin with ATP-dependent chromatin remodelers and histone modification enzymes in a coordinated manner.

Nucleoplasmin 3 (NPM3) was identified as a B23 binding protein from a mouse liver cDNA library using a yeast two-hybrid system (23). NPM3 shows significant structural similarity to B23ΔC and interacts with endogenous B23. Overexpression of NPM3 decreased the rate of pre-rRNA synthesis and processing. It is possible that NPM3 also functions as an inhibitor for the histone binding activity of endogenous B23, as does B23ΔC. We have also observed retardation of rRNA processing by overexpression of B23ΔC (data not shown). Since B23.2 lacking the C-terminal RNA binding domain was found to form an oligomer with B23.1 and inhibit the RNA binding activity of B23.1 (45), it is likely that B23ΔC also inhibits not only the histone chaperone activity but also the RNA binding activity of B23.1. The RNA binding activity of B23.1 could be important to target B23.1 to pre-rRNA to be processed. This could be one of the reasons why overexpression of B23ΔC and NPM3 inhibits the pre-rRNA processing.

From the observation that B23, NPM3, and nucleolin are involved in pre-rRNA synthesis and processing (1, 15, 23, 48, 51), it is likely that the rRNA gene transcription and pre-rRNA processing are coupled and coregulated. This idea is supported by the finding that B23 associates with not only the promoter region but also the region encoding 5.8S rRNA next to ITS2 cleaved by B23 (Fig. 3B). In yeast cells, Pol I transcription and pre-rRNA processing were shown to be linked by pre-rRNA processing factors (13, 16, 52), and the complex containing Pol I drives both transcription and pre-rRNA modifications in vitro (11). In mammalian cells, B23 could be one of the molecules linking rRNA gene transcription and pre-rRNA processing.

We demonstrated that B23ΔC drastically decreased the proliferation rate of 293T cells (Fig. 8A). $B23^{+/-}$, $B23^{hy/hy}$ (homozygously hypomorphic mutant), and $B23^{-/-}$ MEFs show decreased cell proliferation rates compared with wild-type MEFs (17). It was previously reported that B23 binds to p53 directly and enhances its stability and the transcriptional activity of p53, so that overexpression of B23.1 induces inhibition of MEF proliferation (5). B23 interacts with ARF and Mdm2, which are involved in cell cycle regulation in concert with p53 (3, 29, 30). We had suspected that B23ΔC inhibits the cell proliferation through the p53 pathway. However, this possibility was ruled out by experiments using $p53^{-/-}$ MEF (Fig. 8D). This is supported by the report that a deletion mutant of B23 similar to B23ΔC does not bind to p53 in vitro and that the expression of B23.1 increases the proliferation rate of $p53^{-/-}$ MEFs slightly (5).

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